Acta Biomaterialia 7 (2011) 1627–1633

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

In situ forming IPN hydrogels of calcium alginate and dextran-HEMA for biomedical applications

Laura Pescosolido [a,b], Tina Vermonden [b], Jos Malda [c], Roberta Censi [b], Wouter J.A. Dhert [c,d], Franco Alhaique [a], Wim E. Hennink [b], Pietro Matricardi [a,*]

[a] Department of Drug Chemistry and Technologies, "Sapienza" University of Rome, p.le Aldo Moro 5, 00185 Rome, Italy
[b] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Sorbonnelaan 16, P.O. Box 80082, 3508 TB Utrecht, The Netherlands
[c] Department of Orthopaedics, University Medical Center Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands
[d] Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80163, 3508 TD Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 24 August 2010
Received in revised form 15 November 2010
Accepted 29 November 2010
Available online 1 December 2010

Keywords:
IPN
Polysaccharides
Protein delivery
Cytocompatibility
Chondrocytes

ABSTRACT

In situ forming hydrogels, which allow for the modulation of physico-chemical properties, and in which cell response can be tailored, are providing new opportunities for biomedical applications. Here, we describe interpenetrating polymer networks (IPNs) based on a physical network of calcium alginate (Alg-Ca), interpenetrated with a chemical one based on hydroxyethyl-methacrylate-derivatized dextran (dex-HEMA). IPNs with different concentration and degree of substitution of dex-HEMA were characterized and evaluated for protein release as well as for the behavior of embedded cells. The results demonstrated that the properties of the semi-IPNs, which are obtained by dissolution of dex-HEMA chains into the Alg-Ca hydrogels, would allow for injection of these hydrogels. Degradation times of the IPNs after photocross-linking could be tailored from 15 to 180 days by the concentration and the degree of substitution of dex-HEMA. Further, after an initial burst release, bovine serum albumin was gradually released from the IPNs over approximately 15 days. Encapsulation of expanded chondrocytes in the IPNs revealed that cells remained viable and, depending on the composition, were able to redifferentiate, as was demonstrated by the deposition of collagen type II. These results demonstrate that these IPNs are attractive materials for pharmaceutical and biomedical applications due to their tailorable mechanical and degradation characteristics, their release kinetics and biocompatibility.

1. Introduction

Hydrogels are commonly studied in the pharmaceutical field as materials for the controlled release of bioactive molecules, i.e. proteins, as well as in the tissue engineering field for encapsulation of cells [1,2]. Hydrogel networks can be obtained by chemical or physical cross-linking of water-soluble macromolecules [3,4]. Because of their hydrophilic nature, hydrogels are able to retain large amounts of water, thereby mimicking natural tissues. Particularly, hydrogels made of polysaccharides are attractive because of their abundance, possibilities for chemical derivatization and, generally, good biocompatibility [5,6]. Amongst the various hydrogels described in the literature, particular attention has been given in recent years to in situ forming hydrogels [7–10]. These formulations can be administrated by injection in the therapeutic target, after which gelation takes place and the locally formed gel can act as a depot system for release of drugs or bioactive compounds. The use of hydrogels can improve efficacy, efficiency, patience compliance of the therapeutic agent and reduce, in most cases, its toxicity [3,7,11]. In situ gelling hydrogels are also interesting materials for tissue engineering applications, where they can be injected into the defect site and, after gelation, mimic the extracellular matrix acting as scaffold for proliferation and differentiation of entrapped cells [12,13].

In order to modulate the properties of the networks, a new class of hydrogels based on the interpenetration of two different polymer networks (IPNs) has been developed recently [14–17]. An IPN consists of two or more hydrophilic polymers, each forming either physically or chemically cross-linked networks which are entangled with each other. A semi-IPN, instead, is composed of one cross-linked polymer system in which free polymer chains are dissolved.

In IPNs, neither chemical nor strong physical interactions are present between the two networks. Nevertheless, both polymers contribute, sometimes in a synergistic way, to the physico-chemical and e.g. drug delivery properties of the IPN [18].

Recently, we described IPN hydrogels based on calcium alginate (Alg-Ca) and dextran methacrylate derivatives (dex-MA) [19,20]. The physico-chemical characteristics of the semi-IPNs (Alg-Ca

* Corresponding author. Tel.: +39 06 49913226; fax: +39 06 49913133.
E-mail address: pietro.matricardi@uniroma1.it (P. Matricardi).

1742-7061
doi:10.1016/j.actbio.2010.11.040

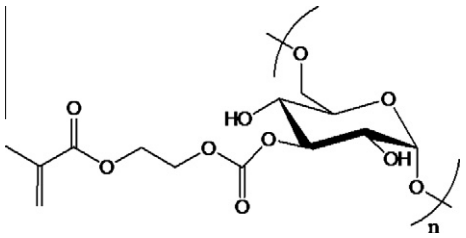

Fig. 1. Chemical structure of dextran derivatized with hydrolyzable hydroxyethylmethacrylate moieties.

and dex-MA before UV curing) and of the corresponding IPNs (i.e. after UV curing) were described [19,20]. These systems showed interesting properties including injectability, improved mechanical strength in comparison to the two separate hydrogel systems, arising from the synergistic interaction between the polymer networks, and tunable protein release properties. The high viscosity of the semi-IPNs, the ease of manipulation and UV curing possibilities make the system useful for in situ hydrogel formation. However the dex-MA hydrogels are not biodegradable under physiological conditions [11], limiting their application in the biomedical and pharmaceutical fields. Therefore, in the present work, polysaccharide IPNs based on alginate and biodegradable dex-HEMA (Fig. 1) [21,22] were evaluated for their mechanical and release properties, cytocompatibility and their ability to act as scaffold for tissue engineering applications.

2. Materials and methods

2.1. Materials

Dextran (dex) samples from *Leuconostoc* ssp. with molecular weight (M_w) of 40×10^3, 4-*N*,*N*-dimethylaminopyridine (4-DMAP), and hydroxyethyl methacrylate (HEMA) were Fluka products. Sodium alginate (Alg) with M_w of 150×10^3 (70% L-guluronic acid and 30% D-mannuronic acid), carbonyldiimidazole (CDI), bovine serum albumin (BSA), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, ethylenediaminetetraacetic acid (EDTA), ascorbic acid 2-phosphate, haematoxylin, DAB, haematoxylin, eosin, fast green FCF and safranin O were all provided by Sigma–Aldrich, USA. Irgacure 2959 (2-hydroxy-4β-(2-hydroxyetoxy)-2-methyl-propiophenone) was obtained from Ciba Specialty Chemicals Inc. Collagenase type II was provided by Worthington Biochemical, Lakewood, USA. Phosphate buffered saline (PBS), DMEM, penicillin, streptomycin, 1× ITS-X and trypsin were obtained from Invitrogen (USA). Fetal bovine serum (FBS) was a Biowhittaker product. βFGF and TGF-β2 were provided by R&D Systems (USA). Human serum albumin was a product of SeraCare Life Sciences (USA). All other chemicals were analytical grade.

2.2. Methods

2.2.1. Dex-HEMA synthesis and characterization

Dex-HEMA batches were prepared by functionalizing dextran according to a previously described method [21]. In short, first HEMA is activated with CDI (resulting in HEMA-CI), which in a second step was coupled to the hydroxyl groups of dextran. The degree of substitution (DS, i.e. the number of HEMA groups per 100 glucopyranose residues of dextran) was determined by ^1H nuclear magnetic resonance (NMR) in D_2O using a Gemini 300 MHz spectrometer (Varian Associates Inc., NMR instruments, Palo Alto, CA). Dex-HEMA with DS = 8, 12, and 16 were prepared.

2.2.2. Hydrogel preparation

In the IPNs, the Alg concentration was fixed at 3% (w/v), while three different dex-HEMA concentrations, 10% (w/v), 15% (w/v) and 20% (w/v), respectively, were used.

Hydrogels (1 ml) were formed in cylindrical vials (diameter 12 mm) as follows. Alg-Na (0.030 g) was dissolved in 0.6 ml of HEPES buffer (100 mM, pH 7.4). Next, an appropriate amount of dex-HEMA was added followed by the addition of 3 μl of a concentrated solution (33% w/v) of the photoinitiator Irgacure 2959. Then, 0.4 ml of a $CaCl_2$ solution (0.025 M) also containing NaCl (0.385 M) was added to the alginate/dex-HEMA solution. The system was mixed by mechanical stirring resulting in the formation of the semi-IPN, which was subsequently irradiated with a BluePoint 4 mercury lamp (Honle UV technology, λ range 350–450 nm, light intensity of 400 mW cm^{-2}), for 5 min to polymerize dex-HEMA and to yield the IPNs. Table 1 gives the compositions of the investigated hydrogels.

For release studies, BSA was loaded into the IPNs by dissolving 10 mg protein in 200 μl of HEPES, which was subsequently added to the alginate/dex-HEMA solution. In order to avoid UV-induced protein degradation, a glass filter was interposed between the sample and the UV lamp.

2.2.3. Rheological experiments

Oscillatory shear experiments were performed using an AR-G2 rheometer (TA-Instruments). Frequency sweeps of the semi-IPNs were carried out using a cone-plate geometry (diameter = 20 mm; cone = 1°) in the range 0.01 ÷ 10 Hz, which is in the linear viscoelastic region as assessed by stress/sweep experiments. To prevent water evaporation from the samples, a solvent trap was used. The polymerization kinetics were followed using a UHP device connected to a BluePoint 4 mercury lamp (Honle UV technology, λ range 230–500 nm, intensity of 50 mW cm^{-2}), setting the gap to 500 μm and starting irradiation after 180 s from the start of the rheological experiment. During the UV irradiation of the samples for 7 min, G' (storage modulus) and G'' (loss modulus) were monitored at a strain of 0.1% and a frequency of 1 Hz. Next, the viscoelastic properties of the IPNs were recorded in the range of 0.01–10 Hz, applying a constant deformation in the linear regime (1%). All measurements were performed at 25 °C.

2.2.4. DMA measurements

The Young's moduli (E') of the hydrogels were determined by dynamic mechanical analysis (DMA), performed with a DMA 2980 dynamic mechanical analyzer (TA Instruments, New Castle, DE, USA) equipped with a compression clamp (upper plate 6 mm, lower plate 45 mm). Experiments were performed at room temperature by imposing a range of frequencies (from 10 to 0.1 Hz), an amplitude of 20 μm with a constant preload force of 0.01 N. For each formulation, the reported data are the mean of four independently prepared samples.

2.2.5. Methacrylic conversion

The hydrogels were incubated in 9 ml of 0.02 M NaOH at 37 °C for 24 h to hydrolyze unreacted methacrylic groups [23]. Next, 1 ml of 2 M acetic acid solution was added and the methacrylic acid concentration was determined using an Acquity UPLC trade, equipped with a UV detector operating at 210 nm, utilizing a BEH C18 1.7 μm, 2.1 × 50 mm column. The eluent was H_2O/acetonitrile/perchloric acid (95/5/0.1%) and the flow rate was 0.5 ml min^{-1}. The methacrylate conversion is defined as (1 − (moles of unreacted methacrylate groups/moles of methacrylate groups originally coupled to dextran) × 100).

Table 1
Composition of the samples prepared.

	% Polymer concentration (w/v)				
Sample	Alginate	Dextran	Dex-HEMA DS 8%	Dex-HEMA DS 12%	Dex-HEMA DS 16%
$A_3D_{10}8$	3	–	10	–	–
$A_3D_{15}8$	3	–	15	–	–
$A_3D_{20}8$	3	–	20	–	–
$A_3D_{10}12$	3	–	–	10	–
$A_3D_{20}12$	3	–	–	20	–
$A_3D_{10}16$	3	–	–	–	10
$A_3D_{20}16$	3	–	–	–	20
A_3Dex	3	20	–	–	–
Alg_3-Ca	3	–	–	–	–
$D_{10}8$	–	–	10	–	–
$D_{20}8$	–	–	20	–	–
$D_{10}12$	–	–	–	10	–
$D_{20}12$	–	–	–	20	–
$D_{10}16$	–	–	–	–	10
$D_{20}16$	–	–	–	–	20

2.2.6. Hydrogel swelling and degradation

IPNs of Alg-Ca and dex-HEMA were weighed and transferred into pre-weighed glass vials. Next, 6 ml of 100 mM HEPES buffer (pH 7.4) with 0.02% NaN_3 was added and the gels were incubated at 37 °C. The hydrogels were weighed at regular time intervals after removal of the excess of buffer, until their complete dissolution in the incubation buffer [24]. After each measurement, 6 ml of fresh buffer was added. The hydrogel swelling is defined as the ratio W_t/W_0, where W_0 and W_t are the hydrogel weight after preparation and at time t, respectively. The swelling/degradation experiments were performed in duplicate.

IPNs were also incubated in 6 ml of EDTA (70 mM) in HEPES buffer pH 7.4 for 48 h, changing the buffer three times per day. Subsequently, the IPNs were weighed.

2.2.7. Protein release

Hydrogels with a BSA concentration of 10 mg ml^{-1} were prepared as described above. The obtained IPNs were transferred into 20 ml glass vials and 6 ml of 100 mM HEPES buffer pH 7.4 plus 0.02% NaN_3 was added and the IPNs were incubated at 37 °C. Samples of the release medium (1 ml) were taken at appropriate time intervals (the first day after 1, 3, 5, and 24 h, and subsequently once a day) and replaced by an equal volume of fresh buffer. The protein concentration in the different samples was determined by using an Acquity UPLC trade, BEH C18 1.7 μm, 2.1 × 50 mm column and a UV detector working at 210 nm. An eluent gradient, from 0% to 100% of eluent A was used, where A is H_2O/acetonitrile/perchloric acid (95/5/0.1%) and eluent B is acetonitrile/perchloric acid (100/0.1%).The injection volumes of the samples were 7.5 μl and the flow rate was 0.25 ml min^{-1}. For an appropriate comparison, BSA release from Alg-Ca hydrogels and from dex-HEMA hydrogels was followed.

2.2.8. Cell culture experiments

Full thickness healthy articular cartilage was obtained from the femoropatellar joints of fresh equine cadavers (n = 2, age: 2 years) under aseptic conditions [25]. Cartilage was digested overnight using 0.15% collagenase type II at 37 °C. The cell suspension was filtered through a 100 μm cell strainer and washed with PBS. Cells were resuspended in chondrocyte expansion medium, consisting of DMEM, supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10 ng ml^{-1} βFGF. Cells were then counted and seeded in 175 cm^3 culture flasks at a density of 5000 cells cm^{-2} in expansion medium and maintained in a humidified incubator (37 °C, 5% CO_2). After the first passage (t = 10 days), the chondrocytes were detached using 0.25% trypsin and embedded into the alginate/dex-HEMA solutions. Expanded chondrocytes were combined with IPNs ($A_3D_{10}8$, $A_3D_{10}12$ and $A_3D_{10}16$) at a density of 1.0×10^7 cells ml^{-1}. For IPN bead cultures, the polymer solution, containing Irgacure, was dripped with a 23G needle into 100 mM $CaCl_2$ solution supplemented with 10 mM HEPES pH 7.4. Subsequently, IPNs were formed by 5 min of UV exposure of the beads. The resulting IPN beads were then cultured in differentiation medium (DMEM supplemented with 0.2 mM

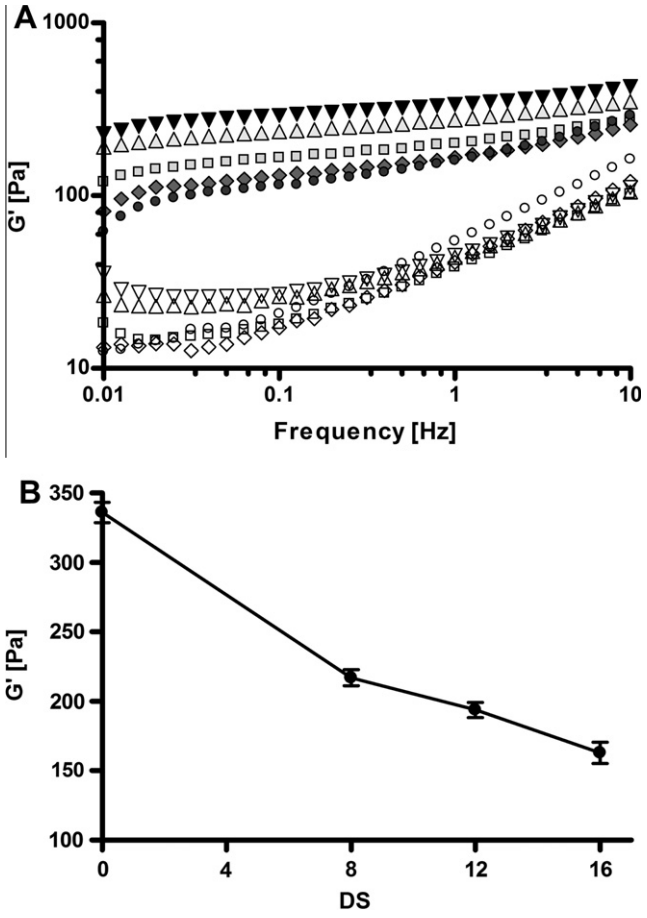

Fig. 2. (A) Storage modulus G' and loss modulus G'' as a function of frequency for (▼) A_3Dex; (Δ) Alg_3-Ca; (■) $A_3D_{10}8$; (♦) $A_3D_{15}8$; (●) $A_3D_{20}8$. (B) Storage modulus G' at 1 Hz of semi-IPNs of Alg-dex-HEMA as a function of the DS of dex-HEMA at a fixed dex-HEMA concentration of 20% (w/v).

ascorbic acid 2-phosphate, 0.5% human serum albumin, 1× ITS-X, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and 5 ng ml^{-1} TGF-β2) for up to 4 weeks. Medium was replaced every 2–3 days.

2.2.9. Cell viability and differentiation

Live/dead viability assay (Molecular Probes MP03224, Eugene, USA) was performed according to the manufacturer's recommendations. The samples were examined using a light microscope (Olympus, BX51, USA) and photomicrographs were taken with an Olympus DP70 camera (USA). The excitation/emission filters were set at 488/530 nm to observe living (green) cells and at 530/580 nm to detect dead (red) cells.

For the evaluation of cellular differentiation, samples were dehydrated through graded ethanol series, cleared in xylene and embedded in paraffin. Embedded samples were sectioned to yield 5 μm sections, which were stained using either haematoxylin and eosin or a triple stain of haematoxylin, fast green FCF (0.001% w/v) and safranin O (0.1% w/v). The sections were examined using a light microscope (Olympus, BX51, USA) and photomicrographs taken with an Olympus DP70 camera (USA). For immunolocalization of collagen type II, sections were rehydrated and endogenous peroxidase activity was blocked using a 0.3% H_2O_2 solution for 10 min. Samples were then washed with PBS/Tween 20 (0.1%) for 5 min, blocked with 5% BSA in PBS for 30 min and incubated overnight with anti-collagen type II (1:100, II-6B3II, Developmental Studies Hybridoma Bank, USA) antibodies. Samples were then incubated for 60 min with the secondary HRP-conjugated goat anti-mouse antibody (1:200, Dako, USA). Staining was visualized using DAB solution for 10 min. Counterstaining was performed with hematoxylin. The sections were examined using a light microscope. Isotype controls were performed by using mouse isotype IgG1 monoclonal antibody at concentrations similar to those used for the stainings.

3. Results and discussion

3.1. Physico-chemical characterization of hydrogels

In Fig. 2A the mechanical spectra of Alg-Ca gel as well as semi-IPNs composed of Alg-Ca and dextran or dex-HEMA (DS = 8, different polymer concentrations) are reported. G' and G'' for the different samples showed a slight dependence from frequency; moreover, the storage moduli were higher than the viscous ones in the whole range of analyzed frequencies and quite parallel each other, thus demonstrating that the semi-IPNs behave like weak hydrogels. The formulation behaves as a plastic system, i.e. a gel at low shear forces, but a liquid at high shear forces due to reversible breaking of the cross-links. Fig. 2A also shows that when dextran was dissolved in the Alg-Ca network a slight increase in G' was observed, which might be explained by the higher polymer concentration of the semi-IPN compared to the Alg-Ca gel. Fig. 2B clearly shows that, at fixed dex-HEMA concentration, G' decreased with increasing degree of HEMA substitution. The decrease in G' of the Alg-Ca/dex-HEMA semi-IPNs with increasing dex-HEMA concentration (Fig. 2A) and increasing DS suggests a disturbing effect of the HEMA moieties on the "egg-box" of the Alg-Ca network, as also found in a previous study on semi-IPNs based on Alg-Ca and another methacrylate derivative of dextran [19]. Because of the low hydrogel strength, the semi-IPNs allow extrusion through a needle, which is an important handling property for in situ hydrogel-forming applications.

The semi-IPNs can be transformed into IPNs by means of UV exposure in the presence of a photoinitiator. In Fig. 3, the G' behavior upon UV irradiation of the $A_3D_{10}8$, $A_3D_{15}8$ and $A_3D_{20}8$ samples are shown. The storage moduli, G', of the samples increased very rapidly when the UV light was switched on (after 180 s from the start of the experiment), reaching an upper plateau level within 10 s. In this short time, the HEMA groups polymerize upon irradiation, leading to a chemically cross-linked dex-HEMA network entangled in the Alg-Ca physical network. It should be emphasized that UV polymerization resulted in an increase of the elastic moduli of the samples of more than one order of magnitude. Fig. 3 also shows that, as expected, the higher the dex-HEMA concentration, the higher the G' values are, as the cross-link density increases.

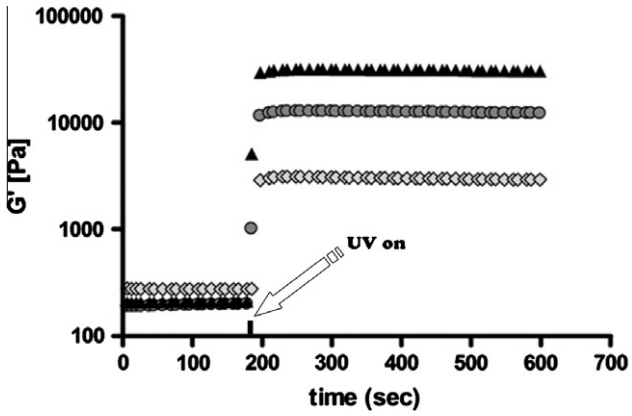

Fig. 3. Storage modulus, G', at 1 Hz, of gels: (♦) $A_3D_{10}8$; (●) $A_3D_{15}8$; (▲) $A_3D_{20}8$ as a function of time. UV irradiation was started at 180 s, as indicated by the arrow.

We investigated whether the hydrogel strength can be modulated by varying the DS of dex-HEMA. The values of the Young moduli, E', at 1 Hz, of different IPNs are reported in Fig. 4; for comparison, E' of the dex-HEMA hydrogels are also reported. Fig. 4 shows that the IPNs had higher E' values than the corresponding dex-HEMA hydrogels. As the polymer concentration increases the E' values increase correspondingly, as also seen for G' (Fig. 3). It should be emphasized that the hydrogel strengths of the IPNs are greater than the sum of the dex-HEMA and Alg-Ca moduli. It suggests a synergistic interaction between the two interpenetrating polymer networks.

The homogeneity of the IPNs was investigated by soaking the IPNs in an EDTA solution which results in dissolution of the Alg-Ca network. DMA analysis showed that the EDTA treatment resulted in a substantial drop in E' (e.g. from 80 to 45 kPa for the $A_3D_{20}12$ IPN). Importantly, the resulting weaker hydrogels maintained the original shape, indicating that a homogeneous chemical network of dex-HEMA was formed after UV curing.

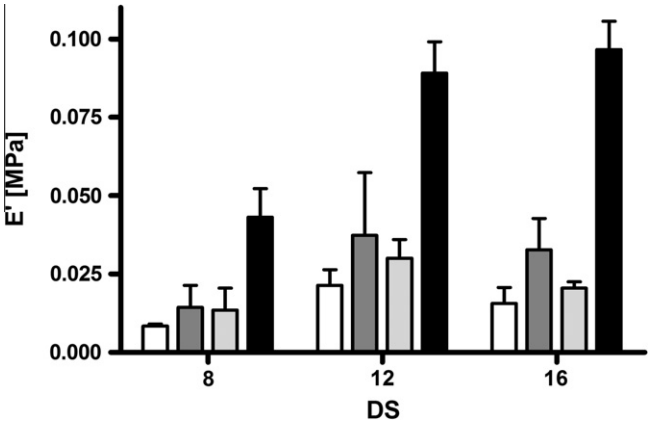

Fig. 4. Young's moduli (E') at 1 Hz of different hydrogels: (□) D_{10}; (■) A_3D_{10}; (■) D_{20}; (■) A_3D_{20}. E' of Alg-Ca is not reported because this hydrogel was too weak.

UPLC analysis showed that the methacrylate conversion for both the dex-HEMA hydrogels and the IPNs was >95%, indicating that the UV-photopolymerization was very effective and, importantly, the Alg-Ca network did not inhibit the polymerization of the HEMA moieties.

3.2. Hydrogel swelling and degradation

Fig. 5A shows that at pH 7.4 and 37 °C , the $A_3D_{10}8$ IPN swelled substantially (almost three times its original weight within 8 days) while the solvent absorption of the $D_{10}8$ hydrogel was lower (two times weight increase in 10 days, Fig. 5B). The higher swelling of the IPN can be ascribed to the higher water-absorbing capacity of Alg-Ca network, which is more hydrophilic than the dex-HEMA hydrogel. Fig. 5A and B also shows that this effect is more pronounced for IPNs with a low DS. In previous studies, it has been reported that at high DS, dex-HEMA gels hardly show swelling due to the relatively high cross-link density [26]. Obviously, in such highly cross-linked hydrogels the Alg-Ca had a low contribution to the swelling capacity of the IPNs.

Fig. 5A shows that the IPNs degradation is influenced by the polymer concentration and the DS of the dex-HEMA. $A_3D_{10}8$ and $A_3D_{20}8$ IPNs showed complete degradation in about 15–18 days, depending on the polymer concentration. The $A_3D_{12}10$ and $A_3D_{12}20$ IPNs fully degraded in about 30 and 60 days, respectively, while the IPNs with the highest DS degraded in 70–180 days. As expected, increasing both the dex-HEMA concentration and the DS resulted in increased degradation times, which can be attributed to the higher cross-link density of the IPNs [22,27]. It appears that the degradation time of the IPNs and the corresponding dex-HEMA hydrogels is about the same. The presence of the alginate network therefore does not affect the degradation rate of the hydrolytically sensitive carbonate esters in the cross-links of the chemical dex-HEMA network.

3.3. Protein release

Fig. 6A shows that the $A_3D_{10}16$ and $A_3D_{20}16$ IPNs displayed a sustained release of BSA for more than 300 h. Surprisingly, the protein showed the same release kinetics in both hydrogels, while a slower release was expected for the IPN with the higher dex-HEMA concentration. Both hydro gels however show a considerable swelling (Fig. 6A, insert), which obviously resulted in an IPN in which the pores are presumably larger than the hydrodynamic diameter of the protein molecule, allowing its free diffusion in the IPN. The release curves were fitted to the Ritger–Peppas equation [28]:

$$M^t/M^\infty = kt^n$$

where M^t/M^∞ represents the fractional release of the loaded protein, k is a kinetic constant, t is the release time and n is the diffusional exponent. Analysis showed that the n-value was 0.46, demonstrating that the release is essentially governed by Fickian diffusion, supporting the suggestion that the pores of the IPNs are larger than the hydrodynamic diameter of BSA.

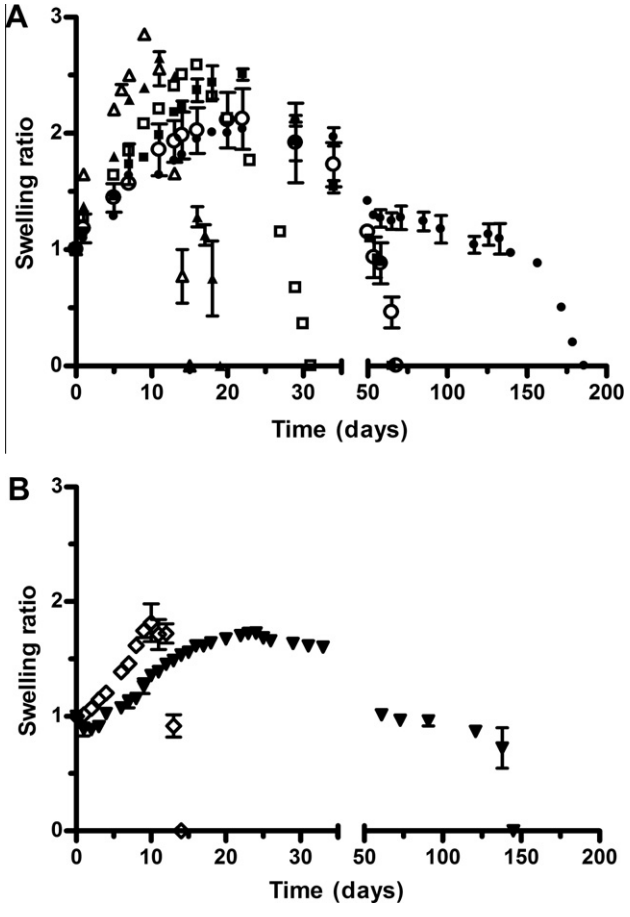

Fig. 5. Swelling and degradation profiles of (A) IPNs (▲) $A_3D_{10}8$; (▲) $A_3D_{20}8$; (□) $A_3D_{10}12$; (■) $A_3D_{20}12$; (○) $A_3D_{10}16$; (●) $A_3D_{20}16$; (B) dex-HEMA hydrogels (◇) $D_{10}8$; (▼) $D_{20}16$, in 100 mM HEPES buffer at 37 °C, pH 7.4.

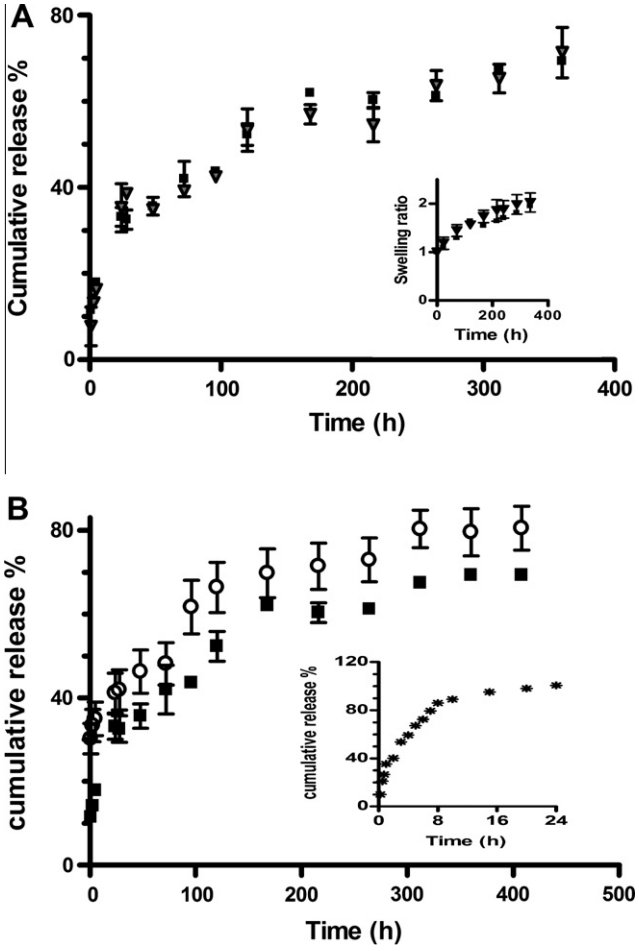

Fig. 6. BSA release in HEPES buffer, 100 mM, pH 7.4 at 37 °C: (A) (▼) $A_3D_{10}16$ and (■) $A_3D_{20}16$; and (B) (○) $D_{20}16$ and (■) $A_3D_{20}16$.

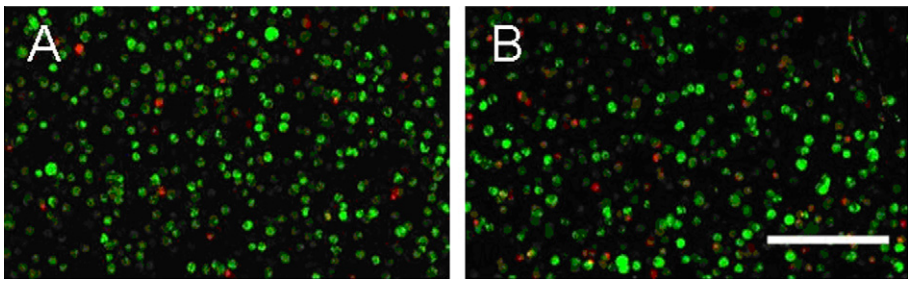

Fig. 7. Cell viability assay (stains live cells green and dead cells red), of $A_3D_{10}8$ (A) and $A_3D_{10}16$ (B) chondrocyte-seeded IPN hydrogels after 4 days of in vitro culture. Scale bar represents 100 μm.

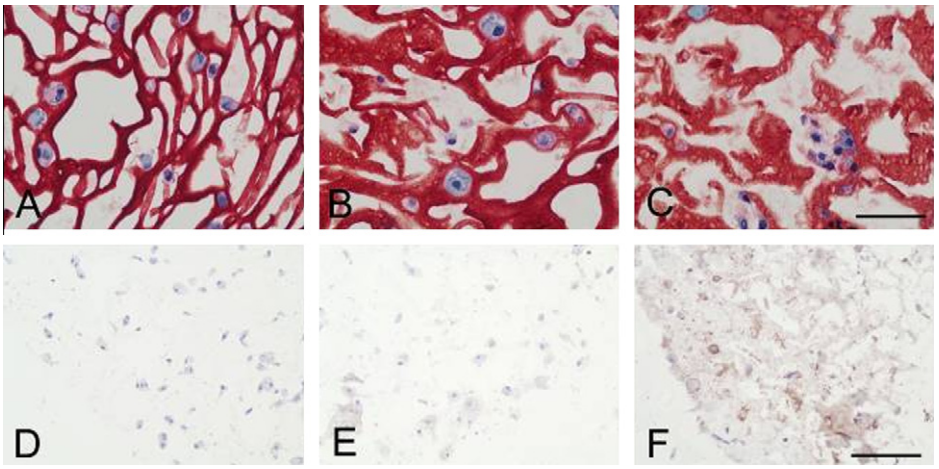

Fig. 8. Safranin O staining (A, B, and C; stains proteoglycans red) and immunolocalization of chondrogenic marker collagen type II (D, E, and F; brown) of $A_3D_{10}8$ (A and D), $A_3D_{10}12$ (B and E) and $A_3D_{10}16$ (C and F) chondrocyte-seeded IPN hydrogels after 4 weeks of in vitro culture. Scale bar represents 50 μm (A–C) or 100 μm (D–F).

In Fig. 6B, the release profiles of the BSA from $D_{20}16$, Alg_3-Ca and $A_3D_{20}16$ are reported. The first mentioned hydrogel showed a burst release of about 35%, which might be explained by the observation that after polymerization the formed hydrogel expelled some water. The Alg_3-Ca, instead, showed a low burst whereas a complete protein release was observed in 24 h. The protein release from the IPN is a superposition of the behavior of the IPN "building blocks" (Alg-Ca and dex-HEMA hydrogels). In fact, the$A_3D_{20}16$ IPN showed a low burst effect, as the Alg-Ca network, and a slow release, governed by the dex-HEMA network.

3.4. Cell encapsulation experiments

Cell therapy is currently clinically applied, for example, to treat cartilage defects [29]. Such technologies require the in vitro expansion of the patient's cells (chondrocytes) to obtain sufficient numbers to treat the defect. During the expansion, chondrocytes lose their chondrogenic phenotype, which is termed dedifferentiation. This process is associated with the acquisition of a fibroblast-like appearance, the loss of proteoglycan production and a switch in collagen synthesis from type II to type I [30]. Therefore, to evaluate cytocompatibility and chondrogenic behavior of the IPNs, in vitro expanded chondrocytes were seeded and cultured in beads of a selected number of compositions of IPNs ($A_3D_{10}8$, $A_3D_{10}12$ and $A_3D_{10}16$). Upon encapsulation of the chondrocytes in the IPN beads, a uniform distribution of cells was observed (Fig. 7). After 4 days of culture, cell viability in the hydrogels was approximately 60–70%, regardless of the composition (Fig. 7). No difference in cell survival between photopolymerized and non-photopolymerized groups was observed (data not shown), which is in line with previously published reports [31,32].

The IPNs preserved their shape for the entire course of culture (4 weeks). It should, however, be specified that this period is longer than the degradation time described in this paper (Fig. 5A) because of the higher Ca^{2+} concentration used to prepare these gels. With culture time, increasing numbers of cell clusters were observed in the beads with increasing DS (Fig. 8A–C). In addition, the embedded cells started to demonstrate signs of redifferentiation, as was revealed by histological examination. The dedifferentiated chondrocytes regained their original round morphology (Fig. 8A–C) and areas of positive safranin O staining were observed around the cells and cell clusters, indicating the synthesis of new proteoglycan-rich matrix (Fig. 8A–C). Most intense staining for proteoglycans was observed around cell clusters in the $A_3D_{10}16$ beads (Fig. 8C). It should be noted that the IPN also stains positive for safranin O, in line with previous reports on Alg-containing hydrogels [33,34]. Positive staining for collagen type II, an additional commonly used marker of chondrogenic differentiation, was also found (Fig. 8D–E) and was again most intense around the cells embedded in $A_3D_{10}16$ (Fig. 8E).

4. Conclusions

This paper reports on a novel class of hydrogels based on the interpenetration of two polysaccharide networks. It is shown that the semi-IPNs and the IPNs based on Alg-Ca and dex-HEMA are suitable for in situ hydrogel-forming applications. The semi-IPNs formed by dex-HEMA chains, with DS 8, 12 or 16, interfered with the Alg-Ca network, resulting in gel formulations with good flow

properties that can be easily injected. These viscous semi-IPNs can be transformed into elastic IPNs using UV polymerization of dex-HEMA. Furthermore, due to the hydrolytically sensitive carbonate esters in the chemical dex-HEMA network, the IPNs were fully degradable and their degradation time can be tailored by varying the dex-HEMA concentration and/or dex-HEMA DS. The IPNs showed a sustained release of BSA for more than 300 h. The IPN showed in contrast to the dex-HEMA gel, a very small burst release. Finally, IPN beads seeded with equine chondrocytes showed good cell survival and differentiation. They can facilitate chondrogenic differentiation, which is influenced by the DS. The IPNs based on Alg-Ca and dex-HEMA have potential for applications in regenerative medicine. In particular, they can be further optimized to enhance specific tissue formation by embedded cells by, for example, the incorporation of specific binding sequences [35] or the controlled release of bioactive compounds. Taken together, the IPNs described in this paper are promising systems as injectable in situ forming hydrogels for protein delivery and tissue engineering applications.

Acknowledgments

Financial support from FIRB, "Fondo per gli Investimenti della Ricerca di Base, Research Program: Ricerca e Sviluppo del Farmaco" (CHEM-PROFARMA-NET), Grant No. RBPR05NWWC_003 is acknowledged. The collagen type II monoclonal antibody (developed by T.F. Linsenmayer) was obtained from the Developmental Studies Hybridoma Bank. Dr J. Malda is supported by VENI Fellowship from the Dutch Technology Foundation STW, Applied Science Division of NWO and the Technology Program of the Ministry of Economic Affairs.

References

[1] Brandl F, Kastner F, Gschwind RM, Blunk T, Teßmar J, Göpferich A. Hydrogel-based drug delivery systems: comparison of drug diffusivity and release kinetics. J Controlled Release 2010;142(2):221–8.
[2] King VR, Alovskaya A, Wei DYT, Brown RA, Priestley JV. The use of injectable forms of fibrin and fibronectin to support axonal ingrowth after spinal cord injury. Biomaterials 2010;31(15):4447–56.
[3] Vermonden T, Fedorovich NE, Van Geemen D, Alblas J, Van Nostrum CF, Dhert WJA, et al. Photopolymerized thermosensitive hydrogels: synthesis, degradation, and cytocompatibility. Biomacromolecules 2008;9:919–26.
[4] Aimetti AA, Machen AJ, Anseth KS. Poly(ethylene glycol) hydrogels formed by thiol-ene photopolymerization for enzyme-responsive protein delivery. Biomaterials 2009;30(30):6048–54.
[5] Rinaudo M. Main properties and current applications of some polysaccharides as biomaterials. Polym Int 2008;57:397–430.
[6] Coviello T, Matricardi P, Marianecci C, Alhaique F. Polysaccharide hydrogels for modified release formulations. J Controlled Release 2007;119:5–24.
[7] Van Tomme SR, Storm G, Hennink WE. In situ gelling hydrogels for pharmaceutical and biomedical applications. Int J Pharm 2008;355:1–18.
[8] Tan H, Ramirez CM, Miljkovic N, Li H, Rubin JP, Marra KG. Thermosensitive injectable hyaluronic acid hydrogel for adipose tissue engineering. Biomaterials 2009;30:6844–53.
[9] Kretlow JD, Klouda L, Mikos AG. Injectable matrices and scaffolds for drug delivery in tissue engineering. Adv Drug Deliv Rev 2007;59:263–73.
[10] Zhao L, Weir MD, Xu HHK. An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. Biomaterials 2010;31(25):6502–10.
[11] Cadée JA, van Luyn MJA, Brouwer LA, Plantinga JA, van Wachem PB, de Groot CJ, et al. In vivo biocompatibility of dextran-based hydrogels. J Biomed Mater Res 2000;50:397–404.
[12] Burdick JA, Anseth KS. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. Biomaterials 2002;23(22):4315–23.
[13] Toh WS, Lee EH, Guo X-M, Chan JKY, Yeow CH, Choo AB, et al. Cartilage repair using hyaluronan hydrogel-encapsulated human embryonic stem cell-derived chondrogenic cells. Biomaterials 2010;31(27):6968–80.
[14] Liu Y, Chan-Park MB. Hydrogel based on interpenetrating polymer networks of dextran and gelatin for vascular tissue engineering. Biomaterials 2009;30:196–207.
[15] Ding F, Hsu SH, Wu DH, Chiang WY. Drug release from interpenetrating polymer networks based on poly(ethylene glycol) methyl ether acrylate and gelatin. J Biomater Sci, Polym Ed 2009;20:605–18.
[16] Kulkarni RV, Sa B. Novel pH-sensitive interpenetrating network hydrogel beads of carboxymethylcellulose-(polyacrylamide-grafted-alginate) for controlled release of ketoprofen: preparation and characterization. Curr Drug Deliv 2008;5:256–64.
[17] Suri S, Schmidt CE. Photopatterned collagen–hyaluronic acid interpenetrating polymer network hydrogels. Acta Biomater 2009;5:2385–97.
[18] Bajpai AK, Shukla SK, Bhanu S, Kankane S. Responsive polymers in controlled drug delivery. Prog Polym Sci 2008;33:1088–118.
[19] Matricardi P, Pontoriero M, Coviello T, Casadei MA, Alhaique F. In situ cross-linkable novel alginate–dextran methacrylate IPN hydrogels for biomedical applications: mechanical characterization and drug delivery properties. Biomacromolecules 2008;9:2014–20.
[20] Pescosolido L, Miatto S, Di Meo C, Cencetti C, Coviello T, Alhaique F, et al. Injectable and in situ gelling hydrogels for modified protein release. Eur Biophys J 2010;39:903–9.
[21] Van-Dijk-Wolthuis WNE, Tsanga SKY, Kettenes-van den Bosch JJ, Hennink WE. A new class of polymerizable dextrans with hydrolyzable groups: hydroxyethyl methacrylated dextran with and without oligolactate spacer. Polymer 1997;38(25):6235–42.
[22] Van-Dijk-Wolthiuis WNE, Van Steenbergen MJ, Underberg WJM, Hennink WE. Degradation kinetics of methacrylated dextrans in aqueous solution. J Pharm Sci 1997;86(4):413–7.
[23] Stenekes RJH, Hennink WE. Polymerization kinetics of dextran-bound methacrylate in an aqueous two phase system. Polymer 2000;41:5563–9.
[24] Censi R, Vermonden T, Van Steenbergen MJ, Deschout H, Braeckmans K, De Smedt SC, et al. Photopolymerized thermosensitive hydrogels for tailorable diffusion-controlled protein delivery. J Controlled Release 2009;140(3):230–6.
[25] Schuurman W, Gawlitta D, Klein TJ, Hoope Wt, van Rijen MHP, Dhert WJA, et al. Zonal chondrocyte subpopulations reacquire zone-specific characteristics during in vitro redifferentiation. Am J Sports Med 2009;37(Suppl. 1):97S–104S.
[26] Hennink WE, Talsma H, Borchert JCH, De Smedt SC, Demeester J. Controlled release of proteins from dextran hydrogels. J Controlled Release 1996;39(1):47–55.
[27] van Dijk-Wolthuis WNE, Hoogeboom JAM, van Steenbergen MJ, Tsang SKY, Hennink WE. Degradation and release behavior of dextran-based hydrogels. Macromolecules 1997;30(16):4639–45.
[28] Ritger PL, Peppas NA. A simple equation for description of solute releaseII. Fickian and anomalous release from swellable devices. J Controlled Release 1987;5(1):37–42.
[29] Bekkers JEJ, Inklaar M, Saris DBF. Treatment selection in articular cartilage lesions of the knee. Am J Sports Med 2009;37(Suppl. 1):148S–55S.
[30] von der Mark K, Gauss V, von der Mark H, Muller P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 1977;267(5611):531–2.
[31] Fedorovich NE, Swennen I, Girones J, Moroni L, van Blitterswijk CA, Schacht E, et al. Evaluation of photocrosslinked lutrol hydrogel for tissue printing applications. Biomacromolecules 2009;10:1689–96.
[32] Bryant SJ, Nuttelman CR, Anseth KS. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. J Biomater Sci, Polym Ed 2000;11:439–57.
[33] Ma HL, Chen TH, Low-Tone Ho L, Hung SC. Neocartilage from human mesenchymal stem cells in alginate: implied timing of transplantation. J Biomed Mater Res A 2005;74(3):439–46.
[34] Chubinskaya S, Huch K, Schulze M, Otten L, Aydelotte MB, Cole AA. Gene expression by human articular chondrocytes cultured in alginate beads. J Histochem Cytochem 2001;49(10):1211–20.
[35] Lee HJ, Yu C, Chansakul T, Hwang NS, Varghese S, Yu SM, et al. Enhanced chondrogenesis of mesenchymal stem cells in collagen mimetic peptide-mediated microenvironment. Tissue Eng A 2008;14(11):1843–51.