Preparation and physico-chemical characterisation of microporous polysaccharidic hydrogels

GEMMA LEONE, ROLANDO BARBUCCI*
CRISMA and Department of Chemical and Biosystem Science and Technologies, University of Siena, Via Aldo Moro no. 2, 53100 Siena, Italy
E-mail: barbucci@unisi.it

ASSUNTA BORZACCHIELLO, LUIGI AMBROSIO, PAOLO A. NETTI
Institute of Composite and Biomedical Materials and CRIB, University of Naples "Federico II", Piazzale Tecchio no. 80, 80125 Napoli, Italy

CLAUDIO MIGLIARESI
Department of Materials Engineering and Industrial Technologies, University of Trento, Via Mesiano di Povo no. 77, 38050 Trento, Italy

A new technique to obtain microporous hydrogels was realised. It permits us to obtain a microporous structure directly on the already cross-linked hydrogel. It consists in stratifying the already cross-linked hydrogel on to a filter with known porosity and forcing the CO_2 bubbles, derived from the addition of HCl to a porogen salt ($NaHCO_3$), to cross through the filter first and then the matrix. By changing the porosity of the filter, it was possible to modulate the porous morphology of the hydrogels. The polysaccharides selected were hyaluronane, alginate, and carboxymethylcellulose. The influence of the porous morphology on the physico-chemical properties of the gel has been evaluated by FT-IR, FRAP, calorimetric, water uptake, and rheological analysis.

Introduction

Hydrogels have been extensively used in medicine, pharmacy, and life sciences [1]. Their morphologies and physico-chemical properties make them suitable for several applications, in particular as controlled release systems of drugs [2, 3] or scaffolds for tissue engineering [4, 5]. For both these applications the presence of pores is a fundamental requirement. In fact, it guarantees easy diffusion of nutrients and clearance of wastes and provides an adequate environment for the growth of cells in a cellular scaffold [6, 7]. In controlled release systems, pore dimension and distribution affect the release kinetics of the substance included in the matrix [8, 9]. Several polysaccharides have been utilised as materials for hydrogel production [10–13]. The preference accorded to polysaccharides is due, first of all, to the critical role played by saccharide moieties in cell signalling schemes and in immune recognition. Furthermore, their chemical structures can be easily modified to introduce new biological properties [14]. In this work, natural polysaccharides, hyaluronate and alginate, and a semisynthetic polysaccharide, carboxymethylcellulose, were cross-linked in order to obtain a water insoluble hydrogel. Cross-linked hydrogels may be morphologically modified in order to obtain a microporous structure, thus increasing their applicability in the biomedical field as scaffolds or drug delivery vehicles. The technique reported here permits us to obtain a microporous structure on the already cross-linked hydrogel [15]. The characterisation of microporous hydrogels in terms of water absorption, rheological properties, FT-IR, thermal analysis, and diffusion resistance allows the important properties of the materials to be defined.

Materials and methods

Materials

Sodium salt of hyaluronic acid (Hyal) (Biophil Italia S.p.A) ($\sim$ 150–200 kDa), carboxymethylcellulose (CMC) (Hercules Italia S.p.A) (carboxymethylation degree of 0.9 ± 0.1 per disaccharide unit, $\sim$ 100 kDa), and alginic acid (AA) (Roth, Germany) ($\sim$ 100 kDa) were used as received. The other reagents were purchased from Fluka Chemie (Switzerland).

Methods

Hydrogel synthesis

Hydrogels were synthesised by cross-linking the polysaccharidic chains through an alkylic bridge derived from the formation of an amidic bond between the carboxylic groups of the polysaccharide chains and the aminic groups of the cross-linking agent, an alkylic

*Author to whom all correspondence should be addressed.

diamine (1,3-diamine-propane) as previously described [16]. Cross-linking derivatisation was controlled to use only 50% of the carboxylate groups (50% hydrogel).

Microporous structure realisation

The porous structure was obtained by stratifying the 50% hydrogel onto a filter with a defined and controlled porosity (40, 70, and 100 μm). The diameters of the filter pore were checked by scanning electron microscopy (SEM). Once a film was obtained, it was rinsed with some drops of chloroform in order to keep the matrix soft, but at the same time preventing an excessive swelling that would alter the pore formation. The filter was placed on a beaker containing 1.5 g of a porogen salt ($NaHCO_3$). By slowly adding 0.1 M HCl solution with a syringe, violent effervescence was observed. The formation of CO_2 bubbles and their passage through the filter first, and then the matrix, induced the hydrogel to assume a porous morphology [15].

Characterisation

Scanning electron microscopy

The morphology of native and porous hydrogel samples was examined by SEM (XL20 Philips, The Netherlands). Five milligrams of each sample was rinsed with 1.5 ml of distilled water and, once the swelling equilibrium was reached, frozen in liquid nitrogen and lyophilised. Samples were cut along the three axes to obtain a complete observation of the pore distribution in all the dimensions.

Samples were mounted on SEM stubs in order to analyse the surface and the internal structure, and gold-sputtered with an automatic sputter-coater (BAL-TEC SCD 050, Balzers, Germany). The hydrogel morphology was then observed by SEM at 10 kV. For the statistical evaluation of pore diameter, thickness, and pore density, 15 specimens for each dimension were used.

Water uptake measurements

Water uptake (WU) was determined for each sample, as calculated with the following formula:

$$\mathrm{WU} = [(W_s - W_d)/W_d] \times 100$$

where W_s and W_d are the weight of the swollen and dried hydrogels, respectively. The procedure follows one that has already been reported [17].

FT-IR ATR analysis

ATR spectra of the samples in dry form were recorded on a Biorad FTS 6000 between 4000 and 750 cm^{-1}. A horizontal ATR accessory with a 456E zinc–selenide crystal was used. An MCT detector was used, and the apparatus was purged with nitrogen. Typically, 50 scans at a resolution of 1.0 cm^{-1} were averaged. The frequency scale was internally calibrated with a helium–neon reference laser to an accuracy of 0.01 cm^{-1}.

Rheological characterisation

The rheological properties of the hydrogels were evaluated on a strain controlled Bohlin VOR Rheometer (Bohlin Reologi AB, Lund, Sweden) at a controlled temperature of 25 °C. The geometry was plate and plate (PP 30 cell).

Small-amplitude oscillatory shear experiments were performed to measure the time dependent response of the samples. The frequency range spanned from 0.1 to 10 Hz. In particular, $G'(\omega)$ (shear storage modulus) and $G''(\omega)$ (shear loss modulus) were evaluated. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character or the energy dissipated as heat.

Strain sweep tests at a fixed oscillation frequency (consisting in monitoring the viscoelastic properties while logarithmically varying the strain amplitude γ_0) were performed on the materials to determine the strain amplitude at which linear viscoelastity is valid.

Diffusion coefficient

Diffusion coefficients were measured using an image-based fluorescence recovery after photobleaching (FRAP) technique. The reliability of this method has been previously established for the measurement of diffusion and partition coefficients in gels, tissue, and aqueous systems [18, 19]. The purpose of the apparatus is to expose a fluorescent sample to a brief pulse of intense focused light to quench, irreversibly, the fluorescent molecules in a small portion of the sample, and then record images of the subsequent changes in the fluorescent pattern. The images are acquired at a spatial resolution of 128 × 128 pixels, with a signal resolution of 256 intensity levels, and a temporal resolution of 10 images/s. The acquired images are then analysed to evaluate the diffusion coefficient. The spatial Fourier analysis (SFA) algorithm [20] used makes full use of the spatial and temporal details contained in those images.

The samples were prepared by immersion in a 0.0058 mg/ml solution of bovine serum albumin (BSA) marked with fluorescein isothiocyanate (BSA-FITC) for the time necessary to reach the stage of complete swelling.

Differential scanning calorimetry

The thermal properties were studied using MDSC (TA 2920) (TA Instruments), heating at a rate of 3°K/min and with a modulation of $\pm$ 0.5°K with a perforated capsule. The modulation technique permits us to collect reversible transitions in comparison with the irreversible ones. The samples were previously lyophilised in order to reduce the water effect.

Results and discussion

Morphological characterisation of microporous hydrogels

The presence, dimension, and distribution of the microporous structure was evaluated by SEM. As shown by the micrographs in Fig. 1, a homogeneous pore distribution was obtained. For all the hydrogels a

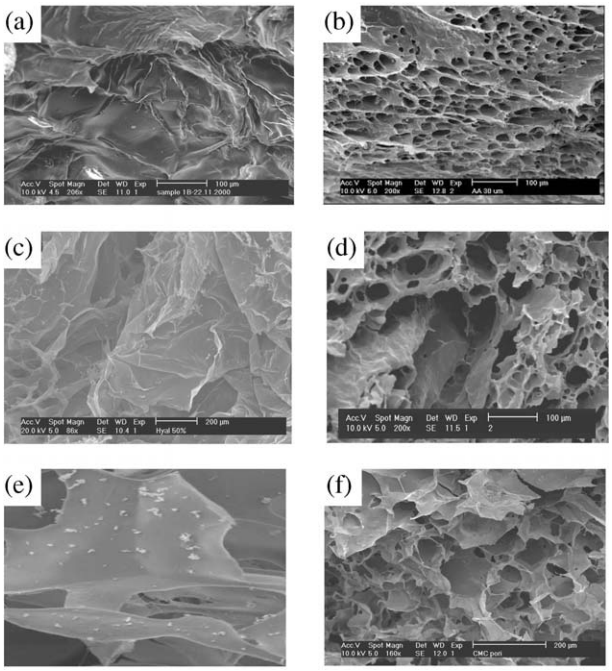

Figure 1 SEM micrographs of native and porous 50% hydrogels: (a) native AA 50%, (b) 30 μm microporous AA, (c) native hyal 50%, (d) 30 μm microporous Hyal, (e) native CMC 50%, (f) 30 μm microporous CMC.

strict correspondence between the porosity of the filter and the porous structure was found. In fact, using the 40 μm filter the hydrogels showed pores of about 15 μm diameter, whereas with 70 and 100 μm filters the gels showed pores with greater diameters, 30 and 40 μm, respectively (Table I). Furthermore, all the porous matrices showed similar thicknesses and a pore density that decreased with the increase of the pore diameter (Table I).

Physico-chemical characterisation of microporous hydrogels

The microporous hydrogels showed a decreased water uptake in comparison with the corresponding native matrices at all the pHs investigated (Fig. 2). The decrease in water uptake was dependent on the polysaccharide chemistry. In fact, the decrease was small for CMC- and alginate-based hydrogels, whereas for Hyal-based hydrogel a substantial decrease of water uptake was observed. In order to evaluate if the mechanism of pore formation induced in Hyal hydrogel a chemical as well as a morphological modification, an infrared analysis was performed. The infrared spectrum of porous Hyal showed a new peak (absent in the IR spectrum of the

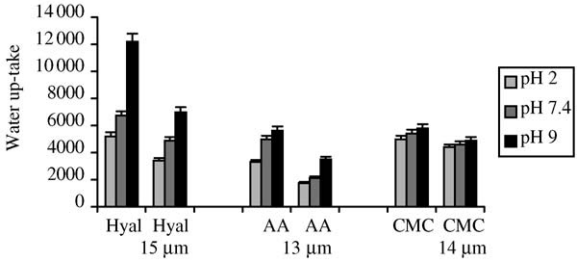

Figure 2 Water uptake behaviour of native and microporous AA, Hyal, and CMC as a function of pH.

native Hyal) at 1729 cm^{-1} (ascribed to protonated carboxylic groups that were hydrogen bonded). The appearance of this peak was followed by the disappearence of the peak at 1600 cm^{-1} ascribed to the unprotonated carboxylate groups (Fig. 3). This change is assumed to be due to CO_2 bubbles which, crossing the matrix, induced a lowering of pH, provoked protonation of COO^-, and, as a consequence, a decrease of the charge density and of the repulsion between the polysaccharide chains was observed. The functional groups capable of forming hydrogen bonds were located at a closer distance and so the hydrogen bonds were formed. The hydrogen bonds formed were strong enough to resist at all the pHs investigated, as demonstrated by the permanence of the peak not only at pH 2 but also at pH 7.4 and pH 9. This phenomenon was not found for CMC- and AA-based hydrogels. In fact, the peak relative to COOH hydrogen bonded was present only at pH 2. Thus, for hydrogels based on these two polysaccharides the small decrease in water uptake can be entirely because of the compaction of the matrix due to the crossing of the CO_2 bubbles.

In fact, by crossing the hydrogel, the CO_2 bubbles provoked the approach of the material of the hydrogels. The surface in contact with the water was drastically reduced, causing decreased water uptake. Furthermore, the compact material reduces the flexibility and freedom of the polysaccharide chains, decreasing their solvation power.

These morphology changes also lead to quantitative differences of the mechanical properties between the porous and non-porous hydrogels. The rheological behavior of all the matrices was the same. They behaved as strong gels [21–22] with both moduli almost frequency independent and with the storage modulus greater than the loss modulus by about one order of magnitude (Table II). The presence of chemical cross-linking among the chains lead to a permanent stable network, the intrinsic mobility of the molecules decreases dramatically and the principal mode of accommodation of the applied stress is through network

TABLE I Pore diameter (expressed as μm), pore density (expressed as pores per mm^2) and thickness of the walls between the pores (expressed as μm)

Cell strainer (μm)	AA			CMC			Hyal		
	Diameter (μm)	Density (pores/mm^2)	Thickness (μm)	Diameter (μm)	Density (pores/mm^2)	Thickness (μm)	Diameter (μm)	Density (pores/mm^2)	Thickness (μm)
ϕ 40	13 ± 4	5.0 ± 1.5	2.2 ± 1.1	14 ± 4	2.00 ± 0.5	2.7 ± 0.9	15 ± 3	3.00 ± 0.5	1.80 ± 0.9
ϕ 70	30 ± 2	1.5 ± 0.5	2.4 ± 0.9	30 ± 4	1.50 ± 0.03	2.3 ± 1.4	35 ± 2	1.00 ± 0.01	2.70 ± 0.8
ϕ 100	40 ± 4	1.5 ± 0.3	2.0 ± 1	40 ± 9	1.00 ± 0.01	3.7 ± 2.1	40 ± 4	0.50 ± 0.02	0.70 ± 0.05

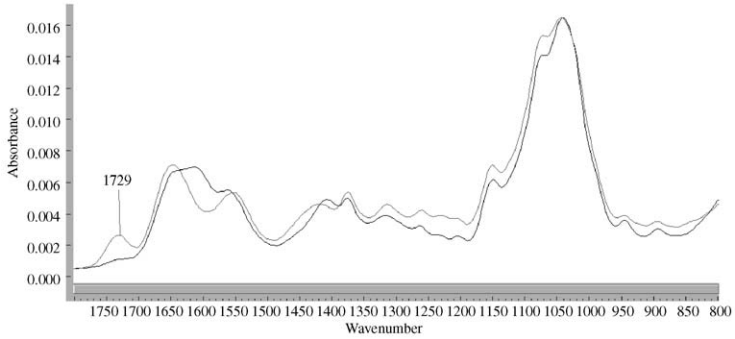

Figure 3 Infrared analysis of native (black) and microporous (grey) 50% Hyal.

TABLE II G' (storage modulus) and G'' (loss modulus) of native and microporous polysaccharide hydrogels detected at 10.00 Hz

	G' (detected at 10.00 Hz)	G'' (detected at 10.00 Hz)
Hyal 50% (μm)	100 ± 5	10 ± 1
15	1500 ± 75	90 ± 5
35	900 ± 45	100 ± 15
40	1000 ± 45	100 ± 10
AA 50% (μm)	2000 ± 110	100 ± 5
13	6000 ± 150	400 ± 60
30	6000 ± 120	400 ± 45
40	6000 ± 180	500 ± 35
CMC 50% (μm)	900 ± 45	90 ± 5
14	1200 ± 50	110 ± 25
30	1500 ± 65	120 ± 15
40	1400 ± 80	110 ± 10

deformation ($G' > G''$). The network is also stabilised by physical interactions, such as hydrogen bonding. The rheological properties of the porous hydrogel are enhanced compared to the hydrogels without pores; indeed, both moduli are increased. This result is in agreement with the decrease in water uptake. A strict correspondence between the decrease of water uptake and improvement in mechanical properties was observed. In fact, regarding water uptake and mechanical properties the influence of the porous structure in the CMC-based hydrogels was negligible, the small decrease of water uptake observed for AA-based hydrogels was followed by an analogous small improvement of the mechanical properties whereas the large decrease of water uptake observed for Hyal was followed by the large improvement of the mechanical properties. Both G' and G'' increased by an order of magnitude (Table II).

The thermal analysis indicated that no significant differences were induced by the formation of the porous structure. The main differences were found among the three different polysaccharides.

The three polysaccharide-based hydrogels showed different behaviours in terms of diffusion coefficient. As shown by the graph in Fig. 4, Hyal- and CMC-based matrices showed an increased diffusion coefficient value with increasing pore diameter. No significant difference was found between native CMC and Hyal 50% and 14 μm CMC and Hyal, whereas a substantial increase was found in 30 and 40 μm filters. AA-based matrices showed a peculiar behaviour. A drastic decrease was observed in 13 μm AA in comparison with native AA. In this case also, by increasing the pore diameter, from 13 to 30 and 40 μm, a large increase of the diffusion coefficient was observed. The presence of pores makes the diffusion easier.

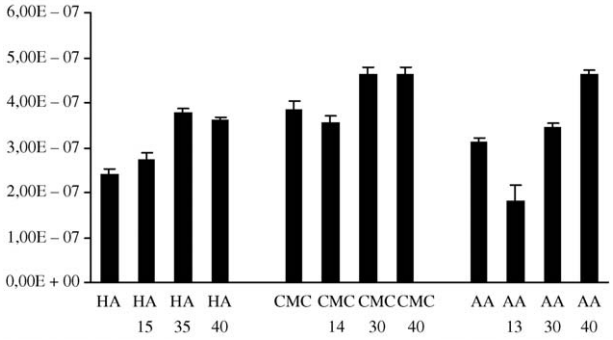

Figure 4 Diffusion coefficient of BSA-FITC through native and microporous polysaccharidic matrices.

Conclusion

Carboxymethylcellulose, hyaluronate, and alginate based hydrogels were synthesised and morphologically modified in order to obtain a microporous structure. The technique utilised guarantees a wide pore distribution and a predictable pore dimension. The presence of pores drastically affected water absorption and rheological properties. Microporous hydrogels showed a reduced water uptake and improved mechanical properties. The presence of pores also enhanced the diffusion coefficient, as ascertained by FRAP measurements. On the basis of the observed modification of the hydrogel properties these microporous hydrogels appear as good candidates for cellular scaffolds.

Acknowledgments

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