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Synthesis of highly substituted amide hyaluronan derivatives with tailored degree of substitution and their crosslinking via click chemistry

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ABSTRACT

Hyaluronan (HA) based hydrogels with well defined 3D-molecular architecture have been synthesized combining chemical modification of hyaluronic acid and 'click chemistry'. The high degree of substitution of HA was obtained after activation of the carboxyl group with ethyl chloroformate and subsequent functionalization of the carboxylic group with primary amine containing either a terminal azido or alkynyl groups. The degree of amide substitution could be controlled by reaction conditions. The chemical modification probed to be highly chemo-selective providing only amide modified HA derivates. The prepared derivates showed higher resistance towards thermal degradation than starting hyaluronan material. The crosslinking reaction of azido- and alkynyl-amide derivates of HA led to the formation of highly organized and porous networks, which due to their high stability against degradation are potential candidates for application as drug delivery systems, or scaffolds in tissue engineering.

1. Introduction

The development of new biomaterials for tissue engineering has accelerated with the increasing demand for tissue regeneration as a substitute for organ transplantation. The new biomaterials can be derived from natural or synthetic polymers. Many of the biopolymers derived from the extracellular matrix (ECM) have been modified and adapted for medical uses. One of the main components of ECM on which attention was focused in the last decade is hyaluronan (HA).

HA is a linear polysaccharide consisting of alternating β-1,4-linked units of β-1,3-linked glucoronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc). HA is a main component of the extracellular matrix in connective, epithelial, and neural tissues and is known to play an important role in organ development, cell proliferation and migration (Lapčik, De Smedt, Demeester, & Chabreček, 1998). Additionally, HA contributes to the lubrication and maintenance of cartilage, where it is a major component of synovial fluid and forms a coating around chondrocytes (Fraser, Laurent, & Laurent, 1997). It can be considered as an attractive building block for new biocompatible and biodegradable materials, having applications in drug delivery, tissue engineering and viscosupplementation (Ilgin et al., 2010; Oh et al., 2008; Zheng Shu, Liu, Palumbo, Luo, & Prestwich, 2004). In fact, biodegradable hydrogels are largely prepared from polysaccharides and in particular from HA (Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009).

Ideally, HA delivery is performed using minimal invasive techniques. However, unmodified HA solutions are rapidly degradated by the body and cleared from the implanted site (Sahoo, Chung, Khetan, & Burdick, 2008). To increase the stability of HA gels which could efficiently encapsulate cells, slowly degradable crosslinks need to be introduced (Chung, Beecham, Mauck, & Burdick, 2009).

Chemical modification of polysaccharides using carbodiimides is a common procedure (Bruneel & Schacht, 1993; Bulpitt & Aeschlimann, 1999; Kuo, Swann, & Prestwich, 1991). However, this reaction is known to be non selective and to produce insignificant or very low chemical modification of the polysaccharide. Activation of polysaccharides by 4-nitrophenyl chloroformate is also well documented (Vandoorne, Vercauteren, Permentier, & Schacht, 1985). Photo-crosslinking of vynilic derivatives of hyaluronan was reported to produce hydrogels with controlled degradation (Burdick, Chung, Jia, Randolph, & Langer, 2004). Methacrylated HA with tailored degree of substitution was synthesized and the authors concluded that the stability of the obtained hydrogels increased with the degree of substitution (Oudshoorn, Rissmann, Bouwstra, & Hennink, 2007). Hydrogels based on HA have been also prepared by crosslinking with trisodium trimetaphosphate. However, the material is reported to be heterogeneous and non porous (Dulong et al., 2004).

Another way of crosslinking is cycloaddition. For example, a very powerful tool for crosslinking of two components, is based

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on the 1,3 dipolar cycloaddition between an azide and an alkyne. This reaction is known as Huisgen reaction and was independently developed into 'click chemistry' by two research groups (Kolb, Finn, & Sharpless, 2001; Rostovtsev, Green, Fokin, & Sharpless, 2002; Tornøe, Christensen, & Meldal, 2002). This reaction was used recently to crosslink dextran (Pahimanolis, Vesterinen, Rich, & Seppala, 2010) and cellulose (Liebert, Hänsch, & Heinze, 2006). Modified polysacharides such as (1 → 3)-β-D-Glucans (Hasegawa et al., 2006) and chitosan (Gao, Zhang, Chen, Gu, & Li, 2009) also underwent click chemistry reactions. Hilborn and coworkers applied click chemistry to synthesize hydrogels based on polyvinyl alcohol. The authors reported that in order to retain the water solubility of the alkyne- and azide-functionalized polymer, only low degrees of substitution (1–5%) were possible to be used (Ossipov & Hilborn, 2006).

The click chemistry reaction proceeds fast in the presence of copper (I) under mild conditions and in water media (Chanda & Fokin, 2009). Since the Cu (I) reaction is very selective, it is highly compatible with almost all the functional groups present in biological macromolecules such as proteins, polysaccharides, and DNA/RNA (Rodionov, Presolski, Gardinier, Lim, & Finn, 2007). The amidation of HA with a terminal azide and alkynyl substituents was carried out using carbodiimide activation (Crescenzi, Cornelio, Di Meo, Nardecchia, & Lamanna, 2007). These amide modified HA-derivatives bearing alkyne and azide terminal groups were used for producing hydrogels via click chemistry. It was reported that the release rate of substances from the hydrogel could be tailored by varying the degree of crosslinking. However, this methodology allowed only lower degree of substitution, due to which high amounts of toxic copper catalyst are required for the crosslinking reaction.

The aim of this work was to provide a suitable substitution of reaction conditions for HA-amidation, yielding highly substituted products so that the subsequent crosslinking requires only a minimal amount of toxic catalyst to produce biocompatible and biodegradable hydrogels. The HA derivates will be analyzed by NMR and FT-IR spectroscopies. The obtained hydrogels will be structurally characterized by scanning electron microscopy (SEM).

2. Materials and methods

HA (M_w = 1.6 MDa) was provided by CPN, Dolni Dobrouč, Czech Republic. Propargyl amine (PA), ethyl chloroformate (ECF), 3-chloro-propylamine, *N*-dimethylsulfoxide (DMSO), sodium azide, potassium bromide, ascorbic acid sodium salt (AANa), copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), ethylendiaminetetraacetic acid (EDTA), and DOWEX 50WX8 resin, were purchased by Sigma–Aldrich and used as received. All the other chemicals were reagent grade and used without purification.

2.1. NMR spectroscopy

About 10 mg of derivatives were solubilized in 750 μl of D_2O. ^1H and ^{13}C experiments were carried out at room temperature on a BRUKER Avance™ III 500 MHz operating at a ^1H frequency of 500.25 MHz, and ^{13}C frequency of 125.8 MHz and equipped with a BBFO plus probe. The ^1H and ^{13}C chemical shift were referenced to 3-(trimethylsilyl)propionic acid sodium salt (TSPA) used as an internal standard. ^1H–^1H COSY spectra were recorded with 2k data points, 16 scans per increment and 128 increments. ^1H–^{13}C HSQC spectra were acquired using gradient pulse sequences and 2 kHz data points, 80 scans per increment, 256 increments, and heteronuclear scalar coupling C–H set at 145 Hz. DOSY (diffusion ordered spectra) were obtained using a stimulated echo pulse sequence with bipolar gradients (STEBPGP). Scans (32) were collected using 2.5 ms sine-shaped pulses (5 ms bipolar pulse pair) ranging from 0.674 to 32.030 G cm^{-1} in 24 increments with a diffusion time of 600 ms, and 8k time domain data points. Apodization was made by multiplying the data with a line broadening of 1.0 Hz, spike suppression factor of 4.0, maximum interactions number set to 100, and noise sensitivity factor of 2, and number of components set to 1.

2.2. FT-IR spectra

FT-IR spectra were recorded with a FT-IR-8400S Shimadzu spectrometer. Samples were studied as KBr pellets (1%) in anhydrous KBr. Spectra (32 scans) were recorded using 3600 cm^{-1} width (between 400 and 4000 cm^{-1}), and 2 cm^{-1} resolution. Samples were analyzed as acid form to avoid superposition of sodium carboxylate peak with hydrogen bonds.

2.3. Thermal analysis

Thermal properties of the modified azide and alkynyl-HA were studied by thermogravimetric analyses (TGA) conducted on a TGA Q5000IR from TA Instruments. The samples were put on a platinum pan and heated, from room temperature up to 300 °C under a dynamic nitrogen atmosphere with a flow rate of 25 ml/min and a heating rate of 10 °C/min in the thermal analyser. The degradation (% weight loss) of the samples was monitored and measured as a function of temperature. Data were assessed using Universal Analysis 2000 software.

2.4. Molecular weight determination

The molecular weight was measured using an HPLC (Model G1310A, Agilent), provided with an isocratic pump model G1310A. The signal was detected using DAWN EOS (Wyatt Technology Corporation) with a differential refractometer Optilab rEX (Wyatt Technology Corporation). The mobile phase consisted of 100 mM of sodium phosphate (pH = 7.5) containing 0.05% of sodium azide. 100 μl of polysaccharide (2 mg/ml) samples dissolved in mobile phase and previously filtered through a nylon membrane with a pore size of 0.2 μm and ratio of 25 mm (Pall Corporation) were injected into HPLC system while maintaining an isocratic flow of 0.5 ml/min. Chromatographic data were processed using ASTRA software, version 5.3.4.1.5 (Wyatt Technology Corporation). The specific dn/dc increment of 0.155 ml/g was used for the calculation of molecular weight.

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) images were obtained using a VEGA-scanning electron microscope operating at 15 kV to characterize the morphology, structure and size pore of the gels. HA hydrogels film samples in dried and hydrated state were prepared by freeze-drying. The sectioned hydrogels or thin films were mounted on SEM stubs and sputtered with gold using an automatic sputter coater.

2.6. Swelling of hydrogels

Hydrogel samples (1 ml) were accurately weighed (wd), 2 ml of phosphate-buffered saline solution (PBS, pH 7.4) was added and the samples were subsequently incubated at 37 °C. At regular time intervals (each 2 h) the buffer solution was removed from the samples and the weight of the hydrogels was determined till constant weight (ws) was reached. The ratio of ws/wd was used to calcu-

Table 1
Reaction conditions, degree of substitution (DS), polydispersity (P) and molecular weight (M_w) of HA, HA-propargyl-amide (HA-Pr) HA-azido propyl-amide derivates (HA-APA) as determined by ^{1}H NMR and SEC-MALLS. All the reactions were carried out using 1% solution of HA and 3 eq of triethylamine.

ECF[a] (eq)	DS (%)	M_w (kDa); P HA before reaction	M_w (kDa), P HA-amide after reaction
HA-Pr			
5	11	24; 1.8	20; 2.9
3	14	36; 1.9	26; 2.9
5	12	43; 1.4	35; 2.9
5	22	75; 1.8	74; 2.8
5	44	100; 1.9	80; 2.8
3	85	248; 1.7	120; 2.8
3	50	366; 1.6	84; 2.7
5	60	393; 1.6	240; 3.2
3	88	485; 1.7	134; 2.7
HA-APA			
3	42	36; 1.9	25; 2.4
5	54	43; 1.4	32; 3.5
5	60	70; 1.9	65; 2.9
5	54	366; 1.6	219; 2.5
5	85	393; 1.6	165; 2.8
3	98	485; 1.7	177; 3.1

[a] ECF = equivalents of ethylchloroformate in respect to HA dimer.

late the swelling ratio (Sw). The experiments were performed in triplicate.

2.7. Uniaxial compression test

Uniaxial compression was performed with an Instron 3343 Universal Testing Machine (Instron Ltd, High Wycombe, UK). The tested hydrogel species were of cylindrical shape having height of 6 mm and diameter of 10 mm. The stress–strain properties were measured using a load frame with 100 N load cell. The test was strain controlled and the loading rate was 0.5 mm/min. No volume changes or barrel distortions were detected. Modulus of elasticity of swollen hydrogel (G) was calculated from nominal stress (σ) (force divided by undeformed cross-sectional area) using the Flory equation $\sigma = G(\lambda - \lambda^{-2})$. The mechanical data were analyzed using Bluehill 2 Software. The measurements were performed in a triplicate.

2.8. Preparation of hyaluronan acid form

In brief, 5 g of hyaluronic acid was dissolved in 500 ml of distilled water. A DOWEX 50WX8 cation resin exchange (H type) was added to the mixture. After about 2 h when the ions were exchanged, the resin was removed by centrifugation at 5000 rpm for 5 min and the resulting solution was frozen at −80 °C and lyophilized. The molecular weight and polydispersity of the polymer after the cationic exchange were determined by SEC-MALLS (Table 1). The obtained HA acid form was further used for the production of the amide derivates of HA.

2.9. Synthesis of 3-azidopropanamine (APA)

In brief, a solution of 3-chloropropylamine hydrochloride (1.0 g) and sodium azide (2.5 g, 3 eq) in water (10 ml) was added to a catalytic amount of KI. The flask was attached to a water condenser and the reaction mixture was heated at 90 °C for 72 h. After cooling to room temperature, sodium hydroxide was added to reach basic pH. The free amine was extracted from the reaction mixture employing ether. The organic fraction was dried over sodium sulfate and concentrated under vacuum avoiding complete dryness. The ^{1}H NMR confirmed the structure and purity of the compound. ^{1}H NMR (500.25 MHz, $CDCl_3$); 1.41 (2H, bs, $-NH_2$), 1.76 (2H, q, J = 6.8), 2.81 (2H, t, J = 6.8), 3.38 (2H, t, J = 6.8). FT-IR (KBr, cm^{-1}): 3363, 2941 (υ $-CH_2$), 2101 (υ –N≡N), 1650, 1593, 1461, 1286.

2.10. General procedure for the synthesis of water soluble highly substituted hyaluronan amides

0.5 g of HA acid form (1.32 mmol) was dissolved in 50 ml of DMSO at 60 °C. After dissolution of the polymer, the solution was allowed to reach room temperature. After cooling down 0.922 ml of triethylamine (6.6 mmol) was added to the reaction solution and allowed to be stirred for 10 min. At this moment 0.4 ml of ethyl chloroformate was added (3 mmol). The mixture was stirred at room temperature for 1 h. After that the corresponding amine (1.32 mmol) was added. Stirring was continued at room temperature for 24 h. Afterwards, the solution was cooled down to 0 °C and diluted with 50 ml of water and dialyzed (M_w cut off = 12–14 kDa) against 0.1 M of sodium chloride for 24 h, and then dialyzed against water for five days. After dialysis, the samples were freeze-dried. The degree of substitution (DS) was determined by NMR as the integral ratio of the diastereotopic protons of methylene α in amide which was introduced relative to the three *N*-acetyl protons of HA (example is shown in Fig. 1). Two derivates were prepared in this way, a propargylamido- and azidopropyl amido substituted-HA. The detailed reaction conditions are described below. The amidation reaction is depicted in Scheme 1.

2.10.1. Propargylamido-substituted-HA (HA-Pr)

Propargyl amido-substituted hyaluronan derivates were synthesized with different degree of substitution (DS): from 42% to 98% depending on the ratio of TEA/ethyl chloroformate and the molecular weight of HA acid form (Table 1). The structure of the only reaction product was assigned and confirmed by NMR (Fig. 3). The FT-IR analysis confirmed the chemical reaction between the glucoronic units and the amine by the presence of two distinct bands at 1652 cm^{-1} attributed to (C=O) stretching band and (–NH) deformation band, respectively. A small band at 2146 cm^{-1} reveals the presence of a terminal alkynyl group.

2.10.2. 3-Azidopropyl amido substituted-HA (HA-APA)

3-Azidopropyl amido substituted-HA derivates were synthesized with different degrees of substitution (DS) from 11% to 88%. This dependence is a function of the ratio of TEA/ethylchloroformate and the molecular weight of HA acid form (Table 1). The structure of the product was fully established by NMR and the data are depicted in Fig. 1.

The FT-IR analysis confirmed the chemical reaction between the glucoronic units and the amine by the presence of two distinct bands at 1652 and 1550 cm^{-1} attributed to (C=O) stretching band and (–NH) deformation band, respectively. The presence of the azide group was as well confirmed by infrared spectroscopy by presence of a strong band at 2102 cm^{-1} characteristic of the azide group.

2.11. Synthesis of hydrogels

HA-Pr and HA-APA components with a molar ratio 1:1 were dissolved overnight (total volume 1.0 ml). The exact amount of reagents was determined on basis of their degree of substitution (DS) so that the final concentration of both reagents was kept at 2% (w/v) in case of DS = 100%. Then varying molar amounts of catalyst $CuSO_4$ and sodium ascorbate were added to the mixture in order to study kinetics of gelation (Scheme 2), in the ratio described in Table 2. Vortexing was used after the addition of each component to ensure a good homogenization. Then, the reaction solution was stirred for few seconds until the formation of a gel. The gelation time was determined by the vial tilting methodology (Domszy, Alamo,

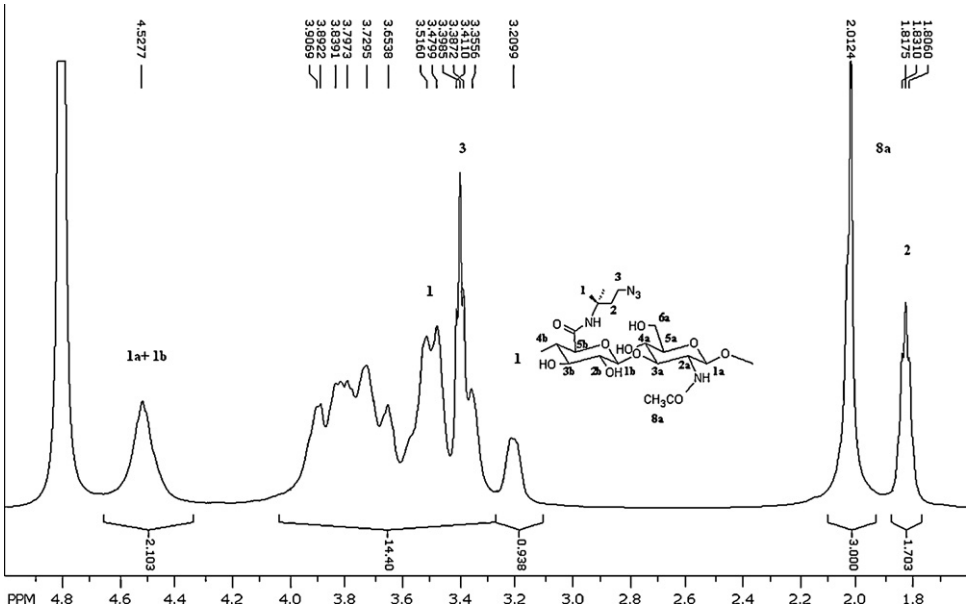
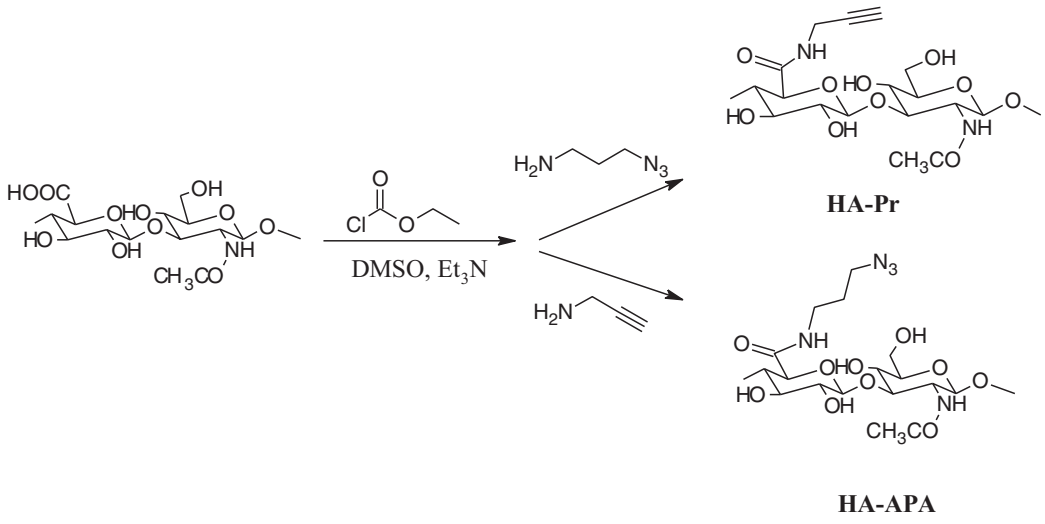
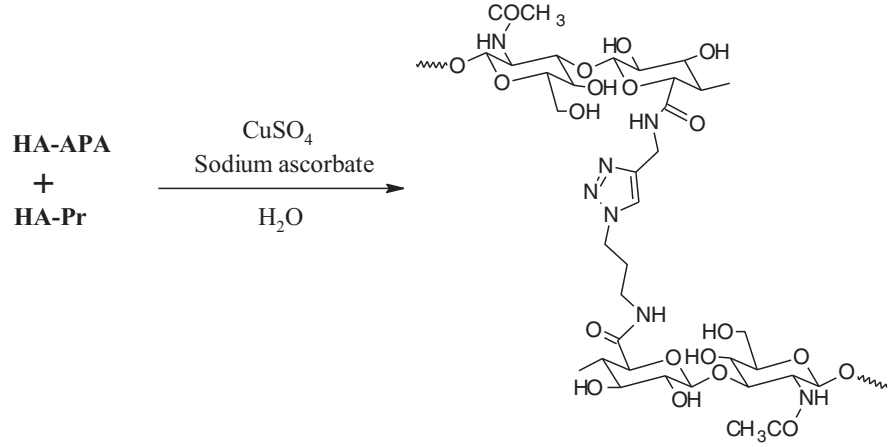


Fig. 1. ^{1}H NMR spectrum of HA-azide propyl amide (HA-APA).



Scheme 1. Modification of hyaluronic acid with primary amines.



Scheme 2. Crosslinking reaction between HA-propargyl amide (HA-Pr) and HA-azide propyl amide (HA-APA) leading to gel formation.

Table 2
Influence of crosslinking reaction conditions of highly substituted HA-APA (DS = 88%), and HA-Pr (DS = 85%) on the gelation time. Reaction was carried out in a total volume of 1 ml of PBS buffer at 37 °C. All gelation experiments were carried out in triplicate.

1a DS (%)	1b DS (%)	c(Cu^{2+})[a] (mM)	c(SA)[b] (mM)	Gelation time (s)
85	88	0.1	1.9	ng
		0.5	1.0	ng
		0.5	1.9	283 ± 32
		0.5	3.9	289 ± 31
		0.5	4.9	274 ± 33
		0.5	5.8	280 ± 37
		1.0	1.9	233 ± 30
		1.5	1.9	225 ± 25
		1.9	1.9	196 ± 30
		2.4	1.9	140 ± 27

[a] Copper sulfate.
[b] Sodium ascorbate.

Edwards, & Mandelkern, 1986). The prepared gel was dialyzed for 48 h against distilled water containing 0.001% (w/v) EDTA (M_w cut off = 12–14 kDa) in order to remove the catalyst. The remaining copper catalyst was determined by ICP-AES following the pharmacopea procedure (Ekolab Žamberk, Czech Republic). The salt-free hydrogels were freeze-dried, and the mass of the freeze dried network was determined. The difference between the total mass of all solid components and the mass of freeze-dried hydrogel was used for the determination of efficiency of crosslinking, which varied from 60% to 80% depending on the reaction conditions, which means that not all the modified hyaluronic acid used for the synthesis was integrated in the hydrogel network and might be washed out during the soaking procedure.

3. Results and discussion

3.1. Synthesis of amide derivates of hyaluronan

Ethyl chloroformate in the presence of TEA and DMSO can be used to activate carboxyl or hydroxyl functional groups in polysaccharides. Using this methodology, two amines bearing azido or alkynyl terminal groups were attached to HA. The activation could yield intermediates such as anhydrides or carbonates, which consequently react with primary amines to give carbamate or amide derivates. In this work the ethyl chloroformate activation of HA was found to be chemoselective and yielded only amide derivates (Scheme 1).

As indicated in Table 1, the degree of substitution of HA was studied using different equivalents of ethyl chloroformate and triethyalmine in respect to HA dimer. The data in Table 1 indicate increasing degree of substitution with increasing M_w of hyaluronan before modification. Thus HA samples of high molecular weight and lower chain flexibility allowed the modification reaction more effectively than HA samples with higher chain flexibility. In fact, the most effective substitution was achieved when hyaluronan of 485 kDa was used as starting material and 3 equivalents of ethyl chloroformate were used. Under these conditions, 88% substitution was obtained for HA-Pr and 98% for HA-APA derivates. Even with such a high degree of chemical modification both derivates were completely soluble in water. The complete structural assignment of the amide derivates, derived from COSY, TOCSY, HSQC and HMBC correlations, is resumed in Figs. 1–4.

For HA-APA derivates, Fig. 1 shows typical HA proton signals at 2.0 ppm belonging to –N–$COCH_3$ group, skeletal signals at 3.4–3.9 ppm and anomeric resonances at 4.4–4.6 ppm. Remaining signals detected at 3.5, 3.4, 3.2, and 1.8 belong to the azido-amine substituent and were attributed as follows. The triplets at 1.8 ppm

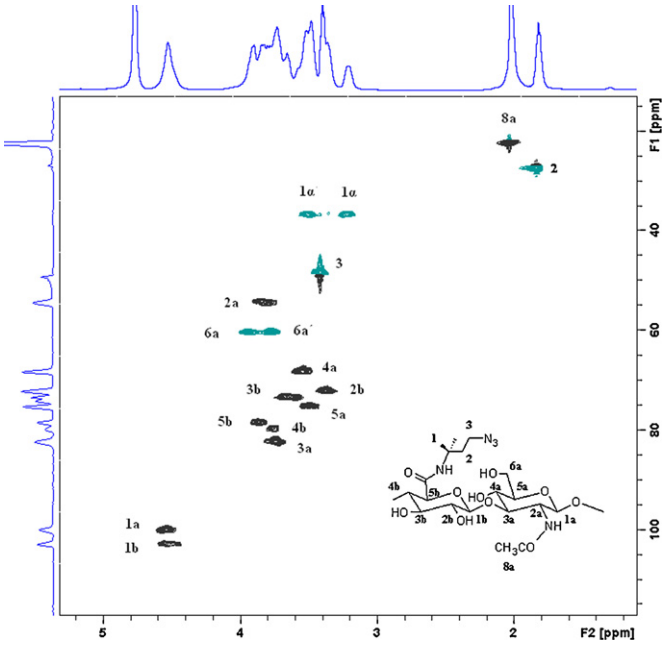


Fig. 2. ^1H–^{13}C HSQC spectrum of HA-azide propyl amide (HA-APA).

and 3.4 ppm were assigned to methylene groups in position 2 and 3, respectively. Both were clearly recognized in edited HSQC spectrum (Fig. 2). This spectrum further indicated the presence of a diastereotopic protons at 3.2 and 3.5 ppm belonging to the third methylene group in position 1. To confirm the proposed structure in Fig. 1, an HMBC spectrum indicated a correlation between a CO group of glucuronic acid at 170 ppm and the two diastereotopic protons of methylene group in position 1.

Similarly, ^1H NMR spectra of HA-propargyl-amide derivate (HA-Pr) indicated two doublets at 4.0 and 4.1 ppm belonging to the diastereotopic protons in position 1 (Fig. 3). Remaining alkyne signals from the substituent have been clearly distinguished in HSQC at 3.7 and 3.4 ppm (Fig. 4).

The linkage between primary amines and HA was further established by DOSY experiment (data not shown). Because of the marked difference between the diffusion coefficients, the DOSY map can easily establish the presence of non attached linkers to HA, which obviously is much faster than the diffusion of the bound amine in solution. In both cases, DOSY experiments showed similar diffusion behavior of all signals between 1.0 and 4.6 ppm and thus indicated that all of the proton signals in this region belonged to one structural complex. There was no free primary amine detected.

It is generally known that high molecular weight HA is susceptible to cleavage during ion exchange and chemical modification steps, therefore the average molecular weight of HA and its derivates were measured by SEC-MALLS during the amidation. While it was found out that ion exchange caused significant HA degradation (Table 1), the applied chemical modification decreased the HA average molecular weight and increased polydispersity of the starting hyaluronic acid (Table 1). As expected, HA with initial high molecular weight was degradaded faster. However, during the optimalization of reaction conditions it was noticed that higher Mw of starting materials yielded products with higher substitution (Table 1). The prepared derivates were degraded at 37 °C by hayluronidase within 6 h.

As compared to the literature data (Crescenzi et al., 2007) the main advantages of the presented methodology of amidation are: (i) relatively high M_w (200 kDa) of prepared derivates, (ii) high degree of substitution (more than 80%), (iii) no byproducts for-

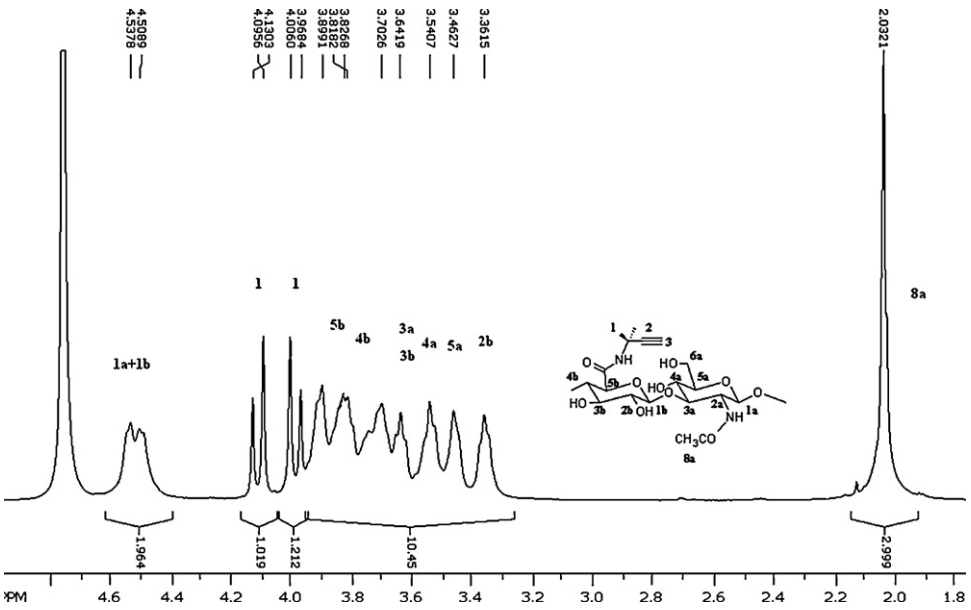


Fig. 3. ^{1}H NMR spectrum of HA-propargyl amide (HA-Pr).

mation, (iv) the reaction proceeds at room temperature and (v) requires short activation time (about 1 h). The only major drawback of this hyaluronan modification is that the reaction must be carried out in a a dry solvent.

3.2. Gel characteristics as a function of polymer components and reaction conditions

Hydrogel properties are expected to be highly dependent on polymer components structure, their concentration, reaction stoichiometry, catalyst concentration and viscosity of the starting solution. An important parameter in the synthesis of crosslinking materials is the way and intensity of stirring, which in an ideal case should allow the formation of homogeneous materials. Here, we are presenting important reaction conditions for the formation of hydrogels involving only the derivates (HA-Pr and HA-APA) with higher DS > 80%, because only these led to the formation of stable hydrogels.

The kinetics of gelation increased with the increasing concentration of all components in the reaction mixture. The values of the gelation times measured by the vial tilting methodology are summarized in Table 2.

The gelation time got shortened, when the click reaction was carried out at 37 °C as compared to room temperature or 0 °C (data not shown). This may be due to the fact that the thermal agitation of the macromolecular chains favors the contacts among side chains resulting in a faster gelation. Then we also noticed that gelation time gets drastically shortened in physiological solution.

Concentration of hyaluronan derivates and their molecular weights significantly affect the gelation time. For example, when a crosslinking reaction was carried out with hyaluronan derivates of lower molecular weight, it was important to keep the total volume of solution as small as possible so that gelation occurs (data not shown). The values of copper catalyst and sodium ascorbate concentrations were tried experimentally from 0 to 6 mmol in order to find the most effective ratio of these two components causing gelation and at the same time not causing oxidative stress to incorporated cells. According to the cytotoxicity of the reagents used

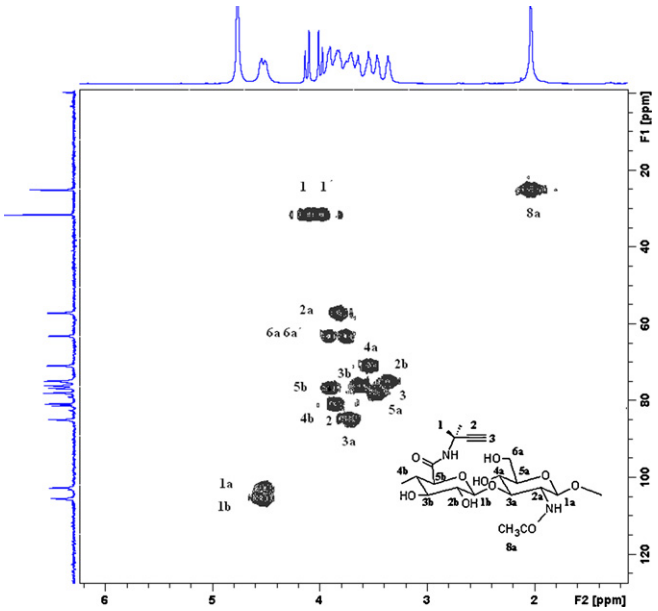


Fig. 4. ^{1}H–^{13}C HSQC spectrum of HA-propargyl amide (HA-Pr).

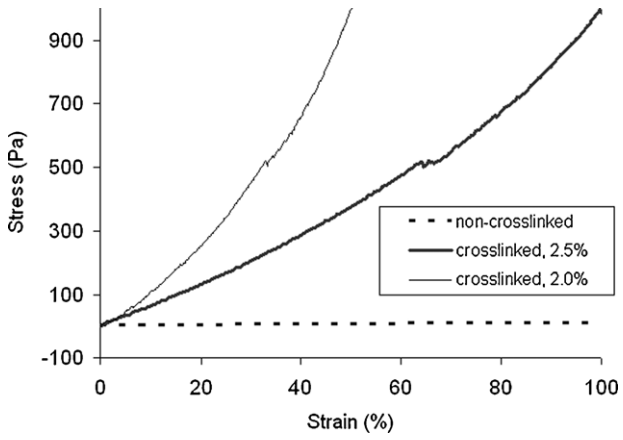


Fig. 5. The stress/strain behavior of the crosslinked and non-crosslinked hydrogel at a concentration of 2.0 and 2.5% w/v.

Table 3
Decomposition temperatures derived from TGA experiments for native HA, HA-APA (DS = 88%), and HA-Pr (DS = 85%), their physical mixture and crosslinked product.

Sample	T_{onset} (°C)	T_{peak} (°C)
HA	176	211
HA-APA	211	226
HA-Pr	213	224
HA-APA + HA-Pr, mixture	192	215
HA-APA + HA-Pr, crosslinked	204	225

during the crosslinking reaction which was first tested in chondrocytes, it was found that the best conditions are 0.5 mmol of Cu^{2+} and 5 mmol of sodium ascorbate (Table 2).

As it was expected, the catalyst and intiator affects the kinetics of the reaction and thus, the reaction is faster when the amount of copper catalyst and initiator (sodium ascorbate) is higher (Table 2). On the other hand, it was observed that at very low concentrations of sodium ascorbate the crosslinking reaction was not completely finished, the reaction mixture was viscous but the gel was not formed. Otherwise, there was no significant influence of sodium ascorbate on the gelation process (Table 2).

It is important to point out that in this work, we were able to obtain a stable hydrogel product when using ten to twenty fold lower concentration of copper sulfate (0.5–1.0 mM, Table 2) in comparison with a published work using 10–20 mM $CuSO_4$ for similar reaction (Testa et al., 2009). After hydrogel dialysis a maximum of 0.9 mg of copper per kg of dry hydrogel was detected. Our discovery is very important from the biological point of view and applications in which gelation *in situ* is involved, where both a fast gelation kinetics is required and low level of copper catalyst is demanded in order to avoid undesirable toxic effects.

3.3. Uniaxial compression test and swelling degree

Fig. 5 shows the stress/strain behavior of the crosslinked and non-crosslinked hydrogels at the concentration of 2 and 2.5% (w/v). The non-crosslinked hyaluronan hydrogel has an initial shear modulus of 20 Pa. As strain is increased, the non-crosslinked hydrogels shows a very low response. Up to the strain 100% no ruptures and flows were detected. Unlike the non-crosslinked hydrogel network, the crosslinked material shows linear stress response over all strains until it ruptures. At the concentration of 2.0% the network ruptured at strain of $35 \pm 8\%$ while at the concentration of 2.5% the network ruptured at strain of $56 \pm 8\%$. The shear modulus of the crosslinked network was found to be around 600 Pa. Future work thus will be directed at increasing the mechanical properties of the hydrogels. The swelling ratio expressed as ws/wd where ws is the weight of the swollen hydrogel and wd is the dry material, was found to be ranging from 500 to 100 and depending on polymer concentration. With increasing polymer concentration the swelling was higher but was independent on reaction conditions.

3.4. Thermal analysis

The thermal decomposition of the synthesized HA derivates and their freeze-dried hydrogel was compared with the native HA using thermogravimetric analysis (TGA). The results, including initial temperature at which thermal decompositions starts (T_{onset}) and the temperature at which maximum degradation is registered (T_{peak}), are summarized in Table 3.

The results (Table 3) clearly demonstrate that chemical modification of HA has led to higher thermal stability against decomposition. The thermal decomposition of HA-APA and HA-Pr was found similar. When the two substrates were physically mixed and underwent heating, the decomposition temperature of this mixture was decreased as compared to single components

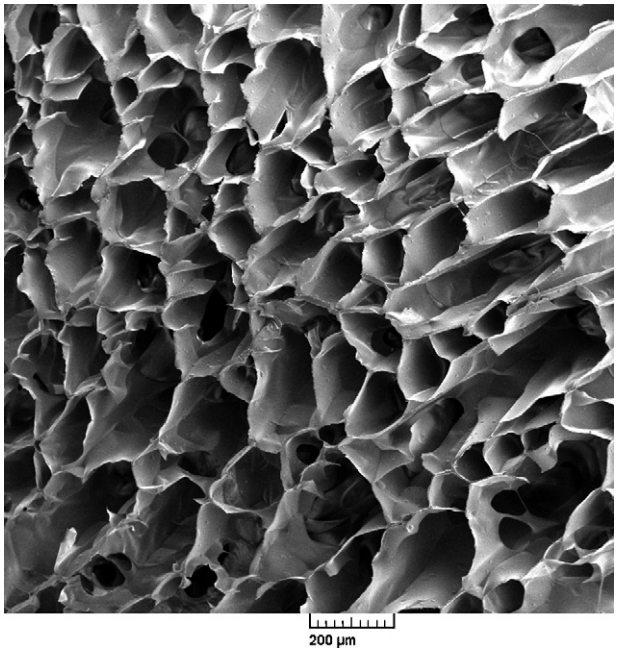


Fig. 6. SEM image of HA gels synthesized by crosslinking HA-Pr (DS = 85%) and HA-APA (DS = 88%). The reaction was performed at 37 °C using 1 mM of $CuSO_4$ and 1.9 mM sodium ascorbate in a total volume of 1 ml of PBS.

(Table 3). However, when crosslinking reaction was employed the resulting material showed comparable thermal stability against decomposition as single components, but still had higher degradation stability than native HA (Table 3). The difference in decomposition between physical mixture and cross linked material may be explained by the fact that water molecules are entrapped more efficiently in the cross linked material than in the mixture of components (Prusová, Smejkalová, Chytil, Velebný, & Kucerík, 2010).

3.5. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to obtain an insight into the 3D structure of the click chemistry obtained hydrogel. Surface images of the hydrogel as well as transversal and longitudinal cuts of the material in swollen state were obtained and are shown in Fig. 6. The SEM study of the interior morphology of the swollen hydrogel clearly showed a network structure. Micrometric drops which are present could be entrapped DMSO remained after the drying process. Other important parameters to be considered for the application of hydrogels in tissue-engineering are porosity and interconnectivity. The amide-hydrogels prepared in this work are highly porous, which means there is enough void space within the network to allow tissue growth, and diffusion of nutrients and waste products. The swollen hydrogels exhibit a smooth and highly interconnected honeycomb-like structure. The pores have irregular size but the walls are well interconnected. In general, larger pores were found for crosslinked derivates with lower DS.

Since efficient crosslinking allows the formation of highly organized networks, the formation of pores within the hydrogel in this work is not due to the expulsion of ice water crystals from the bulk to the surface (Fig. 6). As a result, water molecules entrapped in the bulk could even flow without perforation of the walls and this kind of materials resembles natural networks. In case of deficiency in the network density, which happens for example during short gelation time, the micrograph does not show a very organized comb (data not shown).

4. Conclusions

In this work, the functionalization of the hyaluronan carboxyl groups with two different amines via ethyl chloroformate activation led to the production of azido and alkynyl amide derivates having high degree of substitution. Except for amides, no other reaction products were observed, thus the reaction was chemoselective. The yield of the amide derivatives depends on the nucleophilicity of primary amines and is a function of the molecular weight of HA. Both amide derivates had a higher resistance towards thermal degradation than native hyaluronan. The degree of substitution was given by reaction conditions and could be controlled from 14% to 88%. The amide derivates were successfully applied as precursors for crosslinking reaction via click chemistry with subsequent formation of hydrogels, showing a very organized and porous structure allowing tissue growth and diffusion of nutrients and waste products.

In order to investigate the possibility of application of the obtained hydrogels in tissue engineering, it is planned to seed the amide hydrogels with fibroblast cells in the near future.

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