Resorbable alginate embolic microsphere for Musculoskeletal embolization applications

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Abstract

Resorbable embolic agents can be used for musculoskeletal (MSK) pain relief applications using a Transarterial Embolisation (TAE) technique. However, inconsistent particle size and shape, degradation rates, and unpredictable resorption and excretion of the breakdown products, limit their widespread clinical adoption. The present study reports self-degradable, rehydratable, freeze-dried, resorbable Ca^{2+}-crosslinked alginate microspheres as a novel vascular embolic agent. The alginate lyase enzyme was incorporated into the alginate microspheres to achieve a controlled degradation rate under physiological conditions. Whilst the feasibility of alginate microspheres as embolic agents has been demonstrated, this is the first study where the controlled release is achieved in physiological conditions. To incorporate the alginate lyase enzyme in the alginate matrix, its activity was reversibly inhibited by preparing an alginate lyase-alginate-excipient mixture in a pH 3.6–3.9 buffered solution. An electrostatic encapsulator enabled the scalable preparation of microspheres of size 210 ± 8 µm at room temperature. The results showed that freeze-dried microspheres regained their shape and size within two minutes after reconstitution with saline, along with active alginate lyase. The reperfusion time of alginate microspheres was evaluated using digital subtraction angiography (DSA) and compared with imipenem and celestine (IMP-CS)in a porcine renal arterial embolisation model. It was observed that most of the blood vessels reperfused within 130–210 minutes, and complete vascular blush was recovered within 24 hours. Embolization with resorbable alginate microspheres induced recoverable ischemic necrosis in the targeted renal arteries. This unique feature of resorbable alginate microspheres in inducing ischemic necrosis, potentially in pain-

propagating neovessels, makes it an ideal candidate embolic agent for treating MSK-associated pain using the TAE technique.

1 Introduction

Transarterial embolisation (TAE) is an established minimally invasive techniquused to tree atof chronic K pain[1]. The painful MSK condition is thought to occur due to persistent tissue inflammation caused by the invasion of hyperandrogenic neovascularity and neoinnervation within the osteoarthritic joint space[2]. The neo-hyper vasculature supplies oxygen and nutrients to aberrant endothelial cells. It allows pro-inflammatory cytokines to infiltrate the local microenvironment, causing the tissue to become innervated and more pain-sensitive. TAE can be performed using occluding agents composed of either resorbable or non-resorbable materials, which prevent the blood flow at the target site and create ischemia-hypoxia induced necrosis of neovessels in addition to a decrease in the number of unmyelinated sensory nerves[3,4]. This breaks the pathological loop of neovascularisation, innervation, and chronic pain, reducing pain perception [1,4].

In clinical practice, the exact duration of necrosis depends on various factors, including the targeted vascular structure, the specific embolic material employed, and the clinical context of the embolisation procedure. However, neovessels are generally susceptible to ischemic damage because of their differentiated characteristics from normal blood vessels, such as intercellular gaps and altered basement membranes[3]. Researchers and clinicians have exploited this characteristic feature of neovessel to develop a new class of embolic agents that only transiently occlude the blood vessels to reduce hypervascularity and, subsequently, MSK-associated pain. There are various advantages of resorbable embolic

agents over permanent embolic agents for MSK pain treatment [5]. Firstly, due to the transient nature of the embolic agent, fewer off-target adverse events are likely to occur; secondly, transient occlusion may be sufficient to cause unmyelinated sensory nerve ischemia to relieve pain without creating prolonged localized numbness; and lastly, the use of resorbable agents allows for repeated TAE procedures to be conducted.

Many resorbable embolic materials are being evaluated in clinical practice for MSK pain relief, such as gelatin-based materials (sponge and microspheres), starch microspheres, and off-label IMP/CS particulate antibiotics[4,5]. Both gelatin- and starch-based embolic agents have shortcomings[5]. The gelatin sponge (such as Gelfoam® from Pfizer) must be cut manually, resulting in the formation of a polydisperse, irregular-shaped particle slurry, causing variability in the levels of occlusion. To overcome this problem, several manufacturers have developed gelatin microspheres (Nexsphere™ from Next Biomedical Co. Ltd, Korea, and IPZA™ from Engain C. Ltd, Korea). However, the gelatine-based agents suffer from unpredictability in degradation, as this relies upon local enzymatic action, which varies depending upon the quantity of embolic used, the site of embolization, and the patient's biological response[6]. Indeed, some reports suggest a high proportion of permanent occlusion with gelatin-based embolic agents due to their small size particles[7]. Degradable starch microspheres (DSMs, such as EmboCept® S from PharmaCept GmbH, Berlin) are calibrated 50 µm particles of hydrolyzed starch crosslinked and substituted with glycerol ether groups having a very short half-life (< 40 minutes) [3,8]. These are commonly used in combination with the delivery of chemotherapeutic drugs to liver tumours. However, they also suffer from unpredictable degradation rates when used in low blood flow anatomical territories, as their degradation depends solely on serum amylase. Off-label use of resorbable IMP/CS antibiotic particulates is widely used in clinical practice for pain relief in

osteoarthritis, tendinopathy, adhesive capsulitis, and back pain. The recanalization time for IMP/CS ranges from 1 to 4 hours *in vivo*, which is considered an ideal duration to reduce neohypervascularity for MSK indications [4]. Because IMP/CS particles are formed from an antibiotic mixture, its adoption as a temporary embolic device in mainstream clinical practice is hampered by concerns over antibiotic resistance development, allergic reactions, and significant regulatory challenges [9]. For these reasons, there is a pressing need for a biocompatible resorbable embolic agent with a calibrated size and reliable resorption time. For its target product profile (TPP), the most appropriate size of microspheres for this use is considere0-300 µm, as smaller particles can travel more distally and may result in unwanted off-target embolization to the skin and muscle. A clinical trial is being carried out using permanent Embozene microspheres of a similar size to treat knee pain secondary to moderate to severe osteoarthritis [10]. As for the appropriate recanalization duration for temporary embolic materials, *in vivo* IMP/CS degradation time of 1-4 hours has been shown to be very effective in clinical practice and represents an appropriate target degradation rate for developing new resorbable embolic microspheres.

Alginate is a biocompatible polysaccharide previously used to prepare Ca2+-crosslinked microspheres for TAE [11]. Under *in vivo* conditions, the backbone of the alginate may be degraded slowly and uncontrollably through non-enzymatic hydrolysis of glycosidic bonds. One strategy to enable both an increase in, and level of control over the degradation rate, is to incorporate an alginate-degrading enzyme within the alginate matrix. Leslie *et al* have demonstrated the immobilization of alginate lyase into alginate microspheres to achieve the tunable degradation of alginate microspheres for the controlled release of rat adipose stem cells to improve bone regeneration [12]. However, several technical challenges were not addressed in the reported work to enable its usage for novel applications such as TAE,

methods to preserve the product and provide a shelf-life, and to ensure its sterility for safe administration into the human body. Here, we have therefore, focused on developing stable, reconstitutable, freeze-dried alginate lyase-loaded Ca^{2+}-crosslinked alginate resorbable microspheres that can be terminally sterilized. This study addresses an important challenge, ensuring that alginate lyase-entrapped alginate microspheres can be produced using medical-grade alginate for a sufficient length of time to achieve a good yield and can be preserved at room temperature without being degraded by the enzyme. Other aspects related to using these microspheres as resorbable embolic agents are discussed in this study: (i) characterization of alginate microspheres for the degradation time *in vitro*, (ii) determining the degradation time of resorbable microspheres *in vivo*, (iii) evaluating its effectiveness for inducing recoverable necrosis in renal arteries, and (iv) predicting the biological fate of alginate breakdown products. To the best of our knowledge, this is the first report demonstrating the development of the resorbable alginate lyase-loaded alginate microsphere as an embolic agent for MSK pain relief applications.

2 Material and methods

2.1 Preparation of lyophilized resorbable alginate beads

The method of preparing alginate microspheres using an electrostatic encapsulator was adopted from Leslie *et al*, with appropriate modifications [12]. The alginate beads were prepared by dissolving 2% w/v low-viscosity LVM sodium alginate (Novamatrix Pronova®, Oslo, Norway) in 0.1 M pH 2.8 acetate buffer, mixed with 0.0875U and 0.15U alginate lyase (Nagase Cooperation, Japan) solution to achieve the desired degradation rate. The acetate buffer inhibits the alginate lyase degrading activity, thereby allowing the synthesis of beads without losing the viscosity of alginate over a 30 minute time period at room temperature,

thus improving the throughput of resorbable microspheres. The alginate-alginate lyase precursor solution was dripped through a 0.09 mm ID needle into a 2% w/v calcium chloride gelling bath at different flow rate 2 and 3 mL /hour under a range of potential of 2-5kV for 30 minutes to identify an optimised condition for producing microspheres of size around 200 µm at a high throughput. At least 10 beads of 3 independent batches were used to compute the size of the particles. The yield of each batch was determined by allowing the microsphere to settle in a 3 mL syringe for 20 minutes, and the volume of the microspheres was recorded.

The microspheres were then washed with a proprietary mixture of cryoprotectants and transferred into lyophilizer for freeze drying (Biopharma Process Systems Ltd, Winchester, UK) at -45°C degrees Celsius for 90 hours [13]. The lyophilized microspheres were sealed under a vacuum and sterilized.

2.2 Sterilization using gamma- radiation

The sterilization of the resorbable alginate microspheres using gamma radiation was performed as per ISO 11137-2: 2013[14]. Based on the verification dose of 6.21-7.59 kGy, a sterilization dose of 22.5 kGy +/- 10% was determined to meet the bioburden level of ≤ 220 CFU at SAL of 10^{-6} levels using a sterility test. To determine the verification dose, 3 batches of 10 samples each were used to identify the bioburden levels.

2.3 *In vitro* degradation of resorbable alginate microspheres

This is a quantitative bespoke method that was developed to simulate the degradation of microspheres *in vivo*. The lyophilized microspheres made with 0.0875U and 0.15U were reconstituted with 1.5 mL of saline for 2-3 minutes, followed by addition 6 ml of 0.1M pH 7.4 HEPES buffer (supplemented with physiological levels of calcium, phosphate, and

sodium ions). The hydrated beads were filtered using a 20µm pore filter and removed the excess liquid before weighing not more than 110 mg (100mg, +10/0) of beads as a starting quantity. The weighed beads were dispersed into 6 ml HEPES filtrate, incubated at 37 ^{o}C and gently agitated at 150 rpm (to avoid any mechanical damage) to evaluate the mass loss. The residual mass was recorded every 20 minutes until it reached less than 10 mg of weight. Where necessary, the 6 mL test volume of HEPES was maintained throughout the test period. A volume of 6 mL of HEPES Buffer was selected as it is sufficient to facilitate repeatable rinsing through a filter and creates an environment with sufficient ion exchange to degrade the microspheres to the level required for this test.

A quantitative method was used to evaluate individual microspheres' diameter and perimeter shape (degraded and non-degraded forms). Randomization of the microspheres was achieved by taking a droplet (containing multiple microspheres) from the test vial (using a pipette) and placing it on a glass slide for imaging performed at 10X magnification (Leica stereomicroscope, USA and ECHO Rebel microscope, USA). Once the image was taken, lines were digitally drawn between opposite corners, and the first 10 microspheres on the intersecting lines were selected for measurement. 10 microspheres were taken to obtain a representation of the distribution within the sample. To determine the size of the microspheres, two diameter measurements were taken for each microsphere (vertical and horizontal to remove subjectivity) and averaged to minimise the impact of a skewed result due to non- spherical microspheres. In order to calculate the shape of microspheres, a minimum of twenty (approximately evenly spaced) points were selected along the perimeter of each microsphere to gain a true representation of the outer profile. The area within twenty selected points was digitally calculated and the below equation was used to determine circularity (independent of the size of the microsphere). A perfect circle will

return a value of 1.0. A less than perfect circle will return a numerical value <1 which provides a quantitative result for circularity.

$$\text{Circularity} = \frac{4*\pi*(Area)}{(Perimeter)^2}$$

2.4 Size exclusion chromatography-multiple angle light scattering (SEC-MALS) analysis of resorbable alginate beads breakdown products *in vitro*

2.4.1 Mapping of the breakdown profile

The molecular weight of the alginate breakdown products was determined at different mass loss stages. This study provides the evolution of the molecular weight of alginate breakdown products over 24 hours of degradation time. The 100% mass loss of resorbable beads is compared to the *in vivo* data (described in the subsequent section), to correlate the *in vitro* breakdown mechanism with *in vivo* conditions. To achieve this, a degradation profile was first mapped according to the method described in Section 2.2 to determine 33%, 66%, and 100% mass loss. The degradation times to achieve the stated mass loss are given in Table 1.

Samples	**Degradation Time (minutes)**
0%-mass loss	0
33%-mass loss	39
66%-mass loss	118
100%-mass loss	1440 (24hr)
Alginate control	
Excipient control	
Product control-containing alginate and excipient (without alginate lyase)	

Table 1: Degradation times for Mass loss samples and the control samples used for the molecular weight analysis of the alginate breakdown products.

2.4.2 SEC-MALS analysis of alginate breakdown products

The samples at the respective stage of mass loss were exposed to boiling water for 10 minutes to inactivate the alginate lyase enzyme and to cease the further catalytic breakdown of the alginate matrix [15]. To de-crosslink the degraded alginate breakdown products, the samples were then treated with 0.2 M sodium citrate dihydrate, which chelates the calcium ion crosslinks [16]. These samples were subjected to SEC-MALS measurement without further processing.

Buffer and sample preparation for SEC-MALS measurements

This study followed Aarstad et al. in selecting the mobile phase [17]. To prepare 5.0 L of mobile phase, 35.5 g of Na_2SO_4 and 18.5 g of disodium EDTA dihydrate were dissolved in 4.7 L of water. The solution pH was adjusted to 6.0 with the addition of 1N NaOH. The volume was then adjusted to 5.0 L, and the solution was filtered through a 0.1 µm PES membrane. The injection volumes were chosen to target an alginate amount of 0.2 mg. For the samples not containing alginate, an injection volume equivalent to 200 µL was used to achieve maximum resolution of analytes. The injection volumes are listed in Table in supporting information (SI 1).

SEC-MALS method description

The SEC-MALS measurements were carried out on the Agilent HPLC system with UV, MALS and RI detector. All measurements were conducted at 30 °C and included signal collection by an RI detector and by a UV detector at 235 nm. The columns OHpak LB-804 and OHpak LB-G 6B were used for sample separation. These samples were eluted with a flow rate of 0.5 mL/ min through the column set at 30 °C. The measurement time was 40 minutes.

SEC-MALS data evaluation

Light scattering evaluation was carried out with the refractive index detection (RI) signal as the concentration source. A refractive index increment of 0.146 mL /g was used for the alginate nanoparticle samples. The light scattering data was evaluated by applying a Zimm Plot model. The UV signal at 235 nm was used to identify the signals of the alginate products [18]. The integration ranges applied to determine the molecular weight of the alginate breakdown products are given in Table 2.

Samples	Fraction 1 (min)	Fraction 2 (min)
0% Mass Loss	11.5 – 14.0	14.0 – 20.5
33% Mass Loss	14.5 – 17.5	17.5 – 20.5
66% Mass Loss	16.0 – 19.5	19.5 – 20.5
100% Mass Loss	17.0 – 19.5	19.5 – 20.5
Product Control	11.5 – 14.0	14.0 – 20.5
Alginate control	11.5 – 14.0	14.0 – 20.5
Excipient control	13.5 – 19.0	N/a

Table 2: The integration range for different samples used for the SEC-MALS analysis.

2.5 *In vitro* hemocompatibility of resorbable alginate microspheres

In vitro hemolysis of resorbable alginate microspheres was conducted per ISO 10993-4:2017 (E) and ASTM F756-17[19,20] . Blood pooled from three rabbits had a plasma free hemoglobin concentration of <2 mg/mL (0.171 mg/mL) used in the study. Total blood hemoglobin was adjusted to 10.00 mg/mL using CMF-PBS (Calcium and Magnesium Free Phosphate Buffered Saline). The alginate microsphere extract was prepared at a 0.1 g/mL ratio in CMF-PBS. Triplicate samples for indirect extraction included blank control, negative control (High density polyethylene film), positive control (sterile water for injection), and the test material. All tubes were capped and incubated at 37 ± 1 °C in a shaking incubator for 72

hours. For the direct contact method, reconstituted rabbit blood was added to triplicate tubes at a 1:7 (diluted blood: CMF-PBS) ratio, while for the indirect method, it was at a 1:7 (diluted blood: sample extract) ratio. After 3 hours in a water bath, tubes were inverted every 30 minutes to maintain contact. Following incubation, samples were mixed and centrifuged at 800 × g for 15 minutes at 4°C. Supernatants from the various controls and extracts were examined for color and appearance. The supernatants were then mixed with Drabkin's reagent and allowed to stand for 15 minutes at room temperature, after which absorbance was measured spectrophotometrically at 540 nm.

To see the effect of resorbable alginate microspheres on blood clot formation qualitatively, 0.2 mL of alginate microspheres (without alginate lyase- positive control for resorbable microspheres), resorbable microspheres and Embozene particles (100-300 µm- Varian Medical Systems, Austin Texas, USA, positive control) were taken in glass Pasteur pipettes with one closed end. 200 mg of IMP/CS particles were also used as one of the test samples. To see the effect of these embolic agents on blood clot formation, the anti-coagulant present in the rabbit blood was reversed by adding 10 mM of calcium chloride. This blood was mixed with the samples and allowed to incubate at 37 °C for 24 hours. To determine the stability of blood clot formation after incubation, 5 mL of PBS was added on the clot followed by the opening of the closed end and observation of any fluids collected in tube. Furthermore, the effect of alginate lyase on the blood clotting property was evaluated by mixing a high concentration of 2 U of alginate lyase with anti-coagulant reversed blood (supplemented with 10 mM calcium chloride) and incubated for 20 mins at 37 °C to observe clot formation. The clot alone was considered as a control.

2.6 Degradation of resorbable alginate beads in a porcine renal arterial embolization model

2.6.1 Animal model and husbandry

The swine model was selected for this study. The healthy swine model was chosen as the experimental species for this study because the size and anatomy of the vascular system are clinically relevant for the purpose of testing interventional medical devices. The porcine model has been widely used for vascular and physiology experiments to evaluate MSK embolization. The study was reviewed and approved by an external ethical committee for compliance with regulations prior to study initiation. Animals were individually or group housed in conditions meeting or exceeding the requirements outlined in the Directive 2010/63/EU and as described in the Guide for the Care and Use of Laboratory Animals.

2.6.2 Embolization of the Renal arteries

In this study, 5 Yorkshire swine weighing 55-60 kg were used. After induction of anesthesia, using a standard Seldinger's technique, the femoral artery was punctured, and a 6 Fr arterial sheath (Merit Medical, USA) was placed. 100-200 IU/kg of heparin were administered intravenously before selective catheterization for the test article delivery. Under fluoroscopic guidance, a guide catheter (Medtronic Inc., USA) was advanced through the sheath over a guide wire (Abbott, USA) into the target arteries. A microcatheter (Medtronic Inc., USA) was coaxially inserted through the guide catheter. The catheter tip was positioned at the origin of the target site.

A vial of sterile resorbable microspheres was reconstituted with 1.5 mL of saline and 2 minutes was allowed to dissolve the contents to obtain a clear solution of hydrated beads. To the hydrated beads, 2 mL of non-ionic contrast agent (Omnipaque, GE healthcare) was

added and gently inverted several times to mix the solution. A sterile 21-gauge needle was inserted to withdraw the beads in a 1 mL sterile syringe. Then, the needle was removed, and the syringe was attached to the catheter to start with injections of beads. Across 5 animals, 10 injections were delivered with aliquots ranging from 0.8 mL to 1.4 mL of resorbable alginate microsphere solution to target sites in kidneys under angiographic guidance to achieve 100% complete stasis in all the injections.

A similar procedure was followed to evaluate the embolization of IMP/CS (Imipenem/cilastatin antibiotics) in the kidneys of three animals. A suspension of IMP/CS was prepared by adding 5 ml of non-ionic iodinated contrast in 0.5 g of IMP/CS, followed by 10 secs of vigorous mixing, and was injected with 0.2 mL increments into the 6 different regions of the kidneys across 3 animals to achieve complete stasis. Angiography was performed at various follow-up times to assess the reperfusion. Animals were sacrificed and kidneys were collected and sent for histopathology analyses.

2.7 Histology

Out of 5 animals embolized with resorbable microspheres, 3 animals were euthanized on day 1 and the remaining on day 3 by intravenous injection of a barbiturate-based euthanasia solution[11] . The animal on day 1 was sacrificed to determine the presence of the embolic material as well as their biological fate to correlate with the *in vitro* studies (SEC-MALS). The kidneys were prepared and immersed in 10% NBF for the histology processing. For IMP/CS, three animals were sacrificed on day 3 of the survival period. In the case of IMP/CS, the objective was not to identify the presence of the embolic material but to identify the possibility of observing tissue necrosis.

The harvested kidneys were trimmed to document to assist in maintaining orientation. The tissues were divided into proximal, middle and distal treated regions. The trimmed sections were fixed and embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin [11]. Using light microscopy, histological evaluations were performed to identify the presence of microspheres or their fragments, haemorrhage, inflammation and revascularization.

2.8 Statistical analysis

All the experiments were performed at least in triplicate. The statistical significance was tested for $p < 0.05$ using On-way ANOVA with post hoc Tukey test. The values were calculated as mean ± S.D.

3 Results

3.1 Preparation and *in vitro* degradation of resorbable alginate microspheres

To determine the optimal conditions for producing resorbable microspheres of approximately 200 µm at a high throughput, various conditions related to flow rate and electrostatic potential were screened, as shown in Table 3. For optimization, the screening was conducted with 0.15 U of alginate lyase enzyme, and the optimized condition was then used to prepare alginate microspheres incorporated with 0.0875 U alginate lyase. When the potential increased from 2 to 3 kV at flow rates of 2 and 3 mL /hr, circular-shaped particles were observed, along with an increase in particle size. However, at 5 kV, regardless of flow rate, multiple streams of alginate were observed emanating from the needles, leading to liquid accumulation on the accelerator. This caused interruptions in production runs and

blocked needles. Additionally, flow rates 4 mL/ hr or above resulted in excessive backflow pressure, causing leakage issues.

Iterations	kV	Flow rate (ml/hr)	Particle size, µm (mean ± S.D, n= 3)	Outcome
1	3	2	201 ± 7	Circular beads/yield (0.4-0.6ml)
2	4	2	250 ± 6	Circular beads ((0.5-0.8ml))
3	5	2	--	Blocked needle
4	3	3	210 ± 8	Circular beads (0.7-1.1 ml)
5	4	3	240 ± 5	Circular bead (0.9-1.2 ml)
6	5	3	--	Blocked needles

Table 3: Summary of parameters screened for the optimisation of resorbable alginate microspheres synthesis.

The aim of optimizing the microsphere synthesis is not only to achieve circular microspheres of desired size but also to obtain good yield. A good yield can be achieved at a higher flow rate. Therefore, the optimum conditions of 3 kV and 3 mL/ hr were selected, which provided a relatively higher yield of 0.7-1.1 mL and an acceptable particle size of 210 ± 8 µm (Fig 1a). Using the optimized condition, the size of alginate microspheres made with 0.15U was found to be 211 ± 7 µm, as shown in Fig 1 (b). Both types of microspheres were lyophilized and reconstituted with an optimized volume of 1.5 mL saline. At this volume of saline, the reconstitution time was less than 2 minutes which is an acceptable timeframe for clinical practice. The reconstituted microspheres largely recovered their original shape and size, as shown in Fig 1(a`) and Fig 1 (b`) for 0.0875U and 0.15U-incorporated alginate-microspheres, respectively.

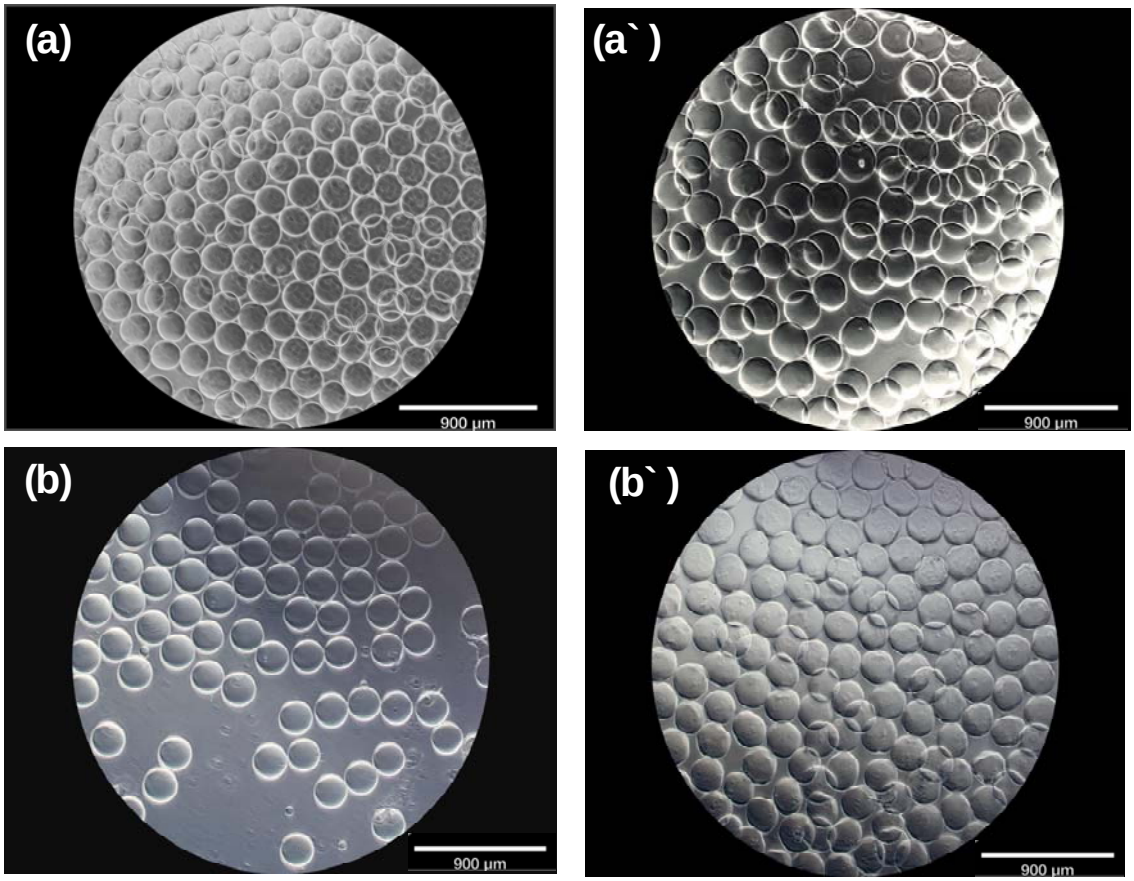
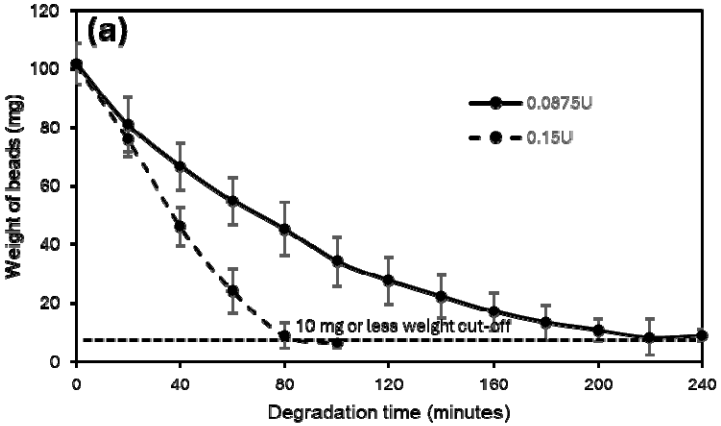
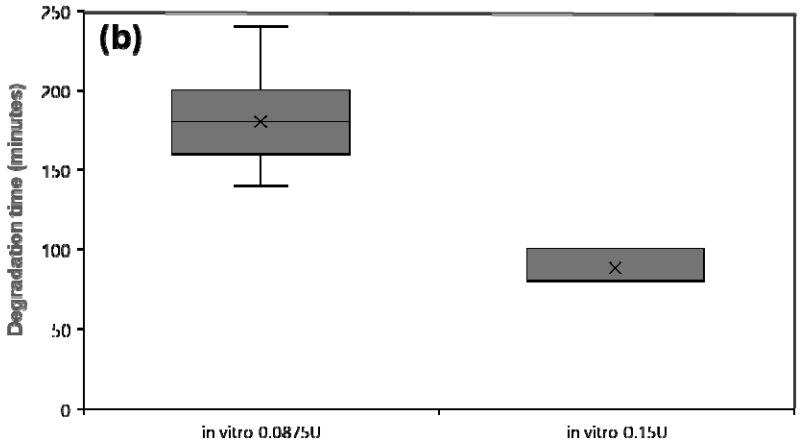


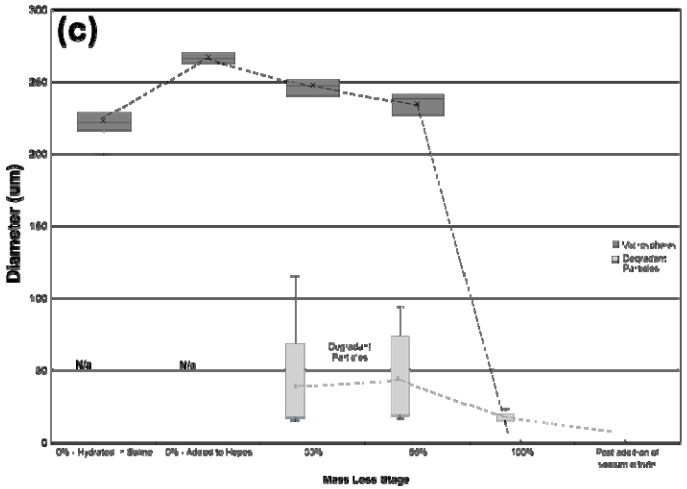
Figure 1: Representative images of pre-lyophilized (a, b) and post-lyophilized rehydrated/reconstituted (a`, b') resorbable alginate microspheres made with 0.0875 U and 0.15 U alginate lyase enzyme, respectively.

The reconstituted microspheres made with 0.0875U and 0.15U alginate lyase were degraded in simulated *in vitro* conditions to develop a mass loss profile over 24 hours as shown in Fig 2(a) using a 20-µm filter with a weight cut-off point of 10 mg or less. A filter pore size of 20 µm was selected as fragments that permeate through the filter are less than 1/l000th of the original volume (volume of 20 µm sphere /volume of 200 µm sphere) resulting in the test capturing 99.9% of volumetric degradation. On the weight cut-off point,

the 10 mg value was selected as it is more difficult to accurately detect a change as the mass approaches zero due to


(a)
Weight of beads (mg)
Degradation time (minutes)
0.0875U
0.15U
10 mg or less weight cut-off
0
20
40
60
80
100
120
0
40
80
120
160
200
240


(b)
Degradation time (minutes)
in vitro 0.0875U
in vitro 0.15U
0
50
100
150
200
250


(c)
Diameter (um)
N/a
N/a
Mass Loss Stage

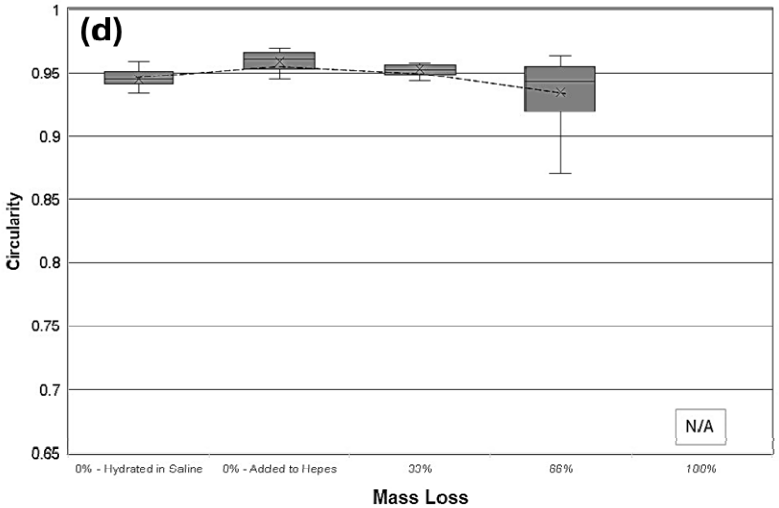

(d)
Circularity
0.65
0.7
0.75
0.8
0.85
0.9
0.95
1
N/A
0% - Hydrated in Saline
0% - Added to Hepes
33%
66%
100%
Mass Loss

Figure 2 *In vitro* degradation of resorbable microspheres (a) Degradation profile to achieve volume reduction by 99.9%, (b) degradation time, change in the diameter (b) and shape (c) of microsphere at different mass loss stage over 24 hours degradation period.

the measurement accuracy of the equipment. This can mask the variability between test samples (details are explained in the SI 2). The degradation time range for 0.15U and 0.0875U resorbable alginate microsphere is shown in Fig 2 (b) The resorbable microspheres encapsulated with 0.0875U and 0.15U alginate lyase were degraded to over 99.9% of the original volume in 140-240 minutes and 80-100 minutes, respectively. However, the latter iteration of 0.15U resorbable microspheres was not deemed suitable for obtaining an occlusion time similar to or higher than IMP-CS resorption *in vivo* (results are given in section 3.4). Therefore, further studies were conducted on alginate microspheres with 0.0875U alginate lyase enzyme. These results demonstrated that the resorbable microspheres loaded with varying contents of alginate lyase can be prepared, lyophilized and reconstituted successfully to obtain the desired degradation time. The degradation of resorbable alginate microspheres in the simulated *in vitro* conditions covering the entire duration of initial, intermediate and final time points was assessed further.

Figure 2(c) shows the trend of the average diameter of alginate breakdown products at each stage of mass loss over a 24-hour degradation period. The microscopic images are provided in SI 3. Two populations of breakdown products can be observed: the first consists of partially degraded large microspheres, while the second comprises relatively smaller-sized particles. At the 33% and 66% mass loss stage, the size of the large microspheres largely remains unchanged, but smaller-sized particles are also present. By the 100% mass loss stage, the large microspheres have fully degraded into smaller breakdown particles. Similarly, the shape of the microspheres was evaluated at each stage of mass loss and found to be largely unchanged until the 66% mass loss stage, whereas no intact microspheres were observed at 100% mass loss as shown in Figure 2(d). Based on the results, it can be deduced that as the total mass of the sample reduces over time, the large microspheres

transition into smaller degradants, continuing to decrease in size until they are completely degraded at 100% mass loss.

3.2 Sterilisation of resorbable alginate microspheres using gamma radiation.

Table 4 shows the average bioburden value at the verification dose that was used to calculate the sterilizing dose to achieve SAL of 10^{-6}. The average bioburden level of 3 batches of the microsphere was found to 16.45 CFU/device. To achieve the SAL of 10^{-6}, the product was sterilized at 22.5 kGy. On irradiation with sterilizing dose, the samples were tested for the sterility, and 9/10 samples were shown to be negative for growth. This substantiates 22.5 kGy as the appropriate sterilization dose for the resorbable alginate microspheres

Samples	Batch 1	Batch 2	Batch 3
1	0	4	2
2	0	53	3
3	4	1	1
4	0	6	1
5	1	1	95
6	18	6	7
7	42	7	3
8	17	6	0
9	5	9	0
10	17	8	9
Total	104	101	121
Average	10.4	10.1	12.1
Recovery Factor (RF)	0.66		
Average CFU with RF	15.76	15.3	18.33
Total Whole Product	15.76	15.3	18.3
Overall average	16.45		

Table 4: Average bioburden level at the verification dose.

3.3 Molecular weight analysis of alginate breakdown products or degradants using SEC-MALS.

Fig 3 shows the SEC-MALS analysis of alginate breakdown products. Fig 3 (a-d) shows the chromatograms of 0% mass to 100% mass loss- alginate sample. The presence of the UV signal (green colour) at 235 nm indicates the elution of degraded alginate (Alginate oligosaccharides containing Mannuronic acid and guluronic acid) after 14.5 mins for all the degraded alginate mass loss samples. No UV signal was detected for the 0% mass loss (Figure 3a), alginate control (Fig 3e), excipient control (Fig 3f) and product control samples (Fig 3g). The evolution of alginate molecular weight throughout the degradation period is shown in Fig 3 (h). The alginate control exhibits two fractions: High molecular weight minor fraction of 124.3 ± 5.0 kDa and Low molecular weight major fraction of 46.7 ± 1.5 kDa. These were observed at the integration ranges of 11.5 – 14.0 mins and 14.0 – 20.5 mins, respectively.

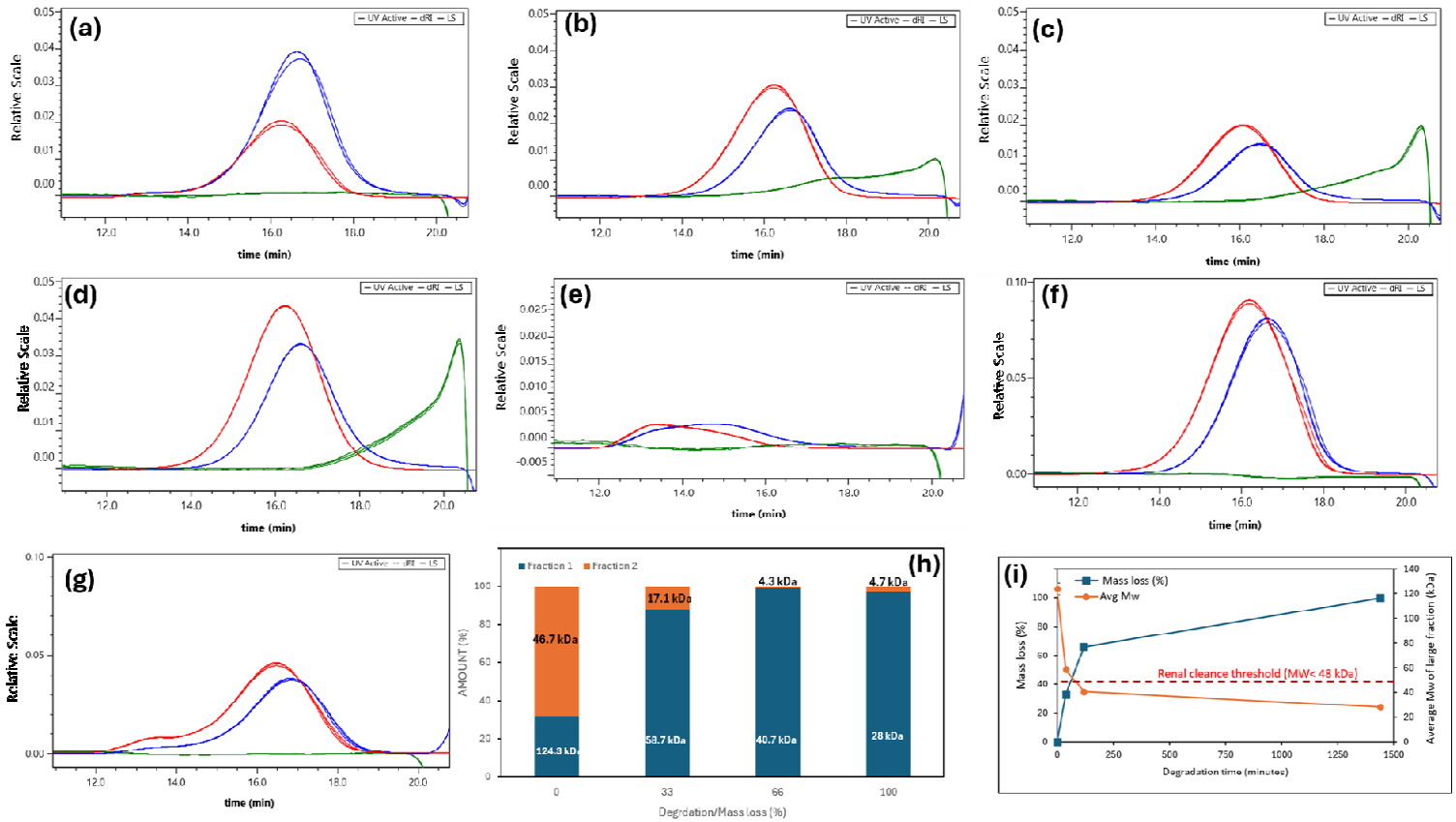


Figure 4 Representative chromatograms of (a) 0% Mass loss, (b) 33% Mass loss, (c) 66% Mass loss, (d) 100% Mass loss, (e) alginate control, (f) excipient control, (g) product control, (h) Evolution of alginate breakdown products' fractions at different mass loss stages and (i) mass loss and molecular weight of alginate breakdown profiles over 24 hours degradation period.

The LS/RI signals from the excipient control are quite broad and both signal masks and interferes with the signals of non-degraded alginate observed at both integration ranges (11.5 – 14.0 mins and 14.0 – 20.5 mins) for the product control and the 0% mass loss samples. This poses an experimental limitation in determining the molecular weight of the non-degraded alginate component of the 0%- mass loss sample. However, the absence of the non-degraded alginate in the 0% mass loss sample is confirmed by the absence of the UV signal, which can also be observed in all the controls, Figure 3 (e-g). As a result, it can be stated that the alginate fractions of the 0% mass loss samples were intact. Therefore, the molecular weight of the alginate control (Fraction 1 and 2) can be ascribed to the alginate fractions of 0% mass loss sample for the comparative analysis of the other mass loss samples. Figure 3 (b-d) shows the fractograms of the 33%, 66% and 100% mass loss samples. It can be observed that the UV signal for fraction 1 shifts (large MW species) from lower to higher retention time: 14.5 mins, 16 mins and 17 mins for the 33%, 66% and 100% mass loss samples respectively, indicating the gradual reduction in the molecular weight of the alginate breakdown products. The respective molecular weights of the large MW alginate fraction (Fraction 1) for the 33%, 66%, and 100% mass loss samples showed a gradual reduction in molecular weight: 58.7 ±0.6kDa, 40.7±1.2kDa, and 28±0kDa, whereas the low MW fraction (Fraction 2) of all degraded samples (33%-100% mass loss) were less than 20kDa. The molecular weight analysis of alginate breakdown products offers insight into the probable biological fate in terms of the renal excretion of alginate breakdown products of the resorbable alginate microspheres. Duncan et al, have demonstrated that the molecular weight threshold for the alginate breakdown products to be renal excreted is below 48 kDa [21]. The MW analysis of alginate breakdown shows that within two hours the average

molecular weight has been reduced to the renal excretion threshold limit (< 48 kDa) and reduced to 28 kDa by 24 hours as summarised in Fig 3(i).

3.4 Haemolytic index of resorbable alginate microspheres of resorbable alginate microspheres

To further understand the degradation mechanism of resorbable microspheres under physiological conditions, the haemolytic assay was performed to evaluate the hemocompatibility of resorbable microspheres both in direct and indirect form, as shown in Table 5. Based on the haemolytic index, the resorbable microspheres are considered as non-haemolytic.

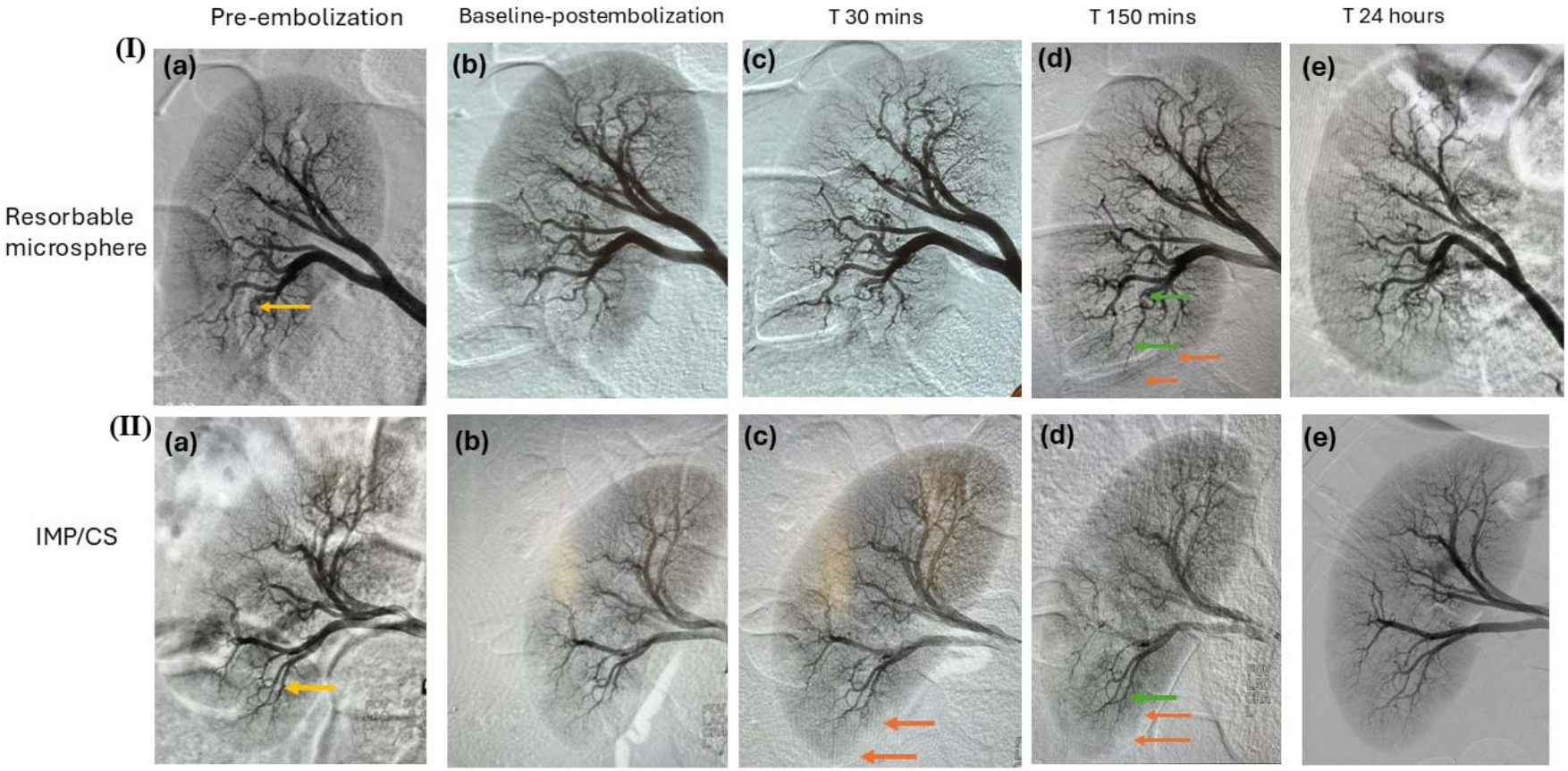
Table 5: Haemolytic index of resorbable alginate microspheres

Samples	Haemolytic index	
	Direct	Indirect
Positive control	100	100
Resorbable alginate microspheres (Test)	0.46± 0.81	1.25±0.54
Negative control	-1.25 ± 0.46	-0.88±0.98

3.5 Porcine renal arteries embolization

The representative DSA images comparing the embolization with resorbable alginate microspheres and IMP/CS, and their reperfusion times are given in Fig 5 (I and II). Here, the reperfusion time *in vivo* is defined as the time required for the complete reperfusion of the parent or main targeted arteries. The renal embolization sites for resorbable microspheres and IMP/CS are shown in Fig 5I(a) and 5 II (a) respectively (denoted by yellow colour arrows). The main or parent arteries were reperfused at 150 minutes for both resorbable

microspheres and IMP-CS (indicated by green arrows) whereas the grey blush area representing the more distal and smaller capillary network was partially reperfused (denoted by orange colour arrows). At 24 hours, complete reperfusion of all the embolized vessels can be observed for resorbable microspheres and IMP/CS antibiotic particles. These results indicate the similarity of the IMP/CS particles and resorbable microspheres in occlusion reperfusion times. The range of reperfusion times of the resorbable microsphere was compared with IMP-CS as shown in Fig 5 (III). In addition, the *in vivo* reperfusion time of RM was compared to the *in vitro* degradation time. It has been observed that the reperfusion time of RM is in the range of 130-210 minutes and was found to be statistically similar to IMP-CS reperfusion time range of 60-247 minutes. Also, *in vitro* degradation time of resorbable microsphere was found to be statistically similar to *in vivo* degradation time. This demonstrates that the method employed to evaluate the *in vitro* degradation simulates the *in vivo* reperfusion trends/conditions, thus it can be used to predict the degradation of resorbable microspheres.


Pre-embolization
Baseline-postembolization
T 30 mins
T 150 mins
T 24 hours
(I)
(a)
(b)
(c)
(d)
(e)
Resorbable microsphere
(II)
(a)
(b)
(c)
(d)
(e)
IMP/CS

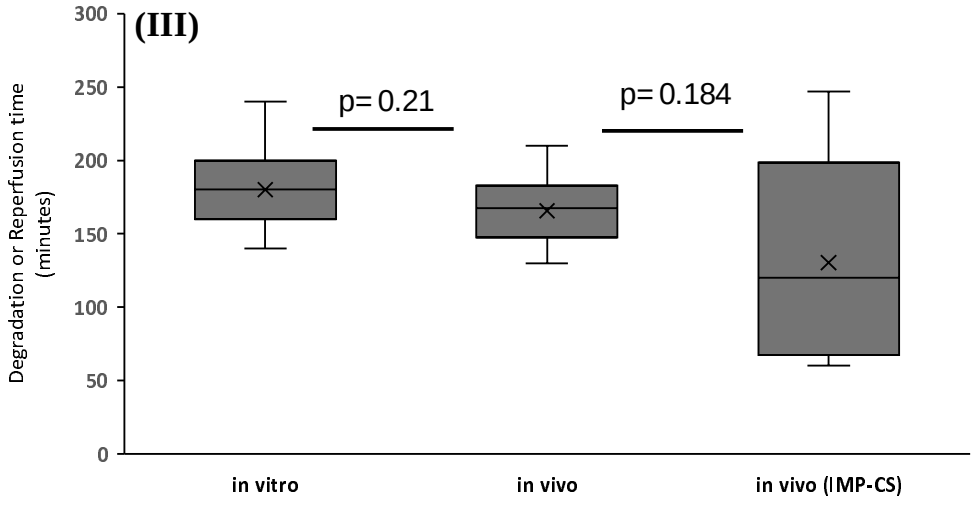


Figure 5: Representative of angiography images showing the occlusion and reperfusion of renal arteries in a porcine animal over 24 hours using (I) Resorbable alginate microsphere and (II) IMP-CS as embolic agents. (III) Comparison of *in vivo* reperfusion time and in vitro degradation time of resorbable microspheres (0.0875U) and IMP. (Site of embolization is denoted by yellow arrows, reperfusion of main or parent arteries is denoted by green arrows and grey blush is denoted by orange arrows).

Representative histology images of renal tissue embolized by resorbable alginate microspheres and IMP/CS are shown in Fig 6. In day 1 histology, traces of resorbable embolic microsphere material were found (indicated by an arrow) as shown in Fig 6 (A).

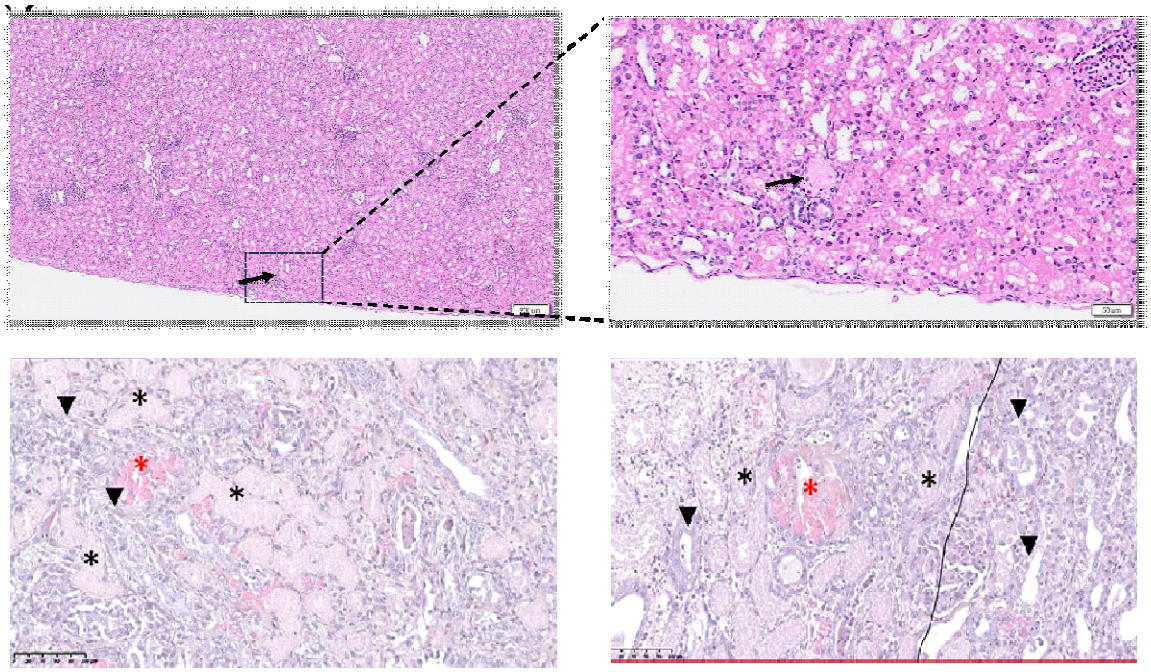


Figure 6: Representative histology images of renal tissue treated with resorbable microspheres after (A) 24-hour (with a higher magnification image on the right), (B) 72-hour, and (C) IMP-CS after 72-hour survival periods. (Denotations: Renal tubular necrosis*, glomerular necrosis* and regeneration▼)

No trace of embolic material was observed on day 3 histology as shown in Fig 6 (B). Likewise, the same observation can be made for the day 3 histology of IMP/CS treated kidney as shown in Fig 6 (C). The day 3 histology of resorbable microspheres shows ischemic lesions with renal tubular necrosis (stars), characterized by renal tubules with few epithelial cells and a homogeneous pink color and glomerulus necrosis (denoted by red stars). Close to these necrotic tubules, tubules under regeneration are observed (denoted by triangles). These tubules have more mitotic epithelial cells indicating the regeneration of renal tissues. The same observation can also be made from the day 3 histology of IMP-CS treated kidney.

4 Discussion

Resorbable alginate microspheres used for the transient embolization of blood vessels to create recoverable ischemic necrosis must be prepared through a process that ensures stability at the point of care for the clinical applications. Both the rate and extent of degradation should be controlled to ensure timely excretion of breakdown products from the body to ensure the ensemble is suitable for clinical application. Leslie *et al* previously demonstrated the preparation of alginate lyase-loaded alginate microspheres, however, several drawbacks of their system render their clinical use as an embolic agent untenable [12]. For instance, no attempts were made to inactivate the enzyme to enable the long-term storage of the alginate microspheres until needed at the point of clinical care. Additionally, the feasibility of preparing microspheres over a prolonged time to facilitate adequate manufacturing throughput was not addressed. Kunjukunju *et al* demonstrated the loading of native or an aggregated form of alginate lyase in alginate microspheres [22]. They also adopted the use of a freeze-drying method (albeit without the use of any excipients or cryoprotectants) to preserve the microspheres for the release of biologics. In their study however, they failed to disclose if the microspheres fully recovered their shape and size after reconstitution; this is an important prerequisite for embolic microspheres, as they must flow through a narrow lumen microcatheter without clumping or blockage during delivery but must subsequently embolize blood vessels predictably and efficiently. The present work used a proprietary excipient mixture, alginate and alginate lyase dissolved in the acetate buffer of pH 2.8. This approach not only reversibly inhibits the alginate lyase activity during the synthesis of microspheres, enabling efficient enzyme encapsulation, but also provides stability to the alginate matrix during the lyophilization process to prevent the collapse of the structure. This process produces an elegant lyophilized cake containing

resorbable microspheres which can be sterilized using gamma-radiation to achieve a sterility assurance level (SAL) of 10^{-6} as required for a medical device. The microspheres can be constituted using saline in less than 2 minutes, offering ease of use for clinicians that fits within their existing clinical workflow. Compared to the existing literature, the method of preparing alginate lyase-loaded calcium ion-crosslinked alginate microsphere is very unique and results in a product well-suited for clinical applications.

This study employed an *in vitro* degradation method which is predictive of *in vivo* conditions, allowing the characterisation of resorbable alginate microspheres throughout the degradation period, including visualisation, mass loss and molecular weight distribution. For simulating *in vivo* conditions, it is important to first understand the degradation mechanism of the alginate lyase loaded alginate microspheres. The degradation of alginate lyase-loaded alginate beads depends on two factors (i) the catalytic cleavage of glycosidic bonds of the alginate by the alginate lyase enzyme at physiological pH and temperature which makes the Ca^{2+} ion crosslinks susceptible to ion exchange with the ions present in the surrounding body fluid; this is followed by (ii) the Ca^{2+} ion outflux through ion-exchange from the weakened hydrogel network leading to the dissolution or erosion of alginate matrix releasing soluble alginate-breakdown products [12]. This process results in the complete degradation (catalytic cleavage activity and dissolution) of microspheres. Leslie *et al*, also showed that the alginate microspheres without encapsulated alginate lyase remain intact under in vitro and in vivo conditions This demonstrates that alginate lyase degradation activity is a pre-requisite to catalytically cleave the alginate polymer chains and the ion-exchange is required for the dissolution of the alginate microspheres. This indicates that as long as the microspheres are suspended in the appropriate medium, the enzyme will continuously cleave the alginate matrix, but the disintegration of microspheres may not

occur in the limited ion-exchange conditions. In the *in vitro* degradation method described herein, there is no biological sink or copious amount of fluid used that would enable sufficient ion-exchange to fully disintegrate the alginate microspheres into their constituent chains. Although excess fluid could be used to mimic the dynamic bodily ion-exchange, but it would dilute the breakdown product, making the molecular weight analysis through SEC-MALS more challenging.

It has been demonstrated that physiological concentrations of PO_4^{3-} and Na^+ ions play a prime role as ion exchangers which affect the disintegration of calcium-ion crosslinked alginate beads [23]. To mimic this aspect, the physiological concentration of these ions was incorporated into the HEPES buffer. However, the effect of ion exchange also depends on the volume of the buffer. With the use of limited buffer volume, the disintegration of microspheres occurs to a certain extent, but it cannot fully mimic ion-exchange conditions offered by the abundance of fluids present in the body. To mimic bodily ion-exchange conditions for the complete dissolution/disintegration of alginate breakdown products into its constituent chains, a 0.2M sodium citrate was added after the completion of the degradation period and the enzyme inactivation step. This completely disperses the breakdown into a soluble form by chelating calcium crosslinks and produces alginate breakdown products in a concentrated form, which can be subjected to the molecular weight analysis through SEC MALS without blocking the columns, thereby facilitating the prediction of the biological fate of alginate breakdown products. Through these approaches, the elements responsible for *in vivo* degradation of microspheres have been incorporated to establish the simulated nature of *in vitro* degradation test method.

Using these *in vitro* conditions, the resorbable alginate microspheres were characterized for degradation and molecular weight analysis. The outcomes of *in vitro* degradation method

were compared with the *in vivo* results from the swine kidney reperfusion model and showed a good in vitro in vivo correlation (IVIVC), validating the appropriateness of the *in vitro* degradation test in predicting *in vivo* performance.

The IMP-CS antibiotic mixture has been widely used as an off-labelled embolic agent for MSK pain relief applications [24]. The reported literature shows that an occlusion time of 1-4 hours is effective in inducing necrosis of neovessels[425]. Therefore, the present work targeted a similar embolization time by investigating the incorporation of different enzyme levels (0.0875U and 0.15U) of alginate lyase in the alginate microspheres. It can be observed in Fig 1 (a) and (b) that the degradation rate of alginate microspheres increases with the increase in the enzyme content. A slower degrading alginate resorbable microsphere was considered preferable to ensure the induction of sufficient ischemic necrosis of the neovessels in the joint space. Thus, 0.0875U of alginate lyase in the resorbable alginate beads was selected over the rapidly degrading 0.15U alginate lyase beads. The degradation time of the slow-degrading microspheres is also more closely aligned to the upper end of the IMP-CS resorption time in vivo.

The physical manifestation of the mass loss in terms of the reduction of particle size is likely to occur through a combination of the microsphere weakening and becoming less dense as the alginate crosslinking is reduced, which is followed by the rapid disintegration of the microsphere structure *via* ion exchange [12]. In the degradation process, the polymer chains of the alginate microsphere are catalytically cleaved by alginate lyase. This weakens the alginate matrix causing some microspheres to collapse into degradants. Evidence of the disintegration into particles of small size during mass loss, can be seen in Figure 1 (c), (d) and SI:4. The relatively sparse presence of degradants in the images taken suggests they

have a very transient existence relative to the overall lifespan of the microsphere. At 20µm size, there is a 100-fold increase in surface area (by comparison to the surface area of a nominal 200µm microsphere) which increases potential for ion exchange and rapidly leads to disintegration/dissolution of the structure.

In the molecular weight analysis of alginate breakdown products/degradants (Figure 3 (i), it can be observed that within 2 hours (at 66% mass loss) the average molecular weight of the larger fractions has been reduced below the threshold considered necessary for renal clearance from the body (<48kDa)[21] and by 24 hours (at 100% mass loss) the molecular weight is 28kDa. These results indicate that even though the microspheres look intact in 66% mass loss, individual chains of low molecular weight are held together by calcium ions. This supports the fact that although alginate lyase catalytically degrades the alginate matrix, complete disintegration of microspheres requires ion-exchange from the surrounding fluids; here, it is provided by HEPES buffer and sodium citrate in the in vitro degradation method employed. The SEC-MALS analysis allows us to look in more detail at the MW distribution of the alginate by analysis of two consecutive fractions that cover the distribution of the alginate, shown in Figure 3(h). The native alginate (Alginate Control Sample) has a small amount of a high MW fraction (32% of a 124.3± 5kDa, fraction 1), together with a major component of a lower MW fraction (68% of a 46.7± 1.5kDa, fraction 2). This is rapidly cleaved by the enzyme and within 39 minutes (33% mass loss) reduces the size of fraction 1 to 59kDa and fraction 2 to 17kDa. Within 2 hours (66% mass loss) the molecular weight of fraction 1 is further reduced to 41kDa and a small quantity (1%) of fraction 2 is detected at 4kDa. At this point, the alginate is below the size required for elimination by renal excretion. By 24 hours the majority (97%) of the alginate has a MW of 28kDa with a small fraction (3%) of < 5kDa. This analysis helps in relating the degradation time to the molecular weight of the

alginate breakdown products and not strictly to the size of the microspheres- i.e. larger partially degraded particles are composed of alginate chains of low molecular weight due to the alginate lyase catalytic activity but remain intact by ionic crosslinks. The resorbable alginate microsphere is used as an embolizing agent. Under *in vivo* conditions, it is difficult to evaluate the mechanism by which an embolic agent forms an occlusion in the blood vessel. The IMP-CS on incubating with the blood prevents the formation of clot and gets completely dissolved in the blood as shown in Figure 4 (c). So, the IMP-CS particles create a temporary physical barrier to create an occlusion and become dissolved without directly promoting thrombus formation. Thus, it also acts as an antithrombic agent and potentially can cause severe adverse events by altering haemostasis. Therefore, these results provide further motivation to avoid the intra-arterial injection of IMP-CS antibiotics for its use as an off-labelled resorbable embolic agent. On the other hand, the resorbable alginate microsphere on incubating with blood did prevent some clot formation whereas the alginate microsphere (without lyase) showed the strong clot formation (Fig 4(C)). Furthermore, the alginate lyase alone did not affect the clot formation shown in Fig 4(d). This indicates that the partial clot formation has likely occurred due to the degrading microsphere which might not allow the stable clot formation. However, under in vivo conditions, the dissolution rate would be higher due to the dynamic ion exchange. So, when the resorbable alginate microspheres are injected into the blood stream, the degrading microsphere may not provide a stable substrate to promote clot formation, but it can form an occlusion by creating a physical barrier at the site of injection. The haemolysis potential of the resorbable alginate microspheres is confirmed by the haemolytic index shown in Table 3. The haemolytic index of the test material in both direct contact method and indirect contact method was within 0 to 2, which is considered non-haemolytic under the

experimental conditions employed using this validated ISO test. These results indicate that alginate-resorbable microspheres have a low propensity for thrombus formation in vivo.

In the renal kidney embolization model, the embolization was induced by intra-arterial injection of resorbable alginate microsphere and IMP-CS as shown in Fig5 I(b) and II (b) respectively. The parent arteries reperfused at the same time for both the embolic agents (Fig 5 I (c) and II (c)), around 150 minutes and a complete reperfusion the distal capillary blush was observed at 24 hours. The range of reperfusion times of resorbable microsphere and IMP-CS were comparable as shown in Fig 5 (III). The variation in the reperfusion times of resorbable microsphere is affected largely by the anatomy of arteries and the depth of the embolization. The narrower blood vessels and distal embolization with resorbable alginate microspheres increase the reperfusion time due to the limited availability of fluid to drive the ion-exchange effect. In the case of IMP-CS, the variation in the reperfusion time is quite broad due to the polydisperse nature of IMP-CS particle. This makes it difficult to inject IMP-CS at the target site in a controlled and repetitive manner, leading to variation in the resorption rate of the particles in the blood. Furthermore, the in vivo reperfusion time of resorbable alginate microspheres was compared with the in vitro degradation time to determine the predictability of the microsphere degradation through in vitro method (Fig 5 III) . The degradation time under *in vivo* and *in vitro* conditions for microspheres was shown to be statistically similar. Thus, it can be said that there is a good correlation between the in vitro degradation method and the *in vivo* conditions.

Through angiography, it is difficult to determine if no material is left at the site of the embolization and thus this must be confirmed by histology. The gross histology provided only scant evidence of suspected resorbable embolic material at 24 hours. No arterial

luminal occlusion was observed, and the unaltered glomerulus is shown in Fig 6 (a). At day 3, no resorbable alginate microsphere embolic material was observed in any sections studies, though necrotic and regenerative tissues were observed, as shown in Fig 6(b). In Fig 6(c), the day 3 histology of IMP-CS treated kidney showed histological observations similar to those of the resorbable alginate microspheres. These results indicate that the majority of the degraded alginate microspheres are expected to be really excreted within 24 hours, with the complete excretion occurring between 24 and 72 hours. The absence of an intact microsphere at 24 hours can be directly related to the MW of alginate breakdown products of the 100% mass loss sample, which contained no intact microsphere but only degradants of size of less than 20 microns. The molecular weights of alginate breakdown products of the 100% mass loss sample were less than the renal excretion MW threshold of less than 48 kDa. The rare suspected alginate-embolic material seen in the histology at 24 hours would be composed of alginate fragments of size less than 48 kDa as the size of the particles (< 50 um) is less than the largest microsphere (around 220 um) observed at 66% mass loss sample, which degraded over 3 days through ion-exchange.

5 Conclusion

A resorbable calcium-crosslinked alginate lyase loaded-alginate microsphere was developed successfully as an embolic agent for MSK pain relief applications. A robust and scalable method was developed to encapsulate the enzyme and produce a gamma sterilized and rehydratable freeze-dried resorbable alginate microspheres. Controlled degradation was achieved under physiological conditions by incorporating alginate lyase into the alginate microspheres. A simulated *in vitro* test method was established and used to predict the degradation rate of microspheres and the molecular weight of the alginate breakdown

products to determine their biological fate in vivo. SEC-MALS studies demonstrated that the molecular weight of alginate breakdown products after a 24-hour degradation period was below the renal excretion threshold molecular weight. The alginate microspheres were found to be non-haemolytic. *In vivo* studies demonstrated that the reperfusion time of resorbable alginate microspheres in a porcine renal embolisation model was similar to that of IMP-CS. Embolization with resorbable alginate microspheres was as efficient as IMP-CS in inducing recoverable ischemic necrosis. These findings provide strong evidence for the suitability of the resorbable alginate microspheres for transarterial embolization. We will also be reporting on the long-term safety of these materials and the first in human experience in treating patients with pain arising from knee osteoarthritis.

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8 Conflict of Interest statement

Liam Farrissey reports financial support was provided by Disruptive Technologies Innovation Fund (Grant number: 174147), Enterprises Ireland. Liam Farrissey reports a relationship with CrannMed

Ltd that includes: board membership and employment. Liam and Sankalp inventors have patent #US 12083242 issued to CrannMed Ltd