Novel Bioresponsive Drug Eluting Microspheres to Enhance Chemoembolisation Therapy

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Abstract

Drug eluting beads (DEB) are employed in the treatment of solid hypervascularised malignant tumours by a method called trans-arterial chemoembolisation (TACE). When the microcirculation to a tumour is blocked, oxygen levels decrease to critically low levels causing the tumour to become hypoxic. Hypoxic tumours are known to be chemoresistant and send out growth factor signals leading to angiogenesis and metastasis of tumour cells to other parts of the body. Commercially available DEB are unable to respond to the conditions of hypoxia and will continue to release drug at a constant rate via ionic exchange through the hydrogel. It is therefore recognised that an avenue for improvement would be the development of novel bioresponsive DEB that are able to react to the conditions of hypoxia to overcome chemoresistance associated with the tumour cells.

Hypoxic tumours provide a reducing environment, and this reducing potential has been used to target cells intracellularly. Disulfide linkages have been exploited as a redox sensitive group in gene delivery systems, with the ability to reduce the disulfide bond to their respective thiol entities, facilitating release of the biomolecule. However, very little literature pertains to the targeting of the redox environment outside of the tumour cell. This thesis focuses on an investigation into the synthesis of a redox sensitive hydrogel DEB, with the potential to respond to a hypoxic tumour by cleavage of the redox sensitive monomer. The properties of this novel device will lead to an increased release of drug with the intention to saturate the tumour cells and combat chemoresistance.

A disulfide cross-linker called Bis(acryloyl)cystamine (BAC) was incorporated into a modified Poly(vinyl) alcohol (PVA) network. The solubility of BAC was limited in water, leading to the synthesis of a water soluble cross-linker called Bis(acryloyl)-(L)-cystine (BALC). The new cross-linker, characterised by NMR and mass spectroscopy, was incorporated within the modified PVA network at different concentrations, varying the amount of BALC within each bead formulation. The beads were characterised to confirm the amount of cross-linker within each formulation and its effects on the bead properties. Elemental and UV/Vis spectroscopic analysis confirmed the incorporation of BALC within the beads and sizing studies showed that in the presence of a reducing agent the beads increased in diameter.

The BALC beads were loaded with doxorubicin hydrochloride (Dox), with high percentage formulations loading in excess of 300 mg of drug per mL of hydrated beads. All formulations, whilst drug loaded, showed an increase in diameter after reduction of the disulfide bonds, providing more potential for increased drug release. Elution of Dox BALC beads in PBS demonstrated a controlled release via ionic exchange through the hydrogel with the amount of drug released increasing in relation to the amount of drug loaded. Some formulations in the presence of a reducing agent exhibited an increased release of drug in comparison to beads in PBS. These beads have therefore illustrated the ability to respond to an in vitro reducing environment resulting in an increase in bead diameter and hence drug release.

A cell culture model demonstrated that mammalian cells in hypoxic conditions can reduce the disulfide linkages within the bead resulting in an increase in diameter. Further work is still required to optimise the formulation with a focus on increased drug release in hypoxic conditions prior to in vivo studies and clinical evaluation.
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Author’s Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. This thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material submitted for a degree.

Signed

Dated
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D COSY</td>
<td>2D correlation spectrometry</td>
</tr>
<tr>
<td>AASLD</td>
<td>American Associations for Study of Liver Diseases</td>
</tr>
<tr>
<td>AMPS</td>
<td>2-acrylamido-2-methylpropanesulfonate sodium salt</td>
</tr>
<tr>
<td>ATP</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AVM</td>
<td>Arteriovenous malformation</td>
</tr>
<tr>
<td>BAC</td>
<td>N,N’-bis(acryloyl cystamine)</td>
</tr>
<tr>
<td>BALC</td>
<td>N,N’-bis(acryloyl)-(l)-cystine</td>
</tr>
<tr>
<td>BCLC</td>
<td>Barcelona Clinic for Liver Cancer</td>
</tr>
<tr>
<td>CAB</td>
<td>Cellulose acetate butyrate</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEB</td>
<td>Drug eluting beads</td>
</tr>
<tr>
<td>DEPT-135</td>
<td>Distortion enhancement by polarisation transfer-135</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxorubicin hydrochloride</td>
</tr>
<tr>
<td>DSM</td>
<td>Degradable Starch Microspheres</td>
</tr>
<tr>
<td>DSP</td>
<td>Dithiobis(succinimidylpropionate)</td>
</tr>
<tr>
<td>DTBP</td>
<td>Dimethyl-3,3’-dithiobispropionimidate</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis(2-nitrobenzoic acid) or Ellmans reagent</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EASL</td>
<td>European Association for Study of Liver</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy Dispersive X-ray</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EWC</td>
<td>Equilibrium water content</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>Iri</td>
<td>Irinotecan Hydrochloride</td>
</tr>
<tr>
<td>KPS</td>
<td>Potassium persulfate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase activity</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistance</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NAAADA</td>
<td>N-acryloyl-aminoacetaldehyde dimethyl acetal</td>
</tr>
<tr>
<td>NaBH₄</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>OLT</td>
<td>Orthotopic liver transplantation</td>
</tr>
<tr>
<td>p(HEMA)</td>
<td>poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>PAA</td>
<td>Polyacrylic acid</td>
</tr>
<tr>
<td>PMASH</td>
<td>Thiolated poly(methacrylic acid)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered serology saline</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>Ref-1</td>
<td>Redox factor-1</td>
</tr>
<tr>
<td>RCTs</td>
<td>Randomised clinical trials</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TACE</td>
<td>Transarterial chemoembolisation</td>
</tr>
<tr>
<td>TAE</td>
<td>Transarterial embolisation</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TEMPO</td>
<td>4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl free radicals</td>
</tr>
<tr>
<td>TMEDA</td>
<td>N,N,N’,N’-tetramethylethlenediamine</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>wt %</td>
<td>Weight percent</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Liver Cancer

The liver is the largest glandular organ within the human body. It is divided into two lobes of unequal shape and size and receives its blood supply from two large vessels called the hepatic artery and the portal vein, with the latter providing 75% of the blood supply (Mitra and Metcalf, 2009; Si-Yuan et al., 2011). These large vessels terminate in very small capillaries within the liver by subdividing repeatedly. Hepatocytes are the predominant cell type within the liver, and it is these cells that give it the unique ability to be the only organ within the human body to regenerate. A diagram of the liver with its blood vessels for hepatic circulation is shown in Fig. 1.1.

![Liver Diagram](image-url)

Fig. 1.1 A diagram of the human liver with its blood vessels. Image courtesy of Biocompatibles UK Ltd [Original in colour].
The liver is vital for a human to survive as there are no current methods for substituting liver function. The liver carries out an array of functions that make it highly susceptible to disease, which in turn can lead to the development of cancer. A recent re-analysis of global cancer statistics placed cancer of the liver as the 5th most affected organ with over 700,000 new cases of primary liver cancer per year and 696,000 deaths annually, and these figures are projected to increase through 2020 (Rusyn and Lemon, 2013; Gish and Finn, 2013; Ferlay et al., 2010). The most prevalent type of liver cancer in today’s society is hepatocellular carcinoma (HCC) which accounts for 90% of all primary liver cancer cases (Alkhouri and McCullough, 2013).

HCC is an aggressive solid tumour. Histological examination described by Sasaki et al (1996), when resecting small HCC revealed small nodular lesions classified as adenomatous hyperplasia (AH), a pre-cancerous lesion which is a definitive precursor of HCC development. The histology of HCC was further analysed by Okuda (2000) who confirmed these findings by identifying the same results observed using microscopy; such as increased cellularity, irregular thin trabecular formation, together with fatty cell changes and cell invasion within the fibrous stroma and vessel cells. These are the foundations for the growth of HCC and it is from these small nodular lesions that the development of a highly vascularised, malignant, solid tumour occurs (Okuda, 2000).

Cirrhosis and chronic hepatic injury are associated with the majority of all HCC cases (McKillop et al., 2006; Dong and Saab, 2008). The damage to the liver is often connected to infection with the Hepatitis B (HBV) and C viruses responsible for 75 - 80% of all cases and Hepatitis C (HCV) related cirrhosis carrying a greater risk of tumour development (McKillop et al., 2006; Zhu et al., 2011). In the situation where a patient is infected with both HCV and HBV, it has been reported that there is potentially a greater risk of developing HCC than through infection with one of the viruses alone (El-Serag, 2012). The progression of chronic liver diseases from hepatic infection is outlined in Fig. 1.2.
The causes of liver cancer are ever-growing and include tobacco smoking, dietary factors, obesity, diabetes, aflatoxin and certain diseases such as Wilson’s disease (Chuang et al., 2009; Wu and Santella, 2012). This list of factors has received much focus for researchers looking at preventative measures, yet an emphasis is still required to treat those who are afflicted with the malignancy.

1.1.1 Diagnosis of HCC

One of the biggest issues involved with the treatment of HCC is the diagnosis of the disease at an early stage. Table 1.1 illustrates the TNM classification of malignant tumours, showing how physicians classify which stage the cancer in a patient has reached. Using the table below, a physician will class the patient’s cancer, dependent on the number of tumours i.e. T1 to T4, and if there has been invasion of the lymph nodes. The later the stage the cancer is diagnosed, the less chance there is of survival for the patient.
<table>
<thead>
<tr>
<th>Primary Tumour ($T$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
</tr>
<tr>
<td>T0</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T3a</td>
</tr>
<tr>
<td>T3b</td>
</tr>
<tr>
<td>T4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional lymph nodes ($N$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant metastasis ($M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
</tr>
<tr>
<td>M1</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Stage Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
</tr>
<tr>
<td>Stage II</td>
</tr>
<tr>
<td>Stage IIIA</td>
</tr>
<tr>
<td>Stage IIIB</td>
</tr>
<tr>
<td>Stage IIIC</td>
</tr>
<tr>
<td>Stage IVA</td>
</tr>
<tr>
<td>Stage IVB</td>
</tr>
</tbody>
</table>

Table 1.1. Cancer staging system for the classification of malignant tumours of the liver used by physicians to determine the treatment and possible prognosis of the patient ($T =$ size of tumour and whether it has invaded nearby tissue, $N =$ regional lymph nodes involved, $M =$ distant metastasis) (Wagman et al., 2011).

With liver cancer, there is frequently a time delay in diagnosis as the organ maintains its full function even when partially damaged (Lin, 2009). The prognosis of untreated HCC is very poor. According to Okuda classification, patients at stage 1 of the disease have a life span of 11.5 months, 3 months at stage 2 and 0.9 months at stage 3 (Dumortier et al.,...
Early diagnosis is of paramount importance not just because it allows treatment to preserve life but it also opens the treatment options available to the patient. If caught at an early enough stage, the patient should be suitable for curative treatments rather than palliative treatments.

1.2 **Current Treatments of HCC**

1.2.1 Curative: Surgical Techniques for the Treatment of HCC

1.2.1.1 **Surgical Resection**

The best current treatment option for a patient with HCC involves the application of surgery (Marrero, 2013). Surgical resection is seen as a curative therapy where the tumour is removed from the liver with some surrounding healthy liver, leaving enough of the liver to maintain normal function (Guglielmi *et al.*, 2008). This procedure is possible due to the regenerative ability of the liver’s hepatocyte cells.

The selection of candidate patients for such a regime is defined by the guidelines set out by EASL (European Association for Study of Liver) and the AASLD (American Associations for Study of Liver Diseases) (Capussotti *et al.*, 2009). Patients that meet the criteria can expect to see survival rates of 60-70% after 5 years. However, it was widely believed that carrying out resection on patients that do not fit the criteria would lead to a reduced prognosis even below the natural history of the disease (Llovet *et al.*, 2000). These criteria were developed by the Barcelona Clinic Liver Cancer group, however, the results have never been confirmed (Jaeck *et al.*, 2004). Studies have been carried out on patients with large HCC which concluded that it became obvious that “huge HCC” was a far more aggressive tumour and had a worse prognosis than small tumours (Choi *et al.*, 2009). However, with the introduction of new surgical techniques, and care taken to avoid tumour spillage into systemic circulation, it seems the outcome with resection is far better than the outcome with nonsurgical treatments (Choi *et al.*, 2009; Capussotti *et al.*, 2009).

1.2.1.2 **Liver Transplantation**

Liver transplantation is another viable option as a curative treatment for HCC and is governed by the guidelines set out in the Milan criteria. The Milan criteria states that
transplantation is only an option if a single tumour is ≤5 cm in size or up to 3 tumours each ≤3 cm in size and have no macro vascular invasion (Jean-François and Philip, 2010). If patients meet these criteria then liver transplantation would be the preferred choice of treatment among early HCC patients. The initial results of orthotopic liver transplantation (OLT) not using the criteria were actually quite disappointing with high recurrence and low survival rates. However with the criteria in place, patients can expect to see 5 year survival rates of 70% (Llovet et al., 2000).

These results are strictly based on short waiting times for liver transplantation (≤ 6 months). As the incidence of HCC increases, so does the demand on the already scarce amount of donor livers available, increasing the waiting period dramatically. In 1 year an untreated tumour has a 70% probability of tumour spread, vascular invasion of 21% and 9% spread of extrahepatic tumours (Llovet et al., 2000). This means that not only will the patient have a decreased chance of survival but at this point the patient is no longer eligible for a transplant. A study of the data showed that for the patients that did experience a transplantation outside of the Milan criteria, although they experienced better results than expected, there was still a direct correlation between increasing HCC size with recurrence and risk of dying (Mazzaferro et al., 2009).

It does seem that the majority of patients are not eligible for a transplant nor do they qualify for surgery, and at this stage there is no standard set treatment. The therapy chosen is currently based on the clinician’s judgement; who will compare the effectiveness of treatment methods while also being guided by patient and institutional factors (Belinson et al., 2013).

1.2.2 Curative: Non-Surgical Therapies for HCC

No tumour classification model has received universal acceptance but the Barcelona Clinic for Liver Cancer (BCLC) staging system seems to be applicable to the majority of HCC patients (Fig. 1.3). The Barcelona Clinic staging system is widely used after a patient has been diagnosed with cancer and the stage of the tumour has been classified. Once the stage of the tumour is known then an algorithm such as the one below can be applied to determine the potential treatments available and most beneficial.
Fig. 1.3 A liver cancer staging system formulated by the Barcelona Clinic Liver Cancer group used to determine the treatment of a patient suffering from HCC. Image courtesy of Biocompatibles UK Ltd. (*PST* = performance status 0-4, *DDLT* = Deceased donor liver transplantation, *LDLT* = Living donor liver transplantation, *OS* = Overall survival).

If patients do not meet the criteria for resection, then there are other curative therapies that can be applied as summarised in Table 1.2. These therapies are non-surgical and their use is becoming increasingly important to treat patients that do not meet the criteria for surgery. These treatments are constantly being optimised with the use of better materials and technology such as new drugs or better equipment.
<table>
<thead>
<tr>
<th>Therapy</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotherapy</td>
<td>The delivery of cytotoxic drugs to interfere with the growth of cancer cells with the intention of killing the tumour. The drug can be administered either systemically or locoregionally with the latter producing better results (Cheong <em>et al.</em>, 2005). Various drugs can be used but recently there has been a return to platinum based agents with promising results (Okuda <em>et al.</em>, 2009). New drugs such as Sorafenib are being evaluated (Llovet <em>et al.</em>, 2008). Side effects can include vomiting, alopecia, and cardiac and liver damage (Buckman <em>et al.</em>, 2010; Monsuez <em>et al.</em>, 2010).</td>
</tr>
<tr>
<td>Percutaneous Ethanol Injection (PEI)</td>
<td>Tumours are very sensitive to alcohol and can be destroyed by directly injecting the cancerous cells with alcohol (Shiina <em>et al.</em>, 2001). The alcohol is toxic to the cells but also kills the cells by dehydrating them (Shiina <em>et al.</em>, 2001). It is a very effective method for the patients that meet the criteria with 80% of patients expecting a complete tumour response, and even better survival and fewer sessions are required with the use of Percutaneous Acetic Acid injection (Cheng <em>et al.</em>, 2008; Germani <em>et al.</em>, 2010). If the tumour diameter is too large there can be a high number of recurrences due to the limited spread of ethanol in the lesions (Shiina <em>et al.</em>, 2001) Multiple lesions are also very difficult to treat.</td>
</tr>
<tr>
<td>Radiofrequency Ablation (RFA)</td>
<td>A thermal ablation technique that relies on the interaction of high frequency alternating current with living tissue and generates heat <em>via</em> ionic vibrations (Ng and Poon, 2005). At temperatures over 60°C intracellular water is evaporated, proteins coagulate leading to irreversible damage of key enzymes and finally coagulative necrosis of the target lesion (Ng and Poon, 2005). Surgery still benefits cancer patients more in terms of survival (Delis <em>et al.</em>, 2009), however RFA is not far behind (Wong <em>et al.</em>, 2009). A major drawback is the pain that the patient endures therefore they must be sedated, and this method is only used to treat smaller tumour nodules ≤ 3cm. Difficulty accessing deep tumours close to blood vessels sometimes rules out the percutaneous approach and this is why an open procedure has been suggested (Delis <em>et al.</em>, 2009).</td>
</tr>
<tr>
<td>Cryotherapy</td>
<td>A treatment which freezes the tumour instead of heating it. Liquid nitrogen is inserted into the lesion <em>via</em> a hollow instrument called a cryoprobe which is inserted either by percutaneous or laparoscopic techniques (Gage and Baust, 2007). The tumour is then frozen in cycles with the initial freeze numbing the nerves so the patient feels very little discomfort (Koyyalagunta and Mazloomdoost, 2010). The longer the freezing the larger the destructive effect with the formation of ice crystals which on thawing disrupt the tissue and create a shearing effect (Gage <em>et al.</em>, 2009). Repeating this process leads to larger ice crystals and the spread of temperature further through the tumour (Hinshaw and Lee Jr, 2007). Due to the ability to treat larger tumours, this technique could find more use than RFA with similar survival rates. The drawback with this technique is that there can be blood coagulation from depressed platelets and in rare cases there has been multiorgan failure due to cytokine release (Gage <em>et al.</em>, 2009).</td>
</tr>
</tbody>
</table>

Table 1.2. A list of non-surgical curative therapies that can be used for the treatment of HCC patients with early or intermediate stage cancer. These treatments can also be used in conjunction with surgical techniques to minimise the recurrence of a tumour.
From the evaluation of these curative methods, surgery still remains the gold standard for HCC patients; for those individuals who fall short of the required surgical criteria, there is however a list of alternative options available for them albeit not necessarily as effective as surgery. If the patient’s cancer is too far advanced for curative treatment, the only remaining options are conservative management or palliative care.

1.3 Palliative Therapies for Patients with Unresectable HCC

Palliative care is a form of treatment with the main aim of reducing the severity of the disease symptoms instead of trying to cure the disease. This form of intervention will attempt to improve the quality of life and extend the amount of time the patient has to live (Fadul et al., 2009). This differs from conservative management which centres on pain relief, the quality of life of the patient and the amount of time they have left.

Palliative care is becoming frequently used in the treatment of cancer in conjunction with curative therapies. The treatments are becoming more and more efficient in extending the patient’s life to the extent that some of the treatments have potential to return the patient back to the stage of having curative treatments. Given the focus of this thesis, this review will focus on treatments that involve embolisation.

1.3.1 Transarterial Embolisation for the Treatment of HCC

As mentioned in section 1.1, the liver receives its blood supply from the portal vein and the hepatic artery, providing 75% and 25% respectively. When a malignancy forms, it receives at least 80% of its blood supply from the hepatic artery (Zhang et al., 2009). A simple procedure has been devised whereby the main vessels supplying the tumour are occluded in an attempt to starve the growing tumour of necessary nutrients, leading to ischemia induced necrosis. This occlusion in oncological terms is called transarterial embolisation (TAE) in which embolising particles are placed by an interventional radiologist in the blood vessels feeding the malignant tumour (Fig. 1.4). This procedure has long been in use for palliation of patients with liver cancer for whom curative treatments are not an option and has now become the recognised standard in loco-regional treatments for unresectable HCC (Forner et al., 2012).
There are two main categories of embolising material that can be applied in the procedure. They are either materials that are used for a transient occlusion which is described in Table 1.3 or material used for a permanent occlusion described in Table 1.4.

Fig. 1.4 A solid malignant tumour having its blood vessels embolised using the technique TAE. The catheter has been placed as close to the tumour as possible, which is followed by the release of the blue embolising material which travels to the blood vessels connected to the tumour and ultimately occlude the vessels. Image courtesy of Biocompatibles UK Ltd [Original in colour].
**Transient Occlusion**

<table>
<thead>
<tr>
<th>Description</th>
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<tbody>
<tr>
<td>Gelatin Sponge Particles</td>
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<tr>
<td>Degradable Starch Microspheres (DSM)</td>
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</tbody>
</table>

Table 1.3 A description of well-known and commonly used materials for transient occlusion of a blood vessel supplying a tumour.

The aim of embolisation is to occlude the vessel to produce ischemic cell death. For this a terminal vessel blockade is imperative to maximise the effects of ischemic-induced necrosis (Brown, 2008). This suggests that materials such as Gelfoam and DSM have limited utility in TAE as occlusion will be temporary and the tumour will not be effectively treated as well as it could be by using a permanent occluding material. In fact, a material such as DSM will have no necrotic effect on the tumour and might better be combined with other forms of therapies. In support of this belief, numerous researchers have turned to permanent occluding materials with examples shown below in Table 1.4.

Also, with a possibility of causing a too proximal or distal occlusion due to the uncertainty in the size of products such as Gelatin particles, devices have been synthesised to exact sizes to provide a more targeted occlusion avoiding any unnecessary complications.
<table>
<thead>
<tr>
<th><strong>Transient Occlusion</strong></th>
<th><strong>Description</strong></th>
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<tr>
<td><strong>Stainless steel coils</strong></td>
<td>A very effective form of TAE for the past 30 years where the coil is formed by using inch guidewire which has its innercore removed and formed into a helix (Rose, 2009). The coils usually target medium to large arteries and can be calibrated to fit the target vessel. It is the attachment of wool strand to the coils which causes the thrombosis and occlusion (Rose, 2009). A negative point is that there have been reports of insecure attachment which has led to procedure related complications (Miller and Thomas, 2009).</td>
</tr>
<tr>
<td><strong>Liquid embolics</strong></td>
<td>A liquid embolic agent called Onyx, made of ethylene vinyl alcohol copolymer dissolved in an organic solvent (dimethyl sulfoxide (DMSO)), is often used and when it comes into contact with aqueous solutions a precipitation occurs forming a spongy soft polymer cast (Abath et al., 2007). Another material with the same action is N-butyl cyanoacrylate (n-BCA or medical grade super glue). Although good results are obtained there is a difficulty in avoiding leakage of the liquid which could potentially mean unwanted occlusions (Abath et al., 2007).</td>
</tr>
<tr>
<td><strong>Poly(vinyl alcohol) (PVA)</strong></td>
<td>PVA is a water soluble synthetic polymer with film forming and adhesive properties. Due to its cost effectiveness and low cytotoxicity it has been widely used as a permanent embolisation material in the treatment of HCC for the past 30 years (Derdeyn et al., 1995). PVA is also beneficial in that it can occlude large vessels as it has the ability to aggregate. At first PVA was used in particle form, and due to varying shapes targeted embolisation was deemed difficult (Derdeyn et al., 1995). However, over the past decade PVA embolisation has become the subject of much interest with the ability to now precisely calibrate PVA into microspheres (Laurent, 2007). This allows a targeted occlusion and application of the microspheres has been shown to be a rapid, minimally invasive procedure which leads to an ischemic induced necrosis of tumours (Brown, 2008). PVA particles have been shown to induce high levels of ischemia but carry a risk of haemorrhagic events, while the microspheres are much safer and still produce similar levels of ischemia (Laurent, 2007).</td>
</tr>
</tbody>
</table>

Table 1.4. A description of well-known and commonly used materials used for the permanent occlusion of a blood vessel supplying a tumour.
1.3.1.1 Transarterial Embolisation Clinical Results

Considerable focus has been given to TAE of HCC and it has revealed that a partial response of 15-55% is achieved in patients which substantially offsets tumour progression and vascular invasion (Llovet et al., 2003). A meta analysis of randomised clinical trials (RCTs) between 1978-2002 included a total of 516 patients and the meta analysis showed a beneficial survival effect of embolisation with a drug over the control group of conservative management, but not embolisation alone (Llovet and Bruix, 2008). Despite this, and the limited number of RCTs over the past 30 years, many clinicians advocate the use of TAE alone and believe it to be a highly effective treatment regime. It would appear that more clinical trials are required to try and further investigate the effects of TAE alone in comparison to conservative management, in order to establish whether there is a survival benefit. This is not to say there have not been a lot of positive results from independent studies. Many have been carried out with bland embolisation which has shown improved survival (Maluccio et al., 2006). Recent studies and evaluation for 322 HCC patients that underwent embolisation between 1997 and 2004 showed median survival times of 21 months with 1, 2 and 3 year overall survival rates of 66%, 46% and 33% respectively which was significantly increased to 84%, 66% and 51% respectively when there was no extra hepatic disease (Maluccio et al., 2008).

This corroborative evidence does push the claim that TAE is highly beneficial in the treatment of HCC, and could very well have a significant survival benefit. The requirement for further research and clinical trials is paramount to confirm these findings and further improve the established treatments. There are some drawbacks however, and over the years other procedures utilising embolisation have evolved in the treatment of HCC to avoid tumour recurrences.

1.3.1.2 Transarterial Chemoembolisation

Treatment of HCC is an area of active research and in recent years has witnessed the emergence of Transarterial Chemoembolisation (TACE). TACE is the combination of an embolising agent, and a chemotherapeutic agent which is mixed with a contrast agent. This technique exposes the tumour to the same effect as experienced with TAE, but with the added enhancement and benefit of the localised toxic effects of the drug. This should lead
to a more efficient and powerful attack on the tumour, with more tumour being destroyed and the patient having an enhanced prognosis over that expected with TAE alone. In cases where there has been a spontaneous tumour rupture (a life threatening complication of HCC), TACE has even been shown to offer increased survival benefits (Kim et al., 2012). Fig. 1.5 below demonstrates how the TACE procedure is used as a locoregional therapy to prevent high levels of chemotherapeutic drug being found in other areas around the body.

Fig. 1.5 Illustration of the TACE procedure, whereby a catheter is guided through the aorta into the hepatic artery. Once close enough to the tumour site the chemotherapeutic agent along with an embolic agent is released leading to tumour necrosis and reduction in tumour volume. Image courtesy of Biocompatibles UK Ltd. [Original in colour]

Orthotopic liver transplantation (OLT) is the best treatment for a patient with HCC. A patient however, is not always eligible for a transplant, either due to the size of the tumour or they are deemed eligible for the procedure but are put on a waiting list due to the sheer demand and shortage of available donors. The excellent results obtained by OLT are being hampered by the increasing waiting times which leads to tumour progression and there are reports of 20% drop out rates (Graziadei et al., 2003). Several therapies have been applied to prevent this tumour progression and one of those is TACE, which has
shown excellent results in a limited number of patients (Graziadei et al., 2003). TACE has now become a popular form of interventional therapy (Sacco et al., 2009). As a consequence of these positive results, TACE has now been recognised as a bridge therapy in liver transplantation in the hope it will prevent and decrease the dropout rate (Heckman et al., 2008).

1.3.2 Chemotherapeutic Drugs

The most common anticancer drug used in the treatment of HCC is doxorubicin hydrochloride (Dox) (Fig. 1.6) with it being used in 36% of HCC cases, closely followed by cisplatin (31%). Other drugs used in the treatment of HCC include epirubicin, mitoxantrone, mitomycin C and styrene maleic acid neocarzinostatin (SMANCS) (Pleguezuelo et al., 2008).

Fig. 1.6 The chemical structure of Doxorubicin hydrochloride (Dox).

The aforementioned drugs can be given in TACE either as part of a single therapy or as part of a combination therapy in a bid to enhance the cytotoxic effects. However, no RCT to date has shown the superiority of one drug over another although a non-randomised clinical trial did show that cisplatin is potentially more effective at treating liver cancer than doxorubicin (Pleguezuelo et al., 2008). There is also no evidence to suggest that combination therapy is better than monotherapy in terms of clinical performance (Seinstra et al., 2010).
1.3.3 Microspheres and Drug Eluting Beads

1.3.3.1 Current Microsphere Formulations

Microspheres are a more appealing option than particles as an embolising agent based on their safety and efficacy (Laurent, 2007). As microspheres can be calibrated to a desired size, accurate targeting of vessels can be achieved and studies demonstrate that microspheres reduce blood flow in vessels quicker and more reliably than particles (Andrews and Binkert, 2003). Microspheres allow more distal penetration of vessels than particles, with particles known to aggregate due to their irregular shape and size. Microspheres can be consistently administered allowing accurate placement, with a wide variety of calibrated microsphere sizes available to target the diameter size of the target vessel (Pelage et al., 2002). An additional benefit of microspheres is that any potential blockage in the lumen of the catheter is minimised, as microspheres have been designed to be compressible and will deform during delivery, but in the absence of any pressure, return to full size undamaged (Lewis et al., 2006a).

With spherical shape, smooth surface and superior performance, microspheres have revolutionised the field of embolisation. So much so, that it has been predicted that in the near future microspheres could completely replace non-spherical particles for embolisation (Laurent, 2007).

Currently there are four main microsphere embolics that are commercially available. The first commercially available was Embosphere ™ (Merit Medical), followed by Contour SE™ (Boston scientific), BeadBlock® (Biocompatibles) and finally Embozene™ (Celanova) (Lewis et al., 2006). There is a variant formulation of Embosphere called Embogold™, which is essentially still the same microsphere as Embosphere but treated with a gold colloid to give a red colouration and ease visualisation of the product (Lewis et al., 2006).

Although these four main competitors’ products share similar shape and size ranges, they have different compositions and therefore they have slightly different mechanical properties (Laurent, 2007). Fig. 1.7 describes the different formulations used to make each product.
Fig. 1.7 A schematic of four commercially available microspheres has been drawn with each microsphere containing its component followed by a description of the method of production. [Original in colour]
The formulations in Fig. 1.7 are based on the information provided in the available patents, but it must be noted that none of the patents directly mention the names of the product. It is assumed, based on the description in the patents and compared against the available literature, that these patents match the products.

Although the microspheres are produced in different manners, they are created for the same purpose. Compression testing of the microspheres shows there are considerable differences between the products with Embosphere having a rigidity of 12 g at 70% compression, with Bead Block being similar with a slightly lower rigidity of a few grams (Lewis et al., 2006a). Contour SE is different as it is very compressible and can flatten with no resistance at low compression forces (Lewis et al., 2006a; Seron et al., 2005). The elastic recovery after compression for Embosphere and Bead Block is instantaneous, while Contour SE takes a few seconds and never fully reforms to its previous shape (Seron et al., 2005).

This demonstration of Contour SE being less elastic than Embosphere and Bead Block, is due to the composition of the beads and how they are manufactured (Fig.1.7). Embosphere and Bead Block are indicative of polymeric hydrogels which can be compressed without releasing water, while Contour SE mimics the actions of a macroporous sponge and as such loses water on compression (Laurent, 2007). The latest addition to commercially available microspheres for embolisation is Embozene and due to its hydrogel core should possess similar compressibility characteristics to Embosphere and Bead Block. The polyzene-F shell has been intensively investigated in the last few years, and in areas such as vascular stent coatings, has shown good biocompatibility (Stampfl et al., 2008).

Comparative studies of the microspheres have shown that with the same sizes Contour SE has a much more distal occlusion and obvious deformation within a sheep kidney, than Embosphere (Laurent et al., 2006). Bead Block also occluded slightly more distally than Embosphere, but the distance travelled was not as pronounced as that of Contour SE (Namur et al., 2005). These results are further supported by a study comparing all four embolics and showed that Embosphere is not as compressible as Bead Block and Embozene, which occludes vessels close to their nominal size (Stampfl et al., 2009; Tam et al., 2011). Embozene was found to be quite the opposite, with low compression resistance and high viscosity yielding a deformed bead under stress that takes time to return to its original dimensions (Hidaka et al., 2011). In the same study, Bead Block was found to be an intermediate between the two, with good elasticity and resistance.
A clinical study comparing Embozene and Embosphere in sheep uterine arteries noted that Embozene produced an inflammation response that led to partial degradation of the microsphere, with signs of re-canalisation, whilst embosphere is not degradable and due to these different properties there are differing inflammation reactions (Verret et al., 2011). In a clinical study, a comparison of Contour SE and Embosphere showed the latter having significantly better results, with patients treated with Contour SE having higher rates of partial devascularisation and clinical failure of uterine artery embolisation for fibroids (Spies et al., 2005). These results suggest that Embosphere and Bead Block are superior embolising agents with larger Contour SE microspheres needed to occlude the same size vessels (Lewis et al., 2006a).

Microspheres and products such as Embosphere and Bead Block have now been set as the benchmark for permanent embolisation. The smaller size ranges (100-300 µm) seem to produce higher responses in HCC patients and are now more favoured among clinicians (Amesur et al., 2008). The results from trials further cement the microspheres credentials as a superior embolic over the use of particles (Scheurig-Muenkler et al., 2010).

More frequently over the past few years the use of microspheres have been investigated in conjunction with a chemotherapeutic agent as a form of TACE treatment with improved results over other embolic agents used in TACE (Scheurig-Muenkler et al., 2010; Ball et al., 2003; Gomes et al., 2009; Burrel et al., 2012). This desire to combine the best embolic agent with an ever improving method to target tumours with drug has led to the synthesis of Drug Eluting Beads (DEB).

### 1.3.3.2 What are Drug Eluting Beads (DEB)?

Conventional TACE is a multi-step process where a chemotherapeutic drug is directly injected into the blood vessel supplying the tumour. The drug can also be mixed with a contrast agent for better imaging. After the drug has been injected, an embolising agent is quickly put in place behind it to occlude the vessel and to trap the drug close to the tumour.

DEB-TACE is a one step process as the chemotherapeutic drug is already held within the embolising agent, therefore the clinician has the ability to administer the drug and embolising agent much quicker and at the same time (Lencioni, 2012). DEB is the optimisation of the microspheres discussed in 1.3.3.1 but now with the added ability to take up drug and elute drug. DEB are able to offer a proximal or distal occlusion,
dependent upon the size of the bead, which is brought about with the ability to precisely calibrate the size of the beads in the manufacturing process (Lewis et al., 2006d). With DEB-TACE combining the benefits of calibrated microspheres with significant drug uptake, then this form of embolic therapy has the potential to surpass other previous embolisation procedures and become the benchmark for all future research into chemoembolisation.

DEB have been designed to uptake drug and release it in a slow and predicted rate. They are administered in the same manner as conventional TACE with more accurate drug delivery and less dispersion of the drug around the body (Reyes et al., 2009b; Varela et al., 2007; Lewis and Holden, 2011).

1.3.4 Current Commercially Available DEB Formulations

Bead Block has been shown to be capable of acting as a drug delivery device (Laurent, 2007). Bead Block has been loaded with the anti-inflammatory drug Ibuprofen with the intention to help to ease the pain associated with TAE (Borovac et al., 2006). The preliminary results showed that Bead Block was able to be loaded with the drug, and demonstrated prolonged release lasting a week after the initial embolisation with low plasma levels of the drug around the body (Borovac et al., 2006; Wassef et al., 2008). Bead Block has also been shown to load minimal amounts of Dox, however Bead Block is not designed and is not approved for the use in patients as a DEB and is only administered as an unloaded embolic agent.

There are three DEB’s that have been approved in Europe and the U.S.A for the treatment of HCC. The microspheres that are available for clinical use in Europe are HepaSphere® (Merit Medical), DC Bead® (Biocompatibles, UK Ltd) and Tandem® (CeloNova Biosciences) (Malagari, 2008; Glynos and Malagari, 2014). The same microspheres are also cleared for use in the U.S.A but under different names. DC Bead is known as LC Bead®, HepaSphere is known as Quadrasphere® and Tandem is known as Oncozene® in the U.S.A with all products cleared as an embolising agent for treating hypervascularised tumours, but not with the addition of drug (Glynos and Malagari, 2014; Jordan et al., 2010). To avoid confusion, the available DEB will be referred to here by their European approved titles.
1.3.4.1 DC Bead Formulation

DC Bead is a hydrogel, with 95% of its weight made up by water (Lewis et al., 2007). Interestingly the components used to produce DC Bead are exactly the same as that of Bead Block. DC Bead is composed of a functionalised PVA macromer (400 g 21 w/w%), 2-acrylamido-2-methylpropanesulfonate sodium salt (AMPS) (140 g 50 w/w %), purified water (137 g), potassium persulfate (5.22 g) and N,N,N’,N’-tetramethylethlenediamine (TMEDA) (6.4 g) as the aqueous phase. The aqueous phase is added to an organic phase of n-butyl acetate (2.7 L), Cellulose Acetate Butyrate (46 g w/w 10%) and purified water (19 ml) (Lewis et al., 2006b).

1.3.4.2 HepaSphere Formulation

Although a commercially available product, the exact composition and formulation of HepaSphere is unknown and is protected by Merit Medical™ as a trade secret. What is known is that HepaSphere is a copolymer of acrylate and PVA (de Luis et al., 2008). The original formulation of HepaSphere was designed by Hori and colleagues, which was later purchased by Biosphere Medical (Gupta et al., 2011; de Luis et al., 2008). It was Hori and his team that carried out the early characterisation of the beads (Jiaqi et al., 1996; Osuga et al., 2002a; Osuga et al., 2002b). However, before the work carried out on this superabsorbent polymer microsphere, Hori was working on hepatic artery embolisation with another freely available superabsorbent polymer (SAP) named Sumikagel (Inoue et al., 1990). Sumikagel is composed of a copolymer of vinyl alcohol and acrylic acid and also has many properties similar to Hori’s SAP. It could be interpreted that HepaSphere is based on Hori’s adaptations of Sumikagel. Theoretically the starting foundation of HepaSphere can be found in US patent 4320040, which is from the manufacturers of Sumikagel (Fujita et al., 1982). However as there is no patent directly relating to HepaSphere, it is possible that changes have been made to its manufacturing process since the earlier patent.

1.3.4.3 Tandem Formulation

Tandem is the latest potential DEB to be released in Europe in 2012. According to patent WO2009014549, the formulation composition is identical to Embozene, with additional...
cross-linking of poly(methyl methacrylate) (PMMA) (Fritz et al., 2009). Using the patent, Fig. 1.8 below has been drawn to illustrate the most likely method for bead synthesis.

![Diagram of Microsphere Formation Reactor and Microsphere Sieve Sizing]

**Fig. 1.8** A schematic illustrating the potential Tandem manufacturing process using literature provided in patent WO2009014549.

### 1.3.4.4 Comparison of DEB

DC Bead, HepaSphere and Tandem are intended for the treatment of hypervascularised malignant tumours and their application with Dox is permitted outside of the U.S.A. DC Bead binds Dox via a mechanism of ion exchange where an interaction takes place between the protonated primary amine group on the sugar moiety of the Dox and the negatively charged sulfonate group of the AMPS (Lewis et al., 2007; Malagari, 2008; Biondi et al., 2012).

Loading of Dox into HepaSphere and Tandem is due to the negative charge held by the sodium and potassium acrylate groups respectively and therefore both formulations also load and release drug via ion exchange, but unlike DC Bead it is the carboxylic acid groups which are responsible for binding drug (Blummel et al., 2012; Malagari et al., 2008). DC Bead loads efficiently with 99% of the Dox loaded within the first 2 hours and 95% loaded within the first hour (Lewis et al., 2007). HepaSphere will load all the drug.
within 1 hour with both products loading Irinotecan in a similar manner (Jordan et al., 2010). Tandem is reported to have faster loading rates with a higher known loading capacity of DC Bead (Blummel et al., 2012).

The products also behave differently when suspended in solution with DC Bead remaining as separate beads, while HepaSphere beads aggregate together forming clumps. Fig. 1.9 below is a picture of the products after loading with Dox, which highlights the aggregation of HepaSphere that consequently leads to the beads in the centre of the clumps being less amenable to loading. Therefore, while DC Bead and Tandem loading of Dox is homogenous, HepaSphere loading is not (Jordan et al., 2010; Eichler et al., 2012).

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Fig. 1.9 (A) The loading of DC Bead with Dox showing consistent loading (B) The loading of Tandem showing consistent loading (C) The loading of HepaSphere with Dox showing inhomogenous loading of Dox, shown by some beads having a dark red colour where maximum loading has taken place, while some beads remain white with very little loading. Images A and C used from Jordan et al., 2010. Image B is from internal data held by Biocompatibles UK Ltd [Original in colour]

Fig. 1.9 demonstrates a key difference in the properties of the three DEB with HepaSphere showing inhomogenous loading which could, in turn, cause difficulties in delivery of the product. Tandem has been reported to retain the same diameter after loading of Dox (Blummel et al., 2012). However when DC Bead and HepaSphere load Dox, a significant decrease in diameter of the beads is observed (Jordan et al., 2010; Biondi et al., 2012; Liapi and Geschwind, 2011).

With homogenous loading, the change in diameter of loaded DC Beads should be similar throughout the population of microspheres. The decrease in diameter when loading DC Bead is caused by the drug displacing the water within the hydrogel with no more than 20% of the water displaced from the bead (Malagari, 2007). The shrinkage is also linked to
the neutralisation of the binding charges which leads to less repulsion of polymer segments, hence exclusion of water. With this loss of water the diameter of beads shrink and they become less compressible, potentially hindering the delivery of the beads through the lumen of a catheter (Lewis and Dreher, 2012). However, Lewis et al (2007), demonstrated that the loaded beads were still deliverable and no obvious aggregation was observed.

HepaSphere does not load homogenously at the same dose as shown in Fig. 1.9. This will lead to a wide distribution in the size of the microspheres meaning an inefficient embolisation of the target vessel. This represents a flaw in the concept of a reproducible and consistent product used as a DEB.

Jordan (2010) also mentions the difficulty associated with the handling of HepaSphere due to the aggregation of the DEB. Fig. 1.10 highlights again the differences in the loading of the beads and eventual difficulty in handling of HepaSphere due to the aggregation of the beads.

Fig. 1.10 (A) An image of DC Bead loaded with Dox with small quantities of beads stuck to the glass. (B) An image of HepaSphere loaded with Dox, demonstrating the beads forming large aggregates which stick on the glass and are buoyant in solution. [Original in colour].
Further work with HepaSphere showed that the DEB gather on the glass and the neck of the vial with its preparation proving inconsistent (Internal Data of Biocompatibles UK Ltd). Jordon (2010) highlights that attempts to separate the microspheres by agitation just led to the formation of more aggregates.

An explanation of this aggregation is offered by Liu (2011), who shows that HepaSphere does not aggregate at lower Dox loading concentrations. Potentially the cause is linked to high concentrations of Dox which is known to self-associate at high concentrations (Liu et al., 2011). Additionally, Dox has a low solubility at physiological pH which subsequently leads to an increase in viscosity at high concentrations meaning that administration of this product may be affected (Liu et al., 2011).

A comparison of the release of Dox in saline solution (0.9%) from the DEB’s showed that they had similar release kinetics although DC Bead released larger quantities of drug as a percentage of its total loading and maintained this elevated percentage of drug release for over 3 days (Jordan et al., 2010). The reproducibility of the release from HepaSphere is questionable and should be further examined because inhomogenous loading should directly correlate to inhomogenous release of drug from the beads. Recent investigations into Tandem eluting capabilities has shown that it is unable to fully release the drug with less than 40% released over a period of 3 days (Internal data of Biocompatibles UK Ltd). As Tandem is a relatively new device, there is very little literature pertaining around its drug eluting capacity, however the inability to release the payload is not a desirable trait for a drug delivery device.

During release, DC Bead maintains its spherical shape, as does Tandem, however HepaSphere is known to deform and there have been reports of damaged microspheres with broken sphere and polymer pieces (Jordan et al., 2010; Liu et al., 2011).

A final comparison of the products involves an investigation of their embolising capabilities. DC Bead has been shown to successfully embolise target vessels and does this by travelling down a narrowing vessel where eventually numerous beads meet in the tightening space and aggregate (Malagari, 2008). It is essential that the exact size of the beads is known before-hand and this is why homogenous drug loading is necessary.

This comparison highlights the crucial differences between the existing DEB formulations available on the market. The results show that DC Bead and Tandem are superior to HepaSphere in terms of consistency, reproducibility and handling of the product. However, Tandem requires further characterisation studies to be able to comprehensively compare it to the other DEB formulations. In addition, results from a
multicentre study has also shown DC Bead to produce higher tumour responses than HepaSphere (Grosso et al., 2008). Therefore for the purposes of this thesis, as the product has been so extensively studied and used worldwide and with characterisation data so widely available, DC Bead will be the focus DEB formulation providing insight into desirable characteristics of microspheres. The studies with DC Bead will also yield the effectiveness of such products into the treatment HCC and will highlight gaps that can be exploited.

1.3.5 Elution of Drug from DC Bead

It is known that DC Bead loads drug through a mechanism of ionic exchange which can be seen in Fig. 1.11 (Biondi et al., 2012).

Fig. 1.11 A schematic showing the loading of DC Bead. On the left the bead is hydrated in saline solution. The bead on the right shows the interaction of Dox with the ionic groups causing a shrinking of the bead by the displacement of water. [Original in colour].

This mechanism of loading is also responsible for the release of drug from the beads and its rate of release is dependent on the presence of ions in the surrounding media (Gonzalez, 2006; Lewis and Holden, 2011). This loading and release data provides
information on a standardised method for the treatment of HCC. The most important point is that these results are reproducible and can be used in clinical trials for further analysis. With the discussed benefits of DC Bead in section 1.3.4.4, it is assumed that better survival benefits might be achieved over previous embolisation techniques when factoring in its drug loading and elution properties. Due to its characteristics, DC Bead has now been extensively evaluated \textit{in vivo}, with a range of models and centres used (Bower et al., 2010; Won et al., 2013a; Namur et al., 2014; Bize et al., 2013).

1.3.6 DC Bead: \textit{In vivo} Studies

Animal studies have demonstrated the efficacy of DC Bead with the results supporting the findings of \textit{in vitro} characterisation studies. Hong et al., (2006), reported the effects of DEB loaded with Dox in a Rabbit (VX-2) model which showed that the greatest concentration of drug within the tumour was achieved on the third day post treatment and remained high until 7 and even 14 days. These results suggest a continuous elution of drug, therefore DEB results were compared against data from rabbits that received drug \textit{via} intra-arterial administration. The study showed that while high levels of Dox was found with the use of DEB, it was not the case with rabbits that received drug \textit{via} intra-arterial administration, with the level of doxorubicin within the tumour being almost undetectable (Hong et al., 2006).

The systemic level of Dox revealed that drug levels were significantly lower in the DEB rabbit model compared to other methods. These results demonstrated that the half-life of Dox release from DEB is between 60 – 100 days and at 3 days post treatment the levels of Dox within the tumour were 400% higher than treatment with conventional TACE (Hong et al., 2006). This is supported by the results of an animal study where the high local levels of Dox were maintained for 90 days post treatment in Yucatan pigs (Lewis et al., 2006c). Interestingly the peak concentration of Dox at the tumour site was 15 times higher when using small beads compared to large beads even though the dose was only 1.5 times greater in the small bead (Lewis et al., 2006c).

The results from the animal studies also reveal that DEB inflicts widespread pan-necrosis, with severe liquefactive and coagulative necrosis (Malagari, 2007). The results suggest that with DEB there is both an ischemic and toxic cause of the death, while bland embolisation leads to no hepatic necrosis but mostly non-necrotic vasculopathy (Lewis et
al., 2006c). It is the cellular necrosis that also aids in the diffusion of doxorubicin. The author also demonstrates that it is the smaller bead range that yields the most necrotic results (Lewis et al., 2006c).

These results demonstrate the potential of chemotherapy for the treatment of tumours in combination with embolising microspheres and the added benefits of combining the two for DEB-TACE treatment. Clinical studies support the same results within humans:

In a Phase I/II clinical trial, Varela and co-workers (2007) showed that with the use of Dox loaded DC Bead, patients had a tumour response rate of 75%, with a median 1 and 2 year survival rate of 92.5% and 88.9% respectively. However there was a limitation to the study as there was a small sample size (n = 27) (Varela et al., 2007).

A key study into the effects of DEB-TACE was carried out by Poon and colleagues (2007) in a phase I/II trial on patients with unresectable HCC. The study correlated with preclinical studies and showed that patients treated with drug loaded DC Bead had a low systemic exposure to Dox within normal tissue and there were no treatment related deaths. Using the modified response evaluation criteria in solid tumours (RECIST) which takes into account the tumour necrosis, it was shown that 63% of patients had a partial response, while 6.7% had a complete response post DEB-TACE treatment (Poon et al., 2007). These findings are corroborated by other studies including a single centre, single arm study in 62 cirrhotic patients (Malagari et al., 2008).

Further work has demonstrated the beneficial impact of drug loaded DC Bead in the treatment of HCC and secondary liver cancer (Cannon et al., 2012; Song et al., 2012; Skowasch et al., 2012; Won et al., 2013b; Fiorentini et al., 2012; Martin et al., 2012). One investigation has also highlighted an increased survival mean for HCC patients using DC Bead in comparison the recommended Sorafenib treatment with notably less side effects (Pinter et al., 2012)

Much of the data supports the use of DC Bead in the treatment of HCC with there being the highest tumour response and lowest systemic exposure to the Dox with this device (Lencioni et al., 2012). It is of interest that the DC Bead formulation is a slight alteration of the Bead Block formulation. This alteration demonstrates that by using the same materials but by altering their concentration within the formulation, the properties of the bead can be significantly altered. This is emphasised by the work performed by Heaysman (2009), who altered the amount of AMPS within DC Bead. AMPS formulation ranging from 0-100% AMPS formulation were used to produce beads which all had
different mechanical properties and drug loading capabilities (Heaysman, 2009). This ability to only slightly manipulate an existing formula, yet achieve vast differences in the performance of the beads yields the potential to make alterations to current formulations. This could be in order to optimise an existing formulation or to completely alter the purpose and properties of the bead. It would seem from the data that DC Bead, with its superior performance over all other formulations, would be the best starting point for the production of a new bead.

The new bead would be required to fulfil a purpose that is not being currently met by any existing formulations. The new bead would have to be novel, to fill a niche and have demand and purpose. It must also offer the potential to better treat sufferers of liver cancer and offer improved survival chances. It must also differ in chemistry and properties from existing formulations to avoid intellectual property issues.

Therefore with the extensive investigation of the all existing DEB products, it has been noted that none of them are bioresponsive. All formulations will release drug as part of an ionic exchange mechanism and this is largely independent of the environment they are within. However no formulation exists that can adapt to the ever changing and dynamic environment provided by a tumour, which is known to have the ability to resist certain treatments (Correia and Bissell, 2012). This presents the opportunity to produce a potential bioresponsive bead that is able to respond to this dynamic environment in a manner that impedes the tumours propensity to spread in spite of treatment. Therefore a study of tumour biology is needed to reveal what sort of stimulus could be used, and therefore how the chemistry of a bead must be altered to respond to the stimulus.

### 1.4 Tumour Biology caused by TAE

#### 1.4.1 Hypoxia

It is known that growing tumours have a lower requirement for oxygen than normal tissue, even in the presence of an abundant amount of oxygen. The current understanding of tumour hypoxia is that the hypoxic tumour cells present a fierce resistance against any form of treatment. Their attempt to survive is quickly followed by a multiplication of the cells which metastasise throughout the body (Brahimi-Horn et al., 2010).

Initially it was thought that as tumour cells proliferate their unrestrained growth would lead cells further away from the blood vessels that are supplying them with nutrients
and oxygen, and as a consequence they would die. This starving of the tumour of oxygen carries the same principles as TAE. It seems however, that as the cells get further away from the nutrient source, instead of cell death taking place, a mechanism is activated that supports cell survival and promotes migration to other parts of the body (Fig. 1.12).

Fig. 1.12 A schematic showing that as the oxygen levels supplying the tumour decrease consequently the presence of growth factors increases while the tumour also increases in size. Image adapted from Brahimi-Horn and Pouysségur (2007). [Original in colour].

Oxygen tension throughout the body varies within healthy tissue from anywhere between 20-120 mmHg, but when the levels are <7 mmHg the tissue is thought to be in severe hypoxia (Vaupel and Harrison, 2004; Vaupel et al., 1989). As the tumour microenvironment is so dynamic, different subpopulations of cells within the same tumour can be exposed to different concentrations of oxygen. Some cells can be exposed to acute hypoxia for a matter of minutes to hours, while some can be exposed to a more prolonged chronic hypoxia. Cells that fluctuate between a state of hypoxia and re-oxygenation are known to undergo intermittent or cycling hypoxia (Bristow and Hill, 2008). However, with the use of embolisation techniques, the cells in the tumour should be placed in a state of severe hypoxia or anoxia with the latter leading to cell death. Therefore the use of
embolisation will avoid placing the cells in a state of flux between high and low oxygen levels due to the restriction of the blood supply.

Without embolisation of a tumour it has been regularly noted that a necrotic core of cells form, which is thought to arise from low levels of oxygen (Brahimi-Horn et al., 2007). This shows that as the tumour grows, untreated, it has a good chance of becoming hypoxic with tumour growth resulting in the increased distance between the cells at the outer edge of the tumour and vascular supply. However, if the tumour is treated with an embolisation technique then there is a more probable likelihood that the tumour will become hypoxic with the vasculature supplying the blood vessel being occluded. In this blood and nutrient stricken state it is now known that the tumour cells have a reduced sensitivity to certain treatments such as radiotherapy and chemotherapy: this is followed by the activation of certain mechanisms that lead to revascularisation of the tumour allowing it to receive nutrients, but also providing a route for the tumour cells to metastasize into other regions of the body (Semenza, 2013).

When HCC is treated with these embolisation techniques, if there is not a complete response, then the remaining tumour cells with very low oxygen concentrations may have the potential to become severely hypoxic and as a result this can lead to the negative consequences described (Xiao et al., 2009). This phenomenon of hypoxia could contribute to the large number of recurrences after treatment (with TAE or TACE) favouring cell survival and migration, and the large mortality rate incurred by HCC sufferers. A further understanding of this aggressive cell survival mechanism is warranted to help elucidate potential future targeting of hypoxic tumours.

1.4.2 Hypoxic Cell Metabolic Shift and Hypoxia-Inducible Factors

Otto Warburg (1956) hypothesised that rapidly proliferating tumour cells consume glucose at a surprisingly fast rate compared to normal cells, metabolising the glucose through an up regulated glycolytic pathway with an accompanying increase in lactate production (Hsu and Sabatini, 2008). What is now gathering recognition is that when cancer cells are placed in hypoxic conditions the rate of glycolysis increases even faster with a stronger emphasis on anaerobic respiration and an increased consumption of glucose compared to normal cancer cells (Parks et al., 2013). This shift in metabolism is supported in hypoxic cancer cells with an increase in lactate dehydrogenase activity (LDH) (responsible for the
conversion of pyruvate to lactate), with Bhaita (2012) demonstrating nearly an 80% increase in LDH activity with cells in 0.1% oxygen. Bowyer (2011) was also able to demonstrate that LDH activity was greater in hypoxic cancer cell lines compared to cancer cells in normal oxygen conditions. This demonstrates that the lower the $O_2$ concentration, the faster and harder the cell is working to survive and therefore the cell metabolism rapidly increases. The literature and these results are putting forward the theory that all cancer cells have the potential to display the aggressive hypoxic phenotype; the trigger for this phenotype is a decreasing availability of oxygen with hypoxic cancer cells increasing on a metabolic scale from 5 to 0.01% $O_2$.

These results reveal that as the $O_2$ levels drop, the metabolic rate of the cancer cells shift and increase, and can be directly correlated with the appearance of the hypoxia-inducible factor (HIF), a transcription factor that is activated under low oxygen conditions. HIF is a highly conserved DNA binding protein that, in the absence of oxygen, will translocate to the nucleus and is a master regulator that activates the expression of multiple target genes containing a hypoxia response element (HRE) (van Pelt et al., 2011). HIF-1 is a protein with over 70 genes known to have binding sites and be activated at the transcription level (Semenza, 2004). However this estimation of involved genes regulated by HIF-1 is probably an underestimate and will most likely increase as research continues.

To date, multiple HIF isoforms have been described, however it is HIF-1α that has received widespread attention and is best characterised, thus in this thesis we have concerned ourselves with activity of HIF-1α. The appearance of HIF-1α in cancer cells is directly dependent on the availability of $O_2$. When cells are in the presence of 20% $O_2$, HIF-1α is not detectable. At 5% $O_2$ it is again barely detectable, however it has been shown that at 2% and 1% $O_2$ the HIF-1α levels dramatically increase and are stabilised in the hypoxic conditions (Holmquist-Mengelbier et al., 2006; Semenza, 2004; van Pelt et al., 2011). This increasing presence of HIF-1α directly correlates with the severity of hypoxia with maximal expression at hypoxic/ischemic environment (Jiang et al., 1996). Therefore the resulting cascade of events that occurs after HIF-1α stabilisation seem to be interlinked, and the metabolic changes associated with some of the responsible proteins upregulated by HIF-1 are shown in Fig 1.13.
Fig. 1.13 Up-regulation of HIF-1 expression induces the expression of different genes used in glycolysis favouring catabolism to lactate. The schematic lists some of the proteins that are upregulated by HIF-1 and induce pathways that maintain the nutrient supply for a hypoxic cell and enhance its survival. Image adapted from Brahim-Horn et al., (2011). [Original in colour].

Fig 1.13 depicts cellular methods that have been described as being efficient in allowing the cell to survive as long as possible in usually inhospitable conditions (Song et al., 2011; Brahim-Horn et al., 2011; Solaini et al., 2010; Burgess, 2013). HIF-1 is the key antagonist and master regulator of these effects in the hypoxic cancer cell, and it is this expression of HIF which triggers these mechanisms and subsequently provides the foundation and time for the cell to send out signals to promote self-survival. Some of the key manifestations of these survival mechanisms associated with hypoxic cancer cells are discussed below.

1.4.2.1 Hypoxic Cancer Cells and Acidosis

With the mechanisms described above there appears to be a large excess of lactate being produced when cancer cells are exposed to hypoxic conditions. Glycolysis is a very inefficient method of producing energy and leads to the production of lactate in anaerobic
conditions which drops the cellular pH if the cells are not well perfused (Solaini et al., 2010). To compensate for the lack of energy being produced by glycolysis, the tumour cells increase glucose uptake and metabolism (Brahimi-Horn and Pouysségur, 2007). The overload in lactate contributes to acidosis within the cells and the extracellular environment. This increase in acidity is detrimental to the survival of the cell and can lead to cell death if not addressed. As the cell enters a more hypoxic environment, so the glycolytic rate increases and hence the production of lactate and therefore H⁺.

In an attempt to balance the intracellular pH, HIF-1 induces intracellular pH regulating systems that extrude lactate from the cells via the H⁺/lactate monocarboxylate transporter (MCT1-4) (Chiche et al., 2010). These systems gather to protect the cytosol from acidification, and are responsible for the maintenance of the intracellular pH at a level that promotes cell survival. The importance of the expression of these pH regulatory systems has been demonstrated with the knockout of MCT1 and MCT4 leading to a significant reduction in tumour cell growth in vitro and in vivo (Brahimi-Horn et al., 2011).

It is known that tumours possess a lower pH than the surrounding tissue and this has provided a strategy for targeting tumours more effectively with loco-regional therapies that respond to a decrease in pH. It is this ever evolving environment of a tumour and its metabolic progression that provides targetable factors for medical devices. Thus far, no DEB formulations are stimuli responsive, however pH responsive polymeric micelles have shown sharp opening transition and release of the drug payload within the aqueous acidic environment of a breast cancer cell line (Min et al., 2010). This approach is shown to be a reproducible technique with positive results achieved by many investigators using the pH of the tumour cell as a target, rather than indiscriminately attacking all cells within reach of the treatment (Yu et al., 2013b). This highlights the potential avenue of using intelligent polymers similar to pH responsive polymers to target the resistant hypoxic tumour cells.

Identifying a potential target that is exhibited from a hypoxic tumour requires an in depth understanding of the multitude of routes that the cells will take to ensure survival. After this well described shift in the cell metabolism, the activation of HIF has already initiated the necessary metabolic changes to allow for the significant physical responses associated with hypoxic tumour. Such specific targeting of hypoxic cells could negate and halt the progression of some of these cell properties.
1.4.2.2 Hypoxia and Cell Proliferation

The treatment of HCC with TAE assumes that in hypoxic conditions cells would be prevented from proliferating by ischemia-induced necrosis. This seems to be in contradiction to the growing number of reports that detail the survival and growth of these HCC cells in the hypoxic microenvironment (Gwak et al., 2005). The stimulation of enzymes such as hexokinase II which is involved in the glycolytic pathway and an enzyme called insulin-like growth factor-2, stimulates the growth of HCC cells (Wu et al., 2007).

Various factors are involved in the proliferation of the hypoxic tumour cells with HIF-1 known to be directly involved with the upregulation of proteins such as Bel-2 (Sun et al., 2011). These changes are known to result in a phenotype change in the hypoxic cells from an epithelial to a mesenchymal state, named the epithelial-mesenchymal transition (EMT) (Brahimi-Horn et al., 2010). This transition gives the cancerous cells stem cell characteristics and the ability to invade non-cancerous tissue by the repression of E-cadherin by HIF induction (Brahimi-Horn et al., 2011). This repression of E-cadherin also activates hepatocyte growth factors and causes the stem-cell like cancer cells to travel in search of nutrients (Bristow and Hill, 2008).

To compare the proliferation rate of normoxic and hypoxic tumours, an animal study was performed where mice had tumour xenografts established in mammary fat pads. The tumours belonged to the cell line of human breast cancer, which were pre-exposed to normoxic and hypoxic conditions (Milane et al., 2011a). Once the cells were implanted, tumour growth was monitored every other day until 100 mm$^3$ in size was achieved. Interestingly, it only took the hypoxic tumour 3 weeks to reach the size of 100 mm$^3$, while the normoxic tumour took 7-8 weeks to reach the same size (Milane et al., 2011a). The results of the tumour growth rate study can be seen in Fig. 1.14.
Fig. 1.14 A graph showing the growth rates of normoxic and hypoxic tumours. The tumour cells were pre-exposed to either normoxic or hypoxic conditions and then implanted with a mouse. The tumour was measured every other day until 100 mm$^3$ in size was achieved. Milane et al., 2011a.

The results of the graph highlight the phenomenon that in hypoxic conditions, the proliferation rate of tumour cells more than doubles. Hypoxia has been heavily linked with quickening cell proliferation and growth (Cui et al., 2012). This aggressive rate of proliferation correlates with the metabolic shift the cells experience and the tumour cells uptake of glucose to compensate for inefficient energy production. Therefore, if the application of embolic procedures to treat HCC does not completely rid the patient of the disease, then there is the potential for a quick recurrence of the tumour.

1.4.2.3 Angiogenesis and Metastasis

It is known that angiogenesis plays an important role in the progression of HCC. Angiogenesis is the formation of new vessels and the establishment of architecturally disarranged blood vessels, the extent of which could have severe consequences on the rate of disease progression (Fernández et al., 2009).
Fig. 1.15 A schematic showing the tumour's ability to grow blood vessels and re-oxygenate the tumour. The tumour sends out growth factor signals, which leads to the formation of new blood vessels followed by growth of the tumour and spread of tumour cells down the new vessels. [Original in colour].

Fig. 1.15 depicts how the hypoxic tumour can re-grow its own blood supply, even when its previous blood supply had been blocked by embolising material. This would explain why the glycolytic shift in metabolism can be maintained for only a certain period of time and that this formation of new blood vessels will lead to nutrients and oxygen being re-routed back to the tumour. These new vessels have been shown by imaging techniques to start growing and forming within three weeks of the tumour becoming hypoxic (Pleguezuelo et al., 2008).

When HIF-1 is activated a whole host of angiogenic factors are switched on but most of the research has focused on Vascular Endothelial Growth Factor (VEGF) (Semenza, 2012a). Results have shown that high levels of VEGF occur in the majority of cancers and that high VEGF expression correlates well with larger tumour size and enhanced local spread (Duffy et al., 2003). As HIF-1α and VEGF are upregulated, important tumour suppressors such as von Hippel-Lindau and p53 that are affected by hypoxia, become down regulated and are unable to prevent tumour progression. Patients
with high levels of HIF-1α expression have much shorter survival times than those with low expression. Increasingly research is now looking at the role of VEGF with the intention of studying its receptors in order to inhibit its actions (Duffy et al., 2003; Semenza, 2012a). This is of clinical importance as high levels of VEGF are also associated with tumour metastasis. Although the exact mechanism is still unknown, hypoxia is associated with metastasis and the spread of the cancer cells around the body. The cells have gained an invasive ability and to sustain their continuous growth must escape the immune surveillance of their host (Xiong et al., 2004).

In a study of a set of patients that received TACE, 70% of those with high VEGF plasma levels developed metastasis of the cancer around the body (Wu et al., 2007). It would appear that these new blood vessel formations are the perfect routes for the cancerous cells to escape and metastasise. Angiogenesis is therefore important to tumour survival. Although TACE is effective in the treatment of HCC, it could in itself be inefficient at targeting the entire tumour, therefore allowing recurrences.

1.4.2.4 Chemoresistance

Hypoxia is now associated with chemoresistance and radioresistance (Wilson and Hay, 2011). Bristow (2008) details how hypoxic cells are 2-3 times more radioresistant than normoxic cells because less free radicals are produced in a low O₂ environment after ionizing radiation. However for the purposes of this thesis, we will focus on chemoresistance.

Numerous factors have been linked to chemoresistance. Highly acidic extracellular tumour environments have been linked to tumour migration and progression, however it has also been linked to the neutralisation of weak base chemotherapeutics making the drug less efficient (Chiche et al., 2010). This is why treatments trying to disrupt cellular pH regulation have received such attention (Parks et al., 2013). Low oxygen tension has also been implicated in reduced effectiveness of drugs such as bleomycin and doxorubicin. Doxorubicin requires the presence of oxygen to be most effective at producing reactive oxygen species (ROS); when oxygen is not available the cycling of Dox is slower but it can still produce free radicals that can alkylate cellular DNA (Bowyer, 2011). Hypoxic stress has also been linked to abnormally large mitochondria in various cancer cell lines,
which has proven to afford the cells resistance to chemotherapeutic treatment (Brahimi-Horn et al., 2011).

However it is again the activation of HIF that has been directly linked to the up-regulation of proteins and mechanisms that induce chemoresistance (Milane et al., 2011b). These mechanisms increase the activity of ion channels and intracellular transporters and many of these key proteins affect the entry and the removal of drugs from cells (Huang and Sadée, 2006). One of the most prominent groups are the ATP-binding cassette (ABC) transporters, such as the multiple drug resistance transporter MDR1 which is known to play a big role in chemoresistance (Li et al., 2012b). There are numerous transporter proteins and drug substrates but very few have been investigated; even those that have been shown to be chemoresistant have not been fully characterised. Of the ABC transporters, it is P-glycoprotein (MDR1) that is the most studied (Milane et al., 2011b).

Interestingly for MDR1 to efflux these drugs out of the cell, 2 molecules of ATP are consumed. This shows that these transport systems require the ATP energy source and this can be abundantly supplied by hypoxic cells and their glycolytic shift. There has been strong evidence linking increased levels of ATP in hypoxic cells to tumour cell drug resistance (Zhou et al., 2012). Potentially under anoxic conditions the cell cannot keep up with the demand for ATP from the MDR mechanisms and succumbs to chemotherapeutic saturation.

If hypoxia causes the cell transporters to become drug resistant then very little drug will enter the cell. If the drug is hydrophobic like Dox, then it may be able to diffuse across the membrane; however transporter proteins would have helped the drug have a more pronounced effect if not in hypoxic conditions (Huang and Sadée, 2006). Interestingly ABCG2 is a protein that is heavily linked in the extrusion of Dox in breast cancer cell lines and has now been found in liver cancer cell lines such as HCC (Sukowati et al., 2012). This would make alkylation of the cellular DNA with Dox more difficult if the drug is being targeted and deliberately extruded from the cell.

It is these energy-dependant transporters that have an increased drug efflux in hypoxia and force any drug back out of the cell. Therefore it is these transport systems that are receiving critical attention and methods to overcome them are being constantly tested. One method shown to overcome this chemoresistance is to flood the tumour cell with drug such as Dox, this has been applied with the use of targeted nanoparticle delivery systems (Kim et al., 2008; Soma et al., 1999). These systems deliver the drug intracellularly, however with this targeted method and excess drug, the cells are unable to export the drug
fast enough to survive. The use of embolic microspheres is not a form of intracellular delivery. However with loco-regional delivery, supplying the hypoxic cell site excess hydrophobic drugs such as Dox may have the potential to overcome MDR.

It is apparent that a hypoxic tumour is very challenging disease state in the treatment of HCC, and brings forth problems that were, until recently, unknown. The ability to overcome this transporter induced chemoresistance is key to overcoming the poor prognosis associated with hypoxic tumours. A large accumulation of these cytotoxic drugs within the hypoxic cells can lead to cell death (Deng et al., 2014). If the microsphere formulation was able to increase the amount of drug released and saturate the cells with drug, it could potentially overcome MDR of hypoxic tumours. The design of an embolic microsphere formulation, with a high drug loading capacity with the ability to specifically target hypoxic cells could find wide use in the treatment of HCC. However a stimuli has not yet been identified and one is needed that distinguishes between a normal cancerous cell and a hypoxic cancer cell with an aggressive phenotype. Section 1.4.3 investigates a potential difference between cancer cells and hypoxic cancer cells.

1.4.3 The Redox Environment of a Hypoxic Tumour

Multiple studies have shown that one of the key changes that hypoxia causes in a tumour is an alteration of the redox status (Solaini et al., 2010, Jaffar et al., 2001, Hu Chen and Shi. 2008, Clanton, 2007, Kuppusamy et al., 2002). Clear evidence has been shown that the redox environment can regulate the HIF-1α protein under the conditions of hypoxia (Guo et al., 2008). There are numerous different elements that contribute to the redox status of the tissue under hypoxia and these reducing species have an impact on how the tissue will respond to the oxidative stresses that will be incurred by drug targeting and other therapies (Kuppusamy et al., 2002).

Redox balance is the key to cellular homeostasis. The balance between oxidation and reduction reactions plays a crucial role in numerous cell signalling mechanisms. This balance can be disrupted by either an increase or decrease in O₂ levels. The use of O₂ for in cellular metabolism has been shown to produce ROS which in large quantities can be toxic by readily reacting with cells, damaging DNA and oxidising lipids and proteins (Grek and Tew, 2010). To circumvent this effect and combat ROS along with other factors that might inhibit cellular growth, the body has produced an antioxidant system to defend itself and
scavenge for ROS. Therefore the redox state is maintained by ROS and antioxidants, although this is not in a state of equilibrium with these redox antioxidants providing a more reductive environment in the intracellular space of the cells (Schafer and Buettner, 2001). This has always been coupled with the belief that the extracellular environment is an oxidising environment and typically redox inert. The extracellular redox environment to this date is still poorly understood, however the view that it is redox inert is beginning to shift (Banerjee, 2012). Some research has begun to show that the extracellular environment is dynamic and in some instances can be affected by the actions of the intracellular redox environment (Kemp et al., 2008; Banerjee and Smith, 2012; Banerjee, 2012). However, what is clear is that this redox balance is most definitely disrupted by cancer initiation and progression, and with the onset of hypoxia in tumours there is a greater emphasis on the production of antioxidants (Karlenius and Tonissen, 2010). This increase in antioxidants provides a highly reducing environment and has been directly linked to benefit the proliferation and the malignant progression of cancer cells by protecting them from damage and allowing them to escape to metastasise (Pani et al., 2010). Therefore high levels of these antioxidants are seen as a marker for malignant phenotypes and as a bad prognostic indicator.

Research has begun elucidating the redox networks involved and have highlighted the redox nodes that are responsible for the maintenance of the redox state. The main antioxidants involved in the reductive intracellular environment are thiol/disulfide based systems. These redox couples have been identified as the thioredoxin, glutathione and cysteine/cysteine couple. These redox nodes are described in the following sections.

1.4.3.1 Thioredoxin

The thioredoxin system contains reduced thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH (NADP+/NADPH being a redox couple itself) (Bhatia et al., 2013). Trx is a 12kDa protein that is ubiquitously expressed and has an active dithiol motif which can reduce disulfides in proteins and peptides. The mechanism of Trx catalyses the reduction of disulfide bonds and in doing so becomes oxidised itself. Oxidised Trx is then recycled back to the reducing form with the use of TrxR, which is at the expense of a NADPH molecule (Mukherjee and Martin, 2008). TrxR is the only known electron donor for Trx to date with a noted higher expression of TrxR in patients that do not fully respond to
chemotherapy. This thioredoxin system contributes to maintaining the cellular reducing environment through the thiol/disulfide exchange reaction. Trx has been shown to do its scavenging work through peroxiredoxins (Prx), which aid in the reduction of peroxides giving an oxidised Prx, which in turn is converted back to the reducing form by Trx (Noh et al., 2001). Initially it was thought that Trx was primarily involved in the scavenging of ROS, however subsequent studies have shown Trx has a major part to play in cancer progression.

Trx is responsible for transferring its reducing equivalents to redox factor-1 (Ref-1). This interaction between Trx and Ref-1 is crucial as it is now known that Ref-1 is responsible for the modification of a cysteine residue within HIF-1α which then allows dimerization with the HIF-1β subunit (Karlenius and Tonissen, 2010; Bhatia et al., 2013).

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Fig. 1.16 Interaction of Trx with Ref-1, which permits stabilisation of HIF-1 and expression of its target genes within hypoxic cells. Image adapted from Bhatia et al., (2012). [Original in Colour].

The scheme in Fig 1.16 illustrates how Trx allows HIF-1 to perform its functions as previously described and therefore outlines the importance of thioredoxin in cancer biology. Without the upregulation of this antioxidant, HIF-1 would not be able to stabilise...
and the consequential reactions of hypoxia in tumours would not take place. Thioredoxin has other key functions that work in favour of tumour growth.

Trx is currently thought to be directly connected to the presence of hypoxia in tumour cell lines with a 14 fold increase of Trx mRNA in hypoxic HT29 cells (Huang and Zhong, 2012). This overexpression of Trx has been noted in hypoxia in multiple tumour cell lines, however why this mechanism is induced is still not understood. What is known is that Trx has been shown to be responsible for the de-regulation of the Phosphatase and tensin homolog (PTEN) which regulates metabolic pathways and PTEN loss of activity has been linked to many human tumours (Mukherjee and Martin, 2008; Weljie and Jirik, 2011).

All of these results show the significant role Trx has to play in hypoxic tumour progression and hence validates research looking at potentially targeting thioredoxin and inhibiting its action in solid malignancies (Ramanathan et al., 2012). Trx’s role as a scavenger in the intracellular environment for toxic oxidants cannot be ignored with patients having over-expression of Trx displaying resistance to cisplatin and other chemotherapeutic agents (Karlenius and Tonissen, 2010). However, what is only coming to light recently is the role that Trx has to play in the extracellular environment.

The strong reducing potential of Trx is maintained in the intracellular environment and levels are known to significantly increase in tumour cells in hypoxic conditions (Karlenius et al., 2012; Grek and Tew, 2010). However research is beginning to elucidate the presence and upregulation of extracellular Trx. Elevated serum levels of Trx have been found in patients with HCC (Miyazaki et al., 1998). Trx can not only be found in the blood surrounding the tumour but is stable in its reducing form and is used as a clinical biomarker to indicate disease (Chaiswing et al., 2008). Its presence in the extracellular environment is thought to be the reason why the disease progresses and spreads. Extracellular Trx has been associated with the matrix metalloproteinase function (MMP). MMP are involved in extracellular matrix (ECM) degradation, a crucial aspect of metastasis, and Trx has been implicated in disrupting tissue inhibitor of matrix metalloproteinase (TIMP) which is required to maintain MMP levels (Bhatia et al., 2013). Trx therefore has a very active role to play in the extracellular environment just as much as the intracellular and adds support to the hypothesis that in a hypoxic tumour the extracellular environment can be a strong reducing environment.

Trx is secreted by hepatocytes and a variety of cancer cells, but is also found in microvessel endothelial cells. The Trx present in these cells is in the active reduced form
The presence of these cells in hypervascularised tumours could provide an abundant supply of the reducing agent. It is known that Trx is released from cells in response to a malignant event which should provide a reducing extracellular environment, but the precise mechanism for the release still remains unknown (Matsuo and Yodoi, 2013).

All this evidence provides weight to the notion that the reduced form of Trx is highly abundant in hypoxic tumours as a protective agent. Its additional roles in further stabilising HIF-1, justifies the attention it is beginning to receive. This upregulation of Trx is a potential marker that could be used to distinguish between a hypoxic tumour and one that has not taken the aggressive phenotype. Therefore the use of Trx to mark hypoxic tumours could be exploited for the benefit of increasing drug release to the malignancy. A microsphere formulation could be synthesised with the use of an intelligent polymer able to respond to the stimulus of Trx. In addition to Trx there are other redox couples involved in maintaining the intracellular reducing environment that could also play a role in the extracellular environment.

### 1.4.3.2 The Glutathione System

The conformational integrity of many proteins is directly dependent on the interconversion of thiols and disulfides. Glutathione (GSH/GSSG) is thought to be the most abundant thiol-disulfide redox buffer in the cell and is integral in maintaining the intracellular reducing environment. It is also responsible for maintaining the integrity of proteins and is thought to maintain thiols of protein disulfide isomerase for protein folding in the endoplasmic reticulum (Circu and Aw, 2010). However a question that remains unresolved is the exact specificity of reduced glutathione (GSH) in its actions other than protecting the cell.

As in the thioredoxin system, NADPH is considered the primary source of reducing equivalents for the glutathione system. NADPH/NADP⁺ itself is a reducing couple within the cell with one of the strongest redox potentials observed of -400 mV. Its actions in replenishing the glutathione system can be seen in Fig 1.17.
Fig. 1.17 A schematic for the glutathione redox cycle showing the supply of electrons from NADPH to the cycle leading to the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH). The process is reversible by the donation of electrons from selenoenzyme to glutathione peroxidase which oxidises 2 GSH to GSSG.

However, unlike the thioredoxin system which is able to maintain its reducing potential at -280 mV, the glutathione potential can fluctuate when the cell goes through cues such as differentiation and apoptosis from -220 mV to -260 mV (Banerjee, 2012).

Fig. 1.18 The chemical structure of reduced glutathione showing the free thiol moiety able to cleave disulfides
Fig. 1.19 The chemical structure oxidised glutathione

GSH (Fig. 1.18) is a reducing agent and will reduce disulfide bonds with the sulfhydryl group acting as an electron donor, converting to oxidised glutathione (GSSG) (Fig. 1.19). The liver is the organ responsible for the synthesis of GSH and that is why high levels of glutathione are encountered there. Similarly to Trx, the role of GSH is to protect the cell from oxidative stress, such as those brought about by reactive oxygen species and has been shown to protect the cell from damage encountered by drug release (Kuppusamy et al., 2002). The GSH/GSSG ratio is maintained at approximately 90% in favour of the reduced form due to cytosolic de novo GSH synthesis, enzymatic reduction of GSSG and exogenous GSH uptake (Circu and Aw, 2010).

The level of naturally occurring GSH within cells is thought to be approximately 10 mM which is significantly higher than extracellular GSH level in normoxic cells (Meng et al., 2009). This has provided the rationale for delivering bioactive molecules by using intracellular conditions as a trigger (Saito et al., 2003). What is of more importance to the current thesis is the fact that although the intracellular environment is highly reducing, a hypoxic tumour is highly reducing when compared to normal tissue and there is at least a 4-fold higher concentration of GSH in the tumour tissue (Meng et al., 2009). This could provide a GSH concentration of almost 40 mM, and what is of great interest is that GSH is known to be exported to the extracellular environment by the ATP-binding cassette proteins such as MDR 1 (Brechbuhl et al., 2012). This extrusion of GSH through these channels is shown to aid the conjugation between the antioxidant and the chemotherapeutic
drugs such as Dox. The extracellular export of GSH has already been correlated to the protection of neurons by astrocytes (Stridh et al., 2010).

This transport of GSH into the extracellular environment is just another redox couple in conjunction with thioredoxin that could provide a highly reducing extracellular environment in a hypoxic tumour. These factors are combining to provide a clear targetable difference that can only be discharged by hypoxic cancer cells.

1.4.3.3 Cysteine/Cystine Redox Couple

Another redox couple involved in the maintenance of the reducing intracellular environment of the cell is the cysteine/cystine couple. L-cysteine is an amino acid commonly found and synthesised within the body. Within the cell it maintains a reducing potential of -160 mV which is the lowest of the mentioned redox couples. This redox couple can also be found in the extracellular environment as it can be extruded via amino acid transporters (Banerjee, 2012). The couple is the most abundant low-molecular weight thiol/disulfide system found in blood plasma. However, in the extracellular environment it only provides a reducing potential of -80 mV (Anderson et al., 2007).

Cysteine/cystine has the weakest reducing potential of the previously mentioned couples and it appears that the couple will act as an oxidising agent in the presence of the glutathione couple (Jones et al., 2004). Cysteine is the predominant form in plasma, which is potentially due to the fact that GSH can reduce cystine leading to cysteine being used along with other amino acids such as glycine to replenish glutathione pools (Anderson et al., 2007; Jones et al., 2004). Cysteine itself can protect from ROS and other harmful species, but what is interesting is that the disulfide moiety in L-cystine is readily reducible in the extracellular environment and this mechanism should be favoured more in the extracellular environment of hypoxic tumour.

From this review of the literature it is clear that there is a highly reducing intracellular environment in a hypoxic tumour. The presence of this reductive environment does lend itself to the potential for a therapeutic approach to target tumour cells which are hypoxic and such therapies are described below in section 1.5. However, the literature is starting to gather strength around the possibility that the extracellular environment is not as unreactive as first thought. An opportunity could present itself for an approach to investigate the potential of targeting an extracellular reducing environment but first a
thorough understanding of the current approaches targeting an intracellular reducing environment is required.

1.5 Possible Therapies for the Treatment of Hypoxic Tumours

Various therapies to address hypoxia-induced effects are being investigated; however, these studies still remain in their infancy and knowledge in the area will continue to expand, as will new therapies. Much of this research is being carried out at the molecular level looking at transcriptional response initiated by the oxygen-dependant stabilisation of the HIF-1 transcriptional factor and ways of down regulating its expression (Magagnin et al., 2006).

Therapeutic agents such as anti-angiogenic drugs are being studied to prevent the vascular formations and ultimately the recurrence of the cancer. A phase II study looked at the antiangiogenic properties of the multikinase inhibitor Sorafenib (Abou-Alfa et al., 2006). It showed that the drug has modest efficacy in HCC with the majority of patients again showing a stable disease, although there was an increase in tumour necrosis (Abou-Alfa et al., 2006). The trial, however, did lead to the conclusion that anti-angiogenic agents have limited single-agent activity and that possibly better results would be obtained if anti-angiogenic agents were combined with cytotoxic drugs. This has led to trials being carried out with Sorafenib in combination with Dox as a single drug agent given intra arterially (Richly et al., 2009). The results of the trial showed that the combination therapy is more successful than any of the agents alone (Richly et al., 2009). As a result many new studies are being carried out with a range of anti-angiogenic drugs and combining them with various cytotoxic drugs to try to establish the best line of treatment. The use of anti-angiogenic factors has also been investigated in combination with DEB to optimise results and to prevent the side effects that can occur with the use of such drugs as Sorafenib (Reyes et al., 2009a). The beneficial results achieved with the combination of Sorafenib and DEB has led to the combination of DEB with other drugs such as Everolimus (Cabrera and Nelson, 2010). There seems to be a large focus in the use of anti-angiogenic treatments, and this research will continue as scientists determine the best combination of treatments to battle the effects of hypoxia and angiogenesis.
1.5.1 Therapies using the Reductive Potential of the Tumour

A lot of early work has focused on the use of bioreductive drugs to specifically target hypoxic cells. Drugs such as Mitomycin C were proposed to be more active in hypoxic tumours as it requires a reductive metabolism from its benzaquinone ring for activity (Brown and Giaccia, 1998). On reduction a cytotoxic bifunctional alkylating agent is produced, however the drug still has the ability to kill aerobic cells (Brown and Giaccia, 1998). Fig. 1.20 demonstrates the effects of hypoxia on the quinone class leading to synthesis of an intermediate compound along with the therapeutic drug. The NAD(P)H dependant cytochrome, P450’s and other haemoproteins have been directly linked to the electron reduction of various substrates and this knowledge has been used on other bioreductive drugs such as banoxantrone where the low toxicity N-oxides are converted to respective tertiary amines (Patterson, 1993).

Fig. 1.20 A schematic showing how hypoxia causes a co-current cyclisation of a quinine system forming a potentially non-alkylating intermediate and a therapeutic drug

Fig. 1.20 shows a drug from the quinine class can undergo a two electron reduction affording the hydroquinone species. These species can potentially cause release of a therapeutic drug. The information regarding bioreductive drugs provides further evidence of the reductive potential of the hypoxic environment that surrounds a tumour and thus, redox as a stimulus for its potential to cause drug release in a hypoxic tumour. The application of prodrugs directly targets the reducing environment of a hypoxic tumour. These drugs can be delivered systemically or loco-regionally and only become active at the target site. If microspheres can be synthesised with a redox responsive mechanism or a polymer, then the microspheres can potentially target hypoxic tumours. The incorporation of redox sensitive linkages in polyplexes has shown cleavage of the linkages, leading to
biodegradation of the device in the reductive environment of the cell (Kumagai et al., 2012; Kim et al., 2013). This shows the application of redox sensitive cross-linkers/polymers and potential to be used to target hypoxic tumours.

1.5.2 Disulfide Systems used for the Delivery of Drugs in Hypoxia

Recently work has been performed in the gene delivery field where non-viral vectors have been developed which comprise of cationic polymers and a redox cleavable disulfide bond (Dai et al., 2010). Dai’s study used a cross-linker called N,N’-bis(acryoyl cystamine) which was shown to be reducible in a reductive environment and the polymer showed it could mediate gene transfection (Dai et al., 2010).

A study by Christensen and co-workers (2007) has provided the evidence that disulfide cross-linkers will reduce to their thiol group in a reductive environment. The intention of the authors was to deliver VEGF to promote neovascular formations in ischemic tissue (Christensen et al., 2007). The delivery system used contained ethylenediamine which was cross-linked with cystamine bisacrylamide. The delivery system was loaded with VEGF and then was transfected into hypoxic cells (Christensen et al., 2007). The results showed that the disulfide cross-linker reduced in the hypoxic conditions and that VEGF expression was 76 times higher in hypoxic cells than in normoxic cells (Christensen et al., 2007). This is the strongest evidence yet that disulfide cross-linkers will reduce in a reductive environment and more importantly that a hypoxic tumour can be targeted using its own redox environment as a trigger for the release of medication as shown in Fig. 1.21.

![Fig. 1.21 A schematic showing the redox sensitive delivery system entering the cell. When within the cell, the device has its disulfide bonds cleaved by GSH; opening its contents within the cell. Image adapted from Sun et al., (2011a). [Original in colour]](image-url)
Already noted is the exploitation of the intracellular reductive levels. Oupicky (2008) describes how the molecular architecture of the disulfide containing polymer is responsible for how the system acts in hypoxia and how its aids either gene transfection or drug delivery. In Oupielky’s paper a poly-l-lysine system is described which contains disulfide links. On reduction of the DNA delivery vehicle there is an overall 60 fold transfection efficiency (Oupicky, 2008).

Protenoid microspheres have been made of thermally condensed amino acids cross-linked with a cross-linking agent containing disulfide functionality (Quirk, 2007). The microspheres are able to encapsulate material and release it slowly when exposed to a reducing agent (Quirk, 2007). The patent confirms that the rate of release is dependent on the number of cross-linking and the rate at which they are removed or opened (Quirk, 2007). The cleavage or the removal of the cross-linker leaves a hole or so-called window and the more windows or the size of the window dictates the rate of release. Although size is not discussed it is believed the invention is of a sub-micron size.

Another system is a biodegradable polymeric capsule based on thiol-disulfide chemistry (Zelikin et al., 2005). Zelkin et al (2005), prepared poly(methacrylic acid) (PMA) cross-linked with disulfides by layer by layer deposition of thiolated PMA (PMASH) and poly(vinylpyrrolidone) (PVP) on silica particles, followed by oxidation of the thiols to crosslink the PMA and removal of the silica and PVP by changing the pH to disrupt hydrogen bonding and form a capsular structure (Zelikin et al., 2005). In a slightly altered form of this product, the capsules were able to retain their contents in normoxic conditions, but in the presence of glutathione the contents was released (Chong et al., 2009). However, of all the systems described in the literature, none are capable of embolising a blood supply vessel to a tumour and not all are designed for the treatment of tumours.

Little information is present in the literature that describes hydrogel beads that contain disulfide groups used for the delivery of small compounds or indeed chemotherapeutic drug for the treatment of cancer or other tumours. Most of the work is developed on the sub-micron level and is not designed for the application of drug delivery. PMASH microcapsules have been described which have been shown to deliver Dox to colon cancer cells, although no demonstration of the redox-triggered release of Dox was presented in the paper (Yan et al., 2010). Again the microcapsules were not of relevant size for embolisation. Therefore the literature to date shows that there is a lack of embolic redox sensitive devices for the treatment of HCC.
1.5.2.1 Disulfide Cross-linkers

As research is currently employing the use of disulfides as cross-linking agents, an abundance of potential candidates are available for use in the intended final product of this research. The structure of some of these cross-linkers can be seen below in Fig. 1.22 below.

Fig. 1.22 List of disulfide cross-linkers that potentially could be used to cross-link with polymers.

The disulfide linkages have been used in some instances to covalently cross-link with polymers, an example being the conjugation of a polycation with both dithiobis(succinimidylpropionate) (DSP) and dimethyl-3,3’-dithiobispropionimidate (DTBP) (Gosselin et al., 2001). The disulfide groups react and form covalent bonds with available amine groups providing a reducible cross-link. DSP is a commonly used disulfide cross-linker as is DTBP and dithiodipropionic acid (Lee et al., 2012; Gouda et al., 2013). However these mentioned linkers would require some form of modification step to covalently bind them to form a copolymer.
The ideal cross-linker would be easy to incorporate into a microsphere system with little modification required leading to homogenous spread of the intelligent polymer throughout the microsphere. One such system is 2-(2-pyridyldithio)ethyl methacrylamide (PDTEMA), a monofunctional monomer with a free vinyl group, which is therefore able to directly polymerise with available sites of the copolymer, but importantly also with itself to ensure even spread of the polymer throughout the system (Zelzer et al., 2013). The use of a bifunctional cross-linker could potentially yield more aggressive alterations in the polymer structure in response to a reductive environment. These bifunctional linkers have the potential to form tightly branched cross-linked systems, placing stress upon the structure. When cleaved this tension could lead to an increase release of drug due to tension pulling the resulting thiols further apart and with the potential of polymer to biodegrade.

Disulfide cross-linking agents therefore have been widely used in the delivery of a payload to cells, and they appear to have a number of clear benefits with knowledge that disulfide groups are highly bio-reducible (Son et al., 2011; Li et al., 2012a; Kim et al., 2013). In addition, they are known to naturally stabilise proteins and have been noted to stabilise polymer structures (Liu et al., 2005). These characteristics of disulfides make them a suitable candidate to incorporate into a polymeric microsphere structure. This polymeric structure is intended to fill a clear niche available for a hydrogel bead that can embolise the tumour supplying blood vessels, while at the same time release drug and alter its release rate in response to hypoxia. In conjunction with the literature that pertains to the redox couples present, there is the possibility to target these hypoxic tumours by using a very real reducing environment in the extracellular space surrounding the malignancies. This PhD endeavours to develop a drug delivery vehicle capable of exploiting the generally unrecognised environment.
1.6 Research Aims

Embolisation is a method currently employed in conjunction with the application of chemotherapeutic drug for the treatment of hypervascularised malignant tumours. This treatment has demonstrated that the tumour has the potential to become hypoxic resulting in chemoresistance and spread of the tumour cells.

The overall aim of this PhD thesis was to synthesise and evaluate a novel, bioresponsive drug eluting bead to enhance chemoembolisation therapy by directly tackling the problems associated with hypoxia. It was hypothesised that incorporating a redox sensitive cross-linker into a hydrogel backbone (or co-polymerising with a hydrogel) would provide an embolic delivery device with a bioresponsive sensitivity to the reducing environment associated with hypoxic tumour leading to an alteration in the structure of the beads. This abrupt change in bead infrastructure will directly alter the rate of drug release from the beads. By varying the incorporation of the cross-linking agent into the polymer structure it could be possible to manipulate the rate of drug release from beads to overcome the chemoresistance associated from hypoxic tumour cells.

The following objectives were established to achieve this aim:

- To identify and synthesise, if necessary, a suitable cross-linking agent with a reducible redox sensitive moiety.
- To efficiently and homogeneously incorporate the reducible cross-linking agent into a hydrogel microsphere structure.
- To determine whether the newly synthesised microsphere structure is redox sensitive.
- To vary the incorporation of the cross-linking agent into the hydrogel microsphere structure, thus providing multiple formulations for characterisation.
- To assess the drug loading and elution capabilities of the microsphere and characterise whether the cross-linking agent alters the rate of release of therapeutic agent in a reducing environment.
- To model the behaviour of the formulations in vitro and determine whether through this formulation variation an optimal design can be selected for in vivo evaluation.
Chapter 2

2 Disulfide Cross-linker Synthesis-BALC

2.1 Introduction

As discussed in Chapter 1, disulfide cross-linking agents have been frequently used to cross-link polymers with their bio-reducibility used as a mechanism for drug delivery. One potentially attractive cross-linker is a bi-functional disulfide compound with two vinyl groups, one at each end of the monomer. Such cross-linking agents have been described and one that is mentioned by Meng (2009) is bis(acryloyl)cystamine (BAC).

![Fig. 2.1 The structure of a redox sensitive disulfide cross-linker (BAC) used in drug delivery devices [Original in colour].](image)

BAC is shown in Fig. 2.1 and its properties have been well documented in the literature (Piest et al., 2008; Kim and Kim, 2011; Pan et al., 2012). Synthesis of BAC can be performed by producing dimethylcystamine and bisacrylamide in two separate reactions and then reacting the two together with a Michael Addition polymerisation to form a poly(amido amine) polymer (Piest et al., 2008). To remove a step, a more appealing method is the addition of the starting material cystamine dihydrochloride to 2 molar equivalents of acryoyl chloride in base conditions (Pan et al., 2012). Conversely BAC is commercially available and therefore synthesis of the material is not routinely required. BAC was chosen as one of two suitable disulfide cross-linking agents for use in the preparation of novel microspheres, and the results of its application are detailed in Chapter 3.
Another interesting disulfide cross-linker is bis(acryloyl)-(L)-cystine (BALC) first detailed by Emilitri (2005) and colleagues. BALC has a very similar structure to BAC, and is symmetrical, with the addition of two carboxylic acid groups (Fig. 2.2)

![Fig. 2.2 The structure of the disulfide cross-linker BALC [Original in colour].](image)

These acid groups have the potential to aid solubility of the monomer in water and if used in a microsphere could contribute to their ability to swell in response to stimuli. BALC has been used as a biodegradable cross-linker in siRNA delivery as well as in swellable hydrogels (Shi et al., 2012b; Jere et al., 2009). However BALC is not available for purchase on the market and as a result must be synthesised to be used in this work. This chapter focuses on the synthesis of BALC and its characterisation using a wide range of analytical techniques to confirm its structure.

### 2.2 Materials

The N,N’-bis(acryloyl)-(L)-cystine (BALC) monomer was synthesised using sodium hydroxide (NaOH), L-cystine dihydrochloride, 2-methoxyhydroquinone, acryloyl chloride and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl free radicals, (TEMPO) purchased from Sigma-Aldrich UK. Hydrochloric acid (HCl) was purchased from Merck (UK), while the water used was purified at Biocompatibles UK Ltd using a Millipore Elix water distribution system. The solvents dichloromethane, acetone, ethyl acetate (EtOAc), ethanol (EtOH) and methanol of super purity solvent grade were bought from Romil Ltd. Universal indicator sticks (pH 0-14) were purchased from Sigma-Aldrich UK.
2.3 Methods

2.3.1 Initial Synthesis of N,N'-bis(acryloyl)-l-cystine (BALC)

The method previously described by Emilitri and co-workers (2005) to form a disulfide cross-linking agent was performed as follows:

A 3 necked 500 mL flask, equipped with an overhead stirrer through the middle outlet, had 12.1 g of l-cystine dihydrochloride and 100 mg methoxyhydroquinone dissolved in 50 mL of water. The flask was placed in a water bath and the mixture was cooled to between 0-5°C with the use of ice. The temperature was observed with a glass thermometer. Two glass dropping funnels were placed either side of the stirrer. One of the funnels contained a 8.1 mL acryloyl chloride solution in dichloromethane (2 mL) and the other a 1 M NaOH aqueous solution (40 mL). Once the temperature reached 0-5°C both solutions were simultaneously added drop wise to the vessel over a period of 1 hour with continuous stirring. The temperature was maintained between 0-5°C. After addition, stirring continued for 2 hours at room temperature.

At this point the pH was adjusted to pH 1-2 with a 2 M HCl aqueous solution. The solution was frozen overnight and then placed on a Copley Hetro freeze dryer. After freeze drying acetone was added and the residual sodium chloride (NaCl) was filtered off and discarded. The acetone was then evaporated using a rotary evaporator.

2.3.2 Final Synthesis of N,N'-bis(acryloyl)-l-cystine (BALC)

The production of BALC was achieved using a modified version of the method described in section 2.3.1. In this method 12.1 g of cystine dihydrochloride and 15 mg TEMPO were dissolved in 50 mL of distilled water. The overhead stirrer was set to 350 rpm and the mixture was cooled and kept between 0-5°C. This was maintained at temperature throughout the reaction with a salt ice bath. Firstly 6 M NaOH was added dropwise until the pH fell between pH 10-12 and that pH was maintained throughout the addition, which accumulated approximately 45 mL of NaOH. Once at the correct pH, 8.1 mL acryloyl chloride solution was dissolved in 10 mL dichloromethane and this was added drop wise, over a period of 1 hour 30 min. Once the addition had finished, the mixture was stirred at room temperature for a further 3 hours. Once stirring was complete, the solution was adjusted to pH 1-2 using 2M HCl. The mixture was then frozen overnight at -20°C and then freeze dried.
Once dry, DCM (75mL) was added to the resulting white powder and allowed to mix. The DCM was discarded and the remaining solid was dissolved in methanol. The solution was filtered through a sintered funnel and placed onto a rotary evaporator until a product was obtained.

2.3.3 Purification of N,N'-bis(acryloyl)-(L)-cystine (BALC)

3 x 30 mL DCM was added to the resulting white powder obtained after synthesis to remove residual acrylic acid. The DCM solution was put through a sintered funnel and discarded. The remaining BALC was dissolved in 3 x 50 mL acetone and the solution passed through a sintered funnel to remove NaCl. The filtered solution was placed under a rotary evaporator and the majority of the acetone removed.

To purify the material, silica gel chromatography was used to separate the BALC from salt and other potential impurities. A volume of between 5-10 mL of BALC solution was left in the flask, to which silica gel was added until all the solution absorbed onto the gel. Then, approximately half to two thirds of a column was dry packed with silica gel. This packed column of silica gel was washed with ethyl acetate solution. The BALC mixture was then packed down evenly on top of the column. A further small amount of silica gel was added on top of the mixture to protect the surface of the mixture from the disturbance of the added eluent. The column was then washed with the non-polar solvent ethyl acetate. After two washes of ethyl acetate, the eluent was changed to a 3:1 ratio of EtOH:EtOAc. The fractions of eluent were collected and placed on a rotary evaporator to remove the solvent. The product was allowed to dry under vacuum over 24 hr.

2.3.4 Final Purification of N,N'-bis(acryloyl)-(L)-cystine (BALC)

The final purification method is similar to that described in section 2.3.3 with the only difference being the substitution of the solvent acetone for ethanol.
2.4 Methods used to Characterise BALC

The following methods were used to characterise BALC and allow determination of the structure of the monomer produced in section 2.3.2.

2.4.1 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for studying molecules at the atomic level. NMR is based on the fact that when magnetic nuclei are placed in an external magnetic field, the nuclei become aligned in a finite number of orientations (Dixon, 1965). In the magnetic field, the nuclei are able to absorb and re-emit electromagnetic radiation at a frequency characteristic of the isotope (Harwood and Claridge, 1997). This re-emission of energy provides detailed information about the electronic structure of the molecule.

For analysis of BALC samples, the Fourier Transform spectra method was used. BALC was dissolved in deuterium oxide. All NMR experiments were conducted on a BRUKER Biospin (Instrument AV360). In this thesis, NMR spectroscopy was used to study the $^1$H NMR, $^{13}$C NMR, 2D correlation spectrometry (2D COSY), distortion enhancement by polarisation transfer-135 (DEPT-135) and the nuclear overhauser effect spectroscopy (NOESY) of the synthesised product.

2.4.2 Electrospray Mass Spectroscopy

Mass spectroscopy was used to confirm the structure of BALC. The sample was vaporised in an ion chamber which converts the molecule into a combination of positively charged and neutral molecules. These molecules then pass along a curved tube (Thomas, 1996). As the ions travel through the tube there is a strong magnetic field which deflects the positive ions onto a detector while the neutral ions are lost (Thomas, 1996). These ions are then sorted according to their charge and a spectrum of their abundance is drawn up which is known as a mass spectrum.

Producing the ions in electrospray mass spectroscopy involves dissolving the sample into a liquid with a volatile organic solvent. The organic solvent is evaporated by a strong electric field and a coaxial flow of N$_2$ gas or with the assistance of slight heating, leading to the formation of a charged droplet (Thomas, 1996; Harris, 2006). This is then
used to achieve the mass spectrum and an understanding of the structure of BALC. The instrument used was a Bruker microTOF electrospray and the solvent was methanol.

2.4.3 Elemental Analysis

The elemental analysis of BALC was undertaken by Medac Ltd UK, who performed combustion analysis on the samples. The BALC sample was prepared by vacuum drying in an oven vacuum over 2 days. Heat was not introduced to dry the product to avoid potential degradation or polymerisation of the product. The product was crushed to powder and given further vacuum drying. The dried product was sent away for analysis.

The product was tested for Carbon, Hydrogen, Nitrogen and Sulfur (C,H,N & S). The combustion analysis was performed on a Thermo EA1108 or FlashEA1112 series elemental analyser. The accuracy of the results should be within the 95% confidence interval, although chemists consider a result to be within a ±0.30% as absolute and the detection limit for each component is <0.1% (Harris, 2006).

Comparison of elemental analysis was compared against the theoretical results which were calculated by using the molecular weights and quantities of used material.

2.4.4 Solubility Test

A solubility test was used to determine which solvents would be suitable to dissolve BALC without extracting salt and other impurities, and which solvents would be useful in dissolving the salt and impurities found as a by-product of the monomer synthesis. Therefore 50 mg of the product and/or NaCl control were added to 1 mL of various chemical solvents. The results were determined by visual analysis, noting the turbidity of the solution. The following polar and non-polar solvents were tested: ethyl acetate, N-butyl acetate, diethyl ether, dichloromethane, acetone, ethanol, methanol and water.
2.5 Results and Discussion

2.5.1 Synthesis of N,N'-bis-acryloyl-(L)-cystine (BALC). Published Method

The synthesis of BALC proceeded according to the method described in section 2.3.1. During the synthesis of the BALC it was noted that actually L-cystine dihydrochloride did not dissolve in water as described in the paper and the solution remained white (Emilitri et al., 2005). This is postulated to be due to L-cystine being retained in its acid form, which is not particularly soluble in water, and therefore needs to be converted to its salt form.

The extraction of the monomer proved unsuccessful, when the acetone was removed by rotary evaporation there was nothing left in the flask. This method was repeated again using the exact conditions mentioned in the referenced paper and again no monomer was achieved. Therefore amendments had to be made to the method in an attempt to produce BALC.

2.5.2 Synthesis of BALC: Modified Method

The synthetic route for the manufacture of BALC is shown in Fig. 2.3
The first change made to the method was to increase the amount of NaOH added to the solution during synthesis. This was based on a molar calculation that showed the amount of NaOH used in the paper was inadequate to allow diacrylation of \( \text{\textit{L}}\)-cystine (calculation presented in Appendix II). The results shows that only one fifth of the required NaOH was being introduced into the mixture and therefore deprotonation could not take place and diacrylation could not occur. Therefore the concentration of NaOH was increased to 6 M for 40 mL. As the method became optimised, pH paper was used to monitor the solution at pH 8-10 during the addition phase.
With the addition of concentrated NaOH, the \(L\)-cystine did dissolve in solution leaving a clear solution. However the solution eventually turned a dark brown colour. After freeze drying, part of the compound was very hard suggesting that the monomer had polymerised. The inhibitor 2-methoxyhydroquinone was tested with the highly concentrated NaOH and a colour change was observed as the NaOH broke down the inhibitor possibly explaining why the product had polymerised. Therefore the inhibitor was changed to TEMPO which was shown to work with NaOH in other syntheses (Internal data of Biocompatibles UK Ltd).

Also in the modified method, the volume of DCM was increased from 2 - 10 mL to slow down the reaction of acryoyl chloride by increasing phase separation and reducing the acryloyl chloride concentration. As a result, the stirring time after the reaction was increased to approximately 3 hours.

2.5.3 Characterisation of BALC Monomer

2.5.3.1 \(^1\)H NMR

Following the synthesis of the BALC monomer using the method described in section 2.3.2, \(^1\)H NMR was used to characterise the sample. The results were compared against that achieved by Emilitri and colleagues (2005). Considering that the workers had made the same cross-linker, the NMR spectra should be nearly identical. On interpretation of their results, it appears that the proton attached to the nitrogen atom (\(H_4\)), and the proton from the carboxylic acid became deuterated and did not give a signal (Fig. 2.4).
This leaves 6 proton signals, which all give different chemical shifts as each proton is in a different chemical environment. The shifts are caused by the electronegativity of substituents (or functional groups) near the proton in question. The more electronegative a substituent, the more deshielded a proton will be as its electron density is attracted to the withdrawing effect of the substituent leaving the proton open to the applied magnetic field (Streitwieser et al., 1992). If all protons are in the same environment there would be a single peak on the spectrum as each proton would be exposed to the same amount of deshielding. An example of this shielding can be seen with the tetramethylsilane (TMS) which is commonly used as a reference compound as it gives a single sharp peak (Floris et al., 2008). The chemical shift is measured on δ scale in parts per million (ppm). TMS is placed at the right hand side of the spectrum and is designated at 0 ppm.

The BALC compound has various different functional groups and therefore should yield chemical shifts on different ranges of the δ scale. The results of the 1H NMR analysis can be seen in Fig 2.5.
Fig. 2.5 $^1\text{H}$ NMR spectrum of BALC synthesised in method section 2.3.2. BALC was dissolved in deuterated water. The graph shows the positioning of the proton peaks allowing determination of the structure.

The results of the $^1\text{H}$ NMR are consistent by providing the same NMR spectra compared with the results achieved by Emilitri and colleagues (2005). This indicates that the BALC monomer has been synthesised. Further analysis was performed to assign the peaks manually to ensure the validity of the results achieved within this research project and those achieved by Emilitri and colleagues (2005).

The structure of BALC is shown in Fig. 2.4 A, and the reactive ends of the monomer are a vinyl group that should contain 3 protons. Alkenes give proton peaks from $\delta$ 4.2 to 7.6 and from the spectrum there are 5 obvious peaks, meaning three could be the
protons in question. Acrylonitrile is almost identical to that of the vinyl group in B ALC, with the slight difference that there is a nitrile group attached to the alkene instead of a carbonyl group. Acrylonitrile gives three peaks, with two between 6.0-6.3 ppm and one between 5.6-5.7 ppm (Silverstein, 2005). This is almost identical to the chemical shifts achieved in Fig. 2.5, and indicates that the peaks achieved at 5.8 ppm and 6.2-6.4 ppm can be attributed to the vinyl group. Acrylamides are known to show on proton NMR at these positions (Atkins, 1986). The slight difference is caused by the weaker deshielding effect of the nitrogen, with oxygen causing a slightly increased left shift down the δ scale (Silverstein, 2005). With the withdrawing effect of the carbonyl group factored in, the results align showing the three protons of the vinyl group positioned at 5.8 ppm and 6.2-6.4 ppm.

The labelling of the protons by Emilitri and colleagues (2005) is correct as it is H₂ that would give the furthest to the left peak as the most strongly deshielded by the carbonyl group. There is a coupling effect as there are multiple peaks, at this range. Coupling is where the spin of a proton is affected by the electrons of another proton spinning in close proximity (Silverstein, 2005). This effect does not usually stretch beyond three bonds and leads to the single peak being split into doublets, triplets or quartets. H₂ is coupled trans across to H₃, giving a doublets of doublets peak, while there is weaker coupling with H₁, and due to its stronger shielding is positioned further on the right than the other two protons of the vinyl group. Under the graph, the integrals for H₂ and H₃ add up to 2, while H₁ has an integral 1. This correlates showing each proton has an integral of 1 and is consistent throughout the spectra.

The peak at position 4.8 on the spectra has an integral of 5, however it is not part of the structure and is actually the signal given by HOD contamination of D₂O (Floris et al., 2008). To the right of it there is another proton peak at 4.7 ppm which is calculated to be the methine group at H₅ (Silverstein, 2005). The peak at 3.3 is that of the solvent methanol which was used in the extraction process for the synthesis of B ALC (Silverstein, 2005). The methanol peak also lies on top of the proton peak from the methylene group. The peak for H₆ is attributed to the proximity caused by the oxygen from the carboxylic acid, thus causing a greater shift of the proton. The difference in shift means that although the protons are attached to the same carbon, they are in different chemical environments with the disulfide group having less of a deshielding effect on the proton H₇, than the deshielding effect caused by the neighbouring carboxylic acid group and its attached subsituents on H₆.
An integral of 1 is taken off the methanol peak which leaves 6 protons on the spectrum in Fig. 2.5, each with an integral of 1. The analysis shows that the desired product has been synthesised due to assignment of the peaks, plus corroborating spectra of BALC by Emilitri and colleagues (2005). BAC which has a similar structure to BALC, provided similar NMR proton positions (Hansen et al., 2007). There has also been repeat synthesis of BALC, with each batch yielding identical spectra in the latter parts of this chapter confirming reproducible synthesis of BALC. Further NMR analysis was performed to gain a better understanding of the structure and its interactions.

2.5.3.2 $^{13}$C NMR

The carbon skeleton of the structure allows analysis with the use of $^{13}$C NMR; however, due to a low abundance of the active $^{13}$C nucleus and its poor sensitivity, more sample and time is required for analysis. Another difference is carbon peak integrals do not correlate to the number of carbon atoms, as in proton NMR. $^{13}$C NMR has a larger chemical shift range of up to 220 ppm in comparison to $^1$H NMR. However, the same principles of deshielding and electronegativity apply in $^{13}$C NMR. Therefore, coincidences in this spectra technique are uncommon, with impurities readily detected, due to the use of a large chemical shift range and the sharpness of decoupled peaks (Silverstein, 2005). Fig. 2.6 is the $^{13}$C NMR of BALC carried out by Emilitri and colleagues (2005) and based on the results of $^1$H NMR, the same results are expected.

![Fig. 2.6 A Carbon numbering of BALC by University of Milan. Fig. 2.6 B $^{13}$C NMR spectrum of BALC in D2O by University of Milan. Image used from Emilitri et al., 2005.](image-url)
Fig. 2.6 reports that there are 6 carbon atoms present which correlates with the chemical structure of BALC. This figure will be compared against that obtained for BALC in Fig. 2.7. The spectrum is identical to that described by Emilitri and colleagues (2005) seen in Fig. 2.6. Using Silverstein (2005), the spectrum was analysed for confirmation and to assign the carbon peaks. The carbonyl group is shown to have a signal at 168 ppm, matching with C₃ from Fig. 2.6. The only functional group that could have a higher signal within the BALC structure is the carbon belonging to a carboxylic acid which is given a δ value range of 165 to 185. This is higher than the range given to carbonyl groups. Knowing that 168 is the carbonyl signal, C₆ is assigned to the peak at 175 ppm.

Fig. 2.7 ¹³C NMR spectrum of BALC synthesised in method section 2.3.2. The BALC was dissolved in deuterated water. The results show the positioning of the proton peaks allowing determination of the monomer structure.
The alkenes are known to hold a wide peak signal range of 80-160 ppm. However, there are only two peaks given in the spectra in Fig. 2.7, which was expected based on the vinyl group in BALC, meaning the 2 peaks must belong to that of the two carbons of the vinyl group. Also with confirmation of proton NMR and the agreement by Emilitri et al., (2005), C₁ and C₂ can be confidently assigned to these peaks. While the peak belonging to the relevant carbon is unknown at the moment, it will be determined later in section 2.5.3.3 with further investigation. For now, peak 129 will be labelled C₁, and 128 labelled C₂ in accordance with Emilitri and colleagues (2005), until proven otherwise.

There are 4 peaks remaining and with the knowledge that the peak at 49 ppm is the methanol signal and that at 0 ppm is the reference compound TMS, the two remaining peaks belong to the methine and methylene group. Both fall in their respective ranges, but the signal at 53 ppm is assigned to C₄ knowing methine groups give a slightly larger chemical shift. This leaves methylene (C₅) with a slightly smaller chemical shift at 39 ppm (Silverstein, 2005).

The results are identical to that achieved in Fig. 2.6, and again this corroborates earlier findings that BALC has been synthesised. Further ¹³C NMR has been performed on newly synthesised BALC samples, all giving the same spectra. Knowing that BALC is being consistently produced, it would be of value to investigate the interactions of BALC to determine what conformation it might take.

2.5.3.3 2D COSY Analysis of BALC

The 2D COSY gives another view of the data already obtained through ¹³C and ¹H NMR. Instead of the side on view that has been seen in Fig. 2.5 and 2.7, an aerial view of the spectrum is achieved looking down on the peaks. What is seen are the off diagonal or cross peaks for all the protons with spin-spin coupling, therefore showing the interactions of protons and the intensity of their coupling with each other (Silverstein, 2005). Fig. 2.8 is a proton 2D COSY of the synthesised BALC showing which protons are coupling with each other. The proton scale is displayed on the bottom running from right to left and the scale is also found on the right hand side running from top to bottom. On the opposite side of the page from the scales are the established proton peaks for reference to establish which peak belongs to which proton.
Fig. 2.8 A 2D $^1$H-$^1$H COSY of BALC showing the interactions between the protons

Following the diagonal from the top right to the bottom left, 0 ppm gives the TMS reference which has no interactions with the BALC. Further down the first cross peaks are intersected at 3.0-3.4 ppm. Using the $^1$H NMR, there are two protons belonging to H$_6$ and H$_7$. These two protons have strong coupling with each other, but at 3 ppm on the horizontal axis and 4.8 ppm on the vertical axis another interaction of these two protons is noted. H$_6$ and H$_7$ has an interaction with H$_5$ and a very slight coupling effect with H$_1$. Further down the diagonal line, H$_5$ has interactions with H$_6$ and H$_7$ but does not have coupling with the vinyl group. H$_1$, H$_2$ and H$_3$ have strong coupling with each other but it is only H$_1$ from the vinyl group that has interactions with other protons within the structure. This result is crucial in showing that BALC is not a linear structure because coupling between protons means that protons will have to be a certain distance apart from each other to have an interaction. This could yield a chair like structure with H$_1$ pointed slightly in towards H$_5$, with H$_2$ and H$_3$ pointing away from the inner section of the structure.
This 2D COSY has also been used for $^{13}$C-$^1$H COSY, which compares the two spectra by showing exactly how many protons are bound to each carbon (Fig. 2.9 and 2.10).

Fig. 2.9 $^1$H NMR vs $^{13}$C NMR COSY. Part 1. Showing the first half, closest to the TMS reference point, of BALC and the proton interactions with the carbon atoms.

The proton scale is on the bottom of Fig. 2.9, running from right to left with the carbon scale on the right side running from top to bottom. At 39 ppm on the carbon scale there are two protons at 3.1 and 3.4 which corresponds with $H_6$ and $H_7$ attached to $C_5$. At 53 ppm, $C_5$ is attached to $H_5$. 
Fig. 2.10 $^1$H NMR v $^{13}$C NMR COSY. Part 2. Showing the second half, furthest away from the TMS reference point, of BALC and the proton interactions with the carbon atoms.

The labelling of carbon atoms at 128 and 129 is now shown to be incorrect as described in 2.5.3.2. Emilitri and colleagues (2005), label peak 129 as $C_1$ and peak 128 as $C_2$. However Fig.2.10 shows $C_2$ being bound to two protons which is not possible as it is a methine. Therefore peak 129 is $C_2$ and peak 128 is $C_1$. Using the COSY NMR results a possible orientation of BALC has been drawn in Fig. 2.11.
Fig. 2.11 The chemical structure of Bis acryloyl-(l)-cystine in its probable orientation [Original in colour].

The use of 2D COSY has been useful in suggesting the orientation of the BALC structure. The BALC structure may be chair-like in nature, with the vinyl groups kept sufficiently separate that reaction at each end could be contemplated. The oxygen of the carbonyl group appears to have an interaction with the hydrogen of the carboxylic acid almost forming a ring structure.

2.5.3.4 DEPT-135

DEPT-135 is a method that was used to confirm the structure of BALC and the results are shown in Fig. 2.12. The structure of BALC has no methyl groups, therefore any peaks in the top side of the spectrum belong to methine groups.
The results confirm the number of methine, methylene and methyl groups within the chemical structure of BALC. Interestingly the DEPT confirms the findings in section 2.5.3.3 in relation to the carbon labelling. It shows the peak at 129 ppm as being CH, revealing that the carbon atom is C_2 from Fig. 2.12. Therefore C_1 is confirmed at peak position 128.

2.5.3.5 NOESY Analysis of BALC

NOESY has been used to confirm the proton interactions of BALC to confirm the position of the vinyl group interactions (Fig. 2.13).
The results demonstrate that the biggest interactions with all protons are from the solvents D$_2$O and that of methanol. The vinyl groups do not have through space interactions with any of the other protons, with H$_1$ having the slightest interaction with H$_5$. This confirms, along with the COSY, that the vinyl groups of BALC should not be sterically hindered by its own interactions or prevented for being used in a polymerisation reaction.

### 2.5.4 Electrospray Mass Spectroscopy

Mass spectroscopy was used on BALC in Fig. 2.14 to determine the molecular weight of the structure and to confirm the synthesis of BALC. The structure of BALC is C$_{12}$H$_{16}$N$_2$O$_2$S$_2$ which gives a molecular weight of 348.
The results do not give a molecular weight of 348, yet yield the largest peak at 371. It is described that in electrospray mass spectroscopy, if the compound has no charge no reading will be obtained. In some instances the compound will create a charge by taking a sodium ion from the glass, known as a sodium adduct (Smith et al., 1990). This charge allows the molecular weight to be determined, and is added to one side of the compound (Han and Gross, 1995). In the case of BALC it can be calculated that the 371 peak is made up of the accounted 348 and an extra 23 which equates to the atomic weight of sodium. Interestingly there are also tiny peaks at 174, which could be BALC in its thiol state and in its dimerised form at 742.

2.6 Elemental Analysis

The analysis so far has detailed the synthesis and characterisation of BALC. The NMR and Mass spectroscopy have provided structural information about the product synthesised in section 2.3. Elemental analysis was carried out to characterise the composition of BALC. In this method the solid obtained after freeze drying was washed with DCM, but the product was dissolved in ethanol as described in section 2.3.4. The sample was analysed by elemental analysis shown in Table 2.1.
Table 2.1 Elemental analysis of final method of BACL extraction using ethanol.

<table>
<thead>
<tr>
<th>ELEMENT</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Original Theory</td>
<td>41.30</td>
<td>5.00</td>
<td>7.30</td>
<td>18.10</td>
</tr>
<tr>
<td>% Found</td>
<td>40.88</td>
<td>5.0</td>
<td>7.25</td>
<td>16.89</td>
</tr>
<tr>
<td>%Found</td>
<td>40.87</td>
<td>4.83</td>
<td>7.26</td>
<td>16.91</td>
</tr>
</tbody>
</table>

The result obtained in Table 2.1 is the closest value to the theoretical obtained thus far showing that approximately 92-93% of the product is BACL. Although the result does not match within the accepted deviation, when the solvents used in the purification step are factored in to the calculation all the results fall within 0.2% deviation. The benefit of using ethanol in place of acetone produces larger quantities of BACL to be used in the synthesis of microspheres.

This data confirms, along with all the other characterisation techniques undertaken on this product, that BACL has been synthesised. The evidence produced allows the BACL synthesised in this thesis to be used in future experiments with confidence.

As mentioned many techniques and solvents were used in the work up to purify the achieved the product. Section 2.7 details the method development of purification of BACL from impurities, and the examination of resulting materials by $^1$H NMR and elemental analysis, and where future work could improve ease of purification of the desired product. The work performed in section 2.7, provided a method that was used for the synthesis of BACL in this thesis.

### 2.7 Purification of BACL

Section 2.7 details the numerous methods developed for the extraction of residuals from the BACL compound. This section includes the initial results obtained and the changes made to reach a stage where BACL could be synthesised routinely.
2.7.1 Acetone and Methanol Method-Original Elemental Analysis

The original method for the purification of BALC involved the use of diethyl ether to wash the solid and remove any acrylic impurities, followed by dissolving the product in acetone. Looking at the structure of BALC and its carboxylic acid groups, the product should be water soluble as detailed by Emilitri (2005) and colleagues. Therefore washing with a non-polar solvent (diethyl ether) should not solubilise the product, only the organic impurities. A polar solvent would then be ideally added to the BALC. However acetone is only a moderately polar solvent and it was quickly discovered that the product BALC was not very soluble in acetone. Small traces of the product were found in acetone after rotary evaporation which was not sufficiently efficient or of a large enough quantity to use for the purposes of development of a novel chemoembolisation system.

The purification step was modified to use acetone to wash the BALC and remove any impurities, followed by a highly polar solvent method to solubilise the product. A very fine white powder was achieved after freeze drying and this product was subjected to elemental analysis. The results can be seen below in Table 2.2

<table>
<thead>
<tr>
<th>ELEMENT</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Theory</td>
<td>41.00</td>
<td>5.00</td>
<td>8.00</td>
<td>18.00</td>
</tr>
<tr>
<td>% Found 1</td>
<td>36.21</td>
<td>4.13</td>
<td>6.54</td>
<td>13.39</td>
</tr>
<tr>
<td>% Found 2</td>
<td>36.38</td>
<td>4.21</td>
<td>6.54</td>
<td>13.76</td>
</tr>
</tbody>
</table>

Table 2.2 Elemental analysis of initial BALC samples in 2.5.1

The results obtained in Table 2.2 do not match with the theoretically calculated element composition. Although the NMR and Mass spectroscopy show that BALC has been made, this result does not match and suggests that the product may not be pure. This initial result has led to a more in-depth understanding of BALC synthesis with further methods to corroborate the results seen by NMR and mass spectroscopy. Table 2.2 demonstrates that all the elements appear to be lower than the calculated theoretical
percentage. This may suggest that there is another compound alongside BALC which does not contain any carbon, hydrogen, nitrogen or sulfur. This compound was not shown on the NMR spectra in section 2.5.3.

When analysing the reaction scheme of L-cystine dihydrochloride with acryloyl chloride, the presence of 2 hydrochloride has been previously overlooked shown in (equation 2.1).

Equation 2.1  \[ \text{HCl} + \text{NaOH} \rightarrow \text{NaCl} + \text{H}_2\text{O}. \]

Using the calculation detailed in Appendix II, every 500mL batch of BALC produced 14.9 g NaCl. This accounts for over 52% of the product before extraction of BALC with methanol. This amount of salt explains why the elements in Table 2.2 were lower than the theoretical values. It was observed during the reaction that there was a white solid that was not particularly soluble in the methanol. It was initially thought that this was the product, but now it is established that this solid is actually the salt produced. This reveals a problem as both the product and the salt have similar appearances and also seem to be soluble in similar solvents making them hard to separate.

An approach to determine the percentage of salt in the product using silver nitrate was not successful as it also reacted with the acid groups of BALC to form a precipitate. However with the use of less polar solvents, salt was effectively removed from the product. To determine the best combination of solvents to use for the extraction of BALC with removal of salt, the solubility of BALC in different solvents was tested.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Ethyl acetate</th>
<th>n-butyl acetate</th>
<th>Diethyl ether</th>
<th>DCM</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility of BALC</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>✓ Limited</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 2.3 Solubility of BALC in various solvents

Table 2.3 shows that BALC is more soluble in polar solvents and this data can be used to devise a method to produce a redox responsive polymer incorporating BALC. A better understanding of why acetone was used in the original method to solubilise the
product instead of methanol. Even though BALC is much more soluble in methanol and hence more product is obtained after evaporation, salt is insoluble in acetone with 0.000042 % NaCl soluble in 1 litre of acetone (Burgess and Burgess, 1978). However, it should be noted that salt is inert and if the BALC monomer were to polymerise and form a water swellable, yet water insoluble hydrogel when placed in water, the salt would dissolve and be washed away whilst the BALC hydrogel should remain solid. This result allows us to use BALC in bead synthesis (Chapter 3), however for the purposes of research more work was carried out to try and determine which method would the yield the purest product.

2.7.2 Method Development for BALC Purification

A solvent method described in section 2.3.3 was used in an attempt to extract BALC, whereby the raw product was washed with DCM to remove any organic impurities. After washing with DCM, acetone was added to dissolve as much BALC as possible. Using the method detailed in section 2.3.3, a white solid was achieved. Although this method was limited by the relatively low solubility of BALC in acetone, a benefit was the reduced solubility of salt in acetone.

The collected solid was subjected to $^1$H NMR and elemental analysis of which can be seen in Fig 2.15 and Table 2.4 respectively.
Fig. 2.15 $^1$H NMR of sample obtained after column chromatography in section 2.5.2 using DCM/Acetone [Original in colour].

<table>
<thead>
<tr>
<th>ELEMENT</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Theory</td>
<td>41.30</td>
<td>5.00</td>
<td>7.30</td>
<td>18.10</td>
</tr>
<tr>
<td>% Found 1</td>
<td>41.81</td>
<td>4.96</td>
<td>7.20</td>
<td>14.61</td>
</tr>
<tr>
<td>% Found 2</td>
<td>41.76</td>
<td>4.92</td>
<td>7.21</td>
<td>14.83</td>
</tr>
</tbody>
</table>

Table 2.4 Elemental analysis of sample obtained after column chromatography in section 2.5.2 using DCM/Acetone.

The generally accepted maximum deviation between the calculated and found values as stated within the instruction for authors in the Journal of Organometallics is 0.4%. The results of the analysis do not fall within the accepted deviation range and results
show that although the product BALC has been synthesised, it is not pure. The results also show that instead of salt being the only inclusion within the sample, there is another element present due to the elevated carbon results. Analysis of the NMR in Fig 2.15 reveals the BALC proton peaks are present; however there are additional peaks also present. Further analysis and reference to the literature established that all the additional peaks seen in Fig 2.15 are attributed to the solvents used in the eluent to wash the product through the column. The peak seen at 2.22 ppm is the chemical shift of the solvent acetone, while the peaks at 1.24, 2.07 and 4.14 ppm are attributed the solvent ethyl acetate (Gottlieb et al., 1997).

The presence of solvent in the sample would contribute to the variances noted in the elemental analysis with possible increases in the carbon and hydrogen contributing to the overall percentage in the sample sent away for analysis, whilst at the same time decreasing the nitrogen and sulfur total. Interestingly it is now recognised that the methanol peak noted in the original NMR spectrum in Fig 2.5 would have a similar effect to the obtained elemental analysis, however the amount of salt within the sample most probably was too great and lowered all the found values in Table 2.2.

With the knowledge that the sample still retains the solvents, a new calculation was carried out by adjusting the theoretical values of the elemental analysis to include the solvents in the final percentage. This manipulation was able to bring theoretical value of BALC to within 0.6% of the achieved BALC percentage by factoring in contributions from impurities. Not only is there a salt contribution to the elemental analysis, but a significant contribution to the sample impurity comes from solvent that has not been removed by evaporation. This demonstrates that the drying technique is inadequate and must be improved to increase confidence in the purity of the sample. Although these variances had mainly been accounted for and a reasonable theory has been put forward, further work was performed to substantiate these claims and increase the reliability of future synthesis of BALC and increase confidence in the findings.

The DCM/Acetone method was repeated but as the solvent factor seemed to be causing such deviation the product was not put through a column with the aid of additional solvents. The product was solubilised in acetone to remove salt. To remove the acetone the powder was left under vacuum overnight to dry. The sample was sent for elemental analysis and the results are shown in Table 2.5 below.
Table 2.5 Elemental analysis of BALC obtained from DCM/Acetone purification left on vacuum pump overnight

<table>
<thead>
<tr>
<th>ELEMENT</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Theory</td>
<td>41.30</td>
<td>5.00</td>
<td>7.30</td>
<td>18.10</td>
</tr>
<tr>
<td>% Found 1</td>
<td>40.85</td>
<td>4.96</td>
<td>7.25</td>
<td>15.37</td>
</tr>
<tr>
<td>% Found 2</td>
<td>41.01</td>
<td>5.00</td>
<td>7.26</td>
<td>15.59</td>
</tr>
</tbody>
</table>

The results shown in Table 2.5 demonstrate that all the deviations are within 0.4% except that of sulfur. However, as shown in the NMR spectrum from a sample with longer vacuum time, the solvent is still present, so the carbon and hydrogen results could be elevated. BALC is also very hydrophilic, potentially hygroscopic and in the acidified form will be able to absorb water from air (Bechaouch et al., 2003). Additional knowledge that freeze drying still leaves 2-5% water in the sample (discussed in Chapter 3), highlights that water must be factored in as a variable in the calculation adjustments.

The DCM/Acetone method was repeated again with no column chromatography. In this case the sample was left under vacuum for a period of 48 hours. The sample was analysed by $^1$H NMR and elemental analysis.
Fig. 2.16 $^1$H NMR of BALC sample purified in DCM/Acetone and left under vacuum for 48 hours.

With the use of acetone to solubilise BALC, there is confidence that the final product does not contain any salt. The spectrum in Fig. 2.16 again shows that BALC has been made, however even after 48 hours of drying under vacuum, there still seems to be a strong acetone peak at 2.22 ppm. BALC appears prone to retaining these solvent peaks. This could be because of the viscous BALC solid preventing solvent from evaporation. The resulting acetone could be trapped within the structure and requires more time to evaporate. The acetone will have an effect on the resulting elemental analysis shown below in Table 2.6.
Table 2.6 Elemental analysis of BALC from DCM/Acetone with 48 hours vacuum

The results from the elemental analysis in Table 2.6, appear to be the closest thus far within terms of value deviation from the theoretical. Therefore the need to continue drying the sample becomes apparent; however heat should not be applied as it may cause polymerisation between the acrylamide groups of the monomer (Stevens, 1999). An adjustment calculation was performed to see (if by including acetone and water) in the theoretical calculation the deviation be brought within 0.3%.

Table 2.7 Inclusion of water and acetone into theoretical elemental analysis calculation for DCM/Acetone sample with 48 hours drying
The results from Table 2.7, show that by considering residual impurities such as the organic reaction solvents and water, all the deviations can be brought within 0.3% of the found result. These are the closest results obtained and add further support to the argument that the product BALC is being synthesised.

Additionally the remaining solvent and salt should not affect any future reactions with BALC as they are in low quantities. Their presence will be reduced further when BALC is dispersed in the solvents in the synthesis of microspheres to be discussed in Chapter 3. Also salt, as an inert structure, along with the mentioned solvent, will be unable to interfere with the polymerisation and cannot be incorporated into the physical structure of the drug delivery device (Stevens, 1999).

However, a problem with the method is that although the results become close to the theoretical, insufficient amounts of product is being retrieved with the use of acetone as discussed previously in this section. For this reason, the ethanol method described in section 2.3.4 was devised to yield larger amounts of the product whilst attempting to remove salt and solvent from the equation.

### 2.8 Conclusion

This chapter has focused on choosing a disulfide cross-linker that could be suitable for synthesising a redox sensitive polymer. Two were chosen and one of these had to be synthesised to be utilised in this research. The work so far has detailed the synthesis and multiple characterisation techniques used to produce BALC and multiple approaches to purifying the product. The NMR spectra along with mass spectrometry confirm BALC has been synthesised using an adapted method by Emilitri (2005) and colleagues. Using polar solvents, yields of 71% were achieved with the DCM/Ethanol method; however this value decreased with use of column chromatography. The use of correlation spectroscopy seems to reveal new information compared to the data obtained to Emilitri (2005) and colleagues in reference to the carbon numbering.

The synthesis was performed multiple times. For each repetition an identical NMR spectra was achieved showing the reproducibility and robustness of the process. This allows confidence in producing product repeatedly for future work. The elemental composition data also aligned with the other characterisation techniques by discovering the effects of the remaining solvent after rotary evaporation and the production of salt in the
synthesis of the product. The solvent effect was corroborated by the NMR and when calculated it offered the only means to ensure that the calculations fell within the accepted deviation. With the elemental analysis data now complementing the other techniques, it can be confidently stated that BALC has been synthesised.

The work described in this chapter set out to produce the cross-linker BALC, with the characterisation data supporting the use of the newly developed method for BALC synthesis. This method allowed repeatable synthesis of BALC, and most importantly synthesis in large enough quantities to permit the investigations reported in the remaining chapters of this thesis. An extraction method was also developed that was able to remove the majority of impurities associated with the synthesis providing a relatively clean product. However it must be stated that the objective of this thesis was not to produce a pure monomer, but the aim was to produce a monomer to a clean enough standard that could be used in future investigations. This was ultimately achieved.

This study has yielded a water soluble disulfide cross-linker which was confidently synthesised and characterised. The product made in this chapter was used in further work in this thesis to produce a redox sensitive drug delivery device. This thesis will focus on the incorporation of these disulfide cross-linkers into potential drug delivery devices and their relevant properties.
3 Synthesis and Characterisation of Novel Bioresponsive Microspheres

3.1 Introduction

Hydrogels have received particular attention from biomaterial scientists since the innovative work of Wichterle and Lim in 1960 on cross-linked pHEMA hydrogels (Wichterle and Lim, 1960). Hydrogels are hydrophilic polymeric networks which can retain large amounts of water. This amount of water can be anywhere from 10-20% up to thousands of times their dry weight (Hennink and van Nostrum, 2012; Hoffman, 2012). In the presence of water, a hydrogel has the ability to hydrate hydrophilic groups or domains within the polymeric network thus creating the hydrogel structure (Hennink and van Nostrum, 2012). Due to their high water contents and soft consistency (similar to natural tissue) hydrogels resemble living tissue more than any other type of synthetic biomaterial (Peppas et al., 2000). These factors contribute to their good tissue compatibility and has led to their use as contact lenses, linings for artificial hearts, scaffolds for tissue regeneration as well as drug delivery devices (Michalek et al., 2010; Kopecek, 2009; Van Vlierberghe et al., 2011).

Hydrogels are composed of homopolymers or copolymers with cross-links present to avoid dissolution of the polymeric network in the aqueous phase (Peppas et al., 2000; Hennink and van Nostrum, 2012). There are multiple methods that can be used to cross-link the hydrogel, including chemically bonded cross-linking methods which covalently link the polymer network together. An example would be the conversion of hyaluronic acid to form aldehyde functional groups which are used to cross-link with the amino groups of a water-soluble chitosan derivative (Tan et al., 2009). Hydrogels can also be formed through physical cross-linking; here the hydrogel is held in place by secondary forces such as hydrogen bonding or hydrophobic forces with an example being amphiphilic block copolymers (Rösler et al., 2012; Prestwich et al., 1998). An efficient and widely used method of chemically bonded cross-linking is the use of free radical polymerisation.

Free radical polymerisation may be used to form a hydrogel by the addition of free radical building blocks which adds monomer units leading to chain propagation and hence chain growth.
Fig. 3.1 Scheme showing the addition of the monomer radical (R) to the vinyl molecule, followed by successive additions of further monomers to the available radical showing the propagation reaction (X = substitute group).

Fig 3.1 shows that once an initiator creates free radicals, it reacts with the polymerisable group which in the majority of cases are unsaturated vinyl groups (Kretlow et al., 2007). If both of the co-monomers possess the same functional group, then there can be an even distribution of both reactants throughout the system (Ning et al., 2012). This method of cross-linking maintains the structural integrity of the hydrogel, allowing swelling without its dissolution. A benefit of the use of free radical polymerisation is the abundance of literature on existing hydrogel systems synthesised using this method. There are multiple initiator systems that can be used and different ways of creating the free radical (Stevens, 1999).

Free radical polymerisation is employed in the synthesis of DC Bead, with its main polymer component being functionalised PVA. PVA is routinely copolymerised with AMPS, however this could be substituted for other vinyl monomers such as the redox sensitive material described in Chapter 2. With this in mind, free radical polymerisation was the method chosen to cross-link the novel hydrogel system with disulfide cross-linkers. Such a system would have the potential to perform as other embolic systems such as DC Bead but at the same time in a redox environment undergo macroscopic responses that could alter the structure and dimensions of the microspheres (Ulijn et al., 2007).

This chapter focuses on the synthesis of a potentially bioresponsive microsphere for the purposes of chemoembolisation therapy. The work detailed below combine’s disulfide cross-linkers discussed in Chapter 2 to form a redox responsive microsphere to treat hypoxic solid tumours. The synthesis of the smart hydrogel was undertaken using a method of reverse suspension polymerisation to form the microspheres, allowing homogenous dispersion of the copolymers throughout the structure. The microspheres were characterised and their mechanical properties tested with in vitro studies.
3.2 Materials

Ethyl acetate, n-butyl acetate and acetone were purchased from Romil UK Ltd (Super purity solvent grade). The cellulose acetate butyrate (CAB) was from Sigma-Aldrich UK. The Nelfilcon B Macromer was manufactured by Biocompatibles UK Ltd while N,N’-bis-(acryloyl) cystamine (BAC) and 2′2′-azobis (2-methylpropionamide) dihydrochloride were purchased from Sigma-Aldrich UK. Balc was synthesised as described in Chapter 2 of this thesis. 2-Hydroxyethyl methacrylate (HEMA), acrylic acid, sodium borohydride (NaBH₄), 5,5′-dithiobis-(2-nitrobenzoic acid), dl-dithiothreitol (DTT) were also purchased from Sigma-Aldrich. Hydrochloric acid was purchased from Fisher Scientific. Frozen section compound (FSC-22) was purchased from Leica Microsystems.

3.3 Methods

3.3.1 Synthesis of BAC Beads

The synthesis of the beads was based on a method used to manufacture DC Bead (Lewis, 2008). A 1 litre jacketed reaction vessel was filled with 600 g n-butyl acetate and 11.5 g CAB 10% w/w in ethyl acetate. This was stirred for 30 minutes to allow sufficient mixing and for the organic phase to be purged with nitrogen. An over-head stirrer was set to 400 rpm and the vessel was maintained at 30°C. After the organic phase had been purged, nitrogen was placed above the oil phase to form a N₂ blanket.

During the purging of the organic phase, the aqueous phase was prepared. The aqueous phase contained 130 g of water. The BAC was dissolved in the amount of water set aside to make up the aqueous phase. The amount of BAC monomer added was 400 mg, 1 g and 2 g to synthesise the 1.7, 4 and 8% microsphere respectively. To increase the solubility of the BAC in water the solution was heated to 70°C. The BAC solution was then added to 100 g PVA macromer solution (21% w/w) and mixed thoroughly. The macromer was manufactured in-house by a process in which N-acryloylaminoacetaldehyde (NAAADA) is attached to PVA chains by transesterification (Lewis, 2008). The thermal initiator 2′2′-azobis (2-methylpropionamide) dihydrochloride (1.3 g) was mixed with the BAC and macromer. The aqueous phase was then quickly added to the organic phase and the temperature of the vessel was set to gradually increase to a temperature of 80°C. The reaction was allowed to continue for 2 hours.
Once reaction was complete the organic phase was removed from the vessel leaving the newly formed microspheres. A cleaning process to remove the organic phase was carried out by adding 125 mL ethyl acetate to the microspheres and was allowed to stir for 10 minutes. After each stirring, the solvent was removed. This was repeated twice and on the third wash the ethyl acetate was added and allowed to stir for 5 minutes before 250 mL of acetone was added to the solution. The ethyl acetate in this instance acted as a buffer to prevent the beads clumping together as the acetone was added, which causes dehydration of the beads. This was allowed to stir for 10 minutes before the solution was removed. Then an additional 250 mL of acetone was added to the beads and allowed to stir for 15 minutes. The solution was removed and another 250 mL of acetone was added to the beads and was left for a minimum of 30 minutes. The solution was then removed and a final 250 mL of acetone was added and allowed to stir for 1 minute to prevent the microspheres from settling and the solution, including the microspheres, was removed and placed in a sintered funnel. The acetone was removed via a pump and the collected microspheres were washed with purified water in order to remove the acetone from the microspheres and to hydrate the beads fully. Once the beads were fully washed they were passed through a number of different size stainless steel sieves (Fisher Scientific UK) in order to separate the BAC beads into different size groups. The beads were passed through a sieve stack with the assistance of a recirculating water loop pressurising the beads through the sieves. The microspheres were hydrated in water for characterisation studies, see section 3.4.

3.3.2 Synthesis of BALK beads

3.3.2.1 High Initiator BALK Formulations

After the development of the BALK monomer (Chapter 2), the monomer was used to form cross-linked microspheres. A similar method to that described in section 3.3.1 was used to create BALK microspheres. The difference in the two procedures is that the BALK was substituted for the BAC monomer. As a direct result no heating was required to dissolve the BALK in water.

Various amounts of BALK were added again to each microsphere formulation therefore giving different degrees of incorporation of the BALK. After synthesis and
hydration the microspheres were analysed using characterisation techniques described from section 3.3.3 onwards.

### 3.3.2.2 Low Initiator BALC Formulations

Further BALC formulations were synthesised using the method described in section 3.3.2.1. The size of the glass reaction vessel was reduced from 1 litre to 500 mL. The initiator concentration was lowered by a factor of 10, which including the vessel change, equalling 0.065 g of thermal initiator added to the low initiator BALC formulations. A range of formulations were synthesised using the modified method (31, 45, 60 and 80% BALC beads). All microsphere formulations used in this thesis are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Sample Entry</th>
<th>BAC or BALC formulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation 1</td>
<td>0% BAC *</td>
</tr>
<tr>
<td>Formulation 2</td>
<td>1.7% BAC *</td>
</tr>
<tr>
<td>Formulation 3</td>
<td>4% BAC *</td>
</tr>
<tr>
<td>Formulation 4</td>
<td>8% BAC *</td>
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<tr>
<td>Formulation 5</td>
<td>0% BALC *</td>
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<tr>
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</tr>
<tr>
<td>Formulation 13</td>
<td>60% BALC **</td>
</tr>
<tr>
<td>Formulation 14</td>
<td>80% BALC **</td>
</tr>
</tbody>
</table>

Table 3.1 Microsphere formulations of BAC and BALC samples used in this thesis. *High initiator (10 mg.mL\(^{-1}\)) BALC formulation. **Low initiator (1 mg.mL\(^{-1}\)) BALC formulation

All microsphere formulations were analysed and characterised using techniques described in section 3.3.3.
3.3.3 Elemental Analysis

The elemental analysis of both the BAC and BALT beads were undertaken by Medac Ltd where combustion analysis was performed on the samples. The beads were prepared by taking 1 mL of each formulation, placing them in a glass vial with as much water as possible being removed. The beads were then frozen and placed on a Copley Heto drywinner, freeze dryer for 24 hours. The vial lids were modified with a semi-permeable membrane allowing removal of water but retaining beads with the vials. The dried beads were sent to Medac Ltd for elemental analysis. A minimum of 200 mg in each vial was prepared for analysis.

The microspheres were tested for carbon, hydrogen, nitrogen and sulfur (C, H, N & S) (N=2). The combustion analysis was performed on a Thermo EA1108 or FlashEA1112 series elemental analyser. The accuracy of the results were noted to be within a ±0.30% absolute and the detection limit for each component is <0.1% according to the service provider.

3.3.4 Sizing of Microspheres

The sizing of beads was performed by taking a microsphere formulation and placing 1 mL in a glass dish. The 1 mL of beads was then placed under an Olympus BX50 microscope with an attached ColorView III camera and sizing was performed manually using the sizing tool of AnalySIS software (Soft imaging system GmbH). A population size of 200 microspheres was chosen at random throughout the dish with the diameter measured. The dish was moved around its axis to ensure that the same beads were not measured twice.

3.3.5 Investigation of Effect of Reduction on Bead Size

In order to evaluate the effects of a reducing environment the BAC beads were placed in a glass vial and 1 mL of 4% NaBH$_4$ was added. The vials with microspheres were placed in a water bath at 37°C and left for one hour. After one hour a repeated process of washing with deionised water (20 mL) was employed to wash out all the NaBH$_4$. This process was carried out 5x to remove the reducing agent. Once the beads were hydrated in water they were placed under the microscope again and another 200 measurements of the diameter
were recorded. To demonstrate a statistically significant change in bead diameter, the same batch of beads were measured before and after exposure to the different environments. The two different sizing populations were compared using a Mann-Whitney test. This is a non-parametric test which was used as the data was not normally distributed, proven through an Anderson-Darling normality test. Levene’s test proved equal variance and the Mann-Whitney test was used as there were only two factors being compared. BALC beads were reduced using dithiothreitol (DTT) in place of NaBH₄. The same washing steps were employed and the beads were sized.

3.3.6 Qualitative Analysis of BAC or BALC Monomer Incorporation into the Beads

After synthesis of the novel beads an experiment was performed to characterise and prove that either the BAC or the BALC had been incorporated into the bead structure. An adapted method from Dai (2010) was used. In this method 1 mL of the BAC or BALC beads was placed in 2 mL of water. 1 mL of a control PVA macromer beads was prepared in the same manner. The beads had 1.5 mL of NaBH₄ added to the vials. The vials were then placed in a water bath at 37 ºC and left for one hour. 5 M HCl (200 μL) was then added to the reaction mixture to consume the remaining NaBH₄ then 1 M NaOH was added drop-wise to the solution to neutralise the HCl. The solution was raised to pH 8 with the use of NaOH. At this point 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellmans reagent) was prepared at a concentration of 0.08 mg.mL⁻¹ (Dai et al., 2010). DTNB solution (3 mL) was added to each vial and left to incubate for 30 minutes in the water bath at 37ºC. After 30 minutes the samples were removed and were tested using a Varian Cary 50 UV/Vis spectrophotometer scanning the range of 350- 450 nm for the NTB anion.

3.3.7 Water Content of Beads

Gravimetric analysis was used to establish the water content of all the various formulation of beads. One gram of the beads was weighed out on pre-weighed aluminium trays. Excess water was then pipetted away from the beads, being cautious not to remove water from the bead itself. This was done in triplicate for each formulation. The beads were placed in an oven at 60ºC under vacuum. The beads were then weighed repeatedly until there was no change in their weight. This point was taken as the dry weight of the microspheres.
3.3.8 Synthesis of BALC Beads with other Monomers.

BALC can also be used to create copolymer microspheres with the use of alternative monomers. The microspheres were made using an equivalent method to that described in section 3.3.2.1 for the BALC formulations, with the PVA being substituted in one study for 2-hydroxyethyl methacrylate (HEMA) and in another study using acrylic acid.

3.3.9 Energy Dispersive X-ray Spectroscopy-sulfur Mapping (EDX)

EDX was used to map the sections of the disulfide cross-linker throughout the bead. The BALC bead sections were prepared firstly by obtaining 10 µm sections from the centre of the beads with the use of a cryostat (Leica CM1860). This was prepared by freezing BALC beads in a PVA gel (FSC-22) on a small metal stub. The stub, was placed in a cryostat and 10 µm sections cut. The sections were mounted on to a glass slide and as the sample is electrically non-conducting, sputter coating with vacuum–evaporated carbon was applied with a Quorum Q150T sputter coating system. The coated samples were placed in the SEM chamber (Zeiss Sigma FEG) and viewed using an accelerating voltage of 10 KeV. Using the X-ray spectrometer attached to the SEM, the elements within the hydrogel were mapped.

3.4 Results and Discussion

3.4.1 BAC Beads

DC Bead is a clinically successful product (Lewis and Holden, 2011) and for this project it was proposed that DC Bead be used as the starting point for the research with the knowledge that small changes in the composition of the bead could yield vastly different properties (Heaysman, 2009). Therefore in this study, the AMPS group was substituted with the redox sensitive monomer BAC (Fig 3.2). The BAC microspheres were produced within a 1 litre jacketed reaction vessel as described in the methods section 3.3.1. BAC is a bis-acrylamide with a disulfide linkage in its main chain.
Fig. 3.2 Schematic of proposed substitution of AMPS found in DC Bead formulation with disulfide cross-linker BAC.

BAC has two acryl groups either side of disulfide chain, and it is assumed that these will allow facile incorporation of the cross-linker to the PVA macromer with the intention of forming a spherical, insoluble bead. BAC was chosen as the starting cross-linker, to be used as a redox sensitive material incorporated in the bead structure. Previous work with BAC has demonstrated its utility in gene delivery as a redox sensitive material and that the cross-linker can react under hypoxic conditions (Christensen et al., 2007).

The difference between the synthesis of the redox sensitive beads and DC bead is the substitution of the initiator potassium persulfate (KPS) for a thermal initiator. The disulfide cross-linker incorporation into the bead was determined by sulfur analysis. However, KPS possesses sulfur in its structure and would therefore act as another source of sulfur in the beads. To limit the only source of sulfur in the beads to that of BAC, the initiator system was changed from the redox initiation system to the thermal initiator 2'2-azobis-(2-methylpropionamidine) dihydrochloride (Fig. 3.3).
With the introduction of a new initiator system, the synthesis of the beads could be adversely affected by incomplete conversion of the monomers and the speed at which the reaction takes place could be severely altered (Wieme et al., 2009). It was known that polymerisation of DC Bead using the redox initiators would take 3 hours at 55°C (Lewis et al., 2006b). Initiator 2,2’-azobis(2-methylpropionamidine) dihydrochloride has a half-life around 10 hr at 56 °C, which has a rate constant of $1.92 \times 10^{-5}$ (Denisov et al., 2005). At 70°C, the rate constant is $1.35 \times 10^{-4}$, showing over a 7 fold increase in the rate of reaction with raised temperature with a half-life of 1.5 hours (U. Rojas Wahl et al., 1998). Pre-engineering runs demonstrated that 76°C formed beads within 1 hour. As a result of the calculations the decision was made to perform the synthesis of the beads at 76°C.

A control bead was manufactured which was composed of macromer only, where no cross-linker was added, and in which the NAAADA groups on the macromer react with one another eventually forming beads. However, based on the molar calculations shown in Appendix II, the chances that the NAAADA groups would come into contact to bind with each other were initially thought to be low.

The calculation reveals that there are over 1500 repeating units of PVA and there are only 7 NAAADA groups available to bind with each other. It would therefore seem unlikely to form a bead based on the sparse number of NAAADA binding groups available. Previous work by Heaysman (2009) however, has shown that the synthesis of PVA macromer only beads was possible and reproducible. This demonstrates that this low amount of cross-linking is sufficient to render the structure insoluble.

Once this bead was produced it was termed as the 0% cross-linked bead and was the starting point and comparison for future experiments. From this point BAC was added to the bead formulation at certain weight percentages to achieve various levels of cross-
linking within the beads with the aim of understanding how differing amounts of cross-linking would affect the property of the beads. The composition of DC Bead can be seen in Fig. 3.4 A, where 53% (21 g) of the bead is made up of PVA macromer with 44 wt% (17.5 g) being made up of AMPS. The whole composition gives a total solid weight of 39.8 g making up approximately 169 mL of aqueous phase. This gives a ratio of oil to aqueous phase of almost 4:1.
Fig. 3.4 A) Pie chart showing the composition of DC Bead (Lewis, 2008). B) Pie chart showing the composition of the 0% Cross-linked bead, comprising of 21 g macromer and 1.3 g initiator. C) A pie chart showing the composition of a 2 weight percent BAC bead [Original in colour].

This ratio alters in the manufacture of the 0% BAC bead whereby the AMPS is removed from the formula, leaving 22.3 g solid weight content. It was of interest to see at this new decreased ratio of solid weight content to water, whether the beads would polymerise or whether the new ratio would have a negative impact on the synthesis and
polymerisation of the control beads. Fig. 3.4 B, shows the composition of the control beads with 94% of the bead comprising entirely of macromer. Although the percentage of the thermal initiator has increased from 3% to 6%, there has been no change in the quantity (1.3 g) added to the system. This percentage change is due to the removal of AMPS from the system, lowering the total aqueous volume and increasing the initiator contribution to the system.

With successful polymerisation of the control beads, a range was established whereby beads could be produced with a solid weight between 22.3 and 39.8 g. Once acquainted with the method of producing beads, an attempt was made to produce 4% cross-linked BAC beads. Initially a weight percentage of BAC was dissolved directly into the additional water and then the macromer. However, it was found that the BAC was very insoluble in water and the mixture turned highly turbid with clumps of insoluble white powder floating in the mixture, this has been observed by other investigations (Andac et al., 2008). A further investigation study showed that BAC has a solubility in deionised water of approximately 0.1 mg.mL$^{-1}$. Therefore it is unlikely to efficiently mix within the aqueous phase and potentially could partition out into the organic phase of the solution.

To increase the solubility of the BAC within the water, small amounts of ethanol were added directly to the BAC to aid in dispersing the cross-linker in solution with the knowledge that ethanol is miscible in water in small quantities (Aliyar et al., 2004; Swatloski et al., 2002). However, it took 9 g of ethanol to dissolve 500 mg of BAC. On that basis to dissolve grams of BAC required for synthesis necessitates the need for large volumes of ethanol and at this point the BAC would most likely partition out into the organic phase.

Another method to dissolve the BAC was used whereby the cross-linker was dissolved in water by heating. However, as BAC has a melting point of 121-125ºC, it was ensured this temperature was not exceeded or reached. This method proved successful, and after the mixture was dissolved in water, the solution was cooled to 30ºC and added to the macromer. The composition of a 1.7% cross-linked BAC bead can be seen in the Fig. 3.4 C. with an optical microscope image in Fig. 3.5.
The next step was to produce more highly cross-linked beads, therefore 4% and 8% cross-linked beads were manufactured. The 4% beads were produced in the same manner as the 1.7% beads, however as there was more BAC in the 4% beads, the temperature had to be increased to 75°C to dissolve all the monomer due to its saturation limit.

When producing the 8% BAC beads the temperature required to dissolve the BAC was 90°C. On cooling the BAC began to precipitate out at 45°C. When added to the reaction vessel and the organic phase, due to the heat, most of the BAC appeared to dissolve. Whether it remained in the organic phase or the aqueous phase is unknown. However at the end of the procedure small circular white soft lumps similar to the BAC monomer were removed from the vessel which demonstrates that there is a solubility issue involved with the use of higher amounts of BAC in the synthesis of the beads. It was also observed that after the beads were removed from the vessel and placed in water they aggregated together. However, the beads were shown to be easily separated from each other.
other. The beads may aggregate together partially due to BAC’s hydrophobic nature (Zeng and Pitt, 2005). This aggregation could prove problematic if the beads were ever to be administered through a catheter as the beads might stick together and occlude the device.

After synthesis, the beads were separated into their respective size ranges and the properties evaluated with the following techniques.

### 3.4.1.1 Elemental Analysis

The results of the combustion analysis are presented in Tables 3.2 A, B, C and D. It was the intention to use the technique to qualitatively determine the composition of the beads which were analysed for C, H, N & S content.
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Table 3.2 Elemental analysis of BAC cross-linked beads compared to the theoretical composition A) 0% BAC B) 1.7% BAC C) 4% BAC D) 8% BAC (n=2)
In the synthesis of BAC Beads, in addition to KPS, AMPS was also removed as the sulfonate group would directly affect the sulfur levels obtained from elemental analysis. In exchange of the initiator and AMPS, the only source of sulfur now pertained from the disulfide cross-linker. Looking at the results it can be seen that in the cross-linked beads there is a sulfur content and this suggests that BAC, being the only source of sulfur, has been successfully incorporated into the bead structure.

The elemental analysis results, however, do not consistently correlate with the theoretical results. In some instances the carbon and nitrogen content seem to be lower than theoretical values. This indicates that the results are skewed and that there could potentially be a slight error partially due to excess water being left in the bead on analysis or inhomogeneous BAC incorporation.

Literature review has shown that the freeze drying technique could be the cause for the beads retaining water (Chan et al., 2011). It has been shown freeze drying is neither robust nor efficient and samples do retain small amounts of water. The amount of water left in the sample can be around 3-4% of the total sample (Internal data of Biocompatibles UK Ltd). Although such small amounts are usually disregarded, in the case of elemental analysis, this could contribute to the deviation from the theoretical value.

However, water does not seem to be the only reason why the elemental analysis does not match; the theoretical nitrogen is much higher than that of the actual results. The mechanism of the thermal initiator, where 2 nitrogen molecules are lost in the radical formation, are taken into account in the calculations. The largest source of nitrogen to the system is the initiator which could signify that all the initiator is not being incorporated into the system. Studies with vinyl chloride suspension polymerisation describe that when an initiator is in excess, there can be a cage effect (Wieme et al., 2009). This cage effect occurs where there are too many free radicals in one area, and instead of incorporating into the structure, the radicals combine and terminate.

Calculation of the moles of initiator introduced into the system showed that at its highest concentration, a 1:6 ratio of initiator to available sites is given. This is in excess of the ratio usually used, with 1:75 being more common (Rikkou and Patrickios, 2011). Although this could potentially explain why there is much less nitrogen in the beads than first assumed, a molar calculation shows that the DC Bead uses a 1:8 ratio of initiator to available co-polymer reactive sites. The elemental analysis results of DC Bead show consistent correlation of the theoretical results against the actual with the high initiator ratio, although a different initiator is being used (Heaysman, 2009).
Different initiators can yield different results. When KPS was used to synthesise sub-micron polystyrene particles containing magnetite, the desired characteristics and properties were achieved (Mori and Kawaguchi, 2007). However, when a thermal initiator 2,2′-azobis isobutyronitrile (AIBN), similar to that used in the bead was applied to the same polystyrene particles, the properties of the bead were adversely affected (Mori and Kawaguchi, 2007).

All these factors could be contributing to the differences between the actual results of the elemental analysis and the theoretical. This gives vast scope to improve the technique in sample preparation, and provides the opportunity to experiment with different initiators and their quantities.

The sulfur content also appears to be slightly higher than the 0.3 variance range applied to results. It appears from the sulfur analysis that there is an increase in the amount of BAC added to the bead formulation as less nitrogen is incorporated due to the drop out of initiator; therefore there are higher levels of sulfur, and thus higher amounts of incorporated BAC.

These results seem to suggest more cross-linker could be incorporated into the bead structure, however the insolubility issues related with BAC still remain and could prove difficult to overcome.

### 3.4.1.2 Sizing of BAC Beads - Reduced Vs Non-reduced Beads

Once the beads were synthesised and separated into different size ranges by sieving, the beads were tested to determine if the BAC had been incorporated into the bead structure and subsequently were sensitive to reducing agents.

As already noted disulfides can be readily reduced to their thiol groups in the presence of a reducing agent. This cleavage of disulfide groups can lead to the appearance of tiny gaps or ‘windows’ within the bead structure, changing from a tight and rigid cross-linked network, to a more open network with space between the thiol groups (Quirk, 2007). It is thought that these beads will be highly rigid because of the nature in which a divinyl cross-linker polymerises (Gao and Matyjaszewski, 2009). Due to the cross-linker having two available reactive functions, a network like polymer will be obtained as the cross-linker can facilitate formation of various arm compositions from either moiety (Gao and Matyjaszewski, 2009). It is postulated in the BAC beads that the cross-linker will
initially bind to the available NAAADA groups and will build a network of BAC bound to itself, polymerising in various directions before it will reach the next available NAAADA group and produce the same result. The polymerised cross-linker will pull all the NAAADA groups together to form the final rigid bead.

The cleavage of BAC turns the bifunctional cross-linker into a more linear polymer (Rikkou and Patrickios, 2011). In conjunction with the gaps between the thiol groups, the formation of a more linear polymer will lead to an increase in the diameter of the beads. As the chains spread out they are not restricted by the rigidity of the disulfide group (Rikkou and Patrickios, 2011). The resulting polymer structure will also be more hydrophilic than the bifunctional polymer due to the increased hydrophilicity of thiol groups over disulfide groups (Floris et al., 2008). This increased absorptive capacity of the thiols will lead to an increased uptake of water, leading to an increased swelling of the beads, hence a larger increase in the diameter of the beads.

However, such a large increase in bead size is not necessarily an advantage. This size increase is well documented with the use of HepaSphere (Lewis and Dreher, 2012). The superabsorbent polymer microspheres can increase to 4 times their original size in the presence of human serum and are designed to adapt to the lumen of the arterial wall leaving no space between them and the wall surface (de Luis et al., 2008). Although there have been reports of successful embolisation of target vessels (de Luis et al., 2008), further investigations into the use of HepaSphere seem to show otherwise (Senturk et al., 2010). Due to its ability to expand, HepaSphere has been reported to cause high levels of vessel wall injury and compared to the other products has the highest levels of extravasation which can result in recanalisation of the blood vessel (Senturk et al., 2010). One can hypothesise that this is the effect of the pressure from the expansion of the bead. This is an effect which is not as pronounced with the use of DC Bead. Therefore, although an increase in size may be desirable, the optimum increase in size is not known and manufacturers must be mindful of this effect when designing products for embolotherapy.

With regards to BAC beads in a reducing environment, the hypothesis is that the disulfide groups should cleave in a reducing environment resulting in a swelling of the beads and an ultimate increase in their diameter. The method of measuring the sizes and exposing the beads to a reducing agent was performed as described in Sections 3.3.4 and 3.3.5. The reducing agent used was NaBH₄ which has been described as highly efficient in its reduction of disulfide bonds (Hansen et al., 2007). The results are presented in Fig 3.6 A, B, C and D.
Fig. 3.6 Sizing of BAC bead formulations before and after reduction, A) 0% B) 1.7% C) 4% and D) 8% (n = 200) [Original in colour].
Statistical analysis was used to determine whether the change in diameter of the beads was significantly different. A two sample Mann-Whitney test was used to reveal whether the median of the population was the same or different in the two groups of the 0% bead. An equal variance was assumed, through the use of Levene’s test. The results of a Mann-Whitney test shows that the null hypothesis can be accepted with the results giving a p-value = 0.88. Therefore any differences in the size of the control beads are not significantly different.

The Mann-Whitney test on the 1.7%, 4% and 8% beads all gave p-value <0.05 showing that bead size increase was statistically significantly. The results confirm that the BAC beads are responsive to the stimulus of a reducing environment and this response leads to a size increase in the diameter of the beads. The results show that the control beads are not altered in size in the presence of a reducing agent. This would suggest that the presence of BAC is responsible for this alteration and this confirms the original hypothesis.

The size increase in the BAC containing beads is approximately 200 μm throughout the different formulations. This appears to be independent of the amount of BAC added. This limited size increase could be due to restriction by the macromer or it could be due to the short chain lengths of the cross-linker due to the high amount of initiator used in the polymerisation (Chern, 2008). The shorter chains will be less flexible and won’t allow as much movement within the bead as a longer winding chain. Even after reduction, this intertwined network means that although the beads will swell, the chain entanglement will restrict the network increasing in diameter at a certain point i.e. 200 μm. This restriction may be a benefit. If the BAC polymerisation was linear like AMPS, when the disulfide groups cleave, segments of cross-linker could be released from the bead structure.

The results of the sizing indicate that the BAC beads could have the potential to respond to a reducing hypoxic environment. If the beads were to respond to the reducing environment of a tumour then they have the potential to induce a more efficient embolisation by increasing in diameter against the walls of the target vessel. Whether this leads to complications for a patient or if the beads increase within the vessel against the resistance of the vessel wall is unknown. However the results do provide the rationale for further investigation.
3.4.1.3 Qualitative Analysis of BAC Incorporation into the Beads

Although the elemental analysis indicates that BAC has been successfully been incorporated into the bead structure, it is desirable to corroborate this and also determine whether the cross-linker has remained intact within the bead structure. This was performed with the use of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) shown in Fig. 3.7.

![Chemical structure of 5,5′-dithiobis(2-nitrobenzoic acid)](image)

Fig. 3.7 Chemical structure of 5,5′-dithiobis(2-nitrobenzoic acid ) (Ellmans reagent).

DTNB is a chemical that can be used to quantify the amount of thiol groups within a sample (Chen et al., 2008). In this case it was used to qualify the presence of the disulfide groups within the bead structure.

When the beads are in a non-reduced state the disulfide groups should not react with the Ellman’s reagent. However, when the beads are reduced the thiol groups will cleave the disulfide bond within the DTNB to give 2-nitro-5-thiobenzoate (NTB’), which ionizes to the NTB^{2-} dianion in water at neutral and alkaline pH (Chen et al., 2008). The mechanism for the cleavage of DTNB can be seen in Fig. 3.8.
The NTB\(^{2-}\) produced a yellow colour which was used to confirm the incorporation of the BAC (King et al., 1999). Therefore DTNB was added to beads which were not reduced to see if a colour change was observed. As no colour change was noted, it implied that either the BAC was in its disulfide form or that no BAC had been incorporated. NaBH\(_4\) was chosen as the reducing agent as, unlike DTT, it can be easily removed from the system by the addition of acid (Hansen et al., 2007). After reduction, the reducing agent was thoroughly washed away, ensuring none was left in the beads, as the reducing agent could yield a false result by reducing the DTNB directly (Hansen et al., 2007). Testing with DTNB demonstrated an immediate colour change to yellow for all formulations containing BAC following reduction.

The use of DTNB can be used to quantify the amount of thiols as it is known that one mole of thiol releases one mole of NTB (King et al., 1999). The NTB\(^{2-}\) can be quantified using a spectrophotometer to measure the absorbance of visible light at 412 nm (Wright and Viola, 1998). This method of quantification was attempted with the BAC beads to determine exactly how much BAC had been incorporated in the beads. It was assumed that, according to the mechanism 50%, of NTB attaches to the bead. However, the results did not match those obtained in elemental analysis. The results were considerably lower than expected and were questionable. An explanation was proposed for the low result, which was that due to the spread of the BAC through the bead structure not all sites were accessible to the DTNB and as a result only the surface BAC could be measured using this method. This has been seen in studies where it has proven very difficult or nearly impossible to quantify hidden cysteinyl residues (Wright and Viola, 1998). The only way of accurately quantifying the amount of disulfide groups within the bead would be by opening the structure to make the reactive sites more accessible (Wright and Viola, 1998).
It is noted that the use of DTNB has been widely used in conjunction with linear polymers and accessible structures (Dai et al., 2010). The use of DTNB has been criticised as being insensitive to low thiol concentration levels and other methods have been proposed (Chen et al., 2008, Hansen et al., 2007). Regardless, the method was abandoned as a quantitative procedure and was used only in the context to qualify the inclusion of BAC.

Another experiment was run whereby the control beads were reduced and had DTNB added and this was compared against BAC beads that were treated in the same manner. The results can be seen in Fig. 3.9 below. The picture shows that BAC has been successfully incorporated into the bead structure. This also proves that the BAC is incorporated in its disulfide form as it does not reduce the DTNB when the beads have not been previously exposed to a reducing agent. Beads were removed from solution and assessed for any colour changes. It was determined that the beads do not possess any colour and it is the NTB$^{2-}$ dianion in solution that appears coloured.

Fig. 3.9 BAC beads vs control beads after reduction with addition of DTNB. BAC beads in the left hand vial. Control beads in the right hand vial. [Original in colour].

The use of BAC has been successful as proof of principle, demonstrating that it is possible to produce a reductive responsive bead and the bead itself could potentially be used to produce a more efficient embolisation of a target vessel. However this approach has limitations as the BAC that can be added to prepare beads is restricted to relatively small amounts due to its poor solubility in water. For the redox responsive beads to become a product on the market, the method to produce them has to be consistent and
reproducible and unfortunately with BAC that is not the case. This gives the incentive to use an alternative new cross-linker in the manufacture of the beads. A thorough literature search was conducted to yield a cross-linker with the dithiol entity but also with the advantage of water solubility. After an extensive search the cross-linker BALC was selected as discussed in Chapter 2.

3.5 BALC Beads

The BALC beads were synthesised using a method based on that used to produce BAC beads. A fundamental difference was that the BALC was very soluble in water and was easily dissolved within the aqueous phase, removing the need for heating. Once the BALC was dissolved within the macromer and initiator formulation, a visually homogenous aqueous solution was obtained. Even when the BALC content was increased to over 10 g, no difficulties were encountered in dissolving the cross-linker in the aqueous phase.

The aqueous phase was added to the organic phase where it immediately took droplet form. With the use of BALC no lumps or insoluble particulates were noticed in the reaction vessel. At the end of the reaction, beads were removed from the vessel, which were transparent. After washing, the new beads were separated into their respective size ranges for characterisation.

3.5.1 High Initiator Formulations and Elemental Analysis

The first formulations of BALC beads were synthesised using the same method and same cross-linker wt% as BAC beads. It was thought that by changing the redox responsive monomer to a more water soluble monomer would lead to better incorporation of all the monomers into the system as seen with AMPS and DC Bead. BALC beads underwent elemental analysis as previously described in section 3.4.1.1. The results can be seen in Table 3.3 A and B.
Table 3.3 Elemental analysis of BALC cross-linked beads with A) 0% BALC, 4% BALC, 15% BALC, 31% BALC (n=2) and B) Theoretical calculations of BALC beads factoring in loss of initiator and retention of water.

The elemental analysis shows successful inclusion of BALC within the microspheres. The original theoretical results do not match the data returned by elemental analysis, however the method employed to dry the beads is the same method used to dry down the BALC monomer in Chapter 2. Therefore the beads most likely have retained a
degree of water within their polymer networks after freeze drying. Once recalculated, the actual results are much closer to the theoretical results in comparison to the BAC bead, with more results falling within the 0.3% variance. However the only element not falling within the accepted deviation is nitrogen.

In each formulation there is always a higher theoretical percentage of nitrogen than that actually found. The highest contributor to nitrogen content in all formulations is the azo initiator. However, after calculating its incorporation, it would appear that all the results fall within the standard deviation once a percentage of the initiator is lost from the reaction. In the case of the 0% BALC it would appear that more of the initiator is lost compared to the 31% formulation. It can be assumed that as there is more BALC within the 31% formulation, then there are more reaction points within the network. This loss of initiator in conjunction with the knowledge that there is a high monomer:initiator ratio shows that there is an excess amount of initiator within the system.

It is known that the production of free radicals in solution is never 100% efficient and as a result theoretical calculations should be adjusted accordingly (Lorand, 2009). In the polymerisation of acrylate monomers such as methyl methacrylate, initiator efficiencies as low as 50% have been documented and this low initiator efficiency has been demonstrated with azo initiators (Arnett and Peterson, 1952; Bevington, 1955). A variety of reasons in the past have been put forward to rationalise this loss of initiator from the system, such as loss because of decay of the excited state, non-radical reaction pathways (Lorand, 2009). However these reasons can only partially account for the loss of initiator, and the cage effect may further explain the incorporation inefficiency (as mentioned in section 3.4.1.1). In a gas phase formed radicals may spread apart in different directions, however in an aqueous phase, likened to the synthesis of BALC beads, the radicals will exist side by side, caged in by surrounding solvent molecules (Denisov et al., 2005). In this caged state the pair of radicals may undergo collisions with other radicals before breaking apart (Lorand, 2009). In the case of the BALC bead synthesis, this cage effect may increase in the presence of excess initiator and therefore lead to a decrease in radical production with more collisions.

Although not all the initiator is reacted during the synthesis, based on the elemental analysis data the initiator contribution the bead composition is relatively low and can be accounted. The majority of BALC and PVA macromer quantities added to the reaction remain unchanged and this would suggest that it is the loss of the initiator that affects the calculation.
These results quantitatively show that the targeted formulation microspheres can be consistently achieved with this method. It is postulated that due to the solubility of BALC, all of the cross-linker is dispersed in the aqueous solution only. The results show that BALC has been incorporated into the bead structure, with Table 3.3 A and B showing that more disulfide groups are held within the structure as more cross-linker is added. With the only source of sulfur within the beads a direct result of BALC inclusion, incorporation of BALC could be quantified within the beads (See Appendix II).

The results show that the incorporation of BALC within the beads is efficient. An attempt was made to make a formulation containing a higher percentage of BALC for further characterisation studies and to determine what differences are present when BALC becomes the major component of the bead. The synthesis was carried out according to the method detailed in section 3.3.2.1.

On addition of the initiator solution to an initial attempt of the 60% BALC formulation, a white precipitate was observed. The precipitate spread throughout the reaction and soon after, as the temperature increased, a polymerised aggregate was obtained from the reaction. Instead of forming microspheres a large agglomerate was obtained. It is assumed that the excess initiator is the cause of the anomaly and can be explained by a theory of nucleated polymerisation.

Although both BALC and macromer are water soluble, BALC has lower solubility. With the addition of high quantities of BALC the aqueous phase becomes saturated, although still homogenous at this stage. The solution may be becoming supersaturated with the addition of excess amount of initiator to the reaction, and an increase in the temperature (which may be an increase above the upper critical solution temperature) (Bang et al., 2013). Supersaturation is used to describe a solution that contains more of a dissolved material than can be dissolved under normal circumstances and is only highlighted when a condition of the solution is altered i.e. temperature in the case of BALC synthesis. Supersaturation has been crucial for crystallisation as it allows precipitated molecules to find each other and start a critical mass that nucleate around the mass (Vogl et al., 2012). Nucleation polymerisation describes the growth of filamentous structures in extremely close proximity (Cohen et al., 2011). It is usually used in molecular biology to describe the assembly of a polymeric structure where a small monomer such as actin can aggregate, stabilise and initiate rapid polymerisation. Nucleation polymerisation has been seen in poly-acrylamide hydrogel systems where a multitude of small crystallites were formed by
nucleation and after polymerisation porous hydrogel structures were obtained (Grassmann and Löbmann, 2004).

Nucleation occurs mainly in heterogeneous samples and this is because an impurity or in this case, supersaturated sample offers an interface where the reaction can commence (Schlüter et al., 2012). It is noted that nucleation will commence as soon as the concentration of the polymer becomes supersaturated, however as nucleation occurs particles are formed, growing outwards from their central core, which relieves the reaction of being in a supersaturated state and therefore nucleation will occur at the beginning of a reaction (Flake et al., 2011).

In the case of BALC it would appear that the resulting aggregates generated in the initial 60% BALC formulation could be a direct consequence of the excess amount of initiator. In the presence of high quantities of BALC, a supersaturated solution could be created and in the presence of free radicals this could lead to nucleation polymerisation. To produce a high percentage BALC formulation, the cross-linker quantity cannot be reduced. However, as discussed the initiator is in excess and this can be reduced to try to avoid producing a potential supersaturated reaction. Thus as discussed in section 3.4.1.1, a drop in initiator concentration would be favourable to firstly bring the initiator ratio in line with other hydrogel systems and to remove the possibility of nucleation.

3.5.2 Low Initiator Formulations and Elemental Analysis

To produce higher BALC containing formulations the monomer:initiator ratio was reduced 10 fold to 1:60. This was applied to all the new formulations of BALC here on in. On addition of the lower quantity of initiator to the monomers in the reaction vessel, any form of precipitation, hence supersaturation, was not observed. The following polymerisation produced spherical microspheres as before. The potential BALC beads were sent away for elemental analysis and the results can be seen in Tables 3.4 A and B
The results of the elemental analysis demonstrate firstly that BALC can be incorporated into a hydrogel structure and more importantly that high percentage formulations can be synthesised with the altered method. Therefore, decreasing the initiator concentration has yielded an efficient method to synthesise highly cross-linked microspheres. The results of the 31% BALC formulation in Table 3.4 A compared to that in Table 3.3 A demonstrate slight differences in values, with higher sulphur and carbon in the low initiator formulation. However, these slight variations can be accounted for with the reduction in the initiator level used during synthesis. In comparison to the composition of other BALC formulations, these are very low deviations. Therefore in the elemental analysis data both sets of beads are very similar in composition.

The results confirm the justification for altering the bead synthesis method as it allows for a larger range of redox sensitive microspheres. The problems encountered early on in the synthesis of the microspheres have been removed and a more reliable method has been established providing accurate results. However, it was of great interest to investigate any differences in properties between the high and low initiator formulations as they could possess slightly different polymer network arrangements. The high and low initiator BALC microspheres were put forward for further investigation, the results of which are documented below.
3.5.2.1 Sizing of BALC Beads - Reduced Vs Non-Reduced Beads

The sizing of the beads was undertaken in the same manner as in Section 3.3.4 and 3.3.5. The BALC beads were sized before and after being placed in a reducing environment. The results are presented in Fig. 3.10 A, B, C, D and E.
Fig. 3.10 Sizing of BALC Bead formulations before and after reduction A) 0% BALC B) 4% BALC C) 8% BALC D) 15% BALC E) 45% BALC (n=200)
<table>
<thead>
<tr>
<th>BALC Formulations</th>
<th>0%</th>
<th>2%</th>
<th>4%</th>
<th>8%</th>
<th>15%</th>
<th>45%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Reduction</td>
<td>705 ± 57</td>
<td>856 ± 62</td>
<td>810 ± 86</td>
<td>869 ± 79</td>
<td>745 ± 146</td>
<td>847 ± 71</td>
</tr>
<tr>
<td>Reduced</td>
<td>704 ± 62</td>
<td>955 ± 47</td>
<td>938 ± 89</td>
<td>1003 ± 76</td>
<td>933 ± 114</td>
<td>1137 ± 84</td>
</tr>
<tr>
<td>% Change</td>
<td>-0.1</td>
<td>10.3</td>
<td>13.6</td>
<td>12.3</td>
<td>20.1</td>
<td>25.5</td>
</tr>
</tbody>
</table>

Table 3.5 Mean diameters of BALC Beads Pre and post reduction with percentage change (µm) (mean ± SD, n = 200).

Table 3.5 highlights the positive correlation between the percentage BALC in a formulation, and the size after reduction. Although there is overlap in some populations, statistical analysis showed that the medians of the populations were statistically different. A two sample Mann-Whitney test was applied, where the macromer only beads have a p-value > 0.05 allowing acceptance of the null hypothesis as there is no significant difference in medians between the two ranges. All the BALC beads give a p-value <0.05 allowing rejection of the null hypothesis showing a significant difference in the variances of the medians.

The results of the sizing mirror the results achieved with the BAC beads. The BALC beads also experience a significant diameter increase after being exposed to a reducing environment. The added benefit of the BALC beads, is that sizing could be performed on more highly cross-linked beads. This was not possible on the BAC beads due to the insoluble nature of BAC. The arguments put forward in Section 3.4.1.2 regarding increased embolisation efficiency, will be exactly the same here with the exception that as BALC is hydrophilic, more swelling could potentially be expected. It is suggested that this size change shown by BALC beads would be beneficial in the embolisation process, unlike the significantly larger increases in diameter experienced by HepaSphere® (Senturk et al., 2010). HepaSphere® is an expanding microsphere that increases to 4 times its original size in the presence of blood. Such a large expansion has been shown to cause severe vessel damage leading to recanalisation. The rigidity of the beads have been shown to increase, with a decrease in compressibility, when delivered to the target vessel (Jordan et al., 2010). The BALC beads, most likely due to their high initiator ratio leading to short chain lengths in the high initiator formulations, can expand to no more than 1/3 of their original size.
Such a small expansion is not likely to cause vessel damage and therefore recanalisation is not expected, while at the same time a more efficient embolisation is expected.

In the low initiator formulations as seen in the 45% BALC bead, an even larger increase in the expansion of the bead was noted. However this is still no more than a 25% increase, therefore even in the higher percentage formulations the size increase is no more than half the original dimension of the bead. Such a small increase should not heighten the chances of vessel damage. This expansion could be due to the longer polymer chains in the lower BALC formulations, however it is most likely due to the increase of the cleavable BALC monomer within the bead. As there is an increase in BALC content, there is an increase in cleavable bonds, resulting in a bigger increase. This effect is most likely limited in the lower percentage BALC formulations, because of the composition of the bead being mainly of the insoluble PVA macromer. However, as the PVA ratio begins to decrease, the limitation on bead size increase also decreases hence the larger increases in bead size. It was hypothesised that as the BALC content increases the beads actually have the potential to biodegrade due to the reduction of multiple disulfide bonds throughout the hydrogel. It would seem that in the higher percentage BALC formulations that size increases are also observed, however they do not increase indefinitely and still hold a spherical shape 24 hours after reduction. It was originally thought high percentage BALC formulations would immediately fall apart in the presence of a strong reducing environment; however it would appear that even with a low ratio of PVA macromer in the formulation this is not the case. This is shown by sizing of the 45% BALC formulation shown in Fig. 3.10 E. It was still possible to size the beads after reduction as they still hold a spherical shape. However after the initial 24 hour sizing changes in the appearance of the high percentage BALC bead formulations have been noted. Any sizing completed after 24 hours is documented in an extensive degradation study in Chapter 6.

### 3.5.2.2 Qualitative Analysis of BALC Incorporation into the Beads

DTNB was again used to confirm whether BALC had been incorporated into the bead structure. The beads were exposed to DTNB before and after reduction.
Fig. 3.11 Qualitative analysis of cross-linker incorporation into the beads with the use of colorimetric analysis. 4% BALT beads are on the left. 0% BALT is on the right of the picture. [Original in colour]

Fig. 3.11 demonstrates the reduction of 4% BALT beads next to a control bead. After being reduced, the NaBH$_4$ was washed away leaving the beads in water. DTNB was then added. The results showed that the BALT beads contain thiol groups, which are known to be present due to the reduction of the disulfide groups. However, this experiment also demonstrated that the DTNB was broken down by the thiol groups on the beads and not by any remaining reducing agent left in solution. This therefore proved that the washing away of the reducing agent was efficient. To confirm that all the reducing agent had been removed the DTNB solution was analysed by UV/Vis spectroscopy. Fig. 3.12 is a UV analysis of two samples. The first sample is DTNB in a non-reduced state represented by the blue line and the second sample is DTNB reduced to its dianion represented by the pink line. If reducing agent had been left in the solution then a peak would be observed at a wavelength of 410 nm for the NTB$^{2-}$ Dianion.
Fig. 3.12 UV/Vis spectroscopy of DTNB in the non-reduced and reduced state. The reduced state gives an absorption peak at 410 nm, while the non-reduced state gives a peak at 323 nm. (n=2) [Original in colour]

Although the peaks have passed the limit of quantification the graph can still be used to demonstrate the position of where the peaks lie. Fig. 3.12 shows the position of where BALC would appear on the graph (410 nm), and the position of the non-reduced DTNB peak equating to no BALC in the beads. UV/Vis analysis shows that as an increased amount of BALC is added to the bead formulations; in turn a higher dianion peak is achieved at 410 nm (Fig. 3.13). This directly relates to the knowledge that more BALC has been incorporated into the bead structure.
Fig. 3.13 UV/Vis spectroscopy of all BALC bead formulations, using a colorimetric method to qualify the incorporation of cross-linker into the beads. (n=2) [Original in colour].

The aim of the UV method was to qualitatively assess and to provide additional support to confirm incorporation of BALC within the microsphere structure. Visual assessment showed an increasing yellow colour with increasing incorporation of BALC. This is corroborated in Fig. 3.13 which demonstrated that as the amount of cross-linker added to each formulation increased, so did the UV absorption which is suggestive of more BALC incorporation into the bead structure. The trend also suggests that if more cross-linker was added to the bead synthesis then more BALC could be successfully incorporated.

3.5.2.3 Water Content of Beads

Analysis of the BALC beads was carried out to determine effect of BALC on the equilibrium water content (EWC) (Fig. 3.14). The amount of weight lost equates to the water content lost from the beads, which was measured using the method described in Section 3.3.7.
Fig. 3.14 Equilibrium water content analysis of all BALC bead formulations. (mean ± range, n = 3)

Fig. 3.14 is a study of the water content of the BALC beads. The results show the BALC monomer to be very hydrophilic, and instead of decreasing in water content as was expected, the lower BALC bead formulations actually increased in water content. The BALC beads seemed to absorb more water than the control beads suggesting BALC to be responsible. The 4% BALC beads absorbed the most water with EWC of 97.6 % ± 0.1 %, with a very noticeable drop from 97.2 ± 0.1 % to 92 % ± 0.2 % water content from 15 – 31% BALC beads. This indicates that although the BALC is hydrophilic, as the wt % of the BALC increases the water content of the beads will decrease. The same trend can be seen by the EWC carried out by Heaysman (2009), whereas increased AMPS were added to bead formulations, the water content began to increase until there was a drop in water content for the highest 100% AMPS formulation. Interestingly both BALC formulations of the 31% had identical water contents demonstrating again the reproducibility of the formulations.

However an anomaly is noted with the 45% BALC bead formulation as the water content increased far above that of the previous 31% formulation. The elemental analysis
shows us that the bead is composed of 45% BALC, however there seems to be a lower solids content of 95.5 % ± 0.1 %. Although BALC is itself hydrophilic, this result does not follow the trend shown in Fig 3.14.

The 60% and 80% BALC formulations define the trend: as the BALC contents increases, the water content decreases. The application of EWC analysis is used in conjunction with other methods to assist in establishing the amount of BALC incorporated in the bead. Determination of the solid content of the microspheres can be accurately used with elemental analysis results to determine the potential maximum loading capacity of the beads (Heaysman, 2009).

3.5.2.4 Synthesis of BALC Beads with Other Polymers

Using the method defined in section 3.3.8, BALC beads with comonomers other than PVA macromer were synthesised. The beads were not characterised in detail but are reported in more detail in Chapter 6.

3.5.3 EDX Sulfur Mapping

The elemental composition of a compound can be determined using EDX. The technique is founded on the principle that each element has a unique atomic structure and therefore the energy of X-rays differ based on the difference between the low and excited energy levels. This allows easy identification of the elements present in a sample being studied (Joy et al., 1986).

However, quantitative analysis requires measuring the line intensity for each element and comparing that against a calibrated standard. This requires high resolution with a high count ratio of the line of each element in the correct ratio to the other element in each sample. A thin sample is also favourable as the electron beam only has a depth of 1-5 µm and therefore would be improved spatial resolution with a thin sample with the beam passing through the sample and at the same time avoiding loss of absorption in bulk samples. However the samples were coated with carbon, therefore elevating the carbon levels in the EDX analysis. As the coating was performed with carbon, an element found in the bead, quantitative analysis of the results is not ideal. Therefore EDX was used qualitatively to confirm the presence and distribution of sulfur within the hydrogel.
Before analysis the beads were frozen in a cluster and placed on a cryostat and then 10 µm sections were cut out and mounted on a silica coated glass slide. The analysis of the 0% BALT bead can be seen in Fig 3.15.

The energies obtained from each element are measured in electron volts (eV) along the X axis with the number of x-ray counts measured along the y-axis. It is known that sulfur has a KeV of approximately 2.3 and can be analysed by EDX in poly vinyl systems (Yoshihara et al., 2010). The 0% formulation revealed no sulfur as expected (Fig 3.15 A). Therefore no mapping images of the elements were presented. Fig 3.15 B is a micrograph image of a section of the bead, however a smooth surface section is not obtained with noticeable gaps observed within the structure. The gaps in the bead are not a property of the microsphere structure, but are an artefact of the sectioning technique. These artefacts proved difficult to remove until the final formulation where a clean surface was presented in the 80% formulation electron image. Factoring in the effect of sectioning, these images show that the beads possess a homogenous internal structure. The analysis was performed on the 15%, 31% low initiator, 45% and 80% formulations. The results of the sulfur mapping can be seen in Fig 3.16, 3.17, 3.18 and 3.19.
Fig. 3.16 A) Point and I.D scan of 15% Bead. B) Electron image of 15% bead. C) Electron mapping of elements carbon, oxygen and sulfur.

Fig. 3.17 A) Point and I.D scan of 31% Bead. B) Electron image of 31% bead. C) Electron mapping of the elements carbon and sulfur.
Fig. 3.18 A) Point and I.D scan of 45% Bead. B) Electron image of 45% bead. C) Electron mapping of carbon, oxygen and sulfur.

Fig. 3.19 A) Point and I.D scan of 80% Bead. B) Electron image of 80% bead. C) Electron mapping of the elements carbon, oxygen and sulfur.
The 15% to the 80% formulations show an even distribution of sulfur throughout the microsphere. The original concern was that at small concentrations of BALC, the monomer would aggregate at NAAADA reactive ends, and that even in higher percentage formulations BALC would form concentrated areas of monomer aggregation while other areas would be void of the monomer. However, this is not the case and the disulfide monomer appears to be evenly distributed throughout all beads. This shows that the bead is homogenous and there is no phase partitioning.

Although the results cannot be used quantitatively, the Point and I.D scans show that as the percentage of BALC increases, so does the sulfur counts in the scan. This again correlates well with the elemental analysis data and the water content.

3.6 Conclusions

The characterisation techniques in this study have been used to prove that a disulfide cross-linker can be successfully synthesised and that it can be incorporated into a hydrogel bead retaining its functionality and redox sensitivity.

All the characterisation techniques demonstrate the incorporation of the disulfide cross-linkers into the bead structure, with the strongest evidence provided by elemental analysis which shows the actual amount of an element incorporated (Kumbar et al., 2003). The elemental analysis shows the efficiency of the synthesis method with very little percentage loss of the cross-linker added to the formulations, and this is realised by matching the theoretical with the achieved results. Encouragingly the optimisation of the elemental analysis method by lowering the initiator concentration improves the synthesis method further and allows for the polymerisation of high percentage BALC formulations. The EDX results further support the elemental analysis. Even though the data is not used quantitatively, the increase in BALC in formulations is recorded by the increasing X-ray counts of sulfur in the EDX scan. Interestingly the location of the redox monomer, highlighted by the sulfur, is also shown in the mapping images with a good distribution throughout the hydrogel noted.

UV/Vis spectroscopy was used to quantify the amount of disulfide cross-links in other studies (Monostori et al., 2009). However, as previously described in section 3.4.1.3, it is postulated that this technique is inadequate for the bead system and not a true indication of the amount of cross-linker incorporated. This technique supports the notion
that the cross-linker has been successfully incorporated but more importantly it shows that the cross-linker remains in its disulfide form in a non-reducing environment. The results show that as soon as the disulfide bonds are exposed to a reducing environment the bond breaks down to two thiols as expected (Emilitri et al., 2007). This suggests that the cross-linker within the system maintains its functionality.

The sizing technique was also used to test the hypothesis that if the cross-linker is co-polymerised within the beads, they should be in their disulfide form. This has now been clearly demonstrated. However, when in the presence of a reducing agent, the disulfide link breaks down to its thiol and it is predicted that this change leads to an alteration of the beads properties. The space in-between the thiols could mean that a more open and less rigid network is produced as described in section 3.4.1.2. The sizing data shows that when reduced, the beads increase in size (diameter). When in a non-reducing environment the bead size does not change. Therefore, beads as a whole respond to the reductive stimuli and the additions of a disulfide cross-linker is responsible for these changes.

All the characterisation techniques correlate with each other and indicate that novel redox monomers can be co-polymerised with other co-monomers to form redox sensitive beads. What has not been tested is whether the beads’ capacity to load and elute cytotoxic drugs has been maintained. A possibility remains that the new cross-linker could affect the PVA hydrogel in its ability to allow the diffusion of drug through its structure. This leads to Chapter 4 where a range of techniques are used to quantify the drug loading of the novel beads.
Chapter 4

4 Drug Loading of BALC Microspheres

4.1 Introduction

The use of conventional intravenous drug administration for chemotherapy, does not usually provide a rate-controlled release, or more importantly, target specificity. In fact, intravenous (I.V.) delivery has the potential to provide systemic drug at toxic concentrations, resulting in numerous adverse side effects (Pennock et al., 1991). Additionally, the dose is eliminated more quickly from the body having less effect on the target region.

Since the employment of polyethylene as a drug carrier in the 1960’s, the research community has investigated the use of a range of polymers for drug loading and controlled release (Desai et al., 1965). Polymer microspheres have been investigated as vehicles for drug loading and drug delivery due to a number of properties which allow them to be tailored to produce a desired release profile. They can be injected or ingested and can even provide organ-targeted release (Freiberg and Zhu, 2004). Early research highlighted numerous mechanisms for loading of drug within these microspheres, such as the solvent evaporation technique where drug can be incorporated into the microsphere during its synthesis or the hot melt microencapsulation method which uses a solvent to disperse drug and the polymer mixture together (Vasir et al., 2003; Soppimath et al., 2001). However these methods expose the drug to unfavourable conditions such as high temperatures, use solvents or surfactants which can be toxic, and can suffer from limited encapsulation capabilities (Mathiowitz and Langer, 1987). One approach to overcome these limitations is encapsulating the drug into pre-formed devices, such as the precipitation method where the microsphere is soaked in a swelling agent with excess drug solution, allowing drug to diffuse into the device (Soppimath and Aminabhavi, 2002). The microsphere is then separated and dried to allow stable storage. However this method has very low loading efficiencies; for example it has been reported that some devices load the drug dexamethasone at only 0.9% w/w (Govender et al., 1999). In addition to poor loading, these methods could result in possible migration of drug within the microsphere during storage and inevitable waste of expensive drug.
Of the different loading methods investigated, the use of microspheres with an ion exchange capability have exhibited the highest loading for ionic drugs such as Dox (Liu et al., 2001). Ion exchange microspheres are essentially a three dimensional network carrying fixed ionic groups which carry a charge that is countered by a mobile ion of the opposite charge (Helfferich and Plesset, 1958). The interaction between the drug and the microsphere does not radically alter the structure and property of the solid, nor does it affect the properties of the drug which is immobilised on the solid (Anand et al., 2001). This process is effective with high drug loading fraction per polymer solid content, for example DC Bead having a Dox loading of 88% w/w (Gonzalez, 2006). Here the ionic interaction occurs between the anionic sulfonate group of the microsphere and the protonated amine group of the Dox structure. The amount of drug loaded is homogenously diffused throughout the microsphere and the amount of drug loaded can be controlled by altering the concentration of the drug loading solution. This loading method does not expose the drug to extreme temperatures neither does it introduce any toxic solvents to the drug or microsphere.

The novel responsive microspheres developed in this thesis are hydrogels with carboxylic acid functional groups running throughout the microsphere. The ion exchange nature of carboxylic acid groups has been investigated before, specifically, its ability to remove metal ions from solution (Saito and Isogai, 2005). The carboxylate groups attached to the disulfide cross-linker shown in Fig 4.1 carry a negative charge and should therefore be able to associate with cationic drugs.
BAC beads were not used in drug loading experiments due to a lack of binding sites and hence an inability to bind ionically charged drugs (Zeng and Pitt, 2005). BALC beads (Fig. 4.1) do have functional groups that would suggest ion-exchange may be possible. The difference structurally between BALC and BAC is minimal; there is an addition of a carboxylic acid group before the amide groups and the disulfide link either side on the BALC. It is these two carboxylic acid groups which actually cause BALC to be soluble in water, while BAC without the carboxylic acid groups remains insoluble in water (Schröder et al., 2010).

The redox sensitive BALC beads produced in this work may therefore have a similar loading mechanism to that of both HepaSphere and Tandem with carboxylic acid groups spread throughout the hydrogel network. This chapter will investigate the potential binding capacity of the beads by immersing them in the solution of suitable chemotherapeutic agents such as Dox and then calculating the extent of ionically bound drug. This binding capacity will ultimately determine whether the product has the potential to be used as a DEB-TACE device and can therefore be used to treat hypoxic tumours with chemotherapy, or whether it may be more suitable for TAE, as a more efficient embolic that can increase in size after reduction.
4.2 Materials

BALC microspheres were prepared as described in Chapter 3 of this thesis. In the characterisation of the BALC microspheres Doxorubicin hydrochloride was supplied from Hisun Pharmaceutical Co. Ltd, China. Irinotecan hydrochloride (Campto®) was supplied by Pfizer, whilst Rapamycin was supplied by LC Laboratories. Phenolphthalein was obtained from Sigma Aldrich. Dimethyl sulfoxide and propan-2-ol were purchased from Sigma-Aldrich. HCl and NaOH was purchased from Fisher Scientific. Deionised water was provided by a Millipore Elix 4 type 1 water purification system.

4.3 Methods

4.3.1 Loading of Ionically Charged Drugs

The method of loading drugs into the microspheres was based on that described by Lewis et al (2007). Dox is the initial drug of choice and is used as an example of cationically charged drug loading. A 25 mg.mL\(^{-1}\) Dox loading solution was prepared in deionised water.

The pre-calculated theoretical maximum loading capacities of the BALC microsphere were used to determine the maximum amount of drug to be added. An excess of the maximum value of the drug solution was chosen and placed within all the vials, irrespective of binding capacity, to ensure maximum binding and consistency. Each 1 mL of BALC bead was immersed in approximately 3 mL of Dox solution. The vials of beads were then placed on a shaker at 200 rpm and left overnight at room temperature. After loading of the drug into BALC beads, it was necessary to determine the precise amount of drug loaded within the beads. This was performed by washing away unbound drug with deionised water (Dox washing). Initially all the free drug solution was removed using a glass pipette and placed in separate glass vials ensuring no beads were removed. The collected drug solution was kept in glass vials for analysis.

Deionised water (10 mL) was then added to the vials to remove any residual drug. After the beads were washed, the added deionised water was removed and collected and a fresh 10 mL deionised water was added. This process was carried out repeatedly until freshly added water was no longer tainted red by the Dox solution.
The collected wash solutions of initially removed drug were then analysed on a Varian Cary 50 UV/Vis spectrophotometer. The absorbance for the highest peak ($\lambda_{\text{max}}$) of Dox was measured at 483 nm. Using a calibration curve, the concentrations were then added together from each wash cycle to give the amount of drug not taken up by the beads. This was then deducted from the total amount added to give the final value of the maximum binding capacity.

4.3.2 Fluorescence Spectroscopy of Dox Loaded BALT Beads

Fluorescence spectroscopy is a standard analytical technique that is complimentary to UV-Vis spectroscopy. However, instead of measuring the excitation of the electron to a higher energy state as in UV-Vis spectroscopy, fluorescence measures the energy released from electrons returning from the excited to the ground state (Skoog, 1984). Different compounds have different chromophores and will provide different intensities of radiation allowing the structure to be analysed (Lakowicz, 2007). A calibration curve was created by plotting the intensity of standards of known concentrations of drug against wavelength (nm). Due to the increased sensitivity of this method over absorption spectroscopy, this method was applied as a qualitative method to corroborate the presence of the drug doxorubicin within the BALT beads.

Three formulations of BALT beads (1 mL), the 4%, 15% and 31% were loaded with 3 different concentrations of Dox (0.01, 0.1 and 0.5 mg.mL$^{-1}$). This was repeated 3 times for each formulation as each concentration of each bead was exposed to three different conditions. The beads were either a) loaded into BALT beads and left in water and placed in the quartz glass cuvette b) Reduced-the BALT beads were loaded with drug then exposed to the reducing agent DTT. After reducing the beads, they were washed with water and placed in the quartz glass cuvette. C) Pre-reduced-before loading the beads were reduced with DTT. The reducing agent was then washed with water and placed in the cuvette. These three variations of the Dox loaded BALT beads were put into a 1 cm macro quartz cuvette, in the Varian Eclipse Spectrophotometer. The solution was scanned at an excitation wavelength of 475 nm and an emission wavelength range of 500 – 700 nm. The excitation and emission slit widths were both set to 10 nm.
4.3.3 Confocal Laser Scanning Microscopy

Current fluorescence microscopy and optical microscopy only provide images at the surface of the beads and no internal information can be gathered without physical sectioning of the beads. Confocal Laser Scanning Microscopy (CSLM) is a non-destructive method which penetrates and retrieves optical sections of samples in their hydrated state (Lewis and Heaysman, 2012). Also, unlike fluorescence microscopy which can appear blurred due to the light emitted outside of the focal plane contributing to the image, CSLM images are focused and clear. This is because CSLM uses a spatial filter (pin hole) to remove unwanted fluorescence above and below the focal plane of interest, with the pinhole limiting detection to the desired plane (Paddock, 1999). The information is passed back to the detector to build an image. A rectangular pattern of parallel scanning lines (RASTER) is used to collect an image of the section, and to obtain full 3D image optical cross sections at different depths (Berkland et al., 2004).

In this experiment two separate formulations, the high initiator 31% and the low initiator 45% were loaded with Dox at maximum loading capacity and at 0.5mg.mL\(^{-1}\). The intention was to determine if the drug had been homogenously loaded throughout the microsphere. The beads were placed in confocal microscope glass bottom plates and the microscope was focused at the centre of the formulations. The settings below were used to analyse the Dox loaded BALC beads. The equipment used was a Leica TCS SP5.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Objective</td>
<td>HCPL FLUOTAR 20.0x0.30 DRY</td>
</tr>
<tr>
<td>Smart gain</td>
<td>813.9</td>
</tr>
<tr>
<td>Frame average</td>
<td>2</td>
</tr>
<tr>
<td>Laser λ (power)</td>
<td>488nm, (25%)</td>
</tr>
<tr>
<td>Zoom magnification</td>
<td>1</td>
</tr>
<tr>
<td>Pinhole size (µm)</td>
<td>70.8</td>
</tr>
</tbody>
</table>

Table 4.1 Leica TCS confocal setting for analysis of Dox loaded BALC Beads
4.3.4 Determination of Drug Binding Sites within BALC Beads

To determine the amount of carboxylic acid groups within the microspheres and hence the potential maximum drug loading capacities, an acid-base titration was performed. This was performed by neutralising the acid attached to the beads with a known concentration of base, therefore quantitatively determining the concentration of unknown acid binding sites within the beads.

BALC beads (1 mL) were mixed with 1 mL of 1 M HCl (n=3), to ensure that all sites were in their acid form. The beads were then washed and agitated in deionised water (20 mL) to remove any free acid. The beads were then collected and placed into a conical flask with 20mL of deionised water. Two drops of 1% phenolphthalein solution (in propan-2-ol) were added to the conical flask with the beads and the flask contents were stirred with a magnetic flea. The titrant used in the reaction was 0.005 M NaOH and this was added using a burette with the titrant added drop wise. The sample was then left to equilibrate by mixing. This method continued until the equivalence point was reached and all the acid groups had disassociated—indicated by a colour change when the solution turned from clear to a pink colour and the change was permanent. The amount of NaOH added was recorded. This approach provided another method to predict the theoretical binding capacity of the beads. This method was also repeated with the same formulation of beads that were washed with deionised water, rather than acid.

4.3.5 Binding Site Neutralisation Loading Technique

Further drug loading studies were performed where 1 mL of the 80% BALC beads were placed in 1 M NaOH solution and left for 20 minutes. The NaOH solution was then removed and the microspheres washed with deionised water to remove any residual NaOH. The microspheres were then loaded according to the same method used in section 4.3.1. The BALC microspheres were immersed in excess Dox solution and allowed to load on a mixing plate over-night. After 24 hours, all excess drug loading solution was removed and analysed by UV.
4.3.6 Molecular Modelling

A molecular diagram of the starting components of the BALC bead was created using chemdraw Accelrys Draw 4.1. The model was then developed with the assistance of Dr. Peter Cragg at the University of Brighton where a computational chemistry system program (Spartan ’06 Wavefunction Inc, Irvine CA 92612) was used to grow the model and predict the possible interaction of the bead structure with chemotherapeutic agents such as Dox and a reducing agent (DTT).
4.4 Results and Discussion

Dox is an anthracycline antibiotic that is widely employed as a chemotherapeutic agent with a high activity against a wide range of solid tumour lines (Weiss, 1992). Dox has the ability to intercalate with DNA, hence preventing its replication by inhibition of the topoisomerase II enzyme and causing damage to DNA through the metabolism of free radicals (Biondi et al., 2013). However when introduced intravenously, side effects of Dox have been well documented with myelosuppression, nephrotoxicity, nausea, arrhythmias and decreased survival with the main chronic effect being cardiomyopathy with precipitant congestive heart failure (Santos et al., 2010; Von Hoff et al., 1979; Mitra et al., 2001). The associated creation of free radicals cause oxidative stress to the heart which is the hallmark for Dox-mediated cardiotoxicity (Daiber et al., 2011). Due to the positive correlation between Dox concentration and systemic toxicity, a maximum cumulative dose limit has been set for the use of the drug (Legha et al., 1982). However, higher doses of the drug may be tolerated in the tumour tissue which has led to research to focus on limiting systemic toxicity while maintaining high local concentrations of drug at the tumour site.

With the use of intra-arterial delivery, and now microspheres such as DC Bead, local delivery of the drug to the tumour and prevention of antegrade flow of the drug can lead to high localised Dox concentrations at the malignant tumour sites. Its application with DC Bead has been well noted and the ability to load and bind to the microsphere via ion exchange previously discussed (Lewis et al., 2006d; Lewis et al., 2007). Dox was chosen as the main candidate drug for characterisation of the BALC microspheres due to benefits in the treatment of tumours described in Chapter 1. Additionally, due to its frequent application in drug delivery, use of Dox will allow comparison of the novel redox responsive system against existing systems.

4.4.1 Theoretical Maximum Binding Capacity

Before attempting to load the BALC beads with Dox, it was necessary to theoretically determine the maximum binding capacities of each formulation so that they can be loaded in excess. This was established using an adapted method from previously used for DC Bead (Gonzalez, 2006).
Dox molecules bind to carboxylate groups in a mole:mole ratio as both molecules are univalent (Sawaya et al., 1987). It is known that DC Bead is 95% water and 5% polymer (Gonzalez, 2006). Within the polymer composition, 55% is PVA macromer and 45% is AMPS, the small amount of initiator was not considered in the calculations (Gonzalez, 2006). For the calculation of B alc bead binding capacity the components used are macromer, while the remainder is B alc and initiator. The B alc concentration varies between formulations and therefore each formulation should bind a different amount of drug based on the amount of B alc introduced in the bead. This difference in B alc concentration alters the composition of the beads in each formulation and as a result alters the weight of actual polymer within the system. Microspheres of a uniform size are known to pack to an extent of 77.8% of a solution (Hales, 1992). Therefore in 1 mL of packed beads, 0.778 mL will be the beads and the remainder is packing solution. Of the 0.778 mL, approximately 5% will be polymer, however with the varying levels of B alc, the results of the water content studies reveals the actual amount of polymer within the beads.

To determine the potential binding capacity of the B alc beads, a mathematical calculation was applied from Gonzalez (2006), with the use of the water content results from section 3.5.2.3. The moles of B alc were calculated in a 2:1 ratio with Dox, as each mole of B alc has two carboxylic acids groups as shown in Equation 4.1.
0.778/ 100 x polymer content (5%) = 38.9 mg of polymer
38.9/ 100 x 2% = 0.778 mg of BALC
Moles = Mass/Mw          0.778/348 = 2.235 \times 10^{-6}

2.235 \times 10^{-6} \times \text{Mw of Dox (580)} \times 1000 = 1.296 \text{ mg of Dox per mL of beads}
\text{X 2 as 2:1 ratio} = \textbf{2.59 mg} \text{ of Dox per mL of beads.}

**Equation 4.1** Theoretical calculations of the potential Dox binding capacity of 2% BALC microspheres, dependent on the percentage of BALC added being fully incorporated.

<table>
<thead>
<tr>
<th>% BALC Bead</th>
<th>2%</th>
<th>4%</th>
<th>8%</th>
<th>15%</th>
<th>31% High Initiator (High I)</th>
<th>31% Low Initiator (Low I)</th>
<th>45%</th>
<th>60%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Solid Content</td>
<td>4.9</td>
<td>2.4</td>
<td>3.3</td>
<td>2.8</td>
<td>8.0</td>
<td>8.4</td>
<td>4.5</td>
<td>17.0</td>
<td>24.7</td>
</tr>
<tr>
<td>mg/mL Dox</td>
<td>2.59</td>
<td>2.5</td>
<td>6.53</td>
<td>10.9</td>
<td>64.8</td>
<td>67.8</td>
<td>52.5</td>
<td>265.1</td>
<td>514.2</td>
</tr>
</tbody>
</table>

**Table 4.2** Table displaying the different BALC Bead formulations and solid contents used to calculate their theoretical Dox binding capacity per mL microspheres.

The results of the theoretical binding capacity calculation are used only as a guide and actually the amount of drug loaded in the bead should be loaded in excess of the value in order not to limit the experiment. It is observed from the calculation that the loading capacity of the different formulations does not follow a trend with an increase of loading based on the percentage of BALC, but that the solid content is an important factor.
Although, the 60% formulation was thought to have a loading capacity twice that of the 31% formulation, the loading is in fact quadrupled based on the increase in the solids content. An increase in solid contents in polymeric devices has been well correlated to large increases in drug encapsulation (Freiberg and Zhu, 2004; Alex and Bodmeier, 1990; Govender et al., 1999). These theoretical values were used as a reference to determine the maximum loading capacity of the BALC Beads in section 4.4.2.

### 4.4.2 Theoretical vs Measured Binding Capacity of BALC Beads

BALC beads were loaded according to the method described in section 4.3.1 and analysed to determine the amount of Dox that could be loaded and held within the different formulations of beads.

Fig. 4.2 Loaded BALC beads in water retaining Dox. From left to right 0, 2, 4, 8 and 15% BALC bead formulations. The BALC 0% shows no drug loading. [Original in colour]

Fig. 4.2, demonstrating the different formulation BALC beads, shows visually that after washing, Dox is retained within the beads that possess the cross-linker BALC. It was observed that different formulations of BALC beads are darker in colour suggesting they retain different amounts of Dox. These results should match the theoretical calculation trend that as the amount of cross-linker incorporated increases, so does the amount of Dox retained. However, for increased accuracy and confidence within the results, BALC incorporation was not taken at a set value of 100%. The theoretical loading capacity was re-calculated taking into account the actual amount of cross-linker incorporated into the bead using the previously discussed elemental analysis results. For example, the high initiator 31% has a sulfur percentage of 5.3%. Using total sulfur found in a formulation
(\textsuperscript{w}S) over the overall total sulfur found in BALC (\textsuperscript{w}total) which is equivalent to \textsuperscript{w}S/BALC (18\%), the percentage incorporation of BALC within the microspheres was determined. Results are shown in Table 4.3.

<table>
<thead>
<tr>
<th>% BALC Bead</th>
<th>15%</th>
<th>31% High I</th>
<th>31% Low I</th>
<th>45%</th>
<th>60%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>% BALC incorporated</td>
<td>15</td>
<td>29</td>
<td>33</td>
<td>44</td>
<td>55</td>
<td>76</td>
</tr>
</tbody>
</table>

Table 4.3 Using elemental analysis sulfur results, the table shows the achieved percentage of BALC incorporation into each formulation.

The results highlight that the incorporation of BALC is efficient and very similar to that of the theoretical, with no more than a 5\% deviation in incorporation to the amount of BALC added. The low initiator formulations appear to have higher percentage disulfide incorporation, demonstrated by the 31\% BALC formulations, which could be due to the points discussed in Chapter 3, section 3.5.1 and 3.5.2.

The data revealed that the control beads did not bind any drug, although 2 mg of Dox remained unaccounted for, which could be explained by the staining of the glass vial and the staining that appeared on the BALC beads. This staining effect (personal observation) has been noted on numerous occasions on glassware when running standards for the UV/Vis spectroscopy and proved difficult to remove. It was at this point assumed that a minimum 2 mg.mL\textsuperscript{-1} should be a variance that is allowed for drug loading on all formulations.

The results of the actual binding of Dox to the BALC beads (Table 4.4) revealed that the amount of cross-linker is directly related to the amount of Dox bound.
Table 4.4 Table with the high and low initiator formulations of BALC bead showing theoretical Dox loading (T) per mL of BALC Beads. New theoretical Dox loading (T.EA) per mL of BALC beads using elemental analysis to determine % BALC incorporation into the microspheres. The actual Dox loading (ACT) per mL of beads into the BALC formulations (mean ± range, n = 3).

Table 4.4

<table>
<thead>
<tr>
<th>% BALC Bead</th>
<th>0%</th>
<th>2%</th>
<th>4%</th>
<th>8%</th>
<th>15%</th>
<th>31% High I</th>
<th>31% Low I</th>
<th>45%</th>
<th>60%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T) Dox mg mL⁻¹</td>
<td>0</td>
<td>2.6</td>
<td>2.5</td>
<td>6.5</td>
<td>10.9</td>
<td>64.8</td>
<td>67.8</td>
<td>52.5</td>
<td>265.1</td>
<td>514.2</td>
</tr>
<tr>
<td>(T.EA) Dox mg mL⁻¹</td>
<td>0</td>
<td>4.9</td>
<td>6.4</td>
<td>11.2</td>
<td>14.6</td>
<td>60.6</td>
<td>68.9</td>
<td>51.8</td>
<td>243.1</td>
<td>489.7</td>
</tr>
<tr>
<td>(ACT) Dox mg mL⁻¹</td>
<td>2.1</td>
<td>5.4</td>
<td>8.5</td>
<td>11.3</td>
<td>14.7</td>
<td>30.3</td>
<td>15.3</td>
<td>25.0</td>
<td>93.8</td>
<td>109.0</td>
</tr>
<tr>
<td>(ACT) Range</td>
<td>1.8-2.2</td>
<td>5.0-5.7</td>
<td>8.2-9.1</td>
<td>10.9-11.6</td>
<td>13.9-15.3</td>
<td>27.9-32.6</td>
<td>15-15.6</td>
<td>24.1-25.5</td>
<td>91.7-97.6</td>
<td>107.8-109.8</td>
</tr>
</tbody>
</table>

The two original hypotheses have been demonstrated to be correct. BALC does bind Dox directly, and as the amount of BALC increases within a bead, so does the amount of Dox. The results also show that the theoretical and actual results do not exactly match. Table 4.4 indicates that the actual amount of Dox loaded in most cases is marginally more than expected for the high initiator formulations, except for the high initiator 31% BALC bead. Already factoring in the variation of 2.1 mg of drug, which is noted to be able to stain the surface of the beads, slightly more drug is loaded in the beads and it is postulated that this is a result of Dox-Dox interaction (Liu et al., 2011). Dox is known to π-π stack with other Dox molecules at high concentrations (Nakanishi et al., 2001).

As the beads are loaded in excess to the amount of available binding sites, this Dox-Dox interaction is feasible. Also, the variances allowed by Heaysman (2009) and Gonzalez (2006) are much larger and yet good correlation is stated between the theoretical and actual as they both take into account the effect of Dox-Dox interaction. The reason that the high initiator 31% BALC bead does not load near its theoretical capacity is explained by Heaysman (2009) with investigations into a 100% AMPS formulation. The high degree
of cross-linking in both formulations affects the diffusion into the beads and although there may be more binding sites within the beads, the positioning and accessibility of the sites coupled with the actual space within the bead limits the loading. This is discussed in more detail within this section 4.4.2.

The Dox-Dox interaction restricts the microsphere structure and can affect the accessibility of the free binding sites (Heaysman, 2009). Heaysman (2009) shows that even though there is a much higher theoretical loading capacity, the AMPS bead formulations are not able to load more than 52 mg.mL\(^{-1}\). While it is possible to create BALC beads that are more heavily cross-linked, there was concern when synthesising the more heavily cross-linked formulations that the loading within the beads might be limited to 30-40 mg.mL\(^{-1}\) Dox. However the results in Table 4.4 allay these concerns; the 80% BALC bead formulation loaded over 100 mg.mL\(^{-1}\) of Dox.

Table 4.4 does however suggest a large difference between the high initiator formulation and the low initiator formulation. This is highlighted by the two 31% BALC bead formulations that have the exact same composition yet their maximum loading capacities are very different. The low initiator BALC beads appear to have a reduced loading capability compared to their high initiator counterpart, with the only difference between the two being the initiator added to the system. The initiator was added in smaller concentrations to aid the incorporation of the larger quantities of BALC required for high percentage BALC beads, resulting in less surplus of the initiator. In doing so this has comparatively decreased the maximum loading capacity. The two original points have not changed, whereby drug is bound directly to the cross-linking agent and as the disulfide linker increases within the bead so does the drug, however there is a definite impact on drug binding and an understanding of why there is a decrease in drug loading is warranted.

Altering the concentration of initiator does impact the rate of polymerisation with increasingly available initiator leading to faster polymerisation rates (Jung et al., 2007; Chung et al., 2005) Initiator concentration also directly affects the length of the polymer chains, with a higher concentration of initiator leading to increased initiator coupling points and therefore shorter polymer chain lengths (Jansen et al., 2013; Kim et al., 2010). Therefore in the case of the low initiator BALC beads, the decreased initiator concentration has led to the formation of longer polymer chains. It is postulated that longer polymer chain lengths could lead to higher chances of chain entanglement (Lee et al., 2002). Just as proteins, composed of amino acids like \(\text{L-cysteine}\), are able to fold and hide functional sites within the structure, polymer chains are able to interlink and tangle with each other.
(Stevens and George, 2005). This increase in length of the polymer chains in the low initiator BALC beads is most likely causing greater entanglement and intertwining of the hydrogel network leading to cloaking of potential drug binding sites. The longer polymer chains make the structure inherently more mechanically stable with high melting temperatures because it is difficult to separate the folded chains (Huang et al., 2000). However, as the BALC content increases so does the entanglement and this can be correlated to Fig 4.3 below which shows a percentage decrease in drug loading efficiency as the BALC concentration increases. This may be because as the BALC concentration increases so does the length of the polymer chains therefore there is more entanglement and a higher percentage of hidden binding sites.

![Graph showing the percentage of Dox loaded in low initiator BALC Bead formulations (mean ± range, n=3)](Original in colour)

Fig. 4.3. Percentage of Dox loaded in low initiator BALC Bead formulations (mean ± range, n=3) [Original in colour].

When the BALC beads are exposed to a reducing environment such as DTT, the disulfide cleavage leads to increases in bead size. It is hypothesised that cleavage of the bonds leads to a change in the network structure and with the expansion of the bead, more binding sites could become exposed and re-emerge from their convoluted structure. To test this hypothesis, 1 mL of the 60% BALC beads were reduced before the addition of Dox. The beads were then washed to remove the reducing agent and the drug added. The maximum loading capacity significantly increased with a value of 126 mg.mL$^{-1}$ of Dox (n=3) for the 60% formulation. This result is a 34% increase in the drug loading formulation of the device, but more importantly demonstrates that the binding capacity of
the beads is not saturated. The results indicate that there are hidden binding sites within the structure as assumed, and that when the hydrogel network is manipulated it is possible to reveal these functional sites. However, based on the theoretical calculations, 50% of the binding sites are still hidden and unavailable for drug binding interactions. Therefore as more BALC is added to a formulation there is an increase in cross linking, less swelling of the microsphere which again could limit the diffusion of the drug, as well as a reduction in the macromolecular mesh size available for drug diffusion (Varshosaz and Koopaie, 2002). These results demonstrate that this chain entanglement affect hinders the drug loading capabilities of increasing BALC percentage formulations and explains the loading results observed in Table 4.4.

Another interesting observation is that BALC beads appear to load drug homogenously, in the same manner as DC Bead, even though it binds via carboxylic acid groups. It was postulated that BALC beads might load drug inhomogeneously as does HepaSphere due to the similarity in their use of carboxylic acid group binding sites (Jordan et al., 2010). However, BALC beads show that the inhomogeneous loading noted in HepaSphere is not due to the carboxylic acid groups, but could be an artefact of the swelling effect experienced with HepaSphere (Lewis and Dreher, 2012). This swelling effect has not been observed with BALC beads during drug loading. For example, Fig 4.4 below is an image of the low initiator 45% Dox loaded BALC beads. Under optical microscopy the beads appear to be evenly loaded and retain their spherical shape after loading. This result of homogenous drug loading is observed for all formulations.

![Image of BALC beads](image-url)

**Fig. 4.4** 45% Dox loaded BALC beads showing even distribution throughout the microspheres
Therefore with higher cross-linked formulations, BALC bead could offer benefits over other systems with high loading capacities achieved with carboxylic acid binding site groups (Liu et al., 2011). In addition, it should not suffer the inhomogenous loading observed in other systems, although it must be noted that optical microscopy only allows observation of the surface of the bead. Therefore it is not known whether drug is truly homogenously distributed throughout the bead, or indeed if the drug is only loaded through surface association with the beads. This was investigated in section 4.5.2.

Hydrogels with high water content allow easy diffusion of molecules throughout the network. However, another factor that may impact the drug loading efficiency in the case of BALC beads is that the addition of increasing BALC to the formulation leads to a decrease in water content. As the water content decreases the ability of the drug to penetrate to the centre and evenly distribute throughout the bead can become compromised (Kumar, 2011). This could result in Dox movement being limited, leading to Dox aggregating closer to the surface of the bead. This effect could be because of a molecular weight cut off, as noted by Heaysman (2009), which most likely decreases with increasing BALC. This reduction in free interstitial volume could cause Dox to come into close proximity with itself leading to self-association and as a result less drug penetration. As more Dox is added in high percentage BALC drug loading formulations, this effect could be amplified. These increases in the solid content could be blocking drug from entering the centre of the bead and/or creating molecular spaces so small that as drug binds to a site, it blocks drug behind it from going deeper in the bead structure.

As seen with Daunomycin as well as Dox, when drug molecules are forced close together, the ability to self-associate increases (Yokoyama et al., 1998). A hypothesis is put forward that as the BALC concentration increases so does the Dox-Dox interaction which leads to the formation of dimers which has been described before and is shown by a slight shift in the Dox peak under fluorescence (Missirlis et al., 2006). It is of interest to understand how the BALC beads impact Dox self-association, and to consolidate the hypothesis that Dox is evenly distributed throughout the structure, further characterisation techniques will be applied on the Dox loaded BALC beads in this chapter.
4.5 Fluorescence Studies

Fluorescence techniques have been used in the past to determine the loading concentration of Dox within microspheres (Biondi et al., 2013). Fluorescence spectroscopy can be used as a quantitative tool when used in conjunction with a calibration curve; however the intent of this method is first to prove the loading of the anthracycline drug into the bead, and second to investigate the interactions of the drug within the bead under different conditions.

At low concentrations, Dox is known to be fluorescent, while at high concentrations a phenomenon known as fluorescence self-quenching reduces the profile intensity (Dai et al., 2008). Dox has a dihydroxyanthraquinone which is common to all the anthracycline antibiotics and this is the chromophore responsible for Dox fluorescence (Karukstis et al., 1998). Fluorescence analysis has shown that at low concentrations of Dox, two different populations of Dox exist with a monomeric form and a dimeric form (Lebold et al., 2009). A study confirmed that Dox has potentially different fluorescence spectra based on different solvents and different pHs which could be due to self-association or even proton transfer between the structure of the drug (Rana et al., 2011). As Dox has shifts in its spectrum, a better understanding of its fluorescence in the BALC bead would give an insight into how the drug and bead interact. This could also help explain how the drug may be released from the device.

In this study, samples were analysed under fluorescence using the conditions described in section 4.3.2. Fig 4.5 A, B and C demonstrates the fluorescence analysis of the three formulations loaded at 3 different concentrations (0.01, 0.1 and 0.5 mg.mL$^{-1}$) of Dox.
Fig. 4.5. BALC Bead formulation loaded with different concentrations of drug analysed by fluorescence with a Varian Eclipse Spectrophotometer with an excitation wavelength of 475nm A) 4% BALC bead B) 15% BALC bead C) 31% High initiator BALC bead.
After analysis of the data in Fig. 4.5, it was necessary to normalise the data to allow comparison between the different formulations. This was due to the pronounced quenching effect of Dox, which took effect from 0.01 mg.mL\(^{-1}\) and led to decreasing intensity as the Dox concentration increased. This quenching effect has been reported in previous studies and has been assigned to the self-association effect between Dox molecules (Karukstis et al., 1998). This self-association, or aggregation of Dox molecules occurs at concentrations greater than 5.8 µg.mL\(^{-1}\) which has also been demonstrated in UV studies (Eksborg, 1978).

However, the lowest concentration used in this study was 0.01 mg.mL\(^{-1}\), which indicates that at that concentration, Dox is in an aggregated form. Therefore, the drug loaded within the beads at the starting point of the experiment, is in an aggregated form. This shows that as the concentration of Dox increases within an environment, so does an increase in \(\pi-\pi\) hydrophobic stacking of the drug with itself. This aggregation and interaction has also been linked to a red shift of the spectrum towards the longer wavelength (Rana et al., 2011; Missirlis et al., 2006).

The next trend present in all three formulations is that as the concentration of drug increases, so does the aggregation, displayed by a slight shift towards the red side of the spectrum with a gradual loss of the peak at 560 nm. This appears to be a common trend for all of the peaks at a concentration of 0.5 mg.mL\(^{-1}\) of Dox. This alteration in the state of Dox, either in a less or very aggregated state, has been noted before with fluorescence spectroscopy where Karuksitis and colleagues (1998) showed that there are differences in the ratios of the \(\lambda_{\text{max}}\) at 560 and 595 nm when the aggregation of Dox is altered. This is what is seen in Fig. 4.5; as the Dox concentration increases, there will be more aggregation and this is followed by a loss of the peak at 560 nm. Although a complete loss of the 560 nm peak is not noted in Karuksitis’ (1998) studies, compared to the results seen in this work, it is thought that this could be due to a function of the much higher Dox concentrations used in this study. It is speculated that the shift is more intense as there is greater Dox presence, resulting in this quenching effect.

It has also been shown that there is a difference in the emission spectra when drug is free in solution compared to drug bound in a drug delivery vehicle (Gallois et al., 1998). This is because drugs such as Dox, which can interact, can do so when free in solution and have a chance of colliding and therefore aggregate. This has been confirmed by work performed at Biocompatibles UK Ltd showing that Dox aggregation and hence the peak loss at 560 nm occurs quicker in water than in a medical device (Internal data of Biocompatibles UK Ltd). The data also revealed that there was greater aggregation of Dox
in DC Bead compared to that of Bead Block and this was thought to be due to the amount of extra binding sites within DC Bead being distributed closer together permitting a greater chance of drug interaction. In Bead Block the binding sites are far fewer and thought to be more distributed with the ability to hold the drug further apart from each other. More importantly, this work demonstrates that in different environments the spectra can be slightly altered and that polymer content or number of binding sites can have an impact.

Taking into account the work performed at Biocompatibles UK Ltd on the difference between the fluorescence spectra obtained from Bead Block and DC Bead at different concentrations, Fig 4.5 appears to agree that as BALC content increases within the formulation, so does the aggregation. This point can be demonstrated with the 560 nm peak visible for the Dox concentration of 0.1 mg.mL in the 4% BALC formulation, physically shifting to the red side of the spectrum and being lost altogether as the concentration of polymer and hence binding sites increase. It is thought that as the binding sites are more abundant and hence closer together, the Dox could be held in closer contact with other molecules to allow π-π stacking.

However the interpretation of the loading of Dox within the BALC beads is not straightforward and just like the different aggregation states noted in different solvents, it is postulated that there are multiple parameters that could affect the spectra. Fig 4.5 A, in comparison to the other graphs, demonstrates that the peaks at 560 nm for 0.01 and 0.1 mg.mL\(^{-1}\) Dox are the same with no aggregation despite an increase in concentration, unlike that seen in Fig 4.5 B and C. However the peak height or ratio is also different, with an increased peak size seen in Fig 4.5 B and C. It is possible that in different drug delivery vehicles, different spectra could be obtained and there is no significant difference between the peaks. However just as altering dielectric constants has different effects on aggregation, so can different polymer contents and their spread throughout the device. It is known that in the 4% formulation there are fewer binding sites, compared to the other formulations used in this study, and these binding sites are more evenly distributed throughout the bead. Therefore Dox molecules are held further apart from each other avoiding Dox-Dox interaction, whilst at the same time it is possible there are fewer binding sites, and the drug is more evenly distributed and diffused with the beads to reach these bindings sites. This causes a reduction in the fluorescence intensity as there are fewer Dox molecules within a defined space within the low percentage formulations than in other formulations.

In the higher percentage BALC formulations, there are more binding sites and these sites are within close proximity of each other. This means that when the lowest
concentration of drug is loaded, the drug is already being held closer together. It is suggested from the data of the 15 and 31% formulation that at 0.01 mg.mL\(^{-1}\) Dox, this abundance of binding site causes a greater fluorescence intensity by bringing the drug closer together, giving a greater peak emission. However as the concentration of drug increases in high BALC concentrations, the drug becomes more aggregated than in lower BALC formulations. This suggests that as the Dox concentration increases so does the aggregation, but that this is different within each formulation of BALC beads based on the percentage of binding sites and its distribution within the bead. The lower percentage BALC formulations will have a lower intensity due to better diffusion of drug in search of less frequent binding sites. The higher percentage BALC formulations will have a higher intensity as although the drug is not highly aggregated at low concentrations of Dox, the drug is still within a closer proximity giving a stronger absorption and emission. As the binding sites are closer together, it is the high percentage formulations that form aggregates because of the close proximity of the binding sites, resulting in a loss of the peak at 560 nm. The low percentage formulation still maintains the peaks as the increasing drug concentrations diffuse further to find more binding sites and therefore the drug is still held further apart. This seems to show that there is a tipping point in polymer content having a direct impact on the drug loaded and the form it is in, suggesting that an increase in polymer content increases the aggregation and a loss of the less aggregated Dox state is symbolised with loss of the peak at 560 nm. This impact of the polymer on the self-association is illustrated in other work using nanocarriers whereby in solution low concentrations of Dox are in the dimeric state (aggregated), however once loaded into vesicles the drug is held further apart and with circular dichroism are able to show a decrease in aggregation (Gallois \textit{et al.}, 1998). These results could explain why analytical analysis is feasible with such low concentration of Dox as it seems concentrations as low as 0.00058 mg.mL\(^{-1}\) are enough to aggregate in free solution (Changenet-Barret \textit{et al.}, 2013). The aggregated form of Dox shown by Changenet and colleagues (2013), yields a fluorescence spectrum equivalent to concentrations of Dox 18 times higher when in BALC beads at 0.01 mg.mL\(^{-1}\) as shown in Fig 4.5 A, B and C. This would support the theory that when Dox diffuses into BALC beads it is held further apart at the binding sites, while in solution there is more ability for the Dox to move, collide and hence aggregate. However, as the Dox concentration increases within the BALC polymers and depending on the number and position of the binding sites the aggregation increases and the fluorescence spectra is altered.
Such a substantial shift or loss of a peak has not been seen before, but this is thought to be related to the higher concentrations of Dox being employed in this study. Li and colleagues (1998) reported on Dox loading results, however throughout their studies they do not refer to a monomeric state and dimeric state. With the use of cryo electron microscopy Li and colleagues were able to image Dox-Dox interaction within liposomes. With the use of Circular Dichroism they were able to show that 99% of Dox loaded into vesicles at a concentration of 0.01 mg.mL\(^{-1}\) is already in an aggregated state. They put forward the theory and showed that at low concentrations Dox does not simply form dimers, but give evidence and foundation to the earlier argument that they have the ability to form aggregated fibres rather than a simple dimer or trimer. These fibres are comprised of Dox molecules and can condense and stack upon each other longitudinally in a hexagonal array to form large bundles as shown in Fig 4.6.

Fig. 4.6 Schematic representation of twisting hexagonally arranged Dox fibres. The image depicts each fibre which is comprised of stacked Dox molecules. The schematic illustrates how the stacked Dox molecules (circles) would appear as viewed end-on. Copyright permission granted (Li et al., 1998). [Original in colour]

The repeating striations observed led Li and colleagues (1998) to suggest that the entire bundle twists 60° approximately every 50 nm which is consistent with a twisting hexagonal lattice. The study showed that the growth of these fibres was dependent upon space available. When space is limited these fibres can coil on themselves and instead form curved and circular bundles. Dox was also shown to be capable of forming fibres that were not organised into bundles but were still aggregated in different pH conditions (Li et al.,
These results add strength to the argument that Dox is not in a monomeric form but in an aggregated form at low concentrations. As the Dox concentration increases so does the aggregation which can form large fibres that are stacked together and take shape of the space permitted by the polymer delivery device.

With BALC beads it is hypothesised that large linear Dox fibre bundles will not form due to the distribution and non-linear positioning of the binding sites. However as the number of binding sites increase within the microsphere, the space between the Dox fibres will reduce with an increasing concentration of bound Dox permitting more interaction of the drug molecules with each other. Although the orientation and spread of binding sites will hinder linear growth, condensed and stacked Dox fibre growth of unrestricted shapes is expected. The shape of the fibre will be dependent on the space permitted by the BALC formulation for fibre growth and the position of other binding sites with Dox bound. This formation of Dox fibres would be sufficient to quench the spectra peak at 560 nm and is the reason why throughout the high Dox concentration all the shoulders are lost from each formulation of BALC bead.

4.5.1 Dox Spectra in Reductive Conditions

With a clearer understanding of the Dox fluorescence spectra and its changes when loaded into BALC beads, it was necessary for the purposes of this thesis to understand the effect of a potential reductive environment on the drug loaded within the bead. Understanding the nature of Dox interactions when loaded within the drug delivery device is of paramount importance. It is assumed that at high Dox concentrations, the drug will self-associate within the beads when delivered to the tumour. However, although the sizing data shows the diameter of the spheres will increase in size, it is not known if this still remains the case when drug is loaded. With this in mind, an experiment was performed to examine the reductive properties of DTT on Dox-Dox interaction within the microspheres. In this experiment one formulation was chosen and loaded at all three Dox concentrations used before. These concentrations were exposed to three different conditions as described in section 4.3.3 and the results monitored by fluorescence. The results of the experiment can be seen in Fig 4.7 A, B and C.
Fig. 4.7 31% BALC beads loaded with A) 0.01 mg.mL$^{-1}$ Dox. B) 0.1 mg.mL$^{-1}$ Dox C) 0.5 mg.mL$^{-1}$ Dox. These beads were exposed to either (N) Normal conditions; (PR) beads reduced with DTT, washed, then loaded; (R) Microspheres loaded then reduced with DTT. The beads were analysed by fluorescence at an excitation wavelength of 475nm. [Original in colour].
The results of the experiment show that exposing the drug loaded beads to a reductive environment alters the fluorescent spectra of the loaded Dox. A Dox control was analysed with just DTT and Dox (data not shown), showing no changes to the Dox fluorescence, revealing that the changes in the spectra are not attributed to the impact of DTT on Dox. The change in spectra is attributed to the DTT reductive effect on the microsphere.

The results show that when the drug loaded microspheres are exposed to a reducing agent, the amount of Dox-Dox interaction is reduced and the drug loaded becomes more separated and less aggregated. By looking at the results from the graph it is speculated that the energy required to reduce the disulfide bonds is greater than the energy that holds the Dox-Dox interaction in place. Due to the tightly cross-linked network of the hydrogel, it is postulated that the structure is under stress, especially as the cross-linking increases. Therefore when the disulfide bridge is cleaved, the stress or energy is released leading to an expansion of the beads as seen in Fig 4.8. This expansion pulls apart the Dox fibres loaded within the bead leaving a less aggregated Dox loaded bead. This is indicated by the return of the peak at 560 nm.

![Graph showing sizing of Dox loaded 45% BALC beads before and after reduction](image)

**Fig. 4.8** Sizing of Dox loaded 45% BALC beads before and after reduction (n=200).

Fig. 4.7A does not show any noticeable difference when the microsphere is exposed to different conditions but this could be a consequence of the Dox concentration being very low and already in a low aggregated state. When the concentration of Dox
increases, the effect of reduction can be clearly seen with an increase in the de-aggregated form of Dox, even when the highest concentration of Dox is used (Fig 4.7C). It is thought that expansion of the microspheres could be pulling the Dox stacked molecules and hence the aggregated fibres apart. Fig 4.8 is the sizing results of a max loaded 45% BALC microsphere, which through the use of the Mann-Whitney test, shows an increase in size after reduction (P<0.05), suggesting that even when the microspheres are loaded to maximum drug loading capacity, the Dox fibre aggregates could still be pulled apart. This result would suggest that the stacking between Dox is broken when the beads expand and that the Dox self-association is not strong enough to hold the bead together.

These results could suggest that elution from the microspheres will be enhanced in a reductive environment, because as the beads expand, the Dox becomes less aggregated. This could allow a faster diffusion of the counter ions within the expanded beads, whilst the less aggregated forms of Dox should have less π-π stacking holding it in place. This could allow quicker diffusion of the drug out of the microspheres. If smaller Dox fibres are formed when the bead expand in size, there should be less steric hindrance affecting the release of the drug from the device. These graphs show the potential for the beads to deliver a higher payload of drug to a tumour in response to hypoxia with a potential increase in ease of diffusion of Dox out of the microspheres.

The fluorescence study has shown that as the BALC concentration increases within a formulation, so does the aggregation of Dox. In the high percentage BALC formulations the lower loading capacity could be due to the BALC preventing the diffusion of drug within the microsphere. The fluorescence confirms that as the Dox concentration increases there is a higher likelihood of forming aggregates, potentially in the form of stacked fibres, which in conjunction with high BALC content could both be blocking access to other BALC binding sites. More studies are required to understand whether aggregation or diffusion is affecting the loading of the BALC microspheres and whether Dox is reaching the core of the beads.

4.5.2 Confocal Microscopy Studies of Dox Loaded BALC Microspheres

Another method used to assess the potential of the BALC microspheres as a drug delivery device and to reveal more about the distribution of drug within the beads is Confocal Laser Scanning Microscopy (CLSM). In this study the 31% BALC microsphere was loaded to
maximum capacity with Dox and then washed to remove any residual from the solution to avoid any interfering fluorescent signals around the microsphere. The CSLM was then used at an excitation wavelength of 488 nm to obtain cross sectional images of the microsphere, starting from the top of the microsphere and working its way to the centre of the microsphere at 10 µm intervals. The different z-sections from the edge of the microsphere are shown below in Fig 4.9.
Fig. 4.9 CSLM sections of 31% Dox maximum (Max) loaded microspheres showing progressive images from outside inwards at 10 µm intervals also known as z-stacking.
Z-stacking is an image processing technique which combines multiple images taken at different focus distances, achieving extended focus and building a composite image. The results of a z-stack of the 31% Dox loaded BALC bead can be seen in Fig. 4.9. Following the multiple images from the top left to bottom right of Fig. 4.9 appears to show that the drug is prevalently localised at the surface of the structure. As the size of the sections increase and the scans move further into the microspheres, the drug is found further at the periphery of the BALC microspheres. In fact the images show that there is no Dox at all within the centre of the microspheres. This would appear to align with work performed by Heaysman (2009) and other recent research looking at Dox loading hydrogels which achieved similar results (Weng et al., 2011). This could be interpreted as limited diffusion of drug into the microspheres with aggregation of Dox at the edges due to high polymer content and the formation of aggregates as suggested from the fluorescence studies. However, the results achieved in Fig. 4.9 are contradictory to recent literature which demonstrates that Dox loading occurs throughout the entire diameter of hydrogel beads (Lewis and Heaysman, 2012).

A report by Biondi and colleagues (2012) investigating the interactions of Dox in DC Bead initially revealed similar confocal results to Fig. 4.9. However in the study multiple drug loading concentrations were applied and when low concentrations of Dox were loaded the apparent distribution was qualitatively different. At concentrations of 5-50 µg.mL\(^{-1}\) Dox, the drug appears to be uniformly distributed throughout the microspheres. The original results, contradicting these later findings, were put down to being an artefact of Dox fluorescence with an excess of drug in the inner regions of the beads leading to quenching of the fluorescent spectra (Biondi et al., 2012). The Dox self associates, just as in the case in the fluorescent studies, and due to the high levels shows no fluorescence.

To confirm whether this was the case with BALC microspheres, the 31% formulation was loaded with Dox at a low concentration of 0.5 mg.mL\(^{-1}\) and scanned under the CSLM as shown in Fig 4.10 below. A z-stack of the loaded BALC formulation performed with images taken at 10 µm intervals.
Fig. 4.10 CSLM sections of a 0.5 mg.mL$^{-1}$ 31% Dox loaded microsphere showing a z-stack at 10µm intervals.

The results of the confocal scanning microscopy directly correlate with the results achieved by Biondi and colleagues (2012) and demonstrate that the earlier lack of Dox penetration was false and due to a quenching effect. The images show that at 0.5 mg.mL$^{-1}$ the drug is homogenously spread throughout the entire structure of the BALC microspheres. Even at a 10 times higher concentration than that used by Biondi and colleagues (2012), a fluorescent spectra was achieved, although the final 2 images of the
bead section do begin to show a darkening in the centre of the microsphere. It is thought that this concentration of Dox could be the upper limit, at which, as the sections move further into the core, there is the beginning of a Dox quenching effect. However, the images clearly demonstrate that the drug is distributed evenly and cast doubt over the formation of a corona of drug around the periphery of the microsphere and explains the previous fluorescence as a result of the proximity of the ever increasing binding sites bringing the Dox molecules in closer quarters.

This result is very important as it shows that even with a high polymer content, the microspheres are able to load drug throughout its structure and therefore even though aggregates are formed, the drug should be able to diffuse within the beads. Although it is thought that increasing polymer content should have an impact, the effects on drug distribution are not so immediate. It is speculated that the small dark speckles noted in the images could be the well discussed fibres that aggregate. At the correct concentration the lack of fluorescence of such aggregates could be visible. The results of the CSLM appear to correlate well with the position of the BALC within the beads which is shown in the EDX work discussed in Chapter 3. The 45% BALC beads loaded at 0.5 mg.mL$^{-1}$ of Dox were also analysed under CSLM and the even distribution of the drug was observed with the results shown in Appendix II. Fig 4.11 below is a linescan of the intensity of Dox throughout the 45% BALC beads showing an even intensity through all regions of the microsphere.
Although these result are seen positively by allowing even distribution of the Dox throughout BALC microspheres, the questions remain why the drug loading capacity doesn’t match the theoretical and why it decreases with an increase in polymer content. The increase in polymer content still most likely has an effect, but it doesn’t prevent diffusion of the drug. The effect of the polymer content still will cause an increase in aggregation, but the most likely effect appears to revert back to the initial theory of chain entanglement, hence blocked off binding sites. Therefore further experiments were conducted to understand and provide a reason why the Dox loading is not as high as expected.

### 4.6 Acid-Base Titration of BALC Microspheres

To determine the loading capacity of the BALC formulations, the carboxylic acid binding sites were titrated with a base. The experiment was performed according to the method described in section 4.3.4, and previous work titrating carboxylic acid groups has been
undertaken previously (Hu et al., 2001). When the titrant was added to the beads, time was given for the base to equilibrate within the structure of the beads. As carbon dioxide can react with the phenolphthalein and turn the solution colourless, lids were placed on the conical flasks to prevent the reaction. A control of phenolphthalein solution in basic conditions was run to monitor any potential changes. For the duration of the experiment, the control solution did not change colour, yielding confidence that any change in colour would be due to the acid groups within the bead. After acidification of the binding sites, the titration was performed and the results can be seen in Table 4.5.

<table>
<thead>
<tr>
<th>BALC Bead Formulations</th>
<th>8%</th>
<th>15%</th>
<th>31% High I</th>
<th>31% Low I</th>
<th>45%</th>
<th>60%</th>
<th>80%</th>
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<td>(ACT) Dox mg.mL⁻¹</td>
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<td>93.8</td>
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<tr>
<td>(T. EA) Dox mg.mL⁻¹</td>
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<tr>
<td>11.2</td>
<td>14.6</td>
<td>60.6</td>
<td>68.9</td>
<td>51.8</td>
<td>243.1</td>
<td>489.7</td>
<td></td>
</tr>
<tr>
<td>(AT) Dox mg.mL⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>15.4</td>
<td>61.9</td>
<td>71.0</td>
<td>44.6</td>
<td>238.0</td>
<td>473.4</td>
<td></td>
</tr>
<tr>
<td>(AT) Range</td>
<td>11.1-</td>
<td>15.0-</td>
<td>59.5-</td>
<td>66.7-</td>
<td>41.9-</td>
<td>228.4-</td>
<td>461.8-</td>
</tr>
<tr>
<td></td>
<td>11.2</td>
<td>16.1</td>
<td>64.3</td>
<td>73.9</td>
<td>46.1</td>
<td>245.6</td>
<td>495.2</td>
</tr>
</tbody>
</table>

Table 4.5 Table of BALC bead formulations. The actual measured Dox loading capacity of BALC beads (ACT). The calculated theoretical maximum Dox loading capacity of BALC Beads (T.EA). The acid base titration results showing the actual loading capacity of the BALC bead formulations to bind Dox (AT) (mean ± range, n = 3).

The results from Table 4.5 show that original theoretical calculations correlate with the calculated Dox loading capacity obtained from the Acid-Base titration. The BALC beads seem to have a very high potential drug loading capacity, >400 mg.mL⁻¹ Dox, although this has not necessarily been reached. The titration results match those of the theoretical and this is most likely due to the Na⁺ ions with their low molecular weight being able to penetrate all areas of the hydrogel. The Na⁺ ions are thus not limited by the chain entanglement. However, a control experiment was performed to ensure that the
results seen were not due to residual acid. The results obtained can be seen below in Table 4.6.

<table>
<thead>
<tr>
<th>BALC Bead Formulations</th>
<th>31% High I</th>
<th>31% Low I</th>
<th>45%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ACT) Dox mg.mL⁻¹</td>
<td>30.3</td>
<td>15.3</td>
<td>25.0</td>
<td>109.0</td>
</tr>
<tr>
<td>(T.EA) Dox mg.mL⁻¹</td>
<td>60.6</td>
<td>68.9</td>
<td>51.8</td>
<td>489.7</td>
</tr>
<tr>
<td>(Non-AT) Dox mg.mL⁻¹</td>
<td>36.4</td>
<td>49.3</td>
<td>30.6</td>
<td>321.5</td>
</tr>
<tr>
<td>Range</td>
<td>34.8-37.3</td>
<td>46.6-54.0</td>
<td>30.1-30.9</td>
<td>312.9-327.4</td>
</tr>
</tbody>
</table>

Table 4.6 Table of BALC bead formulations comparing: the actual measured Dox loading capacity of BALC beads (ACT); the calculated theoretical maximum Dox loading capacity of BALC Beads (T.EA); the acid base titration results showing the actual loading capacity of the BALC bead formulations to bind Dox, where the formulations were not pre-treated with an acid wash to acidify all binding sites (Non-AT) (mean ± range, n = 3).

The results from the non-acidified BALC bead titration demonstrated a large difference compared to the beads that were pre-acidified before titration. The potential drug loading capacity of the BALC beads are considerably reduced when the beads are not acidified. On closer investigation of the tables it can be postulated that the value between the theoretical and the titrated values in the non-acidified beads appear to correlate with the actual loaded values of Dox.

As residual acid is washed away, free acid is not thought to be responsible for the higher values obtained in the acidified titration of BALC beads. The two differing values for the titration obtained in Tables 4.5 and 4.6 are indicative of the potential two forms of the binding group. Unlike DC Bead which has a sulfonic acid group, the BALC bead has carboxylic acid binding sites which are known as weak acids. Ethanoic acid is known to have a pKa of 4.6 and it is assumed that due to the C-H bond to the acid groups in BALC, the pKa might be slightly higher around 5 which is considered a weak acid whilst sulfonic acids are strong acids with a very low pKa. The difference between a weak acid and a
strong acid is that a strong acid is always completely disassociated under neutral conditions and therefore completely ionised and available for binding. A weak base is not completely ionised in neutral conditions as with a higher pKa the acid is not completely disassociated. Therefore the weak acid would be able to take up fewer Na\(^+\) ions in acidic conditions than in alkali conditions (Helfferich, 1962). The ionisation of binding sites is directly dependent upon the pH of the solution, with more basic conditions allowing greater ionisation and hence exchange capability. If the pH value is lower than the pKa then acid will not be able to dissociate and therefore is non-ionic. As the carboxylic groups are weakly disassociated, then the loading of Dox is not as efficient as in strong acid; the weak electronegativity denotes a lower likelihood of the H\(^+\) ions to exchange. Once dissociated, the carboxylate anion is stabilised by resonance with the electron delocalised between the two electronegative oxygen atoms meaning the oxygen’s are less negatively charged. This electronegative structure is now less likely to attract back a positively charged ion than the sulfonate group.

The titration data alludes to the fact that BALC microsphere binding sites are in two forms; the acidified form and the carboxylate form, and a potential reason why loading capacity of the beads is much lower than that of the theoretical is because of the inability of all acid sites to dissociate in the neutral conditions of deionised water. Therefore the loading is not as high as it potentially could be. When acid binding sites, which are treated, and are completely disassociated in the salt form, the optimal range for loading Dox and other amine ions is between pH 4.5-5.5 (Borodkin and Yunker, 1970). However as the pH increases, even though the exchange resin becomes more ionised and susceptible for interaction, the drug itself can be affected by the conditions and can counter the increase in ion exchange at a pH as low as pH 6 which is lower than that of deionised water. Therefore there seems to be multiple parameters affecting the ability for BALC beads to match the speculated theoretical binding capacity with incomplete disassociation of weak acids noted as characteristic of carboxylic acids (Averill and Eldredge, 2006). It is postulated that this incomplete dissociation plays a key role in the prevention of BALC beads meeting their potential binding capacity.

The fact that a large portion of the microspheres is still in its dissociated form not only impacts the drug loading directly by not providing an ionised exchange site, but is postulated to also limit the drug loading through hydrogen bonding of the hydrogen from the OH group to the highly electronegative oxygen on the carbonyl atom (Brooks and Haas, 1967). This electrostatic interaction is known to be weaker than that of a covalent
and ionic bond, however it is directly responsible for increasing the boiling point of carboxylic acids (Streitwieser et al., 1992). Interestingly the hydrogen bonding interaction allows intramolecular bonding, however carboxylic acids are also able to dimerise and form intermolecular bonds with other acid groups (Etter, 1990; Lifson et al., 1979). This intermolecular bonding could be directly responsible for the chain entanglement that was noted previously in section 4.4.2 along with low initiator concentration leading to longer chains causing chain entanglement.

The hydrogen bonding in conjunction with incomplete ionisation of the binding sites and chain entanglement appear to be the main reason why approximately only 20% of microsphere loading capacity is reached. To determine if these effects could be reversed, a study was performed in section 4.7 to force more drug into the beads and provide a high loading bioresponsive DEB-TACE device.

4.7 Binding Site Neutralisation Loading Technique

A method was developed to maximise and utilise the potential binding capacity of the BALC beads. Based on previous work, the intent was to pre-ionise the beads and present the bead binding sites for drug loading in the carboxylate salt form (Borodkin and Yunker, 1970). By adding the NaOH, the \( \text{H}^+ \) ions are displaced allowing formation of the carboxylate anion. This prevents hydrogen bonding and presenting a less entangled hydrogel network. This could increase the ion exchange capacity of the BALC beads and increase the maximum Dox loaded within 1 mL of BALC microspheres. This theory was tested with the pre-prepared 80% BALC microspheres which have the largest theoretical binding capacity. The results of the loading studies demonstrated that the 80% BALC beads pre-neutralised with 1M NaOH, have an actual Dox loading capacity of 322 ± range 300-333 mg.mL\(^{-1}\) (n=3).

The results demonstrate that the loading capacity has increased over three times the value of the original loading capacity. The neutralised beads load up to 68% of their theoretical loading capacity in comparison to 22% Dox loading of the non-neutralised beads, which shows that incomplete disassociation of acid binding sites is directly responsible for previous limited Dox loading values. It is also of interest that the BALC bead formulations offered one of the highest loading capacities with 80% BALC bead
having 130 % w/w Dox loading capacity per polymer solid content, greater than that of the sulfonated DC Beads.

The results demonstrate that the neutralisation of the BALC bead binding sites is necessary to increase their loading capacity by presenting the beads in the carboxylate form. To provide a high drug loading BALC bead, this technique must be performed on the beads. The result, along with the confocal and the EDX, dismisses the idea that there is a drug loading corona effect around the periphery of the beads and that the drug is loaded homogenously throughout the bead because the loading sites are spread homogenously throughout the beads.

The drug still penetrates throughout the device before complete dissociation of the binding sites, but there were limited binding sites available. However the polymer chains are less entangled and more binding sites are available for Dox binding. This is a marked improvement in reaching the values set out by the theoretical results and show that the drug loading is not aggregated in certain areas due to Dox-Dox interaction but is limited by the structure and position of the polymer chains of the device. By dissociating the acid, the process for drug loading has been optimised for future potential use and would allow a further improved distribution of drug throughout the device.

4.7.1 Investigation of Loading Other Drugs In BALC Microspheres

To expand the scope of the potential uses of the BALC microspheres for various tumour treatments, evidence was required to illustrate the use of BALC beads as a multi-drug loading device. Demonstrating the potential loading of various chemotherapeutic agents would broaden the spectra of tumour and cancer types that can be targeted and diversify the use of BALC beads. Therefore BALC beads were exposed to the commonly used anti-cancer drug irinotecan hydrochloride, which is currently applied or being considered for application in the treatment of tumours.

4.7.1.1 Irinotecan Hydrochloride Loading of BALC Beads

DC Bead have been used to load irinotecan hydrochloride and have been applied to the treatment of metastatic colorectal cancer with promising results (Eichler et al., 2012). Irinotecan is considered a prodrug and the active form (SN38) is achieved when the
dipiperidino side chain is cleaved by carboxylesterases that are present in the body (Forster et al., 2010). The active metabolite SN38 is the potent inhibitor of the nuclear enzyme that is involved in DNA replication, topoisomerase I (Slatter et al., 2000). Irinotecan is one of the first drugs along with 5-fluorouracil to demonstrate significant activity against metastatic colorectal cancer (Lewis, 2010; Martin et al., 2009). Safety data on Irinotecan loaded beads also highlighted that maximum plasma levels were 70-75% lower when compared with intravenous administration of irinotecan and below the limit of quantification after 24 hours (Biondi et al., 2013).

Loading irinotecan hydrochloride into DC Bead showed loading up to 50 mg.mL\(^{-1}\), which matched the theoretical value of 52 mg.mL\(^{-1}\) for the microspheres which assumes almost 100% binding (Taylor et al., 2007). The mechanism of binding to DC Bead is via ion exchange and identical to that of Dox, with the protonated amine group of the irinotecan salt interacting with the negatively charged sulfonate group. Dox is known to self-associate and forms dimers due to interactions between the anthracycline rings, however irinotecan does not share this property with the drug only having an interaction with the negative charge provided by the polymer, which therefore slightly alters the properties between Dox loaded and Irinotecan beads (Lewis, 2010). As there is no π-π stacking, the distance between the irinotecan molecules is larger and would explain why DC Bead Irinotecan beads are larger in size than Dox loaded beads and have a lower compressibility modulus, despite being loaded at equivalent drug levels.

It was assumed that BALC beads should also to be able provide a platform to bind irinotecan due to the negative charge of the carboxylate groups and so 3 formulations of the BALC beads: 31%, 45% and 60% were used in loading experiments with irinotecan Hydrochloride (Iri). The theoretical loading capacities were calculated using the molecular weight of Iri, along with the actual sulfur incorporation using previous elemental analysis results and water content. The results of the loading experiment can be seen in table 4.8.
<table>
<thead>
<tr>
<th>BALC Bead Formulations</th>
<th>31% High I</th>
<th>45%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T.EA) Iri mg.mL(^{-1})</td>
<td>65.1</td>
<td>55.7</td>
<td>261.2</td>
</tr>
<tr>
<td>(ACT) Iri mg.mL(^{-1})</td>
<td>36.9</td>
<td>29.8</td>
<td>116.4</td>
</tr>
<tr>
<td>(ACT) Iri Range</td>
<td>34.9-39.4</td>
<td>27.7-31.0</td>
<td>112.6-118.1</td>
</tr>
</tbody>
</table>

Table 4.7 Table of BALC Bead formulations demonstrating the calculated theoretical Iri maximum loading capacity (T.EA). The actual maximum amount of Iri loaded within each BALC bead formulations (Act) (mean ± range, n = 3)

As seen with Dox loaded into BALC microspheres, similar percentages of Iri are loaded compared to the theoretical values. The result is of interest as it demonstrates that changing the drug compound from Dox to Iri does not affect the percentage of the drug loaded. Therefore it is not a matter of diffusion into the microspheres that affects the loading capacity, but it is the effect of the form of the binding site, being in either the acid or salt form, and the impact of polymer chain entanglement. This is highlighted in the 60% BALC bead loading study with Iri. The BALC bead underwent a pre-neutralisation according to the description in section 4.7. When the beads were loaded with Iri the loading capacity again increased, this time yielding a much larger Iri value of 185 ± 14 mg.mL\(^{-1}\) (SD, n=6).

These results suggest that the microspheres can be used to load significant amounts of alternative chemotherapeutic drug and as a result can be used for the treatment of other malignancies. These results also show the significance that the incomplete dissociation has to play in the loading capacity of the BALC microspheres and that this is the main factor that affects the beads. However, there is a still a percentage of binding sites (approximately 30%) that have not bound to the bead. As the binding sites are completely in the carboxylate form after neutralisation, it is assumed that this could be due to steric hindrance.

4.7.1.2 Molecular Modelling of Drug Interaction with BALC Beads

From results of the drug loading of the BALC Beads, it appears that in some BALC bead formulations over 60% of the beads can be loaded with anti-cancer agents and the previous
limited loading has been explained by the interaction of intermolecular forces. However, even after neutralisation, approximately 1/3 of the bead loading capacity remains unaccounted for. It is postulated that the remaining binding sites are unavailable due to the steric hindrance caused by the size of the drug attempting to bind and the small molecular mesh sizes created by the star polymerisation of the BAC microsphere. To demonstrate this point, a small unit of the BAC microsphere was created and was digitally polymerised on the software computational chemistry system program (Spartan '06 Wavefunction Inc, Irvine CA 92612) while monitoring the interaction with Dox and Iri.
Fig. 4.12 Molecular modelling of 80% BALC microspheres binding A) Dox B) Iri. Fig 4.12 shows the modelled interaction of BALC beads with drugs that are highlighted in orange. All sulfur bonds are shown in yellow, with red as oxygen, grey as carbon and white as hydrogen. Courtesy of Dr. Peter Cragg, University of Brighton. [Original in Colour]
The images clearly show that the drug is large and that it overlaps with other binding sites. If the drug is too large and protrudes over the space of other binding sites it is reasonable to assume that this will result in steric hindrance and could prevent other drug molecules from reaching potential binding sites. This model fits with the knowledge that BALC has a molecular weight of 348 D, whilst the smallest drug compound has a molecular weight of 580 D. Therefore with the tightly cross-linked nature of the beads and close proximity of binding sites to each other, space around certain binding sites will be limited and will prevent the beads from reaching their theoretical drug loading capacity.

4.8 Conclusion

BALC microspheres have demonstrated a potential to be used as drug carriers in TACE procedures. They have shown application with a number of chemotherapeutic drugs and have demonstrated active uptake and retention of these agents. This ability to uptake drug via ion exchange could prove useful in the treatment of malignancies as long as the microspheres are able to release the drug in the same manner.

This Chapter has investigated the potential drug candidates such as Dox and Iri that can be used with the BALC microspheres and has characterised the interactions of the drugs with the microspheres using a range of techniques and experiments. The drug of choice for this thesis is Dox and the results from this Chapter have provided a understanding into the mechanisms included in drug loading.

The loading data shows that Dox can be loaded within the microspheres. At high Dox concentrations it has been shown that the drug will aggregate within the beads. The data obtained from the analysis strengthens the understanding of how the drug could be delivered to the target organ. Originally, it was thought that this phenomenon of Dox-Dox interaction was hindering the drug loading due to blocking the diffusion of the drug into the beads. However confocal microscopy demonstrated otherwise, with drug penetrating throughout the bead and the titration results of BALC beads revealing that the formation of Dox fibres have no part to play in the perceived limited drug loading capacity. The actual prevention of increased drug loading was due to the inability of the weak acid binding sites to completely dissociate. With the neutralisation of these binding sites, and further loading studies performed, the question of why the drug loading is limited was answered, whilst simultaneously yielding a high capacity bead. Post neutralisation, the bead polymer
network was in a less entangled state after removal of the intermolecular hydrogen bonding, therefore a more open structure able to load higher concentrations of drug.

BALC bead is therefore a high capacity loaded drug delivery device with drug homogeneously distributed throughout the bead. The experiments performed in this chapter provide an understanding of how the drug is delivered to the target site, along with which mechanisms can influence the state of the drug. The novel information shows how these beads could potentially be applied to treat hypoxic tumours, and how the reductive environment of the hypoxic tumour could affect the potential state of the drug within the microspheres. It appears that the BALC microspheres provide a higher loading capacity bead compared to DC Bead, and a more uniform and homogenously loaded device compared to HepaSphere. These studies also demonstrate that the bioresponsive nature of BALC bead provides a niche not covered by any current formulation and shows how alteration in the polymer structure could affect the presentation of Dox. These drug loaded BALC microspheres were examined for their drug elution properties, and this is described in the following Chapter.
Chapter 5

5 Factors Affecting Drug Elution from BALC Beads

5.1 Introduction

In recent decades there has been a decline in the discovery of new drug entities, with a greater drive focusing on improving the efficacy of existing drugs (Ranade and Cannon, 2011). The delivery method of the active agent can be as crucial as the therapeutic agent itself. How the drug is presented to the target region is crucial to enhancing current therapies in the treatment of disease and maintaining maximal therapeutic activity.

When a drug is administered as chemotherapy intravenously, high concentrations of drug are delivered as a bolus. The delivery of the drug to the tumour is hampered by diffusion across the vasculature. When drug extravasates from the blood vessel to the target region, the distance of diffusion for a drug molecule, across the interstitium or through cells, can be the order of 1cm in the period of a month (Ruoslahti et al., 2010; Sinek et al., 2004). Therefore the chemotherapeutic effects of anticancer drugs can be limited, especially in hypoxic tumours, where large concentrations of drug are washed away (Heldin et al., 2004; Trédan et al., 2007). In the time it takes for drug to penetrate the tumour, the limited drug diffusion rate in conjunction with drug wash out allows time for some cancer cells to become multidrug resistance as previously discussed (section 1.4.2.4) (Chidambaram et al., 2011). These problems accumulate into systemic toxicity due to exposure of healthy tissue to the chemotherapeutic drug, therefore the maximum amount of drug delivered in chemotherapy becomes determined by toxicity to healthy tissue (Lewis, 2009; Lewis et al., 2006c). This is not necessarily effective due to the known high interstitial pressure which makes it more difficult for drug to penetrate and be retained in malignant tissue than in healthy well vascularised tissue (Heldin et al., 2004).

To reduce this systemic toxic exposure, the use of techniques such as intra-arterial chemotherapeutic administration have been used to provide regional treatment of tumours and have demonstrated the beneficial approach of using new routes of delivery to increase the effectiveness of anticancer drugs (Geschwind et al., 2002). Loco regional therapy by definition is a treatment that affects the local area and so in the case of cancer, it would be the targeting of the malignant cells, dramatically reducing the exposure of healthy cells to
any damaging therapy. The use of loco-regional therapies to treat diseases is somewhat predicated on Ehrlich’s concept of the “magic bullet,” and has led scientists to explore more specific delivery to the target sites giving rise to a vast array of nano and microparticulate drug carriers (Lewis and Dreher, 2012).

Polymeric devices such as hydrogel microspheres can have high doses of drug loaded into the carrier which can be implanted directly to the tumour site, increasing the drug exposure to the tumour, with microspheres acting as a depot for drug release (Wolinsky et al., 2012; Lewis and Holden, 2011). These technologies provide high targeted concentrations of drug for a long duration of time leading to significantly lower systemic toxicity levels as well as a benefit in survival (Lammer et al., 2010). Release can be sustained over a period of days, weeks, months, years and these deliveries can be tailored to a target region (Huang and Brazel, 2001). Loco-regional treatment in combination with a controlled release device can be used to reduce the amount of drug necessary to cause the same therapeutic effect in patients (Wolinsky et al., 2012). This is a benefit to patients, as overall, these devices utilise lower concentrations of drug to have the same effect as conventional treatment, leading to increases in patient compliance.

The use of DEB’s are beneficial, as microspheres block the blood flow which reduces the removal of drug through the blood supply and increases the exposure time of drug/cell interaction. Chemically tuning a microsphere permits the control over key properties such as drug release. BALC beads could be used to deliver drugs loco-regionally and embolise the vessel to limit drug wash out. Chemically tuning the polymer can allow a slow release with increased cross-linking within the polymer, however as a bioresponsive element has been included into the hydrogel structure, the intention is for the release to become altered in the presence of a hypoxic tumour as the polymer structure changes. An increased release in drug from the stimuli altered device would provide a higher concentration outside the tumour, potentially creating a larger diffusion gradient, resulting in larger amounts of drug penetrating through to more central regions of the tumour.

In Chapter 4 it has been demonstrated that drugs can be loaded into the BALC beads via ion exchange with chemotherapeutic drugs such as Dox and Iri bound within the microspheres. The aim of the work described in this chapter was to investigate the release profiles of drugs from the previously synthesised formulations of Chapter 3. These studies aimed firstly to identify whether drug loaded BALC beads are bioresponsive, and whether a response to a reducing environment leads to an increase in drug released from the formulations. Secondly, these studies investigated whether varying the amount of BALC
within each formulation affects the release of drug from the beads, and dependent upon the results, sought to determine which BALC formulation offers the greatest difference in drug release before and after reduction. This chapter finally describes the work undertaken to determine whether this stimuli response and drug release of BALC beads is impacted by the different synthesis conditions of the high and low initiator concentrations.

5.2 Materials and Methods

5.2.1 Materials

The microspheres were prepared as described in chapter 3 of this thesis. Doxorubicin hydrochloride (Dox) was supplied by Hisun Pharmaceutical Co. Ltd. China. Phosphate buffered saline (PBS) was supplied by Inverclyde Biologicals, UK. Sodium chloride and calcium chloride were supplied by Fisher Scientific UK. Sodium borohydride (NaBH₄), 5,5′-dithiobis-(2-nitrobenzoic acid), D,L-dithiothreitol (DTT), 2-mercaptoethanol, sodium citrate, citric acid and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were all purchased from Sigma-Aldrich.

5.2.2 Reducing and Non-reducing Environments for Drug Release

The BALC beads loaded with Dox (as prepared in Chapter 4) were used to test the ability of the BALC beads to release drug. The experiments were conducted with one major difference compared to the commonly used drug elution models used by Gonzalez (2006). While one set of BALC beads were placed in a normal environment with standard release media used such as PBS, a second set of beads were subjected to a reducing environment. The objective was to illicit differing release rates. The reducing agents used are listed in Table 5.1, with the concentrations used.
<table>
<thead>
<tr>
<th>Reducing Agent Concentration</th>
<th>Amount in PBS (g)</th>
<th>Conc (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaBH₄</td>
<td>0.45</td>
<td>60</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0.94</td>
<td>60</td>
</tr>
<tr>
<td>TCEP</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>DTT</td>
<td>1.85</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 5.1 List of reducing agents with concentrations used to apply to BALC beads to mimic a reducing environment for drug release in 200 mL.

5.2.3 Drug Release from BALC Beads

The drug release of BALC beads was performed in triplicate on pre-loaded samples as described in Chapter 4. Dox loaded BALC beads (1 mL) were removed from the packing solution and washed in water to remove any excess drug. The water was then removed and the beads left in slurry until the start of the experiment. Using the release media, Dox loaded BALC beads were washed out of the vials and added to a sealable, light-protected vessel with 200 mL of PBS. Another 1 mL of Dox loaded BALC beads of the same formulation was placed in a sealable, light-protected vessel with 200 mL PBS and a reducing agent from the list shown in Table 5.1. At set time points of 10, 30, 60, 120, 240, 360 and 1440 minutes, 5 mL of the solution was removed and placed in a glass vial for analysis. Then a fresh 5 mL of either PBS or PBS with reducing agent was added to the respective vessels. Some Dox loaded BALC bead formulations were added to a larger volume of 500 mL PBS, with the same conditions, described above, applied.

The eluted materials collected from the vessels were analysed again using UV/Vis spectroscopy. This analysis was performed in the same manner as that for determining the amount of drug loaded in Chapter 4, using a Varian Cary 50 UV/Vis spectrophotometer. The absorbance for the λ max of Dox was measured at 483 nm. Using a calibration curve, the concentrations were determined from each sample point to determine the amount of drug released from each BALC bead formulation.
5.2.3.1 Elution of Doxorubicin using a Sink Conditions Model

A known volume of BALC drug loaded microspheres (1 mL) were placed in a container with a 200 mL PBS release media, with a 1 mL replicate formulation placed in 200 mL PBS with a reducing agent, identical to the method described in section 5.2.3. However, in sink conditions, at the pre-set time points of 30, 60, 120, 180, 240, 300, 360 and 440 minutes, 100 mL of the release media was removed with use of a peristaltic pump, which dispensed the solution into a measuring cylinder. The removed solution was replaced with fresh media to allow continual release of the drug and avoid drug saturation in the media. The amount of drug eluted was again monitored by UV spectrophotometry (483 nm).

5.2.3.2 Type 2-Apparatus Dissolution Tester Model

An automated method for drug elution was used to measure drug release from BALC beads overnight. A Type 2-Apparatus dissolution tester (US Pharmacopoeia 28) with 6 independent vessels (Fig 5.1) was used for the studies.

![Type-2 apparatus dissolution tester](image)

Fig. 5.1 Type-2 apparatus dissolution tester. [Original in Colour]

A 1 mL aliquot of 60% Dox loaded BALC beads were placed in 500 mL PBS at room temperature. The beads were maintained in a normal PBS environment with stirring for at least 4 hours. The solutions in the vessels were constantly monitored by UV spectrophotometry at 483 nm, connected to peristaltic pump with recirculating tubing, with
samples scanned every 20 minutes until the 264 minute time point. At this point TCEP was
added to make up a final solution concentration of 60 mM. The addition of the reducing
agent at this point of the elution was to allow any initial burst of drug to level out, clearly
highlighting the impact of a reducing environment on the drug elution properties of the
microspheres. Further, 1 mL aliquot of 60% Dox loaded B ALC beads was allowed to
continue eluting in the vessel without the addition of TCEP as a control. Sample time
points were automatically taken every 2 minutes for a further 120 minutes.

5.2.3.3 TCEP-Reductive Efficacy Testing

1 mL of 60% Dox loaded B ALC beads were placed in a vial with 10 mL deionised water,
with a duplicate sample placed in 10 mL of deionised water with TCEP. A 1 mL sample of
Dox loaded DC Bead was placed in identical conditions. The solution was monitored by
UV Vis spectroscopy at 483 nm.

5.2.3.4 Drug Release from B ALC Beads using Reducing Agent Mimics

1 mL of 60% Dox loaded B ALC beads was placed into 200 mL deionised water in
triple. To one set of B ALC beads 60 mM of sodium citrate was added, to the other set
60 mM of citric acid. The solution of both sets of B ALC beads was monitored by UV Vis
spectroscopy (483 nm), at the same set point indicated in section 5.3.2.

5.2.3.5 Drug Release from B ALC Beads into Media with Different Ionic
Concentrations

Dox loaded B ALC beads were exposed to different ion concentrations in different drug
release media. The first method used 1 mL 80% Dox loaded B ALC beads which was
placed in 500 mL isotonic Ringer’s solution. An additional 1 mL aliquot of 80% Dox
loaded B ALC beads was added to another 500 mL Ringer’s solution with an reducing
agent of DTT (60 mM).

The second experiment involved placing 1 mL of 60% Dox loaded B ALC beads in
500 mL non-buffered salt solutions. The salt solution was made up in deionised water with
NaCl with concentrations of 0.9, 2, 5, 10 and 15%. A duplicate set of samples were
prepared for elution studies at these salt concentrations with an added 60 mM DTT. All drug elution was monitored by UV/Vis spectroscopy at a wavelength of 483 nm.

5.3 Results and Discussion

5.3.1 Effect of Reducing Agent of BALC Beads

Once loaded with drug, BALC beads were characterised to determine if they had the capacity to perform their primary function of releasing anticancer drugs in a controlled manner. The original hypothesis of the elution properties of BALC beads was that beads would release drug in normoxic conditions as per standard DEB-TACE formulations, whilst a reductive environment would cause an altered release rate of drug.

To test the release potential of BALC beads, a drug elution model was required that would provide a suitable reducing environment able to cleave the disulfide cross-linker within the microspheres, permitting the potential increased release of drug from the drug delivery system. Developing this elution model and creating a reducing environment that cleaved the disulfide cross-linkers and did not directly interfere with drug release was key to the experiments. A number of reducing agents were selected that are well known for their reductive potential. These reducing agents were added to the ionic release media solution as described in section 5.2.

The first reducing agent used was NaBH₄ which had shown its reductive potency in section 3.3.5 where it was used to reduce BAC beads. A Dox loaded 15% BALC bead formulation was prepared for investigation according to the method described in 4.3.1 and 5.2.3. However the experiment was stopped when an observation noted that the red colour associated with Dox solution had turned colourless after incubation with the reducing agent. On review of the literature, it was highlighted that Dox can undergo reduction of its quinine structure leading to loss of the red colour associated with the drug, which has also been shown to be susceptible to reduction in tumour tissue (Cummings et al., 1992). Sodium borohydride is known to be an excellent but strong reducing agent (Lu et al., 1997; Li et al., 2010) and was shown capable of reducing Dox, therefore NaBH₄ was removed from all further experiments.

The next reducing agent chosen was 2-mercaptoethanol also known to be a potent reducing agent of disulfides to thiols (Saito et al., 2003). Additionally it is a biological reducing agent that has been used in various experiments as the reducing agent of choice
(Meng et al., 2009). The control showed under UV/Vis spectroscopy that 2-mercaptoethanol did not alter the Dox spectrum, unlike NaBH₄. 2-mercaptoethanol (shown in Fig 5.2) was then applied to all high initiator BALC bead formulations according to section 5.2.3. The results achieved were promising with significant increases of drug released within a reducing environment (data not presented).

![Chemical structure of 2-mercaptoethanol](image)

Fig. 5.2 The chemical structure of 2-mercaptoethanol.

However, when 2-mercaptoethanol was applied to a control experiment, where a DEB with no cleavable cross-linker (DC Bead) was exposed to the reducing agent, an increase in drug release was achieved.

It has been described that 2-mercaptoethanol can be used in the dissolution of drug loaded delivery devices (Ugwoke et al., 2000). The fact that 2-mercaptoethanol has been used to extract drug from other delivery systems can help explain why there is a release of drug from DC Bead. It is not thought that mercaptoethanol is involved in the ionic exchange mechanism from DC Bead. However, mercaptoethanol has been used as a permeation enhancer e.g. in allowing more drug through the transungual barrier by swelling the nail (Kobayashi et al., 1999; Myoung and Choi, 2003). (Analysis of the structure of 2-mercaptoethanol presents the ethanol group which has been highly used in dissolution experiments of Dox from DC Bead (Gonzalez, 2006, Heaysman, 2009)). In the case of DC Bead loaded with Dox (which is hydrophobic and results in a decrease in the diameter of the beads), mercaptoethanol will swell the hydrophobic beads and enhance the ionic exchange and the dissolution of Dox in a mixed solvent system (PBS and 2-mercaptoethanol). Any Dox bound through Dox-Dox interaction will be susceptible to organic solvents (Gonzalez, 2006).

It was noted, however, that the increase in drug release from DC Bead was not to the same extent as the increase observed from BALC beads (data not presented). It is suggested that the disulfide bonds in BALC beads were still being reduced and therefore increasing the elution from the beads regardless of the solvent effect. The fact that all the 8, 15 and 31% beads release a much larger percentage of drug would suggest that the beads
are potentially redox responsive and do have the capacity to release more drug than a non-stimulus responsive bead. However, to prove and be able to quantify this claim, a new reducing agent was investigated. The new reducing agent should not have a solvent effect and should have no direct involvement in releasing drug from the bead, but still be very efficient at cleaving disulfide bonds.

5.3.1.1 Effect of Tris(2-carboxyethyl)phosphine (TCEP) on Drug Elution

Previous reducing agents have proven unsuccessful thus far, however investigations continued to identify a suitable reducing agent to permit \textit{in vitro} modelling of the BALC bead system. The ideal reducing agent would be selectively active towards disulfides without the capacity to reduce the drug. It would provide an irreversible reduction of the disulfide unit. The work of Rüegg and Rudinger (1977) with tributylphosphine showed that trialkylphosphine compounds could be used to reductively cleave disulfide bonds. The phosphorous compound attacks the S-S bond which initially forms a thiol-phosphine intermediate. In the presence of water it undergoes hydrolysis exposing the open thiol groups and the irreversible phosphine oxide (Dmitrenko \textit{et al.}, 2007; Buckler \textit{et al.}, 1962; Rüegg and Rudinger, 1977). Historically, the use of trialkylphosphines has been limited due to their relative insolubility in water and expense, that is until the synthesis of TCEP which is highly water soluble and relatively inexpensive (Burns \textit{et al.}, 1991). TCEP was shown by Burns (1991) and colleagues to be a clean and efficient reducing agent to cleave disulfide bonds.

TCEP is now frequently used as the reducing agent of choice for the reduction of disulfide bonds in nano based drug delivery systems leading to significant increases in drug release (Sun \textit{et al.}, 2013b). For these reasons TCEP was chosen as a potential reducing agent to aid the determination of a predictive modelling system for the release of drug from BALC beads.
Fig. 5.3 Application of TCEP in the reduction of disulfide bonds within a polymer structure in the presence of water. The diagram shows TCEP in its starting form reacting with the disulfide polymer, and the subsequent oxidised state of TCEP after reduction of the labile disulfide bond. [Original in colour].

Fig 5.3 shows the intended use of TCEP in the reduction of the novel bioresponsive microspheres from this thesis. It was postulated that when BALC microspheres were placed in the presence of this reducing agent, the disulfide bonds would be cleaved leading to a significant increase in Dox release. The effect of TCEP on the drug release from Dox BALC beads can be seen in Fig. 5.4. The experiment was performed according to section 5.2.3.2.
Fig. 5.4 Elution graph of 60% Dox loaded BALC beads in the Type 2 dissolution tester. Release of drug in a reducing environment (Red) and a normal environment (Blue). 60% BALC beads loaded with Dox to 93 mg.mL$^{-1}$. [Original in Colour] Addition of TCEP is noted. [Original in colour].

The result in Fig 5.4 demonstrates that there is an immediate reaction between the disulfide groups and the reducing agent TCEP which leads to a 100% increase in release of drug from the system. This release graph clearly shows that the response to the reductive environment is very quick and leads to the desired effect of increased drug release. This would meet the requirement of having an embolisation device that has a slow controlled release when in a normal tumour environment, however if the tumour turns hypoxic this can stimulate extra release from the system. This would introduce a concentration gradient to overcome the interstitial pressure and to penetrate further into the tumour and oversaturate the cells. This action of TCEP on reducing redox sensitive devices to release the anti-cancer payload has been previously demonstrated, with TCEP showing promise as a potential reducing agent to perform in vitro drug elution modelling (Sun et al., 2013a).
5.3.1.2 TCEP-Reductive Efficacy Testing

To verify and validate the results seen in Fig. 5.4, experiments were performed to ensure that the burst release of drug from BALC beads was a real effect and not caused by any phenomena other than the cleavage of the disulfide bonds. Preliminary testing by UV/Vis spectroscopy showed that TCEP does not provide an absorption spectra at 483 nm. However, when TCEP was added to Dox loaded BALC beads in the presence of no ions according to section 5.2.3.3, an unexpected trend was observed as shown in Fig 5.5.

![Graph showing UV analysis of BALC beads and DC beads with and without TCEP](image)

Fig. 5.5 UV analysis of 60% BALC Bead in water (483nm) Reducing agent (Green), No reducing agent (Dark Blue). Dox loaded DC Bead in water reducing agent (Light Blue), no reducing agent (Red). BALC beads loaded to 93 mg.mL\(^{-1}\) Dox, DC Bead loaded to 37.5 mg.mL\(^{-1}\) [Original in Colour].

As soon as TCEP was added to the BALC beads it was immediately observed that a red solution was eluted from the beads. Therefore an aliquot of the solution from the first minute of the experiment was tested by UV/Vis spectroscopy as shown in Fig 5.5. The results indicated that the drug was being released from the BALC beads. This phenomenon was also noted in DC Bead, with sample time of 2 min showing a continued increase in Dox being released into solution, although nowhere near the same rate as BALC bead (graph not shown). The beads with no reducing agent showed no release of Dox in
solution, and it would appear that TCEP could be having a direct impact on drug release from the drug eluting microspheres with a particular impact on BALC beads.

However the question is raised that if the reducing agent is having such an evident impact on the microspheres (Fig. 5.5) by directly elevating the drug release with no ions present to facilitate the ion exchange process, then why is there such a significant difference between the release of BALC beads and DC Bead. One clear difference between the devices is that DC Bead does not possess any labile disulfide bonds; it is unknown whether the release of drug is elevated because TCEP is reducing the cross-linker bonds in BALC bead and this cleavage is causing such a rapid increase in release of the payload.

However further analysis of the reducing agent TCEP shows that in order to aid solubility in water, it has three carboxylic acid groups. It could be possible that the drug is binding to the carboxylate group and as a result is diffusing out of the microspheres attached to the reducing agent. An experiment to test this hypothesis was conducted according to section 5.2.3.4, where two similar compounds to TCEP were employed, but neither compound possessed the reducing phosphine group. These two control compounds can be seen below in Fig 5.6 below.

![Chemical structure of sodium citrate (A) citric acid (B)](image)

Fig. 5.6 Chemical structure of sodium citrate (A) citric acid (B)

The chemicals chosen are almost identical to TCEP, except without the reducing agent in the centralised phosphine atom. Therefore the use of citric acid should not be able to cleave the BALC beads and the only potential interaction will be the binding of the drug on the tri-carboxylate groups. The sodium citrate was also used to study the effect of having a positive charge available that can directly displace bound drug by an ion exchange mechanism. The reaction was monitored by UV/Vis spectroscopy over time and the results can be seen in Fig 5.7.
The results in Fig 5.7 show that both citric acid and sodium citrate are able to elicit release of drug from the bead without reduction of the disulfide groups. The sodium citrate acts as a salt with the positively charged sodium ion and is expected to displace drug as does PBS. The effect of citric acid was, however, not expected; almost all the drug content of the bead is displaced into the water solution. The reaction of both compounds is instant, with citric acid displacing over 70 mg Dox from the beads within 30 minutes, while dissolution of the drug from the beads with sodium citrate is slower than that of citric acid, but a significant amount of drug is still eluted. Such a quick elution would suggest that, although there is an ion exchange process present with the use of citric acid, and therefore an effect present with the use of TCEP, there is an additional factor involved which could result in such a rapid release. It is postulated that carboxylic acid groups on TCEP and citric acid provide a source for ion exchange. Although the Dox is initially bound to acid groups within the microspheres, the movement of drug within the microsphere is not dormant and the drug is in a constant state of interchange between binding sites (Abdekhodaie and Wu, 2008). As TCEP has its own available binding sites when diffusing through the hydrogel and because Dox is in a constant state of movement between available acid binding sites, Dox is able to bind to the increasingly abundant carboxylic acid binding sites of TCEP and diffuse out of the microspheres. The work of Li (1998) also confirms this theory by showing that the citrate-Dox interaction is dynamic with bound
citrate and free citrate constantly exchanging. As citrate possesses carboxylate groups, it
could be argued that it is the reverse situation in the microsphere, and that Dox is
constantly exchanging with itself on the binding sites.

The release of drug with sodium citrate is slower than that with citric acid and there
are two potential reasons. It is suggested that as sodium citrate carries a positive charge, a
process of ion exchange takes place where the sodium ion is donated to the microsphere
binding site, releasing the Dox more slowly than that of citric acid. The second and most
important reason is the pH of the environment created by both compounds. Citric acid
creates an acidic environment, which was measured at pH 4 compared to sodium citrate
which is a well-known buffer, therefore Dox finds itself much more soluble in the acidic
conditions than it does in the pH buffered solution of sodium citrate, while at the same
time the latter sodium salt also decreases the solubility of Dox in solution (Fritze et al.,
2006; Mayer et al., 1990). For these reasons the release of Dox from BALC beads in water
is limited in the presence of sodium citrate, compared to citric acid, despite the structures
being very similar. It is important to note that the use of either compound could cause Dox
to aggregate into different states based on the literature by Li (1998), where Dox forms
organised fibre bundles in the presence of citric acid. However in the presence of sodium
citrate it is assumed Dox fibres (aggregates), which are not bundles and are more
disorganised, are formed. This could also be a contributing factor to the difference in
release between the two samples as seen in work by Heaysman (2009) where the molecular
weight affects the diffusion of drug through the microspheres. Therefore, disorganised and
more aggregated structures could have molecular configurations that impede release
compared to more organised structures.

Another interesting observation is the difference in release of Dox from BALC
Beads and DC Bead in the presence of TCEP (Fig 5.7). The control experiments have
shown that the release of drug is not directly related to the cleavage of the disulfide units
within the microsphere but is to do with a Dox-TCEP complex formation. It is postulated
that the reason why the release is dramatically reduced is due to the composition of DC
Bead with its sulfonate binding groups instead of carboxylate groups. Sulfonate groups
possess much stronger binding sites than carboxylate groups. Therefore BALC bead will
freely exchange Dox with the acid groups of TCEP, however in DC Bead the drug is more
inclined to remain bound to the binding sites of the microsphere.

The results of the use of TCEP, clearly demonstrate that it is not a suitable reducing
agent to form a predictive model for drug release from this bioresponsive system, as the
majority of drug release is not dependant on the cleavage of the disulfide units. However TCEP is still widely used by many researchers as a reducing agent to cleave disulfide links in nanocarriers when investigating the release of drugs such as Dox but with a focus on intracellular delivery (Wong et al., 2011; Fang et al., 2012; Sun et al., 2013a). The research of Wong and colleagues (2011), discusses the issue of redox sensitivity and that cleavage of these Dox conjugates lead to cell death with the release of Dox. However none of these papers mentioned the binding of TCEP with Dox as observed in this thesis. The rapid release of drug from these nanocarriers noted in these papers is not necessarily solely due to the degradation of the redox sensitive links but could potentially be due to the binding of the drug onto the reducing agent. This does call into question whether the use of TCEP in these experiments is appropriate and whether the use of an alternative reducing agent, which does not form a Dox complex, would produce a different result. This action of TCEP warrants further investigation and should be taken into account with all future research, but for the purposes of this thesis this reducing agent is no longer a viable candidate to assist in the understanding of the effect on Dox release when the bead increases in size after cross-linking cleavage.

5.3.1.3 Effect of DTT on Drug Release of BALC Beads

The alternative reducing agent chosen was dithiothreitol (DTT), known to be very efficient at cleaving disulfides to their respective thiol groups (Shirazi et al., 2011). Before being added to the BALC beads, the reducing agent was added to the loaded DC Bead formulation to determine whether it could increase the release of drug in a reducing environment. If it is a suitable reducing agent, there should be no significant increase in drug release in the DC Bead formulation in different environments. However a difference in drug release was expected with the BALC beads. Fig. 5.8 shows a triplicate study of loaded DC Bead being exposed to a normal environment and loaded DC Bead being exposed to dithiothreitol.
Fig. 5.8 A) Dox loaded DC Bead (Dox concentration 37.5 mg.mL$^{-1}$). B) 31% High initiator BALC bead elution graph up till 360 minutes (Dox concentration 30.1 mg.mL$^{-1}$). C) 31% High initiator BALC bead elution graph up till 1440 minutes (Dox concentration 30.1 mg.mL$^{-1}$) (Blue) in a non-reducing environment and (Red) a reducing environment (mean ± range, n=3).
Fig. 5.8 A shows that there is almost no difference in the amount of drug released from DC Bead when exposed to different environments. This would suggest that DC Bead does not respond to the reducing effects of dithiothreitol and that any drug release is due to the ionic exchange mechanism. As there is no redox responsive monomer within DC Bead no change in elution is expected. To determine whether the elutions are significantly different a two paired independent t-test assuming equal variance, was performed giving a p-value = 0.26. This allows acceptance of the null hypothesis and reveals that the elutions are not significantly different.

The loaded 31% BALC beads were exposed to a simulated normoxic environment and simulated hypoxic environment produced by the addition of DTT (Fig. 5.8 B). Fig. 5.8 B shows that there is a difference between the two elutions with the beads in a reducing environment releasing a higher amount of drug throughout the elution. The error bars show the variances do not overlap showing a clear difference between the two elutions. A two paired t-test was performed to determine if there was a significant difference between the two bead types over 500 minutes. The t-test shows that the elutions are significantly different to each other (p-value <0.05). This suggests that the BALC beads are stimuli responsive and are capable of releasing increased amount of drug. Whether this would be the case in a hypoxic tumour is an unknown and needs to be determined, however the in vitro results do demonstrate that the BALC beads have the potential to elute more in a reductive environment.

These results demonstrate there is a difference in the elution of drug from BALC beads in a reducing environment, while there is no difference in elution from DC Bead in a reducing environment. This shows that DTT does not have a solvent effect like 2-mercaptoethanol and therefore any increased release of drug from the BALC beads would be directly linked to the cleavage of the disulfide bonds by the reducing agent. It must be noted that the 24 hour result shows that elution of Dox from the BALC bead system did eventually level out in the reducing environment allowing the non-reducing system to achieve the same Dox levels (Fig 5.8 C). The result in Fig 5.8 C was unexpected as the increased elution was predicted to continue, however potentially there could be a saturation point that has been reached with the inclusion of DTT into the system, which could be causing any further Dox to precipitate (Alhareth et al., 2011). Such loss of Dox from elution models has previously been noted with studies performed with DC Bead. Another possible explanation is that the slower elution should be expected to catch up eventually as the faster elution has a more depleted source of drug. Further work was performed on
higher BALC formulations that load increased amounts of Dox in order to establish whether this difference in release can be further widened.

5.3.2 Effect of BALC Bead Formulation on Drug Release

This application of the potentially predictive in vitro hypoxia model was thus far applied only to the high initiator formulations. The differences between high and low initiator formulations have been described in Chapter 3 and 4, with more entangled networks formed due to longer polymer chains with low initiator concentrations. As differences were noted in the loading of these microspheres, it was important to test the impact of a reducing environment on the drug release properties of these microspheres and to note any potential differences. Therefore, with expectations already formed due to the elution results seen in Fig. 5.8, the 31% BALC bead formulation synthesised with low amount of initiator was loaded to maximum capacity with Dox and placed in the conditions described in section 5.2.3. At this stage, the BALC beads loaded were not pre-neutralised and therefore had lower Dox amounts loaded than potentially possible. The BALC beads in Fig 5.9 only loaded 15.6 mg Dox per mL of beads. Therefore the total eluted was expected to be lower than that of the high initiator formulation.

Fig. 5.9 Low initiator 31% BALC beads in (Blue) reducing environment and (Red) a non-reducing environment (Dox concentration 15.6 mg.mL\(^{-1}\)) (mean, n=2) [Original in colour].
The results of the Dox elution in Fig. 5.9 does not match the results achieved with the high initiator formulations. There appears to be no significant difference between the elution in a reducing or a non-reducing environment. This would suggest that this formulation is not redox responsive, however earlier characterisation studies with the high initiator 31% BALC formulations suggest otherwise (Fig. 5.8). The high initiator 31% BALC bead revealed that the beads were redox responsive with the use of the reducing agent DTT, with significant diameter size increases observed. With the knowledge that the composition of the lower initiator formulations is similar to that of high initiator formulation, the reason for a decreased release in a reducing environment is not clear.

Therefore at this stage, work was not completed on the synthesis of additional novel bioresponsive microspheres, such as other monomers, as further experimentation was required to obtain an understanding of the effect seen above. Further formulations of BALC beads were therefore synthesised for three reasons a) to better understand the implications of increasing the BALC content within the beads b) to increase the drug loading capacity and c) to achieve a significant increase in the release of drug in a reducing environment.

It was initially assumed that this decrease in release of Dox could be due to the different polymerised structure of the beads. With a low initiator formulation it is considered that the intertwining polymer network could be the cause of, not only slower uptake of drug due to less space around the binding sites, but a slower drug release. A more tangled polymer network could also increase the length of time it takes to reduce the disulfide links as the labile bonds are hidden or more difficult to access. Formulations with higher percentages of BALC were investigated to better understand the effect and to identify any patterns related to the formulation’s impact on drug release. The results of the elution are shown in Fig 5.10 A, B and C.
Fig. 5.10 Elution of Dox from A) 45% BALC bead (Dox concentration 25 mg.mL$^{-1}$) B) 60% BALC bead (Dox concentration 93 mg.mL$^{-1}$) C) 80% BALC bead (Dox concentration 108 mg.mL$^{-1}$) in a reducing environment (Red) and a non-reducing environment (Blue). The proposed point, where the reducing agent is thought to have altered the bead structure by reduction is highlighted in graphs B) and C). (mean, n=2) [Original in colour].
Fig 5.10 A, B and C are the results of the elution of the Dox from the BALC beads containing high concentrations of BALC. It is observed that in the 45% BALC Bead formulation there is no significant increase in release of drug in a reducing environment. The release profiles are very similar to each other and again it would suggest the low initiator formulations are non-responsive to a reducing environment, however again this contradicts the characterisation studies performed in Chapter 3. The results of the elution of the 60 and 80% formulations do suggest that the beads are bioresponsive, however the elution profiles are unexpected. It appears that in a reducing environment there is a complete reverse of the desired effect, where in fact the release of drug in a reducing environment becomes almost stunted and is approximately 50% less than that of the drug release in a non-reducing environment. UV analysis of the DTT incubated with Dox over 24 hours showed no change in the Dox spectrum, effectively ruling out reduction of the drug. With the use of DTT, the results demonstrate that as the BALC percentage increases and as the amount of Dox loaded increases, the amount of drug released decreases. This rejects the null hypothesis that increasing amounts of drug would be released from the high percentage BALC beads. Therefore a series of experiments were performed to understand this effect, and explain why the drug release in the low initiator formulations have been negatively impacted in a reducing environment in comparison to the high initiator BALC beads.

The following studies, as well as those from Fig. 5.10, were undertaken as scoping investigations. Therefore, due to the experience gathered over multiple experiments only duplicate sampling was performed at each time point because previous elutions from BALC beads, e.g. Fig. 5.7 and 5.8, were very close together and therefore reproducible. All replicate studies performed in this thesis as n=2 are displayed as mean, with all data points from the duplicate study consistently overlapping.

5.3.2.1 Elution Media Increase Effect Drug Release from BALC Beads

The ionic strength of the release medium is significant in the release of Dox from the beads but the rate of elution is not only determined by the salt concentration but by a concentration gradient for diffusion which is required in order to promote further drug release (Gonzalez et al., 2008; Lewis, 2010). The reaction rate of solute diffusion is believed to be slower than that of ion exchange and thus the reaction is thought to be
immediate (Abdekhodaie and Wu, 2006). Gonzalez (2006) showed that the elution is not only controlled by the amount of ions in solution, but critically by the maximum solubility of the drug in the release media and therefore the more media available the more drug that can be eluted.

An experiment was performed to see whether it was possible to force more drug out of the BALC beads, in particular the beads in a reducing environment. It was assumed that in previous experiments the release media was saturated with DTT, and therefore only a limited amount of Dox could be released or was soluble in the release media. Therefore the 60% and 80% BALC beads were retested in a larger volume of PBS with the results shown in Fig 5.11 A, B and C.
Fig. 5.11 Release of Dox in 500 mL PBS from A) 60% BALC Beads (Dox concentration 93 mg.mL$^{-1}$) B) 80% BALC Beads (Dox concentration 109 mg.mL$^{-1}$) C) Pre-neutralised 80% BALC beads (Dox concentration 250 mg.mL$^{-1}$) in a reducing environment (Red) in a non-reducing environment (Blue) in 500 mL PBS (mean, n=2) [Original in colour].
The results of the elution (Fig 5.11 A and B), in an increased volume of release media does lead to an increased amount of drug eluted by both BALC formulations in their own separate environment, however the rates and the shapes of the elution remain consistent with previous results of the same formulation. This elution graph appears to be very consistent and this unexpected release curve, where more drug is released in the non-reducing environment, appears to be heightened when more drug is involved in the higher BALC bead formulations. Although the media volume increases, the release is still inhibited and does not confirm whether there is a drug-DTT interaction that could be causing the drug to precipitate out or to reach a saturation threshold.

More drug was loaded into the BALC beads in Fig 5.11 C using the pre-neutralisation technique with the intention of removing the possibility of hydrogen bonding interfering with drug release and understanding how increasing Dox levels affected this trend. Fig 5.11 C, shows that when more drug is loaded into the BALC beads, more drug can be released into media with a larger quantity of drug released in a non-reducing environment. The results also indicate that potential hydrogen bonding between the acid groups is not responsible for the limited release of drug in a reducing environment. The impact of free binding sites is thought to play a role, however the number of free binding sites in these beads has been significantly reduced as a result of the loading method and amount of drug loaded and therefore is also not responsible for the poor release rate.

The increase in media does increase the release of drug from the BALC beads formulations; however it does not affect the difference in elution rates between the high and low initiator formulations. Fig 5.11 C highlights a concern that the release of drug in a reducing environment from the low initiator formulations may become affected to such an extent that it is not be possible to release drug from the bead or force any more out into the release media.

5.3.2.2 Sink Conditions Effect on BALC Bead Drug Release

A concern was highlighted in Fig 5.10 C where the release of drug from BALC beads in the 80% formulation was completely stunted and therefore studies were undertaken to confirm whether DTT was preventing BALC beads from releasing drug. A sizing study was performed on Dox loaded 60% BALC beads before and after reduction with the results shown in Fig 5.12.
Fig. 5.12 Sizing of 60% BALC Bead formulation loaded with Dox (93mg.mL$^{-1}$) in a non-reducing environment (Blue) in a reducing environment. (n =200). [Original in colour].

Fig 5.12 shows a size increase in BALC beads when the low initiator formulations are exposed to the reducing environment provided by DTT. A size increase should lead to an increase in drug release, however this does not translate into the results observed thus far with the low initiator BALC bead formulations. To test whether the high loading capacity BALC beads were able to continue to release drug in the presence of a reducing environment, a sink conditions elution study was undertaken according to section 5.2.3.1. The results of the elution can be seen in Fig 5.13.
Fig. 5.13 Elution of 60% BALC beads in PBS sink condition with BALC beads in a reducing environment (Red) and a non-reducing environment (Blue) (mean ± range, n =3) [Original in colour].

Fig 5.13 demonstrates that in a sink condition system, a burst release is noted. This is encouraging as it suggests the beads are different to beads in a non-reducing environment with the only factorial alteration being the addition of DTT. At the 1 hour time point, the BALC bead elution from both sets of beads are almost equivalent, however as time proceeds the distance between both data sets widens. Interestingly the results do show that BALC beads in a reducing environment are still able to release drug despite the slowdown in release still being observed. This increase in release can be attributed to a continuous replenishment of counter ions and therefore there was no longer a fixed volume of ionic solution. This meant that the point of equilibrium was constantly shifting, yet, at the same time not permitting the drug to saturate the release media.

A novel aspect of the release profile of BALC beads in a reducing environment is the slow-down and stop of release of drug in a closed system (Fig. 5.10). Although this graph shows that the release of drug is not completely halted with the addition of DTT, it still does not answer why this effect is observed. The results thus far in this chapter appear to suggest that there is a difference in elution between the high initiator formulations and the low initiator formulation, with more drug being released in a reducing environment from the high initiator formulations. The elution data also demonstrates that as the BALC
percentage increased within the low initiator formulations, decreasing amounts of Dox are released in a reductive environment. Further investigations were undertaken to understand the reasoning behind this effect and more importantly how this trend could be reversed. Elution models thus far in the thesis have focused on using the maximum solubility of the drug in a given volume to drive the elution. The sink conditions model was able to provide this diffusion gradient and was the only method able to continue Dox elution from the low initiator BALC formulations. However, the sink conditions model also provided an increase in available ions, another known driver of drug elution from microspheres (Gonzalez et al., 2008). Factoring in the results seen in Fig. 5.10, where there was no continued elution from the reduced low initiator formulations, it is suggested that the chemical mechanism, dependent upon the concentration of ions, could be used to drive the drug elution from reduced BALC beads. The impact of different ionic concentrations in release media was used to test the effect on drug release from BALC beads.

5.3.3 Effect of Different Elution Media on Drug Release from BALC Beads

It has been repeatedly shown in ion exchange resins that a high concentration of ion-exchange counter ions is required to break the electrostatic affinity of the drug to the polymer and release Dox into the medium (Anand et al., 2001; Lewis, 2010). If the ionic strength of the release media is changed, the rate of release can also be changed with higher ionic solutions providing increased release of drug. As the cross-linking increases within a formulation, the release is slowed (Saway et al., 1988). Therefore in the case of BALC beads, the formulation in the reducing environment should have fewer cross-links as the disulfide bonds are cleaved and should theoretically have a quicker release than that of the non-reduced formulation.

Investigations of drug release using plasma have shown that the elution rate is faster than PBS with over twice the amount released in vivo (Gonzalez, 2006). Plasma contains sodium ions as does PBS, however it also contains higher levels of potassium and calcium salts which provides a higher concentrations of ions to displace the drug. Ringer’s solution is one of the original simulated body fluids and provides conditions with inorganic ions that would normally be found within the body (Jalota et al., 2008). An experiment was prepared placing BALC beads in Ringer’s solution, to determine whether conditions more similar to that in the human body would alter ceasing of BALC bead elution in a reducing
environment. It was considered that a change in release media to one containing more physiological relevant ionic concentrations might provide the answer to the problem of the limited release profile. If there are still free binding sites being exposed after reduction, would the increase in counter ions be strong enough to limit the pull of these binding sites, and draw drug out of the reduced bead quicker? Using the method described in section 5.2.3.5, the drug elution properties of BALC bead were tested with Ringer’s solution with the results shown in Fig 5.14.

![Graph](image)

Fig. 5.14 Elution of 80% BALC beads in 500 mL Ringer’s solution in a reducing environment (Red) and a non-reducing environment (Blue) (Dox concentration 109 mg.mL⁻¹) (mean, n=2) [Original in colour].

The results of the elution in Ringer’s solution show an increased release of drug into the media for both sets of bead in either redox environment which is due to an increased abundance of counter ions. In this case however release of both sets of beads is identical until the four hour time point when again the beads in a reducing environment appear to decrease and stop releasing drug into the media. Interestingly, the amount of time it takes for the observed effect to take place doubles in time and is not as pronounced as previously noted for the elution of the BALC beads into PBS. The impact of a greater quantity of ions was initially thought to be responsible for this increase in release and increase in duration of release. However, even in the PBS solution, as shown by the elution of the beads in a non-reducing environment (Fig. 5.11), the concentration gradient of ions provided is more than sufficient to drive out more drug. Therefore it is assumed that
although concentration of ions could have an impact, it is not thought to be the reason for an improvement in the release profile of reduced BALC beads. A difference between the release of drug in PBS and Ringer’s, other than the ion concentration, is the solution pH and this is thought to be a factor for the obtained release profile. PBS is buffered to a value of pH 7.4 whilst Ringer’s solution can be buffered with sodium bicarbonate (Jalota et al., 2008). In this experiment (Fig. 5.14) it was recorded as pH 6.5.

With this in mind the next set of experiments were performed with the use of different concentrations of salts but without using the phosphate buffer. This would result in the beads eluting into slightly acidic solution with an approximate pH 5.7 (McLean, 1982). The 60% BALC beads were loaded to maximum Dox capacity and placed into a sodium chloride water solution. NaCl solutions of 0.9, 2, 5 and 10% were used. 60 mM DTT was added to create a reducing environment and to cleave the disulfide links in the presence of the beads. The elution was monitored by UV/Vis spectroscopy and the reduced beads and the non-reduced beads were sized before, during and after elution for both sets of beads. The release of the drug in saline solution can be seen below in Fig 5.15.

![Graph showing elution of 60% BALC beads in 0.9, 2 and 5% NaCl concentrations (Dox concentrations 93 mg.mL⁻¹).](Original in colour)
The results of the elution in Fig 5.15 show that after reduction of BALC beads, an increase in release of Dox is seen in all salt conditions. Even with such high drug concentrations and with a high percentage BALC formulation, the halt in drug release is no longer seen.

In all salt conditions, the release profile of the 60% BALC bead is similar with an increased release in the reducing environment. At the 24 hour point in the 2 and 5% NaCl solution, the Dox elution of the non-reducing beads has eventually released the same amount of drug as the beads in a reducing environment. Although protracted, this equal amount of drug release from BALC beads in both release environments is expected, and unlike the previous release profiles, the reduced beads did not immediately stop releasing drug. The manner in which the release of drug from reduced BALC beads slowed down would suggest that maximum saturation had been reached within that release media. This implies that all the counter ions had been utilised in each formulation and therefore no more drug could physically be released or dissolved in the release media. Maximum solubility had been reached and the drug release plateaued.

For the 0.9% w/v NaCl solution there was still an increase in drug release in a reducing environment, however compared to the higher salt concentration elutions, there was less of a diffusion gradient and less of a concentration of counter ions to drive the elution. Therefore more time was needed for the non-reduced drug release to catch up with the reduced beads’ drug release. Greater focus is given to the results of the 60% Dox BALC beads in 0.9% salt solution, as this is the salt concentration used for elutions throughout this chapter and examples of the relative drug release can be seen before. In addition, the release of the 60% BALC beads in the 0.9% salt solution does not encounter the same release inhibition in a reducing environment seen throughout the chapter.

The results show that in non-buffered conditions the release of drug from beads in a reducing environment is not hindered and there is no drop in Dox release. The beads from each elution were sized before, during and after elution. The beads in a non-reducing salt condition did shrink in size. However in the presence of concentrated salt solution where the majority of beads shrink, after reduction the BALC beads were still able to increase in size as shown in Fig 5.16 A and B. A BALC bead was placed in the presence of 15% salt solution to see if the shrinkage would increase as the salt concentration increased.
Fig. 5.16 A) Dox loaded 60% BALC beads in 2% salt solution B) Dox loaded 60% BALC Beads in 15% salt solution before elution and reduction (Blue) and after elution and reduction (Red). Dox concentration 93 mg.mL$^{-1}$ (n=200). [Original in colour].

The sizing data are of interest as they show the BALC beads are capable of expanding in size, even in the presence of a high concentration of NaCl, which hydrogels such as DC Bead have been shown to be sensitive to, causing shrinkages in the bead size (Lewis et al., 2006d). The drug elution results of the 15% w/v NaCl solution are not relevant because at this concentration, the saturation of Dox in solution is heavily influenced. However the sizing data demonstrates that there is no real restriction in bead size increase between the 2 and 15% salt solution before or after elution.
However, it was noted that the average size increases seen in the formulations were not as large as BALC beads reduced in PBS, with the 2 and 5% w/v NaCl conditions resulting in average increases in diameter of only 60 µm. This would suggest that the high concentration of ions could be restricting the overall diameter of the beads, however another explanation that would fit more appropriately is that DTT is not as active in acidic conditions as it is in neutral and alkaline conditions. The reductive power of DTT at pH 8 is shown to be 10 times stronger than that of DTT in a solution at pH 7 (Cleland, 1964). The reducing power of DTT is controlled by the negatively charged thiolate form, and when placed in increasingly acidic conditions, the thiol group becomes protonated due to its high pKa of 9.2, therefore the reducing agent is no longer active with pH levels as high as pH 4.5 demonstrating DTT to be ineffective (Han and Han, 1994). Therefore the reason why the beads did not expand to full capacity may be due to the DTT being very ineffective and slow at breaking the disulfide bonds. This result is of great interest as it shows that when the reducing agent has been nearly inhibited, the beads are able to release in a reducing environment without the release being halted. As such, a high concentration of DTT is added, the beads are still partially reduced and this does aid in the increased release of drug.

5.3.4 DTT-BALC Interaction

The results of all the elution work described so far does directly implicate DTT as the main factor involved in causing cessation of drug release as seen Fig 5.11. DTT is a commonly used reagent in the reduction of disulfides as previously discussed, and its oxidation mechanism is shown in Fig 5.17.
Fig. 5.17 Structure of DTT and the mechanism for the reduction of a disulfide group resulting in the oxidisation of DTT to its cyclic form.

Fig. 5.17 shows the thiolate group cleaving the disulfide bond and in doing so forming an intermediate whereby the reducing agent is temporarily bound to the thiol group of the original disulfide bond of the bead. The intermediate is thought to be unstable as the remaining thiolate is rapid at attacking the formed intermediate disulfide and cyclisation takes place leaving the disulfide bond of the bead in its thiol state. The DTT is then rendered inactive. The use of DTT as a reducing agent is still common place and is commonly used in \textit{in vitro} modelling of the reduction of disulfide groups in nano carriers targeting the intracellular environment of tumour cells (Huang \textit{et al.}, 2013a). The results of models show promising results with rapid release of drug, however most of the intracellular delivery devices are nano particles that immediately disassociate. The BALC beads remain intact for a long period of time and this permits the DTT to interact with the beads in a manner not yet fully understood until now.

It was initially thought that DTT itself possesses two thiols which would cyclise into its oxidised state as shown in the mechanism above, however there is now evidence to suggest that DTT has the potential to form stable mixed disulfide adducts with cysteine residues (Li \textit{et al.}, 2001). After reduction of the disulfide groups, the DTT forms a cyclic structure which itself becomes an oxidising agent (Srivastava \textit{et al.}, 2011). As an oxidising agent, the cyclic DTT has the capacity to form disulfide bonds as has been well documented in the body with thiol/disulfide exchange (Schafer and Buettner, 2001). The cyclic DTT oxidising agent is now able to form a mixed disulfide bond between the cysteine residue as the cyclic DTT structure is opened. The remaining thiolate group will
form another disulfide bond with another cysteine residue creating a bridging DTT compound that is now bound to the BALC bead (Li et al., 2001). Very little literature exists around this mechanism, which is thought to be a rare occurrence, but the work that has been performed used analytical techniques such as MS to prove the existence of this adduct and intermediate structures (Begg and Speicher, 1999). The first report that highlighted this unusual occurrence provided the possible mechanisms for DTT binding to cysteine residues and forming a bridge, as shown below in Fig 5.18.

![Fig. 5.18 Diagram of how a reduced BALC bead with its protruding cysteine residues are oxidised by the cyclic DTT compound. The DTT forms a bridging adduct with the bead, closing the space left by the cleavage of BALC.](image)

As described in the first paper by Li (1998) and colleagues, obligatory intermediates are formed before the adduct is obtained, and it is possible to bind multiple DTT molecules together via disulfide linkages before the loop is closed and attached to an open cysteine thiol (Li et al., 1998b). Once the DTT molecule is opened and bound to one of the cysteine moieties, usually the free thiol would cyclise, however there are unknown specific interactions between the DTT and the BALC which switches the conformation of the structure and makes the disulfide inaccessible to attack from the remaining free thiol of DTT. This leaves the thiol to form a bridge between a cysteine molecule or another DTT molecule.

Looking at the evidence provided and abundance of intermediates discussed by Li (1998) and colleagues, the data would also suggest that the formation of the disulfide can also occur when reduced DTT cleaves the disulfide and forms the intermediate shown in Fig 5.17. This attachment would also be hindered in cyclisation to the oxidised state, and
with DTT as an oxidising agent, the length of the number of disulfide chains is potentially unlimited.

It is postulated in this thesis, that as DTT cleaves disulfide bonds of BALC, it is cyclised into the oxidised DTT form and/or binds immediately to form the discussed intermediate in Fig. 5.17. As more BALC becomes reduced, more oxidised DTT is formed with the cysteine groups now providing binding sites to form disulfide linkages. Therefore DTT cross-links the BALC beads through the formation of these disulfide bonds. Although the new DTT adduct is itself susceptible to cleavage, the bond formed is more stable as the reaction proceeds, with the environment becoming less reducing as the attachment of DTT to the cysteine groups continues to form adducts. Cleavage of the DTT adducts would additionally present further opportunity to incorporate further DTT cross-links on the BALC beads. This is important as it would suggest that the DTT that binds to the bead will remain on the bead.

DTT is also a compound that is highly unstable and known to autoxidise at zero order kinetics in some conditions. Even with the aid of a chelating agent, 15% of a batch will oxidise in 1 week when at 4°C, while over 50% of DTT solution in distilled water oxidised over a period of 24 hours at room temp (Getz et al., 1999). Although the percentage of each is unknown for these experiments, the DTT added is a mixture of both reducing and the oxidised form of DTT. This amount of DTT in the oxidised state should sufficiently drive the attachment of a DTT adduct.

The DTT acts as a bridging agent, and closes the newly opened channels, however the bridging DTT can contain multiple DTT residues and can form bonds with open thiol groups further away therefore causing further cross-linking within the bead and overcrowding the channel from which the drug diffuses out. Fig 5.19 is a diagram showing how the adduct, due to specific interactions or local conformations, will not necessarily reform a disulfide linkage with the closest thiol group or the molecule it was cleaved from, but the interaction will take place with the thiol that is more presentable to the shape of the adduct and offers the least steric hindrance.
Fig. 5.19 Diagram showing forming of DTT adduct bridge between two thiol groups that are not next to each other. [Original in colour].

The explanation of DTT forming an adduct with reduced BALC explains the effect of the elution of the beads in the presence of DTT. The adduct formation can take place within the core of the bead and is potentially capable of blocking the beads from the inside, however as the majority of the DTT is in solution, the majority of the adduct formation will take place at the surface of the bead. When dissolved in solution the DTT will meet the bead and the quickest reaction will be with most available thiols which will be at the surface. This type of reaction has been seen before with the redox couple used to polymerise DC Beads. KPS is distributed throughout the microsphere, however the TMEDA added to the reaction will meet the spherical un-polymerised beads at the surface and therefore the reaction works its way from the outside to the inside leaving a higher amount of binding sites at the surface (Lewis et al., 2007). As the immediately available thiols are present at the surface, the majority of the adduct formation is postulated to take place covering the bead and forming a film. As more DTT reacts, the film becomes thicker and as the percentage of BALC increases, the more adduct formation takes place. This hypothesis of a DTT forming film has been previously noted, where disulfides such as DTT have been used in the synthesis of polymer films that can coat the outside and cover an object (Shu et al., 2003; Antelman et al., 2009). The finding of this research would suggest that a DTT adduct film full of disulfide bonds has formed around the outside of the microspheres.

With a DTT adduct film forming around the outside of the BALC bead, a reduced rate of elution should be expected. As the percentage of BALC increases, especially with the higher percentage BALC formulations, the majority of the surface will be covered with these DTT attachments which most likely would nearly block the bead from releasing its
payload as seen in elution graphs such as Fig 5.11. Also the higher the BALT concentration, the quicker and easier the adduct formation. This formation would explain why the 60% and 80% BALT bead formulations struggle to elute in comparison to the beads not in the presence of DTT. As the BALT concentration reduces, the effect is not as pronounced as BALT covers less of the bead surface and the adducts take longer to form. In the low formulations such as 15% BALT, the effect is not noticeable because of the low percentage of BALT, adversely the desired significant increase in drug release is compromised as there is less drug loading and less cleavage of disulfides.

This effect was not apparent in the high initiator formulations, as the polymer chains are likely to be shorter and therefore less tangled. It is hypothesised that in the low initiator BALT formulations, the disulfide groups are found closely together due to chain entanglements which could be causing longer exposure of DTT to the thiol groups. With the intertwining and cross-linked hydrogel network, slower diffusion is expected in and out of the beads. The close proximity of the BALT groups may force DTT into specific conformations suited for an adduct formation. Such adduct formations could be taking place in the high initiator formulations, however they will take longer to form and will be less common than in the low initiator formulations, so much less pronounced. The Dox release in DC Bead was obviously not affected as there are no thiol groups with which to form an adduct.

The release of Dox in the salt conditions was very informative as it was the only time the release of Dox in a reducing environment was not halted. This key evidence suggests that DTT was directly involved with the unwanted release phenomenon, as DTT activity is limited under pH 7 (Singh et al., 1995). The Ringer’s solution provided an understanding of the results and in conjunction with the Dox-DTT experiments, it inferred that the DTT was having an interaction with the polymer.

All this work suggests that the use of DTT as a reducing agent for BALT beads is not conducive to demonstrating the benefits of the redox responsive microspheres and shows that the reducing agent actually interferes with the polymer hydrogel. Therefore the models presented are not suitable for representing an in vitro modelling system and the capabilities of the redox sensitive microspheres are still not yet fully appreciated. The data does however provide confidence that the same inhibition of drug elution from beads would not be achieved if the microspheres were used in the treatment of a hypoxic tumour. Firstly, the main reducing agents do not provide a dithiol compound such as DTT but most importantly in the body there are several detoxification enzymes such as glutathione-S-
transferases and NADP(H) quinone oxidoreductase that are involved in mopping up oxidising agents and are responsible for protecting the cells from oxidative stress in a cancer environment (Acharya and Das, 2010). With these systems in place, reducing agents will not be able to oxidise onto the bead while the tumour is active and hypoxic. Therefore based on the characterisation studies that demonstrate that the beads are bioresponsive and do expand to an increased size with drug, an increase in release should be possible as demonstrated in an acidic environment and in low BALT formulations. Although this data does not provide the final desired approximate 50% increase in release, it does provide knowledge and understanding around the microspheres and gives the opportunity to continue with further work.

5.4 Conclusion

The results of this chapter have shown unexpected novel interactions not previously recognised or highlighted by others. The studies set out to answer the aims set out at the beginning of this chapter: whether drug loaded BALT beads are bioresponsive; whether differences in initiator concentration affect elution; and whether varying BALT content affects the elution. It was initially hypothesised that the formulations with high BALT percentages, due to their increased drug binding capacity, would offer an optimal release rate. When placed in a reducing environment, these high percentage BALT beads were expected to offer the greatest increase in drug released due to the higher drug content and the increased cleavage of disulfide links. However, the data has shown the difficulty in modelling a predictive in vitro system that can overcome the complexities and dynamism of the human body. Multiple reducing agents were analysed and tested for their ability to provide a reductive environment capable of reducing the disulfide bonds found within the beads, but at the same time does not interfere with the elution of the beads.

With NaBH₄, the reducing agent itself was too powerful and interfered with the UV absorbance of the drug. Mercaptoethanol was actively involved in removing drug from the beads and this was even more obvious with the use of another commonly found reducing agent, TCEP. The interaction between reducing agent and drug did not require the presence of any ions to disassociate the drug from the microsphere and was powerful enough to break up the electrostatic interactions of Dox-Dox.
The final reducing agent used was DTT which was applied throughout the thesis, and is a commonly used disulfide reducing agent. The use of DTT in this thesis has demonstrated that the BALC beads are bioresponsive, however it has also highlighted that DTT can directly affect drug release from BALC beads, especially in high percentage BALC formulations. This is due to the preferable formation of a DTT adduct that cross-links the BALC beads. This adduct was easier to form in low initiator BALC formulations, and as the BALC concentrations increased, so did the adduct formation which resulted in a DTT film covering the microspheres and halting the release of drug. However, in the lower BALC formulations and in more acidic conditions this DTT adduct was less easily formed, with less of an impact on drug elution.

Chapter 5 intended to produce a suitable model to predict the release of drug from BALC beads. However, due to the lack of suitable reductive agent, a true understanding of the BALC bead drug release potential has not been achieved. The results do however suggest that if a suitable reducing agent was identified, increased drug release from BALC beads in a reducing environment is achievable. The results also show that the amount of cross-linker and the length of the polymer chains of the beads directly impact drug release. All these factors must be taken into account in order to optimise the current BALC formulations. These results lay the foundation to future process development of the BALC bead formulation and predictive model to characterise the microspheres.
Chapter 6

6 Degradable Properties of BALC Bead

6.1 Introduction

Biodegradation is defined as the molecular changes that occur due to chain scission inside a polymer matrix and is caused by biological processes, such as those present in the human body (Chen et al., 2011). Microspheres have been designed to biodegrade after implantation, such as DSM described in section 1.3.1, however there are no commercially available biodegradable formulations for TACE. The only commercially available drug eluting microspheres such as DC Bead, are permanent embolics. Adversely, due to its long term presence and inability to degrade, the retention of high concentrations of drug has been shown to lead to chronic inflammation causing further tissue damage (Weng et al., 2013). To avoid long term post implantation inflammation, synthesis of a biodegradable drug eluting bead could overcome such complications.

In the synthesis of a biodegradable microsphere, the hydrogel could be designed to respond to a number of stimuli including: temperature, light, magnetic fields and molecular recognition events (Qiu and Park, 2012). Response to the chosen stimuli could act as a trigger, leading to changes in molecular interactions that lead to macroscopic responses including biodegradation (Ulijn et al., 2007). Hydrolytic degradation is frequently used as a means for polymer degradation, and was observed in a pre-clinical sheep study with Occlusin™ (Owen et al., 2012). Other observed degradation has been through pH and enzymatic degradation of chitosan based films and microspheres respectively (Bonina et al., 2004; Ramachandran et al., 2011). Disulfide cross-linkers are well known for their ability to respond to stimuli by reduction; however it has been shown that substitution of a disulfide group into microspheres allows the device to biodegrade over time (Gu et al., 2013). Disulfides are commonly used as the most notable example of a labile group employed in degradable polymers (Rikkou and Patrickios, 2011). Nano delivery devices containing disulfide groups have been shown to carry drug which is only released after degradation of the device (Ulasan et al., 2014). Therefore, it is suggested that by the inclusion of these labile disulfide groups into BALC beads, that the microspheres of this thesis are not only bioresponsive but potentially biodegradable.
BALC beads have so far demonstrated their ability to load cytotoxic drug and respond to a reductive environment by size increases through cleavage of the disulfide groups. However, long term analysis of BALC beads has not yet been conducted to understand what physical changes ensue post reduction. This chapter describes the work carried out to investigate the degradative properties of BALC beads, and how varying the percentage of BALC in the formulations affects this degradation. It is of interest to note that greater degrees of cross-linking can slow the rate of degradation (Bonina et al., 2004). With the varying degree of cross-linking within each BALC formulation, the work in this chapter will provide a greater understanding of BALC microsphere degradation kinetics. The data could be used to choose a suitable level of cross-linking to provide greater control of embolisation over time and potentially drug release.

6.2 Materials and Methods

6.2.1 BALC Bead Degradation

Each formulation of BALC Beads, 0, 15, 31 (Low I), 45, 80% were dispensed (0.5 mL) into Millipore centrifuge tubes with inserts of a 5 µm pore mesh on the bottom. Four inserts were set aside for each formulation for each time point. The microspheres were placed in the removable inserts from the centrifuge tubes. The samples were placed in a minifuge and centrifuged at 6000g. The samples were spun for 15 seconds (twice) to remove any excess solution through the bottom mesh. Once the solution was removed the inserts were weighed on a calibrated balance noting the starting wet weight of the beads (insert weight already noted). Then 1 mL of 7 mM DTT solution in PBS was added to each sample, the tubing sealed and placed in an oven at 37°C. The DTT solution was removed from the beads at a 7 day time point, with 1 mL of fresh 7 mM DTT solution added. This was repeated every 7 days for the duration of the experiment.

All of the four bead samples prepared for each formulation, at each sampling time point, were then placed in the minifuge, removing the excess DTT solution by centrifuging for 15 seconds (twice). The inserts were placed on the balance taking the post incubation wet weight of each sample (n=4). Three of the samples were then placed in a vacuum oven at 50°C overnight to obtain the dry mass of the volume of beads. The 4th sample was hydrated in water and analysed under the light microscope and was also sized using the
method described in section 3.3.4 and 3.3.5. Images of the bead were then taken with an Olympus BX50 microscope and ColorView III camera for visual analysis.

6.2.2 Drug Loading and Elution of BALEC Bead Synthesised with Alternative Co-monomers

The microspheres were made using analogous methods to that described for the PVA macromer and BALEC formulations (section 3.3.2), with the PVA being substituted in one study for 2-hydroxyethylmethacrylate (HEMA) (21g HEMA + 2.5g BALEC) and in another study for acrylic acid (AA) (21g AA + 2.5g BALEC) as described in section 3.3.8. In both examples, particulate or bead-like products were isolated. BALEC-HEMA beads and BALEC-Acrylic acid beads were loaded with Dox according to the method described in section 4.3.1.

1 mL of Dox loaded beads from each formulation was then placed in 200 mL PBS, while another 1 mL of Dox loaded beads from each formulation was placed in PBS (200 mL) with a reducing environment provided by DTT (60 mM). The drug released by the microspheres was monitored over set time points with samples of solution removed for analysis by UV/Vis spectroscopy (483 nm). Microspheres were also imaged using the Olympus Bx50 microscope with a ColorView III camera.

6.2.3 Incubation of Dox Loaded BALEC Bead in a Reducing Environment

1 mL 60% BALEC bead was loaded to maximum capacity with Dox according to the method in section 4.3.1. The Dox loaded BALEC beads were eluted following the method described in section 5.2.3, with 1 mL of Dox loaded beads (93 mg.mL⁻¹) in 500 mL PBS and another 1 mL in PBS (500 mL) with DTT (60 mM). These results are shown in Fig. 5.11.

After monitoring the elution of the microspheres for 24 hours, the Dox loaded BALEC beads were sealed and stored in the containers at room temperature for 4 weeks. The elution profile was no longer monitored post 24 hours incubation in the PBS solution. At 1 week intervals, 60 mM DTT was added to the BALEC beads already in a reducing environment. The BALEC beads were gently agitated for 5 minutes post DTT addition, then allowed to settle until the next interval. At the 4 week time point BALEC beads from both
containers were imaged using the Olympus Bx50 microscope with a ColorView III camera.

6.3 Results and Discussion

6.3.1 Degradation of BALC Beads

The potential for BALC beads to be biodegradable was highlighted, post elution of a 60% BALC bead loaded with Dox. The samples as described in the method 6.2.3, were retained in their glass containers for 4 weeks with a continuous dose of DTT added at weekly intervals. After this incubation period the beads were visually analysed (Fig. 6.1).

Fig. 6.1 Images of 60% Dox loaded BALC beads after 4 weeks in a non-reducing environment (A) and a reducing environment (B) [Original in colour].

Fig. 6.1 Images of 60% Dox loaded BALC beads after 4 weeks in a non-reducing environment (A) and a reducing environment (B) [Original in colour].

Both sets of samples from Fig. 6.1 were removed from their respective environments and placed in water, to ensure that the microspheres would continue to retain drug within the polymeric structure and not elute into the solution. Fig. 6.1 A shows that the 60% Dox loaded BALC beads stored in PBS over the incubation period retain their spherical shape and show no sign of fragmentation. This suggests that with no reducing agent present, there is no reduction of the microspheres. However Fig. 6.1 B confirms that when BALC beads are retained in a continuous reducing environment, they are degradable and will eventually break down and fragment. These images were the very first indication that the BALC beads synthesised within this thesis were drug loading microspheres with degradable properties.
Fig. 6.1 B demonstrates that a continuous addition of DTT was able to degrade the beads and cleave the potential DTT bridges/adduct formation highlighted in section 5.5. It is thought that DTT adducts still form and will initially bridge the thiols together, however as more reducing agent is added, the DTT bridges themselves could become cleaved allowing a further alteration in bead structure. Although it is thought that the addition of DTT will lead to the formation of further bridges, the high concentration of the reducing agent should initially be able to cleave the existing adducts and drive reduction of the beads. It is suggested that this process occurs because when DTT is added and the bead changes, thiols become further apart making it more difficult for these bridges to form. If these adducts do form it is thought they will be fewer in number, and with continued reduction of the bead through further DTT addition, the numbers of adducts finally formed may not be enough to hold the bead structure together. Thus as more fresh reducing agent is added, the size of the bead should expand and the internal network alter to an extent where the adducts can no longer form or are limited in their ability to form. They should then have no bearing on drug release.

Fig 6.1 B shows the degradation of the BALC bead, however it was previously assumed that as BALC and PVA are both soluble in water there should be nothing observed under the microscope as the BALC beads degrade. This is the case with the PVA macromer starting material used in the synthesis of the bead. However, particulates are seen here and it is believed that although they are dispersing in solution, the Dox still present is holding them together in conjunction with DTT adducts. The explanation for why Dox is still present in degraded BALC beads can be seen in the elution graph Fig. 5.11. The elution graph of BALC beads shows that the media is quickly saturated with Dox and no further release is observed over the 24 hour period. The elution of the BALC beads is performed in sealed containers and over the 4 week incubation period, there is no further addition of PBS to the containers. The only addition is DTT which is added in powder form to the existing elution media. Therefore no significant increases in Dox release is expected from the degraded BALC beads over this 4 week period, even though there is now a greater exposure of drug to the elution media due to bead degradation, as the solution is already saturated and there are no additional ions to drive drug release. The majority of the drug, which has not been eluted according to Fig. 5.11, will therefore still be held within the microspheres and the degraded bead particles. This is demonstrated in Fig. 6.1 B, where the bead particles which are placed in deionised water show the drug is clearly attached to the BALC fragments. This continued binding of drug to the degraded
BALC bead particles could be hindering the solubility and break down of the BALC beads. It is suggested that if the elution media had been replenished at the same time points of DTT addition, the majority of Dox might have been released from the BALC beads along with a greater degree of degradation.

A basic research stability experiment was performed as described in section 6.2.1 to further investigate the degradative properties of BALC bead. The samples were analysed visually and the samples were weighed before and after incubation. The final dry mass volume of the microsphere formulations was taken after incubation of up to a period of 9 weeks, as discussed in section 6.2.1. The results are displayed below in Fig 6.2 and optical images of the microspheres after incubation displayed in Fig 6.3.

![Fig. 6.2 Percentage weight loss of BALC bead formulations after incubation in a reducing environment for set periods of time. (mean ± range, n=3). [Original in colour.]](image-url)
Fig. 6.3 Images of 0, 15, 31, 45 and 80% BALC Bead after incubation for 0hr, 24hrs, 72hrs, 1 week, 3 weeks, 6 weeks and 9 weeks. [Original in colour].

Fig. 6.2 and Fig. 6.3 clearly demonstrate that BALC beads are degradable. The weight data shows that there is a decrease in BALC bead mass for the majority of formulations over the incubation time in the presence of a reducing agent. In Fig 6.3, there is no change in the appearance of the microspheres within the first 7 days, with the weight data showing no change. However at the 21 day time point, there appears to be a change in the weight of the microspheres with the 45% BALC bead formulation showing a >50% mass loss compared to its starting weight. This is supported by the images showing subtle
changes in the overall shape of the 45% and interestingly the 80% BALC formulations. Although the weight data does not support the degradation of the 80% BALC microspheres until the final 9 week time point, the images conclusively support the degradation of the 80% BALC microspheres from the 21 day time point onwards. The weight data and the images confirm that between the 21 and 64 day incubation period, the 31, 45 and 80% BALC microspheres undergo the greatest and fastest degree of degradation of the formulations, with the 80% BALC beads no longer present in the inserts after incubation, having undergone complete degradation. Although Fig. 6.2 shows weight loss of the higher percentage BALC formulation, initial observation of the images show spherical beads. However, on closer analysis of the images in Fig. 6.3, the beads are not as spherical as first assumed. More importantly, from the 42 day time point for the 31% BALC bead and more pronounced in the 45% BALC bead formulation, the microspheres begin to gel together. This joining of the beads through physical attachment can be seen earlier at the 21 day time point for the 80% BALC beads. Gelling of the beads is considered part of degradation process, demonstrated by the image of 31% BALC beads at 64 days in conjunction with the weight loss data. It is suggested that gelling is witnessed due to the constrained environment of the insert that the beads are placed within. As the beads degrade, free thiol groups could be joined together through potential adduct formation. However, as a reducing environment is maintained through continuous DTT addition, the BALC beads eventually fully degrade as shown by the 45 and 80% BALC bead images at the 64 day time point.

The results demonstrate that as the concentration of BALC increases within a formulation, so does the rate of degradation. Unlike the chitosan films, which show that a greater degree of cross-linking slows the rate of degradation, BALC beads experience the opposite with a faster degree of degradation and the introduction of a larger percentage of labile bonds into the overall mass of the formulation. Therefore, as more of the BALC bead composition is made up of disulfide bonds, it experiences a faster degradation rate in a reducing environment. The BALC beads are highly cross-linked structures, however as the BALC increases within the formulation, a larger percentage of the bead with labile bonds is exposed to a reducing environment resulting in further cleavage and gradual disassembly of the internal polymer structure as sections drop out and dissolve.

The 0% control interestingly also shows signs of weight loss, however this should not be unexpected. The 0% BALC beads are microspheres made out of 100% modified PVA (Lewis et al., 2006b). Heaysman (2009) described the polymerisation of such a bead
detailing how few cross-links the beads would contain to hold the bead together. The bead would therefore contain very long PVA polymer chains with no cross-linking. Some PVA could still be soluble and with little polymerisation and cross-linking, the ability for the PVA to dissolve to water increases (Kadajji and Betageri, 2011). It seems highly possible for the 0% PVA beads to lose weight, however it is not thought degradation is responsible. Due to the low percentage of cross-linking it is proposed that there is an abundance of free PVA unbound within the bead. Additionally, as the beads are not put through an extraction process like DC Bead, residuals are expected within the bead. Unreacted PVA is known to be a reactant commonly found within the beads which is difficult to remove due to its long polymer chains (Internal data of Biocompatibles UK Ltd). As the viscoelasticity of the bead increases at higher temperatures, (from room temperature to 37°C in the experiment 6.2.1) over time the beads should swell and cause more unreacted PVA trapped inside the beads to dissolve out of the formulation (Hassan and Peppas, 2000). Therefore it is hypothesised that any weight loss is caused by the removal of unreacted PVA residuals from the 0% BALC beads, and that the beads do not degrade but continue to be held together by polymerisation points. This was demonstrated when the 0% BALC bead was assessed after 3 years storage in saline solution. Spherical 0% BALC beads, fully in tact can be seen in Fig. 6.4.

Fig. 6.4 Optical image of 0% BALC beads after storage in saline solution for 3 years. [Original in colour].
The hypothesis that 0% BALK beads lose weight from residual unreacted PVA is therefore supported by image ‘0% 64 days’ in Fig. 6.3 as well as the 3 year storage data of Fig. 6.4. The results also prove that the degradation of BALK beads is due to the cleavage of the disulfide bonds rather than any hydrolytic degradation as the 0% BALK formulation are not visually altered during the incubation period whilst the 31% formulations with high PVA contents begin to degrade after 6 weeks. Additionally, it is postulated that as the percentage of cross-linker increases within a formulation, the amount of free PVA decreases with more available coupling points. Therefore the weight loss observed in the high BALK percentage formulations is suggested to be caused by biodegradation. As the cross-linking increases, the weight loss attributed to BALK cleavage and fall out increases in tandem.

The data has shown that the BALK bead formulations are degradable, even with the cross-linking adducts formed through use of DTT. Therefore it is postulated that when BALK beads are loaded with Dox, any Dox-Dox interaction will not be able to hold the microsphere together in a reducing environment, as supported by the images in Fig. 6.1. With the use of DTT, drug release from BALK beads was halted (Chapter 5), however when the reducing agent was continuously applied, the microspheres degraded exposing the contents of the beads. Using the images in Fig. 6.3 as a BALK bead degradation timeline, it is suggested that due to the high Dox loading capacity of the BALK beads, the degradative properties of the high percentage BALK bead formulations could have a significant impact on the release of drug. It is proposed that in a reducing environment, the BALK beads will release a controlled, increased amount of drug until the 21 day period. As the microspheres begin to degrade and break apart, there could be a significant increase in release due to a now larger surface area of the fractured hydrogel. Such an elution profile could be desirable in the treatment of hypoxic tumour.

However it has not yet been possible to prove the hypothesis of significant increases in drug release from degraded BALK beads. As the degradation of the microspheres take a minimum of 21 days, it is not possible to monitor the elution of drug up to this time period. Dox elutions from microspheres are generally not monitored past 24 hours and are definitely not monitored past 72 hours. This is firstly because it is difficult to provide a suitable elution environment for the microspheres over a long period of time, with the elution media generally saturated with drug within the first 24 hours. To continue to monitor the drug release from the microspheres, the elution media would need to be replenished on a daily basis. This is in conjunction with daily additions of an agent to
maintain the reducing environment required to continue the degradation and eventual breakdown of the BALC beads. As the elution would have to be monitored for a minimum of 21-64 days, this would be a lengthy and resource intensive study, with the added disadvantage of possible loss of microspheres, therefore therapeutic dose, when replenishing the elution media. However, the main reason why Dox elution from microspheres are not monitored past 24 hours is because when a solution becomes saturated with drug, Dox is known to precipitate out of solution (Internal data of Biocompatibles, UK Ltd). Within saturated elution medias, Dox precipitate has been found on the walls of the glass containers. This represents a loss of drug from the elution system, meaning it is not possible to accurately follow the drug elution profile of the microspheres. These arguments explain why elution has not been monitored past 24 hours, and why the hypothesis surrounding elution from BALC beads has not yet been conclusively proven. However the work in this section so far presents evidence that BALC bead are degradable, even when drug loaded and therefore it can be postulated that significant amounts of drug would be released from BALC beads upon degradation.

To demonstrate that significant amounts of drug are released when the microspheres degrade, the BALC bead formulation should ideally begin to degrade within 24 hours to allow monitoring of the drug release profile. This chapter has already shown that the degradation rate of the microspheres can be controlled by chemically tuning the formulation to contain more BALC to degrade faster or less BALC to degrade slower. However even the highest BALC percentage formulation does not degrade within the set time frame of 24 hours. It was suggested that high molecular weight PVA chains (Mw 67,000) would slow the degradation rate of the microspheres. Therefore microspheres were synthesised in section 3.3.8 using alternative co-monomers in order to increase the degradation rate of the microspheres and therefore potentially monitor the elution profile of the formulations.

These microspheres are representative of the BALC-PVA microspheres but with shorter polymer chains. If BALC-PVA beads are degradable, these should be degradable, and if a significant release of drug payload is expected upon degradation of BALC-PVA beads, it should be demonstrated by the new BALC-alternative monomer beads, but on a much faster time scale. It is suggested that by substituting shorter monomer chains for the long PVA chains faster breakdown properties could be achieved.
6.3.2 Drug Elution and Biodegradation of BALC Microspheres Synthesised with Short Chain Monomers

As described in section 3.3.8, BALC has also been used to create copolymer microspheres/microparticles with the use of alternative monomers. With shorter polymer chain lengths it is postulated that there will be less entanglement of polymer chains, and therefore faster degradation of microspheres. HEMA has a molecular weight of 130 g.mol$^{-1}$, is known to be highly water soluble and has previously been used in the formation of hydrogels (Chu and Ou, 2000; Casadio et al., 2010). The BALC-HEMA particles were shown to load 8.8 mg.mL$^{-1}$ of Dox. The results of the BALC-HEMA microparticle elution can be seen below in Fig 6.5.

![Figure 6.5](image.png)

Fig. 6.5 Elution of BALC-HEMA beads in 200mL PBS solution in a reducing environment (Red) and a non-reducing environment (Blue) [Original in colour].

Fig 6.5 shows that the formulation does release the entire payload 8.8 mg.mL$^{-1}$Dox within a reducing environment. However the release was 2.6 times greater than the non-reducing environment. Images of the microparticles before and during elution, both in a normal and reducing environment were taken (Fig 6.6).
Fig. 6.6 BALC-HEMA microparticles. A) Loaded with drug prior to elution. B) During elution in a non-reducing environment. C) During elution in a reducing environment (420 minutes) [Original in colour].

Fig 6.6 clearly shows that the particles remain completely intact in the normoxic environment; however the drug loaded particles have completely degraded in the reducing environment releasing the entire drug payload. This demonstrates that BALC copolymer formulations with lower molecular weight components can degrade more rapidly in a reducing environment. pHEMA has been used in other hydrogel systems and once the cross-linking labile bond was broken it was shown that HEMA is able to dissolve in water (Casadio et al., 2010; Lim et al., 2000). The results of the BALC-HEMA microparticles supports the hypothesis of section 6.3.1 that high percentage BALC (PVA) microspheres which undergo similar break down to Fig. 6.6, will also have a similar drug release profile when degrading.

In the case of BALC-HEMA, in the non-reducing environment the HEMA component of the microparticles are highly water swellable which allows for the release of drug. However the cleavage of disulfide bonds of BALC in the reducing environment causes the microparticles to fall apart within 24 hours. As HEMA forms short polymer chains, it is able to swell and dissolve in water but this is not the case in BALC-PVA beads, as the PVA macromer has a large molecular weight polymer chain that twists around itself and other chains and still holds itself together.

Further work with other alternative monomers was performed with the synthesis of a BALC-Acrylic acid microspheres. Acrylic acid is another co-monomer that is commonly used in the syntheses of hydrogels, has a low molecular weight of 72.06 g.mol$^{-1}$ and is known to be highly water soluble (Hoffman, 2012). The BALC-Acrylic acid (BAA) beads were shown to load 11.6 mg.mL$^{-1}$ Dox and the results of the elution can be seen in Fig. 6.7.
Within the first 6 hours (Fig 6.7), only a slight increase in release was noted within a reducing environment, and it appears that this formulation has a very slow release. This could be due to the extra drug binding sites added by the acrylic acid and this trend is shown by the beads in a normal reducing environment throughout. However, at the 24 hour point of the release profile, there is again a rapid increase in drug release within the reducing environment. Visual observations showed that there were clear differences again in the appearances of the beads in different environments.

Fig. 6.8 BALC-acrylic acid beads. A) Loaded with drug prior to elution. B) During elution in a non-reducing environment. C) During elution in a reducing environment [Original in colour].
Again, Fig 6.7 demonstrates the beads in a non-reducing environment remain completely intact, but the beads in a reducing environment have degraded again leading to an increase in drug release. This demonstrates that BALC-copolymer beads can degrade in a reducing environment, releasing the entire payload. Hydrogels containing acrylic acid have previously been shown to respond to stimuli and have the capacity to swell and dissolve (Qiu and Park, 2012).

The release profiles of both co-monomer beads offer an alternative to BALC-PVA beads as there is an immediate release of drug in a reducing environment. They both show great promise in the use of BALC as a drug binding and bioresponsive group that can be used with multiple co-monomers. The beads can be synthesised and chemically tuned to provide an expected breakdown rate and therefore a known release period. The BALC-Acrylic acid beads are of interest as their release profile would suggest that they should be able to wait and hold onto drug, lowering any systemic exposure and only once a reducing environment is provided will release the entire payload over a 3-4 day period. With the rate of the breakdown, and the solubility of the co-monomers, adduct formation is not a concern. The results of the BALC-acrylic acid beads also support the hypothesis in section 6.3.1, that once the BALC-PVA beads begin to degrade, the entire payload of drug bound to the original microspheres should be released. Additionally, through the ability to chemically tune the formulations, varying both BALC and co-monomer content, it is possible to design a formulation that has a defined break down rate and therefore control the embolisation time of a vessel as well as the drug release rate.

These are promising results, suggesting significantly increased amounts of drug can be released from BALC beads in a reducing environment with the potential to allow almost instantaneous bead degradation, through percentage BALC incorporation. Such breakdown properties would be able to overcome issues over adduct formation but also allow extremely large loco-regional delivery of drug at the right time to an aggressive hypoxic tumour. The BALC bead technology could permit a timed attack on the disease, using the cancer’s own environment against itself. No other commercially available TACE treatment can monitor the disease 24/7, however a bioresponsive polymer such as BALC bead would be able to actively wait for the conditions to present themselves and then react.
6.4 Conclusion

It has already been demonstrated in Chapter 3 that BALC beads are bioresponsive and increase drug release in a reducing environment. This is despite in vitro studies showing there are many factors that can interfere (discussed in Chapter 5). Chapter 6 revealed that BALC beads have degradative properties and that altering the BALC concentration can affect the rate of drug release. This degradative property is expected to translate into biodegradative properties when the microspheres are applied in vivo. As seen with the alternative-monomer formulations, there is great potential to chemically tune the microspheres, not only to be bioresponsive, but also to rapidly degrade. This further enhances the release to a controlled dumping of the payload within a 24-72 hour time frame.

Once the BALC beads are exposed to a reducing environment, the breakdown process begins, with rapid weight loss before any visual changes are noted. If maintained in a continuous reducing environment, the BALC beads will eventually degrade regardless of Dox-Dox interaction holding the beads together or DTT adduct formation. As the disulfide bonds continue to degrade, so does the polymer structure with the amount of cross-linking controlling the period of time. Using the co-monomer synthesis study as a model for BALC bead drug release after degradation in conjunction with the high drug loading capacities, significantly large quantities of drug could be released upon degradation. However, the cross-linking density of BALC beads would need to be optimised further to reduce the degradation response time seen in the 80% BALC beads, which began to break down from 21 days.

Further characterisation demonstrated the degradative properties of BALC beads, with higher BALC bead formulations not only containing higher drug concentrations, but also faster and more complete degradation properties. In correlation with the BALC-HEMA-Acrylic acid formulations, this would equate to a significantly large increase in cytotoxic drug release to a hypoxic tumour.

However, it should be noted that the rate of degradation of the BALC microspheres is not as fast as it should be due to the formation of the adducts and use of the reducing agent DTT. It was discussed in Chapter 5 that the adducts halt the release of drug from BALC beads, through the formation of DTT cross links throughout the bead. It is therefore hypothesised that the adducts would also slow the rate of degradation through their formation, and a more appropriate reducing agent could offer a better understanding of the
true degradation rate of the BALC microspheres, potentially rapidly increasing the degradation rate.

Although a suitable in vitro reducing agent has not been found and investigated, the results thus far in this chapter have shown that BALC beads are degradable and that this property can significantly affect the release of drug from the microspheres. These results, in combination with the previous characterisation results, demonstrate that the BALC beads are meeting the desired outputs of this thesis; they show potential to target hypoxic tumours through redox sensitive disulfide bonds and, either through the cleavage of these bonds or degradation, lead to a significant release of therapeutic drug, thus providing the foundations for future work to optimise the formulation. The use of a cell culture environment could help demonstrate the conditions necessary to allow BALC beads to release the therapeutic payload without interference and potentially allow more accurate mapping of the BALC bead properties in correlation with their expected actions in vivo.
Chapter 7

7 In Vitro Bioevaluation of BALC Bead

7.1 Introduction

HCC is a leading cause of cancer related fatalities world-wide with the burden expected to worsen in the coming years (Finn, 2013). It is currently the fastest growing cause of cancer-related deaths among men in the US. By studying the malignancy at the cellular level through cell culture investigations, research has attempted to investigate and develop applicable therapies and drug combinations to combat this trend (Bowyer, 2011).

Cell culture is a well-used technique and is a key asset for scientists in the validation of research. Cell culture is regularly used to provide a 3D model studying cell physiology and changes and is particularly used in the screening of various drugs and biotransformation (Meli et al., 2012b). The use of a cell culture model can act as a bridge between laboratory work and in vivo providing important data that could propel the product forward for clinical use. In addition, cell culture could potentially identify weaknesses or limitations in the product that require processing and optimisation.

The HepG2 cell line is often used to produce a cell model for experimentation is derived from the HCC cancer line (Zen et al., 2007). In vivo, HCC is a solid tumour which is the main target of DEB-TACE treatments and therefore is the most suitable cell line candidate for investigation. HepG2 is one of the preferred human in vitro models and is commonly used in biotransformation studies with benefits such as being easy to handle and providing reproducible systems (Wilkening et al., 2003). HepG2 cells are also known to be capable of displaying hypoxic cell survival tendencies when incubated in low oxygen environments (Yan et al., 2009). DEB formulations have been evaluated in cell culture studies focusing on drug release and the effect on hypoxic HepG2 cells (Bowyer, 2011); however, none of the DEB formulations are known to be selective and tailored towards combating hypoxic cancer cells.

The previous five chapters of this thesis have described the possibility of producing a novel bioresponsive DEB, that could target hypoxic tumours such as those found in HCC and enhance chemoembolisation therapy. Such a system would target a niche not currently met by other products. The in vitro characterisation has shown thus far the development of
a bioresponsive microsphere that is able to bind anti-cancer drugs and alter its release rate in the presence of a reducing environment, as is associated with hypoxic tumours.

The aim of this chapter was to investigate the in vitro efficacy of the system in a cell culture-based model of tumours in both normoxic and hypoxic conditions. As the exact intensity of a reducing environment provided by a hypoxic tumour remains unknown, it was the intention to produce a 3D cell culture model that could potentially mimic the internal microenvironment of hypoxic HCC. This would be the next step before an in vivo model could be considered. The information provided here would act as a bridge to take the system further for more clinically relevant testing. The HCC cell culture model could also provide a means of evaluating whether the reducing environment provided extracellularly is robust enough to cleave the disulfide linkers within the delivery device.

### 7.2 Materials and Methods

#### 7.2.1 Materials

The cell line used in all experiments was the human hepatocellular carcinoma cell line HepG2. Cells were provided by ATCC Cell Biology collection and cells were used between the 75th and 120th passage number. Cell culture media of Minimum Essential Medium (MEM) + Earle’s salts + L-Glutamine, 10% Foetal Bovine Serum Heat Inactivated and 1% Non-essential Amino Acids and trypsin-EDTA were purchased from PAA laboratories, GmBH. All cell culture plates ranging from 96 well plates to T75 and T175 flasks were purchased from Nunc™, Denmark. Ellmans Reagents and dithiothreitol was purchased from Sigma Aldrich. Equipment used was laminar flow cell culture hood HERA Safe, Centrifuge Multifuge 3S and a Cell Incubator (Heraeus). A hypoxic glove box with an incubator was obtained from COY Laboratory products Inc. MI. USA. A BioTek® Synergy™ HT microplate reader was used. A NucleoCounter® NC-100™ from chemometec was used in cell counting. An Olympus BX50 microscope was used in the sizing of microspheres.

#### 7.2.2 Cell Culture Conditions

All work involving cell culture was carried out in a laminar flow hood to reduce the risk of cell contamination. All equipment was sterilised before use and the hood was swabbed
down with 70% industrial methylated spirits. For the development and growth of the HepG2 cells, Minimum Essential Medium (MEM) + Earle’s salts + L-Glutamine which was supplemented with 10% Foetal Bovine Serum Heat Inactivated and 1% Non-essential amino acids was added to the cells and provided the culture media. The cells were then seeded on to T75 flasks and placed in the incubator at conditions of 37°C, 95% air and 5% CO₂. Cells were passaged when confluence levels reached 70%, using a sub-cultivation ratio of 1:4-1:6.

7.2.2.1 Hypoxic Incubation

All hypoxic incubations were performed in the COY Hypoxic Glove Box. This chamber produced a controlled environment, more indicative of in vivo conditions, with controlled atmosphere, temperature (37°C) and humidity conditions. The glove box had a direct feed of carbon dioxide and nitrogen along with HEPA filters for sterility and an internal fan to ensure a uniform distribution of the gas throughout the glove box. Humidity was achieved from water in a high surface area tray, which was placed in the chamber to allow cell growth and prevent the cells from drying in the atmosphere. The use of an oxygen sensor allowed constant monitoring and control of the internal oxygen levels. Nitrogen was used to purge the system to reach the desired oxygen levels. Cells cultured within the chambers were placed in conditions of 1% oxygen, 5% CO₂ and 94% nitrogen. The cell culture media used for the hypoxic cells was placed in the chamber 24 hours before use to de-oxygenate the solution.

7.2.2.2 Cell Passage Method

After the cells had reached a confluence level of approximately 70%, the media was aspirated and the cells washed with 10 mL of PBS. 4mL trypsin-EDTA (0.05%/0.02% in PBS) was added to the flask and placed in the incubator for 10 mins at 37°C to detach the cells. Media (8 mL) was then added to the flask to neutralise the trypsin. The solution was then removed and centrifuged at 300g for 5 minutes. After centrifugation, the supernatant was aspirated, leaving a pellet of cells which was resuspended in 1 mL of fresh cell culture media. A 1:10 dilution of cells was made in PBS for cell counting. 10 µL of the 1:10 dilution was added to a haemocytometer slide and the cells were counted. The appropriate
fraction was added to the flasks, with a cell seeding density of $6 \times 10^6$ and a total cell media volume of 12-20 mL for T75 flasks and a cell seeding density of $12 \times 10^6$ and a total cell media volume of 50 mL for T175 flasks. The passaged cells were then incubated at 37°C until use.

7.2.3 Bead Contact with Hypoxic Cells

Once the cells had reached 70% confluence, the cells were detached with trypsin and seeded into 24 microwell plates at a seeding density of 80,000 cells per well. Three plates were placed in the incubator for the 24, 48 and 72 hour time points. Three seeded plates were placed in the hypoxic chamber for the same time points. Before incubation, pre-sterilised 45% BALC beads were placed in the cell culture media and sized. One bead with a pre-known size was placed in each well for each time point. Eighteen wells from each plate were reserved for cells and beads, the other wells were used for the use of positive and negative controls. At each time point the plates were removed from the incubator and the beads were sized and compared directly against their starting size. This method was repeated for the T75 flasks with proportional increases in cell seeding density described in section 7.2.2.2.

7.2.3.1 Cell Counting

A cell viability method, based on cell staining and microscopy, was applied to determine the number of cells within a well or flask, monitoring the growth through the 3 day period, as well as determining the percentage cell death within a well or flask. Cell counting was performed using a Nucleocounter, which is an integrated fluorescent microscope, to determine the total cell population of a solution and the cell population viability. The microscope is able to accurately detect signals from a fluorescent dye, propidium iodide (PI), which is able to bind to the DNA of lysed cells.

To measure the cell population, a 1:10 dilution of cells was prepared with PBS from the resuspended pellet after centrifugation. A cassette containing PI was used to draw up approximately 60 µL of the diluted cell sample. The PI mixes with the sample and stains the nuclei of the cells. However, the dye only stains the non-viable cells and gives a value of the number of dead cells within the solution. To determine the total cell count and
hence the viable cell fraction, 50 µL of the cell dilution was mixed with 50 µL of an acidic lysis reagent which disrupted the plasma membrane allowing the dye to stain the nuclei. 50 µL of a stabilising buffer was added to the mixture, raising the pH and increasing the efficiency of the PI staining of the cellular DNA. The cassettes were inserted into the nucleocounter giving a value for the total cells, total viable cells and total non-viable cells within a population. This method was repeated several times for the larger cell populations.

7.2.4 Thiol Reducing Agent Levels

To determine the reducing environment provided by the cells, DTNB solution mixed with the cell culture media was used in conjunction with different concentration DTT solutions in a 96 microwell plate. The sample wells were run on the synergy microplate reader at an absorbance of 412 nm, producing a standard curve of DTT concentrations in cell culture media. Appropriate controls were run to ensure that the absorbance was directly related to the cleavage of DTNB by DTT.

T75 flasks were seeded with HepG2 and placed in the incubator at conditions described in section 7.2.2. Replicate flasks were then placed in the COY hypoxic box with humid conditions at 37°C, 1% oxygen, 5% CO₂ and 94% nitrogen, with samples prepared for the 24, 48 and 72 hour time points. At the appropriate time point the flasks were removed from incubation and the cell culture media was added to the DTNB solution in a 96 microwell. The plate was placed in the microplate reader which heated the samples up to 37°C and stirred the samples before analysis.

7.2.5 Reducing Environment Effect on BALC Beads

After determination of the reducing environment provided by cells in normoxic and hypoxic environments, the values obtained from the studies of section 7.2.4 were applied to BALC beads. One high initiator concentration BALC bead (31%) and one low initiator concentration BALC bead (45%) was used in the method. 1 mL of beads from each formulation was sized as the 0 hour starting point, the beads were then placed in 10 mL PBS with varying DTT concentrations of 0.07, 0.14 and 0.25mM. A control of PBS was also used. The samples were placed on a plate shaker and were incubated with the reducing DTT solution. At set time points of 1, 2, 7, 14, 21 and 28 days, the beads were removed.
from the vials and sized under the light microscope before placing the samples in a new vial and refreshing the reducing the environment.

7.3 Results and Discussion

7.3.1 Cell Growth

HCC is a good example of a solid tumour that is known to promote and be prone to tumour hypoxia. There is strong evidence that links hypoxia in HCC to invasiveness and metastases (Yan et al., 2012). However it is unknown what percentage of HCC related fatalities are directly associated with the hypoxic phenotype which propels the malignancy. Due to the link between the high mortality rate of liver cancer and the poor prognosis related to hypoxia, it is postulated that a significant proportion of HCC patients progress to the hypoxic phenotype. More recent investigations have now begun to link hypoxia to half of all locally advanced cancers, indicating that hypoxia in tumours is a major cause of mortality and currently its clinical needs are unmet (Semenza, 2012b). Better understanding of hypoxic tumours and the metabolic changes are important to help reverse this accelerating trend.

One of the key differences between a hypoxic tumour and a normal tumour is the rate of cell proliferation. Hypoxia has been linked in vivo to exponential cell growth and spontaneous metastasis, and these exposed cells progress at a more alarming rate than seen by normoxic tissues (Chang et al., 2011). Experiments have shown that within 12 days, cell numbers had doubled under hypoxic conditions, with a significantly higher number of cells detected only after day 2 (dos Santos et al., 2010). HepG2 cells have been exposed to hypoxic conditions in an in vitro model and have shown the clinically aggressive phenotype of hypoxic tumours (van Malenstein et al., 2012). The exponential cell growth of human hepatoma cells during in vitro, studies in comparison to normoxic cells, has also been documented (Gwak et al., 2005). The impact of hypoxia on cells has been discussed in this thesis; the reviewed literature describes the use of 1-2% oxygen to cause tumour cell hypoxia, which is accompanied by an alteration in cell behaviour and promotion in cell growth (Rhee et al., 2007). The ability of the cells to survive under such stress is more clearly demonstrated with the knowledge that even at 0.01% oxygen (anoxic levels), cells can survive for 24 hours (Bartkowiak et al., 2012).
Using the method described in section 7.2.3, 24 microwell plates were prepared and seeded with the cells. The majority of cell cultures are performed at oxygen concentrations of 21%, which in most cases does not mirror the physiological oxygen concentration within the relevant tissue. In an attempt to mimic the in vivo environment as closely as possible, a 1% oxygen environment was set within the hypoxic chamber to expose the cell line to hypoxic conditions. The aim of the experiment was to cause the HepG2 cell line to become hypoxic and in doing so, alter its phenotype, leading to an explosion of cell growth and the enhancement of the extracellular reducing environment as the cell population grows. BALC beads were placed in the wells of hypoxic and normoxic cells and incubated over the desired time period. It was proposed that the bioresponsive beads would increase in size in the presence of hypoxic cells, with no change in diameter in the presence of normoxic cells. Initial results did not reveal any size change in the BALC microspheres after 72 hours under microscopy. Further cell culture studies were performed for 72 hours with BALC beads incubated with hypoxic cancer cells, again revealing no change in the bead diameter.

It was postulated that the cell numbers produced were too low to provide any meaningful reducing environment. In comparison a hypoxic tumour, which up to 1cm³, can contain up to 1 billion cells (Siegmund et al., 2009). Therefore, with potentially too few cells present in the initial cell culture models to reduce BALC beads, the studies undertaken in this chapter changed focus to provide significantly enhanced cell growth in hypoxic conditions compared to normoxic conditions. Larger cell populations would provide a greater amount of reducing agents in cell media, and by altering the volume of the cell media it may be possible to further concentrate the reducing agents. It was also suggested that enhancing the cell numbers, there would be greater intercellular communication, more representative of in vivo conditions (Oktem et al., 2006). Thus by increasing hypoxic cell growth, the aim was to optimise and enhance the reducing environment provided by the HepG2 cell culture, in order to demonstrate whether it is possible to cleave the disulfide bonds of BALC beads in vivo.

Therefore the surface area of the flasks was increased so that the cell number could multiply. It was important to establish the correct cellular model before adding any more bead product to the flasks for testing. The continued development of the cellular model moved into T75 flasks. The results of the cell culture can be seen in Fig 7.1 A and B.
Fig. 7.1 A) The cell count of the viable and non-viable normoxic and hypoxic incubated cells in a T75 flask seeded at 6 million cells per flask with 12 mL of cell culture media. B) The cell count of the viable and non-viable normoxic and hypoxic incubated cells in a T75 flask seeded at 8 million cells per flask with 20 mL of cell culture media (mean ± SD, n=7). [Original in colour].
The results of the cell counting showed an unexpected trend compared to what was expected based on the literature described earlier in this section. The literature suggests hypoxia is a stimulant that propagates rapid cell proliferation against cells in normoxia, along with epithelial-mesenchymal transition, invasion of the extracellular matrix and secondary growth of the metastases (Kim et al., 2001; Wong et al., 2013). However in the two graphs (Fig. 7.1 A and B) it would appear that the HepG2 cells proliferate at a much faster rate in normoxia than the cells exposed to hypoxic conditions. From Fig 7.1 A, the normoxic cells continue to grow over a 3 day period, however the hypoxic cells seem to have almost stopped proliferating. Although microscopy did confirm cell attachment to the flask, there was no real growth of the cells in hypoxia and small floating cell aggregates were noted.

The experiment was repeated, with the intention of providing surplus media to supplement cell growth and try to encourage additional growth and enhance hypoxic cell growth. A small increase in cell number seeding was performed using 8 million cells to try and enhance the potential reducing environment. However as seen in Fig 7.1B the same trend in cell growth was observed with normoxic cells proliferating faster than the hypoxic cells. The addition of extra cell media is a benefit as it allows and encourages additional cell growth in the normoxic conditions with much higher cell numbers reached. The introduction of extra media also appears to initiate cell growth in the hypoxic conditions with an increase in cell numbers up to day 2. However, the numbers decrease again after day 3 and the cell numbers reduce to approximately half that seen under normoxic conditions.

The experiment was repeated with the use of a larger flask (T175), which is double the surface area of the T75 and therefore provides more room for growth. This allowed an increase in the reducing environment, while also enabling more cell media to be added to help propagate cell growth. The results of the cell incubation can be seen in Fig 7.2.
The cell count of the viable and non-viable normoxic and hypoxic incubated cells in a T175 flask seeded at 12 million cells a flask with 50 mL of cell culture media (mean ± SD, n=7). [Original in colour].

The results from Fig 7.2 replicate the results seen in the previous cell incubation experiments and demonstrate that with the use of a 2D culture system, hypoxic cells will not proliferate faster than cells in an abundant supply of oxygen. Fig 7.2 shows cell growth for all cell lines up until day 2 with the normoxic levels reaching a cell number of 45 million, however the hypoxic cells do not reach half the value. As the normoxic cells grow so rapidly it is proposed that the reason for the decline in cell numbers could be due to insufficient nutrients being available to such a large cell population. It is suggested that the media added was not enough to sustain the cells for 72 hours and as a result they entered the lag phase as their viability began to decrease. An explanation for why cell death was not higher on day 3 and matches the decline in cell numbers from day 2 is due to a limitation of the cell passage protocol. When the HepG2 cells die, they begin to detach from the flask and float in the media. The media was aspirated away before the cell count could be performed therefore not all the non-viable cells were included in the cell count. The theory that there was not sufficient cell media to supplement the cells is further supported by evidence that nearly 50% of the hypoxic cells were non-viable by the second day.
The results in Fig 7.1 and 7.2 appear to contradict data that has shown that hypoxia propels cell proliferation. In depth analysis of the literature shows that hypoxia enhances cell proliferation in a breast cancer cell line which was xenografted onto mice with doubling of the hypoxic cell line density (Milane et al., 2011a). However in a sarcoma cell line implanted in rats a direct comparison of tumour volume was measured between cells of normoxic and hypoxia showed a faster proliferation rate of normoxic cells than hypoxic cells (Thews et al., 2004). These results demonstrate that there are different growth patterns for cells in hypoxia within different cell lines.

In HCC, and with a particular focus on the HepG2 line, it has been shown that when the tumour cells are exposed to hypoxic conditions there is a significant increase in cell proliferation; this has also been shown in vivo where TAE has caused hypoxia directly accelerating the growth rate (Cho et al., 2013; Huang et al., 2013b; Kim et al., 2001; Gwak et al., 2005). However, within the same cell line (HepG2), experiments recently conducted have shown the reverse; the proliferative activities of the cells in vitro have been greater in normoxic conditions than in hypoxic conditions (Sun et al., 2011; Yu et al., 2013c). While Sun (2011) and colleagues show that cells briefly exposed to hypoxia then returned to normoxic conditions has greater proliferative activity than both cells in hypoxic and normoxia; the data achieved by Yu (2013) and colleagues directly matches the results achieved above in Fig 7.1 and 7.2.

This shows that the literature pertaining around the topic of HCC (HepG2) in vitro is not conclusive, with different researchers achieving different results. However this is not to be unexpected; within the field the hypoxic impact on cell growth, pathways and metabolic alterations are poorly understood. With multiple genetic targets of HIF, and the exact trigger for HIF unknown, the role that the down regulation of PI3 Kinase/AKT pathway has in the promotion of hypoxic cells is still lacking (Semenza, 2012a; Semenza, 2004; Yan et al., 2009). However what is clear from the literature is that, even if the proliferative ability of the hypoxic cell is not as great as that of a normoxic cell, research has demonstrated that the metabolic activity of the hypoxic cell is significantly greater than that of a normoxic cell, with protein production of known factors enhanced in cancer progression, including VEGF and Hexokinase II (Wong et al., 2013; van Malenstein et al., 2012). Bowyer (2011), studied HepG2 cells in hypoxia and noted a decline in cell proliferative activity after 48 hours, however even though the cell number was less than that of normoxic cells, its protein expression was far greater, with other reports showing greater migration and invasive properties (Sun et al., 2011).
7.3.2 Reducing Levels in the Extracellular Environment

Although enhanced cell numbers were not achieved in a hypoxic environment, cells did multiply and with the use of the nucleocounter it was established that the cells were viable. Therefore as shown below (Fig. 7.3) the cells should still be active hypoxic cells and exhibit characteristics and properties of hypoxic cells as expected. A study was performed according to section 7.2.4, in order to establish whether cells cultured in hypoxia could produce a reducing environment, and demonstrate that the cells shown in Fig 7.1 and 7.2 are not stagnant but are in fact metabolically active.

HepG2 cells were grown in T75 flasks in both hypoxia and normoxia for time points of 24, 48 and 72 hours. At each time point the respective flasks were removed from incubation and the extracellular media was collected from each flask and added to the DTNB which would cleave in the presence of a thiol reducing agent. The results of the experiment are shown in Fig 7.3.

Fig. 7.3 Measurement of reducing agent produced in the extracellular environment of the cells in the media of the T75 flask with the use of DTNB. The media for the normoxic and hypoxic cells are tested at 24, 48 and 72 hour time points with the use of a microplate reader scanning at 412 nm (mean ± SD, n=10). [Original in colour].

Fig. 7.3 reveals that even though the number of cells present in the hypoxic flasks are almost half that of the normoxic flasks, the hypoxic cells provide a reducing
environment that is nearly double that of the normoxia. This confirms that although the hypoxic cells appeared to have limited capacity for proliferation, they are very much active. This was supported by analysis of the pH (pH 6.5) of the media, as the hypoxic cells would be expected to be secreting lactate as mentioned in the discussion in section 1.4.2, and are rapidly consuming glucose through glycolysis. In addition to the pH change, there was a more notable colour change of the hypoxic cell media from the second day. The probable cause of this change is dramatic reprogramming of the cancer cell metabolism, involving the increase of glucose transport into the cell and increased conversion of the glucose to pyruvate before it is released from the cell into the media (Semenza, 2009). This alteration in cell expression and signalling has been the target of much research; silencing certain proteins and target genes decrease the metastatic potential and cell migration properties of hypoxic cells (Emerling et al., 2013; Liu et al., 2010).

Instead of preventing the onset of hypoxia in cells, BALC beads aim to utilise the conditions they create and respond to the reducing environment as a two mode attack of the cancer, preventing the spread of the tumour. The results presented in Fig. 7.3 confirm that there could be a potential use for the BALC beads as there is a much stronger reducing environment provided by the cells in hypoxia. The cells are releasing compounds into the media that are able to reduce DTNB, a disulfide based agent. Therefore it is suggested the same compounds should be able to reduce the disulfides present in BALC beads to the same effect. However, a greater BALC bead incubation period at these reducing levels could be required, as previous 2D cell models did not yield any size changes. This length of time to reduce BALC beads at these reducing concentrations was investigated in section 7.3.3, however since hypoxic cell numbers decline after 72 hours incubation (Fig. 7.1 and 7.2), cell culture systems were not applied to provide the reducing environment.

The strength of the reducing environment achieved from Fig. 7.3 was not as potent as had been expected. This figure, established from the cell culture, could be limited due to the significantly lower number of cells available in the flask compared to an in vivo situation. Also, within the body there would be various other factors such as adjacent cells and a much more complex and interlinked cellular system that contributes to a more dynamic environment. The results achieved thus far can however be taken as a baseline of the lowest possible reducing environment that could be provided to react with the BALC beads.
7.3.3 Reducing Environment Effect on BALC Beads

The results achieved in Fig 7.3.2 confirm that there is a reducing environment produced extracellularly under the conditions of the experiment and confirm that the reducing species are significantly enhanced by the effects of hypoxia on the cells. What is not known, is whether this environment is sufficient to reduce the BALC beads and lead to a size change. The results of the cell culture are taken as the baseline as the lowest possible extracellular reducing environment, and these levels were used to demonstrate whether the BALC bead would respond and increase in diameter to these reductive levels. The experiment was conducted according to the method described in section 7.2.5 with the results shown below in Fig 7.4 A and B. Two formulations of microspheres were used, one from each type of BALC bead initiator concentration to ensure that any increases were seen throughout the spectra of beads.
Fig. 7.4 A) Percentage size increase of 31% high initiator BALC beads after incubation with varying concentrations of DTT. B) Percentage size increase of 45% low initiator BALC beads after incubation with varying concentrations of DTT (mean ± SD, n=200). [Original in colour].

Control experiments were performed in which 31% and 45% BALC bead formulations were incubated in PBS with no DTT. The beads were incubated for the same period of time, 28 days, and showed no change in diameter. The experiment used DTT concentration levels equivalent to those found in the cell culture experiments of this
chapter. A higher concentration of 0.25 mM DTT was also used to show the trend if there was an increase in the redox potential of the reducing environment. Fig 7.4 A and B demonstrate that at much lower concentrations of DTT compared to the drug elution studies, the beads are still responsive to the reducing agent. This is indicated by an increase in diameter. The rate at which the bead increases in diameter is dependent on the amount of reducing agent in solution, with the higher reducing environment having a faster cleavage and hence size increase. The results also show that the more BALC within a microsphere the larger the increase in size with the 45% formulation showing a larger increase in mean diameter. The results verify that the reducing levels provided by the cell culture model is more than adequate to effectively reduce the BALC beads leading to the respective size increase. Fig 7.4 B also shows that for the beads to increase by 10% in size, the beads in normoxic conditions take an additional 2 weeks to reach the same size as the beads in hypoxic conditions. The results show that the size of the beads do begin to increase immediately after incubation with DTT, although the length of time it takes for the increase to occur is drawn out due to the low concentrations. Seven days to increase is not the immediate response to hypoxia that had been desired, although it is not a limitation as DC Bead has shown to have a half-life elution of nearly 2 months, and with the increased loading capacity of BALC beads there should be sufficient drug left within the microsphere to spike the tumour with drug as the beads open (Lewis, 2010). However to prevent a hypoxic tumour from sending out growth and survival signals, a quicker response would be desirable in an attempt to kill any hypoxic cells before they have the chance to escape and invade. It is postulated that with the higher BALC concentrations and stronger reducing environments expected in vivo, this size increase will occur more rapidly and to a larger effect. Also, by introducing shorter polymer chains into the system such as HEMA, there is potential to significantly increase the drug release rate at the site of a hypoxic tumour.

Interestingly, the beads in normoxia also increase in size, but at a much slower rate than those in hypoxia. As discussed in the introduction, hypoxic cells are a more potent form of cancerous cell and as such cancerous cells also have the ability to produce HIF, but in much lower quantities. The glucose metabolism of normoxic cells is slower than that of hypoxic cells, however they are still faster than normal healthy cells. Therefore it appears that even in normoxia, the BALC beads will increase in diameter, though the length of time to increase is much slower than hypoxic cells. If the tumour does not become hypoxic and remains in a normoxic state, then as shown in Fig 7.4 B, it can take up to 28 days
before the bead increases to a large diameter size. The results show that BALC beads can respond to very low levels of reducing agent and that the size and rate of the increase is linked to the concentration of the reducing agent. It is also known that hypoxic cells provide much stronger reducing levels than cells in normoxia. It is assumed with a high percentage BALC formulation, the microsphere should respond to an in vivo hypoxic tumour anywhere between 0-7 days.

The results of this chapter demonstrated that the level of the reducing agent found in hypoxic HCC cells are capable of cleaving the disulfide linkages and opening the BALC bead formulations. The cultured cells placed in the hypoxic chamber offer insight into tumour cells that have undergone TACE, as both conditions starve the cells of oxygen. Although the use of cell culture investigations allows comparison to be made between the in vitro experiments, and what is to be expected in vivo, the reducing agent concentration obtained from the cells cultured in hypoxic conditions was not as high as expected. With the knowledge that 3D spheroid cultures are able to retain more cellular functions than its 2D monolayer counterpart, and that 3D cultures upregulates protein production, then it is postulated that a more vigorous reducing environment would be obtained in more organised and complex models (Chang and Hughes-Fulford, 2008; Meli et al., 2012a). The 3D in vitro culture is more representative of in vivo systems and is able to preserve functional characteristics that 2D models can not (Tang et al., 2011; Schyschka et al., 2013). As studies into HCC continue along with more drug screening, 3D models are being developed which are more capable of mimicking the in vivo, this includes the production of malignant cancer spheroids better able to regulate the behaviour of cells (Fang et al., 2013; Muranen et al., 2012; Liang et al., 2011). Future studies with BALC beads with 3D cell culture models would provide more data on the properties of BALC bead and would aid in bridging the gap between in vivo and in vitro by continuing to develop an in vitro model

7.4 Conclusion

BALC beads are designed to respond to the internal stimuli of a hypoxic tumour and therefore require the associated reductive conditions to lead to the cleavage of the microspheres. The beads have been engineered to be a mop for the reducing agent, such as glutathione, released by hypoxic cells. In this chapter a HCC cell line was incubated in a...
hypoxic chamber exposing the cells to oxygen conditions similar to *in vivo*. The results showed that the cells adapted to the conditions and continued to grow as shown in Fig 7.1 B, however they died quickly as they used the nutrients of the cell culture quicker than the normoxic cells. Conversely, the defensive mechanisms of the cell are more aggressive in hypoxia as it provides a reducing environment twice as strong as that of normoxic cells. It produced this reducing environment in spite of the fact that there were approximately 50% of the numbers of hypoxic cells to normoxic cells. The proliferative function of the cells in hypoxia is limited in comparison, however this could be due to the use of the 2D cell culture models used in the experiment.

This chapter has demonstrated that hypoxic cells can provide an extracellular reducing environment and it is postulated that this reductive environment is capable of acting as a target for drug delivery. The reducing agent concentrations obtained from this chapter were then used on the BALC beads and showed that reducing levels associated with hypoxic cells are capable of reducing the microspheres and cause a significant increase in size. With this information, it is suggested that the BALC beads will be able to respond to the reducing environment of a hypoxic tumour, resulting in an increased release of drug which has the potential to lead to more efficient cell death and to counteract the cell survival associated with hypoxic tumours. This represents a step forward in palliative HCC treatment with an enhancement of chemoembolisation therapy, as there are no other products that are able to biologically monitor and target the tumours in this manner.
Chapter 8

8 General Discussion

The application of drug delivery from embolic agents has continued to attract much attention in recent years, with studies continuing to focus on optimising the treatment regime to enhance survival rates of cancer patients (Prajapati et al., 2013; Feng et al., 2013; Idée and Guiu, 2013). However, none of the commercially available embolic microsphere treatments are able to adapt their response to a tumour that has become hypoxic, with the recognition now that tumour hypoxia is prevalent and frequent in many cancers (Taiakina et al., 2014; Semenza, 2013; Semenza, 2012b). This thesis has described the synthesis and characterisation of embolic hydrogel microspheres that are able to regulate their drug eluting capacity in the presence of hypoxic tumours. The synthesised microspheres used disulfide linkers which were incorporated into the structure to act as a response mechanism to the environment induced by a hypoxic tumour.

Disulfides have been frequently applied in targeting a reducing environment in many polyplex formulations leading to cleavage of the disulfide linkage facilitating release of the payload (Peng et al., 2008; Wang et al., 2006; Son et al., 2011). The advantages of incorporation of a disulfide has been demonstrated with excellent low levels of cytotoxicity achieved when disulfide cross-linkers are introduced into delivery systems (Li et al., 2012a; Shi et al., 2012a; Liu et al., 2012; Vader et al., 2012). The general cell survival rate when treated with disulfide bioreducible polyplexes averages over 90% while the non-reducible controls show less than 20% cell survival (Nam et al., 2012). Another benefit to the use of disulfide linkages in a potential medical device is the ability of disulfides to stabilise the polymer structure (Liu et al., 2005). Disulfides have long been known to maintain and stabilise proteins and as a result have been directly engineered into proteins to improve natural stability (Zhou et al., 1993). When introduced into various polyplexes they have shown comparative and improved stability in terms of salt-induced aggregation (Lai et al., 2012), as well as excellent stability within normal physiological conditions. However, the stability rapidly alters in the presence of a reducing environment (Bauhuber et al., 2009; Tsuchiya et al., 2012).

All these factors contribute to the rationale why disulfides have been so favourable as a bioreducible entity in polyplex devices. They lead the way in low cytotoxicity, biocompatibility, stability, reducibility, targeting of reducing environments and cost
effectiveness. It is for these reasons that the disulfide functional group is the redox sensitive group of choice in this thesis and was utilised to produce the intended final therapeutic device. Due to its increased water solubility synthesis of a disulfide cross-linking structure called BALT was undertaken. The water solubility of the monomer was postulated to pertain to two carboxylic acids groups either side of the disulfide moiety whilst BAC without these acid groups is insoluble in water (Schröder et al., 2010). The use of BALT was also advantageous due to the ability of the carboxylic acid groups’ ability to bind drug. Carboxylic acids have been used in other embolic microsphere systems e.g. HepaSphere which uses acrylic acid. The carboxylic acid loses its proton in water leaving it negatively charged (Hoigné and Bader, 1983). In its negatively charged state, the carboxylate group will bind positively charged ions such as Dox which has a positively charged amine group at the base of the anthracycline (Grosso et al., 2008).

The monomer BALT was synthesised by di-acrylation of an L-cysteine group yielding a symmetrical disulfide monomer with an acrylate group either side. The results of Chapter 2 show that the synthesis according to the published method was not reproducible in this thesis and therefore an adapted method was produced. The results of the NMR and mass spectroscopy spectra conclude that BALT was synthesised. However elemental analysis confirmed that the product was not pure and on further analysis, salt, a by-product of the synthesis reaction, was found to be present with the monomer. The salt was easily removed from the compound by using the solvent acetone, however to ensure absolute purity by synthesis with other solvents a method in the future could be applied where after washing the solid with DCM, the product could be dissolved in water and put through a column packed with an ion-exchange amberlite resin. All salt would bind to the charged amberlite resin in the column as would any other impurity, whilst no solvent would be used. The BALT could then be washed out with deionised water and lyophilized. The only solvent that would have to be factored in would be water, which could be removed with the use of drying in a vacuum desiccator with the use of, phosphorus pentoxide (Finne and Matches, 1976).

In Chapter 3, several formulations of BALT beads were synthesised with the incorporation of different amounts of the acrylated monomer BALT. These microspheres were synthesised by suspension polymerisation using the PVA macromer used in the synthesis of DC Bead. The results of the synthesis produced spherical microspheres that ranged from 40-1200 µm which were dependent upon the stirrer speed applied. However as the percentage of BALT increased within a formulation, the incorporation efficiency
declined. This was postulated to be due to excess initiator leading to nucleation of the reaction. To overcome this effect, the initiator concentration was reduced, resulting in efficient incorporation of the disulfide cross-linker. However this also resulted in two separate BALC-PVA formulations which with high initiator concentrations had short polymer chains, and with low initiator concentration resulted in long polymer chains. Initial analysis between the two sets of microspheres showed no apparent differences, other than better incorporation of initiator with low concentrations and, therefore no cage effect leading to loss of initiator.

Elemental analysis demonstrated incorporation of the BALC monomer throughout all microsphere structures. Chapter 3 highlighted a trend that as the amount of BALC added increased, the solid content of the microspheres also increased. However, a potential limitation of the increase in solid content could be that the beads become less compressible as the BALC content increases. A concern would be that if a 100% BALC bead formulation was synthesised, would the potential microsphere be too rigid and lead to occlusion of a catheter if used *in vivo*? Or with low water content, would a 100% BALC bead be friable and therefore not robust enough to deliver through a catheter, with possible fragmentation of the microspheres. Such beads could be tested with catheter delivery assessment with the use of contrast agents as would be used by an interventional radiologist. The compressibility of the microspheres could be investigated with the use of tensile testing system which has been used previously to determine the resistance to compression of hydrogel microspheres (Heaysman, 2009). This would provide valuable insight into potential *in vivo* deliverability of the microspheres and the end user handleability of such a product. It would also help select the optimum BALC concentration formulation, with the ideal cross-linking to compressibility ratio.

The characterisation techniques employed were able to show that the BALC incorporated was stable in its disulfide form, however when a reducing agent was added the disulfide cleaved forming two thiols which were able to reduce Ellman’s reagent producing a visible yellow solution. This cleavage of the BALC beads was also shown to lead to an increase in microsphere diameter. The increase in diameter of the BALC microspheres is postulated to induce a more efficient embolisation, however no method was used to test the hypothesis. A potential method to test the embolisation efficiency of BALC beads, in conjunction with the robustness of the different formulations could be to use a microfluidic embolisation device as discussed by Carugo (2012) and co-workers. Through the use of a poly(methyl methacrylate) mould they were able to mimic the
internal bifurcation found within the vessels of a human tumour and administer PVA microspheres through these vessels (Carugo et al., 2012). The use of such a device would provide information on the flow behaviour of the BAlC beads, with the aim of avoiding non-target embolisation which been known to lead organ failure (Wolanske et al., 2003). By embolising a channel in the model, it could be possible to reduce the beads with the addition of a reducing agent and then re-testing the flow of fluid through the vessel which could obtain a comparative pressure reading of the beads before and after reduction. Reverse flow could be used to retrieve the beads and assess the microspheres for any damage or differences found through the formulations, and whether higher percentage BAlC bead formulation incurred more damage than the lower percentage formulations.

EDX analysis mapped sections of the BAlC formulations, confirmed that as the BAlC percentage formulation increased within a microsphere, so did the mapping of the element sulfur. The elemental distribution of the sulfur was homogenous throughout all microsphere formulations. Therefore, as BAlC is the only sulfur containing compound within the microspheres, the distribution of BAlC was confirmed as homogenous throughout the microspheres. With an even spread of cross-linker throughout the microsphere it was suggested that the distribution of drug should also be evenly spread due to the potential binding on the carboxylic acid groups. Previous literature had suggested that PVA microspheres were incapable of loading drug homogenously throughout the microspheres, however Chapter 4 provides confocal microscopy evidence that the ionically charged drug Dox could be evenly distributed throughout the microspheres. The BAlC formulations also showed that as the percentage of BAlC increased within the microspheres the amount of Dox that could be loaded increased.

However, this increase in drug loading was not sequential, with high percentage formulations such as the 80% BAlC beads only loading 22% of its potential maximum binding capacity and lower formulations such as the 15% BAlC beads loading closer to their maximum binding capacity according to the titration data. It was also established that there was a considerable difference in drug binding between the high initiator and low initiator formulations, with low initiator formulation drug binding lower than expected. It was suggested that a lower initiator formulation would have longer polymer chains than its counterpart, resulting in more polymer chain entanglement and thus more hidden and sterically hindered binding sites. However, this was not the only factor affecting the loading, with the charge of the binding site shown to have a large influence on the quantities of drug bound. With the carboxylic acid being a weak acid, it does not associate
as strongly with the drug as sulfonate groups found in DC Bead. A method was devised to overcome this limitation by neutralising the acid groups creating a carboxylate salt which showed greater affinity towards drug binding. With the introduction of a carboxylate salt in the microspheres, the BALC beads were shown to be a high capacity formulation loading up to 300 mg.mL\(^{-1}\) Dox. No other commercially available embolic microspheres are capable of reaching these quantities. Such significant amounts of drug introduced at the malignancy site, with little polymer introduction into the body, could be of benefit to the patient to avoid complications. With such a large reservoir of drug \textit{in vivo}, the elution could extend for over a month based on values given by DC Bead eluting up to 28 days with it lower loading capacity. This would ensure that if a tumour becomes hypoxic over time, there should still be drug within the beads able to illicit a change in the elution rate. However, further work is required to assess exactly how the polymer chain lengths impact drug loading, and whether using an initiator level between the two BALC initiator formulations could yield the optimum BALC bead formulation. High BALC incorporation efficiency might be possible without long polymer chains leading to chain wrapping and entanglement.

Importantly the fluorescence results of Chapter 4 that when drug loaded, the BALC beads are still able to increase in size. With this increase in size of microspheres after reduction, the Dox fluorescence shows that the drug becomes less aggregated. The increase in size increases the space within the formulations and limits the amount of Dox-Dox interaction that can occur. This data held promise that when the BALC beads are in the presence of a hypoxic tumour, that the beads would be able to elute significantly more drug, such as Dox. This is suggested to be due to the increased space for the drug to diffuse out of, in addition to the drug being in a less aggregated state. Initial drug elution results of Chapter 5 showed that the hypothesis was correct, with significantly more drug being released from the 31\% high initiator formulation in the reducing conditions provided with DTT. However elution of the 31\% low initiator formulation did not show the same increase in release in different reductive environments, whilst ominously, the higher percentage BALC beads showed a complete reverse in original trend set. The results showed that as the BALC percentage increased within a formulation, more drug was stopped from being released into a reducing environment. To understand these effects, attention was drawn to the reducing agents used and their possible interactions with drug and polymer. Three of the selected reducing agents were shown to have a direct effect on the drug, whilst the final selected reducing agent (DTT) is suggested to have a direct
impact on the BALC bead microspheres. DTT is postulated to form an adduct as discussed in Chapter 5, which adds a film around the body of the microsphere and limits the release of drug in a reducing environment. This result was not ideal as it did not allow accurate analysis of the reducing potential of BALC Bead. The difference in release between initiator formulations was speculated to be due to the entanglement of the polymer chain in the low initiator BALC beads structurally creating more opportunities for the DTT adduct to form. This information was established too late in the thesis to gather further information, but it does provide valuable information to help develop an *in vitro* modelling system for the future. The information from Chapter 5 draws focus around the use of phosphines as potential suitable reducing agents, however the use of TCEP was not successful as it was shown to directly bind ionically charged drug and transport it out of the hydrogel. However, substitution of TCEP with another water soluble reducing agent, such as Tris(hydroxymethyl)phosphine, could provide a more suitable model which should not interact with the polymer or the drug. Re-analysis of the Dox loaded BALC microspheres would be required to test the new reducing agent and establish the amounts of drug that could be released from the bead. Additionally, synthesis of high and low initiator BALC beads at the same BALC percentages would be necessary for a full comparison of the properties of the beads.

Although a suitable model was not found to fully investigate the drug release properties of the BALC beads, what the results did prove was that the beads are bioresponsive and were able to adapt to the environment that they found themselves in. The results of the degradation study put forward the hypothesis that in the right environment, that the response and release of drug from the BALC beads could be more substantial than originally assumed with the beads shown to breakdown in a reducing environment.

Biodegradability is a main focus for many researchers, providing the potential to remove the device after its function is complete to avoid complications. Hydrogels synthesised from poly (lactic acid) (PLA) and poly(lactide-co-glycolide) have been demonstrate to degrade in the presence of water (Anderson and Shive, 2012). However increasing control over the degradation kinetics of these hydrogels is of great importance and therefore sought after (Hennink and van Nostrum, 2012). Introducing BALC as a stimuli responsive element into the polymer backbone has demonstrated bead degradability which offers this greater control. For example; the hydrogels only degrade if there has been a biological response, such as hypoxia, which requires degradation to return oxygen to the
tumour. However, if a tumour is not hypoxic, then degradation is not required and embolisation continues. This would represent an intelligent and bioresponsive hydrogel system.

BALC hydrogels are unlike older controlled release systems, as they can be designed to be sensitive to metabolic changes in the body and are able to modulate drug release (You et al., 2010). A future goal would be for BALC beads to be able to differentiate between diseased and healthy tissue dependent upon the stimulus. The results of the 0% BALC formulation show that the results of the degradation of BALC bead microspheres are by reduction of the disulfide links, unlike current systems which rely on hydrolytic degradation (Weng et al., 2013). As the percentage of BALC increased within the formulation, the rate of degradation also increases, showing the versatility of BALC beads with their ability to synthesise a formulation dependent upon the length of time the occlusion is required. The BALC beads were shown to gel together and dissolve in the images, however a more thorough understanding of its breakdown could be assessed by maintaining the beads in a reducing environment in the specially synthesised embolisation model discussed earlier in this chapter. However, the length of time taken for degradation for the highest BALC percentage formulation was between 6-9 weeks. The length of the PVA macromer is thought to help retain the bead structure much longer than that of a smaller comonomer.

The BALC-HEMA and BALC-Acrylic acid co-polymers demonstrated that they were able to completely degrade in a reducing environment within 24 - 48 hours. This is potentially too rapid, in terms of long term embolisation, however the advantages noted where that, the breakdown matter of the microspheres was completely dissolved in the elution media. Importantly the cleavage of the microspheres was strong enough to allow degradation and not be prevented by DTT adduct formation, with BALC-PVA beads shown to eventually overcome the adduct formation and breakdown. Finally, the BALC-HEMA, BALC-Acrylic acid microspheres were Dox loaded, and when degradation had occurred, 100% of the payload had been released.

This was the desired property that the thesis had set out to achieve. The aim of this thesis was to produce a novel bioresponsive microsphere that could respond to the harsh environment created by a hypoxic tumour, leading to an increase in release to overcome the aggressive cancer phenotype. The BALC-HEMA beads showed that they were able to release drug normally through diffusion in a normal environment, however in the presence of a reducing agent the microsphere degraded leading to complete and rapid delivery of the
payload. Such a mechanism in the body would have the potential to radically alter chemoembolisation treatment of cancer patients, with the potential to overcome the aggressive tumour phenotype.

With such a poor prognosis for patients with hypoxic tumours, BALC microspheres have the potential to provide a breakthrough in treatment with high amounts of drug rapidly delivered to a tumour site that is at the very start of undergoing the metabolic changes to become hypoxic. BALC microspheres have the potential to stop the tumour progression any further, and could result in mass tumour cell death. The quantities released could be enough to saturate cells and overcome the chemoresistance associated with the hypoxic tumours leading to a decrease in metastatic spread of the malignancy. Such a device offers great potential and after process development and optimisation of the microsphere, taking into account optimum cross-linking level, the device could be used by physicians to aid in the treatment of tumours.

Therefore it was desirable to translate the potential of BALC beads into cellular models, with the use of HepG2 cells employed to replicate a hypoxic tumour. However, a suitable cell model could not be established, although the reductive levels provided by the 2D cell culture (0.14 mM DTT equivalent) was shown to be sufficient to induce cleavage of the BALC beads. Future investigations should look to employ the use of 3D cultures with the benefits of growing tumour spheroids (Schyschka et al., 2013; van Zijl and Mikulits, 2010; Liang et al., 2011; Chang and Hughes-Fulford, 2008). The use of a 3D model would represent conditions more closely aligned to those met within the body. The use of a 3D perfusion bioreactor could be used grow the cells, which could be placed in the hypoxic chamber implored in this thesis. Use of a 3D reactor would allow continuous growth of the cells, which would therefore not require passaging every 72 hours, with results showing that growth of spheroids could be maintained for significantly longer durations of time (Szot et al., 2011; Fong et al., 2013).

Ultimately, once optimised, the microspheres will need to be assessed in vivo, with VX2 tumour models a potentially suitable candidate. The use of an in vivo model would provide the most similar environment to that of a human tumour and with embolisation of the tumour leading to the onset of hypoxia. If required it has been shown that intermittent hypoxia can be induced by a cycle of incubating the rats in low and normal oxygen (Yu et al., 2013a). Use of these in vivo models provide the very foundation and justification to take the device forward for clinical trials. The implantation of BALC bead microspheres is
a major milestone in the development of a commercially available hypoxia responsive microsphere.

However, before delivery to the market, a potential improvement could be undertaken to increase the usability of the microspheres. Future work could investigate the potential of designing the BALC bead microspheres to be radiopaque. Currently DEB-TACE lacks the intraprocedural imaging feedback compared to that of conventional Lipiodol-based TACE. The embolisation process with DC Bead is monitored by detecting changes in antegrade flow of soluble iodinated contrast in which the microspheres are diluted. The embolisation is continued until a desired embolisation endpoint is reached or reflux of contrast material into non-target vessels is observed, without specific feedback on microsphere or drug location. In order to address this lack of feedback, the development of BALC bead as an imageable spherical microsphere that can be visualised by X-ray based imaging (e.g., fluoroscopy and CT) would be desirable to physicians. Image-ability is obtained by chemically attaching the contrast agent to the hydrogel backbone. Knowledge of bead distribution during a procedure may provide useful real-time feedback to modify the intervention and tailor the procedure to a specific patient.

The use of radiopaque microspheres in clinical practice may report on embolic location, and since the chemotherapeutics (e.g., doxorubicin and irinotecan) are initially held by the microspheres, imaging microsphere location may also serve as a surrogate to report on local drug levels, or at least for the location of the drug source. The development of radiopaque BALC beads would also aim to tackle the limitations related to the lack of post-procedural imaging feedback, permitting physicians to track the location of the beads and therefore mark the tumour location for subsequent treatment and support other interventions such as resection. However a potential obstacle could be that BALC beads break down, and therefore the body would be exposed to the radiopaque material. This requires the radiopaque material to be biocompatible, or itself could be a compound that targets cancer cells. 4,5,6,7-tetraiodo-1,3-dioxo-2-isoindolineacetic acid is an iodine based compound that is suggested to be a casein kinase inhibitor and in conjunction with Dox could be used to have a biodegradable radiopaque microsphere. The benefit of targeting tumours with Dox in conjunction with iodine has shown positive results in cancer cell lines and has shown potential to overcome chemoresistance (Alfaro et al., 2013).

These potential investigations set out for future work are more advanced than the scope of this thesis. However the data gathered in this thesis has shown great potential for the application of a bioresponsive microsphere able to modulate itself to the environment
of a hypoxic tumour. This thesis has laid foundations for future work to optimise and potentially provide a more advanced commercially DEB-TACE formulation that could evolve the application of chemoembolisation and provide further understanding into the relatively unknown field of hypoxic cancers.
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Appendix I

Publications and Patents


Appendix II

Formulation Calculations

1. BALC Synthesis

Emilitri and colleagues (2005) add 40 mL 1 M NaOH to 12.1 g of L-cystine 0.05 M. However, on the L-cystine, de-protonation is required at 4 sites, which are the two protons on the acid groups and the two amines to allow addition of the acryl groups. Therefore 0.05 mol required at 4 sites:

0.05 x 4 = 0.2 mol of NaOH required, minimum.

However 1 mol at 0.04 L = 1 x 0.04 = 0.04 mol

- Part 1. Initial addition of NaOH to starting material

Mass of L-cystine dihydrochloride = 0.039 Moles

Mw of L-cystine dihydrochloride

As its dihydrochloride multiply by 2 = 2 x 0.039 = 0.078 moles

0.077 x Mw of NaCl (58.5) = 4.5g of NaCl

- Part 2. Addition of acryloyl chloride reacting with NaOH

10 M acryloyl chloride added to reaction in 10 mL DCM.

\[
\frac{10 \text{ M} \times 10 \text{ mL}}{1000} = 0.1 \text{ mole.}
\]
0.1 x Mw of NaCl = 5.85g of NaCl

- Part 3. Final acidification of BALT salt.

Carboxylic acids in salt form
Addition of acid to form carboxylate group.
Again 0.039 moles x 2 = 0.078
0.077 x Mw of NaCl = 4.56 g

Total potential amount of salt produced from BALT synthesis = Part 1 + Part 2 + Part 3
Total = 14.9g of Salt

2. Molar calculations of available NAAADA reactive ends.

The calculation was based on internal data provided by Biocompatibles UK Ltd.

PVA Mw = 67,000
NAAADA Mw = 159

Moles = Mass/Mw

Moles of PVA = 150 g / 67,000 = 2.2 x 10^{-3} moles
Each repeating unit of PVA = 44
Each chain of PVA = 67,000 / 44 = 1522

Moles of NAAADA = 2.49 g / 159 = 0.0157 moles
Number of NAAADA per PVA chain = 0.0157 / 2.2 x 10^{-3} = 7.1
3. **Method to calculate actual BALC incorporation into BALC beads**

31% BALC beads

\[ \frac{S}{\text{total}} = 5.3\% \]

\[ \frac{S}{\text{BALC}} = 18\% \]

\[ \frac{5.3}{18} \times 100 = 29\% \text{ of BALC within beads.} \]

15% BALC beads = \( \frac{2.7}{18} \times 100 = 15\% \) of BALC within beads

4% BALC beads = \( \frac{1.3}{18} \times 100 = 7\% \) of BALC within beads

**Drug Loading Images**

1. **CSLM section of a 0.5 mg.mL\(^{-1}\) 45% Dox loaded Bead**