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RESEARCH ARTICLE

In vivo biocompatibility of p(HPMAm-lac)-PEG hydrogels hybridized with hyaluronan

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Abstract

The present study reports on the biocompatibility *in vivo* after intramuscular and subcutaneous administration in Balb/c mice of vinyl sulphone bearing p(HPMAm-lac1–2)-PEG-p(HPMAm-lac1–2)/thiolated hyaluronic acid hydrogels, designed as novel injectable biomaterials for potential application in the fields of tissue engineering and regenerative medicine. Ultrasonography, used as a method to study hydrogel gelation and residence time *in vivo*, showed that, upon injection, the biomaterial efficiently formed a hydrogel by simultaneous thermal gelation and Michael Addition cross-linking forming a viscoelastic spherical depot at the injection site. The residence time *in vivo* (20 days) was found to be shorter than that observed *in vitro* (32 days), indicating that the injected hydrogel was resorbed not only by chemical hydrolysis but also by cellular metabolism and/or enzymatic activity. Systemic biocompatibility was tested by analysing routine haematological parameters at different time-points (7, 14 and 21 days after administration) and histology of the main organs, including the haematopoietic system. No statistically significant difference between parameters of the saline-treated group and those of the hydrogel-treated group was found. Importantly, a time-dependent decrease of important pro-inflammatory cytokines (TREM1 (Triggering Receptor Expressed on Myeloid cells-1), tumour necrosis factor-α and interleukin-1β) in cultured bone marrow cells extracted from hydrogel treated mice was observed, possibly correlated to the anti-inflammatory effect of hyaluronic acid released in time as hydrogel degraded. Copyright © 2016 John Wiley & Sons, Ltd.

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1. Introduction

Hydrogels formed from cross-linked three-dimensional networks of amphiphilic polymers are widely investigated and, in some examples, applied in the clinic for a broad range of pharmaceutical and biomedical applications, ranging from controlled drug delivery to tissue engineering (Slaughter *et al.*, 2009; Li *et al.*, 2012; Kharkar *et al.*, 2016). One key consideration in the application of hydrogel technologies *in vivo* concerns their ability to perform the specific tasks for which they are intended with an appropriate host response both locally and systemically (Fournier *et al.*, 2003; Jiang *et al.*, 2014; Major *et al.*, 2015). Generally, the introduction of a biomaterial into the body elicits an inflammatory reaction associated to the so-called foreign body reaction (FBR) (Anderson, 2001; Bryers *et al.*, 2012). The initial acute inflammation, caused by the implantation or injection procedure, evolves into chronic inflammation in response to the presence of the biomaterial. The course and the extent of the FBR that follows biomaterial administration largely depends on the biomaterial characteristics and the consequent interactions with the host tissue (Luttikhuizen *et al.*, 2006; Anderson *et al.*, 2008; Morais *et al.*, 2010). It is well established that inflammation and FBR have a strong impact on the ability of a hydrogel, and, more generally speaking, of a biomaterial, to function properly. Therefore, understanding the processes underlying the tissue response to biomaterials is pivotal in finding new ways to optimize and modulate biocompatibility, biosafety and biointegration (i.e. through changes in chemical composition, porosity, stiffness, degradation products) (Luttikhuizen *et al.*, 2007; Franz *et al.*, 2011). Unlike many solid or hydrophobic biomaterials, hydrogels possess important biofriendly features, such as a high water content, a porous and highly diffusional structure, a soft and rubbery nature and a low free energy at the interface, that minimize irritation of surrounding tissues and adherence

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of cells and proteins to their surface. Among all available hydrogel technologies, thermosensitive injectable synthetic hydrogels are particularly promising as they allow for minimally invasive administration and tailorable network characteristics, such as swelling, mechanics, mesh size and degradation (Jeong *et al.*, 2002). A class of polymers exploited as thermosensitive gel-forming biomaterials is composed of poly(ethylene glycol) (PEG) and its copolymers with aliphatic polyesters, such as poly(lactic acid) (PLA), poly(D,L-lactic-*co*-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL) and polyphosphazenes (Kumbar *et al.*, 2006; Kang *et al.*, 2010; Hwang *et al.*, 2013). Polyethylene glycol is used extensively as it is resistant to protein adsorption and cell attachment, potentially reducing tissue adherence and inflammation (Nehrt *et al.*, 2010; Shi, 2013; Klouda, 2015). Some of the aforementioned copolymers have been investigated for their biocompatibility *in vivo*. Results showed that, despite the attractive characteristics of the hydrogels administered, transient inflammatory phenomena of varying duration and intensity often occurred (Shim *et al.*, 2006; Bjugstad *et al.*, 2008; Kim *et al.*, 2010). Novel and proprietary biodegradable and thermosensitive triblock copolymers based on poly(*N*-(2-hydroxypropyl methacrylamide lactate) and poly(ethyleneglycol) (p(HPMAm-lac)-PEG), capable of spontaneously self-assembling in physiological environments have been developed (Vermonden *et al.*, 2006). It was demonstrated that the hydrogels spontaneously jellify at physiological conditions, could be stabilized by chemical cross-linking and displayed superior and controllable degradation, mechanical and swelling properties (Vermonden *et al.*, 2008; Censi *et al.*, 2010b). Furthermore, their great potential as protein/peptide releasing matrices and as scaffolding material for tissue engineering was demonstrated *in vitro* (Censi *et al.*, 2009, 2011b). In the present study, the previously developed thermosensitive polymers were modified with vinyl sulphone moieties and chemically crosslinked by thiolated hyaluronic acid (HA). This is a non-sulphated and enzymatically degradable glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine, participating in the composition of the extracellular matrix. Hyaluronic acid was selected because it is involved in a great number of relevant biological functions, including cell proliferation, wound healing and inflammation (Kogan *et al.*, 2007). The present study investigated for the first time the host response *in vivo* to p (HPMAm-lac)-PEG/HA hybrid hydrogels upon subcutaneous and intramuscular injection in Balb/c mice. The overall aim was to gain new insights into the biocompatibility and safety of the biomaterial developed with a focus on inflammatory aspects of the tissue reaction. This *in vivo* study will establish the suitability of the proposed hydrogel technology as a promising matrix for tissue engineering and drug delivery. The possibility to apply the *in vivo* hydrogel technology developed will potentially bring advances in the biomedical and pharmaceutical fields because of the introduction of a novel functional biomaterial.

2. Materials and methods

2.1. Materials

Unless indicated otherwise, chemicals were obtained from Sigma-Aldrich (Stenheim, Germany) and used as received. Research grade sodium hyaluronate produced from microbial fermentation and hydrolysed to a molecular mass of 37.9 kDa, was supplied by Lifecore Biomedical, LLC (Chaska, MN, USA). Hydroxyl propyl methacrylamide monolactate (HPMAm-lac$_1$) and dilactate (HPMAm-lac$_2$) were synthesized according to previously reported methods (Soga *et al.*, 2004) The synthesis of p (HPMAm-lac1–2)-PEG triblock copolymers was described previously (Vermonden *et al.*, 2006). 3,3′-Dithiobis propanoic dihydrazide (DTP) was synthesized by the method described by Shu *et al.* (2003).

2.2. Synthesis of vinyl sulphonated triblock copolymer

A triblock copolymer composed of a central 10 kDa PEG chain co-polymerized at both sides with thermosensitive side chains of HPMAm-lac$_1$ and HPMAm-lac$_2$, copolymerized at a 1:1 molar ratio, was synthesized by free radical polymerization, as described by Vermonden *et al*. (2006). This triblock copolymer, indicated as VinylSulTC_0 and displaying thermosensitive behaviour in aqueous solutions, was subsequently modified with vinyl sulphone moieties, in order to introduce chemically crosslinkable sites, as previously reported (Dubbini *et al.*, 2015). The degree of substitution (DS) with vinyl sulphone groups is defined as the percentage of the free OH-groups that have been modified. The DS was determined by ^{1}H-nuclear magnetic resonance (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 p.p.m. values (n).

Before vinyl sulphonation: ^{1}H-NMR, dimethylsulphoxide (DMSO)-d_6, δ in p.p.m.: 7.3 (1H, $-NHCH_2$-$CHCH_3$), 5.5–5.2 (1H, $-OHCHCH_3$), 5.0–4.8 (2H, $-NHCH_2CH(CH_3)O$ and -$COCH(CH_3)O$), 4.2–4.1 (1H, $-COCH(CH_3)OH$), 3.5 (909 H, $-OCH_2CH_2$ PEG protons), 3.1 (2H, $-NHCH_2$), 1.5–0.8 (main chain protons).

After vinyl sulphonation: ^{1}H-NMR, DMSO-d_6, δ in p.p.m.: 7.3 (1H, $-NHCH_2CHCH_3$), 6.9 (1H, $-SO_2CH = CH_2$), 6.3–6.2 (2H, $-SO_2CH = CH_2$), 5.4–5.2 (1H, $-OH$-$CHCH_3$), 4.9–4.8 (2H, $-NHCH_2CH(CH_3)O$ and -$COCH(CH_3)O$), 4.2–4.1 (1H, $-COCH(CH_3)OH$), 3.5 (909 H, $-OCH_2CH_2$ PEG protons), 2.7 (8H, $-CH_2CH_2SCH_2CH_2$) 1.7–0.7 (main chain protons).

The ^{1}H-NMR spectra and peak assignment are shown in the Supplementary material online data section. Vinyl sulphone-bearing polymers are indicated as VinylSulTC_10, where 10 is the DS.

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

Hyaluronic acid was functionalized with thiol groups by slightly modifying the procedure described by Shu *et al.* (2002). The extent of thiol derivatization, also called DS is defined as the number of DTP residues per 100 disaccharide units. To obtain a DS of 50%, 1.0 g of sodium hyaluronate (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 2 M HCl 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 a value of 7 with 5 M NaOH. A large excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was then added as reducing agent. The reaction mixture was stirred for a further 24 h at 4°C and subsequently purified by dialysis Molecular Weight Cut Off (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 DS was determined by ^{1}H-NMR (Censi *et al.*, 2010a) and Ellman's (1959) method. Thiolated hyaluronic acid was indicated as HA-SH_56, where 56 indicates the DS.

^{1}H-NMR, D_2O δ in p.p.m.: 4.6–3.2 protons of hyaluronic acid, 2.7 (C*H2*SH), 2.5 (C*H2*CH2SH), 1.8 (NHCOC*H3*).

2.4. ^{1}H-NMR spectroscopy

The NMR spectra were recorded with a Varian Mercury Plus 400 NMR spectrometer, Milano, Italy. The polymers were dissolved in $CDCl_3$, DMSO-d_6 or D_2O.

2.5. 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, Torino, Italy), 7.8 mm internal diameter × 30.0 cm long, pore size 5 μm. A range of PEGs of defined molecular masses ranging from 106 to 1 015 000 Da were used as calibration standards. The eluent was THF (Tetrahydrofuran), 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.

2.6. 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, (Malvern, UK). The samples were dissolved at the 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.

2.7. Hydrogel formulation

The vinyl sulphone bearing triblock copolymer VinylSulfTC_10 was dissolved at a concentration of 15% *w*/w in phosphate buffered saline at 4°C; thiolated HA of a DS of 56% (HA-SH_56) was then added at a final concentration of 4.1% w/w. The molar ratio between vinyl sulphone groups of VinylSulfTC_10 and thiol groups of HA was 1:1. Fifty microlitres of the VinylSulfTC_10/HA-SH_56 mixture was injected into Balb/C mice by a 1 ml syringe both subcutaneously and intramuscularly. Thermal gelation and simultaneous Michael Addition occurred *in vivo*.

2.8. Rheology

Rheological characterization was performed on a Physica-MCR 101 (Anton Paar, Rivoli, Italy) rheometer equipped with a Peltier plate and a 20 mm 1° steel cone–plate geometry. Solutions of VinylSulfTC_10 combined with HA-SH_56 were applied between the cone and plate geometries and analysed immediately upon mixing. A layer of silicone oil of viscosity of 0.05 Pa.s was positioned 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.

2.9. Swelling tests

One hundred microlitres of fully cross-linked hydrogels were submerged in 900 μl of PBS, 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.

2.10. *In vivo* evaluation of biocompatibility

2.10.1. Experimental animals

Balb/c male mice (Harlan Italy SrL, Correzzana Milan, Italy) weighing between 25 g and 30 g were selected. All the animal experiment protocols were approved by

the Institutional Animal Care and Use Committee and were in compliance with local regulatory guidelines. Mice were kept in laminar-flow cage in standardized environmental conditions. Food (Harlan Italy SrL) and water were supplied *ad libitum*.

Mice (7–9 weeks old) were randomized in two groups: experimental and control group (18 mice for each group). Experimental group was injected both intramuscularly (i.m.), into the hind limb and subcutaneously (s.c.) into the loose skin on the back of the neck with 50 μl of 15% *w*/w VinylSulfTC_10/HA-SH_56 hydrogel in sterile physiological saline (0.90% *w*/*v* of NaCl in water). Control groups received saline. Each group of mice was then further divided in two subgroups in order to simultaneously study both the *in vivo* hydrogel polymerization and degradation and the *in vivo* biocompatibility.

2.10.2. *In vivo* evaluation of hydrogel polymerization and degradation

In order to observe the *in vivo* polymerization of hydrogels, the i.m. injections were closely monitored and controlled by ultrasonographic visualization.

Ultrasonography, both in human and veterinary medicine, is the gold standard, non-invasive tool to track the changes in appearance and size of lesions in soft tissue over time. The normal appearance of tissue varies depending on their physical properties and by interaction with ultrasound. Normally, fluid appears black in colour, while hard tissues, such as bone, reflect all beams and are not evaluable. Soft tissues appear in different shades within the grey scale depending on the characteristic of the tissue itself. For ultrasonography, a 13 MHz linear probe (MyLab™30 VET; Esaote, Genova, Italy) set at a depth of 2.0 cm was used. After administration of 4% isoflurane (induction) followed by 2% isoflurane (maintenance) in combination with a 2:1 mixture of O_2/N_2O, mice were placed in lateral recumbence and the ultrasonographic beam was directed parallel to the femur to visualize the caudal thigh muscles at the level of the injection site.

To monitor the *in vivo* depot formation and biodegradability of the hydrogel, ultrasonographic appearance (echogenicity) and dimensions (vertical and horizontal axis) of the injected material were recorded 5, 10, 15 and 20 days after the injection, and the areas of the ellipsoids were calculated; all images obtained were stored to allow later analysis (MyLabDesk™; Esaote, Genova, Italy).

2.10.3. Determination of the vinyl sulphone conversion upon Michael Addition crosslinking in vivo

A group of three mice were killed by CO_2 asphyxiation, 30 min after i.m. injection of 50 μl of 15% *w*/w VinylSulfTC_10/HA-SH_56 hydrogels. The depot site was cut open and the viscoelastic material explanted and placed in NaOH solution 0.02 N at 50°C until complete degradation.

The efficiency of Michael Addition cross-linking *in vivo* was evaluated for the explanted and degraded hydrogels by quantifying the hydrolysed non-reacted vinyl sulphone moieties by high-performance liquid chromatography-mass spectrometry (HPLC-MS) analyses. The degree of vinyl sulphone conversion during Michael Addition was calculated by comparing the unreacted vinyl sulphone groups of the explants to those of non-crosslinked samples. Analyses were performed on a HPLC-DAD, Agilent, Milano, Italy 1100 Series, using a Phenomenex Synergi 4 μm Polar–RP 80 A, 150 × 4.6 mm column set at 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 a wavelength of 210 nm for a run time of 15 min. Mass spectra were recorded to further confirm the correct identification of peaks, using a HPLC-MS Ion Trap, Agilent, Milano, Italy, with a nebulizer pressure of 414 kPa, 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.

2.10.4. Evaluation of short and long local and systemic response of VinlSulfTC_10/HA-SH_56 hydrogels

To study both short and long local and systemic response of VinlSulfTC_10/HA-SH_56 hydrogels, six mice per group were skilled 7, 14 and 21 days after the hydrogel injections by CO_2 narcosis, according to the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators.

2.10.5. Collection of blood samples

Immediately before being killed, mice were anaesthetized with isoflurane (as described above) and blood samples were obtained by cardiac puncture. The following haematological parameters were analysed within 12 h of sample collection: erythrocytes (red blood cells, RBC), packed cell volume (PCV), haemoglobin (Hb), red cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils and platelets (Cell Dyn 3500; Abbott, San Giovanni Teatino, Italy).

2.10.6. Histological examination of tissues and bone marrow cells (BMCs) preparation

Hydrogels and the tissues surrounding the hydrogel depot were explanted 7, 14 and 21 days after injection and fixed in 4% paraformaldehyde (PFA) for 24 h. After washing in PBS, samples were embedded with Tissue-Tek OCT compound (Sakura, Gentaur, Milano, Italy); then, 10-μm thick sections were obtained by a rotary −30°C microtome cryostat (Ames Cryostat Miles, SCHOTT Italglas s.r.l., Genova, Italy), air-dried and stained with haematoxylin and eosin.

Femurs, dissected free of adhering tissue, were fixed as described above, decalcified in 14% ethylenediaminetetraacetic acid (EDTA) solution for 3 days and soaked in 30% sucrose overnight. Samples, quickly frozen in isopentane, were embedded with Tissue-Tek OCT

compound (Sakura). Subsequently, femurs sections (12-μm thick) were obtained and stained as described above.

Organs (spleen, kidneys, liver and long bones) were collected immediately after death and fixed in 4% PFA diluted in PBS for 48 h at 4°C. After dehydration, tissue blocks were embedded in paraffin and cross-sectioned, 5–8 μm thick, by a microtome (Leica Reichert-Jung 2040 Autocat, Millano, Italy). Sections were stained with haematoxylin and eosin or with Gomori's trichrome and mounted on coverslip.

Tibiae from the above mouse groups were dissected free of adhering tissue. The ends were removed, and the marrow cavity was flushed with Dulbecco's modified Eagle's Medium (DMEM) (Invitrogen, Thermo Fisher Scientific, Milan, Italy) as previously described (Moreno *et al.*, 2014).

In order to evaluate the release cytokines and chemokines from BMC populations, cells were cultured for 1 day in 100 mm dishes with DMEM containing 10% heat-inactivated fetal calf serum (HIFCS) (Invitrogen, Thermo Fisher Scientific), penicillin (100 U/ml), and streptomycin (50 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

2.10.7. Cytokine and chemokine assay

The cytokine/chemokine profiles of BMC supernatants were assessed by using Mouse Cytokine Array Panel A kit (R&D Systems, Milan, Italy) according to the manufacturer's instructions. Immunoreactive dots were visualized using LiteAblot Turbo luminol reagents (Euroclone, Milan, Italy) and Hyperfilm-ECL film (Euroclone, Milan, Italy) and quantified densitometrically.

2.11. Statistical analysis

Statistical comparison were made using the Student's *t*-test, with $p < 0.05$ being considered significant.

3. Results and discussion

3.1. Polymer characteristics

A thermosensitive triblock copolymer VinylSulfTC_0 was synthesized by free radical polymerization using a PEG macroinitiator (PEG, 10 000 Da) and HPMAm-monolactate and HPMAm-dilactate as monomers, with a yield of 68% and mass of 47 kDa, according to ^{1}H-NMR data. This polymer was modified with vinyl sulphone groups by a DCC coupling reaction. Ten per cent of the pendant hydroxyl groups of VinylSulfTC_0 were reacted with 3MPA-DVS (3 mercapto propanoic acid-divinyl sulfone) with a yield of 65%. The ^{1}H-NMR and GPC analyses showed that the M_n of the polymer was conserved upon partial modification of the free hydroxyl groups with vinyl sulphone moieties, indicating that no premature polymerization of vinyl sulphone residues had occurred during reaction, workup and lyophilization procedures. It should be noted that the use of PEG standards in GPC resulted in underestimation of Mn values compared to those calculated based on ^{1}H-NMR, as was also observed previously (Vermonden *et al.*, 2006). The vinyl sulphonation reaction led to modification of 10% of the available hydroxyl groups on the lactate side-chains with vinyl sulphone moieties, as shown by ^{1}H-NMR analysis, with a DVS conversion of 50% (feed DVS ratio to OH groups 20%). The vinyl sulphonated triblock copolymer is indicated as VinylSulfTC_10. As shown in Table 1, the CP of VinylSulfTC_10 slightly decreased from 33 to 29°C upon vinyl sulphonation, as a consequence of the slightly increased hydrophobicity (to which the CP is known to correlate) of the polymer. The observed CP value of 29°C upon vinyl sulphonation reaction is in the range between room and body temperature, making the resulting polymer potentially suitable for *in situ* gelation. Vinyl sulphones are therefore promising Michael Addition crosslinkable groups for thermosensitive polymers, as they allow for extensive modification of the thermosensitive polymers without markedly affecting their lower critical solution temperature and consequently their solubility in aqueous medium, as observed with methacrylate analogues (Censi *et al.*, 2010a).

Thiolated hyaluronic acid (Scheme 1b) of approximately 38 kDa was synthesized with a yield of 80%. The degree of HA substitution with thiol groups was determined by ^{1}H-NMR and Ellman's methods, which showed values of 54% and 58%, respectively. The good agreement between these values obtained by two different methods indicated no premature formation of inter- and intra-chain disulphide bonds. The modified polysaccharide is designated HA-SH_56, where 56 is the mean DS values of those obtained by ^{1}H-NMR and Ellman's method.

3.2. Gel formation and biodegradation *in vivo*

When the two hydrogel components (VinylSulfTC_10 and HA-SH_56) were mixed in aqueous medium at a vinyl sulphone to thiol ratio of 1, and heated to body temperature, an immediate increase in turbidity and viscosity was visually observed as a result of the formation of self-assembled hydrophobic domains within the thermosensitive blocks (Vermonden *et al.*, 2006), which was expected as the lower critical solution temperature was 29°C. However, at the triblock copolymer

Table 1. Overview of the characteristics of the synthesized polymers

	M_n*	M_n†	M_w†	PDI†	Cloud point§ (°C)	DS (%)	Yield (%)
VinylSulfTC_0	47	25.7	52.5	2.0	33	0	68
VinylSulfTC_10	54	25.7	54.2	2.1	29	10*	65
HA-SH_56	38	–	–	–	–	54*/58¶	80

M_n, number average molecular weight; M_w, average molecular weight; PDI, polydispersity index; DS, degree of substitution.
*Based on ^{1}H-nuclear magnetic resonance.
†based on GPC using PEG standards.
§Based on light scattering.
**Based on Elman's (1959) method.

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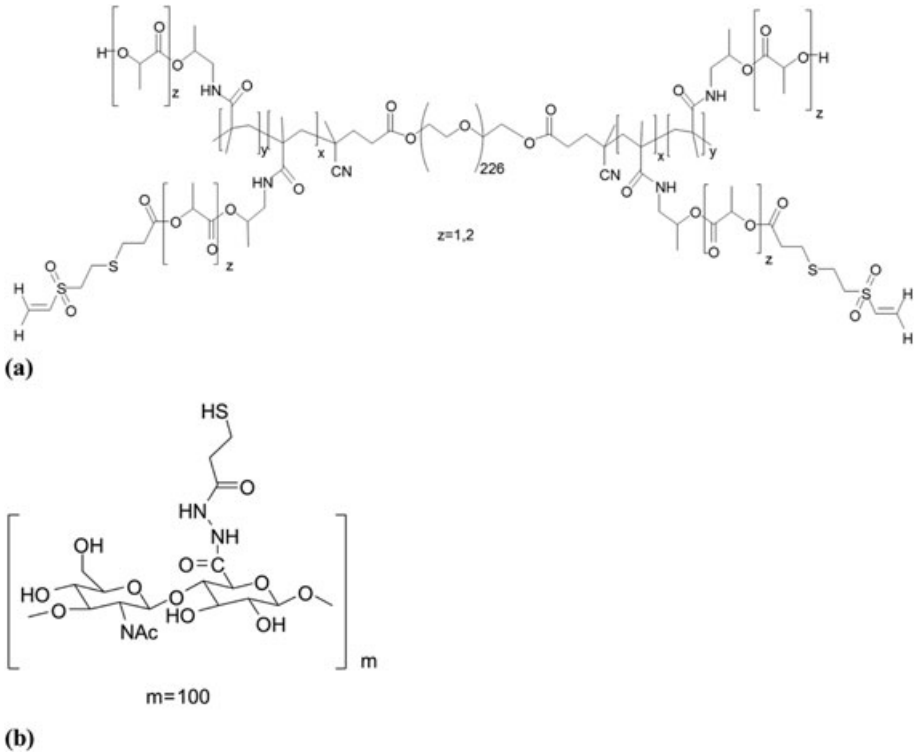

Scheme 1. Chemical structures of (a) vinyl sulphone modified triblock copolymer VinylSulfTC_10 and (b) thiolated hyaluronic acid HA-SH_56

(VinylSulfTC_10) concentration of 15% *w*/w tested, the physical network formed displayed flow behaviour immediately after the temperature rise. During a time-span of approximately 20 min, a rapid and progressive stabilization of the network was observed and through the method of the vial tilting no flow was visible. This observation was confirmed by rheology experiments, where a continuous increase in storage modulus (G′) is observed at increasing Michael Addition reaction time. From Figure 1 it was observed that, at the concentration used, the gel point, defined as the temperature at which the G′ equals the loss modulus (G″), was reached for the polymer concentration tested and the experimental conditions at 28°C, which is potentially suitable for *in vivo* gelation upon injection. During the Michael Addition reaction, chemical crosslinks between vinyl sulphone and thiol groups were formed (Scheme 2), resulting in fast and progressive stabilization of the hydrogel network. The Michael Addition reaction both *in vitro* and *in vivo* showed complete conversion of vinyl sulphone groups after approximately 34 min (data not shown). This reaction time was remarkably faster than that occurring between acrylate and methacrylate derivatives of p(HPMAm-lac)-PEG copolymers, for which flow behaviour was observed for up to 4 h and complete conversion of (meth)acrylate groups occurred after 48 h (Censi *et al*., 2010a). A simultaneous physical (thermal) and chemical (Michael-type reactions) crosslinking mechanism is an attractive and efficacious method to obtain effective gel formation *in vivo*, as it avoids premature dissolution of the polymer chains before chemical crosslinking. Immediate thermal (physical) gelation of the hydrogel upon *in vivo* administration ensures the stability of the hydrogel at the inoculation site

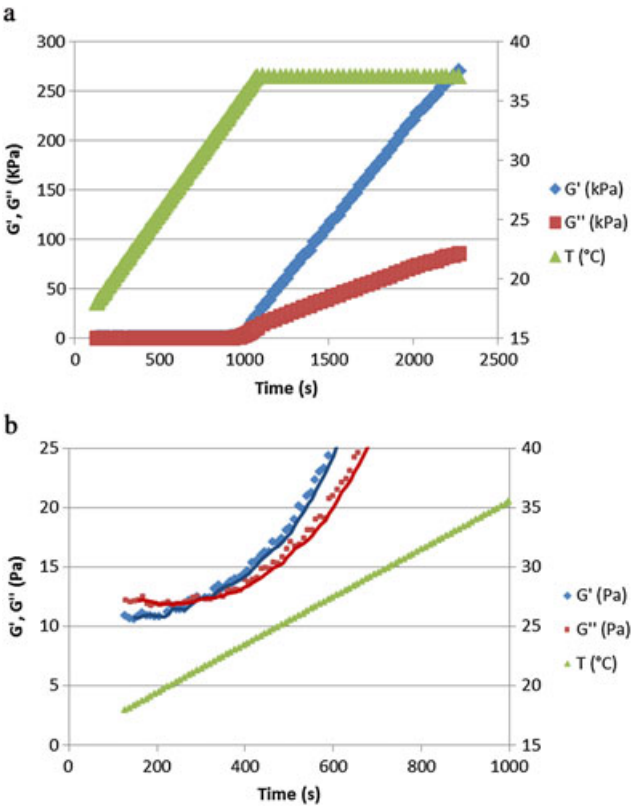

Figure 1. (a) Rheology measurements of VinylSulfTC_10/HA-SH solutions in aqueous medium progressively stabilized by Michael Addition cross-linking. (b) Detail of the gel point at 28°C. G′, storage modulus; G″, loss modulus; T, temperature. [Colour figure can be viewed at wileyonlinelibrary.com]

during the subsequent Michael Addition (chemical) crosslinking, avoiding dilution of the polymer chains in the physiological fluids. The incomplete chemical cross-

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linking, potentially resulting from polymer dissolution *in vivo*, may lead to premature drug/cell release when the hydrogel is used as tissue engineering matrix.

3.3. In vivo hydrogel-HA polymerization and subsequent degradation

VinylSulfTC_10/HA-SH_56 hydrogels, formulated as previously described were maintained at 4°C until administration and were administered i.m. using a 1 ml syringe. Ultrasound imaging was used to monitor both the inoculation procedure and the stability of the hydrogel at the injection site for 20 days.

Once conveyed into the muscle tissue the hydrogels polymerized and immediately formed a hydrogel depot at the site of the injection, as also observed *in vitro*, where temperature increase triggered physical gelation as a result of the self-assembly of the thermosensitive chains. Immediately after injection, the material appeared as a rounded structure, with sharp margins and was slightly hyperechoic in comparison with the surrounding muscular tissue as a result of the high biomaterial turbidity and viscosity induced by the thermal gelation.

In order to evaluate the *in situ* chemical polymerization of VinylSulfTC_10/HA-SH_56 hydrogel, ultrasound analysis was repeated 30 min after i.m. injection (Figure 2a) to verify the stability of the biomaterial at the site of injection. Thirty minutes after inoculation, mice were killed and hydrogels were explanted. No adherence of the material to the surrounding tissue was observed and the gross examination of the explant confirmed the spherical conformation of the hydrogel, as revealed by ultrasonographic analysis, and its fully elastic, three-dimensionally stable structure (Figure 2b).

Immediately after explantation, the hydrogels were weighed and placed into basic aqueous medium to hydrolyse. The unreacted vinyl sulphone groups of the explants were analysed by HPLC-MS. It was found that a negligible amount of Michael acceptors remained unreacted, similar to what was observed during network formation *in vitro* (see the Supplementary material online). This finding indicates that Michael Addition crosslinking was highly effective both *in vitro* and *in vivo*.

Biodegradation of the injected hydrogels was monitored by echography at 5, 10, 15 and 20 days. As time progressed, hydrogels were still well visible as a grey, rounded structure, but their size progressively diminished, as assessed by determination of the depot area, and the gels achieved a more flattened shape in the craniomedial direction (Figure 3a,b) The continuing remodelling of the depot shape may be caused by muscle tissue compressive forces. At day 20 after injection, ultrasonographic monitoring showed complete resorption of the biomaterial. This *in vivo* residence time of the biomaterial was found to be significantly shorter than that observed *in vitro*, where complete hydrolysis of the network was observed after 32 days (Figure 3c). This discrepancy indicates that the accelerated degradation observed *in vivo* was caused not only by chemical hydrolysis but also by endogenous enzymatic degradation (e.g. hyaluronidases) and cellular metabolism. Furthermore, the swelling behaviour of the biomaterial *in vitro*, which doubled its weight in approximately 8 days, was not observed *in vivo*, where no water uptake or increase in size was observed. It is likely that the compressive forces of muscle tissue during limb motions hampered hydrogel free swelling (Figure 3a,b).

3.4. Local and systemic response to VinylSulfTC_10/HA-SH_56 hydrogels

The *in vivo* safety is a crucial parameter for the *in vivo* application of biomaterials and their potential success in the pharmaceutical and biomedical fields. In order to assess the *in vivo* safety of VinylSulfTC_10/HA-SH_56 hydrogels, mice receiving hydrogel administration were evaluated at various time-points, from 5 days to 21 days, for: (1) the tissue response to the implants at the site of injection; (2) possible systemic inflammatory response in distant organs, and compared with control mice that received an equal volume of physiological saline injection. Histological sections of the hydrogel and the tissue surrounding the depot were examined 7, 14 and 21 days post injection.

A slight accumulation of inflammatory cells (macrophages) in the muscle tissue around the VinylSulfTC_10/HA-SH_56 hydrogel was observed at day 7

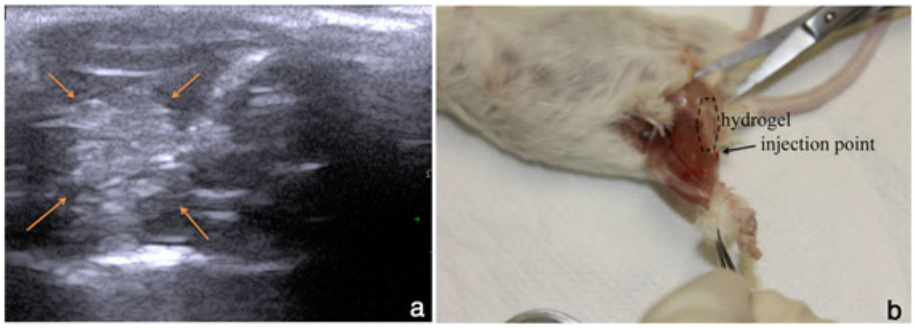

Figure 2. (a) Ultrasonographic image of the hydrogel depot 30 min after intramuscular administration. Arrows indicate the ultrasonographic appearance of the hydrogel, while the background grey shows the normal ultrasound appearance of muscular tissue. The white line on the lower part of image corresponds to the femur surface. (b) Gross image of VinylSulfTC_10/HA-SH_56 hydrogel injected intramuscularly and polymerized *in situ* for 30 min. [Colour figure can be viewed at wileyonlinelibrary.com]

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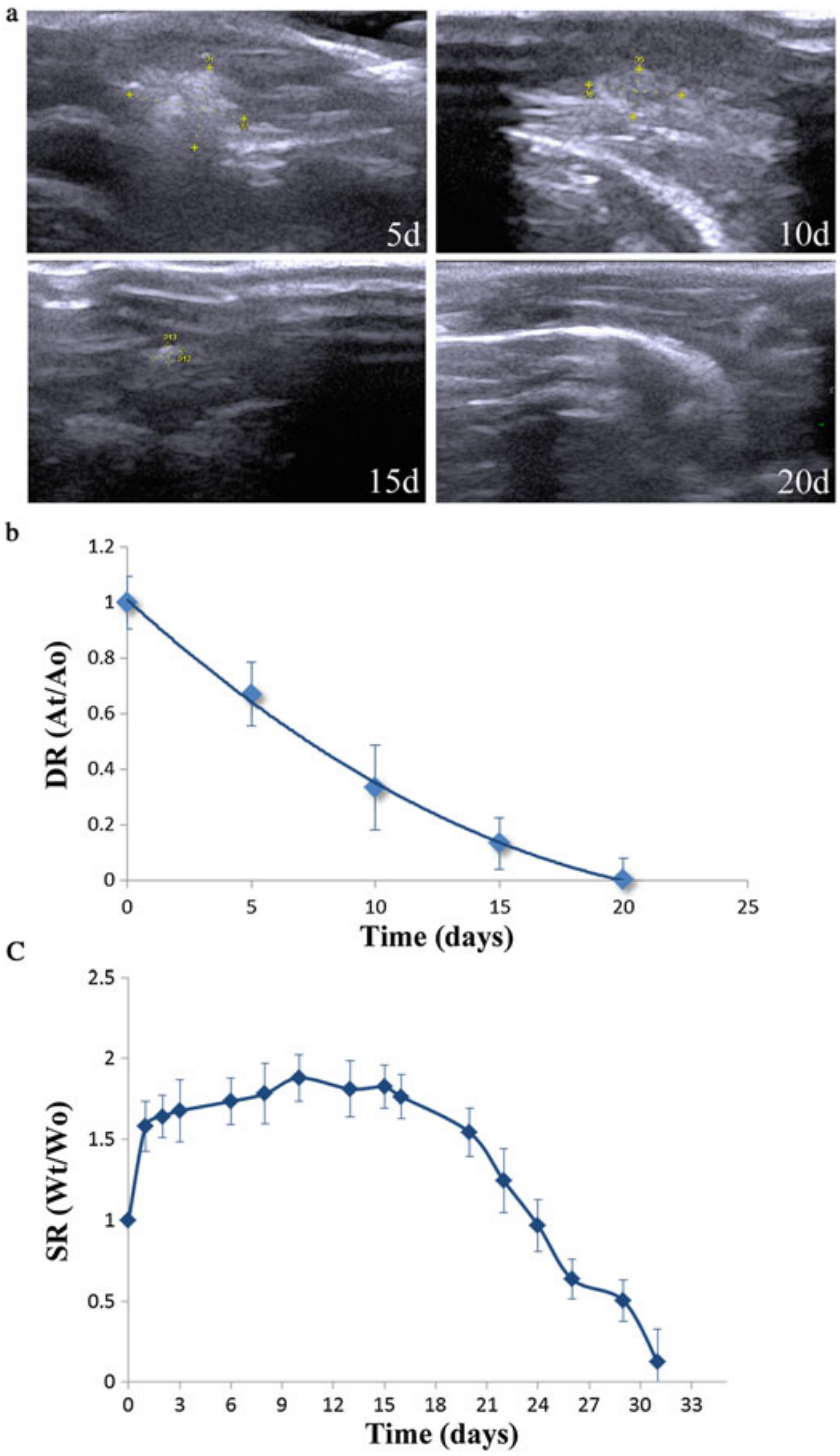

Figure 3. (a) *In vivo* 15% *w*/w VinylSulfTC_10 /HA-SH_56 hydrogel degradation behaviour. Representative ultrasound pictures showing the *in vivo* hydrogel degradation, surrounded by normal muscular tissue, at different time-points: day 5 (5d), day 10 (10d), day 15 (15d) and day 20 (20d). (b) The degradation ratio (DR) *in vivo* of 15% w/w VinylSulfTC_10 /HA-SH hydrogel as a function of time, calculated as ratio between the area of the depot at time *t* (A_t) and the area of the depot at time 0 (A_0). (c) The swelling ratio (SR) *in vitro* of 15% w/w VinylSulfTC_10/HA-SH hydrogel as a function of time, calculated as ratio between the weight of the hydrogel at time *t* (W_t) and the weight of the hydrogel at time 0 (W_0). [Colour figure can be viewed at wileyonlinelibrary.com]

(Figure 4a) that progressively decreased becoming negligible at days 14 and 21 (data not shown). Interestingly, this observation contrasts with previously reported data, where photopolymerized methacrylate derivatives of the p(HPMAm-lactate)-PEG triblock copolymer were subcutaneously implanted in Balb/c mice, resulting in massive chemotaxis of inflammatory cells towards the implantation site and infiltration of such cells in the bulk of the hydrogel material. The initial acute inflammation, upon administration, evolved into chronic inflammation whose extent depended on hydrogel cross-linking density and that tended to resolve in time, being minimized after 21 days (Censi *et al.*, 2011a).

The milder local inflammatory response observed for the newly developed hydrogel reported in this work may have multiple causes. First, the minimally invasive

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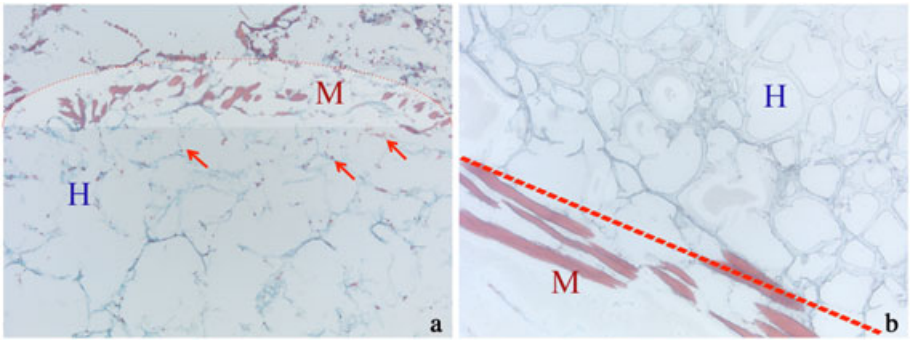

Figure 4. Histological analysis of tissues surrounding VinylSulfTC_10/HA-SH_56 hydrogels. A limited number of inflammatory cells were visible around the material only at 1 week (arrows) × 5 (a) and ×20 (b) magnification. M, muscle; H, VinylSulfTC_10/HA-SH_56 hydrogel. [Colour figure can be viewed at wileyonlinelibrary.com]

administration procedure used in the present work, based on injection through a 1 ml syringe, likely contributed to diminish the tissue inflammation, compared with the previously applied surgical procedure. Importantly, the presence of an endogenous polysaccharide such as HA, as a cross-linker, may also have enhanced the biocompatibility of the hydrogel. The contribution of hyaluronic acid in reducing the inflammatory response to implanted hybrid hydrogels is currently under evaluation in a mouse model of inflammation, using hydrogels at different HA contents and extents of DS. Lastly, hydrogels resulting from Michael Addition cross-linking possessed a more flexible structure compared with their photopolymerized analogues. Photopolymerized hydrogels are cross-linked through the formation of long 'zips' of poly(methacrylic acid) of uncontrolled molecular weight that, linking together several cross-linkable moieties, result in rather rigid and brittle networks. Conversely, Michael addition gels, are formed by cross-links occurring between two reactive groups (one Michael addition donor and one Michael addition acceptor), therefore display higher elasticity and potential capacity to minimize tissue frictions and irritation.

In addition, the evaluation of the routine haematological parameters did not show any statistically significant variations in mice injected with hydrogels as compared with those that were saline treated (Figure 5).

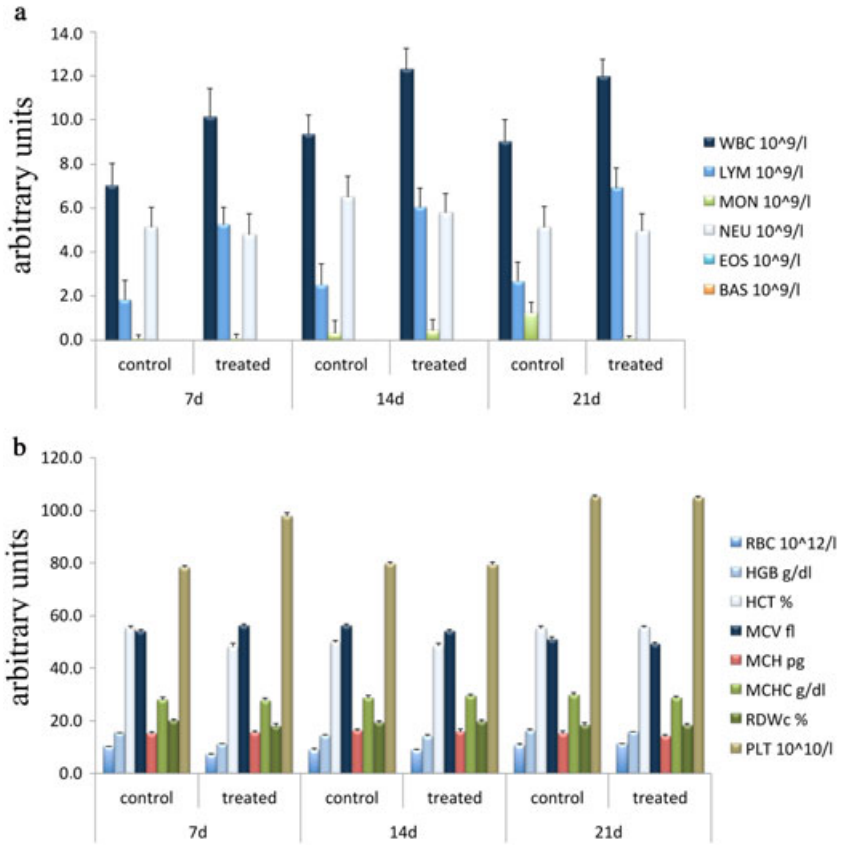

Figure 5. Evaluation of the haematological parameters 7 days (7d), 14 days (14d) and 21 days (21d) after treatment with hydrogel or saline (control). (a) WBC, total leukocytes; LYM, lymphocytes; MON, monocytes; NEU, neutrophils; EOS, eosinophils; BAS, basophils. (b) RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red cell distribution width; PLT, platelets. [Colour figure can be viewed at wileyonlinelibrary.com]

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Accordingly, pathological lesions in liver, kidney, and spleen in comparison with saline treated group were not detected (see the Supplementary material online). The haematopoietic system is also a potential target tissue of external influence, sensitive to chronic inflammation, toxic agents and chemicals. As the bone marrow is a site of intense cell multiplication and maturation, materials that affect cellular proliferation/differentiation can influence this microenvironment non-specifically (Travlos, 2006). Histological evaluation of haematopoietic cellularity at the femur metaphyseal plate level did not show evidence that suggested toxicity and the observations in the experimental mice group were in accord with those of the saline control group (see the Supplementary material online).

An integral part of the systemic inflammatory response to toxic compounds is the activation of bone marrow, which results in the release of inflammatory cytokines (Agas *et al.*, 2015). In the present study, long bones of hydrogel- and saline-treated mice were flushed and the total BMC population was maintained in culture for 3 days. Therefore, supernatants were analysed for the production of cytokines and chemokines (Figure 6). Surprisingly, a time-dependent slight decrease of important pro-inflammatory cytokines in cultured BMCs from hydrogel-treated mice was observed. Specifically, the injected biomaterials downregulated in a statistically significant manner, triggering receptors expressed on myeloid cells 1 (TREM1) and interleukin 1 beta (IL-1β) after 2 weeks of treatment (Figure 6b). Notably, after 3 weeks of treatment, tumour necrosis factor alpha (TNF-α) was also decreased (Figure 6c). All of these compounds are well known to play important roles in a number of chronic and acute inflammatory diseases (Dinarello, 1996; Sasaki *et al.*, 2002; Sabbieti *et al.*, 2015). In this context, it is assumed that this peculiar anti-inflammatory effect of the VinylSulfTC_10/HA-SH_56 hydrogels administered resulted from HA release during the hydrogel degradation. Indeed, it was previously demonstrated that HA is able to reduce the release of pro-inflammatory cytokines *in vitro* and *in vivo* (Campo *et al.*, 2011; Neuman *et al.*, 2011). Furthermore, the level of pro-inflammatory cytokines and chemokines decreased as hydrogel degradation progressed, supporting a possible

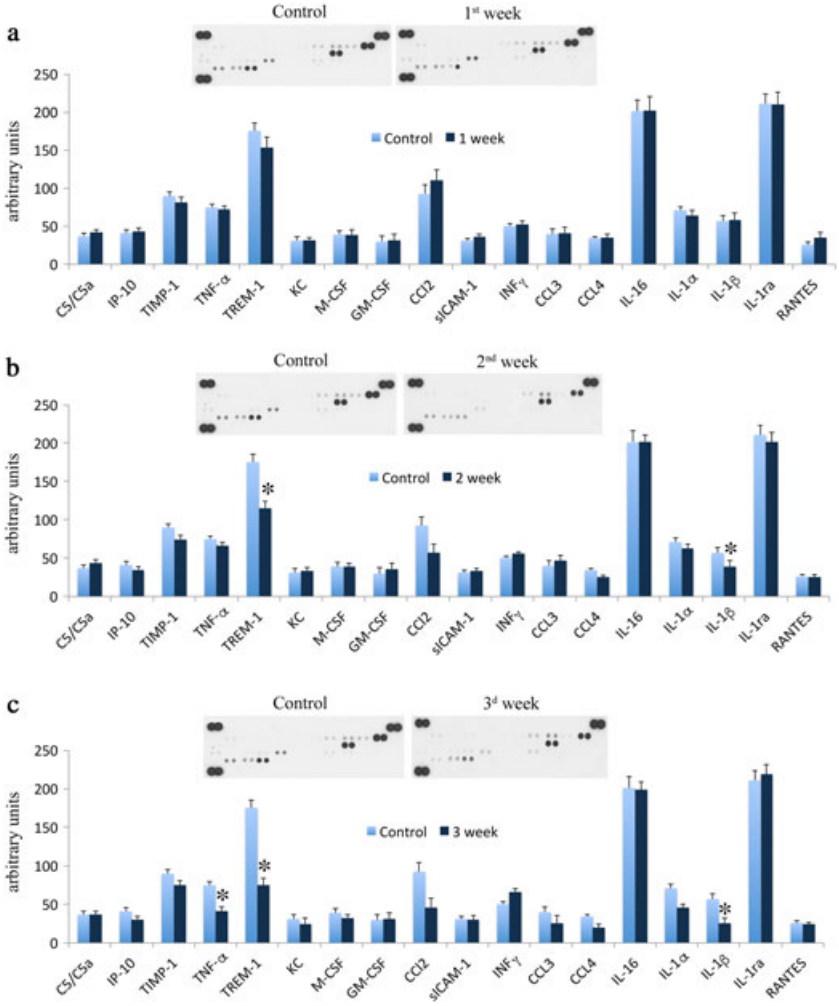

Figure 6. Cytokines and chemokine release was analysed in medium from bone marrow cell (BMC) cultures obtained by hydrogels or saline (control) treated mice killed at 1 week (a), 2 weeks (b) and 3 weeks (c). Insets represent the Immunoreactive dots. Each spot is in proportion to the amount of cytokines and chemokines released. VinylSulTC_10/HA-SH_56 hydrogel statistically significantly decreased the release of pro-inflammatory cytokines and chemokines tumour necrosis factor (TNF)-α, TREM-1 and interleukin (IL)-1β (*$p < 0.05$). C5/C5a, Complement component C5a; IP-10, Interferon gamma-induced protein 10; TIMP-1, Tissue inhibitors of metalloproteinases -1; TREM-1, Triggering Receptor Expressed on Myeloid cells-1; KC, Keratinocyte chemoattractan; M-CSF, Macrophage Colony- Stimulating Factor; CCl2, C-C motif chemokine ligand 2; SICAM-1, Soluble intercellular adhesion molecule-1; INF-&gamma, CCL3, C-C motif chemokine ligand 3; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted. [Colour figure can be viewed at wileyonlinelibrary.com]

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relationship between HA release from the degrading network and the observed anti-inflammatory effect. The histological observations were fully consistent with the biochemical findings derived from the BMC population of hydrogel-injected mice. Further studies will focus on a deeper investigation of the downregulation of pro-inflammatory factors in induced inflammation animal models.

4. Conclusions

The newly proposed thermosensitive *in situ* jellying triblock copolymer based on vinyl sulphone bearing p (HPMAm-lac1–2)-PEG- p(HPMAm-lac1–2) in combination with thiolated hyaluronic acid was studied upon intramuscular and subcutaneous injection in Balb/c mice with the aim of gaining new insights into the process of gel formation *in vivo*, the biocompatibility and its safety. It was found that the hydrogel was an easily injectable material both intramuscularly and subcutaneously, where it formed a stable spherical depot immediately after administration. The gelation and biodegradation of the administered hydrogels were monitored by ultrasonography, which revealed a progressive decrease in the hydrogel volume over time until complete resorption after 20 days. The observed degradation *in vivo* was accelerated compared with *in vitro* testing, indicating that polymer hydrolysis, enzymatic degradation and cellular metabolism were concomitant. The ultrasonography examination proved to be a suitable investigation method to monitor the evolution of the material after intramuscular injection. To the authors' knowledge, this is the first time that this non-invasive procedure has been used for this purpose, and may represent an innovative tool for *in vivo* monitoring as it is safe for the operator, and quick and relatively simple to implement. It allowed the recording of accurate and reliable measurements and decreased the need for a large number of animals to be used and then killed. Evaluation of the routine haematological parameters and histological examination of the tissue surrounding the injection site and the main organs as well as the haematopoietic system did not show any important systemic reaction to the biomaterial. Notably, a decreased release of pro-inflammatory cytokines by BMCs from hydrogel treated mice was observed, possibly because of the anti-inflammatory effect of hyaluronic acid released in time as hydrogel degraded. The present study showed that vinyl sulphone-bearing p(HPMAm-lac1–2)-PEG–p (HPMAm-lac1–2)/thiolated hyaluronic acid hydrogels showed good gelation properties and stability *in vivo*, an excellent biocompatibility *in vivo* and a potential anti-inflammatory effect owing to the release of HA as a hydrogel degradation product. The potential anti-inflammatory effect will be further investigated in future studies where hydrogels containing different amounts of HA and characterized by different DS will be tested in mouse inflammation models.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. HPLC chromatograms of (a) standard solution in DMSO of DVS-3MPA (b) degraded and non-crosslinked VinylSulTC_10 (c) degraded VinylSulTC_10/HA-SH cross-linked *in vivo* (d) degraded VinylSulTC_10/HA-SH hydrogel crosslinked *in vitro*.

Figure S2. Liver, spleen, kidney and femurs were explanted from hydrogel-treated mice 7 (b), 14 (c) and 21 (d) days post-injection.

Figure S3. ^{1}H-NMR spectra in DMSO-d6 of (a) VinylSulTC_0, (b) VinylSulTC_10.

Figure S4. Frequency sweep experiment for an aqueous solution of VinylSulTC_10/HA-SH.