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Influence of the degree of methacrylation on hyaluronic acid hydrogels properties

Sidi A. Bencherif [a], Abiraman Srinivasan [b], Ferenc Horkay [c], Jeffrey O. Hollinger [b], Krzysztof Matyjaszewski [a], Newell R. Washburn [a,d,*]

[a] *Department of Chemistry, Carnegie Mellon University, Pittsburgh, PA 15213, USA*
[b] *Bone Tissue Engineering Center, Carnegie Mellon University, Pittsburgh, PA 15213, USA*
[c] *Section on Tissue Biophysics and Biomimetics, Laboratory of Integrative and Medical Biophysics, NICHD, National Institutes of Health, Bethesda, MD 20892, USA*
[d] *Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA 15213, USA*

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Abstract

The properties of hyaluronic acid (HA) hydrogels having a broad range of methacrylation are presented. Increasing solubility of glycidyl methacrylate (GM) in a co-solvent mixture during the methacrylation of HA with GM was shown to produce photopolymerizable HAGM conjugates with various degree of methacrylation (DM) ranging from 14% up to 90%. Aqueous solutions of HAGM macromonomers were photo-cross-linked to yield hydrogels with nearly full vinyl group conversions after 10 min exposure under ultraviolet light (UV). Hydrogels were characterized by uniaxial compression and volumetric swelling measurements. Keeping the DM constant, the shear modulus was varied from 16 kPa up to 73 kPa by varying the macromonomer concentration. However, at a given macromonomer concentration while varying the DM, similarly the shear modulus varied from 22 kPa up to 65 kPa. Preliminary *in-vitro* cell culture studies showed that GRGDS modified HAGM hydrogels promoted similarly cell interaction at both low and high DMs, 32% and 60%, respectively. Densely cross-linked hydrogels with a high DM have been shown to be more mechanically robust while maintaining cytocompability and cell adhesion.

Keywords: Hyaluronic acid; Cross-linking; Hydrogels; Mechanical properties; Cell response

1. Introduction

Natural polysaccharides such as hyaluronic acid (HA, hyaluronan) have been extensively studied in medical applications since they can provide intrinsic biological activity when used as the basis for biomaterials [1,2]. HA is a naturally occurring, biocompatible, and biodegradable linear polysaccharide composed of unbranched repeating units of glucuronic acid and *N*-acetyl glucosamine linked by β 1−3 and β 1−4 glycosidic bonds. Furthermore, HA is an important component of synovial fluid and extracellular matrices, therefore, it could be an attractive building block for new biocompatible and biodegradable polymers for drug delivery, tissue engineering, and viscosupplementation [3,4].

HA plays a prominent role in lubrication, cellular processes, wound healing, and it is naturally angiogenic when enzymatically degraded to small fragments [5−7]. Cellular interactions with HA occur through cell surface receptors and influence tissue formation, inflammation, and morphogenesis [8−11]. This evidence suggests that HA is an ideal candidate material for promoting wound healing and tissue regeneration if it can be modified to improve its mechanical properties.

A variety of modifications of native hyaluronan have been devised to provide mechanically and chemically robust materials through chemical cross-linking. For example, modification of HA can be achieved by covalent derivatization of either the carboxylic acid or hydroxyl functionalities of the polymer [5,7,12−16]. The resulting hyaluronan derivatives

* Corresponding author. Tel.: +1 412 268 2130; fax: +1 412 268 6897.
E-mail address: washburn@andrew.cmu.edu (N.R. Washburn).

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Table 1
Photopolymerizable methacrylated-HA derivatives reported in literature

References	MW HA (10^6 g mol^{-1})	Degree of substitution (%)	Concentration (wt %)	Modulus (Pa)
[5,15]	2	≤11	2	≤155
[7]	0.35–1.1	≤7	≤5	$\leq 25 \times 10^3$
[12]	0.49–1.3	≤25	2	$\leq 5.5 \times 10^3$
[13]	1.7	≤30	2	$\leq 10.5 \times 10^3$

have physicochemical properties that are significantly different from native polymer, yet most derivatives are biocompatible and biodegradable.

Reducing the degradation rates of HA-based materials is necessary for many biomedical applications. HA is enzymatically degraded by hyaluronidase and is resorbable through multiple metabolic pathways [17]. Although HA polymer is broken down *in-vivo*, cross-linking individual HA polymer chains decreases their degradation rates. An effective strategy for cross-linking HA is photopolymerization [17]. Photopolymerization may increase spatial and temporal control over cross-linking and biocompability with possible *in-situ* polymerization [18].

Chemical modification of HA with methacrylated derivatives in aqueous environment has been used to yield photopolymerizable conjugates for gel preparation [2,5,7,12,13,15,16]. However, as shown in Table 1, low percentage gels (≤5%) and low degree of modification (≤30%) for high molecular weight macromonomer precursors have resulted in low cross-linking density for HA-based hydrogels, limiting mechanical properties and stability over a long period of time against enzymatic degradation [5,7,12,13,15]. There is a general consensus that such material properties strongly affect the cell response. A typical approach to control hydrogel mechanical properties is to tailor the network cross-link density. For example, Bryant and Anseth have demonstrated the benefits of cross-linking density on mechanical properties and cell differentiation on the chondrocytes' ability to produce cartilaginous tissues [19]. This suggests that a broader range of cross-linking density on the gel properties could result in a corresponding increase in the range of cellular responses.

In this article, we describe the effect of cross-link density via the DM on the properties of HAGM hydrogels (swelling and mechanics) and evaluate cell response. To this end, we have developed and characterized a series of photopolymerizable HAGM macromonomers with various DM. Proton nuclear magnetic resonance (^{1}H NMR) was used to investigate the mechanism of methacrylation of HA, and to calculate the DM and vinyl conversion during photopolymerization. In addition, an evaluation of the effects of the DM on the swelling behavior, mechanical strength, and cellular responses were conducted on the gels.

2. Experimental methods

2.1. Materials

Hyaluronic acid ($\sim 1.6 \times 10^6$ g/mol), monomethoxy-PEG ($\sim 5 \times 10^3$ g/mol), glycidyl methacrylate (GM), and triethylamine (TEA) were purchased from Sigma–Aldrich and used as received. Monomethoxy-PEG-carboxymethyl ($\sim 5 \times 10^3$ g/mol) was purchased from Laysan Bio, Inc. Acryloyl–PEG–*N*-hydroxysuccinimide (ACRL–PEG–NHS, 3400 g/mol) was purchased from Nektar Therapeutics. GRGDS peptide was purchased from Bachem Bioscience Inc. Photoinitiator Irgacure 2959 (I2959) was obtained from Ciba Specialty Chemicals and used as received. All other chemicals were of reagent grade and were used without further purification. C2C12 mouse myoblast cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with fetal bovine serum and penicillin/streptomycin, all obtained from Invitrogen (Carlsbad, CA). Live/Dead® Viability/Cytotoxicity Kit were purchased from Invitrogen – Molecular Probes, Inc. (Eugene, OR).

Table 2
Reaction conditions for the synthesis of HAGM macromers with a broad range of degrees of methacrylation (from 14% up to 90%)

Reactions	Solvent (vol. %)		Temp (°C)	Time (days)	DM (%)
HA/GM	PBS	DMF			
1	1/50	100	0	45	10	14
2	1/50	100	0	25	10	21
3	1/50	75	25	25	10	32
4	1/50	50	50	25	5	60
5	1/100	50	50	25	10	90

2.2. Model reactions: GM with monomethoxy-PEG and monomethoxy-PEG-carboxymethyl

In two separate vials, monomethoxy-PEG and monomethoxy-PEG-carboxymethyl (1 g, 0.2 mmol) were reacted with glycidyl methacrylate (0.71 g, 5 mmol) and TEA (0.25 g) in 5 mL of PBS:DMF co-solvent (50:50) for 6 d at room temperature. Detailed experimental protocol, reaction scheme, characterization, and analysis of resulting oligomeric products are described in the supporting information.

2.3. Synthesis of HAGM conjugates

Photopolymerizable methacrylate groups were added to HA to yield HA–glycidyl methacrylate (HAGM) conjugates. As reported in Table 2, we prepared a series of HAGM polymers by treating a 0.5% wt/v solution of fermentation-derived HA ($\sim 1.6 \times 10^6$ Da) in phosphate buffer saline (PBS), and dimethylformamide (DMF) with a 50- or 100-fold molar excess of GM in the presence of excess triethylamine. The reactions were carried out at two different temperatures (25 °C or 45 °C) for 5–10 d. An example of the synthesis of HAGM with a DM of 32% is as follows (reaction 3). HA (1.0 g) was first dissolved in 200 mL phosphate buffer saline (PBS, pH ~7.4) and 67 mL of dimethylformamide (DMF), and subsequently mixed with 13.3 g of GM and 6.7 g of TEA. After 10 d reaction, the solution was precipitated twice in a large

excess of acetone (20 times the volume of the reaction solution), filtered, dried in vacuum oven overnight at 50 °C, and dialyzed for 3 d against d-H_20.

2.4. Synthesis of ACRL–PEG–peptide

GRGDS peptide was dissolved in anhydrous DMF containing 4 M excess of TEA. ACRL–PEG–NHS was also dissolved in anhydrous DMF and immediately after, mixed with 1.1 M excess of peptide. After incubating for 3 h at room temperature, ACRL–PEG–GRGDS was precipitated twice in cold anhydrous ether and dried in a vacuum oven overnight at room temperature. The peptide coupling reaction and molecular mass of the product was monitored by MALDI-TOF MS.

2.5. Characterization of HAGM by ^{1}H NMR

High-resolution, 300 MHz proton NMR spectra were taken on a Bruker Avance™ 300 spectrometer. Deuterium oxide (D_2O) was used as solvent, and the polymer concentrations were varied between 0.5% and 3% by mass fraction. All spectra were run at room temperature, 15 Hz sample spinning, 45° tip angle for the observation pulse, and a 10 s recycle delay, for 128 scans. The standard relative uncertainty for calculation of reaction conversion via ^{1}H NMR arises from the choice of the baseline and is estimated to be 8%. ^{1}H NMR spectroscopy was used to determine the methacrylation conversion on modified HA, as shown in Table 2. The DM is defined as the amount of methacryloyl groups per one HA disaccharide repeat unit and was calculated from the ratio of the relative peak integrations of the methacrylate protons (peaks at ~6.1, ~5.6, and ~1.85 ppm) and HA's methyl protons (~1.9 ppm).

2.6. Characterization of ACRL–PEG–peptide by MALDI-TOF MS

The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a PerSeptive Biosystems Voyager STR in reflectron mode. The MALDI matrix, dihydrobenzoic acid (DHB), and the macromonomer were dissolved in 1 mL of mix solvent methanol/water (50:50). All MALDI samples were hand spotted on the target.

2.7. Hydrogel preparation

HAGM macromonomers of 2, 5, 7, and 10% by mass fraction were mixed in deionized water. 5% HAGM macromonomer was combined with ACRL–PEG–GRGDS to a final concentration of 4 μmol/mL. The hydrogels were made by exposing the aqueous solution of HAGM to UV-light in the presence of the photoinitiator Irgacure 2959 (0.1% by mass fraction). Cylindrical samples of 15 mm in diameter and 1 mm in height were cured under a UV source (365 nm, 300 μW/cm^2) for 10 min to obtain bioactive hydrogels. The HAGM liquid cross-linked into materials ranging from soft to stiff and brittle solids, depending on the DM and mass fraction of macromonomers used.

2.8. Characterization of vinyl group conversion of the gels

Macromonomer solutions with various DM were prepared by mixing 5% wt/v HAGM with D_2O in the presence of photoinitiator (I2959, 0.1% by mass fraction). 1 mL of each solution was transferred in an NMR tube before being cured under a UV source (365 nm, 300 μW/cm^2) for 0.5–15 min to form cross-linked hydrogels. ^{1}H NMR spectroscopy was used to characterize vinyl group conversion during photopolymerization. All spectra were run at room temperature, 15 Hz sample spinning, 45° tip angle for the observation pulse, and a 10 s recycle delay, for 128 scans. The vinyl conversion was calculated from the ratio of the relative peak integrations of the cross-linked methylene protons and benzyl peaks (used as reference) from the photoinitiator compound.

2.9. Swelling measurements

To investigate the relative degrees of cross-linking in the hydrogels, the swelling ratio of each sample was determined. The equilibrium swelling ratio is inversely related to the extent of cross-linking and was calculated by dividing the gel mass after swelling to equilibrium in physiological conditions (pH ~7.4, T ~37 °C) by the dry gel mass. The swelling data were corrected for the PBS by subtracting the soluble fraction of salt from the gel. After polymerization and swelling in PBS for 2 d to equilibrium, hydrogel disks were weighed (M_s) and then dried in a vacuum oven overnight at 80 °C (M_d) to determine the equilibrium mass swelling ratio ($Q_M = M_s/M_d$).

2.10. Mechanical testing

Uniaxial compression measurements were used on hydrogels to assess the mechanical strength. The shear modulus was determined using uniaxial compression measurements performed by a TA.XT21 HR texture analyzer (Stable Micro Systems, UK). This apparatus measures the deformation (±0.001 mm) as a function of an applied force (±0.01 N). Cylindrical hydrogels (height = diameter = 5 mm) were deformed (at constant volume) between two parallel glass plates. The shear modulus (G) was calculated from the nominal stress, σ (force per unit undeformed cross-section), using Eq. (1) [20].

$$\sigma = G\left(\Lambda - \Lambda^{-2}\right) \tag{1}$$

Where Λ is the macroscopic deformation ratio ($\Lambda = L/L_0$, L and L_o are the length of the deformed and undeformed specimen, respectively). Measurements were carried out in triplicate at deformation ratios $0.7 < \Lambda < 1$. No volume changes or barrel distortions were detected.

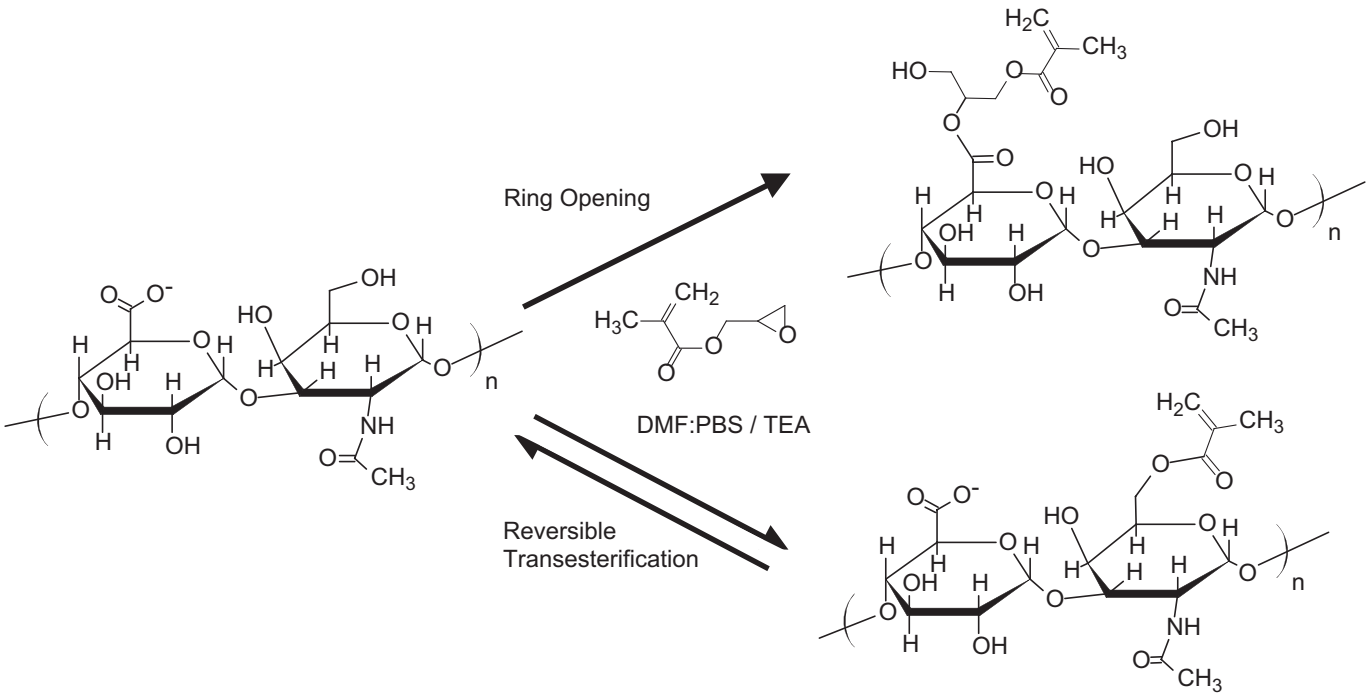

Fig. 1. Methacrylation of HA with GM to yield photopolymerizable HAGM by a competition reaction between ring-opening and transesterification.

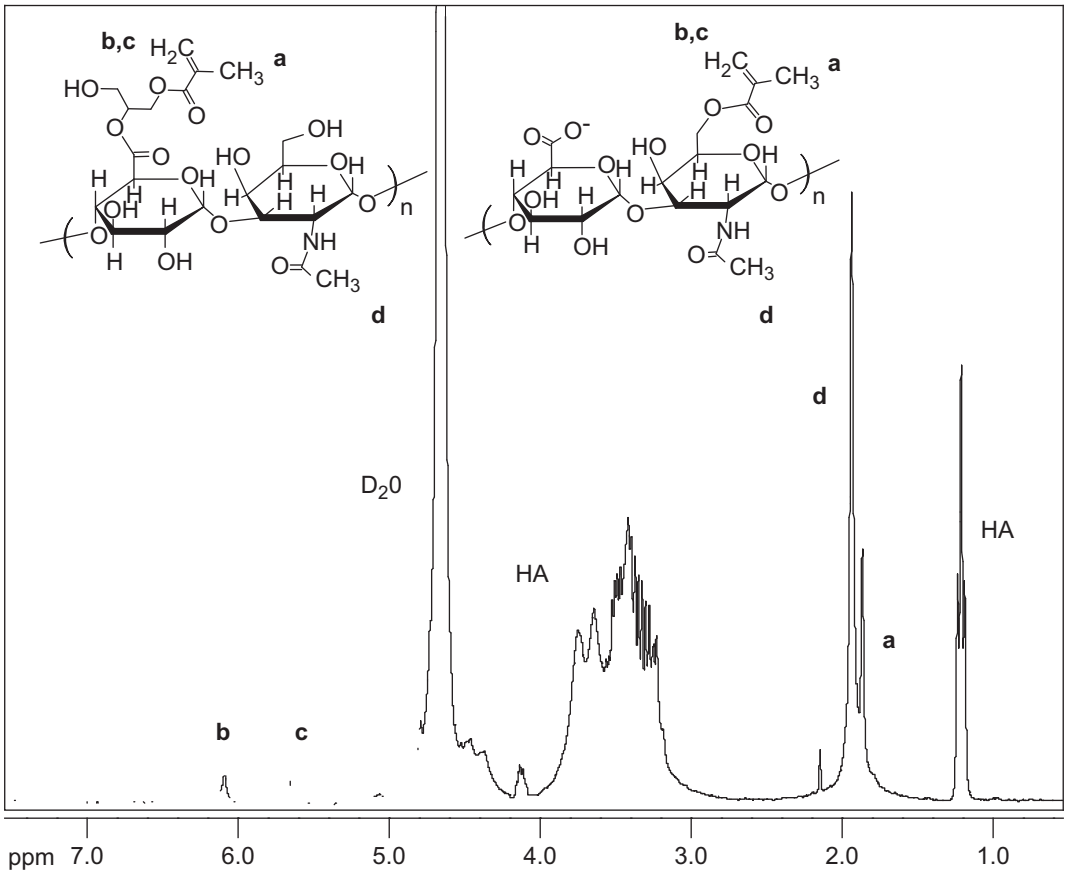

Fig. 2. ^{1}H NMR of HAGM (reaction 3, DM 32%).

2.11. Cellular responses in-vitro

Mouse muscle fibroblast C2C12 cell lines were seeded onto the hydrogel disks at a density of 50,000 cells/gel. The cells were cultured for 1, 3, and 5 d, to assess cell attachment and cytotoxicity. Cell attachment, morphology and spreading were examined using phase contrast light microscopy (Zeiss Axiovert). The Live/Dead® Viability/Cytotoxicity Kit contains Calcein AM to measure the intracellular esterase activity. Live cells fluoresce green at 494–518 nm, while ethidium homodimer-1 enters cells with damaged plasma membrane and fluoresces bright red at 528–617 nm. For live/dead staining, the cell culture media was aspirated and the wells rinsed with PBS, and the cells were incubated in the dark for 30 min at 37 °C with live/dead stain (Calcein1:2000 and ethidium homodimer 1:500 diluted in PBS). After incubation, the cells were observed under a fluorescent microscope (Zeiss Axiovert 200 microscope) and the images were captured using a monochrome CCD camera and pseudocolored.

3. Results and discussion

3.1. Synthesis and characterization of HAGM

The synthesis of HAGM (including both methacrylated compounds) depicted in Fig. 1 relied on the reaction of GM with HA in the presence of triethylamine as catalyst. An excess of GM with respect to HA was used because of the slight solubility and hydrolysis of GM in aqueous medium. As shown with two model reactions between GM and monomethoxy-PEG/monomethoxy-PEG-carboxymethyl (see supplemental information) and in agreement with the reaction of GM with chondroitin sulfate reported by Li et al., two reactions occur simultaneously at pH 7.4, including a reversible transesterification through the primary hydroxyl group and an irreversible ring-opening conjugation through the carboxylic acid group toward the highest substituted carbon of the epoxide [21]. However, as the reaction proceeds, there is a decline in the amount of transesterification products, while the concentration of ring-opening products gradually increases with time. This suggests that the reaction of HA with GM requires a long reaction time (at least 5 d) to promote ring-opening vs transesterification products.

^{1}H NMR spectroscopy confirmed the HAGM reaction, showing methacrylate peaks at ~6.1, ~5.6, and ~1.85 ppm. Fig. 2 shows a proton NMR spectrum characteristic for reaction 3. It is possible to approximate the percentage of methacrylation, which was calculated from the relative integrated intensities of the methacrylate protons and methyl protons in HA acetamide (peak at ~1.9 ppm). Batches of HAGM macromonomers for reactions 1, 2, 3, 4, and 5 were found to have approximately 14, 23, 32, 60, and 90% methacrylation, respectively (Table 2). The reaction conditions show that temperature and solvent mixture with DMF are predominant parameters in enhancing the DM of HA. A high degree of modification is obtained when reaction conditions are carried out at low temperature or in co-solvent with DMF. This is illustrated in Table 2, where the temperature seems to have an impact on the hydrolysis of GM given that a lower DM of 14% was obtained at 45 °C for reaction 1 when compared to reaction 2 where a DM of 23% was obtained for similar conditions but at a lower temperature (25 °C). We noticed that all the reactions performed in co-solvent with DMF have a higher DM. The introduction of DMF enhanced dissolution of GM in the mixture solvent than in pure PBS. The DM increased (up to 90%) as we increased the fraction of DMF, before reaching the solubility limit of HA in the co-solvent mixture (50:50).

3.2. Hydrogel preparation

The hydrogels were prepared from HAGM precursors with various DM and macromonomer mass fractions to evaluate the effect of these parameters on network properties. The gels formed in PBS appear transparent and rubbery; and they swelled further when incubated in aqueous media. During photopolymerization, HAGM macromonomers underwent free radical polymerizations in the presence of light and a photoinitiator to form a cross-linked network. The HAGM liquid cross-links into materials ranging from softer and more pliable to harder and more brittle hydrogels, depending on the macromonomer mass fraction, DM, and exposure time under UV. Highly methacrylated hydrogels yielded gels with increased rigidity.

The final aspect of the gel preparation consisted of the introduction of a cell-adhesive peptide, comprising the RGD sequence, and this was accomplished as reported in literature by coupling covalently GRGDS to ACRL–PEG–NHS with the formation of a peptide bond [15,22]. The remaining acrylate groups in the peptide-grafted PEG monoacrylate were then used for the polymerization reaction at a concentration of 4 mM. For the swelling and mechanical studies, hydrogels containing no peptide were prepared. Previous studies have shown that the physical properties of the peptide-containing hydrogels were statistically indistinguishable from the hydrogels not containing the peptide, presumably because of such small fraction of acrylate groups used do not alter the gel microstructure [16].

3.3. Vinyl group conversion

The kinetic study of vinyl group conversion during photopolymerization from HAGM was monitored by proton NMR analysis. HAGM and I2959 were dissolved in deuterium oxide and photopolymerized under UV exposure directly in an NMR tube. Cross-linking HAGM directly in an NMR tube allows us to characterize easily the sample at different time points during photopolymerization. Fig. 3 depicts the ^{1}H NMR spectrum of the reaction kinetics of uncross-linked (Fig. 3a) and expended views of cross-linked 5% wt/v HAGM (DM ≈ 32%) hydrogels (Fig. 3b). Results reveal a decrease in the methacrylate peaks; additionally, a broadening of the methylene peak in the cross-linked HA network is observed. The splitting of aromatic peaks from the initiator can be attributed to ketyl radical formation induced under UV exposure. We noticed that vinyl groups at ~6.1 and ~5.6 ppm decreased during UV irradiation while the integration of aromatic peaks from the

Table 3
Vinyl groups conversion for 5% HAGM hydrogels as a function of UV exposure time

5% HAGM		NMR vinyl conversion (%)				
		UV exposure time (min)				
		0.5	1	5	10	15
DM (%)	14	–	–	–	83 ± 4	–
	23	–	–	–	97 ± 2	–
	32	38 ± 3	55 ± 4	85 ± 4	>99	>>99
	60	–	–	–	>99	–
	90	–	–	–	>99	–

initiator at ~7.9 and ~7 ppm remained constant. Thus, it is possible to quantitatively evaluate the cross-linking conversion by calculating the ratio of the relative integration of cross-linked vinyl groups to the aromatic protons from the initiator, which is used as a reference. As shown in Table 3, high vinyl conversions (≥97%) can be achieved after 10 min irradiation for the photopolymerization reaction of HAGMs having various DM, with the exception of the 14% DM. This is in agreement with the swelling ratio data where a lower cross-link density was achieved for the low methacrylated HAGM hydrogels. As shown in Fig. 3b, the reaction kinetic of the cross-linking of 5% wt/v HAGM (DM 32%) is monitored. The photopolymerization process appears to be initiated within 15 s exposure under UV since a low vinyl conversion was obtained (<5%, data not shown), while during propagation ~40% was reached after 1 min, increasing to over 99% after 10 min, and nearly full conversion after 15 min. The ^{1}H NMR spectrum shows that the polymerization is not spontaneous and a high cross-linking conversion can be achieved up to 99% after 10 min exposure under UV.

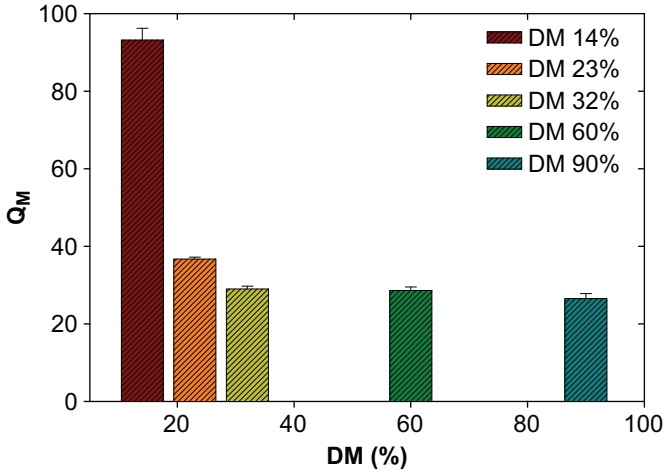

Fig. 4. Influence of the degree of methacrylation on the swelling ratios for 5% wt/v HAGM hydrogels. The hydrogel swelling ratio, calculated by dividing the swollen gel mass by the dry gel mass, is an indicator of the degree of cross-linking in the gel. Each point represents the average ± standard deviation.

After photopolymerization, 5% wt/v HAGM (DM ≈ 32%) hydrogel was dialyzed against deionized water to remove unreacted initiator. The hydrogel was dried to remove the water

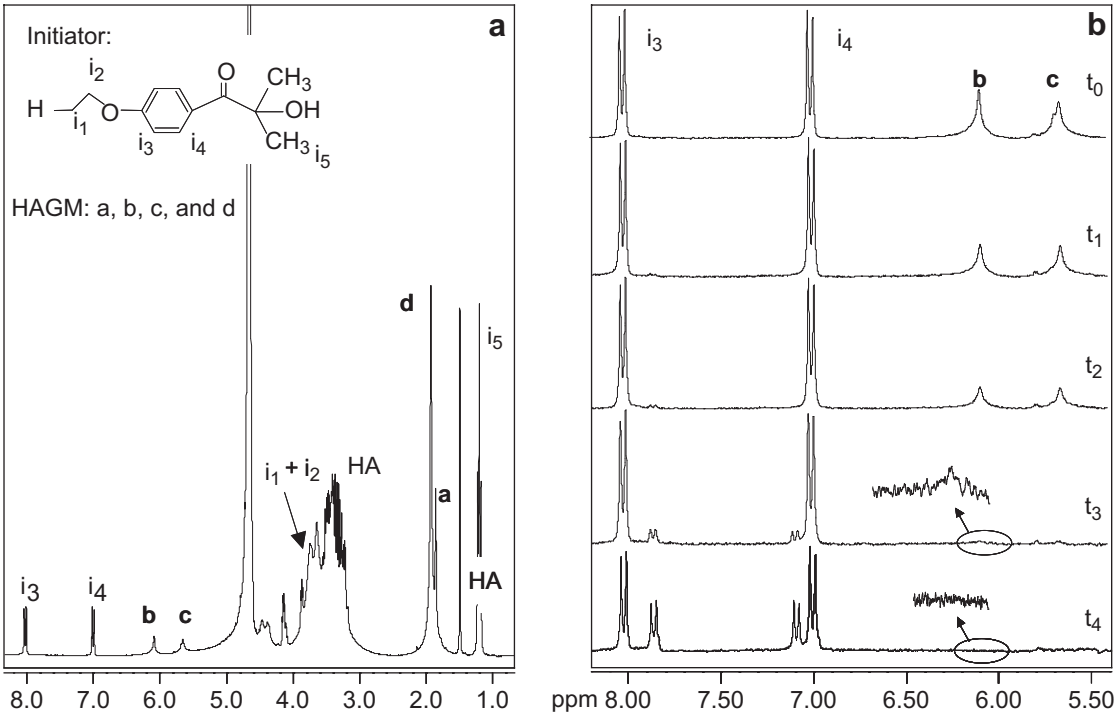

Fig. 3. ^{1}H NMR of uncross-linked (a) and expended views of cross-linked 5% HAGM (DM 32%, b) in D_2O. Photopolymerization is induced directly in an NMR tube. 1 mL of macromer solution (HAGM, I2959) was transferred into the NMR tube before curing with a long wavelength UV source (365 nm, 300 μW/cm^2) to obtain hydrogel. The methacrylate peaks monitored as a function of UV exposure are shown in Fig. 3b at different time points ($t_0 = 0$ min, $t_1 = 0.5$ min, $t_2 = 1$ min, $t_3 = 5$ min, and $t_4 = 10$ min).

content and subsequently re-swollen in D_2O. Surprisingly, ^1H NMR of the purified hydrogel showed a tremendous decrease of peak intensities from the initiator (data shown in the supporting information). These suggest that upon UV irradiation, the aromatic ketone initiator undergoes hydrogen abstraction most likely from the hyaluronic acid backbone to generate a ketyl radical and a donor radical. The initiation of photopolymerization occurs mainly through the H-donor radical while the ketyl radical undergoes either radical coupling with the growing macromolecular chains or radical termination with another free radical species.

3.4. Swelling behavior

To investigate the effect of cross-link density on the swelling behavior of the gels, we determined the swelling ratio of hydrogels with various DM. The swelling tests performed on the HA cross-linked samples in PBS are reported in Fig. 4.

When the macromonomer mass fraction was held constant, the swelling ratio decreased with increasing the DM. Conveniently, by controlling the DM of HAGM macromonomers, the degree of cross-linking can be manipulated. Generally, as the percentage of methacrylation increased, the gels showed

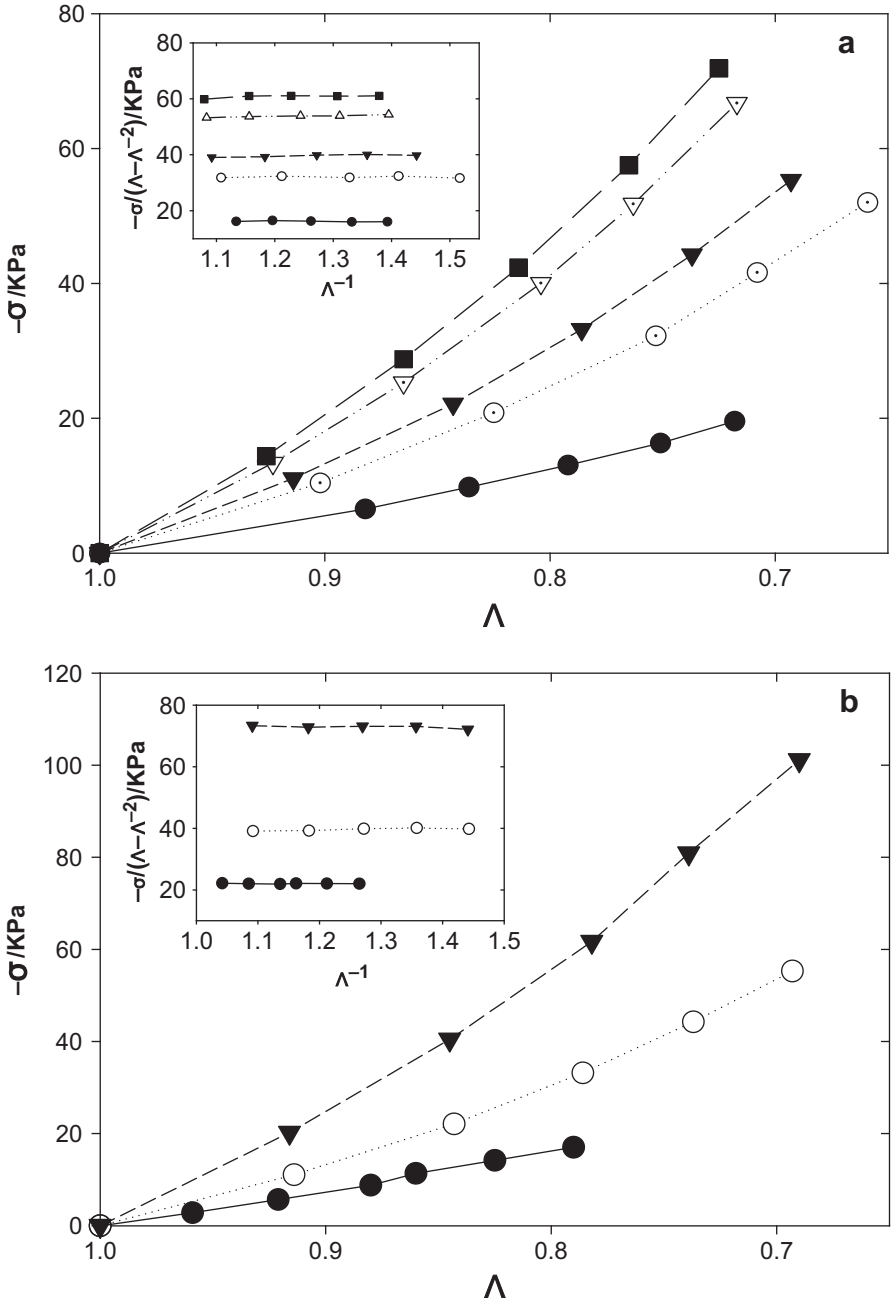

Fig. 5. Nominal stress (σ) measured as a function of the deformation ratio (Λ) for HA-based hydrogels: 5% [DM 14% (●), 23% (.), 32% (▼), 60% (△), and 90% (■)] (a) and 2% (●), 5% (○), and 10% (▼) [DM 32%] (b). The insets show the Mooney–Rivlin representation plotting the reduced stress vs $1/\Lambda$. Note that σ is negative due to the compressive forces used.

decreasing swelling ratios, indicating increased levels of cross-linking. For 5% wt/v hydrogels with DM of 14, 23, 32, 60, and 90%, the swelling ratios were 93.2, 36.7, 29, 28.6, and 26.5, respectively (Fig. 4). We observed that the hydrogels with the lowest DM (14%) exhibited the highest swelling ratio ($Q_M = 93.2 \pm 3$). We presume that in this case the number of photopolymerizable sites available on HAGM is insufficient to support high cross-linking conversion. Furthermore, the DM is correlated to the cross-link density and a low DM results in the formation of hydrogels with low cross-link density, resulting in the absorption of more water. We also observed that the swelling trend for hydrogels with DMs above 23% have lower swelling ratios when compared to the 14% hydrogel and decreased by a much more gradual decline presumably due to the non-classical network structure of these HA-based hydrogels.

As expected, for samples with higher DM, the amount of water uptake in the hydrogel decreased, reaching the lowest water content for the gel with DM $\approx$ 90%. Furthermore, HAGM hydrogels with the lowest DM ($\approx$14%) showed the highest water absorption capacity due to a higher molecular weight between cross-link. We hypothesize that at the lowest methacrylation, the hydrogel network is insufficient to form uniformly dispersed clusters of methacrylic groups, thereby creating large defects in the network. Large inhomogeneities in the network structure lead to larger pore size. An evaluation of the material parameters (e.g. mesh size, molecular weight between cross-links) using Flory–Rhener calculations with appropriate Flory polymer–solvent interaction parameter (χ) for HA in correlation with the swelling and mechanical data is currently under investigation.

3.5. Mechanical testing

The shear moduli (G) of hydrogels were measured by uniaxial compression and calculated using equations derived from the strain energy function [23]. Fig. 5a,b show, respectively, that the nominal stress σ as a function of the deformation ratio Λ for 5% HAGM (DM from 14% up to 90%) and (2% up to 10%) wt/v HAGM (DM 32%) hydrogels. Note that in compression σ is negative. The insets of Fig. 5a,b plot the normal stress vs the deformation ratio according to the Mooney–Rivlin representation (Eq. (2)).

$$\sigma = 2\left(\Lambda - \Lambda^{-2}\right)(C_1 + C_2/\Lambda) \tag{2}$$

where C_1 and C_2 are constants. In agreement with many previous findings reported for swollen polymer networks, the value of C_2 is approximately zero.

Shear moduli of HAGM hydrogels prepared with different mass fractions (2, 5, and 10%) and DM (14, 23, 32, 60, and 90%) are shown in Figs. 6 and 7. As expected, in Fig. 6, G monotonically increases as the DM increases. The mechanical integrity of the hydrogels indicates that the shear modulus increased as the degree of cross-links increased suggesting that the network becomes more resistant to change when a load is applied with more cross-links. However, the variation between the DM and shear modulus is less pronounced for highly methacrylated hydrogels (ie., 60% and 90%). As shown in Fig. 7, for HAGM (DM 32%) hydrogels, the shear modulus also appears to depend on macromonomer concentration while the methacrylation is kept constant. As expected, the macromonomer mass fraction also affects the mechanical strength of the gels. The shear modulus monotonically increases as the macromonomer concentration increases, presumably due to an increase in cross-link density.

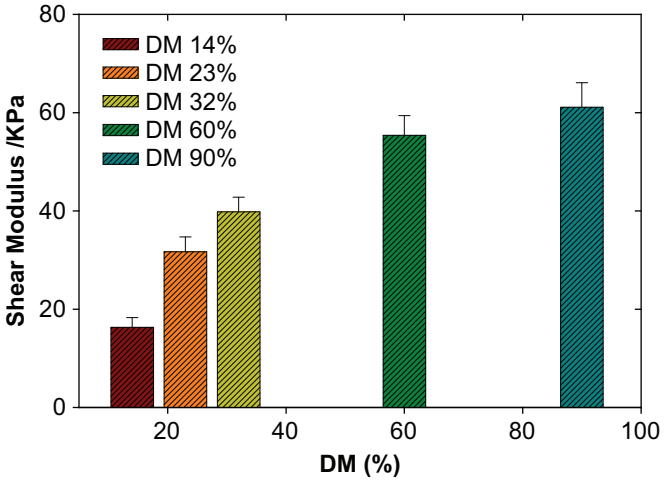

Fig. 6. Influence of methacrylation on mechanical properties. Shear modulus of 5% HAGM hydrogels was measured with various degree of methacrylation.

The uniaxial compression testing showed that 5% wt/v HAGM hydrogels are mechanically robust and showed varied shear moduli from 16 kPa to 65 kPa depending on the DM. Also, when keeping the DM constant (DM 32%), the shear modulus varied from 22.3 kPa up to 73 kPa while the macromonomer concentration was increased between 2% and 10%. One striking observation is that the DM in comparison to the macromonomer mass fraction seems to affect the shear modulus similarly. For HAGM (DM 32%) hydrogels, an increase of the mass fraction from 2% to 10% affects the shear

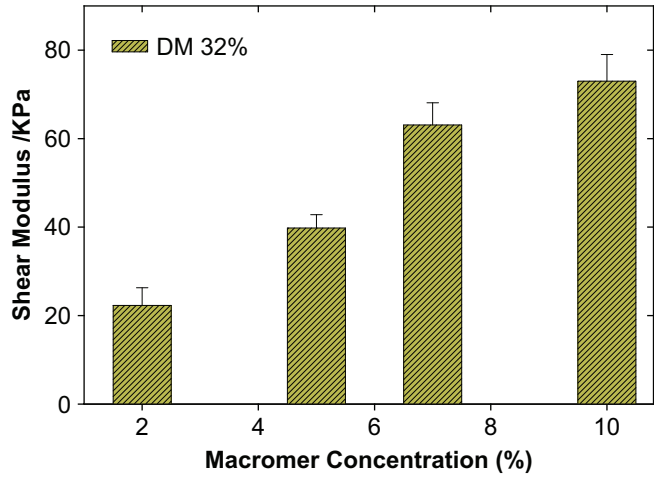

Fig. 7. Influence of hydrogel composition on mechanical properties. The shear modulus (G) of HAGM (DM 32%) hydrogels was measured as a function of the macromer mass fraction (2, 5, 7 and 10%).

modulus by approximatively a factor of 3.3 ($G = 22.3$ kPa vs 73 kPa) and comparably an increase by a factor of 3.8 ($G = 16$ kPa vs 61.1 kPa) was obtained when the DM was increased from 14% to 90% for 5% HAGM hydrogels.

3.6. Biocompatibility and cell response on GRGDS modified HAGM hydrogels

C2C12 myoblast cells seeded on HAGM hydrogels were used as a preliminary assessment of cellular responses to the hydrogels. Cells were examined after 1, 3, and 5 d post-seeding using phase contrast and fluorescent microscopy. Live/dead cell staining (Fig. 8) show HAGM hydrogels are biocompatible and non-toxic as more than 80% viable cells were scored after 5 d of incubation. The polymerized hydrogels present a relatively non-adherant surface to C2C12 cells, presumably due to poor protein adsorption to the hydrophilic polymer surface [24]. The incorporation of the GRGDS peptide allowed modulation of the HA properties from cell non-adhesive to adhesive. More than 25% of the HAGM surface was covered by C2C12 cells by day 1, 50% by day 3, and 80% by day 5, suggesting the cells attached well and proliferated on the GRGDS modified HAGM gels. C2C12 cells cultured on unmodified HAGM gels showed very few adherent cells (Fig. 8) without any sign of spreading over long durations in culture. In the absence of the peptide, most cells remained round, spindle-shaped cells were rare, in comparison with the cells attaching and reaching to confluence on polystyrene surface and modified gels. The short peptide sequences responsible for cell surface adhesion receptor binding have proven to be sufficient for cell adhesion and spreading when chemically incorporated into biomaterial surfaces in sufficient numbers [25].

3.7. Effect of the degree of methacrylation on cell response

The trend of increased cross-linking with increased methacrylation was apparent to improve the physical properties of HA-based hydrogels and supposedly the biostability against enzymatic degradation. Thus, the hydrogels could be tailored to a wide range of degradation times depending on the desired application. Slower degradation rates may be achieved by preparing highly methacrylated gels. While a prominent DM

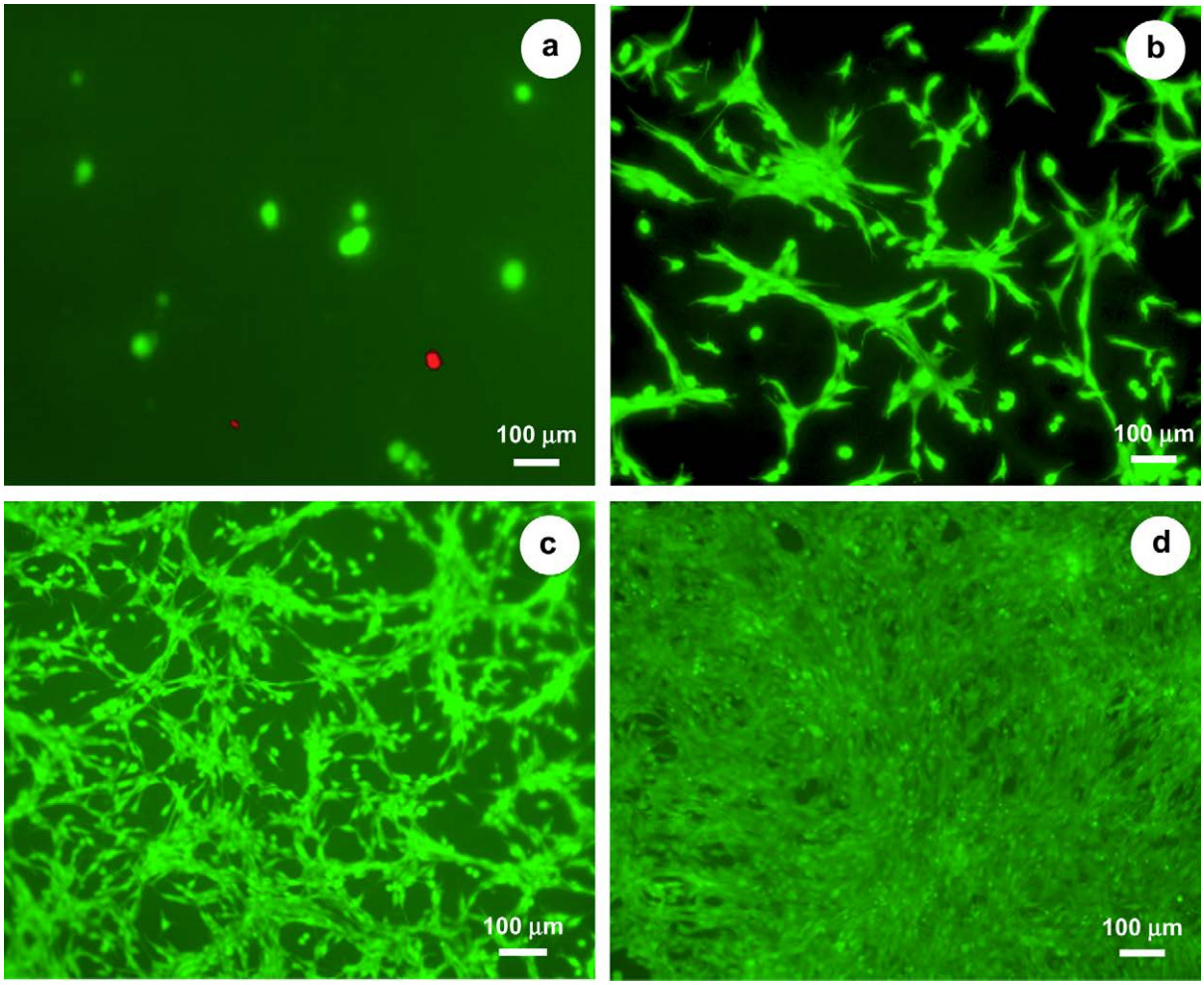

Fig. 8. Mouse myoblast cells (C2C12) seeded on 5% HAGM (DM 32%) gels with the live stain (green) and dead cell stains (red/blue). Live/dead stain of HAGM (a) and GRGDS modified HAGM at different time of incubation, 1 day (a, b), 3 days (c), and 5 days (d).

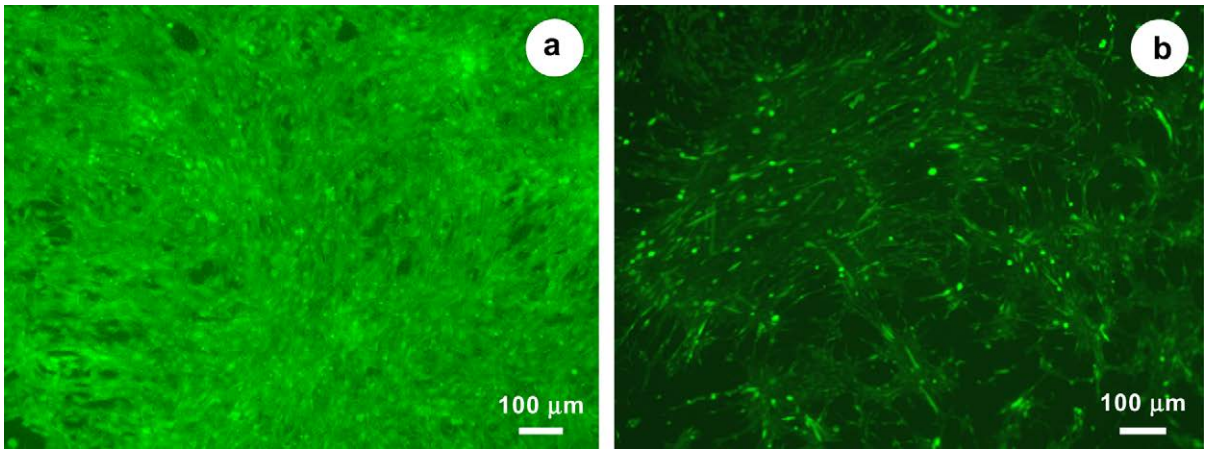

Fig. 9. Influence of methacrylation on cell response. Live/dead staining of mouse myoblast cells (C2C12) seeded in GRGDS modified 5% HAGM (DM 32%, a) and 5% HAGM (DM 60%, b) hydrogels after 5 d of incubation.

seems to improve HAGM hydrogels properties, the recognition of highly modified HA by cells is to investigate. It is known that peptides containing the RGD sequence can mimic cell adhesion proteins in two ways: when coated onto a surface, they promote cell adhesion, whereas in solution they act as decoys, preventing adhesion [26]. Cell adhesion and spreading on the gels were examined to identify whether the biological signals of a HA-based scaffold are conserved or not while using various methacrylation. Response of myoblast cells to hydrogels with two different DM (low, and high) is shown in Fig. 9. The DM had a minimal effect on the cell response, since both types of gels (DM ~32% and 60%) showed good cell attachment and proliferation, eventually covering more than 80% of the HAGM surface by day 5. The efficiency of cell adhesion on HAGM/PEG–GRGDS composites seems not to be altered at different degree of cross-linking when the peptide concentration is kept constant.

4. Conclusions

A new method is presented to modify HA with GM with a good control over the DM (from 14% up to 90%). ^{1}H NMR spectroscopy showed that hydrogels with high vinyl conversions could be formed after 10 min irradiation under UV reducing the cytotoxicity of unreacted methacrylate groups. Determination of the material properties indicated that the hydrogels could be tailored to a wide range of physical properties depending on methacrylation levels. Increasing levels of methacrylation could fine-tune the cross-linking density, the mechanical properties and swelling ratios. Finally, the excellent swelling ability of these HA-based hydrogels suggests they could be efficacious as a drug delivery system where the degradation would be mediated by endogenous enzymes present at the site of tissue repair.

Preliminary *in-vitro* data confirmed HAGM hydrogels were cytocompatible, and the introduction of GRGDS promoted adhesion and proliferation of cells to confluence after 5 d of incubation. Moreover, the DM parameter has shown to have a minimal affect on the cell–scaffold interaction: cell morphology, attachment, and proliferation were similar for both low and high cross-link density hydrogels.

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Appendix. Supplementary material

Supplementary material associated with this article can be found at, doi: 10.1016/j.biomaterials.2007.11.047.

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