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Hyaluronan scaffolds via diglycidyl ether crosslinking: Toward improvements in composition and performance

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ABSTRACT

A novel approach for hyaluronic acid (HA) crosslinking via diglycidyl ether (DGE) was investigated for scaffolds fabrication. In particular, HA sponges were obtained by lyophilization and then reacted with 1,4-butandiol diglycidyl ether (BDDGE) in heterogeneous conditions. Insoluble matrices with 4–20% of the reactive sites of HA modified were produced. The hydrogels showed high swelling capability depending on external stimuli; when equilibrated in physiological solution, pore ranged from 70 to130 μm and G' values were in the range 2–10 kPa. The matrices proved highly stable in cell culture conditions and to enzymatic degradation. A biological evaluation revealed good cellular viability within the scaffolds in two weeks experiments. The main achievement consists in that the novel conditions (BDDGE and heterogeneous reaction) permitted to obtain insoluble, directly structured and potentially applicable scaffolds with the lower content of crosslinker reported to date. The in vitro characterization outcomes propose the hydrogels as promising substrates for soft tissues regeneration.

1. Introduction

Hyaluronic acid (HA) is one of the most diffused biomaterials for tissue engineering due to a unique combination of properties such as high hygroscopicity, biocompatibility, non immunogenicity and safety of degradation products (Schiraldi, La Gatta, & De Rosa, 2010). Actually HA has been widely used in the development of scaffolds, where it has to be crosslinked or derivatized in order to avoid solubility and to achieve resistance to degradation and mechanical performance in the biological environment (Schiraldi et al., 2010; Xu, Jha, Harrington, Carson, & Jia, 2012).

Many crosslinking strategies have been employed in order to produce either HA networks or hybrid matrices in which HA is combined with other macromolecules (e.g. collagen, gelatin, chondroitin sulfate, alginate, etc.) (Bulpitt & Aeschlimann, 1999; Chang, Liu, Lin, Chou, & Lin, 2003; Choi et al., 1999; Chung et al., 2011; Hwang et al., 2012; Ibrahim, Kang, & Ramamurthi, 2010; Kang et al., 2009; Kim et al., 2007; Segura et al., 2005; Shu, Liu, Palumbo, & Prestwich, 2003; Tan, Wu, Lao, & Gao, 2009; Tomihata & Ikada, 1997; Wang, Sun, Wu, Huang, & Lin, 2007; Yoon et al., 2011).

The majority of the reported strategies target the carboxylic groups of HA chains for chemical modifications generally using the carbodiimides (or carbodiimides/carboxylic group co-activators) chemistry (Bulpitt & Aeschlimann, 1999; Chang et al., 2003; Choi et al., 1999; Shu et al., 2003; Tan et al., 2009; Tomihata & Ikada, 1997; Wang et al., 2007). On the contrary, a few examples are reported in the literature of crosslinking processes, based on divinyl sulfone (DVS) and diglycidyl ethers (DGE), involving HA hydroxyl groups, thus preserving the polyanionic nature (Chung et al., 2011; Hwang et al., 2012; Ibrahim et al., 2010; Kang et al., 2009; Kim et al., 2007; Segura et al., 2005; Yoon et al., 2011). In particular, the stabilization of HA via diglycidyl ether crosslinking, though widely employed for the production of commercial HA based fillers for soft tissue augmentation (Allemann & Baumann, 2008; Andre, 2004; Beasley, Weiss, & Weiss, 2009; Falcone & Berg, 2008), only recently was applied for scaffolds construction (Chung et al., 2011; Hwang et al., 2012; Kang et al., 2009; Kim et al., 2007; Segura et al., 2005; Yoon et al., 2011). Scaffolds for cartilage repair were fabricated by Kang et al. (2009) and Hwang et al. (2012) crosslinking HA with poly (ethylene glycol) diglycidyl ether (PEGDGE). The matrices described tuned their mechanical behavior from "flexible" to rigid gels depending on the crosslinker amount (Hwang et al., 2012; Kang et al., 2009). A porous interpenetrating network in which HA was crosslinked with PEGDGE and alginate with calcium chloride was proposed as a scaffold for chondrocyte cultures by Chung et al. (2011). In this case, alginate permitted a better interconnected architecture and improved both the mechanical and the biological responses. Segura et al. (2005) prepared HA/collagen scaffolds using PEGDGE as the crosslinker obtaining porous structures that proved cytocompatible and highly resistant to enzymatic

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degradation. Finally, Yoon et al. (2011) developed a PEGDGE-crosslinked HA scaffold able to induce proliferation and chondrogenic differentiation of human adipose-derived mesenchymal stem cells.

In synthesis, in all the reported processes: (1) a high molecular weight diepoxide (PEGDGE 8860 Da) was used as the crosslinker, from a minimum of 0.9/1 to a maximum of 47.9/1 (w/w) ratio with respect to the biopolymer (influencing the mechanical performance and the stability of the final material); (2) the crosslinking reaction was performed in homogeneous conditions: the biopolymer is solubilized in a highly alkaline aqueous solution and then added with the DGE; 3) the sponges form was obtained after crosslinking by freeze-drying the resulting material swollen in water or by casting the solution containing the product.

The aim of this research work was to assess novel reaction conditions to lower the excessive amount of crosslinker needed so far for the production of scaffolds while maintaining or even improving their performances. In particular, a shorter chain DGE (BDDGE, 202 kDa) was used in order to have the same equivalents in a forty-fold lower weight amount and, heterogeneous conditions were applied expecting a higher crosslinking efficacy.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (Lot. N. 10001) was produced by fermentation and kindly provided by Altergon srl (Italy). According to producers, it has a molecular weight of 200–220 kDa. Bovine testicular hyaluronidase, BTH (EC 3.2.1.35), essentially salt free lyophilized powder with a specific activity of 1492 U/mg (Cat. N. H3884), benzyltrimethylammonium hydroxide (TMBAH) 40% (w/w) water solution (Cat. N. 246034), acetone (Cat. N. 650501) and 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat. N. M5655) were purchased from Sigma-Aldrich (Milan, Italy). Dulbecco's Phosphate Buffered Saline (PBS) without calcium and magnesium was purchased from Lonza Sales Ltd., Switzerland (Cat. N. BE17-512F). 1,4-Butanediol diglycidyl ether (BDDGE) was purchased from Polysciences, Inc. (Warrington, PA) (Cat. N. 01795-50).

Dulbecco's Modified Eagles Medium (DMEM), Calf Bovine Serum (CBS), Swiss NIH 3T3 murine fibroblasts were ATCC products, purchased from LGC Standards (Milan, Italy). Penicillin–streptomycin (Cat. N. DE17-602E) and fungizone (Cat. N. 17836E) were purchased from Life Technologies (Breda, The Netherlands) and used at concentrations equal to 100 units/mL, 100 μg/mL, 2.5 μg/mL, respectively.

2.2. HA hydrodynamic characterization by SEC–TDA system (Viscotek)

The chromatographic analysis of the biopolymer was performed using the SEC–TDA (Size Exclusion Chromatography-Triple Detector Array) equipment by Viscotek (Lab Service Analytica, Italy). A detailed description of the system and the experimental conditions for the analysis are reported elsewhere (La Gatta, De Rosa, Marzaioli, Busico, & Schiraldi, 2010). Sample molecular weight (M_w, M_n, M_w/M_n), molecular size (hydrodynamic radius – R_h) and intrinsic viscosity ([η]) distributions were derived. The MHS curves ($\log[\eta]$ vs. $\log M_w$) and, thus, the related constants ("a" and "$\log k$") were also directly obtained (La Gatta et al., 2010).

2.3. Scaffold preparation

12.48% (w/w) HA aqueous solutions were prepared and lyophilized according to the following procedure: the samples were frozen at −20 °C for 3 h followed by lyophilization overnight using a Christ Epsilon 2-6D LSC Vers. 1.13 freeze-dryer. 1.0 g of the lyophilized HA sponge material, cut to a specific shape, was added with mixtures containing water (2.3 mL), TMBAH (2.3 mL), BDDGE (either 0.22, 0.45, 0.67, 0.90 or 1.12 mL) and acetone to a final volume equal to 23.28 mL (pH = 14). The mixtures were incubated at 60 °C under stirring (900 rpm) in an orbital shaker for 3 h. The amounts of BDDGE used correspond to BDDGE/HA equivalent ratios equal to 22%, 45%, 67%, 90% and 112%, respectively. Reactions were stopped by lowering the pH of the suspension to 7.4 through the addition of a phosphate buffer (0.06 M). The reaction products (XHA) were washed in PBS and then, in highly purified water. Purification process with PBS was monitored spectrophotometrically at 216 and 240 nm, until extracted contaminants were reduced to 0.1% of the initial amount. Purification process with water was monitored by measuring the reduction in conductivity, the final target was set to a conductivity value of 10–20 μS/cm. The materials were then treated with 20 mL of acetone for three times and, then, dried under vacuum at 40 °C. The reaction products will be referred to as XHA22, XHA45, XHA67, XHA90 and XHA112. Materials were sterilized (20 min at 120 °C) before characterization.

Equivalent reactions (using the same BDDGE/HA equivalent ratios) were performed under homogeneous conditions, for comparison. In particular, 1.0 g of the HA sponge was added with 23.28 mL of 0.25 M NaOH water solution containing the specific BDDGE amount. The reactions were carried out and the final samples were purified as reported for the heterogeneous approach.

2.4. NMR analyses

Linear and water insoluble crosslinked HA samples were swollen in a HCl solution (pH = 2) and kept at 70 °C until dissolution. The solution was neutralized by adding Na_2HPO_4 and then lyophilized. The lyophilized samples were dissolved in D_2O. ^1H NMR analyses were performed on a Bruker DRX-600 spectrometer (BrukerBioSpin GmBH, Rheinstetten, Germany). The NMR data were processed using the UXNMR software.

2.5. Swelling studies

Swelling behavior (water uptake kinetic and equilibrium swelling) of the materials was evaluated in different media (highly purified water, PBS, NaCl solutions, buffered solution, DMEM–CBS 10%) by means of gravimetric measurements using an analytical balance (Mettler Toledo, XS105 Dual Range). In particular, samples were immersed into the swelling solution (200 mL aqueous medium/g of sample) and kept in a thermostatic bath at 37 °C. Specimens were withdrawn at fixed intervals, 2–4 min up to 2 h for kinetic studies, or after 3 h to assess the equilibrium swelling degree (equilibrium studies). Wet samples were then blotted with filter paper to remove surface water and finally weighed. The swelling ratio was calculated as follows:

$$\textit{swelling}\ \text{ratio}\ \left(\frac{\text{g}}{\text{g}}\right) = \frac{w_s(\text{g})}{w_d(\text{g})} \times 100 \quad (1)$$

where w_s is the swollen sample weight and w_d is the dried initial sample weight.

2.6. Morphological analyses

The unmodified HA sponge and the crosslinked materials, obtained as described in Section 2.3, were observed by SEM. Specifically the samples were directly mounted on a stub and coated with gold (Denton Vacuum Desk V) before observation under a Scanning Electron Microscope (Supra 40 ZEISS; EHT = 5.00 kV, WD = 22 mm, detector inlens).

Additionally, XHA specimens were observed, while at the equilibrium swelling in physiological solution, at a time lapse optical videomicroscopy station (OkoLab, Naples, Italy). A detailed description of the station is reported elsewhere (La Gatta, Schiraldi, D'Agostino, Papa, & De Rosa, 2012). Specifically, an inverted optical microscope (Axiovert 200; Zeiss, Oberkochen, Germany) was used for the observation. For each sample, a statistically significant number of images was captured and analyzed using the Image Pro Plus 5.1 software for measuring pore and fiber dimensions.

2.7. In vitro stability to enzymatic degradation

Samples of XHA were incubated with a BTH solution in PBS (1000 U/mL or 100 U/mL) at a concentration of 5 mg/mL (3 mL total volume) at 37 °C. 200 μL of the medium were taken at predetermined time points and replaced with fresh BTH solution (200 μL). The collected samples were opportunely diluted in PBS, filtered on 0.45 μm and carbazole assay was used to evaluate the soluble HA content (Bitter & Muir, 1962). The degradation was monitored by following the increase in soluble HA percentage during enzymatic incubation. At each time point, the soluble HA percentage was calculated as:

$$\text{soluble HA}\,(\%) = \frac{\text{HA mass in solution}\,(\text{mg})}{\text{total HA mass}\,(\text{mg})} \times 100 \quad (2)$$

where total HA mass corresponds to the amount of HA in solution when the hydrogel was completely degraded.

2.8. In vitro stability in cell culture medium

Samples of XHA were weighed and incubated in DMEM–CBS 10% at 37 °C in humidified atmosphere at 5% CO_2 at a concentration of 15 mg/mL. At predetermined time intervals (up to 21 days), samples were withdrawn, washed in de-ionized water in order to remove substances absorbed from the medium, dried and then weighed. Degradation was evaluated by measuring the amount of residual insoluble XHA (mg) at each time point with respect to the initial sample amount (%). The swelling degree of the sample was also evaluated during incubation.

2.9. Mechanical measurements

Rheological measurements were performed on hydrogels swollen at the equilibrium in PBS at 37 °C. A Physica MCR301 oscillatory rheometer (Anton Paar, Germany) equipped with a parallel plate geometry (plate diameter of 25 mm) and a Peltier temperature control were used. In particular, strain sweep tests were performed at a constant oscillatory frequency of 1.59 s^{-1} over a strain amplitude (γ) range of 0.001–100%. The storage modulus (G'), the loss modulus (G'') and the value of critical strain, γ_{cr} (strain value at which G' and G'' cross over) were evaluated.

2.10. Biological evaluation

2.10.1. Cell culture

NIH3T3 fibroblasts were routinely cultured in DMEM supplemented with CBS (10%, v/v), nonessential aminoacids (1%, v/v) and antibiotics. Cells were maintained at 37 °C in a 5% CO_2, 95% air, humidified atmosphere and media were changed every 48 h.

2.10.2. In vitro cytotoxicity tests

The in vitro cytotoxicity was evaluated by means of the elution test method (ISO 10993-5): NIH3T3 fibroblasts grown to near confluence were exposed to fluid extracts from the materials under investigation as accurately described elsewhere (La Gatta et al., 2005); cell viability was evaluated after 48 h of incubation with respect to the control (cells incubated in fresh culture medium) by MTT assay (La Gatta et al., 2005; Slater, Sawyer, & Strauli, 1963). Additionally, the cells were observed throughout incubation in fluid extracts at the optical microscope.

2.10.3. Cell adhesion and proliferation

Cytocompatibility of the developed hydrogels was evaluated using NIH3T3 fibroblasts. In particular, weighed samples of each hydrogel were placed in a 12-well plate and (carefully) added with a cellular suspension (NIH3T3 suspended in fully supplemented DMEM without phenol red at a concentration equal to 3.5×10^5 cells/mL) until (the) equilibrium swelling was reached. Seeded materials were then incubated in cell growth conditions for 1 h to allow cell attachment and then, 2 mL of cell culture medium were added. At predetermined time intervals (24, 48, 72 h, 7 and 14 days), samples were withdrawn from the medium, treated twice with PBS (in order to remove non adherent cells) and transferred in a new 12-well plate. 2 mL of a MTT solution (5 mg/mL in DMEM without phenol red-CBS10%) were added and samples were incubated again for 3 h. The medium was then removed from each well and 1 mL of HCl 0.1 M in 2-propanol was added. The suspension was recovered, centrifuged and absorbance of the supernatant was read at 570 nm vs. a control (matrices incubated without cells). Proliferation curves (A_{570} normalized with respect to the number of seeded cells and the volume of the sample vs. incubation time) were derived (Kang et al., 2009; Mosmann, 1983). A proliferation curve on Tissue Culture Polystyrene (TCP) was also obtained.

Morphology of adherent cells was also examined by SEM. In particular, specimens were removed from the medium, washed twice in PBS, fixed with 2.5% paraformaldeyde in PBS for 1 h at 4 °C. Successively, the samples were dehydrated with increasing ethanol percentages, namely 30%, 40%, 50%, 60%, 70%, 80% and 90% in water for 5 min, twice with 100% ethanol for 15 min. The samples were then dehydrated with a Critical Point Dryer (Emitech K850), sputter coated with gold (Denton Vacuum Desk V) and observed under a SEM (Supra 40 ZEISS; EHT = 5.00 kV, WD = 22 mm, detector inlens).

2.11. Statistical analysis

Each crosslinking reaction was performed at least in triplicate and each resulting material was analyzed at least in triplicate, therefore, a minimum of nine samples was analyzed for each product. A Student t test was used for statistical analysis and p values lower than 0.05 accounted for significant differences.

3. Results

3.1. HA hydrodynamic characterization by SEC–TDA system

The report of the HA hydrodynamic characterization, accomplished by SEC–TDA analyses, is presented in Table 1. HA presented a weight average molar mass (M_w) of 207 ± 4 kDa, a polydispersity index of 1.5 ± 0.1 and a hydrodynamic radius of 26 ± 1 nm. The intrinsic viscosity and the MHS constants ("a" and "log k") for the biopolymer are also reported and are consistent with the literature data (La Gatta et al., 2010).

3.2. Manufacturing and morphology of HA scaffolds

Results of scaffolds manufacturing and morphological analyses are illustrated in Figs. 1–3. In particular, the values of HA insolubilization degree achieved after heterogeneous and homogeneous crosslinking are reported in Fig. 1a. As shown, when reacting HA in the non solvent, the resulting products presented final insoluble residues, even if at diverse extent: 26 ± 2, 70 ± 1, 75 ± 1, 78 ± 1 and 82 ± 1% insolubilization degree values were registered when

Table 1
Values of weight average molar mass (M_w), numeric average molar mass (M_n), polydispersity index (M_w/M_n), intrinsic viscosity ([η]), hydrodynamic radius (R_h) and Mark–Houwink constants "a" and "log k" for HA (Lot. N. 10001) used for scaffold manufacturing, as obtained by SEC–TDA analyses.

Biopolymer	M_w (kDa)	M_n (kDa)	M_w/M_n	[η] (dL/g)	R_h (nm)	a	log k
HA	207 ± 4	134 ± 4	1.5 ± 0.1	5.9 ± 0.1	26 ± 1	0.80 ± 0.02	−3.49 ± 0.09

using BDDE 22, 45, 67, 90 and 112%, respectively. On the contrary, when performing the reaction in 100% aqueous medium, products completely solubilized in water regardless of the BDDGE amount.

In Fig. 1b, a scheme illustrating the macroscopic evidences during the processes is shown. An image of the un-modified HA sponge, as obtained by lyophilization, cut to a specific polyhedric shape, is reported on the left side. If reacted with BDDGE 45–112%, the sponge maintained its form during crosslinking (data not shown) and, during washing in PBS and in water, swelled while maintaining its shape; the last photograph shows that, after drying, the material returned to its original dimensions. When BDDGE 22% was used, the macroscopic structure of the sponge weakened during crosslinking (data not shown); furthermore a collapse of the 3D-structure was observed during the purification process and, finally, a powder-like material was obtained after drying. During crosslinking in homogeneous conditions, the sponge dissolved in fact no insoluble product could be quantified.

SEM micrographs of the un-modified sponge and of the heterogeneous reaction products are reported in Fig. 2. A porous structure with interconnected open pores was evidently obtained after lyophilization (Fig. 2a). SEM images of the crosslinked materials (Fig. 2b–f) confirmed the collapse of the substrate structure for the 22% BDDGE product also at the microscopic level (Fig. 2b) while a porous three-dimensional architecture appeared preserved for all the other samples (Fig. 2c–f).

XHA67 swollen at equilibrium in physiological solution is shown in Fig. 2g; the micrograph, obtained by optical microscopy observation, highlighted the interconnected microporosity exhibited in conditions closer to the application. A similar morphology was observed for the XHA45–112 products (data not shown). The analyses performed at the Time Lapse Station revealed the presence of pores ranging from 70 to 130 μm with the more crosslinked materials presenting a higher percentage of smaller pores; the fibers width was in the range of 6–45 μm.

Results of the ^{1}H NMR analyses performed on the linear HA sample and on the insoluble XHA materials are reported in Fig. 3. In particular, the ^{1}H NMR spectra for HA and XHA67, as representative of the XHA samples, are shown. A signal around 1.6 ppm due to the $-(CH_2)_2$ moiety of the BDDGE molecule was detected in all the crosslinked materials proving the occurrence of reaction (Guarise, Pavan, Pirrone, & Renier, 2012). The integration of the signal at 1.6 ppm with respect to the acetylglucosamine signal at about 2.0 ppm gives the amount of BDDGE bonded to HA. In Fig. 3c, the amount of BDDGE in the final network (normal percentage with respect to HA) was reported in function of the BDDGE

(a)

Reaction conditions	HA insolubilization degree (w/w%)				
	BDDGE 22	BDDGE 45	BDDGE 67	BDDGE 90	BDDGE 112
heterogeneous	26 ± 2	70 ± 1	75 ± 1	78 ± 1	82 ± 1
homogeneous	0	0	0	0	0

(b)

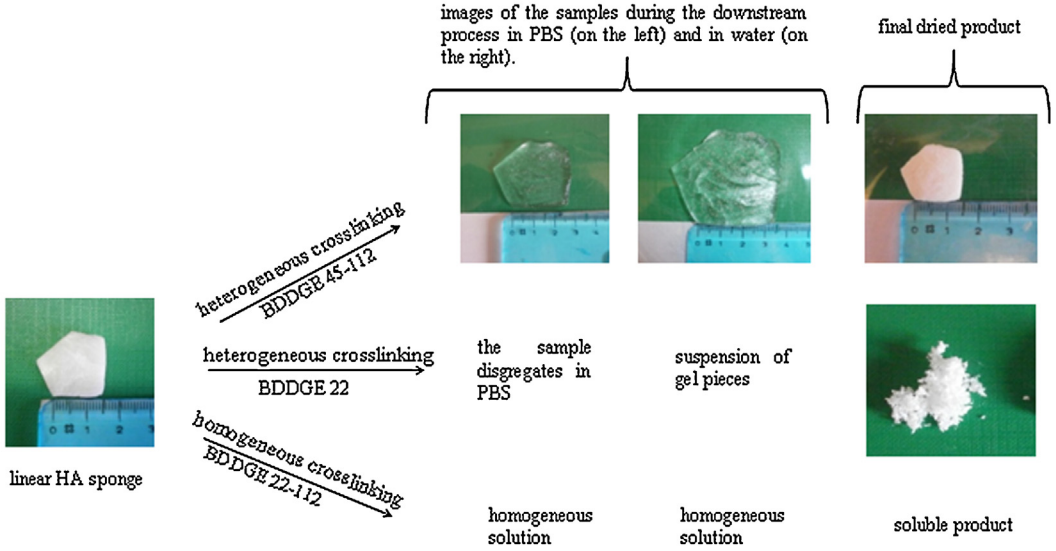


Fig. 1. Results for HA sponges crosslinking under heterogeneous and homogeneous conditions. (a) HA insolubilization degree (weight percentage of insolubilized HA with respect to the reacted amount) obtained at the BDDGE amounts tested (22–112 normal percentage with respect to the biopolymer); (b) schematic report of the materials throughout their processing: photographs or authors observation.

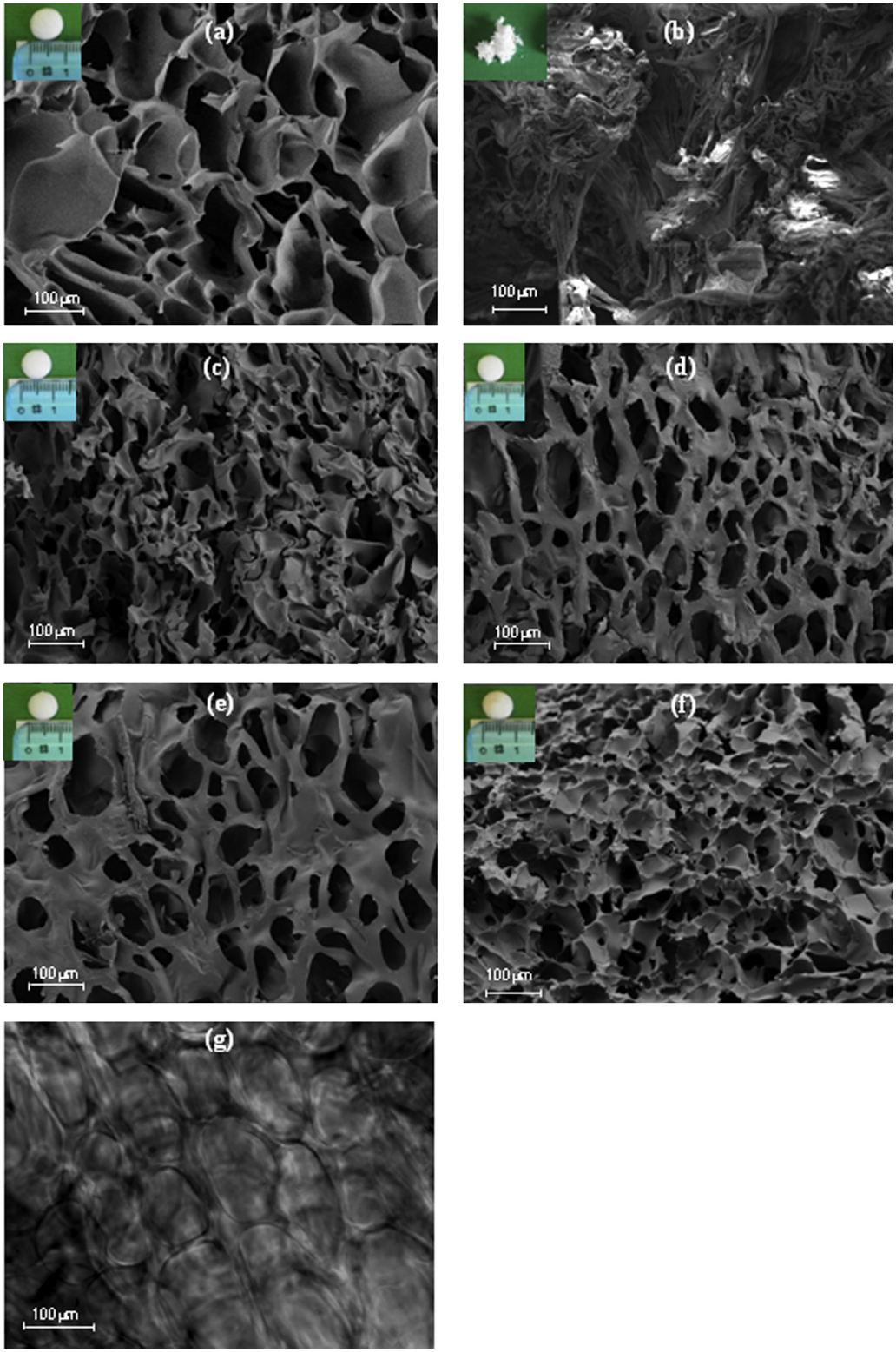


Fig. 2. SEM micrographs of (a) the un-modified HA sponge obtained by lyophilization, (b) XHA22, (c) XHA45, (d) XHA67, (e) XHA90 and (f) XHA112 in their dried form; (g) image at the optical microscope for XHA67 swollen in physiological solution. In the insets, photographs of the samples are shown.

amount added demonstrating a linear trend in the range tested. Only 17–20% of the crosslinker used was incorporated within the HA network.

Finally, considering the yield values in insoluble HA and the results of the morphological analyses, XHA45–112 were selected for further characterization.

3.3. Scaffolds characterization

3.3.1. Swelling studies

Kinetic studies revealed that the hydrogels swelled rapidly reaching equilibrium within few minutes (data not shown). Swelling ratio values at equilibrium in water and PBS are shown in

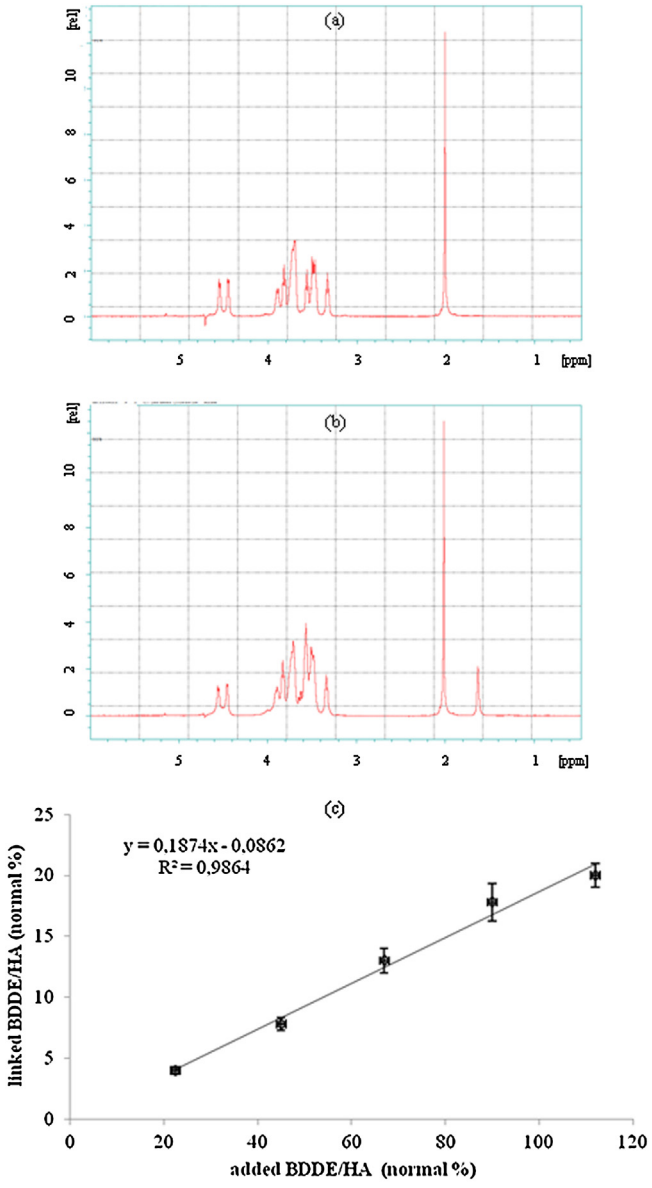


Fig. 3. ^{1}H NMR spectra of un-modified HA (a) and XHA67 (b); (c) plot of the amount of BDDE linked (normal percentage with respect to HA) as a function of the BDDE amount added (normal percentage with respect to HA).

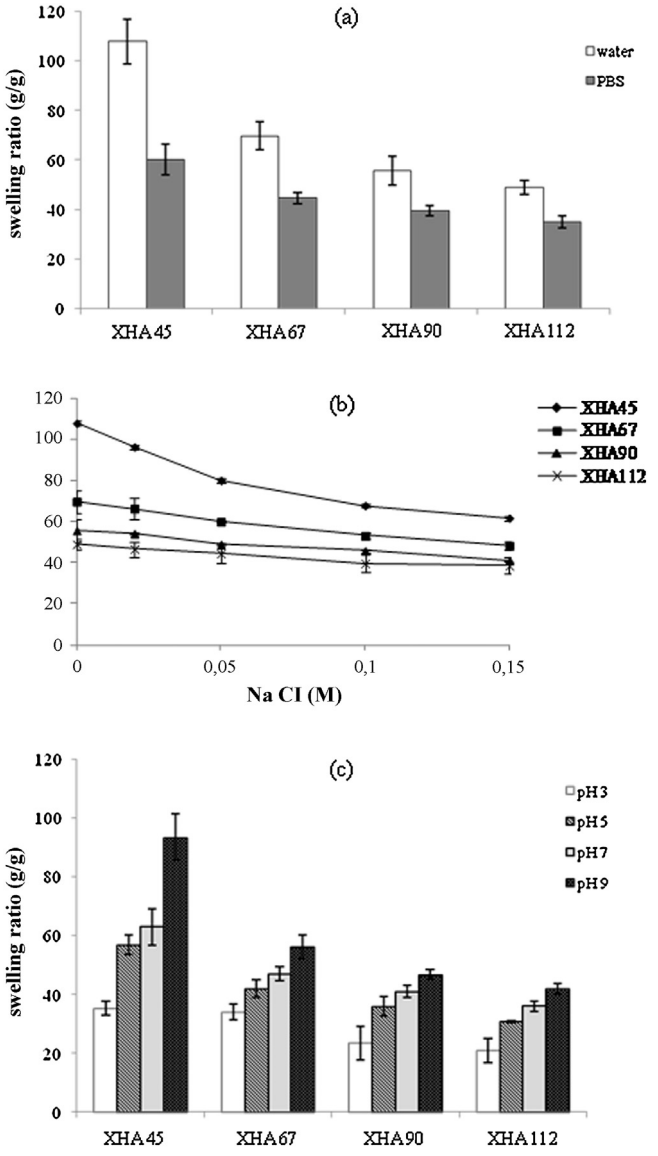
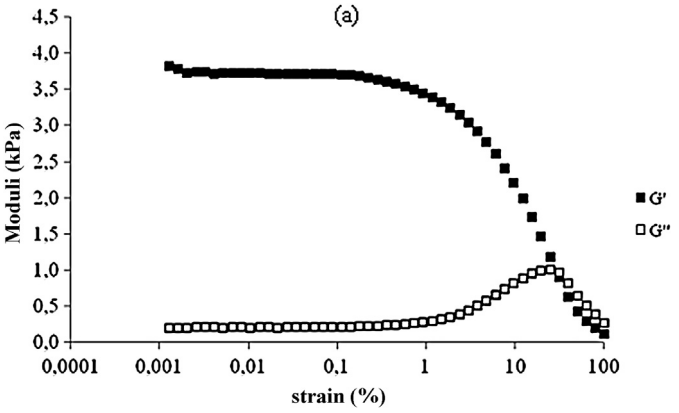


Fig. 4. (a) Swelling ratio values (hydrated material weight/dried material weight) at equilibrium for the hydrogels in highly purified water and in PBS (0.165 M ionic strength); (b) swelling ratio plotted as a function of the ionic strength in NaCl solutions (0–0.15 M); (c) swelling ratio for the hydrogels as a function of pH of the swelling medium (determinations were carried out at ionic strength of 0.165 M). All measurement were performed at 37 °C.

Fig. 4a: hydrogels exhibited a high water uptake capability increasing their dry weight 35–110-fold in aqueous medium. The swelling extent was found to decrease with the increase in the amount of BDDGE used for the reaction; specifically when using BDDGE from 45 to 112%, the swelling ratio values diminished from 108 ± 9 to 49 ± 3 g/g and from 60 ± 6 to 35 ± 3 g/g in water and PBS, respectively. Differences in the water uptake capability of XHA90 and XHA112 were found not significant ($p > 0.05$). As evident from the images reported in Fig. 1b, the samples showed a isotropic volume increase when swollen in aqueous medium. In Fig. 4b the swelling extent as a function of the ionic strength of the medium is reported: the amount of absorbed water decreased with the increase in NaCl concentration in the medium and the difference in the swelling behavior of the hydrogels became less marked, with a minor effect for the less crosslinked material. Results of the swelling studies as a function of pH of the medium ($I = 0.165$ M) are shown in Fig. 4c: the swelling degree decreased with the decrease of pH, in particular, with the reduction of pH from 9 to 3, the swelling ratio values became 40–50% of the initial ones.

3.3.2. Mechanical analyses

Results of the mechanical analyses are shown in Fig. 5. In particular, Fig. 5a shows G' and G'' as a function of the strain amplitude (constant frequency = 1.59 s^{-1}) for XHA67: the hydrogel exhibited an elastic behavior, the storage modulus G' (3.81 ± 0.13 kPa) was far higher than the loss modulus G'' (0.21 ± 0.02 kPa) within the linear viscoelastic range. The same trend was observed for the other hydrogels: G' values were 15–30-fold higher than G'' values for XHA45–112 (data not shown). G' and γ_{cr} values for all matrices, as resulted from the strain test, are reported in Fig. 5b: hydrogels exhibited elastic moduli in the range 1.90–9.74 kPa with XHA45 being the most elastic ($G' = 1.90 \pm 0.09$ kPa) and, thereby the most deformable matrix ($\gamma_{cr} = 29.7 \pm 2.1\%$) while the material obtained at the highest crosslinker percentage, resulted the most rigid ($G' = 9.74 \pm 0.32$ kPa) and therefore, the less deformable network ($\gamma_{cr} = 8.3 \pm 0.9\%$).



(b)

Sample	G' (kPa)	γ_{cr} (%)
XHA45	1,90 ± 0,09	29,7 ± 2,1
XHA67	3,81 ± 0,13	22,4 ± 1,9
XHA90	7,92 ± 0,21	10,9 ± 0,9
XHA112	9,74 ± 0,32	8,3 ± 0,9

Fig. 5. Results of the strain test: (a) G' and G'' as a function of the strain amplitude for XHA67 at 1.59 s^{-1}; (b) storage modulus and critical strain for the developed hydrogels as resulted from the strain test.

3.3.3. Materials stability in cell culture medium

Hydrogels stability in DMEM–CBS 10% was studied by means of gravimetric analyses up to 21 days incubation: a weight loss minor than 2% was detected at the longer incubation time for XHA67–112 materials, while, in the same conditions, 10% (w/w) of XHA45 dissolved. It is worth underlining that macroscopically, the former set of materials retained their shape during incubation while XHA45 finally transformed into a viscous gel. A modification in the swelling extent of materials during the experimentation was observed. In particular, at the longer incubation time, a swelling increase of about 40–45% was registered for XHA45 and XHA67 while XHA90 and XHA112 incremented their water uptake capability of about 10–15% in the same conditions (data not shown). No significant differences were found within the two sets of materials ($p > 0.05$).

3.3.4. In vitro enzymatic degradation

Fig. 6 shows the degradation curves for the hydrogels due to in vitro hydrolysis catalyzed by BTH. In particular, the percentage of soluble HA was reported as a function of the incubation time with a BTH solution 1000 U/mL (Fig. 6a) and 100 U/mL (Fig. 6b). As shown, the degradation rate decreased at the increasing of the crosslinker percentage and XHA112 proved the most stable gel. In particular, when incubated with BTH 1000 U/mL, the hydrogel formed at the lower crosslinker percentage solubilized within 24 h; XHA67 completely solubilized in 7 days while, at the same time, XHA90 and XHA112 showed about 90% and less than 50% of degradation, respectively. All hydrogels were found completely soluble at 13 days of incubation. The degradation curve of XHA45 was derived using a lower BTH concentration (100 U/mL). Results are reported in Fig. 6b: as shown, complete degradation occurred within 9 days.

3.3.5. Biological evaluation

Hydrogels were found non cytotoxic regardless of the crosslinker percentage employed for the reaction (data not shown). The cytocompatibility of the matrices was studied using NIH3T3 fibroblasts in 14 days experiments. Cell viability was evaluated at predetermined time intervals after seeding (24 h, 48 h, 72 h, 7 days and 14 days) by means of the MTT test as described in the Section 2.

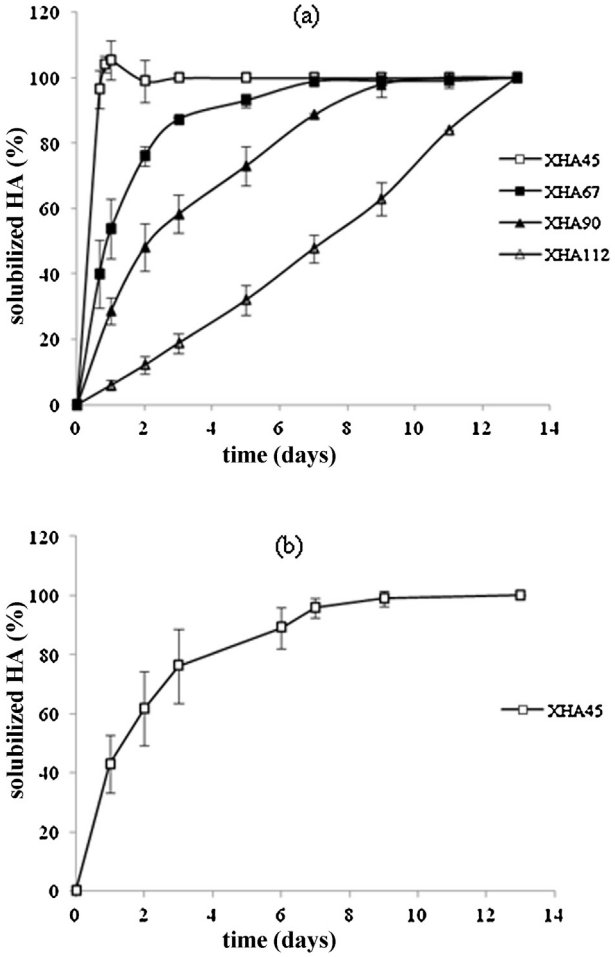


Fig. 6. Hydrogels degradation catalyzed by BTH 1000 U/mL (a); degradation curve in the presence of BTH 100 U/mL for XHA45 (b). The experiments were performed at 37 °C.

At a macroscopic observation, hydrogels appeared homogeneously colored after the MTT treatment at each tested time thus supporting the presence of living and metabolically active cells. Normalized absorbance values at 570 nm were reported as a function of the incubation time in Fig. 7: it is evident, for all materials, an increase in the amount of formazan salt formed (therefore in viable cells number) at increasing time within 72 h of incubation. In particular, XHA67 promoted proliferation twofold better than XHA45 and 20% higher with respect to the other materials. When considering

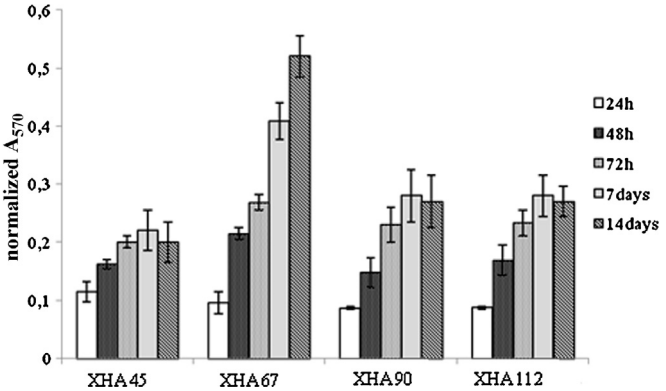


Fig. 7. Proliferation of NIH3T3 on XHA matrices: normalized absorbance values at 570 nm are reported as a function of the incubation time.

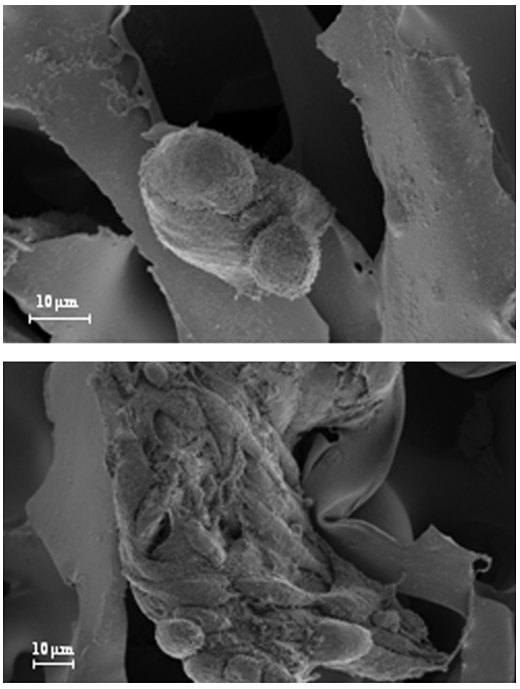


Fig. 8. SEM micrographs of 3T3 on XHA112 at 7 days after seeding.

longer incubation times (7 and 14 days), proliferation, notwithstanding a lower rate, continued on XHA67 while a not significant increment in cells number was detected on the other hydrogels ($p > 0.05$) the worst performing being XHA45.

Fig. 8 shows SEM micrographs of 3T3 fibroblasts cultured within XHA112 at 7 days of incubation, however, the SEM analyses of other samples at other incubation times showed very similar images: good adhering cells along with cells exhibiting a rounded shape morphology may be seen on the materials surface (Fig. 8).

4. Discussion

HA was crosslinked with a diglycidyl ether under innovative conditions aiming at producing hydrogels suitable for tissue engineering purposes while overcoming the disadvantages related to the high amounts of crosslinker currently used. The novelties consisted in reacting HA with BDDGE (202 Da) instead of PEGDGE (8886 Da) and in replacing sodium hydroxide with an organic base (TMBAH) which allowed to perform the reaction in heterogeneous conditions (i.e. the high pH values needed for the reaction could be reached in a non solvent for the polysaccharide).

Since subjected to the chemical modification while in the solid state, HA was processed to a sponge structure before crosslinking. In particular, by lyophilizing concentrated aqueous solutions of the biopolymer, the microporous architecture shown in Fig. 2a was achieved. The shaped sponges underwent crosslinking with BDDGE under both heterogeneous and homogeneous conditions for comparison. As reported in Fig. 1a, despite equivalents of BDDGE (22–112% with respect to HA) similar to the ones reported in the literature for PEGDGE (2–109%) were used, no insolubilization was obtained when HA was reacted in the aqueous medium. Reasonably, the shorter chain DGE was less efficient than PEGDGE in crosslinking HA chains that, while solubilizing, increase their average distance in a faster way with respect to the bridge formation. On the other hand, the effective HA crosslinking achieved under the heterogeneous conditions (Fig. 1a) could be rationally due to the occurring of the reaction within the hydration sphere of the biopolymer in which the chains are close enough to bridge. Finally, the use of BDDGE combined with the heterogeneous approach allowed to produce water-insoluble HA matrices even if using considerably lower DGE weight amounts (more than 40-fold lower than the literature data).

Interestingly, the macroscopic and microscopic observation indicated that the heterogeneous crosslinking with BDDGE from 45 to 112% "froze" the HA chains to almost maintain the original shape and architecture of the lyophilized sponge. This permits to draw the scaffold prior to crosslinking (diverse physical forms like fibers, films, cylinder could be considered) and to obtain the final insoluble material consistent with the original design, that is an interesting technological novelty for the specific HA chemical modification.

Further morphological analyses confirmed that materials exhibited interconnected microporosity also in swollen state more similar to the application conditions (Fig. 2g). Additionally, the range of pore dimensions obtained differed from the ones reported in the literature for the XHA-PEGDGE scaffolds, and the actual range (70–130 μm) is generally considered suitable for the regeneration of soft tissues (larger pores are needed for the engineering of hard tissues) (Chang & Wang, 2011; Chung et al., 2011; Hwang et al., 2012; Murphy, Haugh, & O'Brien, 2010; Segura et al., 2005).

^{1}H NMR analyses confirmed the incorporation of BDDGE in the final network (Fig. 3) also proving, that not all the crosslinker used for the reaction bonded to the biopolymer; this could be explained considering the high susceptibility of the diepoxide to hydrolysis. Specifically, the extent of modification found corresponds to a BDDGE weight fraction in the final products lower than 20%.

Since it is a key parameter in view of application, the swelling behavior of the materials was extensively investigated also in dependence on external stimuli, such as pH and ionic strength. Consistently with the specific crosslinking reaction which preserves the polyanionic nature of the biopolymer, a high water uptake capability was registered: weight increase values up to 110-fold and 60-fold were registered in water and in PBS, respectively (Fig. 4a). These values were much higher (up to 50-fold) than the ones reported for the matrices crosslinked with comparable equivalents of PEGDGE. This difference may be due to the higher weight fraction of the hydrophilic DGE with respect to the charged hyaluronan in these last materials (Chung et al., 2011; Kim et al., 2007; Segura et al., 2005). The XHA swelling extent coherently decreased at the increasing of the crosslinker amount (Fig. 4a) and of the ionic strength of the medium (Fig. 4b); in the first case due to the higher corresponding crosslinking degree and in the second because of the reduction in ion osmotic swelling pressure. Finally, an increase in the water uptake was registered with the pH increase (Fig. 4c) consistently with the presence of the carboxylic groups in the network (pK_a COOH = 2.9). Overall, the scaffolds behave as super-absorbent hydrogels able to maintain the original shape even after extensive swelling (Fig. 1b). Such characteristic could be useful in allowing a minimally invasive surgical incision for the implantation of the dry device that in vivo will swell following a predictable expansion.

As shown in Fig. 5, when equilibrated in physiological solutions, the materials exhibited an elastic behavior with a rigidy increasing, as expected, with the increase of the BDDGE amount used for the reaction (G': 2–10 kPa). The matrices resulted tenfold stiffer than the ones obtained using comparable weight amounts of PEGDGE while higher values of rigidity were reported by Segura and coworkers that used up to 38-fold higher DGE amounts (Hwang et al., 2012; Segura et al., 2005). According to some reports, the XHA materials mimic the stiffness of biological tissues such as brain, muscle and cartilage thus resulting promising supports for the regeneration of soft tissues also considering the mechanical function (Engler, Sen, Sweeney, & Discher, 2006; Levental, Georges, & Janmey, 2006). It is worth noting that this is the first case of HA-DGE scaffolds exhibiting both morphological and mechanical properties suitable for soft tissues regeneration.

Materials stability was sound, in fact, no significant solubilization was observed during 3 weeks of incubation in cell culture conditions, however, an increase in the swelling capability was detected indicating the occurring of hydrolytic processes at some extent (data not shown). The hydrogels degraded in the presence of BTH indicating that the chemical modification did not prevent biodegradation. As expected, the degradation rate was inversely dependent on the crosslinker percentage (Fig. 6). The matrices demonstrated higher resistance to enzymatic hydrolysis comparing to previously reported similar hydrogels: XHA112 and a comparable PEGDGE crosslinked HA network were found to completely degrade within 13 days of incubation with BTH but a tenfold lower enzyme concentration was used in this last case (Segura et al., 2005). The different rates of solubilization measured for the tested materials indicate that the hydrogels could be tailored to match different stability requirements widening applications.

In vitro biological experiments proved the matrices were not toxic with a good cellular viability verified in two weeks incubation (Fig. 7). As shown, XHA67 performed better in promoting cell proliferation possibly due to the concurrent action of different parameters such as pore sizes, elasticity and swelling which elicits a good response of the cell line tested. These evidences were expected and in line with the cytocompatibility reported for the XHA-PEGDGE matrices (Chung et al., 2011; Hwang et al., 2012; Kang et al., 2009; Kim et al., 2007; Segura et al., 2005; Yoon et al., 2011).

5. Conclusions

Insoluble and cytocompatible hyaluronic acid sponges were successfully produced by combining a freeze-drying technique with crosslinking performed in heterogeneous conditions using BDDGE. The approach improved on the conventional procedures in that allowed the production of outperforming hydrogels with tunable properties while using lower crosslinker amounts. The results suggested the heterogeneous crosslinking procedure as an interesting technological alternative for the manufacturing of HA-DGE based scaffold with pre-defined shape. The characterization highlighted the hydrogels potential as supports for the regeneration of soft tissues.

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