Alginate-polymethacrylate hybrid hydrogels for potential osteochondral tissue regeneration

P. Stagnaro[a, *]

paola.stagnaro@ge.ismac.cnr.it

I. Schizzi[a]

R. Utzeri[a]

E. Marsano[b]

M. Castellano[b, *]

maila.castellano@unige.it

[a]Istituto per lo Studio delle Macromolecole, Consiglio Nazionale delle Ricerche, Via De Marini 6, 16149 Genova, Italy

[b]Dipartimento di Chimica e Chimica Industriale, Università di Genova, Via Dodecaneso 31, 16146 Genova, Italy

*Corresponding authors.

Abstract

Porous scaffolds based on alginate-polymethacrylate hybrid hydrogels intended for bone and cartilage regeneration were prepared through controlled calcium ions diffusion from an agar mould. The double interconnected network of such materials combines into a single porous structure maintained by both noncovalent crosslinks (calcium ions for alginate) and covalent crosslinks (polymethacrylate crosslinked by the addition of mixtures of mono and bifunctional monomers). The alginate component ensures the appropriate micro-environment to mimic the extra-cellular matrix, whereas the polymethacrylate improves the mechanical performances of the hybrid hydrogels, helping to overcome the mechanical limitations of the alginate component. Morphological characterization and porosity analysis of the hybrid scaffolds were assessed by scanning electron microscopy and micro-computed tomography. Relative concentration and distribution of calcium ions were evaluated by atomic absorption and dispersive X-ray analysis, respectively. Uniaxial compressive mechanical tests were conducted to evaluate the compressive elastic modulus of the hybrid hydrogels that was correlated with their swelling ratio and crosslinking degree. As was envisaged a much higher modulus (about seven times) was obtained for the hybrid Alg/HE hydrogel than with alginate alone.

Chemical compounds studied in this article: Sodium calcium alginate (PubChem CID: 6850754); Calcium chloride (PubChem CID: 5284359); 2-Hydroxyethyl methacrylate (PubChem CID: 13360); Ethylene glycol dimethacrylate (PubChem CID: 7355)

Keywords: Sodium alginate; Hybrid hydrogels; Mechanical properties; Double network; Crosslinking degree

1 Introduction

For the two last decades, tissue engineering aimed at studying bone and cartilage regeneration has focused on self-supporting materials suitable to construct 3D porous structures, or scaffolds, which ensure long-term tissue regeneration through a controllable degradation rate (Hutmacher, 2000; Nooeaid, Salih, Beier, & Boccaccini, 2012; Rezwan, Chen, Blaker, & Boccaccini, 2006; Temenoff and Mikos, 2000; Wagoner Johnson & Herschler, 2011). To this purpose, an ideal scaffold should be not only biocompatible and bioresorbable, but, in order to efficaciously support in vitro and/or in vivo cell growth, it should mimic the extracellular matrix (ECM), thus facilitating cellular adhesion and expression, promoting vascularization, and favouring exchange of nutrients and growth factors (Coluccino, Stagnaro, Vassalli, Schizzi, & Scaglione, 2012; Coluccino, Stagnaro, Vassalli, & Scaglione, 2016; Malafaya, Gomes, Salgado, & Reis, 2003; Van Vlierberghe, Dubruel, & Schacht, 2011).

Besides these essential aspects, materials specifically destined to bone and cartilage regeneration have to bear physiological loads and stresses (Allan, Pilliar, Wang, Grynpas, & Kandel, 2007; Calvert, 2009; Hutmacher, 2000; Rezwan et al., 2006; Sun et al., 2012; Wagoner Johnson & Herschler, 2011).

In this framework, polymer-based materials have been increasingly investigated due to the unique possibility they offer of obtaining engineered scaffolds with: (i) adequate porosity and pore interconnection to allow both cell-mediated matrix deposition and vascularization; (ii) specific biodegradability/bioresorbability characteristics; (iii) suitable surface chemistry for cell adhesion and differentiation; (iv) tunable mechanical properties to match those of the tissues to be repaired (Dhadayuthapani, Yoshida, Maekawa, & Kumar, 2011; Hutmacher, 2000; Liu & Ma, 2004; Sachlos & Czernuszka, 2003). Indeed, degradable polymers, either natural or synthetic, such as polysaccharides, collagen, poly(lactic acid), polycaprolactone, etc., were used to prepare porous scaffolds for osteochondral regeneration (Abdel-Fattah, Jiang, El-Bassiouni, & Laurencin, 2007; Gloria, De Santis, & Ambrosio, 2010; Glowacki & Mizuno, 2008; Hutmacher, 2000; Liu & Ma, 2004; Park, Lih, Park, Joung, & Han, 2017; Park, Yu, Chun, Chun, & Kim, 2009).

In particular, hydrogels obtained from polymers, such as poly(ethylene glycol), agarose, alginate and chitosan, due to their capability of giving porous 3D constructs are very promising candidates to mimic the ECM natural environment supporting tissue regeneration (Chicatun et al., 2013; Chuah, Peck, Lau, Heec, & Wang, 2017; Coates, Riggin, & Fisher, 2013; Eslahi, Abdorahim, & Simchi, 2016; Van Vlierberghe et al., 2011).

Among the polysaccharides, alginic acid is a naturally abundant polysaccharide obtained from algae already exploited for various medical applications (Draget, Smidsrød, & Skjåk-Bræk, 2005; Pawar & Edgar, 2012). It possesses a copolymeric linear structure constituted by (1-4)-*beta-D*-mannuronic (M) and (1-4)-*alpha-L*-guluronic (G) acid residues arranged in M-rich blocks, G-rich blocks, and alternating or random sequences of M and G units (Pathak, Yun, Lee, & Paeng, 2010), depending on the specific natural source.

Sodium alginate, that is the water-soluble sodium salt of alginic acid, by partial replacement through ionic exchange of Na^+ counter ions with alkaline-earth cations, such as Ca^{2+} and Sr^{2+}, forms crosslinked structures (Mitchell & Blanshard, 1976; Pathak et al., 2010; Pawar & Edgar, 2012; Vicini, Castellano, Marsano, & Mauri, 2015; Vicini, Mauri, Wichert, & Castellano, 2017) where the physical crosslinks are the divalent cations chelated by the G-rich blocks. From sodium alginate grades with high content of G units, due to the strong interaction of the G-rich blocks with the above mentioned divalent cations, water-stable and relatively tough hydrogels can be easily achieved.

In a previous study, some of the authors realized highly porous alginate scaffolds monolithic in their structure, to avoid construct delamination, but bi-layered in terms of biochemical functionalization. In fact, the two layers were functionalized with specific biochemical signals for cell differentiation: namely, hydroxyapatite mineral phase and transforming growth factor TGF-beta1 for the bony and chondral layer, respectively (Coluccino et al., 2012; Coluccino et al., 2016).

However, the mechanical performance of engineered scaffolds only based on alginate hydrogels is often poor and inadequate to endow the final constructs with desirable elasticity and toughness (Bailey, Mitchell & Blanshard, 1977; Calvert, 2009; Sun et al., 2012). Hydrogels with enhanced toughness have been recently obtained by incorporating self-healing physical crosslinks in a chemically crosslinked gel network (Long, Mayumi, Creton, Narita & Hui, 2014; Sun et al., 2012).

In the present work, in order to overcome the mechanical behaviour limitations of alginate materials, scaffolds obtained from alginate-polymethacrylate hybrid hydrogels, which combine into a single porous structure both ionic (from calcium-alginate electrostatic interaction) and covalent (from methacrylate radical polymerization) crosslinks were prepared. Within the double interconnected network being formed the alginate portion would provide the appropriate micro-environment mimicking the ECM, whereas the methacrylate portion should improve the mechanical performances of the resultant hybrid hydrogels.

Morphological characterization, in terms of pore dimensions and their interconnectivity, was performed by scanning electron microscopy and micro-computed tomography. The mechanical performances of the hybrid hydrogels were evaluated by uniaxial compressive mechanical tests. Coupling the mechanical results with swelling measurements allowed to estimate both the polymer-solvent interaction parameter and the elastically effective degree of crosslinking, according to the classical elasticity theory (Flory, 1950; Flory, 1953).

2 Experimental

2.1 Materials

Sodium alginate used was Manugel GMB (FMC BioPolymer), a commercial grade, having a (1-4)-*alpha-L*-guluronic acid (G)/(1-4)-*beta-D*-mannuronic acid (M) ratio G/M ≥ 1.5 (density = 1.6 g/cm^3). $CaCl_2$ (Sigma-Aldrich, ≥ 97.0%, powder) was used as alginate crosslinker. Difco™ Noble Agar powder used for the realization of the well moulds was purchased by BD. Physiological saline (0.9% w/v NaCl) was used as aqueous medium. Methacrylate monomers, namely, 2-hydroxyethyl methacrylate, HEMA (Polysciences, 98%, density = 1.07 g/cm^3), ethylene glycol dimethacrylate, EGDMA (Sigma-Aldrich, 98%, density = 1.05 g/cm^3), and poly(ethylene glycol) 400 dimethacrylate, PEGDMA 400 (Polysciences, MW of PEG block = 400, n = ~ 9, density = 1.12 g/cm^3), when necessary, were purified before use on Sigma-Aldrich columns of basic alumina (30,631-2). Ammonium persulfate, $(NH_4)_2S_2O_8$ (Sigma-Aldrich, 98%), and *N,N,N′,N′*-tetramethylethylene diamine, TEMED (Sigma-Aldrich, 99.5%), respectively initiator and accelerant of the radical methacrylate polymerization, were used as received. All other chemicals used were of analytical grade.

2.2 Alginate hydrogels preparation

A mould of agar gel was previously prepared as follows: a proper amount of agar powder was dissolved in physiological saline enriched with $CaCl_2$ (0.5 or 0.7 M) in order to have an agar concentration of 1% w/w. After boiling

for 10 min, the hot agar solution was poured into a supporting plaque; properly shaped wells were formed by using copper cylinders (diameter ≈ 0.85 cm and length ≈ 1.5 cm) as moulds. The solution was left to cool and kept at room temperature until complete gelification (2 h).

Sodium alginate solution (2% w/w) was prepared by dissolving sodium alginate powder in physiological saline. This solution was poured in the agar wells prepared as described above; the system was then maintained in oven at 37 °C; after a gelification time of 4 or 8 h the so formed alginate hydrogel samples were demoulded from the agar wells (see Table 1).

Table 1 Composition of the alginate-based hydrogels.[a]

alt-text: Table 1

Sample	Ca^{2+} in agar (mol/L)	HEMA[b] (% w/w)	EGDMA[b] (% w/w)	PEGDMA[b] (% w/w)
Alg_05	0.5	–	–	–
Alg_07	0.7	–	–	–
Alg/HE[c]	0.5	4	4	–
Alg/HP[c]	0.5	4	–	4

[a] Sodium alginate 2% w/w in physiological saline.

[b] In physiological saline.

[c] $(NH_4)S_2O_8$ and TEMED, 2 and 0.5% w/w with respect to HEMA, respectively.

2.3 Hybrid alginate/polymethacrylate hydrogels preparation

Sodium alginate solution (2% w/w) in physiological saline, prepared as above described, was added with the proper amount of the monofunctional methacrylate monomer, HEMA, to have a concentration of methacrylate monomer of 4% w/w. The difunctional monomer, PEGDMA 400 or EGDMA, was then added in amount of 100% w/w with respect to the monofunctional methacrylate. Initiator, $(NH_4)S_2O_8$, and accelerator, TEMED, of radical polymerization were also added in quantities of 2 and 0.5% w/w with respect to the monofunctional methacrylate, respectively (see Table 1).

The obtained mixture was first homogenized by magnetic stirring, then centrifuged for 5 min at 3000 rpm to remove air, and finally placed in a Falc LBS1 sonicator bath at 50 kHz for about 10 min at 20 °C to favour methacrylate radical prepolymerization. The hybrid mixture so obtained was introduced in the agar wells and maintained for 4 h at 37 °C, as described above for the alginate hydrogels, to achieve ionic crosslinking while completing as much as possible methacrylate polymerization, thus accomplishing covalent crosslinking of the methacrylate portion (Fig. 1).

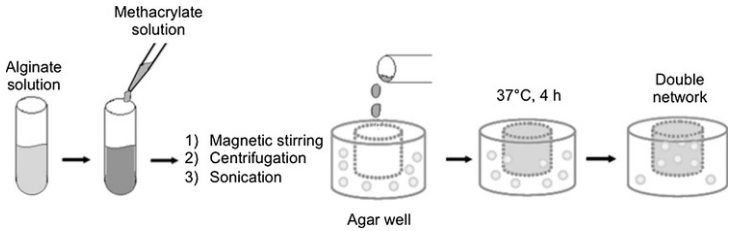

Fig. 1 Step procedure for the preparation of alginate-polymethacrylate hybrid hydrogels.

alt-text: Fig. 1

Hybrid hydrogel samples were then demoulded from the agar wells, and kept in an appropriate aqueous medium or freeze-dried, depending on the successive characterization they have to undergo.

2.4 Freeze-drying

The final highly porous 3D structure of the scaffolds was generated by freeze-drying using a Flexi-Dry instrument. To prevent structure collapsing, the hydrogels, after being removed from the agar moulds, were frozen at

−20 °C, kept at this temperature for at least 12 h, and then freeze-dried.

2.5 Calcium evaluation

A Variant Spectra AA55 B Atomic Absorption Spectrometer (AAS), operating with the internal standard methodology, was used to evaluate the content of calcium ions in the samples. These, after freeze-drying, were mineralized using a standard method (EPA Method 3050B, 1996), which involves a treatment with strong acids, namely, nitric acid (HNO_3, J.T. Baker, 65%) and hydrochloric acid (HCl, J.T. Baker, 37%).

2.6 Morphological analysis

Morphology and structure of the scaffolds were investigated by SEM (Scanning Electron Microscopy) performed on a Hitachi TM 3000 Benchtop SEM instrument operating at 15 kV acceleration voltage, equipped with a SwiftED3000 probe (Oxford Instruments) for EDX (Energy Dispersive X-ray) microanalysis.

Observations were carried out on fragile fractures (in liquid nitrogen) of freeze-dried samples sputter-coated with gold. For each sample the mean pore size was evaluated from 30 measurements on acquired SEM images. EDX mapping analysis was performed to determine calcium ions distribution on the longitudinal and cross-sections surfaces of the scaffold.

2.7 Micro-computed tomography

On a previously freeze-dried hybrid sample, pore morphology and ~~their~~ size distribution were determined by micro-computed tomography (micro-CT). The measurements were performed using a commercially available micro-CT (μCT 100, SCANCO Medical AG, Brüttisellen, Switzerland) cabinet scanner operating with a cone beam originating from a 5 μm focal-spot X-ray tube. The photons were detected by a CCD-based area detector and the projection data were computer-reconstructed into a 3072 × 3072 image matrix. The samples were segmented based on their grey scale values in the CT slices. The wall thickness was computed by the maximum fitted spheres method (Hildebrand & Rüegsegger, 1997). The pore diameters were then computed applying the same method on the inverse segmented image.

2.8 Swelling measurements

The as-prepared hydrogel samples were immersed in physiological saline, until the maximum swelling equilibrium was attained.

Hydrogel sorption properties were evaluated in terms of weight swelling ratio SW_w defined as follows:

$$SW_w = W_{swollen}/W_{dry} \quad \textbf{(1)}$$

where $W_{swollen}$ is the weight of the completely swollen sample and W_{dry} is the weight of the dry sample.

The SW_w value, for each sample, was averaged over five measurements.

From SW_w it is possible to calculate SW_v (volumetric swelling ratio), provided that the density of the solvent δ_1 and the polymer δ_2 are known, and assuming additivity of the volumes.

$$SW_v = \left[1 + \delta_2/\delta_1 \left(SW_w - 1\right)\right] \quad \textbf{(2)}$$

The polymer volume fraction, ϕ_2 variable necessary in the theoretical approach of the swelling phenomena, was obtained by:

$$\phi_2 = \frac{1}{SW_v} \quad \textbf{(3)}$$

The density of the hybrid systems was calculated as the volumetric average of the components.

Due to the low-crosslinking degree of the samples, it was acceptable to assume that the polymer density was the same in the network and in the uncrosslinked state.

Each value of the swelling ratios reported in Table 2 (see below) was the average of three measurements. The error was evaluated to be ± 3%.

Table 2 Weight (SW_W) and volumetric (SW_V) swelling ratio, compressive modulus (G), moles of elastic effective chains for unit of network dry volume (ν), and polymer/solvent interaction parameter (χ) for the alginate-based hydrogels.

alt-text: Table 2

Sample	SW_w	SW_v	G (kPa)	ν (mol/m^3)	χ
Alg_05_1	12.1	18.8	35	223	0.47
Alg_05_2	9.4	14.4	38	223	0.47
Alg_05_3	8.3	12.7	40	223	0.47
Alg/HE	7.1	7.7	245	765	0.49
Alg/HP	5.3	6.1	47	116	0.56

2.9 Uniaxial compressive mechanical tests and crosslinking degree evaluation

The evaluation of the compressive elastic modulus (G) of the prepared hydrogels was performed by a mechanical tester (Instron Series 5500) using a load cell of 2.5 N on cylindrical specimens of 1.5 cm length and *ca.* 0.85 cm diameter, placed between dynamometer plates. As commonly practiced in compressive measurements on hydrogel samples (Czerner, Sanchez Fellay, Suárez, Frontini & Fasce, 2015; Tang, Tung, & Zeng, 1996), the machine plates were coated with Teflon to avoid barrelling phenomena (Groover, 2010). The samples, having parallel limit surfaces, were obtained from the gel cylinder, using a device equipped with a sharp steel blade. The crosshead speed of the tester was set at 10 mm/min. Mechanical parameters, were measured on 10 specimens and the average values were calculated. G was measured at different swelling values, and the error was evaluated to be ±10%.

Starting from these data, a complete characterization of gels in terms of ν (moles of elastic effective chains for unit of network dry volume) and χ (Flory-Huggins interaction parameter) can be made on the basis of theoretical considerations.

2.10 Theoretical approach

In the assumptions of an ideally perfect polymer network and no volume change occurring upon compression, Flory (1953) derived the relation between the compressive stress and the compressive deformation of a swollen crosslinked polymer that can be written as:

$$\sigma = RT\nu\left(\frac{\phi_{20}}{\phi_2}\right)^{\frac{2}{3}}\phi_2\left(\lambda - \frac{1}{\lambda^2}\right) = G\left(\lambda - \frac{1}{\lambda^2}\right) \quad \textbf{(4)}$$

where σ is the uniaxial compressive stress, R the universal gas constant, T the absolute temperature, $\lambda = L/L_i$, being L and L_i the lengths of the compressed and the initially swollen sample, respectively. ϕ_2 and ϕ_{20} are the polymer volume fraction in the totally swollen gel and in the gel formed in the agar wells, respectively.

The modulus G was evaluated from the curve of the mechanical stress (σ) *vs.* $(\lambda - 1/\lambda^2)$.

From the Eq. (4), for a specific set of values ϕ_{20} and T, known ϕ_2 and G from experiments, it was possible to calculate ν.

Taking into account that during the compression measurement the volume changes because part of the water is squeezed out of the swollen sample, the Eq. (4) allows to evaluate the crosslink density only in the case of small deformations ($\lambda \to 1$).

For networks of conventional polymers, following the Gaussian statistics, the well-known basic hypothesis is that the total variation of the Gibbs free energy ΔF of the system, from the unswollen to the swollen state, is the sum of two terms; the former related to the free energy of mixing (ΔF_m) and the latter to the elastic expansion of the network (ΔF_{el}).

The corresponding variation of the chemical potential of the solvent, $\Delta\mu_1$, can be obtained by the derivative of ΔF with respect to the number of solvent moles n_1 at constant T and P (Flory, 1950). When the crosslinking process is performed in solution, at polymer volume fraction ϕ_{20}, it was demonstrated that:

$$\Delta\mu_1 = RT\left(ln\left(1-\phi_2\right) + \phi_2 + \chi\phi_2^2 - \frac{V_1\nu\phi_2}{2} + \phi_{20}^{\frac{2}{3}}\phi_2^{\frac{1}{3}}V_1\nu\right) \quad \textbf{(5)}$$

where V_1 is the molar volume of the solvent, and $(1-\phi_2)$ is the solvent volume fraction in the fully swollen hydrogel.

At the equilibrium swelling $\Delta\mu_1 = 0$, known ϕ_{20}, ϕ_2, T, V_1 and ν, it is possible to calculate the polymer-solvent interaction parameter, χ, which is a dimensionless quantity. On increasing χ value decreases the polymer-solvent

affinity.

3 Results and discussion

3.1 Hydrogel preparation and calcium evaluation

In order to obtain an interpenetrated network (IPN) with the envisaged improved mechanical proprieties, sodium alginate and methacrylate monomers were used (Table 1).

Firstly, hydrogels based on neat alginate were prepared, as described in the Experimental section, using a sodium alginate grade with a high content of guluronic acid units, that is able to form strong and stable network structures in the presence of calcium ions as crosslinking agents.

The procedure followed for alginate gelification is an innovative method, recently set out (Coluccino et al., 2012; Coluccino et al., 2016) to achieve a controlled diffusion, by concentration gradient, of calcium ions from the agar wells. In order to obtain reproducible content and quite homogenous distribution of calcium ions within the samples, experimental conditions in terms of calcium concentration in agar and gelification time were varied.

Atomic absorption measurements, carried out on freeze-dried samples, evidenced no remarkable difference in the content of calcium ions between samples obtained using 0.5 or 0.7 M $CaCl_2$ in agar, as well as using 4 or 8 h as gelification time.

On the basis of what said above, $CaCl_2$ 0.5 M and 4 h gelification time were chosen as convenient experimental conditions to prepare alginate-polymethacrylate hybrid hydrogels with the foreseen structure characterized by a double ionic and covalent network.

To this purpose alginate-polymethacrylate hybrid hydrogels were realized properly modifying the procedure used for neat alginate hydrogels (Fig. 1). Among synthetic polymers, polyacrylates and polymethacrylates, due to their proved biocompatibility as well as versatility and easiness of preparation have been and are largely used in various in-body applications (Chen et al., 2012; Chuah, Peck, Lau, Heec, & Wang, 2017; Derkaku, Avramoglou, Barbaud & Letourneur, 2008). The methacrylic hydrophilic monomer 2-hydroxyethyl methacrylate (HEMA), already employed for applications in the biomedical field (Elouali & Maschke, 2011) and easily polymerizable, was used to form the covalent network. Difunctional hydrophilic monomers, namely ethylene glycol dimethacrylate (EGDMA) or polyethylene glycol dimethacrylate (PEGDMA), were chosen as crosslinker moieties characterized by different spacer lengths.

The optimized formulations reported in Table 1 were selected after several preliminary tests carried out by varying HEMA concentration, type and amount of difunctional monomer crosslinker. Conditions of radical prepolymerization (temperature and sonication time) were optimized as well.

3.2 Morphological and structural analysis

Morphology and structure of scaffolds obtained by freeze-dried hydrogels were investigated by SEM analysis.

The micrographs shown in Fig. 2a and b, related to the neat alginate samples, reveal the formation of a highly interconnected porous structure (pores mean size 300–400 μm), adequate for cell proliferation as well as for vascularization. Moreover, SEM-EDX microanalysis carried out on both cross and longitudinal sections show a homogeneous calcium distribution already after 4 h of permanence in agar wells (Fig. 2).

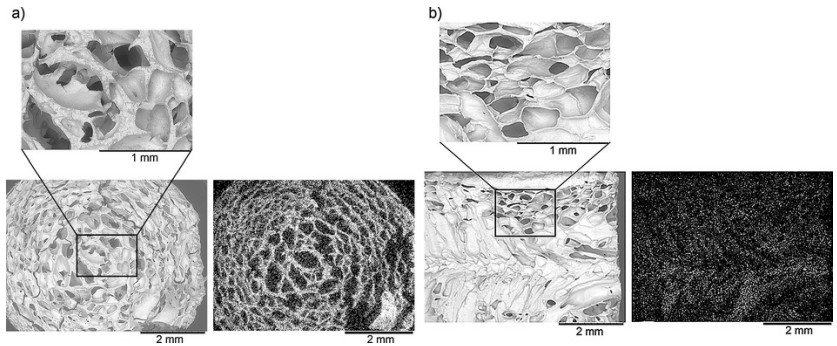

Fig. 2 SEM micrographs of cross (a) and longitudinal (b) sections of the freeze-dried neat alginate sample Alg_05. White dots, in the right portions of the figures, show calcium distribution from EDX mapping analysis.

alt-text: Fig. 2

A similar highly porous structure with interconnected pores was observed also for the hybrid hydrogels, with pore mean size of about 80–100 µm, depending on formulation (Fig. 3). This value, although smaller than that measured for samples of neat alginate, is nevertheless adequate for cell proliferation and tissue vascularization. SEM-EDX mapping analysis confirmed a uniform calcium distribution also for the alginate-methacrylate hybrid samples (Fig. 3).

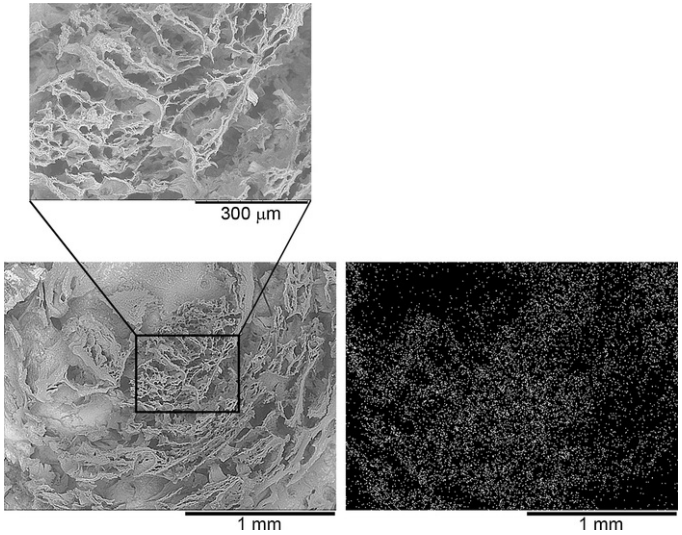

Fig. 3 SEM micrographs of the freeze-dried hybrid hydrogel sample Alg/HE. White dots, in the right portions of the figure, show calcium distribution from EDX mapping analysis.

alt-text: Fig. 3

Micro-computed tomography (micro-CT) performed on a few selected samples showed that pores occupy about 40% of the scaffold total volume. In Fig. 4 are reported the pore mean size (Fig. 4a) and the wall thickness (Fig. 4b). The pore mean size and the wall thickness are about 110 and 50 µm, respectively, in good agreement with SEM results.

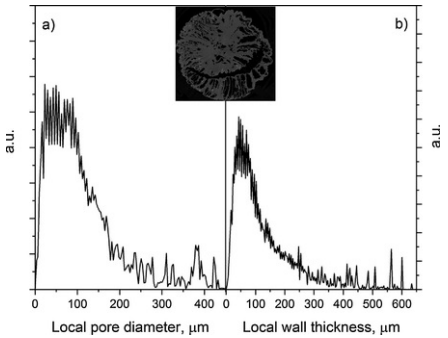

Fig. 4 Micro-CT results for the freeze-dried hybrid hydrogels sample, Alg/HE. The inset shows a grey level image of the sample slice.

alt-text: Fig. 4

3.3 Hydrogel characterization

Uniaxial compression mechanical tests were performed on both neat alginate and alginate-polymethacrylate hybrid hydrogels. In Table 2 the results obtained for the five gels examined, i.e., SW_w, SW_v, G, ν and χ values, were collected.

The G values as a function of SW_w for the investigated hydrogels are reported in Fig. 5; the theoretical curves, calculated according to the theory valid for flexible polymers, are also reported. These curves were estimated taking the ν values that best fit the experimental data.

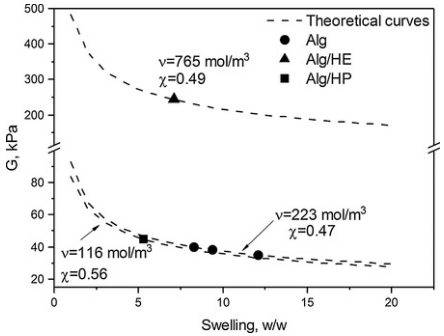

Fig. 5 Compressive modulus vs. weight swelling ratio of the alginate-based hydrogels.

alt-text: Fig. 5

As one can observe, for the neat alginate hydrogels prepared at three different SW_w (Alg_05_1, Alg_05_2, Alg_05_3) the G modulus, as expected, increases with decreasing SW_w.

The theoretical curve, calculated using $\nu = 223$, perfectly matched all three experimental datasets. On this base, the theoretical approach here used can be considered correct to describe the dependence of G on the swelling degree. As a consequence, also the ν value, calculated for the hybrid hydrogels, can be taken as a significant parameter to describe the degree of crosslinking of the systems.

However it can be noticed that the less swollen neat alginate hydrogel ($SW_w = 8.3$) shows poor mechanical properties in terms of G modulus, which barely reached 40 kPa. By observing the theoretical curve, in order to obtain hydrogels with improved mechanical properties, it would be necessary to further decrease the swelling degree. But also for a lower swelling degree ($SW_w = 2.5$) the resultant modulus would be not much more than 60 kPa. Besides, at this SW_w value the porous interconnected structure collapses leading to a drastic porosity reduction, undesired for cell permeation.

In order to increase the crosslinking degree of the hydrogels, the HEMA/PEGDMA (Alg/HP) system was used. However, the obtained hybrid hydrogel exhibited both crosslinking degree and modulus values comparable with those of neat alginate (Table 2). This could be explained by taking into account that PEGDMA, actually being a macromonomer, i) does not lead to a sufficient crosslinking degree in the methacrylate portion of the system, and ii) partially hampers the ionic crosslinking of the alginate counterpart.

Moreover, it is worth noticing that the maximum swelling degree of this hybrid system is lower than that of neat alginate gels (Table 2 and Fig. 5); this may ascribed to its lower affinity with the solvent, which is highlighted from the increasing of the χ values from 0.46 to 0.56 for neat alginate and hybrid system, respectively.

On the contrary, using the EGDMA/HEMA (Alg/HE) system the hybrid hydrogel obtained shows a significant higher crosslinking degree (Table 2). This could be explained by considering that EGDMA monomer has a much lower molecular weight with respect to PEGDMA and, as a consequence, has a higher mobility in the reaction medium, and at the same weight content a much higher concentration of reactive groups.

The hybrid hydrogel obtained shown~~n~~s good mechanical properties in terms of G modulus, which reache~~d~~s values of about 250 kPa, comparable or superior with respect those reported in literature for other hydrogel systems suitable as precursor of scaffolds in osteochondral tissue engineering (Allan et al., 2007; Hyland, Taraban, Hammouda, & Bruce Yu, 2011; Killion, Geever, Devine, Kennedy, & Higginbotham 2011; Sun et al., 2012). The desired improvement of the compression elastic modulus is ascribable to the double network, where ionic and covalent crosslinks play a synergistic action.

Furthermore, it is possible to observe that the maximum swelling degree for this hybrid system is quite good, being around 700% w/w; ascribable to high polymer-solvent affinity, as highlighted from the χ value equal to 0.49, very close to the value obtained for neat alginate (0.47).

The high modulus in addition to the relatively good swelling degree are the condition necessary to obtain the suitable porous interconnected structure suitable for cell permeation.

4 Conclusions

Novel alginate-polymethacrylate hybrid hydrogels, with an interpenetrated network structure characterized by both ionic and covalent crosslinks, were formulated and successfully prepared trough a simple and innovative step-wise method involving prepolymerization of the methacrylate portion and gel shaping in an agar mould containing Ca^{2+} ions as crosslinking agents for the alginate portion.

Ensuing porous materials (scaffolds) obtained by freeze-drying, irrespective of the various formulations, exhibited uniform calcium distribution as well as pores dimensions and interconnectivity adequate to cell adhesion and proliferation as well as tissue vascularization.

With respect to the hydrogel based on neat alginate, the hybrid hydrogel Alg/HE showed very good mechanical properties in terms of *G* modulus (seven times higher), ascribable to the double network, where ionic and covalent crosslinks play a synergistic action. Moreover, a high effective degree of crosslinking, evaluated by compressive uniaxial tests, was estimated. Furthermore, the maximum swelling degree observed for this hybrid system is quite good and ascribable to high polymer-solvent affinity, as highlighted from the χ value comparable to that of neat alginate.

Results gathered so far indicate that hybrid hydrogels based on alginate-polymethacrylate (Alg/HE) double crosslinked system are very promising candidates to obtain 3D scaffolds suitable for osteochondral regeneration.

Uncited reference

~~Microspheres (2017).~~

Acknowledgments

The authors warmly thank Dr. E. Zamparo and Mrs. Lisa Falco of SCANCO Medical AG, CH-8306 Bruettisellen, Switzerland for MicroCT analysis. The authors also thank Dr. M. Maugeri and Dr. E. Carmeli, for their helpful contribution.

References

Abdel-Fattah, Jiang, El-Bassiouni and Laurencin, Synthesis and characterization of chitosans and fabrication of sintered chitosan microsphere matrices for bone tissue engineering, *Acta Biomaterialia* **3**, 2007, 503–514.

Allan K.S., Pilliar R.M., Wang J., Grynpas M.D. and Kandel R.A., Formation of biphasic constructs containing cartilage with a calcified zone interface, *Tissue Engineering* **13**, 2007, 167–177.

Bailey E., Mitchell J.R. and Blanshard J.M.V., Free energy calculations on stiff chain constituents of polysaccharide gels, *Colloid and Polymer Science* **255**, 1977, 856–860.

Calvert P., Hydrogels for soft machines, *Advanced Materials* **21**, 2009, 743–756.

Chen Y.-C., Lin R.-Z., Qi H., Yang Y., Bae H., Melero-Martin J.M., et al., Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels, *Advanced Functional Materials* **22**, 2012, 2027–2039

Chicatun F., Pedraza C.E., Muja N., Ghezzi C.E., McKee M.D. and Nazhat S.N., Effect of chitosan incorporation and scaffold geometry on chondrocyte function in dense collagen type I hydrogels, *Tissue Engineering Part A* **19**, 2013, 2553–2564.

Chuah Y.J., Peck Y., Lau J.E.J., Heec H.T. and Wang D.-A., Hydrogel based cartilaginous tissue regeneration: Recent insights and technologies, *Biomaterials Science* **5**, 2017, 613–631.

Coates E.E., Riggin C.N. and Fisher J.P., Photocrosslinked alginate with hyaluronic acid hydrogels as vehicles for mesenchymal stem cell encapsulation and chondrogenesis, *Journal of Biomedical Materials Research Part A* **101**, 2013, 1962–1970.

Coluccino L., Stagnaro P., Vassalli M., Schizzi I. and Scaglione S., *Bioactive TGF-beta - CaP - alginate based scaffolds for osteochondral tissue repair: disign, realization, and multi-level characterization,* *Journal of Tissue Engineering and Regenerative Medicine* **6**, 2012, 225, [SI].

Coluccino L., Stagnaro P., Vassalli M. and Scaglione S., Bioactive TGF-β1/HA alginate-based scaffolds for osteochondral tissue repair: Design, realization and multilevel characterization, *Journal of Applied Biomaterials & Functional Materials* **14**, 2016, e42–e52.

Czerner M., Sanchez Fellay L., Suárez M.P., Frontini P.M. and Fasce L.A., Determination of elastic modulus of gelatin gels by indentation experiments, *Procedia Materials Science* **8**, 2015, 287–296.

Derkakui S.M., Avramoglou T., Barbaud C. and Letourneur D., Synthesis and characterization of a new polysaccharide-graft-polymethacrylate copolymer for three-dimensional hybrid hydrogels, *Biomacromolecules* **9**, 2008, 3033–3038.

Dhadayuthapani B., Yoshida Y., Maekawa T. and Kumar D.S., Polymeric scaffolds in tissue engineering applications: A review, *International Journal of Polymer Science* 2011, [290602-1-290602-19].

Draget K.I., Smidsrød O. and Skjåk-Bræk G., Alginates from algae, *Biopolymers Online* **6**, 2005.

Elouali F.Z. and Maschke U., Kinetics and equilibrium swelling properties of hydrophilic polymethacrylate networks, *Macromolecular Symposium* **303**, 2011, 71–77.

Eslahi N., Abdorahim M. and Simchi A., Smart polymeric hydrogels for cartilage tissue engineering: a review on the chemistry and biological functions, *Biomacromolecules* **17**, 2016, 3441–3463.

Flory P.J., Statistical mechanics of swelling of network structures, *Journal of Chemical Physics* **18**, 1950, 108–111.

Flory P.J., Principle of polymer chemistry, 1st ed., 1953, Cornell University Press; Ithaca, New York.

Gloria A., De Santis R. and Ambrosio L., Polymer-based composite scaffolds for tissue engineering, *Journal of Applied Biomaterials & Biomechanics* **8**, 2010, 57–67.

Glowacki J. and Mizuno S., Collagen scaffolds for tissue engineering, *Biopolymers* **89**, 2008, 338–344.

Groover M.P., Fundamentals of modern manufacturing, 4th ed., 2010, J. Wiley & Sons Inc..

Hildebrand T. and Rüegsegger P., A new method for the model-independent assessment of thickness in three-dimensional images, *Journal of Microscopy* **185**, 1997, 67–75.

Hutmacher D.W., Scaffolds in tissue engineering bone and cartilage, *Biomaterials* **21**, 2000, 2529–2543.

Hyland L.L., Taraban M.B., Hammouda B. and Bruce Yu Y., Mutually reinforced multicomponent polysaccharides networks, *Biopolymers* **95**, 2011, 840–851.

Killion J.A., Geever L.M., Devine D.M., Kennedy J.E. and Higginbotham C.L., Mechanical properties and thermal behaviour of PEGDMA hydrogels for potential bone regeneration application, *Journal of the Mechanical Behavior of Biomedical Materials* **4**, 2011, 1219–1227.

Liu X. and Ma P.X., Polymeric scaffolds for bone tissue engineering, *Annals of Biomedical Engineering* **32**, 2004, 477–486.

Long R., Mayumi K., Creton C., Narita T. and Hui C.-Y., Time dependent behavior of a dual cross-link self-healing gel: Theory and experiments, *Macromolecules* **47**, 2014, 7243–7250.

Malafaya P.B., Gomes M.E., Salgado A.J. and Reis R.L., Polymer based scaffolds and carriers for bioactive agents from different natural origin materials, *Advances in Experimental Medicine and Biology* **534**, 2003, 201–233.

~~Microspheres, 2017.~~

Mitchell J.R. and Blanshard J.M.V., Rheological properties of alginate gels, *Journal of Texture Studies* **7**, 1976, 219–234.

Nooeaid P., Salih V., Beier J.P. and Boccaccini A.R., Osteochondral tissue enigineering: scaffolds: Stem cells and applications, *Journal of Cellular and Molecular Medicine* **16**, 2012, 2247–2270.

Park S.J., Yu S.M., Chun M.H., Chun M.J. and Kim C.H., Effect of hyaluronic acid on attachment and proliferation of chondrocytes on chitosan/hyaluronic acid bead scaffolds, *Journal of Tissue Engineering and Regenerative Medicine* **6**, 2009, 438–444.

Park S.-B., Lih E., Park K.-S., Joung Y.K. and Han D.K., Biopolymer-based functional composites for medical applications, *Progress in Polymer Science* **68**, 2017, 77–105.

Pathak T.S., Yun J.H., Lee J. and Paeng K.J., Effect of calcium ion (crosslinker) concentration on porosity, surface morphology and thermal behavior of calcium alginates prepared from algae (Undaria pinnatifida), *Carbohydrate Polymers* **81**, 2010, 633–639.

Pawar S.N. and Edgar K.J., Alginate derivatization: a review of chemistry: properties and applications, *Biomaterials* **33**, 2012, 3279–3305.

Rezwan K., Chen Q.Z., Blaker J.J. and Boccaccini A.R., Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering, *Biomaterials* **27**, 2006, 3413–3431.

Sachlos E. and Czernuszka J.T., Making tissue engineering scaffolds work: review on the application of solid free form fabrication technology to the production of tissue engineering scaffolds, *European Cells & Materials* **5**, 2003, 29–40.

Sun J.-Y., Zhao X., Illeperuma W.R.K., Chaudhuri O., Oh K.H., Mooney D.J., et al., Highly stretchable and tough hydrogels, *Nature* **489**, 2012, 133–136.

Tang J., Tung M.A. and Zeng Y., Compression strength and deformation of gellan gels formed with mono- and divalent cations, *Carbohydrate Polymers* **29**, 1996, 11–16.

Temenoff J.S. and Mikos A.G., Review: Tissue engineering for regeneration of articular cartilage, *Biomaterials* **21**, 2000, 431–440.

US Environmental Protection Agency, EPA Method 3050B. Acid digestion of sediments, sludge, and oils, 1996, US EPA Office of Solid Waste; Washington D.C, [December].

Van Vlierberghe S., Dubruel P. and Schacht E., Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review, *Biomacromolecules* **12**, 2011, 1387–1408.

Vicini S., Castellano M., Marsano E. and Mauri M., Gelling process for sodium alginate: New technical approach by usingcalcium rich micro-spheres, *Carbohydrate Polymers* **134**, 2015, 767–774.

Vicini S., Mauri M., Wichert J. and Castellano M., Alginate gelling process: Use of bivalent ions rich, *Polymer Engineering and Science* 2017, https://doi.org/10.1002/pen.24552.

Wagoner Johnson A.J. and Herschler B.A., A review of the mechanical behavior of CaP and CaP/polymer composites for applications in bone replacement and repair, *Acta Biomaterialia* **7**, 2011, 16–30.

Highlights

- Alginate-polymethacrylate hybrid hydrogels are achieved by a simple procedure.
- Formation of a double ionic and covalent interconnected network is envisaged.
- Mechanical performance of the hybrid hydrogels is enhanced due to the double network.
- Swelling ratio and crosslinking degree are correlated to the hydrogel properties.
- From hybrid hydrogels scaffolds suitable for osteochondral regeneration are obtained.

Queries and Answers

Query: The author names have been tagged as given names and surnames (surnames are highlighted in teal color). Please confirm if they have been identified correctly.
Answer: Yes

Query: Please check the hierarchy of the section headings and correct if necessary.
Answer: The hierarchy of the sections is correct

Query: “Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact r.pio@elsevier.com immediately prior to returning your corrections.”
Answer: Yes is correct

Query: Uncited reference: This section comprises references that occur in the reference list but not in the body of the text. Please cite each reference in the text or, alternatively, delete it.
Answer: it is an error, it has been canceled

Query: Please provide article title for Ref. "Coluccino et al., 2012".
Answer: Done

Query: Please provide full bibliographic details for Ref. "Microspheres, 2017".
Answer: was deleted