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ORIGINAL PAPER

Injectable and in situ gelling hydrogels for modified protein release

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Abstract A novel injectable polysaccharide system based on calcium Alginate (Ca-Alg) hydrogel and two Dextran methacrylate derivatives (DexMA) was recently developed. The resulting Interpenetrating Polymer Network showed a synergistic mechanical behavior that can be exploited to target the hydrogel properties towards specific biomedical needs. In the present paper, hydrogels composed of 3% (w/v) Ca-Alg and Dextran (Mw 40×10^4 and 500×10^4), derivatized with methacrylic groups (derivatization degrees 5 and 30%) at concentrations 5% (w/v), were characterized. The data reported here evidenced that Mw and derivatization degree of Dex chains can deeply affect the mechanical as well a model protein (Horseradish peroxidase) delivery rate. The enzymatic activity of such model protein was never significantly altered by the adopted experimental conditions.

Keywords Alginate · Dextran derivatives · Interpenetrating Polymer Network · Protein delivery · Rheology

Introduction

In the last years, injectable formulations that form macroscopic hydrogels at the site of injection have been developed for a broad range of pharmaceutical and biomedical applications because of their high patient compliance and ease of manipulation. Many routes can be adopted to reach this goal: temperature responsive polymers, self-assembling systems or external stimuli responsive polymers (Klouda and Mikos 2008; Schmidt et al. 2008; Van Tomme et al. 2008a, b). In order to modulate the physico-chemical as well as the drug delivery properties of these hydrogels, an interesting approach can be also represented by the development of interpenetrating polymer networks (IPN) and semi-IPN. Furthermore, hydrogels obtained by mixing two different polymeric systems can lead to new materials that can show completely different properties, with respect to the starting polymers, due to a synergistic effect (Bajpai et al. 2008).

Within the above-depicted framework, polysaccharides can represent an opportunity because of their natural abundance, generally low cost, ease of manipulation, facile derivatization and quite general biocompatibility. Among polysaccharides alginates (Alg) possess a relevant interest for their numerous possible applications (Draget et al. 2002; Rehm 2002; Tønnesen and Karlsen 2002). Alg are a family of polyuronates, derived from algae or, more recently, from bioengineering source. They are copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked (1 → 4), containing homopolymeric regions of M or G (M and G blocks, respectively) and alternated MG blocks, extremely various in composition and monomer sequences. Alg are able to form physical hydrogels by simple addition of calcium ions to a water solution of such polymers; calcium ions, in fact, are able to interact with glucoronic acid moieties forming the so-called "egg-box" structures in which two adjacent macromolecules are linked by means of a physical bond.

Dextran (Dex) is another polysaccharide extensively employed in pharmaceutical and biomedical applications.

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Dextrans can be defined as glucose homopolysaccharides that feature a substantial number of consecutive α-(1 → 6) linkages in their main chains, usually more than 50% of the total linkages (Coviello et al. 2007). These α-D-glucans possess also side chains stemming from α-(1 → 2), α-(1 → 3), or α-(1 → 4) branch linkages. Dextrans and their derivatives are among the main promising candidates for the preparation of networks capable of giving a sustained release of proteins. In particular, functionalizing the polymer by introducing reactive groups, such as methacrylate moieties in the side chain; the obtained DexMA derivatives can be crosslinked by means of UV irradiation, leading to chemical hydrogels. These hydrogels have been widely investigated and their biocompatibility has been assessed (Cadée et al. 2000; De Groot et al. 2001). Recently, it was described a new injectable hydrogel IPN-based on Ca-Alg and DexMA derivatives (Matricardi et al. 2008) that showed improved properties with respect each of hydrogel component.

The aim of the present work is the characterization in terms of mechanical and protein delivery properties, of new IPNs based on Ca-Alg and DexMA derivatives. Two IPN systems are compared:

1. $AlgDex_{40}MA_5$ = Dex (Mw 40×10^4), with a methacrylate substitution 5% at a 5% (w/v) concentration (Cp), interpenetrated with Ca-Alg hydrogel (Alg Cp 3% (w/v));
2. $AlgDex_{500}MA_{30}$ = Dex (Mw 500×10^4), with methacrylate substitution 30% at a Cp = 5% (w/v) interpenetrated with Ca-Alg hydrogel [Alg concentration Cp 3% (w/v)]

In the present work, we followed a different preparation procedure with respect the one previously described (Matricardi et al. 2008), thus obtaining IPN hydrogels at pH 7.0, in physiological solutions [NaCl 0.9% (w/v)], and with a reduced content of $CaCl_2$, in order to increase the biocompatibility of the novel IPNs.

The rheological and protein release data collected, evidenced the versatility of the new IPN hydrogel systems and their suitability for protein delivery as well as for tissue engineering applications.

Experimental

Materials

Dex samples from *Leuconostoc* ssp with Mw 40×10^3 (Dex_{40}) and 500×10^3 (Dex_{500}), 4-*N*,*N*-dimethylaminopyridine (4-DMAP), glycidyl methacrylate (GMA), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and silicon oil AP 150 Wacker were Fluka products. Sodium Alg salt, (70% L-guluronic acid and 30% D-mannuronic acid), Irgacure 2959 (2-hydroxy-4′-(2-hydroxyetoxy)-2-methyl-propiophenone) and horseradish peroxidase type I (HRP) was provided by Sigma. Quick Start Bradford protein assay kit was from Bio Rad. All other chemicals were analytical grade.

Synthesis of methacrylated dextran

Methacrylated derivatives of dextrans (DexMA) were synthesized by modifying Dex samples according to the methodology previously described (De Smedt et al. 1995; Van Dijk-Wolthuis et al. 1995, 1997). Different amounts of GMA were added in order to obtain two degrees of substitution (DS): 5 for Dex_{40} and 30 for Dex_{500}. Briefly 5 g of Dex were dissolved in 50 ml of anhydrous DMSO and 1 g of DMAP (4-dimethylaminopyridine) was than added. After the DMAP dissolution, GMA was added to the solution and the reaction was carried out at room temperature for 48 h. pH was then adjusted to 8 with HCl to neutralize DMAP and the solution was exhaustively dialyzed against distilled water at 4°C, lyophilized and placed in an air-tight container, protected from light. DS of the samples were determined by proton nuclear magnetic resonance (^{1}H-NMR) recorded in D_2O with a Bruker AC-400 instrument.

Hydrogels preparation

The IPN hydrogels were prepared following a three-step procedure: (1) polymer solution preparation, (2) semi-IPN formation by addition of $CaCl_2$ (i.e Ca-Alg hydrogel formation) and (3) IPN hydrogel formation by photocross-linking the DexMA derivatives.

All obtained IPNs had an Alg Cp of 3% (w/v) and a DexMA Cp of 5% (w/v).

1. *Polymer solution*: the polymer solution was prepared by dissolving Alg in distilled water. After complete solubilization, DexMA was added. For the preparation of the IPN aimed to the evaluation of release properties, horseradish peroxidase was also added during Alg dissolution.
2. *Semi-IPN formation*: the initiator Irgacure 2959, which allows in the third step a rapid photo-crosslink reaction of Dex-MA which avoids loaded protein degradation, was added to the polymer solution (Williams et al. 2005) (15 µl of a 33% (w/v) solution for each ml of polymer solution 1). $CaCl_2$ 0,025 M and NaCl 0.9% (w/v) solution were then added. The obtained semi-IPN hydrogels were sterilized by autoclaving at 121°C for 30 min.
3. *IPN hydrogel formation*: the semi-IPN has been finally irradiated with a mercury UV lamp (λ maximum

365 nm). Such irradiation allows the radical polymerization of DexMA and the IPN formation.

Rheological characterization

Rheological experiments were carried out by means of a controlled stress Haake RheoStress 300 Rotational Rheometer, provided with a Haake DC10 thermostat. Frequency sweep experiments were performed on the hydrogels at 25°C in the range 0.01–10 Hz, in the linear viscoelastic region, assessed by preliminary stress sweep experiments. The mechanical spectra were then recorded applying a constant deformation in the linear regime: usually a $\gamma = 0.01$ maximum deformation was used. In order to reduce the extent of the wall slippage phenomena (Lapasin and Pricl 1995) a grained plate-plate device (Haake PP35 TI: diameter = 35 mm) was used.

The hydrogels, with a thickness of 1.0–2.0 mm, were removed with the aid of a small spatula from the petri dish where they were prepared and were laid with care on the lower plate of the rheometer. The upper plate was then lowered until it reached the hydrogel surface. Gap setting optimizations have been undertaken according to the procedure described elsewhere (Kuijpers et al. 1999). A layer of silicon oil was poured on the lateral free hydrogel surface in order to prevent water evaporation.

Flow curves of the solutions and of the semi-IPN hydrogels were performed using a cone-plate geometry (Haake CP60Ti: diameter = 60 mm; cone = 1°; gap = 0.053 mm) in the range 0.001–1,000 s^{-1}. A stepwise increase of the stress was applied, with an equilibration time of 30 s. All measurements were made at 25°C.

Texture analysis

For the mechanical characterization of the hydrogels a software-controlled dynamometer, TA-XT2i Texture Analyzer (Stable Micro Systems, UK), with a 5 kg load cell, was used (Alves et al. 2000).

The compression modulus of the IPN hydrogels was measured at room temperature using a steel cylinder probe (P/35, diameter of 35 mm). The pre-test speed, the test speed and the post-test speed were set up at 1 mm/s; the trigger force was set up at 0.002 N. The hydrogel samples height was always in the range 4.5–5.0 cm. The Young's modulus was determined as described by Meyvis (Meyvis et al. 2002).

Injectability of the hydrogels

The injectability of the semi-IPN hydrogels was investigated using the TA-XT2i Texture Analyzer. The samples were introduced into syringes (5 ml) equipped with a needle of 18 Gauge (1.2 mm) by means of a spatula. The syringe was fixed between the lower plate and the steel cylinder probe. The experiments were carried out and recording the force needed to eject the hydrogel through the needle (Lee et al. 2006; Van Tomme et al. 2008a, b) at a plunger rate of 1 mm/s. The measurements were repeated nine times and the average of the plateau data was taken as the final value.

In vitro protein release

For the in vitro release of model proteins horseradish peroxidase type I (HRP) was used. The protein solution was added to the samples during the hydrogel preparation (step I) to a typical final concentration of 1 mg/ml. After the UV curing, the hydrogel loaded with the enzyme was immersed in 200 ml of 1 mM Hepes at 37°C. The solution was kept under constant magnetic stirring. Samples of 5 ml of the buffer solution were taken at regular time intervals and replaced by an equal volume of fresh buffer. The concentration of HRP was determined by means of Bradford Micro Assay, according to manufacturer's protocol, at λ 595 nm using a Perkin-Elmer, lambda 25, UV–Vis spectrophotometer with quartz cell (pathlength 1.0 cm). Standard protein solutions were prepared and analyzed to generate a calibration curve (Tang et al. 2002; Ginty et al. 2008). All the measurements were performed in triplicate and the obtained values always laid within 10% of the mean. HRP enzymatic activity was measured using H_2O_2 as oxidizing substrate and ABTS as reducing substrate; radical ABTS cation concentration was measured (Rojas-Melgarejo et al. 2004; La Rotta Hernandez et al. 2008). A 30 µl of sample was added to 3 ml of ABTS solution (4.14 mg ABTS in 100 ml of H_2O) in a spectrophotometric cell; 30 µl of H_2O_2 (0.034 mg/ml) were then added. The kinetic spectra were recorded for 5 min at $\lambda = 414$ nm. The values of enzyme activity were calculated according to Rojas-Melgarejo (Rojas-Melgarejo et al. 2004).

Results and discussion

In the present work, two IPN systems that can be proposed as in situ gelling systems have been taken into account in order to assess their physicochemical properties and their protein delivery capabilities. In particular, rheological and mechanical properties were determined as well as the ability to carry horseradish peroxidase without reducing significantly its enzymatic activity.

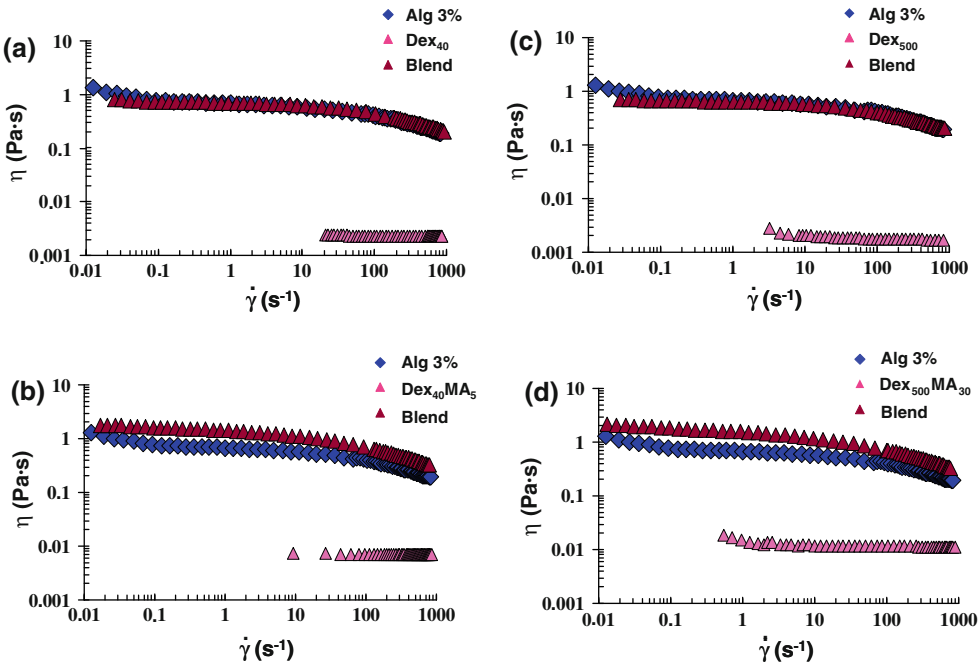

Fig. 1 Flow curves at 25°C of polymer solutions of **a** Alg 3% w/v (*filled diamond*), Dex_{40} 5% w/v (*filled triangle*), Blend Alg 3% w/v + Dex_{40} 5% w/v (*filled triangle*), **b** Alg 3% w/v (*filled diamond*), $Dex_{40}MA_5$ 5% w/v (*filled triangle*), Blend Alg 3% w/v + $Dex_{40}MA_5$ 5% w/v (*filled triangle*), **c** Alg 3% w/v (*filled diamond*), Dex_{500} 5% w/v (*filled triangle*), Blend Alg 3% w/v + Dex_{500} 5% w/v (*filled triangle*), **d** Alg 3% w/v (*filled triangle*), $Dex_{500}MA_{30}$ 5% w/v (*filled triangle*), Blend Alg 3% w/v + $Dex_{500}MA_{30}$ 5% w/v (*filled triangle*)

Rheology

Polymer solutions

In order to test the interactions in water solutions between Alg and DexMA in the experimental conditions adopted for the hydrogel formation, rheological experiments were performed both in steady shear and in oscillatory regime.

Flow curves were registered for 3% (w/v) Alg, 5% (w/v) $Dex_{40}MA_5$ and 5% (w/v) $Dex_{500}MA_{30}$; flow curves were also registered for the solutions prepared blending with 3% (w/v) Alg each of the two prepared DexMA. For an appropriate comparison, the same measurements were carried out with Dex_{40} and Dex_{500} alone, i.e. without chemical derivatization.

Flow curves, reported in Fig. 1, clearly evidence that the blends of Alg with Dex have the same viscosity (i.e. flow curve profile) of the solution of Alg, irrespective of the Mw of Dex. Indeed, the viscosity of Dex_{40} and Dex_{500} water solutions are comparable to the viscosity of the solvent. When methacrylate moieties are introduced on the Dex chains, the viscosity of the water solutions raised up significantly. In fact, the viscosity of water solutions of $Dex_{40}MA_5$ and $Dex_{500}MA_{30}$ increased more than one order of magnitude with respect to Dex_{40} and Dex_{500}.

Also, the blends with Alg showed an increase in the viscosity. This increase, that is a synergistic effect rather than a simple additive increase, can be ascribed to the interpenetrations of the polymer chains in semi dilute regime. In fact, C^*, i.e. the coil-overlap concentration between polymer coils, are in the range of 1.3–5% (w/v), as previously reported for analogous systems (e.g. $Dex_{40}M_{20}$ and $Dex_{500}MA_{20}$; Matricardi et al. 2008). It is to point out that for Dex_{40} and $Dex_{500,}$ the C^* is well above the adopted experimental concentration.

Semi-IPN

The addition of $CaCl_2$ to the blend of Alg and $Dex_{40}MA_5$ or $Dex_{500}MA_{30}$, as described in the experimental section, led to the formation of the semi-IPNs.

In order to measure the injectability of these new systems, flow curves were collected in the range 10^{-4}–10^3 s^{-1}. As evidenced in Fig. 2, a strong pseudoplastic behavior can be observed for the two semi-IPN systems $AlgDex_{40}MA_5$ and $AlgDex_{500}MA_{30}$, as the viscosity values span from 6×10^3 to 1 Pas in the experimental conditions. In particular, the viscosity at the shear rate usually adopted in the extrusion through an 18 G needle (10^2–10^3 s^{-1}) is comparable to that of an usual pharmaceutical formulation.

The injectability of the prepared semi-IPNs was also tested by means of a Texture Analyzer; using the "ad hoc" apparatus reported in Fig. 3a, the strength necessary to extrude the semi-IPNs through a needle (18 gauge)—the plateau region in Fig. 3b—was measured. Water and silicone were used for an appropriate comparison. In Fig. 4, data obtained are reported. As it can be seen, the data obtained clearly show the injectability of the semi-IPN, as the forces involved are of the same order of magnitude of silicone.

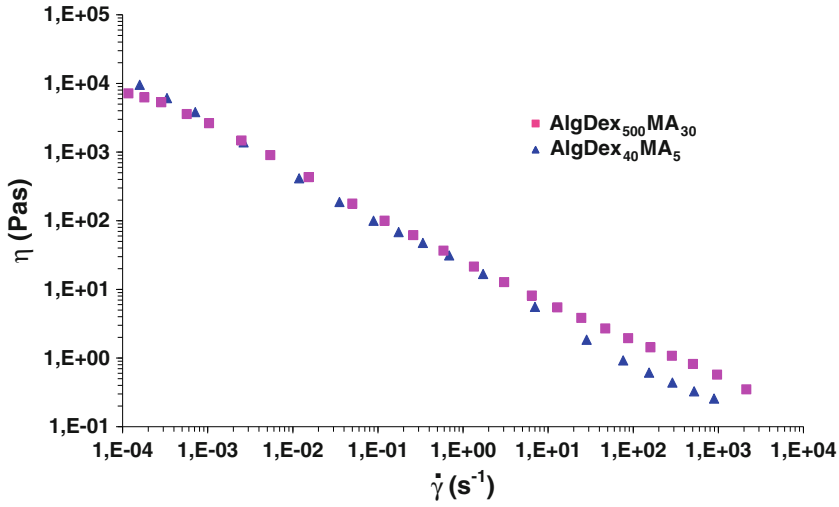

Fig. 2 Flow curves of semi-IPNs $AlgDex_{40}MA_5$ (*filled triangle*) and $AlgDex_{500}MA_{30}$ (*filled square*) recorded at 25°C

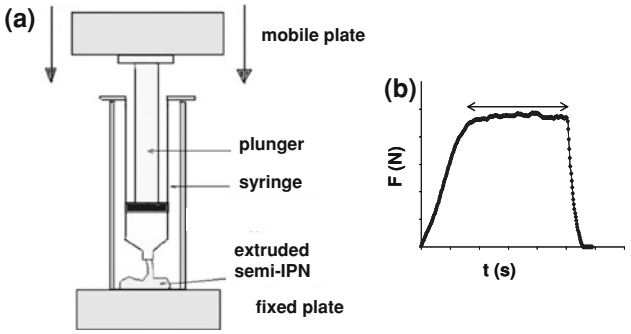

Fig. 3 **a** Schematic representation of the Texture Analyzer apparatus used for injectability tests. **b** A typical force/time profile obtained during the semi-IPN extrusion test

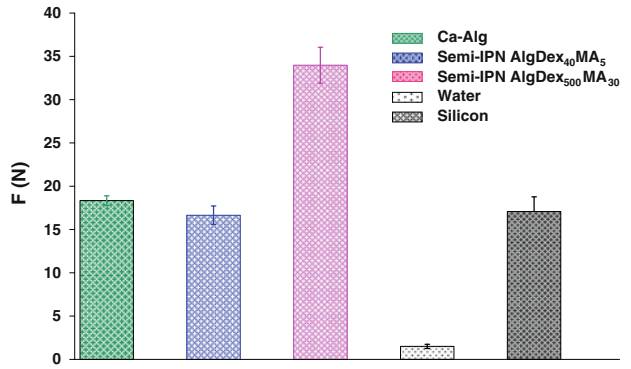

Fig. 4 Forces of extrusion of various samples. The values are arithmetic mean of nine different tests; standard deviations are reported

IPN

UV irradiation of the semi-IPNs leads to the IPNs. Mechanical spectra of the systems were recorded. In Fig. 5 the spectra of two IPN (10 min of UV irradiation) are reported. A typical hydrogel behavior is clearly evidenced, with G′ and G′′ (the elastic and the dissipative moduli) quite parallel and differing of roughly 1 order of magnitude.

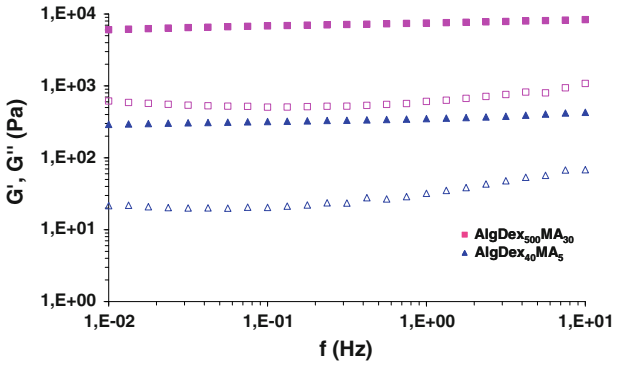

Fig. 5 Mechanical spectra of IPNs $AlgDex_{40}MA_5$ (*triangle*) and $AlgDex_{500}MA_{30}$ (*square*), obtained with 10 min of UV irradiation; G′ (*filled symbols*), G′′ (*open symbols*)

UV irradiation determines the extent of the cross-linking of the methacrylate moieties. In Fig. 6, the elastic modulus G′ at 1 Hz, that is proportional to the number of crosslinks in the matrices, increased for the two systems as the irradiation time increases and reaches a plateau after 10 min for $AlgDex_{500}MA_{30}$. After the same time, $AlgDex_{40}MA_5$ reaches a G′ value, that is lower more than one order of magnitude with respect to the previous reported sample, and double its value after about 30 min of irradiation. It must be pointed out that the adopted experimental conditions, i.e. the presence of the photoinitiator Irgacure and the power of the UV lamp, are chosen in order to increase the potential biocompatibility of the hydrogels with living cells (Nguyen and West 2002).

The Young modulus (E) of the hydrogels prepared at the lowest irradiation time (i.e. 10 min as above reported) were calculated. For an appropriate comparison, the Young modulus of the hydrogels obtained by photopolymerization, in the same experimental conditions, of $Dex_{40}MA_5$ and $Dex_{500}MA_{30}$, i.e. without Ca-Alg, were measured. The E value of Ca-Alg, in the same conditions of the IPN, was not measurable. In Fig. 7 obtained data are reported. As

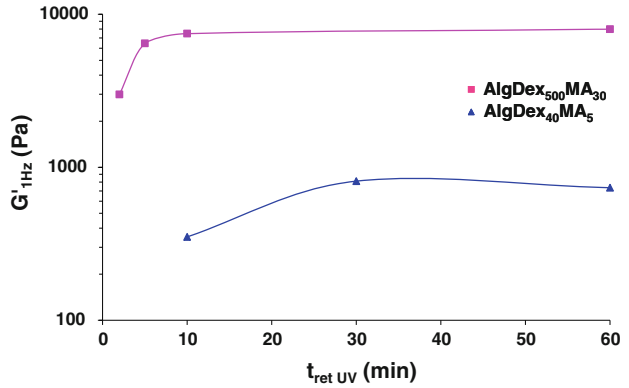

Fig. 6 Elastic moduli, G′, values at the frequency of 1 Hz, for IPNs obtained at different times of UV irradiation

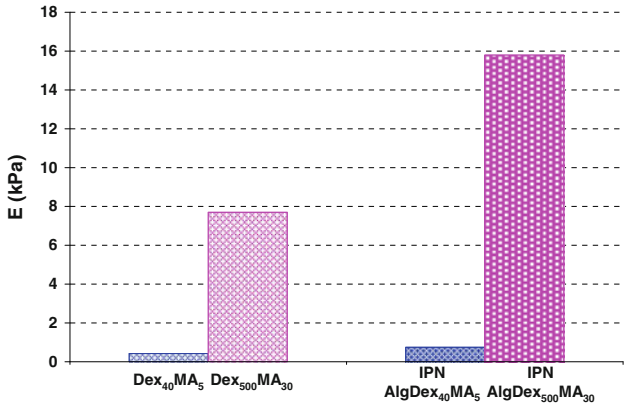

Fig. 7 Young Modulus (*E*) of different hydrogels prepared (10 min of UV irradiation)

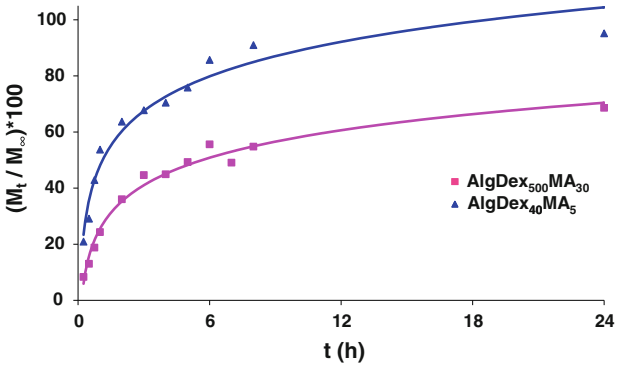

Fig. 8 Release at 37°C of HRP from IPNs (10 min of UV irradiation). The *curves* are reported only for an eye-guide

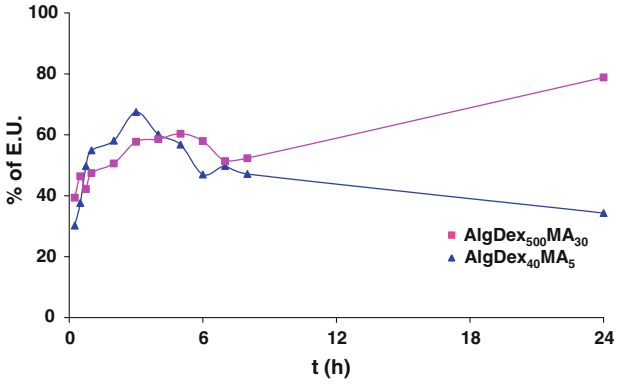

Fig. 9 Percentage of the enzymatic activity of the released HRP with respect to the free enzyme. The curves are reported only for an eye-guide

expected, the IPNs show *E* values that can be considered synergistic with respect to the E of the starting polymers. As reported for the G′ values, also *E* values are higher for the IPN composed by the Dex with the higher DS value. These results are in accordance with the more rigid structure related to higher crosslinking densities among Dex chains.

Protein release

The IPN hydrogels obtained by UV irradiation for 10 min were loaded with the protein HRP, in order to test the delivery behavior of the two IPNs. Testing the enzymatic activity of the protein during the release from the matrices, in which it was physically entrapped, it was also possible to demonstrate that the conditions adopted to build up these new hydrogel systems are not so harsh to inactivate enzyme activity.

In Fig. 8 the release of the protein from the two hydrogels are reported. As expected, the release from the more loose network (i.e. $AlgDex_{40}MA_5$) was faster and more than 90% of the loaded protein was released after 10 h. For the more crosslinked hydrogel (i.e. $AlgD_{500}MA_{30}$), the release was quite slower and no more than 70% of the loaded protein was delivered after 24 h. In this last case, it can be assumed that the higher crosslinking reduces the diffusivity of the protein molecules in the hydrogel matrix.

The enzymatic activity of the released HRP was tested, at fixed time intervals, using H_2O_2 as oxidizing substrate and ABTS as reducing substrate, in the solutions in equilibrium with the hydrogel. As reported in Fig. 9 the activity remains always comparable with that of the free enzyme.

Conclusions

In the present study, two innovative hydrogel systems were compared in terms of their rheological and protein delivery properties. These hydrogels are based on Ca-Alg and DexMA derivatives; the adopted experimental procedure allowed the two networks to be interpenetrated. Two types of DexMA samples were chosen: the first one with Mw 40×10^3 and methyacrylation degree of 5%, and the

second one with Mw 500 × 10^3 and methacrylation degree of 30%. These features of the macromolecules were chosen in order to compare the effects on the physicochemical characteristics and of the protein delivery properties of the obtained semi-IPNs and IPNs. Collected data evidenced that the increase of Mw and of methacrylation degree induce a dramatic effect on the mechanical properties; in fact the two hydrogels significantly differ in their elastic modulus, G′, as well as in their Young modulus, *E*, of more than one order of magnitude. As far as the mechanical properties are concerned, obtained data showed always a strong synergistic effect with respect to the starting polymer properties.

The studied hydrogels appear to be suitable for protein delivery, because the preparation conditions were sufficiently mild to retain the activity of loaded HRP. The HRP delivery profile is also influenced by the Dex polymer features: the release is slower for the hydrogel prepared with Dex with higher Mw and degree of methacrylation.

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