HYALURONIC ACID BASED HYDROGELS FOR THE REPAIR OF CARTILAGE LESIONS

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ABSTRACT
Injectable methacrylated hyaluronic acid (MeHA) hydrogels for the repair of cartilage lesions were synthesized using a matrix metaloproteinase 7 (MMP7)-degradable peptide as crosslinker. The effects of the crosslinker concentration and biofunctionalization of MeHA (i.e., with a chondroitin sulfate (CS) binding peptide) on the end-use properties of the synthesized hydrogels were experimentally investigated. It was found that as the concentration of the peptide crosslinker increased the hydrogel onset time decreased as well as the degradation rate of the synthesized hydrogels while their storage modulus (G') increased. The functionalization of MeHA with a CS binding peptide resulted in an increase of the gelation onset time as well as in a decrease of the hydrogel crossilinking density (i.e., resulting in the formation of softer hydrogels).

INTRODUCTION
Hyaluronic acid (HA) is a natural polysaccharide which is found natively in cartilage tissue. HA based hydrogels favor the differentiation of mesenchymal stem cells (MSCs) to chondrocytes and, thus, the formation of cartilaginous matrix for cartilage repair [1]. In recent studies [2,3], enzymatically degradable peptides have been employed as crosslinking agents in hydrogel formation. The use of MMP-degradable HA hydrogels has been shown to increase the chondrogenic differentiation of human MSCs (hMSCs) and suppress their hypertrophy [4]. In particular, MMP7 aids hMSCs chondrogenesis via the controlled bioavailability of chondrogenic factors and enhanced maturation of collagen type II [5]. MMP7-sensitive peptides were used as cross-linkers in the synthesis of PEG hydrogels. It was shown that the synthesized hydrogels degraded during chondrogenesis by the action of MMPs secreted by the hMSCs [6]. Additionally, a recombinant Streptococcal collagen-like 2 (Scl2) protein was effectively crosslinked by means of an MMP7-degradable peptide [5]. Moreover, CS, an adhesion binding peptide covalently attached to a hydrogel matrix, has been shown to enhance the chondrogenic differentiation of hMSCs [5].
In the present study, the synthesis and characterization of injectable HA based hydrogels for repair of cartilage lesions is reported. To our knowledge, this is the first time that MeHA and CS-MeHA based hydrogels were synthesized using an MMP7-degradable peptide as crosslinker and their end-properties were experimentally assessed [7].

EXPERIMENTAL
Synthesis of methacrylated hyaluronic acid (MeHA)
HA (MW 41-65 & 66-99 kDa, Lifecore) was modified with methacrylic anhydride (MA) in a mixture of water/dimethyl formamide (DMF) (1/1 v/v) [8]. More specifically, HA was dissolved in ultrapure water at 4°C. DMF was then added dropwise to the HA solution until a 1/1 v/v water/DMF ratio was reached. Subsequently, MA (at 2, 5 and 10 molar equivalents with respect to the moles of repeating unit of HA) was added while maintaining the pH between 8 and 9, via the addition of 0.5M NaOH, for 4h. The reaction was kept under continuous stirring for 24h at 4°C. The synthesized MeHA was recovered from the reaction solution via precipitation with ethanol, followed by centrifugation (at 4°C), subsequent washing cycles with water/ethanol mixtures, and dialysis against water (MWCO 7kDa) for 48h with frequent changes of water [9]. Finally, the purified MeHA was recovered by

lyophilization. Infrared spectroscopy (*Perkin Elmer, Frontier FTIR spectrometer equipped with UATR*) was employed to qualitatively assess the formation of MeHA, removal of DMF as well as to quantify the amount of residual MA. The DM of the synthesized MeHA was determined by ^{1}H NMR spectroscopy (*Varian 600 MHz spectrometer*) [10].

Functionalization of MeHA with a chondroitin sulfate (CS) binding peptide

The CS binding peptide (CGGGYKTNFRRYYRF) [5] was synthesized manually at Pepscan (*Lelystad, The Netherlands*) using an Fmoc solid phase peptide synthesis method. MeHA was functionalized with the CS binding peptide via a thiol-methacrylate chemical reaction [11]. In particular, MeHA (0.02 g) was dissolved in ultrapure water containing triethanolamine, TEA (0.2 M) under magnetic stirring at room temperature. A predermined quantity of the CS binding peptide (0.00087 g or 0.00046 mmol; 0.0043 g or 0.00226 mmol), stoichiometrically corresponding to 1 and 5% of the methacrylates in MeHA was also dissolved in ultrapure water containing TEA (0.2 M) and added to the macromer solution. The reaction mixture was kept under magnetic stirring at 37^{o}C for 24h. CS-MeHA was purified by dialysis against water (MWCO 7kDa) over a period of 24h with frequent changes of water [11] and was finally recovered by lyophilization. The conjugation of the CS binding peptide with MeHA was verified by ^{1}H NMR spectroscopy (*Varian 600 MHz spectrometer*) [12].

Formation of MeHA hydrogels using an MMP7-degradable peptide as a crosslinker

The MMP7-degradable peptide (CGGGPLELRAGGGC) [5] was synthesized manually at Pepscan (*Lelystad, The Netherlands*) using the Fmoc solid phase peptide synthesis method. For the formation of hydrogels, a specific quantity of MeHA (16 mg) was dissolved in 400 μL of ultrapure water containing TEA (e.g., 0.3M) at pH = 8.0 [13]. A predetermined quantity of the synthesized MMP7-degradable peptide, stoichiometrically corresponding to 20-60% of the methacrylates in MeHA was dissolved in 100 μL of ultrapure water containing TEA (e.g., 0.3M) at pH = 8. Subsequently, the two solutions were homogeneously mixed at 37^{o}C so the crosslinking of MeHA chains could be effected via a TEA catalyzed Michael type addition reaction [14]. The finally synthesized hydrogel had a 5 w/v% content. The crosslinking kinetics were monitored by measuring the storage (G') and loss (G'') moduli using a dynamic stress rheometer (*Rheometric Scientific, SR-5000*) at 37^{o}C under 1% strain and a frequency of 1 rad/s in a cone and plate geometry.

Formation of CS-MeHA based hydrogels using an MMP7-degradable peptide as a crosslinker

16 mg of CS-MeHA were dissolved in 400 μL of ultrapure water containing TEA (e.g., 0.3M) at pH = 8. A predetermined quantity of the synthesized MMP7-degradable peptide, stoichiometrically corresponding to 40% of the available methacrylates in CS-MeHA, was also dissolved in 100 μL of ultrapure water containing TEA (0.3M), at pH = 8. By homogeneously mixing the two solutions at 37^{o}C, a CS-MeHA hydrogel of 5 w/v% solids was formed via a TEA catalyzed Michael type addition reaction. The gelation kinetics were studied by measuring the storage (G') and loss (G'') moduli using a dynamic stress rheometer (*Rheometric Scientific, SR-5000*).

Characterization of (CS-)MeHA hydrogels

To assess the swelling properties of the synthesized hydrogels, MeHA and CS-MeHA hydrogel discs were formed in custom-made teflon molds (diameter: 10 mm, width: 2 mm) and weighed. After drying, the hydrogel discs were weighed (W_d), and subsequently incubated in PBS, at 37^{o}C. At specified time intervals, a hydrogel disc was removed from the swelling medium (i.e., PBS), wiped with filter paper and weighed (W_s). Thus, the weight increase of the swollen hydrogels was monitored as a function of incubation time, and the swelling kinetics were assessed [15]. The degree of hydrogel swelling was calculated by the following equation [16]:

$$\text{Degree of swelling (\%)} = [(W_s - W_d)/W_d] \times 100 \quad (1)$$

The average molecular weight between crosslinks (M_c) and the mesh size (ξ) of the swollen MeHA and CS-MeHA hydrogels were calculated according to Peppas et al. [17].

The kinetic degradation of MeHA hydrogels was studied by monitoring the weight loss of hydrogel discs formed in custom-made teflon molds (diameter: 10 mm, width: 2 mm) incubated in PBS, at 37°C for three days (W_0) [18,19]. The enzymatic degradation of the formed hydrogels was examined. Hydrogels were incubated in PBS (2 mL) with recombinant human MMP7 (30 ng/mL) at 37°C [5]. The degradation medium was refreshed during the experiments every 48h to ensure a continuous enzymatic activity [19]. At specified time intervals, hydrogels were removed from the MMP7 solution, wiped with filter paper and weighed (W_t). Thus, the weight loss of swollen hydrogels was monitored as a function of incubation time, and the degradation kinetics were assessed [19,20].

RESULTS AND DISCUSSION

Synthesis of methacrylated hyaluronic acid (MeHA)

The removal of DMF from the synthesized MeHA was verified by qualitatively monitoring by ATR spectroscopy the DMF concentration in the collected supernatants. In Table 1, the effects of HA MW and MA molar excess on the DM of MeHA as determined by ^{1}H NMR spectroscopy, are shown. As can be seen, as the molar excess of MA increases the DM of MeHA increases independently of the MW of HA. Table 1 also shows the residual MA (mol%) as determined from the measurement of the characteristic C-O-C peak absorbance at 1056 cm^{-1} of the collected supernatants, using a calibration curve constructed from known MA solutions in water/DMF (1/1 v/v). It is apparent that the residual MA results as determined by ^{1}H NMR and ATR spectroscopy are in very good agreement.

Table 1. *DM of MeHA as determined by ^{1}H NMR spectroscopy and measurement of residual MA (mol %) in the collected supernatants by ATR spectroscopy. Results are presented as mean values of three measurements with their respective ± SDs.*

HA MW (kDa)	MA ME	DM(%)	Residual MA (mol%)	
		^{1}H NMR	*^{1}H NMR*	*ATR*
41-65	2	21.9 ± 6.8	89.0±3.4	88.0±4.7
	5	38.6 ± 4.5	92.3±0.9	91.2±2.8
	10	47.6 ± 2.0	95.2±0.2	94.5±3.1
66-99	2	15.7 ± 3.9	92.1±1.9	92.1±3.3
	5	35.9 ± 6.5	92.8±1.3	92.9±2.6
	10	46.5 ± 5.5	95.3±0.6	97.7±1.9

Biofunctionalization of MeHA

The conjugation of the CS binding peptide with MeHA was confirmed by ^{1}H NMR spectroscopy. More specifically, the ^{1}H NMR spectra of CS-MeHA revealed a decrease of the peak values at 6.1 and 5.7 ppm of the CH_2 groups in methacrylates in comparison to the ^{1}H NMR spectra of MeHA. The measured decrease in the number of CH_2 groups (i.e., the number of crosslinking sites) was due to their conjugation with the CS binding peptide [12]. Thus, as the number of free crosslinking sites decreases (i.e., from 41.3 to 35.1%) the degree of biofunctionalization increases from 1 to 5%.

(CS)-MeHA hydrogels formed using an MMP7-degradable peptide as crosslinker

The effect of the MMP7-degradable peptide concentration (i.e., the peptide moles theoretically required for reaction with a specific percentage of available methacrylates in MeHA) on the storage modulus (G') and the degree of swelling of MeHA hydrogels is depicted in Figure 1. It is apparent that an increase in the amount of the crosslinker (i.e., increase in the theoretical reaction extent of methacrylates) results in an increase in G' up to a plateau value, meaning that beyond a specific value of MMP7-degradable peptide concentration, the degree of hydrogel crosslinking does not change and so does the degree of hydrogel swelling. The observed behavior can be attributed to the restricted accessability of the methacrylates residing on HA chains caused by limited chain mobility and steric hindrance effects. Accordingly, the moles of MMP7-degradable peptide were set equal to the theoretical reaction of 40% of methacrylates moles in MeHA that resulted in a gelation onset time of 469 sec. The effect of MMP7-degradable peptide concentration on the degree

of hydrogel swelling is also depicted in Figure 1. Note that an increase in the amount of the peptide crosslinker up to a specific value (corresponding to 40% of the theoretical reaction of methacrylates) leads to a decrease in the degree of hydrogel swelling up to a plateau value of 2850 %, see Figure 1.

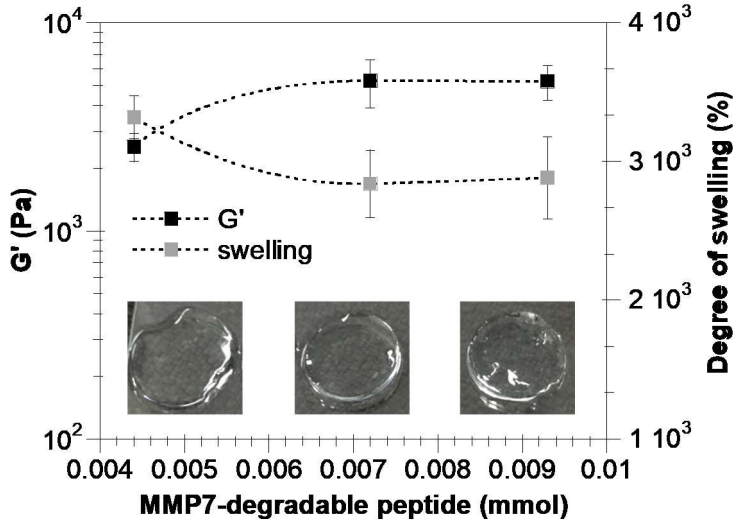

Figure 1. *Effect of MMP7-degradable peptide concentration (i.e., moles of peptide crosslinker required for the theoretical reaction of a specific percentage of methacrylates in MeHA) on G' and degree of hydrogel swelling. MW of HA: 66-99kDa, DM of MeHA: 50.7%, MeHA hydrogel solids content: 5w/v%. Results are presented as mean values of three experiments (squares) with their respective ± SDs.*

Figure 2 illustrates the effect of the crosslinker concentration on the average molecular weight between crosslinks, M_c, and mesh size, ξ. It is apparent that an increase in the amount of the crosslinker (i.e., increase in the theoretical reaction extent of methacrylates) results in a decrease in both M_c and ξ up to a plateau value, indicating that M_c and ξ do not change above a specific value (e.g., 0.007 mmol) of MMP7-degradable peptide concentration. Note that the calculated values of M_c and ξ are in qualitative agreement with the experimental measurements on G' and degree of swelling reported in Figure 1.

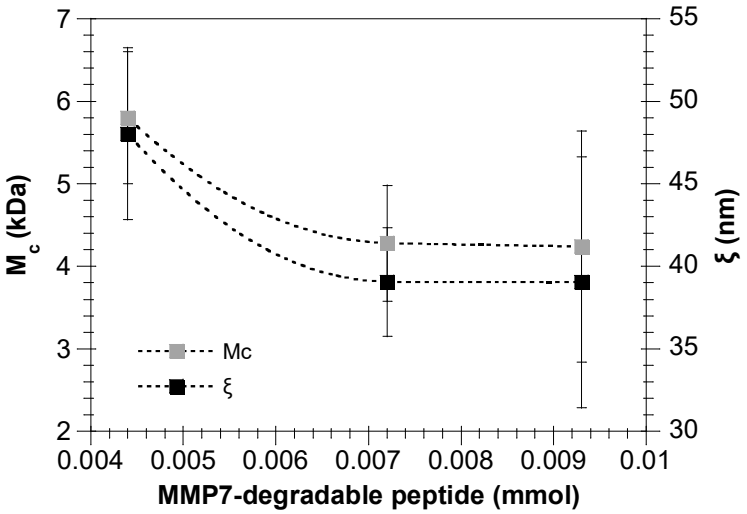

Figure 2. *Effect of MMP7-degradable peptide concentration (i.e., moles of peptide crosslinker required for the theoretical reaction of a specific percentage of methacrylates in MeHA), on Mc and ξ. MW of HA: 66-99kDa, DM of MeHA: 50.7%, MeHA hydrogel solids content: 5 w/v%. Results are presented as mean values of three experiments (squares) with their respective ± SDs.*

Figure 3 shows the degradation kinetics of the synthesized MeHA hydrogels in a PBS-MMP7 enzyme solution (30ng of MMP7 enzyme per mL PBS) at 37°C. The hydrogel degradation was attributed to the cleavage of the peptide crosslinker by the recombinant MMP7 enzyme [5]. In all cases, the

hydrogel degradation exhibited a zero-order kinetic model, that is, the variation of W_t/W_0 with respect to degradation time followed a straight line. As can be seen in Figure 3, the hydrogel degradation rate decreases (i.e., the hydrogel degradation time increases) as the degree of hydrogel crosslinking increases. Note that beyond a specific value of MMP7-degradable peptide concentration (i.e., a specific degree of crosslinking), the degradation rate reaches a plateau value.

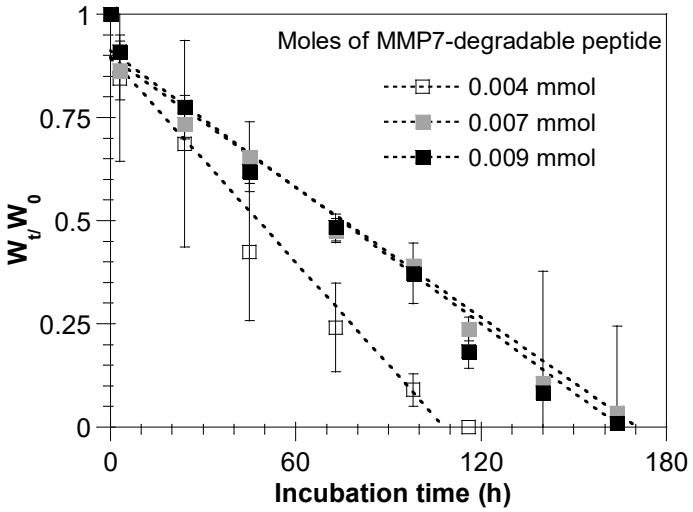

Figure 4. *Effect of MMP7-degradable peptide concentration (i.e., moles of peptide crosslinker required for the theoretical reaction of a specific percentage of methacrylates in MeHA), on the degradation kinetics. MW of MeHA: 66-99kDa, DM of MeHA: 50.7%, MeHA hydrogel solids content: 5 w/v%. Results are presented as mean values of three experiments (squares) with their respective ± SDs.*

Figure 4 shows the effect of MeHA biofunctionalization with the CS binding peptide on the gelation onset time, G', and degree of swelling of the synthesized hydrogels. As can be observed, the gelation onset time increases and the G` value decreases as the theoretical degree of biofunctionalization increases from 0 to 5%, due to the decreased number of available crosslinking sites as well as due to the restricted accessability of the methacrylates residing on HA chains caused by limited chain mobility and steric hindrance effects. As a result, the crosslinked CS-MeHA hydrogels have a lower crosslinking density and, thus, are less rigid. Note that these observations on the effect of the degree of biofunctionalization on the degree of crosslinking are in agreement with the hydrogel swelling measurements shown in Figure 4.

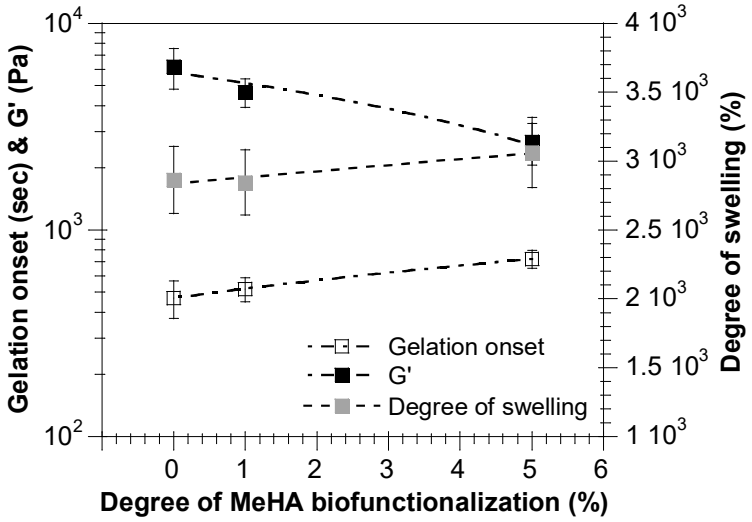

Figure 4. *Effect of degree of biofunctionalization (0 to 5%) on the gelation onset time, G', and degree of swelling. MW of HA: 66-99kDa, DM of MeHA: 50.9%, MeHA hydrogel solids content: 5 w/v%, moles of MMP7-degradable peptide: 0.0072 mmol. Results are presented as mean values of three experiments (squares) with their respective ± SDs.*

CONCLUSIONS

MeHA of various DM (e.g., 15.7-47.6%) was synthesized in the presence of H_2O/DMF (1/1 v/v) and was effectively biofunctionalized with a CS binding peptide. MeHA injectable hydrogels with tunable properties (i.e., G', degree of swelling, degradation kinetics) were successfully synthesized using an MMP7-degradable peptide as crosslinker. It was found that as the concentration of the crosslinker increased, the gelation onset time decreased as well as the degradation rate of the synthesized hydrogels while their G' increased. The measured gelation onset time (e.g., 469 sec) is considered to be appropriate for the in vivo administration of the injectable hydrogel formulations. The biofunctionalization of MeHA with a CS binding peptide resulted in an increase of the gelation onset time as well as in a decrease of the hydrogel crosslinking density (i.e., resulting in the formation of softer hydrogels). The results of the present study show that the synthesized MeHA hydrogels impregnated with hMSCs could be applied for the repair of cartilage lesions via topical injection.

ACKNOWLEDGMENTS

The present research has received funding from the EU Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 642687.

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