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Synthesis of methacrylated hyaluronic acid with tailored degree of substitution

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Abstract

The aim of this work was to develop a new method to derivatize hyaluronic acid (HyA) with polymerizable methacrylate residues with precise control over the substitution degree. The synthesis of methacrylated HyA (HyA-MA) was performed in dimethyl sulfoxide (DMSO) using glycidyl methacrylate (GMA) and 4-(*N*,*N*-dimethylamino)pyridine as a catalyst. HyA was rendered soluble in DMSO by exchanging the Na^+ ions by the more lipophilic tetrabutylammonium ions. HyA-MA with a fully controlled degree of substitution (DS, defined as the number of methacrylate groups per 100 disaccharide units), ranging from 5 to 30, was obtained at 50 °C after 48 h. Hydrogels were obtained upon radical polymerization of aqueous solutions of HyA-MA using potassium peroxodisulfate (KPS) as initiator and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) as catalyst. Almost complete methacrylate conversion (95%) was achieved for hydrogels obtained by polymerization of HyA-MA with a degree of substitution of 15. At lower DS (DS 8.5 and 5) the methacrylate conversion was 82% and 68%, respectively. Rheological characterization showed that with increasing DS the storage modulus of these HyA-MA hydrogels increased. Swelling experiments showed that HyA-MA gels with a DS of 15 or above were dimensionally stable, whereas HyA-MA gels with DS 5 and DS 8.5 swelled 1.6 and 1.4 times their initial weight, respectively. In conclusion, this paper shows that the DS of HyA-MA can be tailored by the reaction conditions and that consequently HyA-MA hydrogels with different characteristics can be prepared.

Keywords: Hyaluronic acid; Methacrylation; Hydrogels

1. Introduction

Hyaluronic acid (HyA) is an endogenous polysaccharide, i.e. present in the vitreous body, synovial fluids and the extracellular matrix, which consists of repeating disaccharide units composed of β(1−4) linked *N*-acetyl-D-glucosamine and β(1−3) linked D-glucuronic acid [1]. Its biocompatibility, biodegradability and immunoneutrality make HyA an attractive polymer for biomedical and pharmaceutical applications. Currently, HyA is applied to treat joint diseases such as in osteoarthritis and in eye surgery as replacement fluid and is under investigation for drug delivery and tissue engineering applications [2–4]. For these applications besides unmodified HyA derivatized HyA is also used. Modification of HyA can be performed as hydroxyl and carboxy groups can be used for chemical derivatization [5,6] and many chemical modifications of HyA have been described in literature [1]. One chemical modification concerns the derivatization of HyA with polymerizable methacrylate groups. This crosslinkable HyA can be used to form hydrogels for drug delivery and tissue engineering purposes [4,5,7,8]. The synthesis of methacrylated HyA (HyA-MA) is performed in an aqueous environment with an excess of methacrylic anhydride with respect to the hydroxyl groups of HyA [3,7,9]. The major drawback of this

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synthesis lies in the aqueous basic (pH 8) reaction conditions: methacrylic anhydride can react with water to yield methacrylic acid and additionally the covalently linked methacrylic ester can be hydrolyzed during the reaction [10]. This makes it difficult to control the degree of substitution. Hence, there is need for a method in which the DS of methacrylated HyA can accurately be controlled as was achieved in the past for the modification of polysaccharides [11–13] allowing the preparation of hydrogels with tailored properties.

This paper reports on the synthesis of methacrylate derivatized HyA with precise control over the substitution degree in a suitable aprotic solvent by substitution of the parent polysaccharide with glycidyl methacrylate. Additionally, the characteristics of the hydrogels obtained with glycidyl methacrylate derivatized HyA are studied.

2. Experimental section

2.1. Materials

HyA sodium salt (from *Streptococcus equi* sp., MW ~1,700,000 Da), tetrabutylammonium fluoride trihydrate (TBA-F), dimethyl sulfoxide (DMSO, H_2O ≤0.005%), glycidyl methacrylate (GMA, purity ≥97%), methacrylic acid and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Fluka (Buchs, Switzerland). 4-(*N*,*N*-Dimethylamino)pyridine (DMAP), 37% hydrochloric acid, 70% perchloric acid and acetic acid were purchased from Acros Chimica (Geel, Belgium). Sodium hydroxide (NaOH) pellets, sodium chloride, potassium peroxodisulfate (KPS) and ethanol absolute were provided by Merck (Darmstadt, Germany). Diethylether and acetonitrile (HPLC grade) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands).

2.2. HyA modification in aqueous solution

Methacrylation of HyA was performed following the procedure described by Smeds et al. [3]. In brief, 0.5 g HyA sodium salt was dissolved in 25 ml H_2O after which a 20-fold excess of methacrylic anhydride (3.7 ml) relative to primary HyA hydroxyl groups was added. Next, the pH of the reaction mixture was adjusted to 8 with 5 N NaOH and the two-phase system was stirred for 24 h at 5 °C. A second synthesis was performed in a similar way as described above, however, at 50 °C to prevent phase separation. For both reactions the polymer was precipitated in ethanol and washed with the same solvent for three times. The sample was dried before characterization.

2.3. Dissolution of HyA in DMSO

To render hyaluronic acid soluble in DMSO, the sodium ions of HyA were exchanged with the lipophilic tetrabutylammonium (TBA) ion. Ion exchange was performed using Dowex® 50W-X8 cation exchange resin (5.1 mmol/g exchange capacity; BioRad Laboratories, Veenendaal, The Netherlands). The Dowex® resin (40 g) was incubated with a large excess of TBA-F (137 g, 435 mmol) dissolved in RO-water (500 ml) for 1 h and washed extensively with water. Next, the resin was transferred into 1000 ml of a 1% (w/w) HyA solution in water and mixed for 2 h at room temperature. The mixture was then centrifuged for 2 min at 5000 rpm to remove the resin. The obtained HyA-TBA solution was lyophilized and used for chemical modification with GMA.

2.4. Synthesis of methacrylated HyA in DMSO

The kinetics of the reaction was studied as follows. A 1% (w/v) solution of HyA-TBA in DMSO (100 ml) was prepared at 50 °C. After dissolution of HyA-TBA, 0.2 g of DMAP and 86 μl of GMA (corresponding to a molar ratio GMA:primary OH of 0.4:1) were added. At regular time intervals samples of 2 ml were taken, precipitated in diethylether and washed three times with the same solvent. Samples were dried overnight at room temperature prior to determination of the degree of methacrylate substitution (DS, defined as the number of methacrylate groups per 100 disaccharide units; i.e. a DS of 10 indicates that 10 out of 100 hydroxyl groups of a HyA molecule are esterified with methacryloyl groups) by reversed-phase (RP) HPLC (Section 2.10.). Based on the results of the kinetic study, the following protocol was used to routinely synthesize methacrylated HyA (HyA-MA). One gram of HyA-TBA salt was dissolved in 100 ml of DMSO at 50 °C under a nitrogen atmosphere. Subsequently, 0.2 g of DMAP and a calculated amount of GMA (43 μl to 430 μl), depending on the requested degree of substitution (DS), were added. The solution was stirred for 48 h at 50 °C. Next, an equimolar amount of concentrated HCl with respect to DMAP was added to neutralize this catalyst. The reaction mixture was transferred into a dialysis membrane (MWCO 12-14,000 Da, Medicell International Ltd, London, Great Britain) and dialyzed at 4 °C for 3 days against 150 mM sodium chloride in RO-water and subsequently for 4 days against RO-water to ensure the exchange of TBA^+ by Na^+ ions and to remove DMSO. Methacrylated hyaluronic acid (HyA-MA) was obtained after freeze-drying and the degree of substitution of the polymer was determined by RP-HPLC analysis (Section 2.10.).

2.5. Polymerization kinetics of HyA-MA

Hydrogels were obtained by free radical polymerization of aqueous solutions of HyA-MA initiated by KPS and using TEMED as a catalyst. In detail, a 2% (w/w) solution of the derivatized polymer (17 mg, DS 5, 8.5 or 15) in 750 μl phosphate buffer (10 mM, pH 7.2) was prepared. Subsequently, KPS (60 μl, 50 mg/ml) and TEMED (24 μl, 20% (v/v), adjusted to pH 7 with 2 M HCl) were added to start the polymerization reaction. At regular time intervals, the samples were quickly frozen into liquid nitrogen to stop the polymerization and then lyophilized. The dried samples were rehydrated in 10 ml 0.02 M NaOH and incubated at 37 °C for 30 min to hydrolyze unreacted methacryloyl groups. RP-HPLC was used to determine the concentration of methacrylic acid (Section 2.10.).

2.6. Preparation of hydrogels

A 2% (w/w) solution of HyA-MA (17 mg, DS varying from 5 to 30) in 750 μl 10 mM phosphate buffer (pH 7.2) was prepared and transferred into a cylindrical mould of 10 × 7 mm (diameter × height). The radical polymerization was started after addition of 60 μl KPS (50 mg/ml) and 24 μl TEMED (20% (v/v), adjusted to pH 7 with 2 M HCl). The resulting solution was allowed to polymerize for 1 h at room temperature.

2.7. Rheological analysis of HyA-MA hydrogels

The rheological properties of the polymerizing HyA-MA solutions were determined on a rheometer (AR1000-N, TA instruments, Etten-Leur, The Netherlands) using a 40 mm 1° steel cone geometry. A 2% (w/w) solution of HyA-MA (DS 5, 8.5, 15, 18 or 30) in phosphate buffer (10 mM, pH 7.2) was prepared and directly after addition of the polymerization reagents KPS and TEMED, ~350 μl was placed between the plates. During the polymerization of HyA-MA, the G' (shear storage modulus) and the G'' (loss modulus) were monitored at 20 °C maintaining a constant strain of 1% and a constant frequency of 1 Hz. The extent of deformation and recovery of a HyA-MA gel were evaluated by a creep experiment. A shear stress of 10 or 100 Pa was applied on the system during 2 min while the strain was monitored and after removal of the stress the recovery was analyzed for 2 min.

2.8. Swelling behavior of HyA-MA hydrogels

HyA-MA hydrogels (0.85 g; 2% (w/w); DS 5, 8.5, 15, 18 and 30) were prepared as described in Section 2.6. The hydrogels were weighed (W_0) after being transferred into preweighed glass vials. Next, 10 ml isotonic 100 mM PBS (pH 7.2) with 0.02% NaN_3 was added and the vials were placed in a water bath at 37 °C. At regular time intervals, the buffer was replaced by fresh PBS and the gels were weighed (W_t) to determine the swelling ratio defined as W_t/W_0.

2.9. ^{1}H NMR spectroscopy

^{1}H NMR spectra were recorded in ^2H_2O (99.9% ^{2}H, Sigma–Aldrich) on a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA) using ^{2}HOH at 4.8 ppm as reference line. A 1% (w/w) solution of HyA-MA in ^2H_2O was prepared for analysis. The substituted methacrylate groups were identified by the signals of the methacryloyl group at 5.6 and 6.0 ppm (protons of the double bond H_a and H_b, respectively) and of the methyl resonance at 1.8 ppm (H_c).

2.10. Reversed-phase HPLC analysis

The concentration of methacrylic acid was determined by RP-HPLC as described by Stenekes and Hennink [14]. In brief, HyA-MA (15 mg) was dissolved in 10 ml 0.02 M NaOH and incubated at 37 °C for 30 min to hydrolyze the polymer-bound methacrylate groups. Prior to analysis, 2 ml of a 2 M acetic acid solution was added. Ten microlitres of this mixture was injected onto a RP-18 column (Lichrospher, Merck, Darmstadt, Germany). Analysis was carried out with a Waters system (Waters Associates Inc.), including a 600A HPLC pump, a Model 717 autoinjector, and a UV detector Model 2487. The mobile phase consisted of acetonitrile/water (10/90 (w/w)) adjusted to pH 2 with perchloric acid. The flow rate was 1.0 ml/min and the detection wavelength was set at 210 nm. A calibration curve was obtained by injecting varying volumes (0.5–150 μl) of a 100 μM methacrylic acid in eluent. Empower Pro software (Waters Associates Inc.) was used to determine the peak areas which, in turn, were used to quantify methacrylic acid in the samples.

3. Results and discussion

3.1. Synthesis and characterization of methacrylate modified HyA

The synthesis of methacrylated HyA was previously described by Smeds et al. [3]. In their procedure, methacrylated HyA was prepared by reacting a 20-fold excess of methacrylic anhydride relative to primary HyA hydroxyl groups in an aqueous environment (pH 8) at 5 °C. Under these conditions a two-phase system is formed. It was shown that by varying the reaction time and the amount of methacrylic anhydride, methacrylated HyA with 3%, 8%, or 17% degree of substitution could be obtained [3]. Attempts were made by us to perform this reaction following this procedure. In addition, the reaction was carried out at 50 °C to prevent phase separation. We were able to obtain methacrylated hyaluronic acid, however, we were unable to control the degree of substitution at both temperatures. Methacrylic anhydride can hydrolyze in water, yielding methacrylic acid, which does not react with HyA. Since this hydrolysis and additionally the hydrolysis of methacrylated HyA are dependent on both pH and temperature, control over the DS is difficult to achieve. Therefore, we developed an alternative method for the synthesis of methacrylated HyA with full control over the DS. Our procedure consists of the reaction of HyA dissolved in DMSO with GMA using DMAP as catalyst, under dry and oxygen free conditions, yielding methacrylated HyA and glycidol (Fig. 1). To render HyA soluble in DMSO, the sodium salt of this polymer was converted into a tetrabutylammonium salt by ion exchange. Methacrylated HyA, after reaction with GMA, in sodium salt form was obtained following dialysis and freeze-drying after which samples were characterized by ^{1}H NMR spectroscopy. Lack of characteristic signals around 1 ppm showed that TBA^+ [$(CH_3–CH_2–CH_2–CH_2)_4N^+$] ions were fully exchanged by Na^+ ions during dialysis. Methacrylate modification was confirmed by ^{1}H NMR, although the degree of substitution (DS) could not be accurately determined as the signals for the methyl groups of both the polymer backbone and the coupled methacrylate overlapped at 1.8 ppm (spectra not shown). Broadening of signals, which may result in an overlap as in our case, is a normal phenomenon for

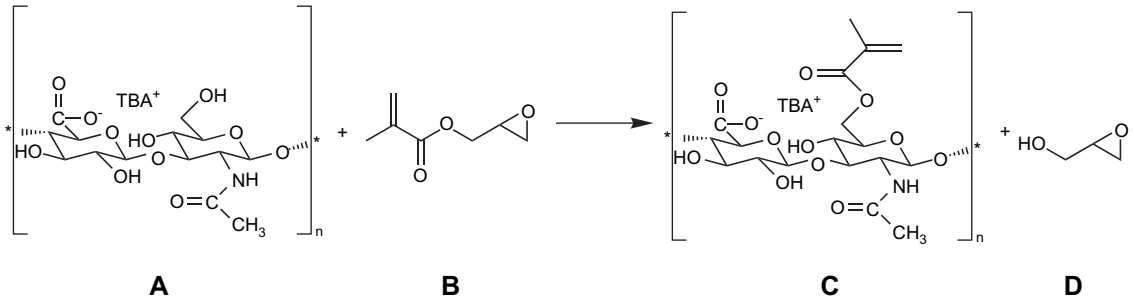

Fig. 1. Synthesis of methacrylated hyaluronic acid. HyA (A) reacted with glycidyl methacrylate (B) in DMSO to obtain the crosslinkable product HyA-MA (C) and glycidol (D).

macromolecules [15]. Also NMR analysis of partially degraded HyA as described in literature [3,12] did not result in better resolution. Therefore, RP-HPLC was used to determine the DS of the HyA-MA. Hence, the polymer-bound methacrylate groups were hydrolyzed under alkaline conditions and the formed methacrylic acid was quantitatively determined using RP-HPLC.

A kinetic study showed that the reaction of HyA with GMA, at a feed ratio of 40%, reached a DS of 8.5 within 48 h at 50 °C. Modification occurred immediately after addition of GMA and longer incubation time did not result in an increased degree of methacrylate substitution. The relation between the percentage of GMA to the primary hydroxyl group of HyA in the feed and the DS of the modified polymer is presented in Table 1. It is shown that for every feed ratio up to 200%, HyA was derivatized with approximately 20% of the added GMA. This indicates that HyA-MA (DS ranging from 5 to 30) can reproducibly be prepared with good control of the DS. The rather low incorporation of GMA was shown previously in literature [11,16] and can be attributed to the occurrence of equilibrium during the reaction of HyA with GMA. Rheological analysis (see next section) shows that the G'' of HyA and HyA-MA (Fig. 2B) did not statistically differ from each other indicating that under the selected reaction conditions no chain scission occurred.

3.2. Preparation and characterization of HyA-MA hydrogels

The hydrogels were obtained by crosslinking aqueous solutions of HyA-MA in the presence of KPS and TEMED as initiator and catalyst, respectively. Modified HyA was well soluble in phosphate buffer. The radical polymerization resulted into opaque hydrogels, independent of the DS used. The opacity of the hydrogels indicates that phase separation (i.e. water-poor domains containing relatively hydrophobic polymerized methacrylates and water-rich hydrated HyA domains) has occurred. The kinetics of the polymerization was studied using RP-HPLC to determine quantitatively the amount of unreacted methacrylate groups. Polymerization

Table 1
Storage modulus G', loss modulus G'' and tan δ of HyA-MA hydrogels (2% (w/w) initial solid content) as a function of the obtained methacrylate substitution, which is related to the molar ratio of GMA to HyA hydroxyl groups in the feed (%)

Feed ratio (%)	Obtained DS (%)	G' (Pa)	G'' (Pa)	tan δ
20	5.4 ± 0.4	1300 ± 90	2 ± 0.1	0.002 ± 0.000
40	8.5 ± 1.5	2000 ± 280	2 ± 0.1	0.001 ± 0.000
80	15.4 ± 1.4	3900 ± 150	4 ± 0.1	0.001 ± 0.000
100	18.0 ± 2.0	4600 ± 410	28 ± 2.5	0.005 ± 0.001
200	30.0 ± 4.0	10,500 ± 850	34 ± 2.1	0.003 ± 0.001

The data are shown as average ± SD, $n = 3$.

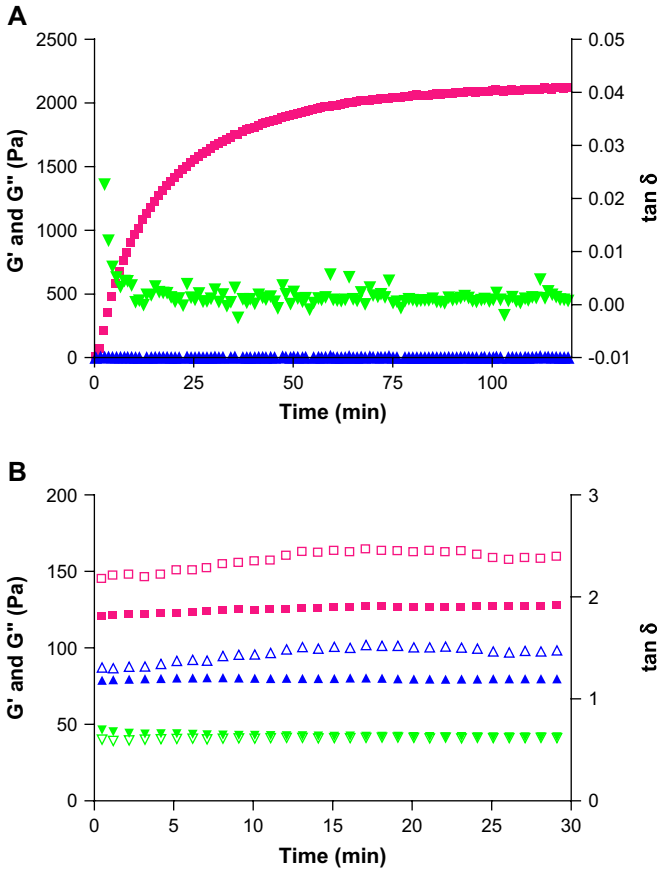

Fig. 2. (A) Storage modulus G' (■), loss modulus G'' (▲) and tan δ (▼) of a gelating HyA-MA solution (2% (w/w), DS 8.5) as function of time following radical polymerization using KPS and TEMED. (B) Storage modulus G' (■ □), loss modulus G'' (▲ △) and tan δ (▼ ▽) of a 2% (w/w) HyA-MA (closed symbols) and 2% (w/w) HyA (open symbols) aqueous solution as function of time.

started immediately after addition of KPS and TEMED and showed that more than 95% of the methacrylate groups were converted within 1 h for hydrogels containing HyA-MA with a DS of 15. It was observed that with lower degrees of substitution (DS 8.5 and 5) the methacrylate conversion decreased to 82% and 68%, respectively, indicating that with higher DS the methacrylate groups have a higher probability to react with each other.

The viscoelastic properties of the HyA-MA hydrogels (2% (w/w) initial solid content) with various methacrylate substitutions were evaluated using controlled strain experiments. In Fig. 2A the formation of an elastic network of HyA-MA with DS 8.5 is shown. It can clearly be seen that the storage modulus G' gradually increased in time to 2100 Pa while the loss modulus remained low (2 Pa). Moreover, the tan δ was below 0.01 indicating that the obtained gel was fully elastic. For comparison, a solution of HyA-MA (DS 8.5) with the same solid content was analyzed without the addition of KPS and TEMED (Fig. 2B). It was shown that both G' and G'' remained low at 120 and 100 Pa, respectively, whereas the tan δ was substantially higher at 0.7, which demonstrates that an elastic network does not exist in an aqueous, but viscous, solution of HyA-MA. The results of a creep experiment confirmed that a fully elastic HyA-MA network was obtained upon the addition of a radical polymerization initiator and catalyst (Fig. 3A). It was shown that application of a shear stress (100 Pa) on the system to which KPS and TEMED were added resulted in a deformation up to 5% strain. The hydrogel recovered completely when the stress was removed. On the other hand, an aqueous solution of HyA-MA showed a continuous flow (Fig. 3B) and no recovery after stress removal at a shear stress of already 10 Pa, again demonstrating that it behaves as a viscous solution. In addition, this aqueous solution of HyA-MA showed a similar deformation (>500% strain; Fig. 3B) as an aqueous solution of initial HyA. Rheological analysis also showed that increasing degree of methacrylation of HyA resulted in gels with increased shear storage modulus and slightly increased loss modulus while the tan δ stayed low (Table 1). As an increased number of methacrylate groups are linked to the hyaluronic acid chains, more covalent crosslinks can be formed resulting in an increased elastic modulus. Nevertheless, the gelation time for all gels remained low: within 1 min G' already exceeded G''.

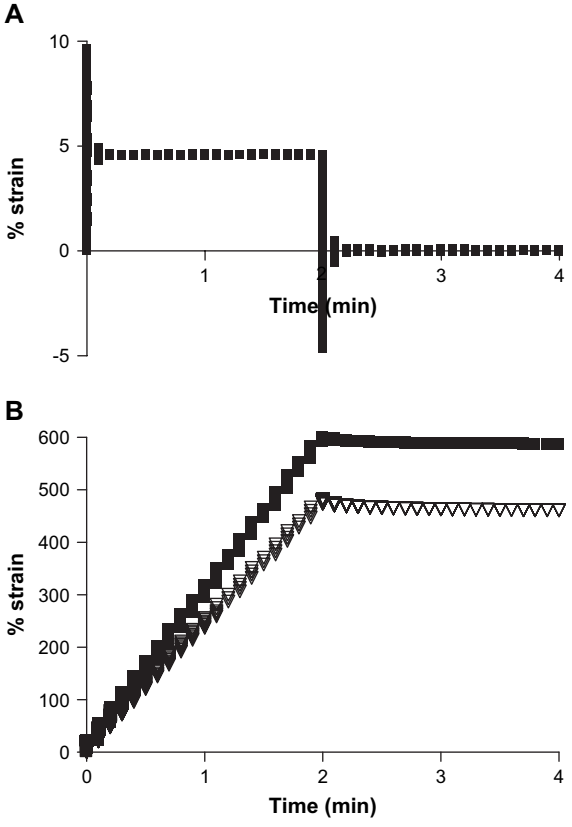

Fig. 3. (A) Creep experiment on a 2% (w/w) HyA-MA hydrogel (DS 8.5, applied stress 100 Pa) as function of time. (B) Creep experiment on a HyA-MA DS 8.5 (closed symbols) and a HyA (open symbols) aqueous solution (2% (w/w), applied stress 10 Pa) as function of time.

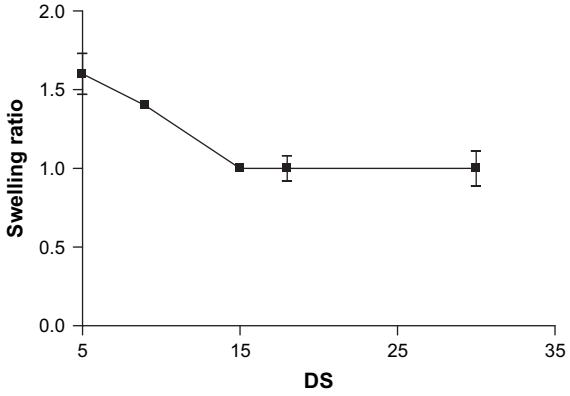

Fig. 4. Swelling ratio of 2% (w/w) HyA-MA hydrogels as function of the DS. The data are shown as average ± SD, $n = 3$.

Besides their rheological characteristics, also the swelling behavior of HyA-MA hydrogels was studied. HyA-MA gels with an initial solid content of 2% (w/w) and various degrees of methacrylate substitution were prepared at room temperature and their swelling evaluated at 37 °C and pH 7.2 (Fig. 4). It was observed that with increasing DS the swelling of the gels decreased: HyA-MA gels with DS 5 and DS 8.5 swelled 1.6 and 1.4 times their initial weight, respectively, whereas gels with DS 15, 18 and 30 did not swell. The gels with DS 5 and DS 8.5 started to swell immediately upon immersion in isotonic 100 mM PBS (pH 7.2) and reached their equilibrium swelling within 2 days. The limited swelling at high DS indicates that rather dimensionally stable hydrogels are obtained.

4. Conclusion

In conclusion, a new method is presented to synthesize methacrylated HyA with full control over the DS. Radical polymerization of aqueous solutions of HyA-MA resulted in opaque elastic hydrogels. Characterization of these HyA-MA hydrogels showed that the elastic modulus and the dimensional stability of the gels increased with higher substitution

degree. Consequently, this novel method indicates that HyA-MA hydrogels are potential application systems for drug delivery systems and tissue engineering purposes.

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