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Injectable hyaluronic acid/PEG-p(HPMAm-lac)-based hydrogels dually cross-linked by thermal gelling and Michael addition

Alessandra Dubbini [a,1], Roberta Censi [a,1,*], Maria Eugenia Butini [a], Maria Giovanna Sabbieti [b], Dimitrios Agas [b], Tina Vermonden [c], Piera Di Martino [a]

[a] School of Pharmacy, Drug Delivery Division, University of Camerino, Italy
[b] School of Biosciences and Biotechnologies, University of Camerino, Italy
[c] Utrecht Institute for Pharmaceutical Sciences, Department of Pharmaceutics, Utrecht University, The Netherlands

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ABSTRACT

Fast in situ forming thermosensitive hydrogels consisted of vinyl sulfone bearing p(HPMAm-lac$_{1-2}$)-PEG-p(HPMAm-lac$_{1-2}$) triblock copolymers and thiol modified hyaluronic acid were prepared via a dual cross-linking strategy based on thermal gelation at 37 °C and simultaneous Michael addition cross-linking between vinyl sulfone and thiol moieties. The formation of a chemical network was varied within a time period of 9–60 min by controlling the degree of vinyl sulfone derivatization, the triblock copolymer concentration and the degree of thiolation. The extent of thiol substitution on the polysaccharidic hyaluronan chain markedly affected the physical and mechanical properties, as well as the swelling and degradation behavior of the resulting networks, as confirmed by rheology, water uptake experiments and degradation tests. In addition, the developed hydrogels showed a good cytocompatibility in vitro during a timeframe of 21 days both for mouse bone marrow stromal cell and for NIH 3T3 mouse fibroblasts. The developed hydrogels showed potential as promising injectable biomaterials with tunable gelation kinetics, adjustable mechanical properties, swelling and degradation times. These biomaterials could find application both as a regenerative cell matrix and as controlled drug delivery system.

1. Introduction

Hydrogels are a class of biomaterials based on cross-linked 3-dimensional polymeric networks displaying viscoelastic behavior and tissue-like mechanical properties [1]. Polymeric hydrogels have the ability to absorb significant quantities of water, reaching water uptakes up to 100 times the dry polymer mass. This high water content makes them compatible with most living tissues. Their viscoelastic and rubbery nature permits their administration into a living host with minimal damage to surrounding tissues, and, ultimately, their mechanical properties can be tailored to closely match those of natural tissues [2,3]. Because of their appealing characteristics, hydrogels, alone or combined with cells, have been used to engineer a variety of tissues in vitro and in vivo [4,5] and, loaded with drugs, found application in the controlled drug delivery field [6,7]. Among all types of hydrogels, in situ-gelling injectable hydrogels have been extensively employed as cell and drug

* Corresponding author at: School of Pharmacy, Drug Delivery Division, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy.
E-mail address: roberta.censi@unicam.it (R. Censi).
[1] Authors equally contributed to this work.

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carriers for in vitro and in vivo applications [8]. The advantages of using injectable hydrogels lie in that they have high moldability, are capable of filling irregular-shaped defects, can be delivered to the in vivo environment by minimally invasive administration methods, such as injection or laparoscopy [9] and processed in a number of shapes from macro to nanoscale [10]. Injectable hydrogel gelation can be accomplished by a number of physical methods including thermal gelation, ionic cross-linking, hydrophobic interactions, host–guest complexation [11,12] and chemical cross-linking, such as photopolymerization, click chemistry, Michael addition and native chemical ligation [13,14]. Thermal crosslinking is applicable for thermosensitive hydrogels that undergo phase separation in response to a temperature change in aqueous solutions [15–17]. The gelation can be achieved simply by raising the temperature to above the lower critical solution temperature (LCST) of the polymer. Various thermosensitive hydrogels such as methylcellulose [18], poly(N-isopropylacrylamide) (PNIPAAm) and copolymers [19], poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO), poly(ethylene glycol) (PEG)/biodegradable polyester copolymers [20,21] and peptide-based polymers [22] have been developed. PNIPAAm is one of the most studied thermosensitive polymers for pharmaceutical and biomedical applications due to its relatively low LCST (32 °C), allowing it to form a gel at body temperature [23,24]. The major limitations for PNIPAAm hydrogel is that it is non-biodegradable, displays syneresis and has intrinsically hard and brittle mechanical properties. Efforts have been made to prepare degradable PNIPAAm by introduction of biomacromolecules or peptide sequences into the PNIPAAm-based polymers, or copolymerization with monomers possessing degradable polyester segments [25–27]. Michael addition polymerizations have been extensively applied for gelation of injectable hydrogels, as it allows the formation of stable chemical bounds in aqueous media and physiological conditions without the use of catalysts that may raise cytotoxicity issues [28–30]. Often, physical and chemical cross-linking methods are combined to achieve an immediate physical gelation that quickly forms the gel network, followed by a chemical stabilization of the system that ensures an adequate residence time of the hydrogel in the physiological environment [31–33].

Our group has previously reported the development of a novel hydrogel system based on (meth)acrylated triblock copolymers consisting of a central PEG chain flanked at both sides by copolymers of PNIPAAm and poly(hydroxypropyl methacrylamide dilactate) (p(HPMAm-lac$_2$)) for the controlled release of peptide-based drugs [34]. When combined with thiolated hyaluronic acid, used as a cross-linker, these polymers formed thermally responsive, biodegradable, non-shrinking hydrogels chemically cross-linked by Michael addition [34]. The above described hydrogel technology was designed to form physically cross-linked hydrogels at physiological temperature, followed by chemical cross-linking in situ through Michael type reaction. The main advantage of this system is that the thermal gelation and favorable Michael addition cross-linking reaction occur through mild processes and require no exogenous, cytotoxic initiators, thus potentially allowing for efficacious drug delivery in vivo. Furthermore, the use of a cross-linker of natural origin is likely to enhance the biocompatibility and positive biological properties of the hydrogel technology. In our previous work, the chemical cross-linking process was found complete after 50 h, with acrylate derivatives reacting faster than the methacrylate analogues [34]. Also, the system was proven effective at releasing bradykinin in vitro in a tailorable and diffusive fashion according to initial solid content. To improve upon the previous hydrogel system and enable the thermosensitive polymer to undergo a more rapid chemical gelation and a controlled degradation pathway, we investigated the gelation kinetics, the mechanical properties and the swelling/degradation profiles of a vinyl-sulfone modified thermosensitive triblock copolymer combined with a series of thiolated hyaluronic acid ranging in degree of thiol substitution from 23% to 72%. Fast gelation is a particularly important characteristic as it prevents the hydrogel from premature dissolution upon administration, allows for immobilization of cells and drugs at the target site and offers a more predictable translation of gel properties in vivo. In the present study, new ways to accelerate Michael addition cross-linking kinetics were investigated by using vinyl sulfone moieties as Michael addition acceptor and by varying the degree of substitution of the Michael donors bearing polysaccharide. Vinyl sulfones were selected as cross-linking groups for their renowned higher reactivity in Michael addition reactions as compared to (meth)acrylates. Additionally, cytocompatibility studies were performed in vitro to assess the capacity of the developed hydrogel to positively interface biological entities, sustaining cell viability and preserving tissue integrity and function.

2. Materials and methods

2.1. Materials

Unless indicated otherwise, chemicals were obtained from Sigma–Aldrich and were used as received. Research grade sodium hyaluronate produced from microbial fermentation and hydrolyzed to a molecular weight of 37,900 Da, was supplied by Lifecore Biomedical, LLC. Hydroxyl propyl methacrylamide monolactate (HPMAm-lac$_1$) and dilactate (HPMAm-lac$_2$) were synthesized according to a previously reported method [35]. Briefly, HPMAm was synthesized by Schotten–Baumann reaction in water at pH 9–11, using methacryloyl chloride and dl-1-amino-2-propanol as reagents at 0 °C. The pure product was purified by extraction in dichloromethane and isolated by rotary. HPMA was subsequently reacted for 20 min with L-lactide in the presence of dimethyl amino pyridine (DMAP) as catalyst and 4-methoxyphenol as inhibitor at the temperature of 130 °C and in dry conditions. The HPMAm-lac$_{1-2}$ workup was performed by VersaFlash chromatography [35].

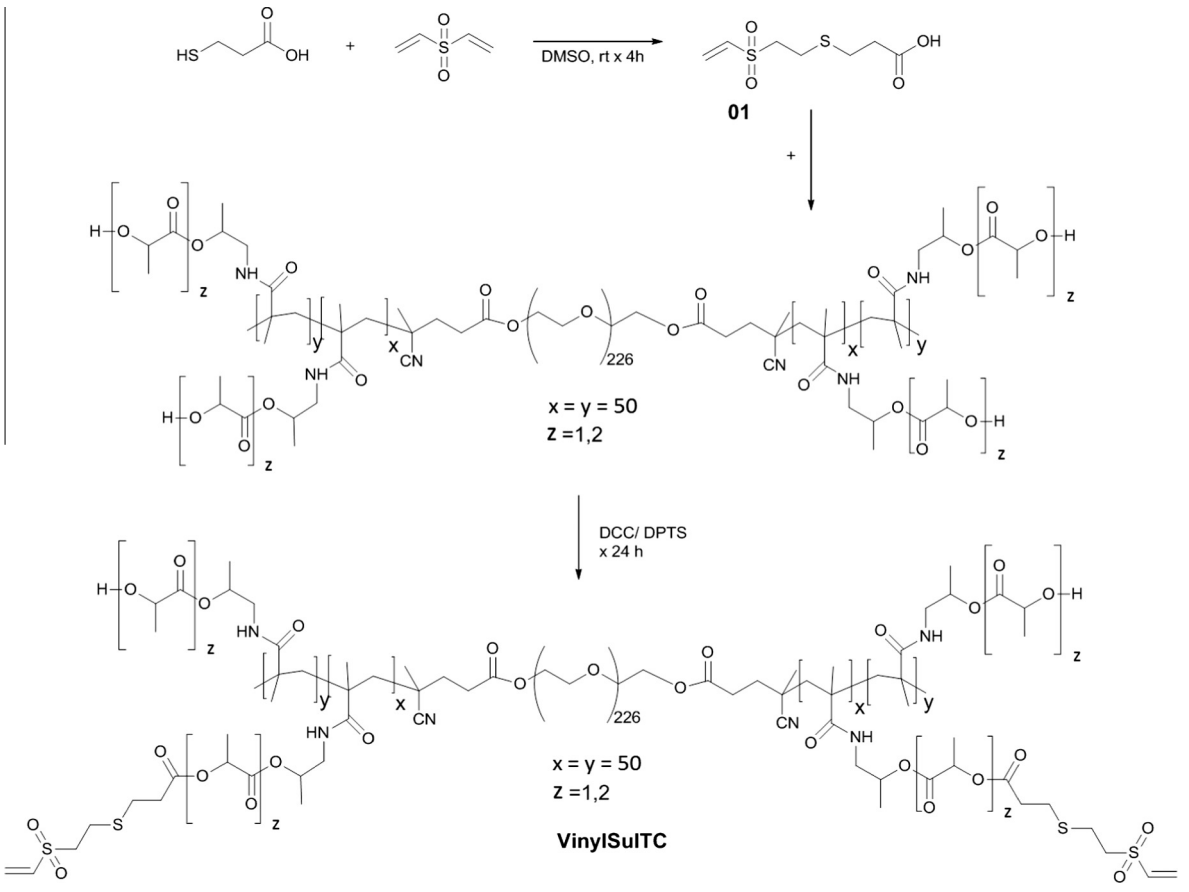
The synthesis of p(HPMAm-lac$_{1-2}$)-PEG triblock copolymers was carried out by free radical polymerization using a macroinitiator of PEG and 4,4′-azobis(4-cyanopentanoic acid) (ABCPA) (PEG-ABCPA)n. The (PEG-ABCPA)n macroinitiator

was synthesized by *N*-*N*′-dicyclohexylcarbodiimide (DCC) coupling [11]. Both p(HPMAm-lac$_{1-2}$)-PEG triblock copolymers and (PEG-ABCPA)n macroinitiator were purified by extensive dialysis in water and obtained as white powders after lyophilization. The synthesized (PEG-ABCPA)n displayed M_n and M_w values of 84 and 117 kDa, respectively according to the gel permeation chromatography (GPC) method described below and a ratio between ABCPA and PEG of 1.93/1 according to ^{1}H NMR in $CDCl_3$. P(HPMAm-lac$_{1-2}$)-PEG triblock copolymer had M_n and M_w values of 25.7 and 52.5 by GPC and a ratio HPMAm-lac$_1$/HPMAm-lac$_2$ of 1/1 according to ^{1}H NMR in $CDCl_3$.

3,3′-Dithiobis(propanoic dihydrazide) (DTP) was synthesized by the method described by Vercruysse et al. [36].

2.2. Synthesis of (vinyl sulfonate) triblock copolymer

A triblock copolymer composed of a central PEG chain of a molecular weight of 10 kDa flanked at both sides by thermosensitive side chains of HPMAm-lac$_1$ and HPMAm-lac$_2$ copolymerized at a 1:1 M ratio was synthesized by free radical polymerization as described by Vermonden et al. [11]. This triblock copolymer, indicated as VinylSulTC_0 and displaying thermosensitive behavior in aqueous solutions, was subsequently derivatized with vinyl sulfone moieties, according to the synthetic route reported in Scheme 1, in order to introduce chemically cross-linkable sites. The degree of substitution (DS) of vinyl sulfone groups was defined as percentage of the free OH-groups that have been modified. Vinyl sulfone bearing polymers are indicated as VinylSulTC_n, where n is the DS. VinylSulTC_10 was synthesized by dissolving divinyl sulfone (DVS) (114 mmol) in 100 ml dimethyl sulfoxide (DMSO). Subsequently, 3-mercapto propionic acid (3-MPA) (5.7 mmol), was added dropwise to the previously prepared solution at a molar ratio of 1:20 compared to DVS and the reaction was stirred at room temperature for 4 h. Separately, p(HPMAm-lac$_{1-2}$)-PEG (0.221 mmol, corresponding to 28.5 mmol of free OH-groups), 4-(dimethylamino)pyridinium 4-toluensulfonate (DPTS) (0.85 mmol) and N,N′-dicyclohexylcarbodiimide (DCC) (8.5 mmol) were dissolved in DMSO (10 ml per gram of triblock copolymer) and added dropwise to the previous mixture at a molar ratio between free hydroxyl groups of p(HPMAm-lac$_{1-2}$)-PEG and 3-MPA of 5:1. The reaction was stirred for 24 h at room temperature. VinylSulTC_15 was synthesized by an analogous procedure, using a 50% higher amount of DVS and 3-MPA. The reaction products VinylSulTC_10 and VinylSulTC_15 were purified by extensive dialysis (MWCO 12–14 KDa) at 4 °C against deionized water and isolated as white powder upon freeze-drying. The vinyl sulfone modified



Scheme 1. Synthesis route of vinyl sulfonated thermosensitive triblock copolymer VinylSulTC_n.

triblock copolymers were characterized by ^{1}H NMR, GPC and light scattering. The DS was determined by ^{1}H NMR and calculated according to the equation:

$$((I_{6.3-6.2} + I_{6.9}/3)/(I_{6.3-6.2} + I_{6.9}/3 + I_{5.4-5.2}) \times 100.$$

where I_n is the integral of ^{1}H NMR peaks at different ppm values (n).

2.2.1. Before vinyl sulfonation

^{1}H NMR, DMSO-d$_6$, δ in ppm: 7.3 (1H, –N*H*CH$_2$CHCH$_3$), 5.5–5.2 (1H, –O*H*CHCH$_3$), 5.0–4.8 (2H, –NHCH$_2$C*H*(CH$_3$)O and –COC*H*(CH$_3$)O), 4.2–4.1 (1H, –COC*H*(CH$_3$)OH), 3.5 (909 H, –OC*H*$_2$C*H*$_2$ PEG protons), 3.1 (2H, –NHC*H*$_2$), 1.5–0.8 (main chain protons).

2.2.2. After vinyl sulfonation

^{1}H NMR, DMSO-d$_6$, δ in ppm: 7.3 (1H, –N*H*CH$_2$CHCH$_3$), 6.9 (1H, –SO$_2$C*H* = CH$_2$), 6.3–6.2 (2H, –SO$_2$CH = C*H*$_2$), 5.4–5.2 (1H, –O*H*CHCH$_3$), 4.9–4.8 (2H, –NHCH$_2$C*H*(CH$_3$)O and –COC*H*(CH$_3$)O), 4.2–4.1 (1H, –COC*H*(CH$_3$)OH), 3.5 (909 H, –OC*H*$_2$C*H*$_2$ PEG protons), 2.7 (8H, –C*H*$_2$C*H*$_2$SC*H*$_2$C*H*$_2$) 1.7–0.7 (main chain protons).

The M_n values of the thermosensitive blocks of the synthesized copolymers calculated according to ^{1}H NMR ranged from 18.5 to 22 kDa, while the M_n of the central PEG block was 10 kDa.

2.3. ^{1}H NMR spectroscopy

NMR spectra were recorded with a Varian Mercury Plus 400 NMR spectrometer. The polymers were dissolved in CDCl$_3$ or DMSO-d$_6$ or D$_2$O.

2.4. Gel permeation chromatography

The weight average molecular weight (M_w), the number average molecular weight (M_n) and the polydispersity index (PDI) were determined by gel permeation chromatography (GPC) using a TSKgel G4000HHR column (TOSOH BIOSCIENCE), 7.8 mm ID × 30.0 cm L, pore size 5 μm. PEGs of defined molecular weights ranging from 106 to 1,015,000 Da were used as calibration standards. The eluent was THF, the elution rate was 1.0 ml/min and the column temperature was 35 °C. The samples were dissolved in THF at a concentration of 5 mg/ml.

2.5. Determination of the cloud point

The cloud point (CP) of the polymers was determined by means of light scattering, using a Zetasizer Nano-S90, Malvern Instruments. The samples were dissolved at a concentration of 3–5 mg/ml in ammonium acetate buffer 120 mM pH 5.0 in order to minimize the polymer hydrolysis. Light scattering measurements were performed at a fixed scattering angle of 90° during temperature ramps from 5 to 40 °C, at a heating rate of 1 °C/min. The CP was determined as the onset of increasing light scattering intensity.

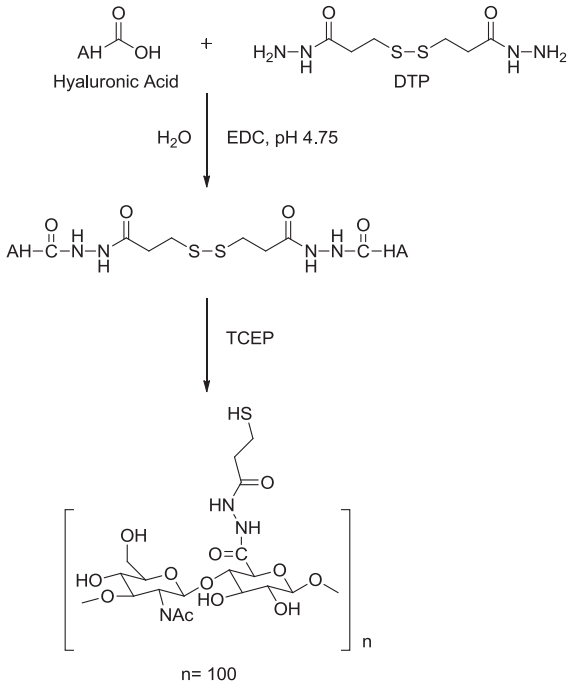
2.6. Synthesis of thiolated hyaluronic acid (HA-SH)

Hyaluronic acid was derivatized with thiol groups, to a varying extent, slightly modifying the procedure described by Shu et al. [37]. The extent of thiol derivatization, also called degree of substitution (DS), is defined as the number of 3–3′-dithiobis propanoic hydrazide (DTP) residues per 100 disaccharide units. As a typical reaction procedure, to obtain a DS of 50%, 1.0 g of sodium hyaluronate (M_n 37.9 kDa) was dissolved in 100 ml sterile water and 482 mg of DTP was added while stirring. The pH was adjusted to 4.75 with HCl 2 M and, subsequently, 388 mg of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) was added while keeping the pH at 4.75. The solution was stirred at room temperature for 48 h and the reaction was stopped by increasing the pH to 7 using 5 M NaOH. Then, a 20-fold excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added as reducing agent. The reaction mixture was stirred for additional 24 h at 4 °C and subsequently purified by dialysis (MWCO 12–14 kDa) against dilute HCl (pH 3.5) containing 100 mM NaCl and finally against water at 4 °C. The final product was obtained as a white powder after lyophilization. The synthetic route for thiolated hyaluronic acid is depicted in Scheme 2. The DS was determined by ^{1}H NMR [34] and Ellman's method [38]. Thiolated hyaluronic acid of different DS is indicated as HA-SH_n′, where n′ indicates the DS, that was chosen as the average between the Ellman's and ^{1}H NMR values.

^{1}H NMR, D$_2$O δ in ppm: 4.6–3.2 protons of hyaluronic acid, 2.7 (C*H*$_2$SH), 2.5 (C*H*$_2$CH$_2$SH), 1.8 (NHCOC*H*$_3$).

2.7. Hydrogel formulation

VinylSulTC_n and HA-SH_n′ were dissolved separately in phosphate buffer solution (PBS) (pH 7.4, 150 mM, supplemented with 0.02% w/vol NaN$_3$ to prevent bacterial contamination) at 4 °C. When both polymers were completely dissolved, the HA_SH_n′ solution was added to the triblock copolymer solution. The final concentration of triblock copolymers was 15 and 20% w/w, while the HA-SH_n′ concentration was calculated in order to obtain a 1:1 ratio between vinyl sulfone and thiol



Scheme 2. Synthesis route of thiolated hyaluronic acid.

groups. Immediately after mixing, the final solution containing VinylSulTC_n and HA-SH_n′ was placed in a preheated oven at 37 °C to allow thermal gelation and simultaneous Michael Addition cross-linking between thiol and vinyl sulfone groups.

2.7.1. Rheology

Rheological characterization was performed on a Physica – MCR 101 (Anton Paar) rheometer equipped with a Peltier plate and a 20 mm 1° steel cone-plate geometry. Solutions of p(HPMAm-lac)-PEG_n combined with HA-SH_n′ were applied between the cone and plate geometries and analyzed immediately upon mixing. A layer of silicone oil of viscosity of 0.05 Pa s was wrapped around the edge of the conical geometry to prevent water evaporation. A temperature sweep test from 18 to 37 °C at a heating rate of 1 °C/min followed by a time sweep test at 37 °C were performed. For both experiments a frequency of 1 Hz and 1% strain were used.

2.7.2. Gelation time and swelling tests

To determine the gelation time, solutions of VinylSulfTC_n and HA-SH_n′ of different DS in PBS buffer (pH 7.4, 150 mM, supplemented with 0.02% w/vol NaN_3 to prevent bacterial contamination) were mixed at a 1:1 M ratio of thiol to vinyl sulfone groups, at a VinylSulfTC_n concentration of 15 and 20% w/w and at the temperature of 37 °C. The gelation time was determined by the tilting vial method [13]. When the sample showed no flow, it was considered a gel. Subsequently, 100 μl of fully cross-linked hydrogels were submerged with 900 μl of PBS buffer solution, and the hydrogels were allowed to swell at 37 °C. The swollen hydrogels were weighted at regular time intervals after removing the buffer. Upon each weighing, the buffer was replenished. The swelling ratio of the hydrogels was calculated from the initial hydrogel weight after preparation (W_0) and the swollen hydrogel weight after exposure to buffer (W_t) according to the following equation:

$$\text{Swelling Ratio (SR)} = W_t/W_0$$

Experiments were performed in triplicate.

2.8. Determination of the vinyl sulfone conversion upon Michael addition cross-linking

The efficiency of Michael Addition cross-linking was evaluated by quantifying the non-reacted vinyl sulfone moieties as follows. Hydrogels were cross-linked at 37 °C until a three-dimensionally stable gelled material was obtained, as assessed by the vial tilting experiments described above. The networks (100 μl), cross-linked by Michael addition at 37 °C for 10, 25, 45 and 60 min for VinylSulf10HA23, 33, 56 and 72, respectively were subjected to basic hydrolysis at 50 °C in 9 ml NaOH 0.02 N until complete hydrogel dissolution. The solutions were neutralized with 1 ml acetic acid 2 M and the unreacted vinyl sulfone groups were quantified. High Performance Liquid Chromatography-Mass (HPLC-MS) analyses were performed for the detection of vinyl sulfone groups and the degree of vinyl sulfone conversion during Michael addition was calculated by

comparing the unreacted vinyl sulfone groups of cross-linked hydrogels to those of non-crosslinked samples. A positive control experiment whereby a freshly prepared mixture of vinyl sulfonated triblock copolymer and thiolated hyaluronic acid was hydrolyzed prior to cross-linking in NaOH 0.02 N for 30 min at 50 °C in order to assess the potential of Michael addition cross-linking occurring during degradation (Supporting Information). Analyses were performed on a HPLC-DAD, Agilent 1100 Series, using a Phenomenex Synergi 4 μm Polar – RP 80 A, 150 × 4.6 mm column set at the temperature of 35 °C. Isocratic elution of a 80:20 mixture of formic acid 0.1% and acetonitrile/formic acid 0.1% at a flow rate of 1 ml/min was applied to run 10 μl volume samples. Detection was performed at the wavelength of 210 nm for a runtime of 15 min. Mass spectra were recorded to further confirm the correct identification of peaks, using a HPLC-MS (Ion Trap), with a nebulizer pressure of 60 psi, a drying gas flow of 12 l/min, a drying gas temperature of 350 °C at a range of 105–800. The target mass was 223 *m*/*z*, negative polarity.

2.9. In vitro toxicity evaluation

2.9.1. Experimental animals

Three-month old male Balb/c mice (Harlan Italy SrL, Correzzana Milano, Italy) were used. Mice were kept in laminar-flow cage in a standardized environmental condition. Food (Harlan, Italy) and water were supplied ad libitum. Mice were sacrificed by CO_2 narcosis and cervical dislocation according to the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators.

2.9.2. Bone marrow stromal cell (BMSCs)

Upon sacrificing the above mentioned mice, long bones (femurs, tibiae and humeri) were dissected free of adhering tissue. The ends were removed and the marrow cavity was flushed and cells were pooled as previously described [39]. Only adherent cells were used for experimental procedures.

Toxicological tests were performed using mouse bone marrow stromal cell (BMSCs) and NIH 3T3 mouse fibroblasts. Studies were performed by assessing the growth of cells exposed to leachables extracted from hydrogel or grown on hydrogel substrates.

2.9.3. Assessment of cells growth on hydrogels

Culture dishes were evenly coated with a thin layer of hydrogel. Subsequently, BMSCs and NIH 3T3 cells were seeded on top of the hydrogel at the density of 5000 cells/well in 96 wells. In parallel experiments, BMSCs and NIH 3T3 cells were plated on hydrogel coated 6 wells culture dishes at the density of 15,000 cell/cm^2. Control cultures were grown on hydrogel uncoated dishes.

Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, Milano, Italy), supplemented with 10% heat inactivated fetal calf serum (FCS, Invitrogen, Milano, Italy), penicillin and streptomycin was used as culture medium. After 5 days of culture, cell viability and morphology was evaluated by MTS assay and toluidine blue staining.

2.9.4. Assessment of cells growth in presence of leachables

The leachables were extracted from hydrogel composite from 24 h to 21 days using DMEM. Dilution of the stock leachables (100%) was performed to vary the concentrations (100%, 50%, 25%) in culture medium [40].

BMSCs and NIH 3T3 cells were plated at a density of 5000 cells/well in 96 wells culture dishes and at the density of 15,000 cells/cm^2, in 6 wells culture dishes, and incubated with the leachables of different concentrations (0%, 25%, 50%, 100%) up to 21 days. BMSCs and NIH 3T3 cells plated at the same density and incubated in culture medium were taken as control. The effects of hydrogel on cell viability and morphology were evaluated by MTS and hematoxylin/eosin staining.

2.9.5. MTS assay

Cells viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium] as previously described [41]. In this assay, the number of viable cells is reflected by the activity of NAD(P)H-dependent cellular oxidoreductase enzymes, that reduce an intermediate electron acceptor reagent (phenazine methyl sulfate), which, in turn, converts MTS into the soluble formazan product. The latter compound is soluble in culture medium and gives a purple color that directly correlates to the number of viable cells and is measured spectroscopically at the wavelength of 490 nm.

2.9.6. Toluidine blue – hematoxylin/eosin staining

BMSCs and NIH 3T3 fibroblasts were fixed with 4% paraformaldehyde (PFA) for 20 min. After washing cultures were stained with toluidine blue (Sigma–Aldrich Co., Milano, Italy) or with hematoxylin/eosin (Sigma–Aldrich Co., Milano, Italy) following the manufacturer's instructions. Cells were monitored by a Leica DM 2500 optical microscope.

3. Results and discussion

3.1. Design and synthesis of polymers for the preparation of rapidly in-situ gelling hydrogels

The main characteristics of the synthesized polymers are overviewed in Table 1. A thermosensitive triblock copolymer VinylSulfTC_0 was synthesized with a yield of 60% and an M_n of 47 kDa, as determined by ^{1}H NMR. GPC analysis revealed a M_n value of 25.7 kDa with a rather broad PDI of 2.04, typical of free radical polymerization procedures [42,43]. The use of PEG standards for triblock copolymers based on PEG and HPMAm-lac$_{1-2}$ resulted in an underestimation of the M_n values compared to those calculated according to ^{1}H NMR, as observed previously [11]. VinylSulTC_0 showed a ratio between HPMAm-lac$_1$ and HPMAm-lac$_2$ close to the feed ratio of 50%, as calculated according to ^{1}H NMR. Such ratio resulted in a thermosensitive polymer with a cloud point, determined by light scattering, of 33 °C, in line with previously observed values [43,44]. VinylSulfTC_0 was subsequently functionalized, to a varying extent, with vinyl sulfone moieties. The synthesized vinyl sulfone-bearing polymers VinylSulfTC_10 and VinylSulfTC_15 displayed 10% and 15% of their free hydroxyl groups on lactate side chains modified with cross-linkable moieties, respectively.

The partial modification of the triblock copolymer with vinyl sulfone moieties led to a decrease in the CP, which dropped to from 33 to 29 and 26 °C, for VinylSulfTC_10 and VinylSulfTC_15, respectively, due to an increase in the polymer hydrophobicity. The dependence of CP on hydrophobicity was observed earlier [44], however, differently from previous results, the extent of temperature decrease is less pronounced with vinyl sulfone derivatives as compared to their methacrylate and acrylate analogues [43]. This observation indicates that the use of vinyl sulfones as reactive groups in Michael Addition cross-linking allows a higher extent of polymer derivation without markedly affecting the polymer lower critical solution temperature and consequently, its solubility in aqueous medium, as compared to methacrylate or acrylate derivatives.

GPC analyses revealed that M_w's and PDI's were constant upon partial modification of the free hydroxyl groups with vinyl sulfone moieties, indicating that no premature polymerization of vinyl sulfone residues had occurred during DCC coupling reaction, workup and lyophilization procedures. The comparison between ^{1}H NMR spectra of VinylSulfTC_0/10/15, as seen in Fig. 1, clearly showed the appearance of new peaks between 6 and 7 ppm for both VinylSulfTC_10 and VinylSulfTC_15 upon DCC coupling reaction. The mentioned peaks are assigned to the protons of the vinyl group and demonstrated the successful derivatization of VinylSulfTC_0. From the obtained DS, calculated according to ^{1}H NMR, it was observed that the conversion of 3-MPA-DVS during DCC coupling was approximately 50%.

Thiolated hyaluronic acid of a molecular weight of 37.9 kDa was used as a cross-linker and synthetized with a yield of approximately 80% and DS values ranging from 23% to 72%. As shown in Table 2, there was good agreement between DS values calculated according to ^{1}H NMR and determined by Ellman's method, indicating no premature formation of inter- and intra-chain disulfide bonds.

3.2. Hydrogel fabrication and gelation kinetics

Vinyl sulfone-modified triblock copolymers of different DS (10% and 15%) were cross-linked using stoichiometric amounts of thiolated hyaluronic acid at varying degrees of substitution from 23% to 72%. Table 3 overviews the polymer composition of the tested hydrogels.

Flow behavior was observed for all the stoichiometric mixtures at room temperature immediately after mixing (below the CP of VinylSulfTC_10) and the difference in HA-SH solid content did not remarkably affect the initial viscosity, possibly because of the low molecular weight of the polysaccharide. When the polymer solutions were incubated at 37 °C, a slight increase in viscosity and turbidity was observed, owing to the thermal gelation of the thermosensitive chains that self-assembled into hydrophobic domains above their CP. However, at the concentration of 20% w/w, VinylSulfTC assembly led to the formation of an extremely weak physical network, yet displaying flow behavior. As Michael addition progressed, the polymer solution became thicker and increased its mechanical strength at rates that strongly depended on the degree of substitution of HA-SH. Particularly, the fastest Michael addition kinetics were observed for HA-SH of lower DS. Fig. 2a shows a picture of a hydrogel composed of 20% w/w VinylSulfTC_10 and HA-SH_23 after four minutes Michael addition cross-linking, where flow behavior was no longer visible. However, when the formed viscoelastic material was expelled from the cylindrical mold by means of a plunger, the pressure applied caused a deformation of the biomaterial that could not be totally recovered, indicating that the flexible network still contained reversibly assembled domains that could be easily

Table 1
Overview of the main characteristics of VinylSulfTC_n copolymers, as determined by ^{1}H NMR, GPC and light scattering.

	M_n[a] (kDa)	M_n[b] (kDa)	M_w[b] (kDa)	PDI[b]	Cloud point[c] (°C)	Feed molar ratio 3-MPA/OH	Obtained DS[a] (%)	Yield (%)
VinylSulTC_0	47	25.7	52.5	2.04	33	0	0	60
VinylSulTC_10	54	25.7	54.2	2.1	29	0.20[a]	10.7	98
VinylSulTC_15	53	25.9	54.1	2.09	26	0.30[a]	14.8	96

[a] Based on ^{1}H NMR.
[b] Based on GPC using PEG standards.
[c] Based on light scattering.

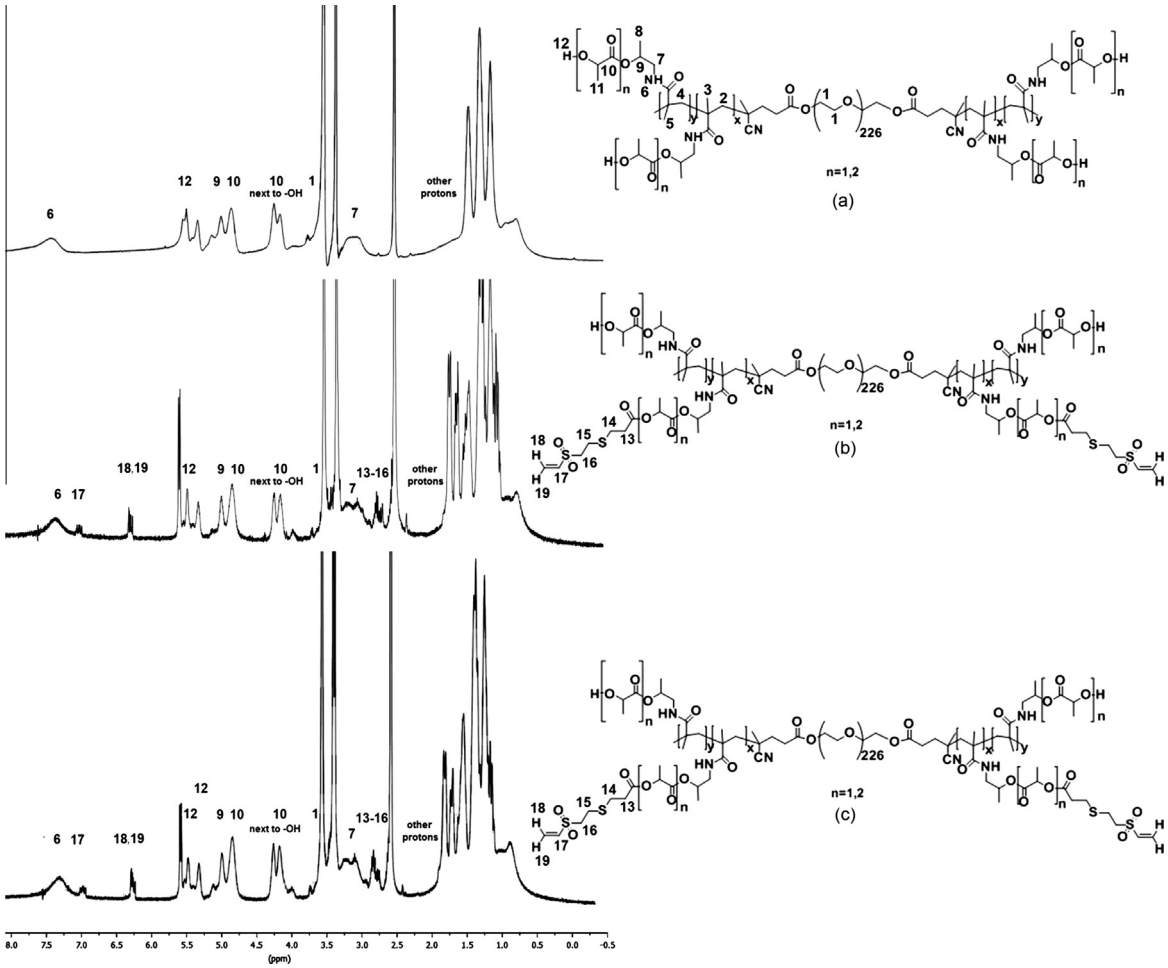


Fig. 1. [1]H NMR in DMSO-d6 of (a) VinylSulTC_0, (b) VinylSulTC_10 and (c) VinylSulTC_15.

Table 2
Overview of the main characteristics of HA-SH_n′, as determined by [1]H NMR and Ellman's method.

	DTP Feed ratio (%)	Obtained DS[a](%)	Obtained DS[b](%)	Conversion%	Yield (%)
HA-SH_23	35	21	25.3 ± 2.2	33	81
HA-SH_33	40	30	35.8 ± 3.1	43	85
HA-SH_56	75	59	52.6 ± 3.7	37	82
HA-SH_72	80	74	70.5 ± 3.3	46	79

† As reported by the producer.
[a] Based on [1]H NMR.
[b] Based on Ellman's method.

destabilized by the application of an external force. The redistribution of internal chain assembly led to the rearrangement of the hydrogel shape and size. In contrast, after 9 min Michael addition cross-linking, dimensionally stable networks able to retain their shape and size were formed. When subjected to the extrusion process, indeed, these hydrogels showed a small transient deformation followed by full recovery of their initial cylindrical shape and dimensions, indicating the formation of elastic hydrogels (Fig. 2b). For all formulations, the formation of a network with no-flow behavior was achieved within a rather quick time-span, ranging from 4 to 24 min. However, the formation of dimensionally stable networks when subjected to expulsion process from a cylindrical mold, were achieved in a time period from 9 to approximately 60 min. The gelled cylinders obtained after cross-linking were similar in shape and appearance for all formulations, regardless of HA-SH thiolation extent solid content, however, a higher flexibility and capacity for elastic recovery when subjected to a stress was observed for hydrogels containing HA-SH of lower DS. Although all formulations displayed the same number of cross-links per volume of gel, the shorter distance between thiol groups in highly modified HA resulted in more rigid and

Table 3
Overview of the polymer composition of the prepared hydrogels. In all formulations a 1:1 ratio between vinyl sulfone and thiol groups was used.

Hydrogel	Components	DS (%)	C (% w/w)	No VS = SH groups
VS10HA72	VinylSulfTC HA-SH	10 72	20 3.4	$6.48 * 10^{-3}$
VS10HA56	VinylSulfTC HA-SH	10 56	20 4.4	$6.48 * 10^{-3}$
VS10HA33	VinylSulfTC HA-SH	10 33	20 7.5	$6.48 * 10^{-3}$
VS10HA23	VinylSulfTC HA-SH	10 23	20 10.7	$6.48 * 10^{-3}$
VS15HA72	VinylSulfTC HA-SH	15 72	20 5.1	$9.72 * 10^{-3}$
VS15HA56	VinylSulfTC HA-SH	15 56	20 6.6	$9.72 * 10^{-3}$
VS15HA33	VinylSulfTC HA-SH	15 33	20 11.2	$9.72 * 10^{-3}$
VS15HA23	VinylSulfTC HA-SH	15 23	20 16.0	$9.72 * 10^{-3}$

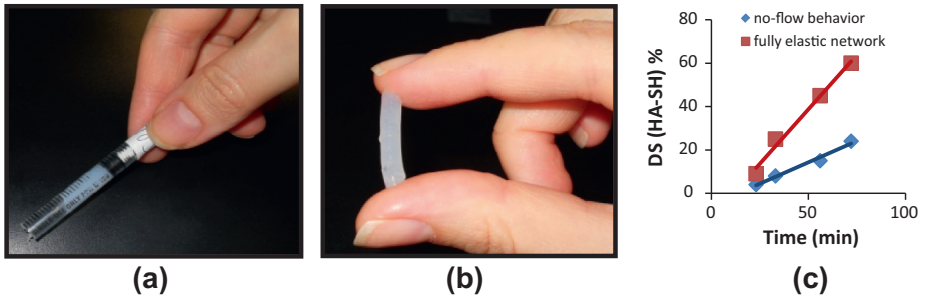


Fig. 2. Gross pictures of VinylSulfTC_10/HA-SH_23 hydrogels dually cross-linked by thermal gelation and Michael addition reaction at 37 °C. (a) After 4 min cross-linking, a viscoelastic material not displaying flow behavior was obtained (b) after 9 min cross-linking, a dimensionally stable, fully elastic gel was formed. (c) Relationship between gelation time and DS of HA-SH evaluated by the tilting vial method. Blue squares refer to the formation of a viscoelastic network displaying no flow behavior; red squares represent the formation of a fully elastic and dimensionally stable hydrogel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

brittle networks as compared to hydrogels based of HA-SH of lower DS. In Fig. 2c the relationship between gelation time and degree of thiolation is shown. A linear dependence between gelation kinetics and thiolation extent of HA-SH was observed. When HA is heavily modified with thiol groups, the steric hindrance, poor flexibility and short distance between Michael addition donors led to slower cross-linking kinetics, while the use of HA modified to a lesser extent showed more rapid chemical hydrogelation. By comparing Michael addition kinetics of previously studied (meth)acrylate modified polymers with vinyl sulfone derivatives, it is clear that, in line with expectations, the higher reactivity of the latter results in faster formation of viscoelatic materials. This evidence may be also due to the slightly higher hydrophilicity of vinyl sulfones as compared to (meth)acrylate residues, that facilitated the access of thiols into the slightly more hydrophilic self-assembled thermosensitive domains.

3.3. Hydrogel mechanical characterization

Mechanical characterization of hydrogels composed of HA-SH of different thiolation degree showed results in good agreement with what observed by means of the vial tilting experiments. Particularly, hydrogels consisting of 20% w/w VinylSulfTC_15 demonstrated faster gelation than networks based on VinylSulfTC_10. Fig. 3a and b show the storage and loss moduli (G' and G'') as a function of time during temperature sweep experiments from 18 to 37 °C followed by time sweep analysis at the constant temperature of 37 °C. It turned out that networks made of 20% w/w VinylSulfTC_10 and HA-SH_72 displayed a temperature at which G' equals G'' of 31 °C, while for the analogous hydrogels having 15% of vinyl sulfone derivatization, G' crossed G'' already at the temperature of 26 °C. Furthermore, the latter reached a G' value higher than 80 kPa, that was nearly twofold higher than that seen for VinylSulfTC_10 based hydrogels and a $\tan\delta$ value of approximately 0.4, indicating a stronger viscoelastic gel as compared to VinylSulfTC_10 hydrogels, displaying a $\tan\delta$ value, upon the same gelation

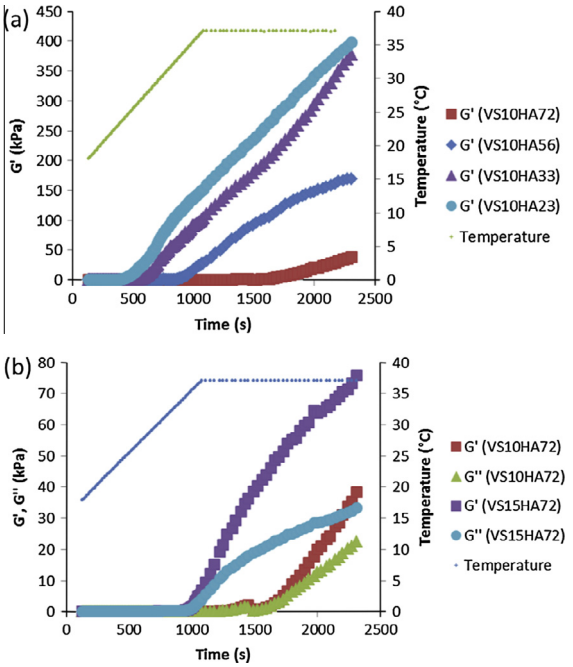


Fig. 3. Mechanical characterization of aqueous solutions of VinylSulfTC_n and HA-SH_n′ combined at a 1:1 ratio between vinyl sulfone and thiol groups. (a) Temperature sweep test from 18 to 37 °C followed by a time sweep test of hydrogels containing 20% w/w VinylSulfTC_10 and HA-SH_n′ of different DS (23%, 33%, 56% and 72%) performed during dual cross-linking (thermogelling and Michael Addition). (b) Temperature sweep test from 18 to 37 °C followed by a time sweep test of hydrogels containing 20% w/w VinylSulfTC_n of different DS (10% and 15%) and HA-SH_72 performed during dual cross-linking.

time, of 0.5. Hydrogel formulations possessing HA-SH of different extent of thiolation confirmed progressively faster gelation kinetics as the DS of HA-SH decreased (Fig. 3a). The initial G' value measured immediately after the combination of the two polymers showed no significant differences among formulations, indicating the variation in HA-SH solid content did not contribute to changes in initial viscosity and stability of the network, as the chain entanglement of low molecular weight hyaluronic acid is negligible at the studied range of concentration. During rheological experiments, elastic moduli never reached plateau values due to partial water evaporation of the small volume hydrogel samples (100 μl) that were subjected to the temperature of 37 °C for long timescales, resulting in progressive increase of solid content.

3.4. Vinyl sulfone conversion upon Michael addition cross-linking

For all hydrogel formulations no chromatographic peak corresponding to unreacted DVS-3MPA was detected (Fig. 4), demonstrating that, within the experimental error, Michael addition crosslinking progressed to a large extent during a time period of 9–60 min and that there was no formation of inter and intra-chain disulfide bonding.

3.5. Hydrogel swelling and degradation

The studied hydrogel formulations displayed remarkably different degradation profiles, with HA-SH_23 based hydrogels reaching a maximum SR value of approximately 2.5 in 15 days and degrading in 28 days. HA-SH_56 gels swelled up to SR values of about 2 and degraded after 50 days and, finally, HA-SH_72 networks showed a water uptake up to 1.7 folds their initial weight and dissolved completely in medium after more than 70 days (Fig. 5). The presence of chemical cross-links holds the hydrogels from dissolution; swelling and degradation phenomena occur when, at physiological pH, the hydrolysis of the ester bonds on the thermosensitive blocks causes increase in polymer hydrophilicity and consequent water uptake upon the degradation of the free lactates. Ultimately, the hydrolysis of the polymer side chains modified with vinyl sulfone residues leads to progressive weight loss until complete polymer dissolution. In the present swelling/degradation study, the investigated hydrogels displayed the same number of chemical cross links but different initial solid content of HA-SH. The observation that hydrogels composed of HA-SH of lower thiolation degree displayed shorter degradation times may be attributed to the higher polysaccharide content and distance between cross-links, that led to greater internal osmotic pressure and water uptake. Furthermore, the faster degradation rate of hydrogels composed of HA of lower DS is likely due to the higher release rate of HA chains. A lower number of cross-links, indeed, needs to be hydrolyzed before a HA chain is released from the network for HA-SH_23 networks compared to HA-SH_56 and 72 hydrogels.

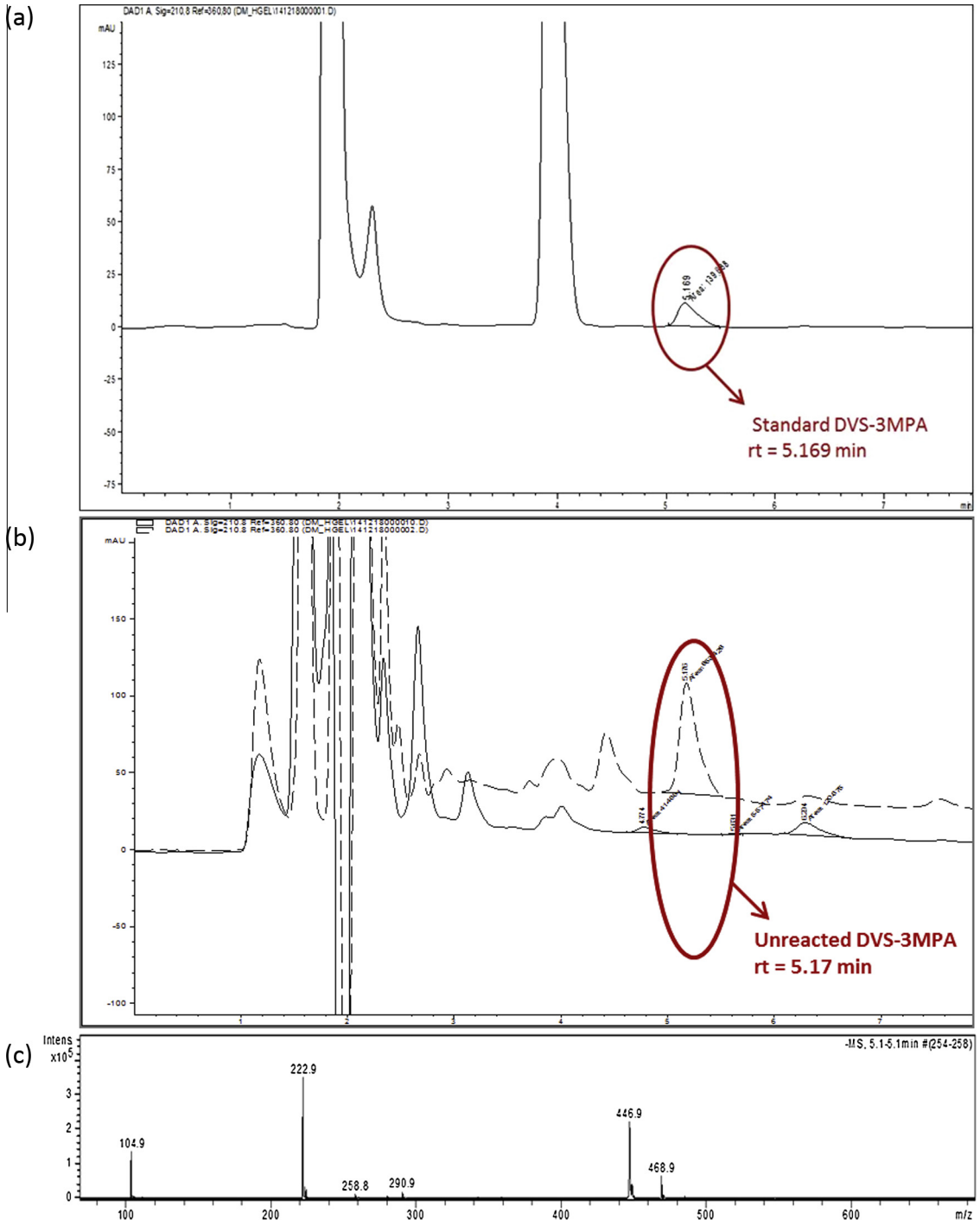


Fig. 4. HPLC chromatograms of (a) DVS-3MPA, synthesized by adding dropwise 3-MPA to a DVS solution in DMSO at a molar ratio of 1:20 between 3-MPA and DVS, respectively. The reaction was stirred at room temperature for 4 h before injection. (b) VinylSulfTC_10 hydrolyzed in NaOH solution (dashed line) and VinylSulfTC_10/HA-SH_23 hydrogel hydrolyzed in NaOH solution after 10 min cross-linking. (c) Negative ions mode electrospray ionization-mass (ESI/MS) spectrum of hydrolyzed VinylSulfTC_10 at the retention time of 5.1 min. The peak appearing at a retention time of approximately 5.17 min in (a) and (b) corresponds to the compound DVS-3MPA, as also confirmed in (c) ($M–H^+$ = 223 Da).

3.6. Cytocompatibility testing: cell viability and homeostasis

In Fig. 6 the cellular viability is expressed as a function of formazan optical density (O.D.) at 490 nm. Fig. 6 shows that cell viability and morphology of BMSCs cells seeded and grown on culture dishes coated with 20% w/w

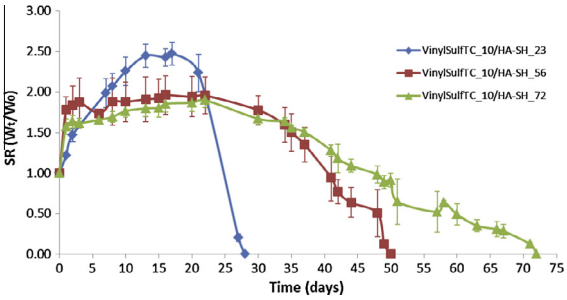


Fig. 5. Degradation profiles in PBS at pH 7.4 of dually cross-linked hydrogels containing 20% w/w VinylSulfTC_10 and HA-SH_n′ of different DS (23%, 56% and 72%). Hydrogels were formulated in order to have a 1:1 M ratio between vinyl sulfone and thiol groups, that translated in a HA-SH_n′ solid content of 4.2, 5.4 and 13.4% w/w, for HA-SH_72, 56 and 23 respectively. The hydrogel degradation is expressed as SR, calculated as ratio between the weight of the swollen hydrogels, upon buffer removal, and initial gel weight.

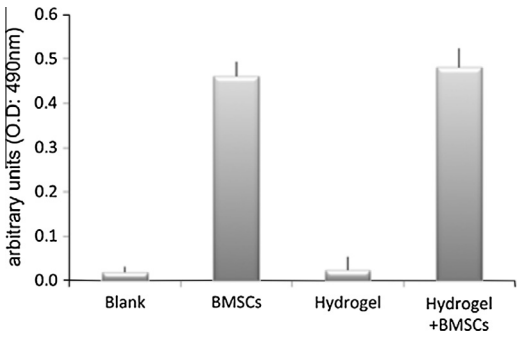


Fig. 6. Viability, evaluated by MTS assay, of BMSCs cells seeded on culture dishes coated and uncoated with 20% w/w VinylSulfTC_10/HA-SH_56 hydrogels. The graphic shows the comparison between Dulbecco's modified culture medium (blank), BMSCs cells culture in Dulbecco's modified culture medium (BMSCs), VinylSulfTC_10/HA-SH_56 hydrogel coated dish in the presence of Dulbecco's modified culture medium (Hydrogel) and VinylSulfTC_10/HA-SH_56 hydrogel coated dish in the presence of BMSCs cells and Dulbecco's modified culture medium (Hydrogel + BMSCs).

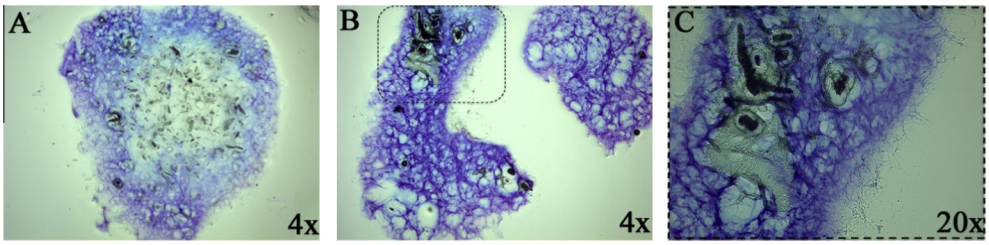


Fig. 7. Toluidine blue staining of BMSCs cells seeded on and infiltrated into 20% w/w VinylSulfTC_10/HA-SH_56 hydrogels. (A) and (B) show a 4× magnification of two different areas of the hydrogel coated dish, (C) shows a 20× magnification of the dotted box in figure (B). In all pictures the hydrogel is visible as white/gray material underlying the blue staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

VinylSulfTC_10/HA-SH_56 hydrogels as substrate were comparable to those observed for BMSCs cells on uncoated culture dishes. Toluidine blue, a sensitive "vital" staining having metachromatic properties, specifically bound the nuclear material of the BMSCs, that were found present both on top of the hydrogel material and infiltrated within the bulk, through diffusion into the porous 3D network of the hydrogel (Fig. 7). The blue staining, indicating the presence of cells, showed that BMSCs clustered in some regions of the hydrogel material, visualized in the microscopy pictures of Fig. 7 as gray/white material.

MTS assay also showed that the medium rich in hydrogel degradation products preserved nearly complete NIH 3T3 cell viability at all time intervals and at all concentrations tested (Fig. 8, top panel). Moreover, supporting MTS data, hematoxylin/eosin cytochemical staining (that marks the nuclei, cytoplasm and extracellular matrix of living cells) showed that the fusiform or polygon shape of the cells was preserved (Fig. 8, bottom panel). Similarly, BMSCs growth and morphology were not affected by leachables extracted from the degrading hydrogel composite consisting of 20% w/w VinylSulfTC_10/HA-SH_56 (Supporting Information).

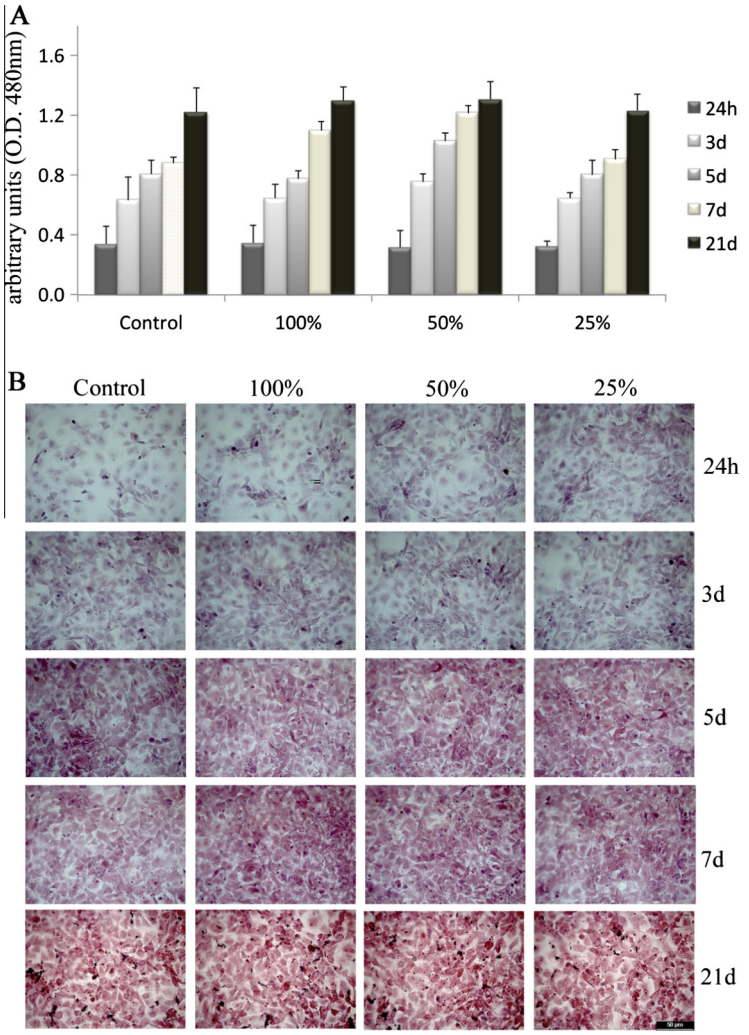


Fig. 8. Viability by MTS assay (top) and microscopy pictures of NIH 3T3 cells stained with toluidine blue (bottom) exposed for a time frame of 21 days to the degradation products of 20% w/w VinylSulfTC_10/HA-SH_56 hydrogels submerged in Dulbecco's medium at 37 °C. Cells were exposed to different dilutions of the leachates (100%, 50% and 25%) in Dulbecco's medium. NIH 3T3 cultured for 21 days in Dulbecco's modified culture medium were taken as control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In conclusion, MTS assay and cytochemical approaches unveiled that VinylSulTC_10/HA-SH_56 hydrogels are foremost compounds not affecting cell viability and, more importantly, they did not affect the steady state behavior of the investigated cells.

4. Conclusions

In this study, we have developed a dually cross-linked hydrogel network consisting of thermosensitive vinyl sulfone bearing p(HPMAm-lac$_{1-2}$)-PEG-p(HPMAm-lac$_{1-2}$) triblock copolymers and thiol modified hyaluronic acid, that combined in aqueous solution at physiological conditions undergo immediate thermal gelation and simultaneous Michael addition cross-linking between vinyl sulfone and thiol groups. We have demonstrated that the modification with vinyl sulfone moieties yielded triblock copolymers with moderate decrease of the cloud point of the triblock copolymer and faster and more efficient formation of chemical networks, as compared to acrylate and methacrylate derivatives [34]. Furthermore, it was shown that the gelation kinetics, the physical and mechanical properties and the swelling and degradation behavior can be easily tailored by polymer content, degree of vinyl sulfonation and degree of thiolation. Importantly, high viability and proliferation of mouse bone marrow derived stem cells and NIH 3T3 fibroblasts seeded on top of hydrogels was observed for 21 days. The tested cells preserved for the whole duration of the experiment (21 days) their shape and conformation and showed a certain mobility, being able to infiltrate the bulk of the biomaterial. In conclusion, the developed hydrogels proved promising candidates for future developments in the biomedical and pharmaceutical fields.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eurpolymj.2015.07.036.

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