Carbohydrate Polymers 90 (2012) 1704–1711

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Reductive alkylation of hyaluronic acid for the synthesis of biocompatible hydrogels by click chemistry

Gloria Huerta-Angeles*, Miroslava Němcová, Eva Příkopová, Daniela Šmejkalová, Martin Pravda, Lukáš Kučera, Vladimír Velebný

Contipro Biotech, Dolni Dobrouč 401, 561 02 Dolni Dobrouč, Czech Republic

ARTICLE INFO

Article history:
Received 10 May 2012
Received in revised form 20 June 2012
Accepted 2 July 2012
Available online 28 July 2012

Keywords:
Hyaluronic acid
4-Ac-TEMPO-mediated oxidation
Reductive alkylation
Click chemistry

ABSTRACT

Hyaluronan (HA) based hydrogels have been synthesized combining chemical modification of the polysaccharide by partial oxidation, reductive amination and 'click chemistry'. HA was oxidized by 4-acetamido-TEMPO-mediated reaction, using sodium hypochlorite as primary oxidant and NaBr in buffered pH, so that the produced aldehyde moieties (hemiacetals) were trapped in situ by adding primary amines containing azide or alkyne-terminal groups. The structure of the reaction products, oxidized-HA and primary amines bonded to HA, was elucidated using 2D NMR spectroscopy. SEC-MALLS analysis of the modified substrates showed a negligible degradation of the polysaccharide using this procedure. Furthermore, azido- and alkynyl derivatives underwent cross-linking by click chemistry into hydrogels, which were characterized by NMR, FT-IR, swelling degree and mechanical properties. Possible application of the material as scaffold for tissue engineering was tested by seeding and proliferation of chondrocytes for up to 15 days.

1. Introduction

Hydrogels, due to their unique biocompatibility, flexible methods of synthesis, range of constituents, and desirable physical characteristics, have been material of choice for many biological applications (Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009). Polysaccharides are potential candidates as scaffolds for tissue engineering and carriers, since they are biodegradable and abundant in nature (Malafaya, Silva, & Reis, 2007). Among them, hyaluronic acid (HA) is a valuable starting material for these hydrogels because of its inherent hydrophilicity, biodegradability and compatibility (Mortisen, Peroglio, Alini, & Eglin, 2011). However, HA solutions present a short half-life, therefore, HA is preferentially stabilized by chemical modification in order to obtain hydrogels with longer residence time and mechanical robustness. HA based hydrogels have been obtained by using highly reactive cross-linking agents such as butanediol diglycidyl ether, divinylsulfone or glutaraldehyde (Collins & Birkinshaw, 2007). However, there have been some reports of allergic reactions to the products (Matarasso & Herwick, 2006). Instead of using highly reactive reagents, modification of the polysaccharide and cross-linking can be used (Schanté, Zuber, Herlin, & Vandamme, 2011; Yeom et al., 2010). Different chemical modification methodologies have been studied so far, among them oxidation reaction. For example, the oxidation of C(6) in HA mediated by 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) have been described previously by Crescenzi's group aiming at the synthesis of fully oxidized products (Crescenzi, Francescangeli, Renier, & Bellini, 2001). Furthermore, application of this chemistry has been extended to the selective oxidation of primary alcohols in various carbohydrates (de Nooy, Besemer, van Bekkum, van Dijk, & Smit, 1996; de Souza, Lucyszyn, Ferraz, & Sierakowski, 2011). However, in all the cases excess of carboxylic groups were obtained. Therefore, this way of oxidation can not be recommended for some applications, where high carboxylic content is undesirable.

Click chemistry is a very powerful tool for crosslinking of two components and is based on the 1, 3 dipolar cycloaddition between an azide and an alkyne. Click chemistry is a reaction of great importance because of its reliability, efficiency and stability and had shown great success in fabricating various functional materials (Malkoch et al., 2006; Mortisen et al., 2011; Ossipov & Hilborn, 2006; Rostovtsev, Green, Fokin, & Sharpless, 2002). The reaction can be also applied to chemically modified polysaccharides, such as cellulose (Liebert, Hänsch, & Heinze, 2006), chitosan (Gao, Zhang, Chen, Gu, & Li, 2009), dextran (Pahimanolis, Vesterinen, Rich, & Seppala, 2011) and HA (Crescenzi, Cornelio, Di Meo, Nardecchia, & Lamanna, 2007).

In recent work, Crescenzi et al. (2007) reported amidation of HA with a terminal azide and alkynyl substituents carried out using

* Corresponding author. Tel.: +420 465 519 573; fax: +420 465 543 793.
E-mail addresses: huerta-angeles@contipro.com, huertang77@yahoo.com (G. Huerta-Angeles).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.07.054

carbodiimide activation. Highly substituted amide derivates of HA were obtained by ethyl chloroformate activation under anhydrous conditions by our group (Huerta-Angeles et al., 2011). However, our previously reported methodology requires an initial conversion of HA to its acidic form, which makes it difficult for the reaction scale up, because it significantly degradates the polysaccharide. Interestingly, both of the amide modified HA-derivatives mentioned above were used to prepare scaffolds for tissue engineering applications. However, up to now there is lack of evidence regarding its biocompatibility. Neither the hydrogels have been seeded with cells capable of creating new extracellular matrix (ECM), such as chondrocytes. From the facts stated above, it is clear that, new precursors for click chemistry reaction should be provided and biocompatibility should be tested.

In the present work an alternative way for HA oxidation is presented. The tested reaction should provide a reliable and non-degradative oxidative route for preparation of HA-aldehyde derivatives, wherein the amount of carboxylic groups can be minimized. The synthesized aldehyde derivatives will undergo coupling with primary amines containing-terminal alkyne or azide groups to obtain functionalized polysaccharide suitable for cross-linking via click chemistry, which will be further used for hydrogel preparation. The biocompatibility of the hydrogel as well as its potential use for tissue engineering will be tested by growing chondrocytes on the prepared scaffold. The precursors and hydrogel characterization will be provided by NMR, FT-IR, SEM, and rheology.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt was provided by Contipro-Pharma, Dolní Dobrouč. Propargyl amine (PA), sodium azide, sodium chloride, ascorbic acid sodium salt (AANa), copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), picoline borane (pic-BH_3), 4-acetamido-2,2,6,6-Tetramethylpiperidine-1-oxyl (4-Ac-TEMPO), sodium hypochlorite (NaClO), ethidium homodimer-1 and calcein AM were purchased by Sigma–Aldrich and used as received. Modified Eagle Medium (DMEM), Glutamax and F12 media were provided by Gibco-Invitrogen. Antibiotics–antimycotic solution and the Quantipro BCA assay kit were purchased from Sigma (St. Louis, MO).

2.2. Measurements

2.2.1. Infrared spectroscopy

Fourier transform-infrared spectroscopy (FT-IR) spectra were recorded on a FT-IR-8400S Shimadzu spectrometer. Samples were studied as KBr pellets (1%) using 32 scans width (between 400 and 4000 cm^{-1}), and 2 cm^{-1} resolution.

2.2.2. Nuclear magnetic resonance

^1H and ^{13}C NMR spectra were carried out at 25 °C on a BRUKER Avance™ III 500 MHz operating at a ^1H frequency of 500.25 MHz, and ^{13}C frequency of 125.8 MHz. The ^1H and ^{13}C chemical shift were referenced to 3-(trimethylsilyl)-propionic acid sodium salt as internal standard.

2.2.3. SEC-MALLS

Size exclusion chromatography (SEC) coupled with a multi-angle light laser scattering (MALLS) detector, was used for determination of molecular weight of the samples after chemical modification. A refractive index increment (dn/dC) of 0.155 mL g^{-1} was used for calculation of molecular weight and polydispersity (M_w/M_n) of HA and derivatives according to the methodology described before (Podzimek, Hermannová, Bilerová, Bezaková, & Velebný, 2011). Data are enlisted in Table 1 and resume the weight-average molar masses (M_w) obtained by using intrinsic viscosities of HA and derivatives, which have been calculated in base of the entire chromatographic peak. Every sample was determined three times. Data acquisition and processing were performed using the Wyatt Technology Corporation ASTRA software, Version 5.3.1.4.

Table 1
Characteristics of N-alkylated HA products (propargyl HA (HA-CAPr) and azidopropyl-amine HA (HA-CAPA)) prepared from oxidized HA form. The characteristics include molecular weight of HA before (M_{w_1}), and after modification (M_{w_2}), polydispersity ($P = M_w/M_n$), degree of substitution of reductive alkylation ($DS_{N\text{-}Alk}$) determined by ^1H NMR and degree of substitution of carboxylic group (DS_{ox}) determined by reaction with methylene blue.

Entry	M_{w_1} (kDa)	$DS_{N\text{-}Al}$ (%)	DS_{ox} (%)	M_{w_2} (kDa); P	Yield (%)
HA-CAPr					
1	18	12	10	17; 1.25	82
2	90	12	10	84; 1.49	88
3	130	17	8	92; 1.38	92
4	202	12	10	139; 1.20	95
5	498	14	8	226; 1.75	95
6	800	15	10	334; 1.5	81
7	1600	19	5	683;1.4	82
8	1800	15	10	900;1.7	78
9	1940	14	10	673;1.47	90
HA-CAPA					
10	90	10	10	93;1.54	81
11	130	20	5	89; 1.37	85
12	202	10	10	128; 1.25	95
13	498	13	8	238;1.6	97
14	1800	12	10	728;1.7	83
15	2000	15	10	268;1.5	88

2.2.4. Scanning electron microscopy

Scanning electron microscopy images were obtained using a VEGA-scanning electron microscope operating at 15 kV to characterize the morphology, structure and size pore of the gels. Specimens were freeze-dried in liquid nitrogen in order to maintain the original structure. The sectioned hydrogels and thin films were mounted on SEM stubs and sputtered with gold using an automatic sputter coater.

2.3. Physical methods

2.3.1. Degree of swelling

A gravimetric method was employed to measure the swelling ratios of the hydrogels in distilled and in phosphate buffers of pH 1.7, 7.4 and 9.0 at constant ionic strength (0.06 mol L^{-1}). Hydrogels were prepared as described in Section 2.4.4, dried and reswelled by immersion at 25 °C. Swelling degree (Q) was calculated according to the equation $Q = (W_s - W_d/W_s)$, where W_s is the weight of the swollen gel at 25 °C and W_d is the weight of dried gel. The swollen gels were dried for 24 h at 60 °C and weighted again to obtain W_d. To gain insight into the swelling mechanism, curves of $(W_s - W_d)/(W_s)$ against time were plotted in a logarithmic scale, and a linear fitting, conducted in the initial phases of swelling ($W_s/W_d \leq 60\%$) were used. The obtained diffusional exponent values (n) and the coefficients of determination (R^2) were obtained by application of Ritger–Peppas model (Peppas & Sahlin, 1989; Ritger & Peppas, 1987).

2.3.2. Rheology

Rheological measurements were performed on a TA rheometer whose temperature had been previously set to 25 °C, with the following geometries: (1) cone and plate, 40 mm diameter. The optimized gels showed a broad linear viscoelastic region and withstood mechanical loadings of up to 10,000 Pa and $f = 1$ rad s^{-1}. Solutions of HA-CAPA and HA-CAPr (Table 1; entries 1 and 10) were

prepared at concentrations of 2 and 4% (w/v) and transferred to the rheometer, wherein a constant volume of freshly prepared solution of ascorbic acid (0.5 M, 10 μL), was mixed with 10 μL of copper sulfate at concentrations 0.01, 0.05, 0.1, 0.2 and 0.5 M. Catalyst, sodium ascorbate and polymeric mixtures were incorporated by fast mixing into the tested solution just before the measurement started to follow the kinetics of gelation depicted in Scheme 2. The time dependence of the storage modulus (G') and the loss modulus (G'') was recorded.

2.3.3. Mechanical testing by uniaxial compression

Compression experiments were measured on an Instron Universal Testing Machine (Model 3343) using a 0.01 kN load cell. In a typical experiment, stainless steel punch was brought in contact with the hydrogel at a constant crosshead speed of 2 mm min^{-1}. Loading and displacement data were collected during the experiment. Stress relaxation experiments were performed by keeping the strain constant over a period of time, typically 1000 s. After the maximum compressive load was reached, the change in the compressive load was recorded over time under constant strain.

2.4. Synthetic part

2.4.1. Preparation of oxidized hyaluronic acid

Regioselective oxidation of HA was carried out by a modification previously patented by our group (Buffa et al., 2011). Sodium hyaluronate (10.0 g, 25 mmol, and number of moles of —OH) of different molecular weights (1.7 MDa, 498 kDa, 202 kDa) was dissolved in water (500 mL). Sodium bromide (2.57 g, 2.5 mmol) and sodium phosphate (38.8 g) were added to the reaction mixture. The pH was adjusted to 9.0 with 0.1 M of sodium hydroxide. After the solution was cooled to 5 °C, it was bubbled with nitrogen and added with 4-acetamido-TEMPO (53.3 mg, 0.25 mmol) dissolved in 1 mL of water. Then, sodium hypochlorite was added to the reaction mixture in order to have 25% of molar equivalents in respect to HA dimers. The oxidation was carried out for 15 min and stopped by addition of ethanol. Then, the solution was diluted with water (1000 mL) and ultrafiltered. The product was recovered by precipitation with isopropanol. Wet samples were decantated and dried in an oven at 60 °C for 24 h. The degree of substitution of the aldehyde was calculated by integration of the signal at 5.2 ppm (geminal diol) with respect to the N-$COCH_3$ group at 2.0 ppm (DS = 10–15%). The percarboxylated groups obtained during oxidation (10%) were determined by the interaction of the oxidized-HA with methylene blue, following a reported literature procedure (Wei, Min, Xuejun, Ping, & Songqin, 2008). Yield of the reaction was 98.5%. FT-IR (KBr, cm^{-1}): 3419 (υ, C—OH), 2923, 1656, 1614 (—C═O), 1413 (—O—C═O), 1153, 1080 υ (C—OH),1039, 607. NMR ^{1}H (500 MHz, NaOD, δ ppm): 3.4–4.0 (m, 10H, sceletal), 4.5 (d, 2H, 1a and1b), 5.23 (s, 1H, —*H*—C═O—). NMR HSQC: 5.23 (s, 1H, *H*—C—OH_2), 4.65, 4.3 cross-peaks 90, 98 and 96.

2.4.2. Synthesis of HA-CAPr

Sodium hyaluronate (10.0 g, 25 mmol) of different molecular weights (Table 1) was oxidized as described in Section 2.4.1. After 15 min of oxidation; pH of the reaction was adjusted to 5.5 by addition of acetic acid and the reaction solution was mixed with propargyl amine hydrochloride (0.362 g, 3.98 mmol). The reaction was stirred for 5 h to allow imine formation. Picoline borane (0.424 g, 3.98 mmol) was added and reaction was stirred overnight at room temperature (25 °C). The reaction mixture was diluted with water and purified by ultrafiltration. The product was isolated after precipitation and drying. Molecular weight of the reaction products, measured by SEC-MALLS are resumed in Table 1, as well as, the reaction yields. The FT-IR and ^{1}H NMR are described as follows: (KBr, cm^{-1}): 3379 (υ, —O—H), 2894, 2131 (υ, C≡C), 1614, 1407, 1078, 613. NMR ^{1}H (500 MHz, NaOD, δ ppm): 2.0 (s, CH_3—CO—NH), 2.85 (m, CH_2), 3.1 (m, CH_2), 3.4–4.0 (m, 10H), 4.5 (d, 2H).

2.4.3. Synthesis of HA-CAPA

The product was synthesized as described in Section 2.4.2 only changing the linker to 3-azido-propylamine. The synthesis of this linker was described in our previous work (Huerta-Angeles et al., 2011). The structure of the synthesized compound was confirmed by NMR analysis. SEC-MALLS molecular weight values obtained after reaction are resumed in Table 1. Full structural assignation is depicted in Fig. 1 (HSQC). FT-IR (KBr, cm^{-1}): 3363, 2941 (υ-CH_2), 2100 (υ-N≡N), 1650, 1593, 1461, 1286. NMR ^{1}H (500 MHz, NaOD, δ ppm): 1.72 (m, —$CH_2CH_2CH_2$—N_3), 2.0 (s, CH_3—CO—NH), 2.68 (—$CH_2CH_2CH_2$—N_3), 2.85 (m, CH_2), 3.1 (m, CH_2), 3.4–4.0 (m, 10H), 4.5 (d, 2H).

2.4.4. Synthesis of hydrogels by click chemistry

Derivatives HA-CAPA and HA-CAPr were dissolved overnight in total volume of 1.0 mL of PBS at constant ionic strength (0.1 mol L^{-1}). The exact amount of components was determined based on their degree of substitution (DS) so that the final concentration of both reagents was kept at 2% (w/v) in case of DS = 100%. Thus, a solution of copper sulfate in water was added to the polymer mixture to produce a total concentration of 0.1 mmol L^{-1} of solution. Consequently, a freshly prepared solution of sodium ascorbate was added in a molar concentration of 0.5 mmol L^{-1} of solution. The prepared gel was dialyzed for 48 h against distilled water containing 0.01% (w/v) EDTA in order to remove the catalyst.

2.5. 2D-chondrocytes culture on hydrogels

Human chondrocytes were isolated from full thickness human hip cartilage specimens that were obtained from the surgical procedure after joint replacement in five probands. The protocol was approved by a local ethical committee. Chondrocytes were grown in DMEM/F12 media supplemented with 10% FBS, 1% ITS supplement and 1% antibiotics–antimycotics onto 75 cm^2 tissue culture flasks in incubator with humidified and hypoxic atmosphere (37 °C, 5% CO_2, 5% O_2). After a confluence cell layer was formed the cells were cultured and removed by trypsin (Trypsin/EDTA solution, Gibco). Scaffolds for 2D-cell culture were prepared by dissolving sterilized derivatives in PBS (pH 7.4) at 2% (w/v) concentration. Material was crosslinked using low concentrations of copper sulfate (3 μL, 10 mM) and sodium ascorbate (400 μL, 2.5 mM). The concentration of catalyst and reducing agent were previously optimized by using chondrocytes before their use in the biological test. Gelation took place in a thermobox at 37 °C for 2 h. The hydrogels were soaked using a solution of EDTA (10 mL, 1 mM), washed 3 times using PBS buffer, and 4 times using water. The material was frozen at −80 °C and lyophilized under sterile conditions. The scaffold was transferred into separate wells (24-well test plate) and seeded with 200 μL of cell suspension (10^6 cells/mL). Cell suspension was absorbed into the dry scaffold for 1 h at 37 °C, and then the whole plate was centrifuged (1200 rpm for 10 min). Then, 1 mL of the cultivation medium was added to each scaffold and placed in a thermobox at 37 °C, 5% CO_2 and humidified atmosphere. Next day each scaffold was transferred to a new well with 1 mL of fresh medium. The medium was replaced every 2–3 days. As cells were pre-cultured for 5 days and seeded on the 6th day, cell viability onto scaffolds under study was characterized on the 6th, 13th and 20th days of culture, by incubating cells with a Live/Dead (Invitrogen) assay (calcein AM/ethidium homodimer-1 in DPBS) for 20 min.

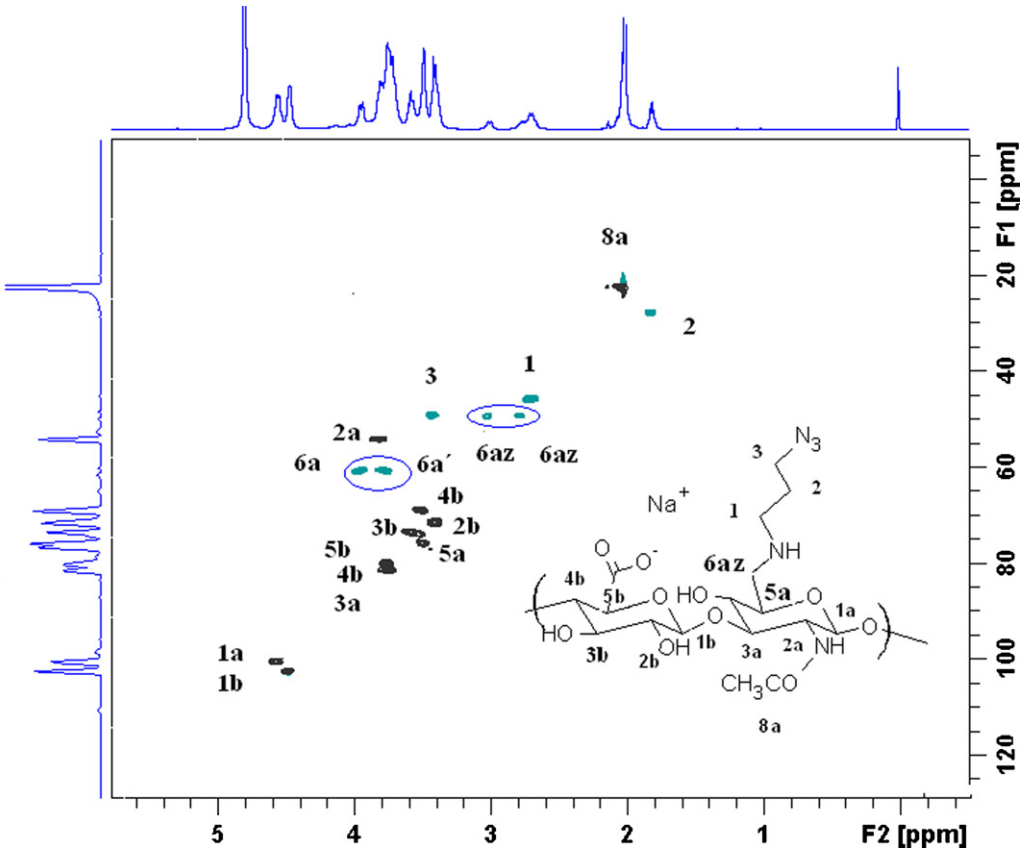


Fig. 1. HSQC spectrum of HA-CAPA.

2.6. Statistical analysis

Statistical differences were determined by Wilcoxon paired test (STATISTICA, version 9.1., StatSoft Inc., USA). Paired *t*-test, statistical significance: $p \leq 0.05$, $n = 5$, ±SEM.

3. Results and discussion

3.1. Preparation of oxidized hyaluronic acid

To oxidize HA and in this way introduce linkers into the polysaccharide backbone 4-Acetamide-TEMPO was used as oxidation catalyst. The oxidation primarily occurred in position C (6) of N-acetyl-D-glucosamine (Scheme 1) and could have resulted either in the formation of hemiacetal and/or carboxylic acid. The TEMPO-mediated oxidation of alcohols in polysaccharides is a very complex reaction (Jiang, Drouet, Milas, & Rinaudo, 2000). Formation of carboxylic moieties should be suppressed because it can negatively affect the cationic/anionic balance of the functional groups. Some authors had mentioned in previous works that the oxidation of primary alcohols to carboxylic moieties depends on the equivalents of oxidant added to the reaction mixture (Crescenzi et al., 2001). Bo Jiang et al., and Crescenzi's group have used a molar amount of sodium hypochlorite corresponding to 50–280% of the moles of HA dimers present in the reaction mixture (Crescenzi et al., 2001; Jiang et al., 2000) and they reached very high degree of substitution of COOH groups (DS_{OX} around 95%). In contrast, the main goal in this work was to trap the hemiacetals with primary amines avoiding peroxidation, therefore, the molar equivalents of sodium hypochlorite were kept at lower concentration than $c \leq 25\%$ (in ratio to HA dimers). The maximum amount of carboxylic groups produced during the reaction was determined spectrophotometrically using methylene blue (Wei et al., 2008) and was found to range from 5 to 10% (Table 1). Furthermore, we have noticed that under our experimental conditions using 25% of molar equivalents of sodium hypochlorite had repeatedly produced an almost constant amount of hemiacetals (aldehydes) from 10 to 20% (to HA dimers).

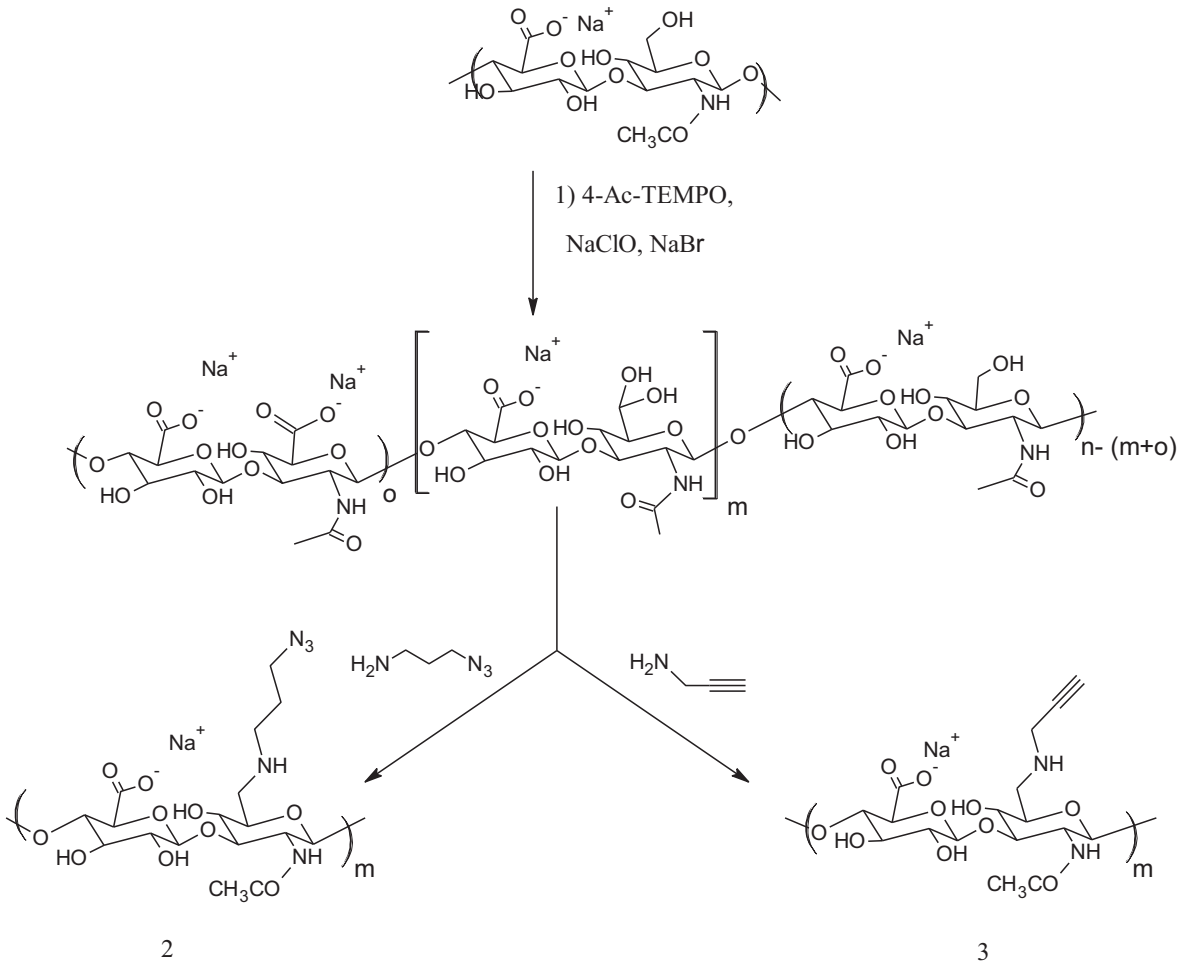
Once, the suitable amount of oxidant was determined, the influence of pH on HA degradation was estimated in the range of pH from pH = 7 to pH = 9. The fastest degradation occurred at pH 7, where the starting M_w = 1.6 MDa was decreased to 223 kDa, while the slowest degradation was detected at pH 9, where the final M_w of oxidized HA was estimated to be 563 kDa. For this reason, HA oxidation (reactions in Table 1) was preferentially performed at phosphate buffer adjusted to pH 9. ^{1}H NMR analyses indicated that the optimum reaction time under these conditions was only 15 min, because after this reaction time, no changes in DS_{OX} were noted.

The effect of the temperature was studied as well, and the reaction was effected from −5 to 5 °C. However, it is preferred to use 5 °C, due to dramatic increase of HA solution viscosity at lower temperatures.

Chemical structure of oxidized hyaluronic acid was fully confirmed by NMR and FTIR analyses. Details are given in Section 2.4.1.

3.2. Synthesis of secondary amine derivatives of hyaluronic acid

The selectively oxidized HA from Section 3.1 was grafted with primary amines carrying alkyne groups via shift imine intermediates mediation. Formation of a stable secondary amine linkage between the amine and HA was enabled by addition of a reductive agent (picoline borane) in the reaction mixture. Degree of substitution after reductive alkylation ($D_{SN\text{-}al}$) and yields of the final products (alkynyl-HA (HA-CAPr) and azidyl-HA (HA-CAPA)) as a function of molecular weights of the native HA are reported in



Scheme 1. Oxidation of HA mediated by 4-Ac-TEMPO in the presence of sodium hypochlorite and sodium bromide at pH = 9, followed by the addition of primary amines and reduction by picoline borane at pH = 5 yielding click chemistry substrates **2** (HA-CAPA) and **3** (HA-CAPr).

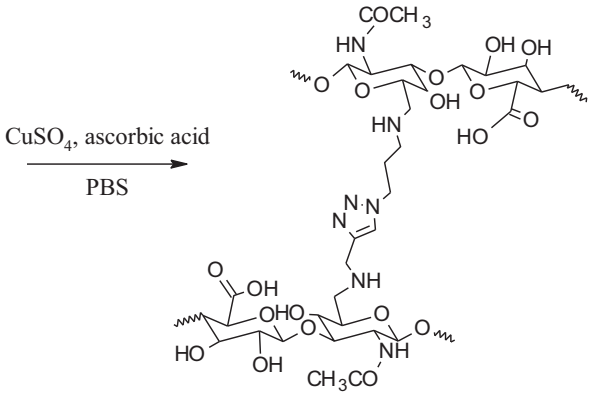
Table 1. The data indicate that the reaction effectively modifies both low and high molecular weight HA (Table 1). As it is expected, the M_w of final products is smaller as compared to native HA due to chain scission. However, N-alkylated derivatives with M_w as high as 700–900 kDa are possible to be prepared. We have also tested this reaction performed in one pot, where the oxidation product is not isolated but is directly used for subsequent reductive alkylation. The DS and M_w of final derivatives were comparable to those shown in Table 1.

The structural characterization of HA-CAPA derivative is fully assigned in the edited HSQC spectrum illustrated in Fig. 1. The spectrum depicts the typical HA proton signals at 2.02 ppm corresponding to —N—$COCH_3$ group, skeletal signals at 3.4–3.9 ppm and anomeric resonance at 4.4–4.6 ppm. Remaining signals detected at 2.7 and 1.8 ppm belong to the azido-ligand. Signal at 2.7 ppm was assigned to methylene located in position 1 (Fig. 1), which is shifted to low frequency due to the presence of the heteroatom, whereas the signal corresponding to the methylene 3 close to the azide moiety is deshielded and located to 3.5 ppm. Protons in position 2 (Fig. 1) present a typical shift of methylene groups at 1.8 ppm. Interestingly, the signals corresponding to the diastereotopic methylene protons in position 6 originally positioned at 3.8 and 3.9 ppm (moved upfield to 2.8 and 3.1 ppm (6az and 6az′, Fig. 1)). This upfield shift confirms the successful formation of a covalent linkage between the primary amine and HA in position 6. Additionally, the signal with chemical shift at 5.2 ppm, corresponding to the geminal diol into HA was not presented. Thus, a complete reduction of the aldehyde (Scheme 1) has been achieved. The structural characterization of HA-CAPr was achieved in a similar way as for HA-CAPA. Detected NMR and FTIR signals are given in Section 2.4.2.

As compared to literature data (Crescenzi et al., 2007; Huerta-Angeles et al., 2011; Testa et al., 2009), the main advantages of the presented methodology for preparation of components for their use in click chemistry are: (i) chemical modification undergoes only the primary hydroxyl group in HA, while the carboxylic group which is known to be the recognition site of CD44 and hyaluronidase remains intact (Banerji et al., 2007). This was confirmed by a complete degradation of hydrogel during 1 h incubation with hyaluronidase at 37 °C in pH 5.5. (ii) The possibility of preparation high molecular weight derivatives (900 kDa) of HA, (iii) the oxidation uses non-expensive reagents. (iii) The reaction is carried out in buffered aqueous solutions.

3.3. Structural characterization of the hydrogel

Hydrogels were prepared by click reaction between HA-CAPA and HA-CAPr at different concentrations (from 0.5 to 10%) in PBS or water (Scheme 2). A concentration of 2% (w/v) was found to produce homogeneous hydrogels. To enable a good characterization of the hydrogel and determine the efficiency of the crosslinking reaction, the produced material was degraded by hyaluronidase and the soluble part was characterized by NMR spectroscopy (data not shown). ^{1}H NMR spectrum showed a peak at 8.47 ppm, attributed to the 1*H*-1,2,3 triazole ring proton obtained after crosslinking, so



Scheme 2. Schematic representation of crosslinking of substrates **2** (HA-CAPA) and **3** (HA-CAPr), leading to gel formation.

that a semi-quantitative estimation of crosslinking degree (11%) was calculated by integrating the resonance of N-acetyl group of HA at 2.0 ppm and the peak at 8.47 ppm. Additionally, FT-IR spectrum indicated that the azide characteristic band at 2109 cm^{-1} had completely disappeared after crosslinking and thus confirmed the cross linking.

The morphology of a freeze-dried and swollen hydrogel is shown in Fig. 2. The hydrogel was frozen in liquid nitrogen to preserve the structure after 2 h of cross-linking. The non-crosslinked components were washed out by swelling of the hydrogel in water for 24 h. The hydrogel presents a defined porous and interconnect network, however, the structure is very irregular. In general, the average pore size of the scaffolds was found to be 200 μm, which fells into the required value for an effective application in tissue engineering, where a well interconnected structure may effectively allow cell migration and proliferation, structural matrix production, as well as a correct supply of nutrients for cell grown and effective transport of metabolic waste products (Gross & Rodríguez-Lorenzo, 2004).

3.4. Gelation behavior and mechanical properties

To monitor the gelation time and the storage modulus of the formed hydrogels, a time-sweep measurement for viscoelastic properties of each system was carried out at 25 °C, and the dependence of the storage modulus (G') and loss modulus (G'') were studied (Fig. 3). All samples exhibited a similar rheological behavior. The crosslinking components at the beginning behaved as viscous liquid, wherein G' was smaller than G'', after which G' increased more rapidly than G'' with prolonging time and the system behaved as gel state. This crossover ($G' = G''$) is a well-known phenomenon in cross-linking reaction, and it could be described as gel point. The gel point time was mainly affected by total concentration of copper sulfate in the reaction mixture (Fig. 3). While copper sulfate concentration of 0.0025% (w/v, Fig. 3A) produced a gelation point at 6 min, an increased concentration of $CuSO_4$ (0.025%, w/v) decreased the gel point time to 45 s. As expected, the concentration of copper sulfate influences only the kinetics of gelation but not the elastic moduli of the final product. As it can be noted, very low concentrations of copper sulfate (from 0.002 to 0.12% (w/v)) were required for gelation in this study. The applied concentrations of catalyst are much lower than the amount of $CuSO_4$ used in previously published works (Crescenzi et al., 2007; Testa et al., 2009). The low concentration of copper sulfate in the reaction mixture is preferred due to a possible toxicity of copper to chondrocytes. Increasing the concentration of HA-CAPA and HA-CAPr from 2.0 to 4.0% (w/v) and using 0.0025% $CuSO_4$ (w/v) had decreased gelation point from 250 to 362 s. The concentration of sodium ascorbate was not determining the kinetics of gelation, which is fully in agreement with other works (Testa et al., 2009). High molecular weight derivatives of HA have produced highly viscous solutions, for which it was difficult to follow the kinetics of gelation by rheology.

On the other hand, the mechanical properties of hydrogels have been determined by compression test. The study was performed using precursors 7 and 10 from Table 1, wherein one possess high and the second one low molecular weight. The compressive modulus was found to be 3350 and 9900 Pa for 2% and 4% (w/v) solution, respectively. Typical compressive modulus–strain curves of hydrogels are reported in Fig. 4A. Stiffness of the gels, or the initial slope of the stress–strain curve, had increased with increasing components concentration. Compressive strain to failure had increased from 7 to 15 N with increasing concentration, whereas the strain at failure was not significantly different for both concentrations (circa 50%). Thus, hydrogels prepared using the secondary amine derivatives and click chemistry were found to be stiff but brittle. Stiffness of the hydrogels could be explained by the fact that the highly hydrophilic polymeric chains after crosslinking were highly stretched and very susceptible to fracture.

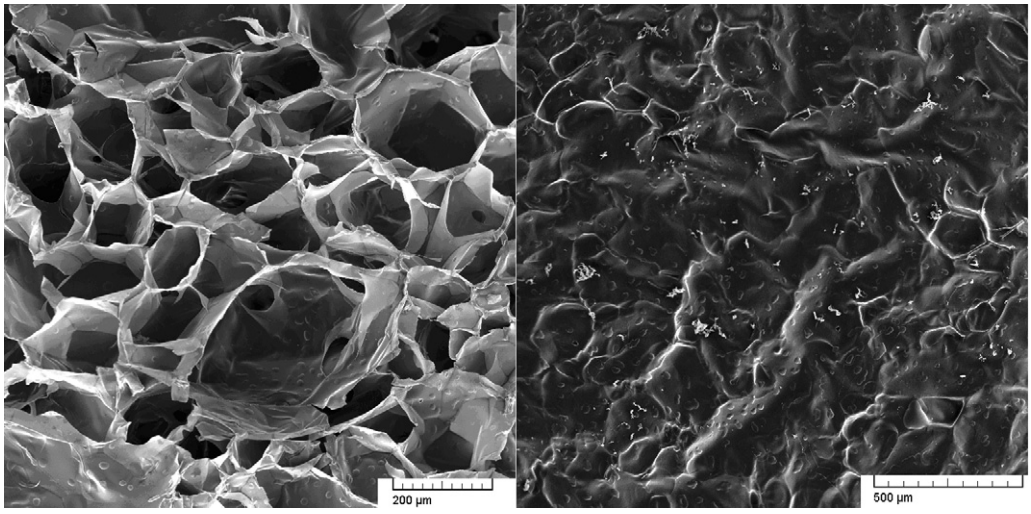


Fig. 2. SEM micrographs of freeze-dried gels obtained by click chemistry: cross-sectional (A) and surface view (B).

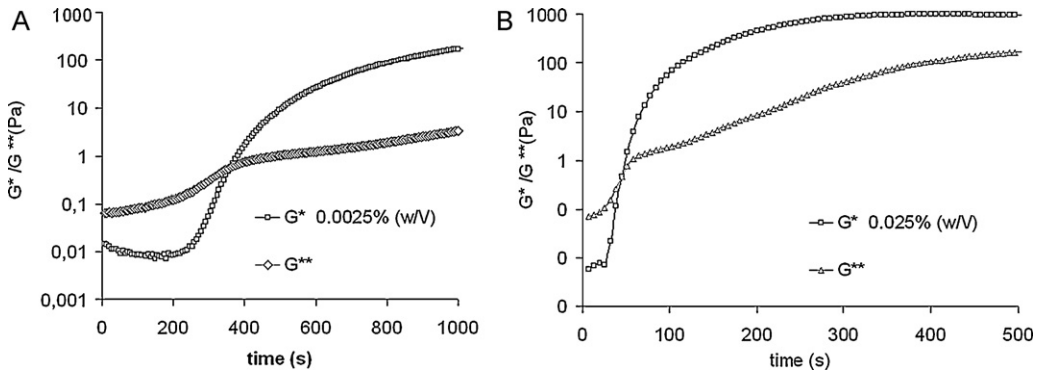


Fig. 3. Sol–gel transition for cross-linking reaction mediated by $CuSO_4$ in the presence of 0.0025% (w/v): (A) and 0.025% (w/v) and (B) $CuSO_4$.

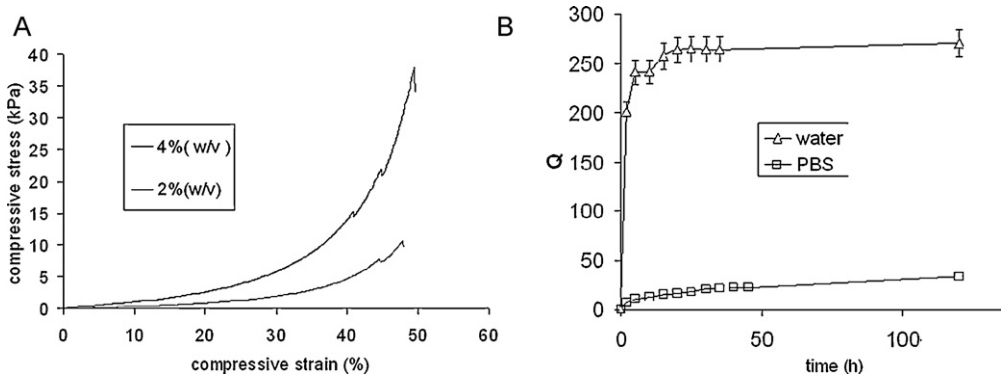


Fig. 4. (A) Curve stress–strain for hydrogels prepared from two different concentrations of HA-CAPA and HA-CAPr and (B) corresponding swelling degree of hydrogels in water and phosphate buffer (PBS).

3.5. Swelling degree

Fig. 4B illustrates the swelling kinetics of click hydrogels expressed as function of the swelling ratio (Q) against immersion time of the hydrogel. Equilibrium swelling ratio for hydrogels prepared from precursors 7 and 10 (Table 1) drastically changed from 250 for distilled water to 25 for phosphate buffer (pH 7.4). Furthermore, compared with previous works (Crescenzi et al., 2007), the swelling of hydrogels containing-secondary amines is higher than the reported one for amide derivatives. Conversely, elastic moduli were found to be lower in our hydrogels. Ritger–Peppas model indicated that the swelling kinetics parameter (n) is a function of the pH of the swelling media, suggesting that water uptake follows an anomalous diffusion at lower pH (n; 0.72), whereas at high pH (pH = 9.0), the value falls into a Fickian diffusion (n = 0.48). This effect can be attributed to an increased amount of ionizable groups of HA after oxidation reaction, which contributed to electrostatic repulsions in the polymeric network, as well as the introduction of partially positive charges due to basicity of the secondary amines moieties present in the polymeric backbone.

3.6. Cell viability and proliferation of cells on the hydrogel

The freeze-dried hydrogel was examined for its suitability as scaffold by culturing chondrocytes on it. Cell number was assessed by quantifying ATP. The results of the cell culture experiment showed that chondrocytes seeded in scaffolds increased in number over the tested period.

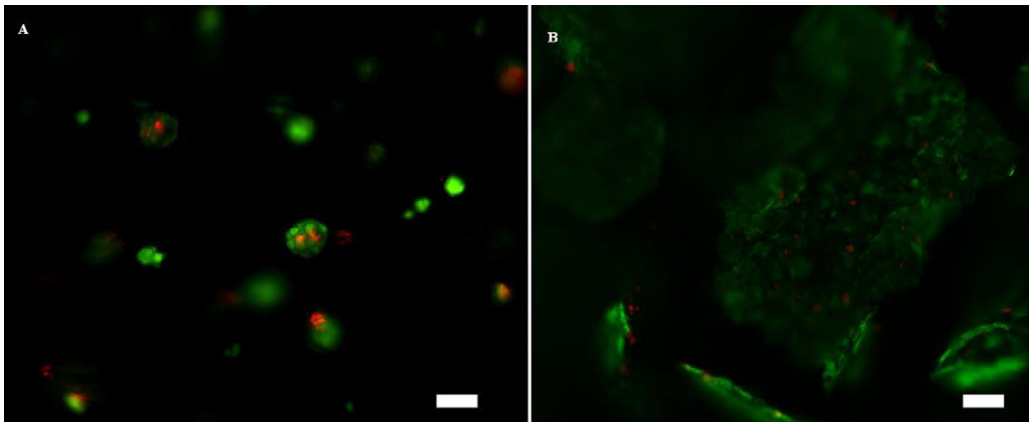


Fig. 5. Live dead assay of chondrocytes cultured on the scaffolds after 1 day (A) and after 15 days (B) (scale bar represents 60 μm).

After 9 days of seeding, there had been a significant increase in proliferation between the tested hydrogel and control groups. This difference in cell numbers persisted until day 15, wherein the cells have proliferated 2.7× more for concentration 0.1 g mL^{-1} and 3.0× more for 1 g mL^{-1}. A comparison of detected living cells on the scaffold after 1 and 15 days of seeding is depicted in Fig. 5. Although, the composition of the formed scaffolds have played an important role for stimulating cell viability and proliferation, the cells were not able to infiltrate and grow inside the scaffold, maybe due to the reduced pore size. Hydrogels seeded with chondrocytes and were cultured for a period of 21 days. LIVE/DEAD assay have showed that >95% of the cells were viable. The results from this study show that chondrocytes can be cultured and successfully expanded in number on the scaffold.

4. Conclusions

Hyaluronic acid was successfully modified by sequential reactions: oxidation, reductive amination and crosslinking via click chemistry. The use of the system 4-acetamido-TEMPO/sodium hypochlorite/NaBr proved to be a good alternative allowing modifications of the C-6 of hyaluronic acid. The reaction conditions have caused relatively low degradation of the polysaccharide in the case of modification of HA with molecular weight lower than 200 kDa. This methodology can be performed under mild conditions and allows an effective scale up. Hydrogels prepared from the derivatives synthesized in this work were found to be biocompatible and could be used as a part of a new material. Further studies should be carried out in order to optimize the properties of scaffolds such as organized porosity and good mechanical integrity.

Acknowledgements

Authors are grateful for funding from a project supported by the Ministry of Industry and trade of the Czech Republic under contract TIP-FR-TI1/150 and Dr. Lucie Wolfová for the determination of mechanical properties.

References

Banerji, S., Wright, A. J., Noble, M., Mahoney, D. J., Campbell, I. D., Day, A. J., et al. (2007). Structures of the Cd44-hyaluronan complex provide insight into a fundamental carbohydrate–protein interaction. *Nature Structural & Molecular Biology*, *14*(3), 234–239.

Buffa, R., Kettou, S., Pospisilová L., Huerta-Angeles, G., Chladkova D., & Velebny, V. (2011). A method of preparation of an oxidized derivative of hyaluronic acid and a method of modification thereof (WO/2011/069475).

Collins, M. N., & Birkinshaw, C. (2007). Comparison of the effectiveness of four different crosslinking agents with hyaluronic acid hydrogel films for tissue-culture applications. *Journal of Applied Polymer Science*, *104*(5), 3183–3191.

Crescenzi, V., Cornelio, L., Di Meo, C., Nardecchia, S., & Lamanna, R. (2007). Novel hydrogels via click chemistry: Synthesis and potential biomedical applications. *Biomacromolecules*, *8*(6), 1844–1850.

Crescenzi, V., Francescangeli, A., Renier, D., & Bellini, D. (2001). New hyaluronan chemical derivatives. Regioselectively C(6) oxidized products. *Macromolecules*, *34*(18), 6367–6372.

de Nooy, A. E. J., Besemer, A. C., van Bekkum, H., van Dijk, J. A. P. P., & Smit, J. A. M. (1996). TEMPO-mediated oxidation of pullulan and influence of ionic strength and linear charge density on the dimensions of the obtained polyelectrolyte chains. *Macromolecules*, *29*(20), 6541–6547.

de Souza, C. F., Lucyszyn, N., Ferraz, F. A., & Sierakowski, M. R. (2011). Oxidation and N-alkylation at the C-6 position of galactomannan extracted from Caesalpinia ferrea var. ferrea seeds. *Macromolecular Symposia*, *299–300*(1), 66–73.

Gao, Y., Zhang, Z., Chen, L., Gu, W., & Li, Y. (2009). Synthesis of 6-N,N,N-trimethyltriazole chitosan via click chemistry and evaluation for gene delivery. *Biomacromolecules*, *10*(8), 2175–2182.

Gross, K. r., & Rodríguez-Lorenzo, A. L. M. (2004). Biodegradable composite scaffolds with an interconnected spherical network for bone tissue engineering. *Biomaterials*, *25*(20), 4955–4962.

Huerta-Angeles, G., Smejkalová, D., Chládková, D., Ehlová, T., Buffa, R., & Velebný, V. (2011). Synthesis of highly substituted amide hyaluronan derivatives with tailored degree of substitution and their crosslinking via click chemistry. *Carbohydrate Polymers*, *84*(4), 1293–1300.

Jiang, B., Drouet, E., Milas, M., & Rinaudo, M. (2000). Study on TEMPO-mediated selective oxidation of hyaluronan and the effects of salt on the reaction kinetics. *Carbohydrate Research*, *327*(4), 455–461.

Liebert, T., Hänsch, C., & Heinze, T. (2006). Click chemistry with polysaccharides. *Macromolecular Rapid Communications*, *27*(3), 208–213.

Malafaya, P. c., Silva, B., & Reis, G. A. R. L. (2007). Natural origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. *Advanced Drug Delivery Reviews*, *59*(45), 207–233.

Malkoch, M., Vestberg, R., Gupta, N., Mespouille, L., Dubois, P., Mason, A. F., et al. (2006). Synthesis of well-defined hydrogel networks using Click chemistry. *Chemical Communications*, *26*, 2274–2276.

Matarasso, S. L., & Herwick, R. (2006). Hypersensitivity reaction to nonanimal stabilized hyaluronic acid. *Journal of the American Academy of Dermatology*, *55*(1), 128–131.

Mortisen, D., Peroglio, M., Alini, M., & Eglin, D. (2011). Tailoring thermoreversible hyaluronan hydrogels by click chemistry and RAFT polymerization for cell and drug therapy. *Biomacromolecules*, *11*(5), 1261–1272.

Ossipov, D. A., & Hilborn, J. N. (2006). Poly(vinyl alcohol)-based hydrogels formed by click chemistry. *Macromolecules*, *39*(5), 1709–1718.

Pahimanolis, N., Vesterinen, A.-H., Rich, J., & Seppala, J. (2011). Modification of dextran using click-chemistry approach in aqueous media. *Carbohydrate Polymers*, *82*(1), 78–82.

Peppas, N. A., & Sahlin, J. J. (1989). A simple equation for the description of solute release. III. Coupling of diffusion and relaxation. *International Journal of Pharmaceutics*, *57*(2), 169–172.

Podzimek, S., Hermannova, M., Bilerova, H., Bezakova, Z., & Velebny, V. (2011). Solution properties of hyaluronic acid and comparison of SEC-MALS-VIS data with off-line capillary viscometry. *Journal of Applied Polymer Science*, *116*(5), 3013–3020.

Ritger, P. L., & Peppas, N. A. (1987). A simple equation for description of solute release II. Fickian and anomalous release from swellable devices. *Journal of Controlled Release*, *5*(1), 37–42.

Rostovtsev, V. V., Green, L. G., Fokin, V. V., & Sharpless, K. B. (2002). A Stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective ligation of azides and terminal alkynes. *Angewandte Chemie International Edition*, *41*(14), 2596–2599.

Schanté, C. E., Zuber, G., Herlin, C., & Vandamme, T. F. (2011). Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications. *Carbohydrate Polymers*, *85*(3), 469–489.

Slaughter, B. V., Khurshid, S. S., Fisher, O. Z., Khademhosseini, A., & Peppas, N. A. (2009). Hydrogels in regenerative medicine. *Advanced Materials*, *21*(32-33), 3307–3329.

Testa, G., Di Meo, C., Nardecchia, S., Capitani, D., Mannina, L., Lamanna, R., et al. (2009). Influence of dialkyne structure on the properties of new click-gels based on hyaluronic acid. *International Journal of Pharmaceutics*, *378*(1–2), 86–92.

Wei, W., Min, W., Xuejun, K., Ping, W., & Songqin, L. (2008). Spectrophotometry study of interaction of hyaluronic acid with methylene blue and its analytic application. *Analytical Letters*, *41*(4), 599–607.

Yeom, J., Bhang, S. H., Kim, B.-S., Seo, M. S., Hwang, E. J., Cho, I. H., et al. (2010). Effect of cross-linking reagents for hyaluronic acid hydrogel dermal fillers on tissue augmentation and regeneration. *Bioconjugate Chemistry*, *21*(2), 240–247.