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Ion-responsive alginate based macroporous injectable hydrogel scaffolds prepared by emulsion templating
Ion-responsive alginate based macroporous injectable hydrogel scaffolds prepared by emulsion templating†

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Ion-responsive biocompatible macroporous hydrogels with a well-defined highly interconnected open porous structure were synthesised using oil-in-water (o/w) high internal phase emulsion (HIPE) templating. Methacrylate-modified alginate was crosslinked in the continuous minority water phase and the oil internal phase removed to produce macroporous hydrogel monoliths. The physical dimensions, pore and pore throat size as well as water uptake of the alginate polyHIPE hydrogel can be controllably tuned by ion-responsive behaviour towards Ca²⁺ ions. The ion crosslinks formed are fully reversible and be dissolved using sodium citrate to remove Ca²⁺ ions through chelation. The polyHIPE hydrogels possess mechanical properties with storage moduli up to 20 kPa and are biocompatible as shown by cytotoxicity assays. The hydrogel can be extruded through a hypodermic needle causing it to break into small pieces (about 1 to 3 mm in diameter) while retaining the interconnected pore morphology after injection. Furthermore, these hydrogel fragments can be reformed into a coherent scaffold under mild conditions using an alginate solution containing Ca²⁺ ions.

Introduction

Alginate is a particularly attractive naturally derived ion-responsive polysaccharide, which has been studied widely for its biocompatibility, biodegradability and reversible aqueous gelation chemistry with di- or trivalent cations.1-3 Applications include adsorbents for removal of heavy metals from contaminated environments,4-6 scaffolds for tissue engineering,7-9 wound dressing10,11 and as delivery vehicles for drugs.12,13 Alginate is a block copolymer composed of (1→4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units).12 Divalent cations such as Ca²⁺ ions cooperatively bind between the G-units of adjacent alginate chains, creating ionic interchain links, which cause gelation of aqueous alginate solutions.14-15 The typical level of Ca²⁺ concentration in the human body is 1.8 mM16,17 the Ca²⁺ concentration in a human knee joint however is equivalent to 4.0 mM CaCl₂,18 so that the Ca²⁺ needed for gelation of alginate solutions could be supplied directly by the human body. Thus the Ca²⁺-induced gelling of alginate under very mild aqueous conditions makes alginate a very attractive stimuli-responsive material for use in vivo.14,15

Ion-responsive hydrogels that have been studied previously include poly(acrylic acid-co-2-hydroxyethylmethacrylate),19 poly(methacrylic acid-co-acrylonitrile)20,21 and poly(acryl-amide-co-maleic acid).22 One of the simplest methods to prepare ion-responsive hydrogels is to polymerise ion-responsive water soluble monomers in an aqueous environment.20,21 Other ion-responsive hydrogels have been prepared from lyotropic surfactant phases19 and by crosslinking of nanofibres.22 Most of these hydrogels possess relatively low porosity (usually less than 60%) or have a poorly interconnected pore morphology, which limits their application as scaffolds for tissue engineering.19,22

Scaffolds for tissue engineering offer physical support as well as a 3-dimensional (3D) environment to create living tissue through cell proliferation to regenerate, repair or replace biological functions.23,24 These highly porous matrices are used to create tissue in vitro or in vivo to repair or replace damaged organs.25,26 Interconnectivity of pores in scaffolds is necessary and important for nutrient and waste diffusion to allow cell proliferation.23,24

As high internal phase emulsions (HIPE) can be used to produce highly interconnected porous materials, they have been studied extensively to prepare scaffolds for tissue engineering in recent years.27-29 HIPEs are defined as emulsions with a dispersed or internal phase volume ratio of 0.74 or greater.30,31 If monomers in the continuous phase of a HIPE are polymerised, the emulsion droplets act as template for pores and once removed (usually through solvent drying or extraction) result in a macroporous polymer foam characterised by a final
high porosity in the range 70% to 90+%.\textsuperscript{40} Besides the high porosity, polyHIPEs have regular interconnected pores with pore sizes ranging from several micrometres to several hundred micrometres,\textsuperscript{32,34,42,43} which makes them potential candidates for the fabrication of tissue engineering scaffolds.

In tissue engineering, constructs of living cells can be formed either by seeding cells onto a preformed scaffold or by injection of a solidifiable porous scaffold together with a cell mixture into the tissue to be regenerated or repaired.\textsuperscript{23,24} Compared with preformed scaffolds, injectable scaffolds possess many attractive features from a clinical perspective, such as minimising the cost of treatment, patient discomfort, risk of infection and scar formation.\textsuperscript{26,44} In addition, cells, drugs or growth factors can be loaded quite simply into the scaffold by mixing prior to injection.\textsuperscript{26} Moreover, injectable scaffolds are capable of filling irregular defects, which is difficult to be achieved with preformed scaffolds.\textsuperscript{44} However, it is usually acknowledged that covalently crosslinked polyHIPEs are not injectable; therefore, most activities concerning the use of HIPE-templated macroporous polymers as scaffolds for tissue engineering concentrated on preformed scaffolds\textsuperscript{32–46} in contrast to our work reported here.

The preparation of Ca\textsuperscript{2+}-crosslinked alginate based polyHIPEs was reported,\textsuperscript{26,45} but the focus was on ionic crosslinking as substitute for the conventional covalent crosslinking used for polyHIPEs.\textsuperscript{46,47} Neither the ion-response behaviour nor injectability was investigated in any detail. Our approach to prepare ion-responsive alginate polyHIPEs is novel as the interconnected highly porous structure of polyHIPEs can be combined with the excellent biocompatibility and ionic cross-linking feature of alginate. Covalently crosslinked methacrylate-modified alginate polyHIPE hydrogels (MAPHs) were prepared. The covalent crosslinks give these hydrogels permanent porosity. Ion-responsiveness is achieved via additional ionic crosslinking of alginate segments, leading to hydrogels which can be swollen and shrunk controllably and reversibly while possessing a high degree of permanent porosity and a well-defined interconnected pore structure. Besides scaffolds for tissue engineering, other potential uses for these hydrogels are controlled drug release,\textsuperscript{48} microactuators,\textsuperscript{49} filtration or separation devices to remove heavy metal ions\textsuperscript{50} and generally biomedical applications requiring a responsive material which can change its dimensional shape controllably.

**Experimental**

**Materials**

The following chemicals were purchased from Sigma-Aldrich Company Ltd. (Poole, UK): alginate from brown algae (viscosity of 2% solution at 25 °C = 250 mPa s), methacrylic anhydride (94%), Triton X-405 solution (polyethylene glycol tert-octylphenyl ether, 70% in H\textsubscript{2}O), 2,2′-azobisisobutyronitrile (AIBN) (98%), glutaraldehyde (25%), sodium phosphate monobasic (99+%), sodium phosphate dibasic (99+%), sodium citrate (99+%) was obtained from Acros (Thermo Fisher Scientific Ltd., Loughborough, UK). Toluene (99+%), calcium chloride (94+%), sodium hydroxide (99+%), ethanol (99.7+) and 12-well BD Falcon™ Cell Culture Insert Companion Plates (BDH-brand, Cat. no. 353503) were purchased from WVR (WVR, Leics., UK). Cellulose Soxhlet extraction thimbles (inner diameter = 19 mm, outer length = 90 mm) were bought from Whatman (Whatman International Ltd. Maidstone, UK). A549 human type II tumour cells (A549) were obtained from American Type Culture Collection (ATCC CCL 183; American Type Culture Collection, Virginia, USA). Dulbecco’s Modified Eagle Medium (DMEM) was purchased from Invitrogen Ltd (Paisley, UK). Foetal Bovine Serum (FBS) was obtained from PAA Laboratories, (Somerset, UK). Osmium tetroxide aqueous solution (4%) was purchased from TAAB (TAAB Laboratories, Berkshire, UK). 1,1,1,3,3,3-Hexamethyldisilazane (HMDS) (98%) was obtained from Avocado (Avocado Research Chemical Ltd., Lancashire UK). Fresh pork muscle (dead) was purchased from local Sainsbury’s Supermarket Ltd (London, UK). WST-1 reagent was obtained from Roche Applied Science (Herts, UK). All materials were used as received.

**Synthesis of methacrylate-modified alginate**

The synthesis and characterisation of methacrylate-modified alginate followed the procedure reported by Smeds and Grinstaff.\textsuperscript{51} Briefly, after alginate (4.0 g) was dissolved in distilled water (200 ml) to make a 2.0% w/v solution, methacrylic anhydride (15 ml) was added in one portion at room temperature. The pH of the solution was adjusted to 8 by dropwise addition of concentrated aqueous NaOH (5.0 M). The pH of the solution was monitored by using a CyberScan pH 11 pH meter (Eutech Instruments, Singapore). The solution was incubated at 5 °C for 24 h. The polysaccharide was purified by precipitation into ethanol and subsequently washed with ethanol (about 1 l). The sample was dried in vacuum at room temperature for 3 days. The yield of methacrylated alginate was 3.0 g (74%).

The degree of substitution of methacrylate groups was determined by comparing the \textsuperscript{1}H-NMR spectra (in D\textsubscript{2}O) of alginate and methacrylate-modified alginate. \textsuperscript{1}H-NMR spectra were recorded on a Bruker DRX 400 (400 MHz) in D\textsubscript{2}O at room temperature. \textsuperscript{1}H-NMR spectra were processed using the software MestReNova (version 6.1.0). The relative integrations of the anomeric protons of the glucose ring of alginate at δ = 4.9 ppm to methacrylate proton peaks (methylene protons at δ = 6.1 and 5.7 ppm and the methyl group peak, δ = 1.9 ppm) were used to determine the degree of substitution, which was determined to be 1.00:0.44.

**Preparation of MAPHs**

Methacrylate-modified alginate (0.35 g) was dissolved in distilled water (5.0 ml) together with Triton X-405 (0.44 g) at room temperature to form the continuous HIPE phase. A solution of AIBN in toluene (1.0% w/v, 20 ml) was added under stirring (500 rpm) at room temperature over a 4 h period. The shear force necessary for dispersing the organic phase into the aqueous phase was provided by an IKA RW 20 Digital overhead stirrer (IKA Labortechnik, Germany) with a D-shaped PTFE paddle (width of paddle ~3.5 cm). An o/w HIPE, also called inverse HIPE (i-HIPE), was obtained after completion of the addition of the organic phase. The resulting i-HIPE was transferred to a soda glass
sample tube with 2.5 cm outside diameter to obtain samples for oscillatory mechanical measurements. The filling level of the emulsion (liquid) in the sample tube was 0.5 cm resulting in a cylindrical polyHIPE hydrogels with a diameter of about 22 mm and a height of 5 mm. Samples were cured in an oven at 60 °C for 24 h in air. The polyHIPE was Soxhlet-extracted with methanol for 24 h, then with acetone for a further 24 h. Finally, the MAPH were dried in an oven at 60 °C for 24 h. It was immersed in distilled water (100 ml) for 6 h to reach its equilibrium level of swelling (determined by observing the dimensional change visually) and then freeze dried for 2 days.

**Ionic crosslinking of MAPHs**

A piece of freeze-dried MAPH (diameter ≈ 22 mm, thickness ≈ 5 mm) was equilibrated in 100 ml distilled water for 6 h. The hydrogel was taken out of the water and thoroughly shaken to remove excess water until no water could be visually observed on the surface of the hydrogel. MAPH was then soaked in 200 ml either 1.8 mM, 4 mM or 100 mM aqueous CaCl$_2$ solution for 24 h. The ionically crosslinked sample was soaked in about 100 ml distilled water for 2 h before being freeze dried for at least 3 days.

**De-crosslinking of ionically crosslinked MAPH**

A piece of freeze-dried ionically crosslinked MAPH (prepared as described in 2.4.1) was equilibrated in distilled water (100 ml) for 6 h. The hydrogel was taken out of the water and thoroughly shaken to remove excess water until no water could be visually observed on the surface of the sample. It was then soaked in 200 ml 100 mM sodium citrate aqueous solution for 24 h. The ionically de-crosslinked sample was soaked in distilled water (about 100 ml) for 2 h before freeze drying.

**Rheological characterisation of MAPHs**

Cylindrical MAPH with diameters of 22 mm and heights of 5 mm were used for oscillatory rheology. A TA Instruments AR1000 rheometer equipped with a 20 mm standard steel parallel plate was used to determine the storage modulus (G') and loss modulus (G'') of MAPH at room temperature. The frequencies scanned ranged from 20 to 120 rad s$^{-1}$. This method is similar to that used by Fernandez et al.$^{[a]}$ and Bajomo et al.$^{[a]}$ The ionically crosslinked MAPHs were prepared by soaking in 100 ml 100 mM aqueous CaCl$_2$ solution for 24 h. After ionic crosslinking, the hydrogel shrunk to 18 mm in diameter and to a height of 3 mm. The crosslinked hydrogels were then stored in distilled water (50 ml) at room temperature. Similarly, to disrupt the ionic crosslinking, the ionically crosslinked samples were soaked in aqueous sodium citrate solution (100 ml, 100 mM) for 24 h. The hydrogels recovered their initial physical dimensions of 22 mm in diameter and 5 mm in height. The resulting sample was washed with distilled water (50 ml) and stored by soaking in distilled water (50 ml) at room temperature.

**Equilibrium water uptake ratio of MAPH**

The water uptake of MAPH was determined gravimetrically. The weight of the dry sample was measured directly. The weight of swollen samples was measured by weighing MAPHs, which were equilibrated in 200 ml distilled water for 24 h after padding them dry individually using tissue paper. The water uptake ratio ($R_w$) was calculated as follows:

$$R_w = \frac{W_w - W_d}{W_d} \times 100\%$$

where $W_w$ is the weight of the hydrogel swollen/shrunk to equilibrium in distilled water and $W_d$ the weight of the freeze-dried hydrogel. The water uptake of MAPH was 8000 ± 250% w/w in the fully swollen state and 3000 ± 100% w/w in the fully shrunk state.

**Rate of shrinking and swelling of MAPH**

Cylindrical MAPH with a diameter of 21 mm were used for these experiments. A Sony W55 digital camera (in video mode) was used to record the shrinking behaviour (please see ESI†) of the samples in 100 ml 100 mM CaCl$_2$ and swelling behaviour in 100 ml 100 mM sodium citrate at room temperature. The diameter of the samples was measured from the video image every minute during shrinking and every 30 s during swelling. The dimensions of MAPHs were calibrated using a ruler placed next to the monolith in the video. After 3 h, the dimensions of the sample did not show any further changes in either swelling or shrinking.

**Injectability of crosslinked MAPH**

Several pieces (about 3 ml in volume) of MAPH prepared as described in Section 2.3 were immersed in distilled water for 24 h. The hydrogels were loaded into a 5 ml syringe (Becton, Dickinson U.K. Limited, Oxford, UK) and gently pressed through a hypodermic needle with an inner diameter of 1.1 mm into a round bottom flask containing about 10 ml distilled water. The resulting injected hydrogel fragments were lyophilised for 3 days prior to SEM characterisation. To demonstrate injectability, we injected some hydrogel MAPH into a diced piece of pork muscle (about 15 mm × 15 mm × 15 mm) at room temperature. In order to observe the injected hydrogel within the muscle by SEM, one part of the muscle was sliced away using a scalpel. The injected hydrogels were then lyophilised for 3 days together within the muscle for SEM characterisation.

**Reforming injected hydrogel scaffold MAPH**

Several pieces (about 3 ml in volume) of crosslinked MAPH obtained as described in Section 2.3 were immersed in distilled water to reach equilibrium and subsequently soaked for 24 h in 50 ml aqueous alginate solution employing individually two different alginate concentrations (0.2% w/v and 1.0% w/v). The hydrogel pieces were loaded into a 5 ml syringe and gently passed through a hypodermic needle (inner diameter = 1.1 mm) into a cellulose extraction thimble (inner diameter = 19 mm, outer length = 90 mm, Whatman International Ltd, Maidstone, UK). The injected polyHIPE hydrogel fragments were confined within the closed end of a Soxhlet thimble using a plastic syringe plunger (diameter = 1.3 cm), which was tightly fixed inside the thimble (acting as a shape template) with the
help of two plastic cable ties. The whole set-up was soaked in a 100 ml 50 mM aqueous CaCl₂ solution for 24 h to trigger the solidification of the alginate solution in these polyHIPE hydrogel fragments. After the Soxhlet thimble was peeled away from the sample, the injected polyHIPE hydrogel fragments had formed a single cylindrically shaped hydrogel monolith (diameter ≈ 13 mm, height ≈ 10 mm). This reforming procedure is illustrated in Fig. 1.

Cell culture and cytotoxicity assessment of MAPH

A piece of MAPH was cut into cubes of about 4 × 4 × 4 mm³ using a scalpel and subsequently sterilized under UV light for 90 min at 0.120 J and 80 W (BLX-254, Vilber Lourmat, France). The scaffold was immersed in DMEM supplemented with 10% FBS (2 ml per well) in a 12-well plate containing polyHIPE hydrogel MAPH and left in an incubator (37 °C, 5% CO₂) overnight. The following day, a suspension of A549 cell (2 µl, 1 × 10⁵ cells per µl) was seeded on top of the scaffolds and incubated at 37 °C, 5% CO₂. The culture medium (DMEM supplemented with 10% FBS) was changed every 2 days by removing and replacing the inserts.

On day 4, after the culture medium was removed, scaffolds were washed twice with phosphate buffer (1 : 5.25 of 0.2 M NaH₂PO₄ to 0.2 M Na₂HPO₄, pH 7.5) and fixed with 2.5% glutaraldehyde in phosphate buffer (as above) for 40 min at 37 °C. For the purpose of cell fixation, the phosphate buffer was then removed and replaced with phosphate buffer (as above) containing 1% osmium tetroxide and left at 37 °C for a further 1 h. Subsequently, the scaffolds were dehydrated by 3 sequential washes with ethanol (100%) for 5 min each and then incubated twice in HMDS for 5 min each.

SEM characterisation of MAPH and MAPH with entrapped cells after cell culture

The internal structure of MAPH and MAPH with entrapped cells after cell culture were studied by SEM (Hitachi S-3400N, Berkshire, UK). All samples were sputtered with gold (Edwards Pirani 501 sancosat) for 2 min in an argon atmosphere to guarantee sufficient electrical conductivity.

The pore size and pore throat size of those polyHIPE hydrogels were determined from SEM images using Image-Pro Plus 6.0. Several SEM micrographs of the specimens were taken and more than 100 pores and pore throats were measured to determine range of pore and pore throat sizes.

WST-1 assay for A549 cell proliferation in MAPH scaffolds

4 days after A549 cells were seeded on MAPH, 2 ml fresh culture medium (DMEM supplemented with 10% v/v FBS) containing 5% v/v of WST-1 solution was added to the well containing MAPH. A well containing only A549 cells in medium without scaffold and a well containing only medium without A549 cells and scaffolds were used as positive and negative controls, respectively. After 2 h, 100 µl of the medium from each well was transferred to a 96-well plate and sampled using a BioRad micro-plate reader (Model 3550; Global Medical Instrumentation Inc., MN, USA) with the test filter at 405 nm and the reference filter at 650 nm.

Results and discussion

Covalent crosslinking is required in our i-HIPE approach to achieve macroporous polyHIPE hydrogels with permanent porosity. The resulting macroporous hydrogel monolith should possess dimensional stability even after removal of the oil phase template. We therefore chose to turn alginate itself into a crosslinker, which can be polymerised by radical chemistry. Polymerisable methacrylate groups were introduced through esterification of only a proportion of the available hydroxyl groups with methacrylic anhydride, which is possibly the simplest method of enabling the covalent crosslinking of alginate.⁵¹ ⁵⁴ The synthesis of methacrylate-modified alginate was carried out following an established procedure by Smeds et al. (Fig. 2).⁵¹ By controlling the degree of methacrylate substitution the methacrylate-modified alginate remains water soluble. In our case the degree of substitution was 44% (based on the ratio of glucose to methacrylate groups) as determined by ¹H-NMR, a toluene-in-water (i-)HIPE was used as template to prepare MAPHs. Compared with other water-soluble monomers, such as acrylic acid⁵⁶ or dextran,⁵⁷ used to prepare inverse polyHIPEs
from i-HIPEs, the alginate solution here was significantly more viscous. A compromise had to be found between the need for a high concentration of methacrylate-modified alginate to obtain a mechanically robust inverse polyHIPE on the one hand and a sufficiently low viscosity to be able to prepare a stable and homogeneous i-HIPE on the other hand. A combination of experience, trial and error allowed the identification of 7.0% w/v of methacrylate-modified alginate as suitable concentration for the continuous aqueous phase of the i-HIPE. Above this concentration, the viscosity of the aqueous continuous phase was too high for the overhead stirrer to produce a well-dispersed and homogeneous emulsion. Also the duration of the dropwise addition of the organic phase had to be extended from 30 min to 4 h (500 rpm, room temperature) in order to avoid phase inversion during emulsification. Any faster addition rates for the oil phase led to phase inversion. Once stable i-HIPEs were produced they were placed in an oven and polymerised at 60°C for 24 h. The crosslinked alginate polyHIPE was Soxhlet-extracted with methanol followed by acetone to remove the oil phase (toluene) and dried at 60°C for 24 h to remove any acetone. Finally the polyHIPE was re-swollen in deionised water (Fig. 2a & b).

Alginate can be ionically crosslinked in the presence of divalent cations, such as Ca^{2+}, to form stable hydrogels. A purely Ca^{2+}-crosslinked alginate based polyHIPE hydrogel has already been reported. In their study ionic crosslinking served solely as a non-covalent mode of crosslinking instead of the more conventional covalent approach and, therefore, no ion responsive behaviour of the hydrogel was reported.

In our work, the main purpose of introducing methacrylate functionalities into alginate was to allow the hydrogel to be crosslinked in two different ways: (i) covalently to maintain the macroporous polyHIPE microstructure generated by emulsion templating and (ii) ionically through formation of ionic crosslinking sites between alginate chains, which imparts the ability to respond to divalent or higher valent cations in an aqueous environment and consequently to allow us to control the external dimension and internal pore and pore throat size in the porous structure as well as the mechanical properties of macro-porous hydrogels. MAPH starts to shrink after immersing it into aqueous CaCl_2 solution with Ca^{2+} concentration ranging from 1.8 mM (the typical level of Ca^{2+} concentration in the human body) to 100 mM (to achieve high levels of cation induced shrinking). Upon exposure to Ca^{2+}, the hydrogel polyHIPE turned from translucent to pale white (Fig. 3). As shown in Fig. 3a, the diameter of the polyHIPE gradually decreased from 21 mm to 12 mm in a 100 mM CaCl_2 aqueous solution. A series of experiments indicated that, as long as there was excess Ca^{2+}, the final shrunk dimension of MAPH was constant independent from whether it had been immersed in 1.8 mM or 100 mM CaCl_2 solution. Before ionic crosslinking, the maximum water uptake of the just covalently crosslinked alginate polyHIPE hydrogel MAPH was approximately 8000% w/w (determined gravimetrically comparing the
weight of fully swollen hydrogel before and after freeze drying, meanwhile the water uptake of other porous alginate based materials ranged from 500% w/w to 3000% w/w.\textsuperscript{16–18} Consistent with the volume shrinkage during the ionic crosslinking, water was expelled from the hydrogel and the water content decreased to approximately 3000% w/w (Table 1).

Fig. 4 displays a juxtaposition of SEM images of MAPH before and after Ca\textsuperscript{2+}-crosslinking. The typical polyHIPE pore structure is clearly observed in all cases. Consistent with the volume shrinkage (50% in diameter), the pore sizes of the polyHIPE hydrogel, which were determined from the SEM images of the freeze dried hydrogels (in their swollen state in water before freeze drying), decreased from 14–31 μm (Fig. 4a) to 7–12 μm (Fig. 4b–d) after Ca\textsuperscript{2+}-crosslinking, Ca\textsuperscript{2+}-crosslinking also led to a similar decrease (about 50%) in the pore throat size (Table 1). The decrease in pore and pore throat size of MAPH is consistent with the change of the diameter change before and after ionic crosslinking. Interestingly the macroporous alginate hydrogel was a closed cell polyHIPE immediately after its preparation (Fig. 4a).

An interconnected pore structure was only produced gradually after the first sequence of a shrinking and swelling cycle. Further swelling-deswelling cycles increased the number of open cells and, therefore, the degree of interconnectivity. Though after 1 swelling-deswelling cycle, a certain amount of pore throats remained covered by a thin polymer film.

Sodium citrate was employed as a strong multi dentate chelator\textsuperscript{29} to remove the ionic crosslinks that formed after exposure of MAPH to a 100 mM aqueous CaCl\textsubscript{2} solution under mild conditions. It allows the complete removal of the Ca\textsuperscript{2+}-induced ionic crosslinks (100 mM sodium citrate). This was evidenced by the re-swelling of the MAPH monolith to its original size, which is defined by the covalently crosslinked alginate network.\textsuperscript{40,61} In these swelling experiments, the excess water from the Ca\textsuperscript{2+}-crosslinked monolith was firstly wiped off using tissue paper before the monolith was immersed in 50 ml 100 mM aqueous citrate solution. After about 3 h of being exposed to sodium citrate solution, its diameter finally recovered to 21 mm (Fig. 4b). The SEM image (Fig. 4f) confirms that also the pore sizes of the MAPH recovered from about 2–6 μm to its original dimension before Ca\textsuperscript{2+}-crosslinking. The swelling–deswelling cycle could be repeated more than three times without observing any volume loss or changes in colour or physical dimensions.

The results of the oscillatory mechanical measurements on cylindrical MAPHs are shown in Fig. 5. As prepared (i.e. before any shrinking–swelling cycle) the storage modulus of the MAPH was approximately 20 kPa. After the first exposure to Ca\textsuperscript{2+} ions, a

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**Table 1** Properties of cylindrical MAPH with 80% nominal pore volume in its fully swollen state and fully shrunken state

<table>
<thead>
<tr>
<th>MAPH</th>
<th>Pore size range\textsuperscript{e} (μm)</th>
<th>Pore throat size range\textsuperscript{e} (μm)</th>
<th>Water uptake\textsuperscript{a} (% w/w)</th>
<th>Diameter of MAPH monolith (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully swollen</td>
<td>14.4–31.6</td>
<td>2.5–12.3</td>
<td>8000 ± 250</td>
<td>2.1</td>
</tr>
<tr>
<td>Fully shrunken</td>
<td>6.8–11.8</td>
<td>1.9–5.9</td>
<td>3000 ± 100</td>
<td>1.2</td>
</tr>
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\textsuperscript{a} Pore size and pore throat sizes of the MAPH were determined from SEM micrographs. \textsuperscript{b} Determined by gravimetrically comparing the weight of the fully swollen MAPH with the freeze dried state.
sharp decrease in the shear storage modulus to 5 kPa was observed. The shear storage modulus remained at this value after subsequent swelling–shrinking cycles. It is evident from the SEM images that after 3 shrinking–swelling cycles, both in the shrunken (Fig. 4e) and in the swollen state (Fig. 4f), the pore walls became thinner compared to the as synthesised MAPH (Fig. 4a). The reason for the fourfold drop of the shear storage modulus after the first Ca\(^{2+}\)-crosslinking is that many of the thin films initially separating the pores in the polyHIPE ruptured. Once ruptured the more interconnected pore structure is mechanically weakened missing the stabilisation originally provided by the thin films. Another possible reason might be that the MAPH was initially conformationally strained and the first Ca\(^{2+}\)-crosslinking/Ca\(^{2+}\) sequestration process allowed for the relaxation of the alginate chains, leading to a lower modulus.

In order to assess the biocompatibility of the hydrogel, an in vitro cytotoxicity evaluation was performed by cultivating A549 human alveolar adenocarcinoma cells onto MAPHs. A549 cells are human pneumocyte-like cells derived from an alveolar cell carcinoma, which have previously been used as a model to study cell–material interactions.\(^{32,43}\) Extensive colonisation of cells was clearly observed on the surface of the porous hydrogel (Fig. 6a) and even inside of the hydrogel (Fig. 6b). Most cells had taken on squamous epitheloid morphologies. But compared with A549 cell size, the relative small pore throat diameter of the polyHIPE limited the penetration/migration of cells into the interior of the porous scaffold resulting in fewer cells to be found within the scaffold. However, judging from the rapid colonisation of the scaffolds by A549, and the typical squamous, epithelial morphologies, A549s were able to establish connections with the hydrophilic network of MAPH.

Cell proliferation was measured 4 days after A549 cell seeding, using the cell proliferation assay reagent WST-1, which is a colourimetric assay to evaluate metabolic activity, proliferation and viability of the cells.\(^{34,45}\) A well, containing only A549s in the medium without scaffolds and a well containing only medium without A549s or scaffolds were used as positive and negative controls, respectively. Increased cell proliferation of A549s grown in the MAPH scaffold was observed (Fig. 7). The cell proliferation on the hydrogel MAPH was found to be around 300% of that observed on the positive control material, which indicates MAPH offered a conducive environment for cell proliferation.

An injectable scaffold should be able to solidify in vivo after injection without causing any irritation to the surrounding tissue and possess a suitable interconnected 3D porous structure with sufficient mechanical strength to withstand different biomechanical loadings.\(^{28}\) A very simple experiment was conducted in order to establish if covalently crosslinked methacrylate-modified alginate polyHIPE hydrogel MAPH were injectable (i.e. retain pore size and pore morphology after injection and are able to fill defect sites in tissue). Several pieces of MAPH (diameter > 10 mm) were loaded into a syringe and gently squeezed through a hypodermic needle (inner diameter = 1.1 mm). As expected from a covalently crosslinked hydrogel, the gel particles broke up into smaller fragments during extrusion through the needle. The SEM images (Fig. 8a) of the extruded gel show that the diameter of the hydrogel fragments produced by extrusion of the polyHIPE gel through the needle ranged from 1–3 mm. Sizes greater than the needle diameter are a result of the very compliant nature of the macroporous and soft hydrogel, which can deform and be compressed reversibly upon exerting physical pressure. As is
explored; here we utilised the Ca\textsuperscript{2+}-responsive feature of the hydrogel fragments and hold them together. In an exploratory study MAPH was injected into a piece of fresh dead pork muscle (purchased from a local supermarket), which subsequently was freeze dried within the muscle. A pork muscle (purchased from a local supermarket), which was still clearly observable in the pork muscle (Fig. 8c & e). No gap could be observed at the boundary between the surrounding pork muscle and the hydrogel (Fig. 8d). This experiment demonstrated that MAPH is injectable and although fractured in the process produces an interconnected porous structure after injection.

Considering the possibility that the target application for injectable scaffolds is not to fill a confined defect, another potential approach of producing a monolithic structure from fragmented polyHIPE hydrogel particles after injection was explored; here we utilised the Ca\textsuperscript{2+}-responsive feature of the alginate. If a sufficient number of interparticle Ca\textsuperscript{2+} crosslinks could be introduced by exposure of the polyHIPE fragments to a CaCl\textsubscript{2} solution the MAPH should form a coherent monolithic scaffold after injection. However the simple injection of MAPH into Ca\textsuperscript{2+} solution did not lead to any significant interparticle crosslinking, only hydrogel fragments were found (Fig. 9d). As soon as the particle came in contact with the Ca\textsuperscript{2+} ions, they will crosslink at the surface of the individual particle, which causes them to shrink which did not seem to allow for sufficient interparticle crosslinks to produce a macroscopic polyHIPE hydrogel from polyHIPE fragments. There is a combined effect with particles constantly shrinking and crosslinking sites being used up intramolecularly, which makes the consolidation into a single monolithic particle very unlikely and other measures (confined environment/extra alginate) are needed.

In order to reform a hydrogel monolith from the extruded hydrogel fragments under relatively mild conditions, a solution of unmodified alginate was used as adhesive to bind hydrogel fragments together in the presence of Ca\textsuperscript{2+} ions (Fig. 9a & b). A cellulose Soxhlet thimble was chosen as the mould for forming a monolithic scaffold because the pores of the thimble material are small enough to offer confinement for the extruded particles but permeable to Ca\textsuperscript{2+} ions and alginate (Fig. 9c). MAPH hydrogel were first placed in aqueous alginate solution for 24 h to absorb “alginate glue”. Then it was loaded into a 5 ml syringe

Fig. 8 SEM images of MAPH after injection through the needle: (a & b) hydrogel passed through a needle with 1.1 mm inner diameter (a) low magnification, (b) high magnification; (c–e): hydrogel after being injected into a dead pork muscle.

Fig. 9 Attempts of reforming injected MAPH fragments by bringing the hydrogel particles into contact with either an aqueous Ca\textsuperscript{2+} solution alone or a combination of an aqueous Ca\textsuperscript{2+} solution plus extra alginate solution. (a & b) A schematic of using Ca\textsuperscript{2+} crosslinkable alginate to bond polyHIPE hydrogel fragments. (a) Aqueous alginate solution filling the interparticle space between polyHIPE hydrogel fragments; (b) aqueous alginate solution gelled in the presence of Ca\textsuperscript{2+} bonding polyHIPE hydrogel fragments together. (c) Reforming set-up composed by a syringe plunger and a cellulose extraction thimble; (d) MAPH being injected into a CaCl\textsubscript{2} solution (50 mM); (e) one piece of scaffold after the reforming step (the width of the spatula is 1.0 cm); (f) reformed scaffold immersed in distilled water; (g) the same hydrogel MAPH being injected into a thimble and soaking in CaCl\textsubscript{2} solution (50 mM) but without using alginate and Ca\textsuperscript{2+} as binder to stick the hydrogel fragments produced during extrusion together; (h & i) SEM pictures of the porous structure of a reformed scaffold.
and gently passed through a hypodermic needle into the cellulose extraction thimble. During this process the hydrogel was broken into small fragments. Then a plastic syringe plunger confined those fragments within the closed end of the cellulose thimble. Finally the whole set-up was soaked in CaCl₂ solution to trigger the solidification of the alginate solution in these polyHIPE hydrogel fragments. (Detailed experiment information can be found in experimental part: reforming injected hydrogel scaffold MAPH.) The alginate solution acted as adhesive which the CaCl₂ solution crosslinked and so the hydrogel monolith fragments were held together and formed one cylindrical piece of hydrogel (diameter = 13 mm, height = 10 mm) (Fig. 9e & f). When handling the reformed hydrogel, it seems to have a similar strength and toughness to the touch compared with the same polyHIPE hydrogel before injection. Increasing the concentration of alginate from 0.2 wt% to 1.0 wt% led to a slight enhancement of the mechanical performance. The reformed scaffold did maintain its shape in water (Fig. 9f) without breaking into the original polyHIPE hydrogel fragments after vigorously shaking the glass tube. In sharp contrast, only hydrogel fragments could be observed when the same hydrogel was injected into thimble and soaked in CaCl₂ solution but hydrogel fragments could be observed when the same hydrogel was injected into thimble and soaked in CaCl₂ solution without using an alginate solution as binder to hold together the individual hydrogel fragments produced during extrusion (Fig. 9g). Ca²⁺-crosslinked alginate thin films (Fig. 9h, dashed circle), which act as binder for the whole scaffold, could be clearly observed between individual hydrogel fragments. Meanwhile, the interconnected macroporous structure could be clearly observed throughout the reformed scaffold without significant filling of the pores and obvious pore size change compared with the polyHIPE before injection (Fig. 9i). When the concentration of alginate binder used was 1.0% w/v, Ca²⁺ crosslinked alginate films also could be observed inside some of the pores of MAPH and the pore walls became slightly thicker. However when the concentration of the alginate “adhesive” was decreased to 0.2% w/v, the no significant differences between the pore morphology of the original and reformed MAPHs.

Ca²⁺-crosslinked alginate is degradable in vivo within several days after implantation,⁶⁶,⁶⁷ which means these thin films will gradually disappear and, therefore, not affect the growth of cells loaded into the porous scaffolds before reforming or migrating into the scaffold from the surrounding tissue. It has also been reported that the mechanical strength of the cell-seeded scaffolds increases substantially with the continuous construction of living tissue,⁶⁸ which means the loss of alginate “adhesive” may be compensated for by the growing tissue, which therefore may not necessarily lead to an overall loss of mechanical strength of the scaffolds during cell growth.

Conclusions

Highly porous and biocompatible covalently crosslinked methacrylate-modified alginate polyHIPE hydrogels MAPH with a well-defined porous structure were prepared. The MAPH retained the known ability of alginate to be crosslinked ionically and it was demonstrated that the Ca²⁺ induced crosslinking is fully reversible under mild conditions using sodium citrate as chelating agent. Reversibility was shown for the external dimensions of a MAPH monolith as well as at the microscopic level where the changes in the pore and pore throat dimensions of the hydrogel directly reflected the dimensional changes observed macroscopically.

The controllable ion-responsive feature and well-controlled pore morphology makes this MAPH promising scaffolds for soft tissue engineering. Biocompatibility was demonstrated through successful proliferation of A549 cells in the presence of the scaffold. Other potential applications in bioseparation, drug delivery, as artificial muscles, biosensors, actuators and immobilization of enzymes and cells are attractive due to the reversible ion-responsive behaviour of the hydrogel.

We also demonstrated that MAPH maintained its pore structure after extrusion through a hypodermic needle and developed a solution based aqueous alginate binder system to allow individual particles to form a monolithic structure post injection (extrusion). This approach has the potential to become a generic and versatile method to produce porous hydrogels intended as injectable scaffolds for soft tissue engineering.

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Notes and references
