



Research paper

Towards Hypoxia-responsive Drug-eluting Embolization Beads



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ABSTRACT

Drug release from chemoembolization microspheres stimulated by the presence of a chemically reducing environment may provide benefits for targeting drug resistant and metastatic hypoxic tumours. A water-soluble disulfide-based bifunctional cross-linker bis(acryloyl)-(L)-cystine (BALC) was synthesised, characterised and incorporated into a modified poly(vinyl) alcohol (PVA) hydrogel beads at varying concentrations using reverse suspension polymerisation. The beads were characterised to confirm the amount of cross-linker within each formulation and its effects on the bead properties. Elemental and UV/visible spectroscopic analysis confirmed the incorporation of BALC within the beads and sizing studies showed that in the presence of a reducing agent, all bead formulations increased in mean diameter. The BALC beads could be loaded with doxorubicin hydrochloride and amounts in excess of 300 mg of drug per mL of hydrated beads could be achieved but required conversion of the carboxylic acid groups of the BALC to their sodium carboxylate salt forms. Elution of doxorubicin from the beads demonstrated a controlled release via ionic exchange. Some formulations exhibited an increase in size and release of drug in the presence of a reducing agent, and therefore demonstrated the ability to respond to an *in vitro* reducing environment.

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1. Introduction

The practice of locoregional delivery of chemotherapeutics from embolic agents to treat hypervascular tumours has continued to attract much attention in recent years, with several different commercial offerings now on the market (de Baere et al., 2016; Pereira et al., 2016). Product innovation in this area is progressing by virtue of added functionality being designed into the embolic agent, such as radiopacity to allow them to be imaged during and after administration (Duran et al., 2016; Levy et al., 2016) and biodegradability to allow these agents to disappear over time (Weng et al., 2011a,b, 2013a; Verretet et al., 2014). Regardless of these added features, the primary mechanism of action of Drug-eluting Beads (DEB) is to physically block the blood supply and create an ischaemic insult to solid hypervascularised malignant tumours, followed by provision of a sustained local release of a chemotherapeutic agent at the tumour site, in a procedure known as transarterial chemoembolisation (DEB-TACE) (Liapi et al., 2007; Malagari 2008; Liapi and Geschwind, 2011; Nicolini et al., 2011;

Lewis and Dreher, 2012). However, when the microcirculation to a tumour is blocked, oxygen levels decrease to critically low levels causing the tumour to become hypoxic. Whilst the ischemia may kill many of the tumour cells, some may survive within this hypoxic milieu and are known to become chemoresistant and secrete growth factors that promote angiogenesis and metastasis of tumour cells to other parts of the body (Kunz and Ibrahim, 2003).

Hypoxic tumour cells have been shown to overexpress intracellular glutathione (GSH) (Kuppusamy et al., 2002) which has been associated with enhanced cellular proliferation (Kang and Enger, 1990), reduced apoptosis (Hall, 1999) and increased resistance to chemotherapy (Traverso et al., 2013). The associated increase in reducing potential compared to normal tissue however, has been used to target tumour cells intracellularly using a variety of redox-responsive nanocarriers such as dendrimers (Kurtoglu et al., 2009), micelles (Lv et al., 2014), polymersomes (Nahire et al., 2014) and liposomes (Sun et al., 2015). The extracellular tumour microenvironment is also highly reducing in nature (Bobko, et al., 2012; Khramtsov and Gillies 2014) and could provide a stimulus for targeting drug delivery from DEBs delivered intra-arterially and present in areas of severe hypoxia. Nanoparticulate non-viral vectors have been developed for gene delivery which comprise of

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cationic polymers and a redox-cleavable disulfide bond (Dai et al., 2010). In this study the investigators used a cross-linker called N, N'-bis(acryloyl cystamine) in which the disulfide link was shown to be cleaved in a reducing environment to the corresponding thiols and that the polymer could subsequently mediate gene transfection (Dai et al., 2010). This same compound has been used to crosslink ethylenediamine to form a polymer system for the delivery VEGF to promote neovascular formation in ischemic tissue by transfecting hypoxic cells (Christensen et al., 2007). Again, the N, N'-bis(acryloyl cystamine) was reduced in the hypoxic conditions and VEGF expression was 76 times higher in hypoxic cells than in normoxic cells (Christensen et al., 2007).

On a larger length scale, protenoid microspheres have been made from thermally condensed amino acids cross-linked with an agent containing disulfide functionality (Quirk, 2007). The microspheres were able to encapsulate material and release it slowly when exposed to a reducing agent, the rate of release being dependent on both the number of cross-links and rates of cleavage. Similarly, biodegradable polymeric microcapsules based on thiol-disulfide chemistry have been described by Zelkin and prepared from poly(methacrylic acid) (PMA) cross-linked using 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 GSH the contents were released (Chong et al., 2009). Whilst hypoxia-responsive nano and microspherical carrier systems have therefore been described previously, to our knowledge, there has not been a report of a disulfide-based microsphere system designed to embolise vessels and respond to the stimulus of the hypoxic environment of a solid tumour. Herein we describe the synthesis and characterisation of the first embolic hydrogel beads designed to regulate their drug eluting capacity in response to hypoxic environments. The design rationale was based upon the reduction of disulfide cross-linking groups, leading to an increase in drug release, with the intention to saturate the tumour cells and combat the chemoresistance associated with those areas of highest ischaemic insult.

2. Materials and methods

2.1. Materials

Solvents used in the synthetic procedures included ethyl acetate, n-butyl acetate, acetone, dichloromethane (DCM), ethanol, methanol, dimethyl sulfoxide (DMSO) and propan-2-ol, which were all purchased from Romil UK Ltd (Cambridge, UK, super purity solvent grade). Cellulose acetate butyrate (CAB), 2'-2'-azobis (2-methylpropionamide) dihydrochloride, sodium borohydride (NaBH_4), 5,5'-dithiobis-(2-nitrobenzoic acid), dl-dithiothreitol (DTT), phenolphthalein and universal indicator sticks (pH 0–14) were all purchased from Sigma-Aldrich UK (Poole, UK). For the bead synthesis, polyvinyl alcohol (PVA) macromer was manufactured by Biocompatibles UK Ltd (Farnham, UK) using PVA partly saponified, 88% hydrolysed, 12% acetate content, average molecular weight of 67,000 Da (Mowiol 8–88, Honeywill & Stein, Ltd. UK) and N-acryloyl-aminoacetaldehyde dimethyl acetal (NAAADA, Biocompatibles UK Ltd, Farnham) by a published method (Lewis et al., 2008). The N, N'-bis(acryloyl)-(1)-cystine (BALC) was synthesised as described in Section 2.2 using sodium hydroxide (NaOH), l-cystine dihydrochloride, 2-methoxyhydroquinone, acryloyl chloride and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl free radicals, (TEMPO) all purchased from Sigma-Aldrich UK. HCl and NaOH were purchased from Fisher Scientific, while the water used in all

experiments was purified at Biocompatibles UK Ltd using a Millipore Elix 4 type 1 water purification system. Frozen section compound (FSC-22) was purchased from Leica Microsystems and doxorubicin hydrochloride (Dox) for the drug loading and release experiments was supplied from Hisun Pharmaceutical Co. Ltd, China.

2.2. Synthetic methods

2.2.1. Synthesis and purification of N, N'-bis(acryloyl)-(1)-cystine (BALC)

The synthesis of BALC was achieved using a modification of the method described by Emilietri and co-workers (Emilietri et al., 2005). In this method 12.1 g of cystine dihydrochloride and 15 mg TEMPO were dissolved in 50 mL of distilled water. The solution was mixed using an overhead stirrer (350 rpm) and then cooled and kept between 0 and 5 °C using a salt ice bath. 6 M NaOH was then added drop-wise to adjust the solution to pH 10–12 followed by the drop wise addition of 8.1 mL acryloyl chloride solution dissolved in 10 mL dichloromethane. The mixture was stirred at room temperature for 3 h. The solution was then adjusted to pH 1–2 using 2 M HCl, frozen over night at –20 °C and then freeze dried. DCM (75 mL) was then added to the resulting white powder and mixed. 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.

DCM (3 × 30 mL) was added to the resulting white powder obtained after synthesis to remove residual acrylic acid. The DCM solution was passed through a sintered funnel and discarded. The remaining BALC was dissolved in 3 × 50 mL acetone and the solution passed through a sintered funnel to remove NaCl. The filtered solution was rotary evaporated to remove the acetone. To purify the material, silica gel chromatography was used to separate the BALC from salt and other potential impurities. Silica gel was added to the BALC solution (5–10 mL) until all the solution absorbed on to the gel. A column was dry packed with silica gel and washed with ethyl acetate solution. The BALC mixture was then packed down evenly on top of the column. Silica gel was then added on top of the mixture to protect the surface. The column was then washed twice with ethyl acetate and the eluent changed to a 3:1 ratio of acetone: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 h. The final product was characterised by ^1H NMR, ^{13}C NMR, 2D correlation spectrometry (2D COSY), Distortion Enhancement by Polarisation Transfer-135 (DEPT-135) and Nuclear Overhauser Effect Spectroscopy (NOESY), electrospray mass spectroscopy and elemental analysis. Characterisation data were fully consistent with BALC structure (Fig. 1) (Emilietri et al., 2005).

2.2.2. Synthesis of BALC beads

The synthesis of the beads was based on a method used to manufacture DC Bead[®] (Lewis et al., 2008). A 1 Litre jacketed reaction vessel was filled with 600 g n-butyl acetate and 11.5 g CAB

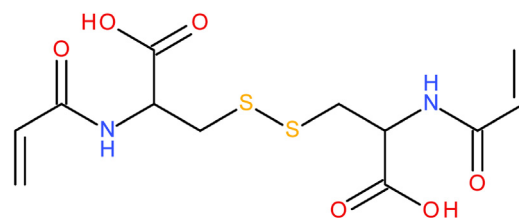


Fig. 1. Chemical structure of N, N'-bis(acryloyl)-(1)-cystine (BALC).

10% w/w in ethyl acetate. This was stirred for 30 min 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 it to form a N₂ blanket.

BALC was dissolved in water to prepare the aqueous phase, the amount of monomer added being dependent upon the desired degree of incorporation in the polymer formulation (0–80 wt%). The BALC solution was then added to 100 g PVA macromer solution (21% w/w) and mixed thoroughly, except for the 0 wt% BALC formulation which was made using exactly the same method but without BALC added to the formulation. The macromer was manufactured in-house using a process by which N-acryloylaminoacetaldehyde (NAAADA) was attached to PVA chains by transesterification (Lewis et al., 2008). The thermal initiator 2,2'-azobis (2-methylpropionamide) dihydrochloride (10 mg/mL for formulations 0–15 wt% BALC and 1 mg/mL for ≥31 wt% BALC) was mixed with the BALC and macromer. The aqueous phase was then added to the organic phase and the temperature of the vessel increased to 80 °C. The reaction was allowed to continue for 2 h.

Once the 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 three sequential washes with 125 mL of ethyl acetate, three washes with 250 mL of acetone, followed by filtration using a sintered funnel to remove the acetone. The collected microspheres were washed with purified water in order to remove the acetone and 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 BALC 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. The proposed generic chemical structure for BALC Beads is shown in Fig. 2.

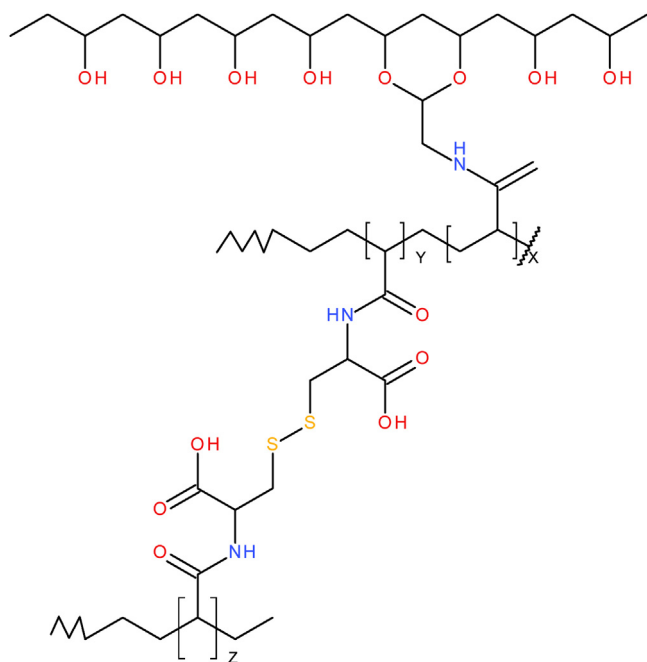


Fig. 2. Proposed chemical structure for BALC Beads (where the values for x, y and z are dependent upon the various weight ratios of PVA macromer to BALC in the reaction mixture).

2.3. Characterisation methods

2.3.1. Elemental analysis

Elemental analysis was conducted on the dried beads by Medac Ltd. (UK). Beads from each formulation (1 mL) were placed in a glass vial. The water was removed, the beads frozen and the vial placed on a Copley Heto drywinner freeze dryer for 24 h. The vial lids were modified with a semi-permeable membrane allowing removal of water but retaining beads with the vials. A minimum of 200 mg in each vial was prepared for analysis. The beads subjected to combustion analysis for carbon, hydrogen, nitrogen and sulfur (C, H, N & S) (N = 2) performed on a Thermo EA1108 or FlashEA1112 series elemental analyser. The accuracy of the results were within a ±0.30% absolute and the detection limit for each component is <0.1% according to the service provider. The theoretical elemental composition used in Table 1 was calculated from the target composition used in the synthesis of each formulation and assuming a 100% conversion of all components.

2.3.2. Energy Dispersive X-ray spectroscopy-sulfur mapping (EDX)

EDX was used to map the sections of the disulfide cross-linker throughout the bead (Ashrafi et al., 2017; Hagan et al., 2017). 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 sample 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 samples were electrically non-conducting, sputter coated with vacuum-evaporated carbon using 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.

2.3.3. Bead sizing

A sample of beads (1 mL) was placed in a glass dish and observed using an Olympus BX50 microscope with an attached ColorView III camera. Sizing was performed manually using the sizing tool of ANALYSIS software (Soft imaging system GmbH). A population of 200 beads was chosen at random throughout the dish and the diameter of each bead in the sample measured (Lewis et al., 2006a,b).

Table 1
Elemental analysis of a range of BALC bead formulations.

Formulation		C	H	N	S
0% BALC	Theoretical%	52.2	9.1	0.7	0.0
	Measured average (n = 2) %	52.2	9.0	0.3	<0.1
2% BALC	Theoretical%	54.2	9.0	0.4	0.4
	Measured average (n = 2) %	54.4	9.1	0.4	1.1
4% BALC	Theoretical%	53.1	8.8	1.7	0.9
	Measured average (n = 2) %	51.9	8.7	1.2	1.3
8% BALC	Theoretical%	53.3	8.7	0.9	1.6
	Measured average (n = 2) %	53.1	8.8	0.8	1.9
15% BALC	Theoretical%	51.8	8.4	2.4	2.8
	Measured average (n = 2) %	50.7	8.3	1.9	2.7
31% BALC	Theoretical%	50.2	7.6	2.8	6.1
	Measured average (n = 2) %	48.6	7.4	2.9	6.1
45% BALC	Theoretical%	48.6	7.1	3.7	8.3
	Measured average (n = 2) %	45.8	7.3	3.7	8.0
60% BALC	Theoretical%	46.6	6.4	4.9	11.0
	Measured average (n = 2) %	42.7	6.3	4.7	9.7
80% BALC	Theoretical%	43.9	5.5	6.5	14.8
	Measured average (n = 2) %	40.4	5.5	6.2	13.7

2.3.4. Qualitative analysis of BALC monomer incorporation into the beads

BALC incorporation into the polymer structure was analysed using a method adapted from Dai et al. (2010). BALC beads (1 mL) were 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 which were then placed in a water bath at 37 °C and left for one hour. 5 M HCl (200 µL) was then added to the reaction mixture to consume the remaining NaBH₄ followed by the drop-wise addition of 1 M NaOH to neutralise the HCl. The solution was raised to pH 8 with NaOH. 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 min in the water bath at 37 °C. After 30 min the samples were removed and analysed using a Varian Cary 50 UV/Vis spectrophotometer scanning the range of 300–500 nm for the 2-nitro-5-thiobenzoate anion (NTB⁻).

2.3.5. Investigation of the effect of reduction on bead size and degradation

In order to evaluate the effect of reducing conditions the BALC beads were placed in a glass vial and 1 mL of 4% dithiothreitol (DTT) was added. The vials with microspheres were placed in a water bath at 37 °C and left for one hour followed by washing (x 5) with deionised water (20 mL) to wash out remaining DTT. Once the beads were hydrated in water they were placed under the microscope again and another 200 measurements of the diameter were recorded.

Potential degradation of BALC formulations from 0 to 80% was evaluated by dispensing samples of each bead type (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 beads were placed in the removable inserts from the centrifuge tubes. The samples were placed in a minifuge and centrifuged at 6000 g. The samples were spun for 15 s (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.

The bead samples at each sampling time point were then placed in the minifuge, removing the excess DTT solution by centrifuging for 15 s (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 previously described. Images of the bead were then taken with an Olympus BX50 microscope and ColorView III camera for visual analysis.

2.3.6. Bead water content determination

Gravimetric analysis was used to establish the water content of the various formulations of beads. One gram of the beads was weighed on pre-weighed aluminium trays. Excess water was then pipetted away from the beads. The beads were placed in an oven at 60 °C under vacuum and weighed repeatedly until there was no change in their weight. This point was taken as the dry weight of the microspheres. From these measurements Equilibrium Water Contents (EWC) and the corresponding solids content of the various hydrated bead formulations were obtained.

2.3.7. Loading of doxorubicin

The method of drug loading into the beads was based on that described by Lewis et al. (2007). A Dox solution was prepared at 25 mg/mL 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 (700–900 µm size fraction) 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. 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. Following washing, the added deionised water was removed and collected and 10 mL of fresh 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 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. Theoretical dox loading was calculated based upon the BALC content present derived from the actual elemental compositions of the bead formulations obtained (Table 1) and the solids content present in each of the corresponding hydrated bead formulations (Section 2.3.6), together with the assumptions that beads would pack to a 77.8% efficiency in solution (Hales, 1992) and that one carboxylic acid group from the BALC would interact with one dox molecule.

2.3.8. Determination of drug binding sites within BALC beads

An acid-base titration was performed to determine the amount of carboxylic acid groups within the beads and hence the potential maximum drug loading capacities. 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 20 mL 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 method was repeated with the same formulation of beads that were washed with deionised water, rather than acid.

2.3.9. Binding site neutralisation loading technique

Further drug loading studies were performed where 1 mL of BALC beads were placed in 1 M NaOH solution and left for 20 min.

The NaOH solution was then removed and the microspheres washed with deionised water to remove any residual NaOH. The beads were then loaded according to the same method used in 2.3.7. The BALC beads were immersed in excess Dox solution and allowed to load on a mixing plate over-night. After 24 h, all excess drug loading solution was removed and analysed by UV.

2.3.10. Drug release from BALC beads

The drug release of BALC beads was performed in triplicate on samples as prepared in 2.3.7. 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 60 mM DTT reducing agent (Meng et al., 2009; Shirazi et al., 2011) at 37 °C. At set time points of 10, 30, 60, 120, 240, 360 and 1440 min, 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 (Varian Cary 50 UV/Vis spectrophotometer, UK) 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.

3. Results

3.1. Bead synthesis and characterisation

With some modifications to the published synthetic method, sufficiently large yields of BALC were prepared that allowed multiple suspension polymerisation reactions to be performed yielding a range of bead formulations with different BALC contents. The beads were mechanically sieved into size fractions and most the experimental work was carried out with 700–900 μm size range, as the largest volume of beads was collected in most instances from this fraction. The BALC cross-linker proved to be very soluble in the aqueous phase, allowing preparation of beads with BALC contents up to 80 wt%. It was noted however, that formulations of greater than 30 wt% BALC had a tendency to begin to precipitate during polymerisation, which was solved by reducing the initiator concentration in the formulation from 10 to 1 mg/mL. Spherical and smooth hydrogel beads were produced in good yield from the suspension polymerisation of various formulations ranging from 0 to 80 wt% BALC with PVA macromer (Fig. 2). The incorporation of the BALC monomer was shown to be efficient in all cases with elemental sulfur analyses corresponding closely to the theoretical compositions, albeit a little higher than predicted for the very lowest BALC contents (Table 1).

Optical microscopy of the beads showed that all formulations ranging from 0 to 45% BALC had a similar smooth and round morphology with optical transparency and no colour hue (see Fig. 3 top row (a)–(c) for examples of the 15, 31 and 45 wt% BALC formulations). The 60 and 80% formulations were still transparent but with a notable yellow tint and the hint of tiny particulates inside the bead structure (see Fig. 3 top row (d) for the 80 wt% BALC

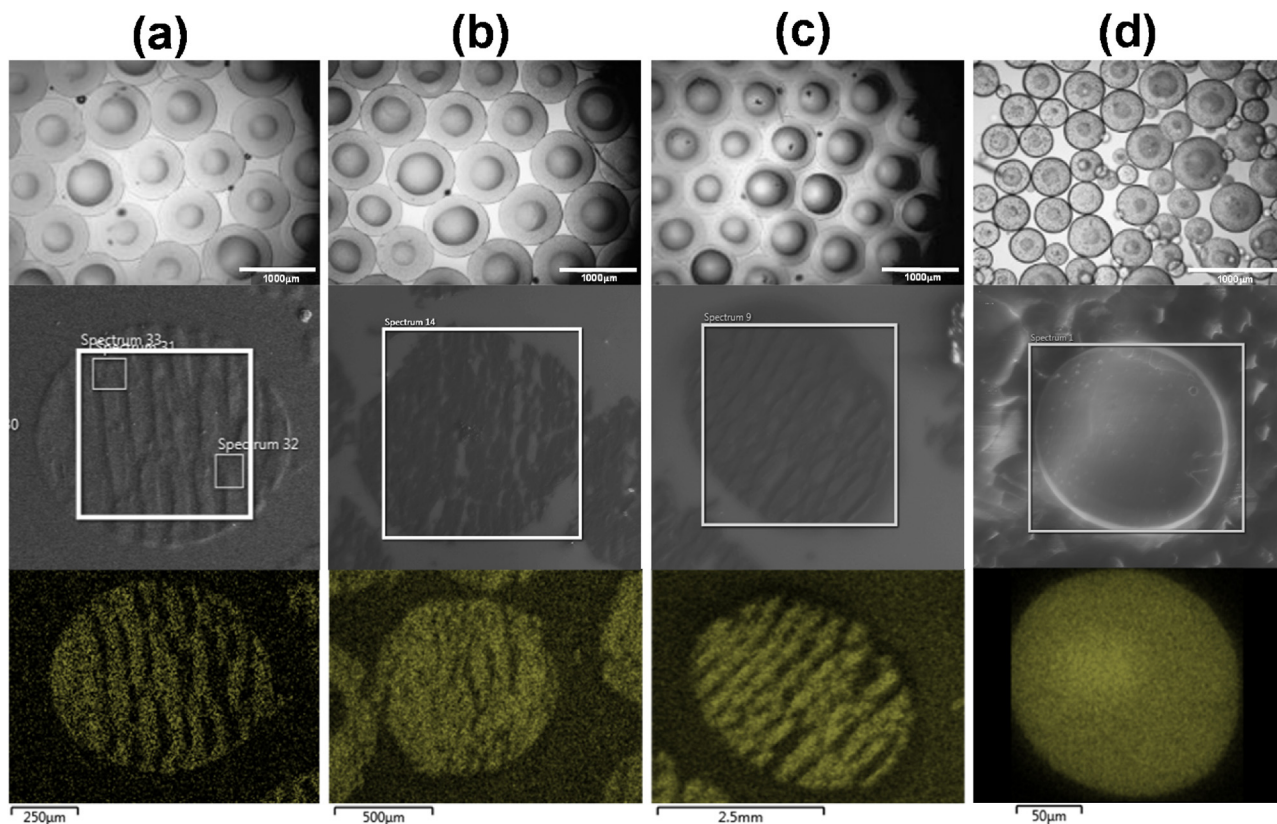


Fig. 3. Example formulations containing 15 (a), 31 (b), 45 (c) and 80 wt% BALC. Top row: Optical micrographs (scale bars = 1000 μm); Middle row: scanning electron micrograph of bead sections; Bottom row: SEM-EDX sulfur mapping image (individually scaled).

formulation). Measurement of the equilibrium water contents of the 0–31% BALC beads showed them to be of relatively high water content ranging from 92 to 97.6% (Fig. 4). This is evident from the optical micrographs in Fig. 3 whereby these softer, swollen beads deform slightly under their own weight, the bottom of the bead contacting the bottom of the glass dish to give the appearance of an inner spherical area resembling frogspawn. The solids content of beads >45 wt% BALC was higher, producing a stiffer structure for which this contact phenomenon is less evident. Moreover, whereas the 0–45%wt BALC formulations were successfully sieved into size fractions (as seen by the uniformity of size in the optical micrographs in Fig. 3, top row (a)–(c)), formulations >45% wt% were more friable to handle and could not be sieved without significant fragmentation of the beads (hence the range of bead sizes present in the optical micrograph for the 80 wt% BALC formulation in Fig. 3, top row (d)).

Beads of each formulation were embedded and sectioned using a microtome to produce a thin section for SEM-EDX elemental mapping analysis. The softer formulations proved difficult to section without the microtome blade forming “chatter-line” artefacts (Fig. 3, middle row (a)–(c)) whereas the stiffer 80% formulation produced a smooth, evenly cut surface. Subsequent SEM-EDX analysis was still possible and revealed no sulfur present for the 0% formulation as expected (not shown), whereas there were uniform distributions of increasing sulfur content for the BALC formulations, consistent with their proposed BALC concentration and the elemental analysis presented in Table 1.

3.2. Effects of a reducing environment on the BALC beads

To demonstrate that the disulfide bond of the BALC had remain intact during the synthesis of the beads, the compound 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, or Ellman's reagent) was added to a sample of the beads and observed for any colour change. DTNB is used to quantify the amount of thiol groups within a sample, as they will cleave the disulfide bond within the DTNB to give NTB⁻, which ionizes to the yellow-coloured NTB²⁻ dianion in water at neutral and alkaline pH (Fig. 5(b)) (Chen et al., 2008). In this case no colour change was noted and confirmed that the disulfide groups had remained intact within the bead structure. NaBH₄ was chosen as the reducing agent as it can be easily removed from the system by the addition of acid (Hansen et al.,

2007). After reduction of the bead sample, 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 again with DTNB demonstrated an immediate colour change to yellow for all formulations containing BALC following reduction (Fig. 5(a), insert images). UV-vis spectrophotometry using a cuvette of sedimented beads demonstrated the shift in the λ_{\max} for unreacted DTNB at 323 nm in the 0% BALC formulation, to 410 nm for the NTB²⁻ dianion generated for the BALC containing formulations, the absorbance increasing in conjunction with increasing BALC content (Fig. 5(a)).

The diameters of beads from a range of BALC formulations that had been selected from the 700–900 μm sieve fraction were measured optically to obtain a size distribution histogram. The beads were then exposed to DTT in order to reduce the disulfide bonds cross-linking the structure, thoroughly washed and then the size distribution was measured again. In the 0 wt% BALC bead formulation there was no statistical difference between the size distributions before and after exposure to the reducing environment (Fig. 6(a), Table 2). In the case of those formulations containing BALC, the bead size distribution was seen to increase by a statistically significant amount following reduction ($P < 0.05$ in all cases, see Fig. 6(b) for example of the 45 wt% BALC beads). Table 2 shows the mean sizes for formulations from 0 to 45 wt% BALC pre and post reduction and the percentage increase in the diameter. In general, the higher the percentage of BALC the greater the percentage increase in the mean bead diameter following reduction. For the lower BALC formulations, the increase in size post reduction is ~ 10 –14% as entanglement of the higher levels of PVA chains in the structure will limit the bead swelling. With much higher levels of BALC in the composition (and hence lower amounts of PVA), the effect of cleavage of the cross-linking points is more predominant than the PVA chain entanglement and the increase in bead swelling is more dramatic post reduction.

When beads are incubated with DTT to induce a hypoxic environment over several weeks, changes to the beads could be monitored by measuring weight loss and their appearance under the microscope. Fig. 7(a) shows the loss in mass over 64 days for 0–80 wt% BALC bead formulations, which was minimal over the first 3 days. At 7 days the 45 wt% formulation had >20% weight loss which accelerated to >50% at 21 days, >90% at the 42 and 64 day

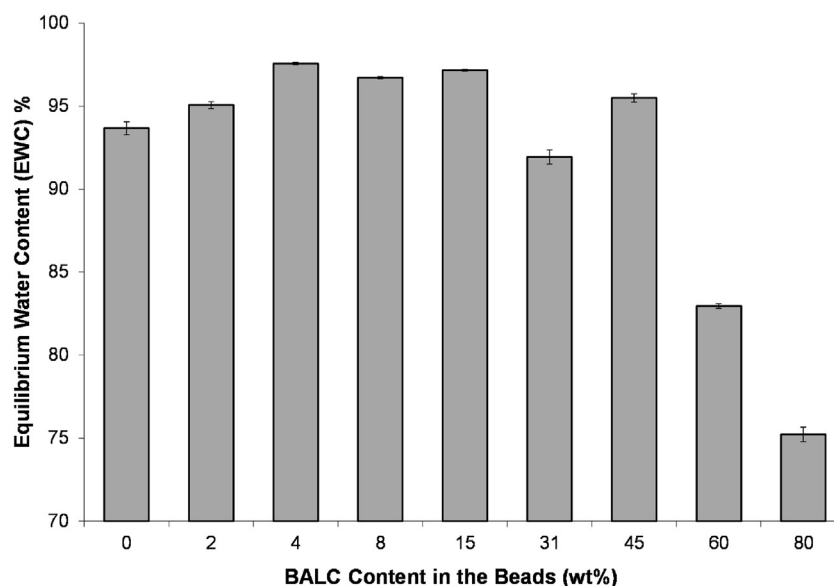


Fig. 4. Variation in Equilibrium Water Content with BALC content in the bead.

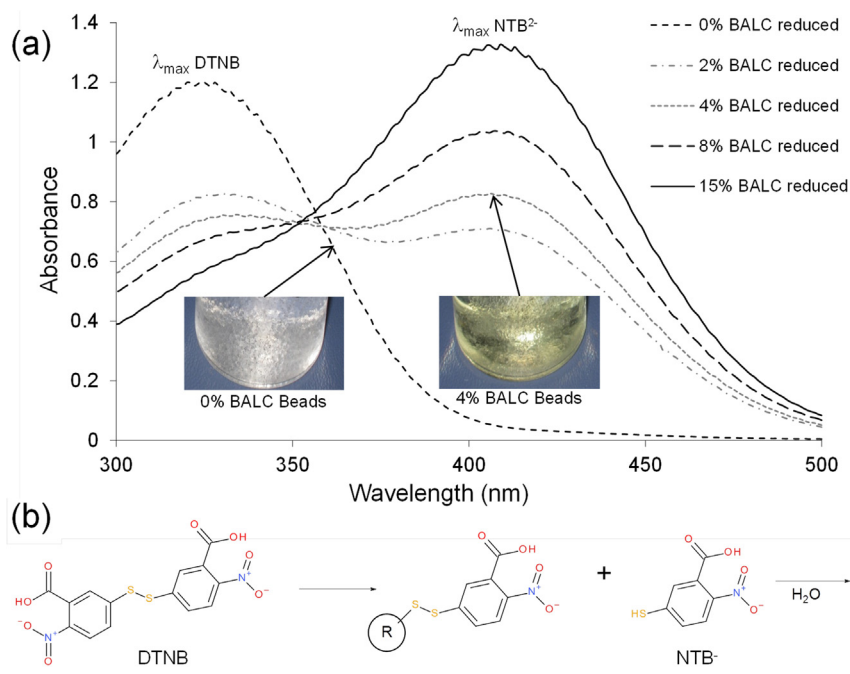


Fig. 5. UV-vis Spectra for bead formulations ranging from 0 to 15 wt% BALC reduced using NaBH_4 and then treated with DTNB. Generation of the yellow colour indicates the presence of thiol groups in the bead structure arising from reduction of the disulfide cross-links. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

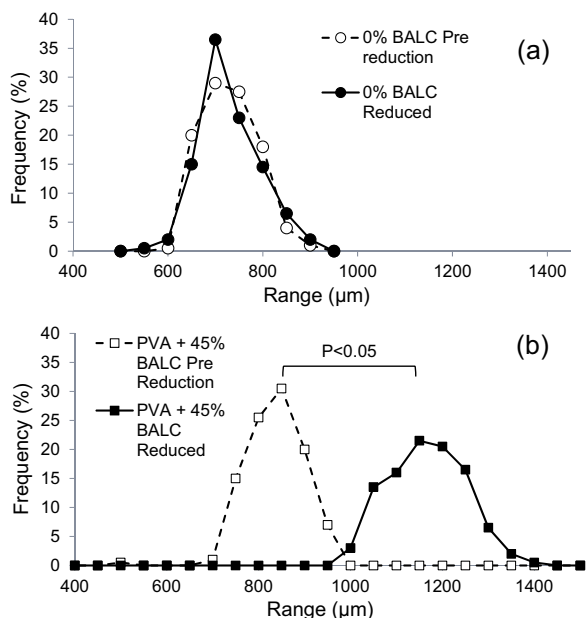


Fig. 6. Bead size distribution histograms for (a) 0 wt% BALC beads and (b) 45 wt% BALC beads pre and post reduction with DTT.

time point, as confirmed by the optical micrograph in Fig. 7(b). Whilst formulations <45 wt% BALC follow a similar trend the degradation was slower the lower the BALC content. The 31 wt% BALC beads for instance had >70% mass loss but appeared as a sticky mass of beads in the optical micrograph at 64 days, which is typical for BALC beads as they degrade. The 15 wt% BALC beads still looked largely intact at day 64 despite ~65% mass loss, although some slight deformation of the spherical shape was evident. The 0 wt% BALC also showed some mass loss, although the beads appeared largely intact at 64 days, as unreacted PVA macromer was lost from the structure. This was supported by the fact that no more mass loss was experienced over several years' storage (data not shown). Oddly, although the 80 wt% BALC formulation clearly showed signs of stickiness at 21 and 42 days, the mass loss was minimal. Yet by 64 days degradation was complete.

3.3. Drug loading studies with BALC Beads

When the BALC Beads were immersed in a doxorubicin solution above that of their theoretical maximum loading, the beads actively sequestered the drug from solution, turning a uniform red colour indicative of homogeneous drug loading (see Fig. 8 (a + b) for an example with 45 wt% BALC beads). Bead morphology remained smooth and spherical for all formulations and the average bead size was seen to decrease with dox loading by 25–30% (Fig. 8(c), similar to DC Bead[®]). Interestingly, upon placing the loaded beads in a reducing environment, the average diameter of

Table 2

Mean diameters of BALC Beads Pre and post reduction with percentage change (μm) (mean \pm SD, $n = 200$).

BALC Formulations	0%	2%	4%	8%	15%	45%
Pre-Reduction	705 \pm 57	856 \pm 62	810 \pm 86	869 \pm 79	745 \pm 146	847 \pm 71
Reduced	704 \pm 62	955 \pm 47	938 \pm 89	1003 \pm 76	933 \pm 114	1137 \pm 84
% Change	-0.1	10.3	13.6	12.3	20.1	25.5

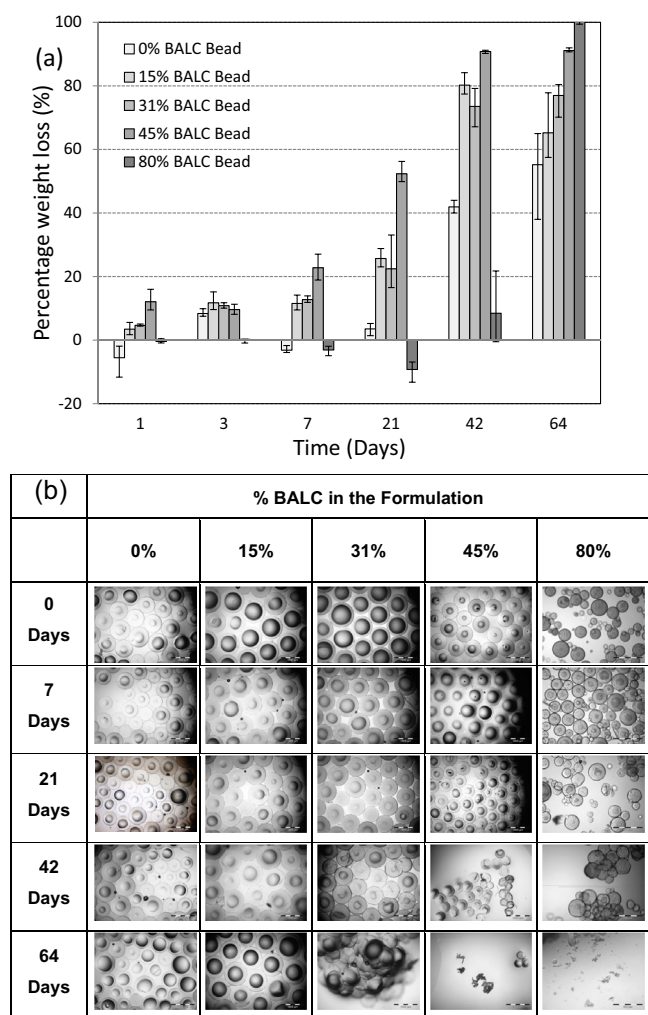


Fig. 7. Degradation of 0–80 wt% BALC bead formulations over 64 days monitored by (a) mass loss and (b) optical microscopy (scale bars = 1000 μm).

the beads was seen to increase again to a size close to that prior to drug loading and reduction of the disulfide links (Fig. 8(c)).

When comparing the maximum dox loading achieved for each BALC bead formulation with that of the theoretical maximum (Table 3), the two figures agree within error for formulations from 0 to 15 wt% BALC and where they exceed 100% is due to a slightly higher incorporation of BALC than targeted. For formulations ≥ 31 wt% BALC, the amount of dox loaded is 20–50% less than the predicted theoretical amount. An acid-base titration was carried out for each formulation to confirm the number of drug binding sites and yielded results close to that of the theoretical maximum $\pm 5\%$. There is therefore a clear disparity between the actual number of binding sites in the ≥ 31 wt% BALC formulations and the amount of dox that these beads can bind.

In a further drug loading experiment, 60 wt% BALC beads were reduced prior to the addition of dox. The beads were then washed to remove the reducing agent and the drug added. The measured maximum loading capacity was significantly greater when compared to those loaded prior to reduction (126 mg/mL of dox ($n = 3$) if reduced, compared to 93.8 mg/mL if pre-reduction). This result represents a 34% increase in the drug loading capability of the device attained by cleaving the disulfide units in the bead structure, with now 52% of drug binding sites accessed. It should also be noted that beads synthesised from BALC have carboxylic acid residues in their acid form, which are weak acids and will not be completely ionised at the pH used to bind the dox. An

experiment was performed in which the acid groups were converted to their sodium salt form following the protocol in Section 2.3.9. In this case, the dox loading capacity of 80 wt% BALC beads was shown to increase from the 109 mg/mL found for those in the acid form, to 322 mg/mL. This represents an increase from 22% to 66% drug binding site access when the sodium salt form is used.

Dox elution from BALC bead formulations was evaluated and compared to that achieved with a commercial standard product, DC Bead[®]. Under these test conditions, drug release rates were dominated by the drug solubility in the limited elution volume and therefore similar total amounts of drug were released in each case (Fig. 9). When release was compared using DC Bead[®] in both unreduced and reduced conditions, as anticipated, there was no difference between the elution curves obtained. For comparison and demonstration of proof of concept, we select the example of the 31 wt% BALC formulation, where there was a clear difference in the rate of release over the first 400 min, the reduced BALC bead releasing dox more quickly in the initial phase (about a 28% increase in dox), although the eventual cumulative release of drug eventually reaches a plateau for both the unreduced and reduced beads at around 1500 min.

4. Discussion

Disulfide chemistry has been frequently employed as a strategy to target reducing environments. There have been many reports of polyplex formulations for instance (Polymer and nucleic acid interpolymer complexes), that have been designed to undergo cleavage of the disulfide linkage under reducing conditions, facilitating release of the nucleic acid payload (Wang et al., 2006; Peng et al., 2008; Son et al., 2011). A further important advantage of such systems has been the low levels of cytotoxicity achieved when disulfide cross-linkers are introduced into delivery systems (Li et al., 2012; Liu et al., 2012; Shi et al., 2012; Vader et al., 2012). Hence, due to the low cytotoxicity, biocompatibility, stability, reducibility and hence ability to target a reducing environment, disulfide functional group chemistry was the route of choice selected in order to engineer redox-sensitivity. At the outset of this work we looked to identify a disulfide compound that carried bifunctional vinylic groups to enable copolymerisation with a preformed polyvinyl alcohol (PVA) macromer that is the basis of DC Bead[®], a well-established drug-eluting embolic microspheres with over a decade of clinical usage and proven safety profile. In an initial feasibility study, the compound N,N'-bis(acryloyl cystamine), which has been described previously in a number of reported systems (Christensen et al., 2007; Dai et al., 2010), was of limited success due to poor solubility of this compound in the aqueous phase of the suspension polymerisation (Ashrafi, 2014). N,N'-bis(acryloyl)-(1)-cystine (BALC) was identified as an alternative option which possessed acidic groups that offered the potential of overcome the solubility issues (Fig. 1). Moreover, as carboxylate groups have been utilised previously in a number of drug eluting embolic bead compositions to provide drug binding sites (de Baere et al., 2016; Pereira et al., 2016), the presence of these groups in BALC could also potentially introduce drug-binding capability into the bead formulations without the need for additional ionic comonomers. The carboxylic acids lose their protons in water leaving them negatively charged (Hoigné and Bader, 1983), allowing them to bind positively charged drugs such as doxorubicin hydrochloride which has a positively charged amine group on its sugar moiety (Grosso et al., 2008).

Suspension copolymerisations appeared to be feasible with a wide range of BALC contents (Fig. 2), although microscopy revealed a suggestion of some very fine particulate formation in the higher BALC compositions (Fig. 3). This was thought to be crosslinked

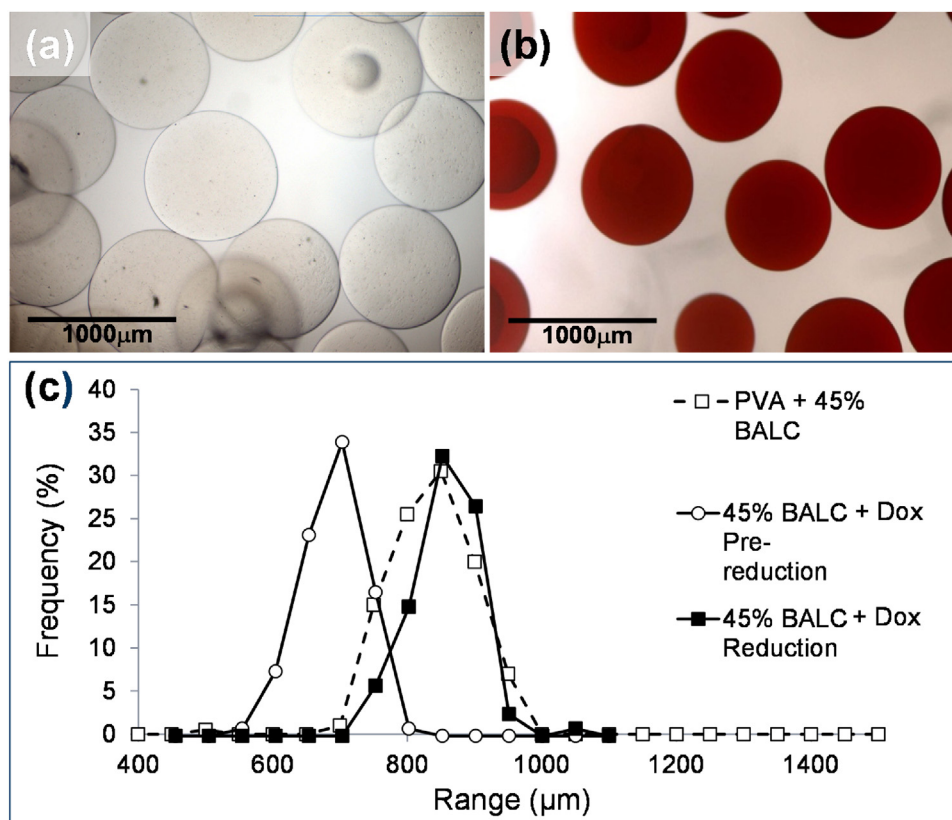


Fig. 8. Optical micrographs of (a) 45 wt% BALC beads and (b) Dox-loaded 45 wt% BALC. (c) Size distributions of 45 wt% BALC beads: unloaded unreduced; loaded unreduced; loaded and reduced.

Table 3

Theoretical versus actual maximum dox loading capacity for a range of BALC beads.

% BALC Bead	0%	2%	4%	8%	15%	31%	45%	60%	80%
Theoretical Dox mg/mL	0	4.9	6.4	11.2	14.6	68.9	51.8	243.1	489.7
Actual Dox mg/mL	2.1	5.4	8.5	11.3	14.7	15.3	25.0	93.8	109.0
Theoretical No. of Binding Sites/mL	NA	8.45×10^{-6}	11.03×10^{-6}	19.31×10^{-6}	25.17×10^{-6}	11.88×10^{-5}	89.31×10^{-6}	41.91×10^{-4}	84.43×10^{-4}
Actual No. of Binding Sites Accessed/mL	NA	9.31×10^{-6}	14.55×10^{-6}	19.48×10^{-6}	25.34×10^{-6}	26.38×10^{-6}	43.1×10^{-6}	16.17×10^{-4}	18.79×10^{-4}
% Binding Sites Accessed	NA	110	133	101	101	22	48	39	22

BALC oligomer precipitation as it was induced more severely with higher initiator levels, which would have tended to favour more frequent chain termination over longer chain network formation. Successful bead fabrication and elemental analysis confirming achievement of target composition (Table 1) demonstrated that, despite BALC being a relatively short bifunctional cross-linker, its acrylamide functional groups could participate favourably in free-radical copolymerisation with both other BALC monomer and the PVA macromer to form stable, swollen hydrogel beads which were transparent and mostly colourless in appearance (Fig. 3). The transparency suggested that no phase-separation was occurring during the synthesis, a phenomenon that has been observed in this type of suspension polymerisation with other water-soluble monomers (Heaysman et al., 2016, 2017). This was confirmed by SEM-EDX which showed an homogeneous sulfur distribution within the bead interior for the various formulations synthesised (Fig. 3). Water content was seen to range between 93 and 97.5% for 0–15 wt% BALC formulations as there is a balance between the added hydrophilicity and the increase in cross-linking. For formulations >15 wt% BALC the EWC began to decrease (and

hence solids content of the beads increase) more significantly as cross-linking density became the more predominant effect on constriction of the swelling of the hydrogel matrix (Fig. 4). Under normoxic conditions, the BALC incorporated in the bead was stable in its disulfide form, effectively covalently cross-linking the structure and able to resist the swelling forces within the hydrogel (Maitra and Shukla, 2014). When a reducing agent was added however, the disulfide bridges were cleaved, each forming two thiol groups (confirmed by the reduction of Ellman's reagent to produce a visible yellow colouration, Fig. 5). The cleavage of the disulfide bridges led to a relaxation of the previously constrained polymer network, allowing the BALC beads to swell to an increased bead diameter (Fig. 6 and Table 2).

The PVA macromer has on average only about 7 NAAADA acrylamide functionalities per polymer chain. Homopolymerisation of the macromer (0 wt% BALC formulation) did result in the formation of microspheres but it was noted that over an incubation period of 64 days some of the PVA had not become incorporated into the structure and diffused out of the cross-linked matrix, leaving an insoluble microsphere network that was stable to any

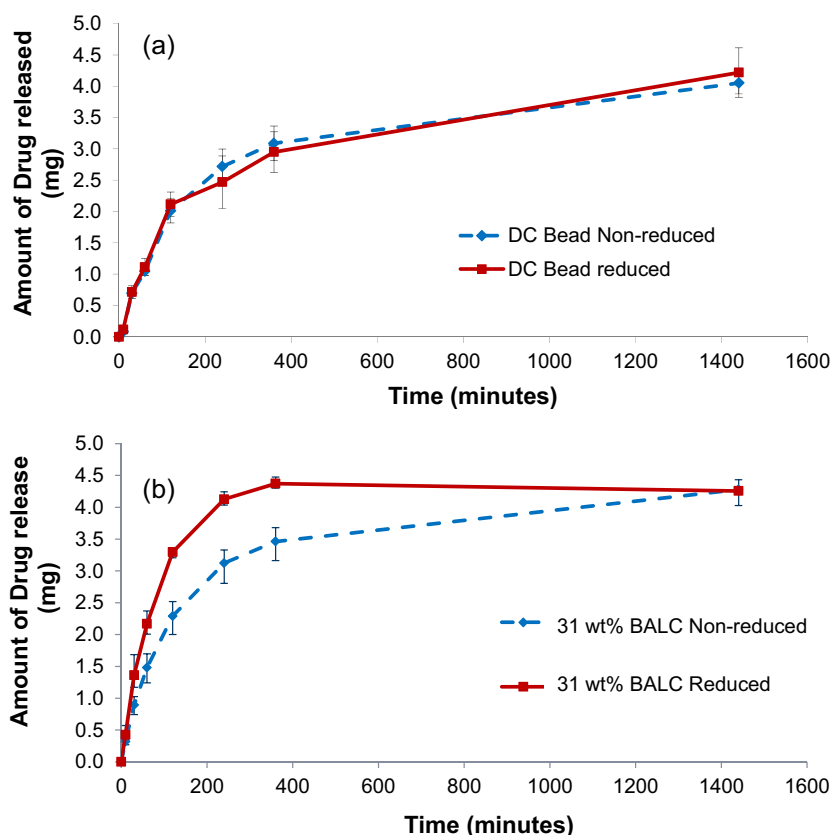


Fig. 9. Demonstration of the feasibility of hypoxia-responsive drug delivery for BALC Beads. (a) Comparison of dox release from DC Bead (700–900 μm size loaded at 37.5 mg/mL) in unreduced and reduced conditions. (b) Release of dox from 31 wt% BALC bead formulation (700–900 μm size loaded at 30.1 mg/mL) in unreduced and reduced conditions.

further mass loss. For those formulations copolymerised with 15–60 wt% BALC, the higher the BALC content, the faster rate of degradation of the bead structure; the disulfide bonds were becoming completely cleaved and the polymer chain fragments gradually extracted from the diminishing network matrix (Fig. 7). Interestingly, the 80 wt% BALC composition is very highly cross-linked and maintained its structure with little mass loss up to 42 days despite the disulfides cleaving over the incubation period. At 64 days however, sufficient chain cleavage had occurred that the entire network had dissolved away demonstrating complete degradation within a 2 month *in vitro* incubation time in a reducing environment. This property of the BALC Beads makes them an attractive option as a tumour embolising device, as there is a great deal of current focus on the design of bioresorbable embolic bead systems (Weng et al. 2011a,b; Owen et al. 2012; Weng et al. 2013a,b,c; Verret et al. 2014) because treating physicians feel this may be advantageous for repeat treatments requiring re-access *via* the same vessels.

Dox was shown to interact with the BALC beads (Fig. 8), the drug loading capacity increasing with increasing content of BALC as this component carries the carboxylic acid drug binding sites (Table 3). Interaction of with the drug resulted in a reduction of the mean bead size by 25–30%, a phenomenon observed previously with DC Bead[®] due to displacement of water from the hydrogel matrix and tightening of the structure (Lewis et al., 2006a,b, 2007). Whilst formulations containing up to 15 wt% BALC loaded close to their theoretical maximum of drug, the high percentage formulations only loaded between 20 and 50% of the theoretical value. An acid-base titration demonstrated the theoretical number of binding sites were indeed present in the bead formulations and could interact with small ions during the titration but not with the

larger drug molecules. The reasons for this were shown to be twofold: Firstly, conversion of the carboxylic acid groups to the sodium salt form was shown to increase the amount of drug that could interact, as the binding sites do not interact with the drug in their acid form. Secondly, it was shown that if the beads were reduced prior to drug loading, an increase in drug loading capacity was attained relative to the unreduced beads, as more of the binding sites could be accessed in the more swollen, less cross-linked hydrogel matrix upon disulfide cleavage. The higher the BALC content, the more cross-linked the structure with a reduction in the macromolecular mesh size available for drug diffusion (Brown, 2008). BALC beads were therefore shown to have the potential for high drug loading capacity in the range 300 mg/mL dox, which is much higher than the current commercially available embolic microspheres.

Elution of the drug from the BALC-containing systems was far more complex than we anticipated and whilst discussed in more detail elsewhere (Ashrafi, 2014) is beyond the scope of this feasibility study. The pH of the elution medium, salt concentration, volume of eluent and effect of the DTT reductant were found to be major contributors influencing the rate of drug elution. Multiple reducing agents were investigated and although DTT was thought to interfere least in the elution process, was implicated in retarding drug elution from high-BALC containing formulations due to adduct formation at the surface of the beads which could interfere with drug diffusion processes (Begg and Speicher 1999; Li et al., 2001; Shu et al., 2003; Antelman et al., 2009; Srivastava et al., 2011). In a simple proof-of-concept study it was demonstrated that the BALC bead systems were able to respond to the reductive environment by cleavage of disulfide cross-links, opening of the hydrogel matrix and subsequent increase in the percentage of drug

released in the early phase of drug delivery, exactly where it is proposed as being essential for overcoming the chemoresistance mechanisms.

5. Conclusion

This study has demonstrated that Drug-eluting Embolisation Beads based on BALC have the potential to be bioresponsive to metabolic changes in the body and to modulate drug release accordingly (You et al., 2010). They offer a novel mechanism of hypoxia-mediated biodegradation, tunable by virtue of the BALC content and unlike current systems which rely on hydrolytic and/or enzymatic degradation (Weng et al., 2013a,b).

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