Available online at www.sciencedirect.com

ScienceDirect

Carbohydrate Polymers 69 (2007) 344–352

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Preparation and characterization of biodegradable alkylether derivatives of hyaluronan

Petra Mlčochová [a,*], Veronika Hájková [a], Bohumil Steiner [b], Slavomír Bystrický [b,*], Miroslav Koóš [b], Martina Medová [a], Vladimír Velebný [a]

[a] CPN spol. s r. o., Dolní Dobrouč, Czech Republic
[b] Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia

Received 17 March 2006; received in revised form 18 October 2006; accepted 20 October 2006

Abstract

Series of partially hydrophobic and water soluble alkyl ether hyaluronans were prepared. The conditions of chemical reaction of alkyloxymethyloxiranes with hydroxyl groups of hyaluronan (HA) were examined. Varying reagent ratio, amount of DMSO, pH, original molecular weight (M_w) and reaction time affected the formation of the products and derivatives with various degree of substitution (*DS*) and M_w can be obtained. All novel HA derivatives were fully biodegradable with testicular hyaluronidase. Tests for chondrocyte adhesion, proliferation, as well as their differentiation marker production on the surfaces containing HA derivatives showed better or equal quality as original HA.

Keywords: Hyaluronan; Alkyl ethers; Chondrocyte; Degradable biopolymers

1. Introduction

Hyaluronan (HA) is a glycosaminoglycan composed of the repeating disaccharide β-(1 → 3) linked glucuronic acid and *N*-acetylglucosamine. The disaccharides are linked by β-(1 → 4) bonds to form the polysaccharide linear chain (Ambrosio, Borzacchiello, Netti, & Nicolais, 1999). It is present in connective tissues and plays a vital role in many biological processes such as tissue hydration, proteoglycan organization in the extracellular matrix and cell differentiation. HA influences several aspects of cell behaviour, e.g. phagocytosis, motility, adhesion and detachment. Unmodified HA has found important applications in viscosupplementation, drug delivery and surgery (Benedetti, Topp, & Stella, 1990). The derivatization of HA allows tailoring of physicochemical properties according to the desired applications. The interest in this macromolecule has grown during the last two decades because of its associative properties and its potential applications in pharmaceuticals, cosmetics, paints and coatings, oil and recovery.

To present, the alkyl derivatives of HA were prepared mostly in the form of esters or carbamates. The esterification of carboxyl groups of hyaluronic acid with therapeutically important alcohols produces water insoluble biopolymers with physical properties that are significantly different from those of hyaluronic acid itself (Vercruysse & Prestwich, 1998). The esterification involves generation of tetraalkylammonium salts of hyaluronic acid in DMSO or DMF, and its subsequent reaction with alkyl bromides (e.g. ethyl, propyl, pentyl, benzyl or dodecyl). Analogously, octadecyl ester was prepared using octadecyl bromide (Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000). Another possibility for the preparation of esters is based on the reaction of HA with palmitoyl chloride in DMF in the presence of pyridine (Kawaguchi, Matsukawa, & Ishigami, 1992). Other methods of hydrophobization are based on the reaction of hydroxyl groups of glycosaminoglycan with fatty acid in trifluoroacetic acid

---

* Corresponding authors. Tel.: +420465519569; fax: +420465543793.
E-mail addresses: mlcochova@contipro.cz (P. Mlčochová), chembyst@savba.sk (S. Bystrický).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.10.015

anhydride (Hariki, Matsuzaki, Yamaguchi, & Akima, 1994) or acetylation with acetanhydride in the presence of acetic acid and sulfuric acid (Oka, Yanaki, & Yamaguchi, 1996). The reaction of quaternary ammonium salt of hyaluronic acid in DMSO with dodecylisocyanate in the presence of di-*n*-butyltin dilaurate lead to the formation of biodegradable hydrophobic polymer which can form micelles in low critical micelle concentration. Therefore, a pharmaceutically active or bioactive molecule was used in the hyaluronic acid derivative micelles to form a bioactive composition with a controlled released effect (Chen et al., 2003). The *n*-alkanoylated HA or its salt was prepared by reacting a complex of hyaluronic acid or its salt with an *n*-alkanoyl halide in a non-aqueous solvent. The alkanoyl group represented a chain with 4–18 carbon atoms (Yui, Otani, & Nakama, 2004). *n*-Hexyl, *n*-decyl, *n*-tetradecyl and *n*-hexadecyl ether derivatives having an equal degree of substitution have been prepared by the reaction of linear alcohols with 6-*O*-tosylated HA in DMSO under basic conditions (Benešová, Pekař, Lapčík, & Kučerík, 2006). New *n*-alkylated derivatives of HA were also obtained by the reaction of HA with adipic dihydrazide followed by the coupling with an aldehydic chain (8, 10, 12, 14, 16 carbon atoms) in the presence of $NaCNBH_3$ (Creuzet, Kadi, Rinaudo, & Auzély-Velty, 2006). Recently, some alkyl carbamates were prepared by the reaction of HA with alkyl amines using cyanogen bromide activation method (Mlčochová et al., 2006).

The HA-esters were also used for production of material for drug delivery, tissue engineering and viscosupplementation. Hydrocortisone was physically incorporated into HA-esters microspheres and the rate of hydrocortisone release *in vitro* was reported. Likewise, the release of chloropromazine from the films of ethyl and benzyl total esters was studied. The ability of HA-esters to form artificial skin was studied with cultivation of fibroblast and keratinocytes on this composite. Similar studies were performed with cells of other tissues, i.e. mesenchymal stem cell, chondrocytes (see review: Campoccia et al., 1998). The insoluble HA-esters are suitable for the preparation of fibers or films used for suturing and protecting the wounds (Hariki et al., 1994).

Glycosaminoglycan derivatives modified by a covalently bound primary amine to the reducing terminal sugar moiety can be used for forming of antithrombotic surface on medical instruments and inhibiting the proliferation of vascular smooth muscle cells to prevent narrowing or obstruction of blood vessels as prevention to arteriosclerosis (Matsuda, 2000).

The aim of our study was preparation of the water soluble alkyl derivatives of HA with high biocompatibility, with convenient degradation rate and new physical properties. In this regard, we have prepared a series of alkyl ethers of HA by the reaction of 2-alkyloxymethyloxirane with HA under basic conditions. In the first step, alkyl alcohols reacted with epichlorohydrin in aqueous sodium hydroxide solution, using phase-transfer catalyst (tetrabutylammonium hydrogen sulphate) to give corresponding 2-alkyloxymethyloxiranes in high yields (Mouzin, Cousse, Rieu, & Duflos, 1983). These were used for etherification in the next step (see Fig. 1).

2. Materials and methods

2.1. Materials

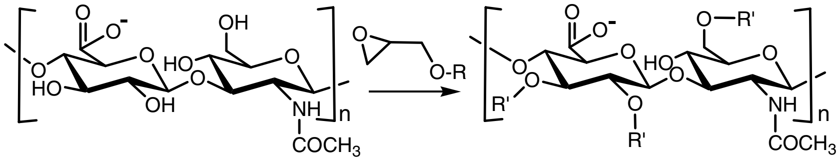
Hyaluronan was obtained from CPN spol. s r.o., Czech Republic. Epichlorohydrin, decyl alcohol, hyaluronidase type 1S Bovina Testes H 3506 were purchased from Sigma–Aldrich. Hexanol was from Fluka and tetradecanol from Merck. Butanol and octanol were products of Riedel de Haën.

2.2. Preparation 2-alkyloxymethyloxiranes (R-EP, R = alkyl)

2-Alkyloxymethyloxiranes (alkyl = butyl, hexyl, octyl, decyl or tetradecyl) were prepared analogously to the literature (Mouzin et al., 1983). Typically, a mixture of 50% aqueous sodium hydroxide (400 mL), epichlorohydrin (250 mL) and tetrabutylammonium hydrogen sulphate (8.4 g) was vigorously stirred at room temperature. Alkyl alcohol (0.6 mol) was gradually added during 30 min with cooling in ice water to keep the temperature below 25 °C. Then, the reaction mixture was poured on ice/water. The aqueous phase was extracted with diethyl ether and organic phase was washed with brine to neutrality, dried with sodium sulphate, filtered, and the product was distilled under reduced pressure.

2.3. Synthesis of hyaluronan-alkylethers

Etherification of HA was performed at alkaline pH with excess of 2-alkyloxymethyloxirane where alkyl was butyl, hexyl, octyl, decyl or tetradecyl as follows: 0.5 g of HA was dissolved in 25 mL of demineralized water and 25 mL of DMSO, pH of solution was adjusted to 9.0–10.0 with 0.1 M



R' = H or/and $CH_2CH(OH)CH_2R$, R = alkyl

Fig. 1. Preparation of alkyl derivatives of HA.

NaOH. 2-Alkyloxymethyloxirane (R-EP) in DMSO was added to the HA solution. The mixture was stirred overnight at 30 °C and the product was isolated by dialysis and freeze-drying. To remove unreacted 2-decyloxymethyloxirane or 2-tetradecyloxymethyloxirane, the product was washed with diethyl ether.

2.4. Determination of substitution degree

The *DS* was defined as a molar ratio of substituent and dimer unit of HA. This means that one alkyl substituent per one dimer unit represents a reference value (100%). ^{1}H NMR was used for its determination (Fig. 2). The *DS* was calculated as a ratio of integration of signal for terminal methyl of alkyl at around 0.9 ppm and methyl of acetylamino group at 2.0 ppm.

^{1}H- and ^{13}C NMR measurements (in D_2O) were performed on Bruker Avance DPX 300 instrument operating at 300.13 (^{1}H) and 75.46 MHz (^{13}C) working frequencies, respectively. Infrared spectra (in KBr pellets) were obtained on FT-IR 8400S Shimadzu spectrophotometer and correspond with substitution pattern.

2.5. Molecular weight determination

The M_w, gyration radius and polydispersity were determined by size exclusion chromatography (SEC). The SEC was performed using a Shimadzu LC-10ADVP pump equipped with Shimadzu SIL-10AD autoinjector. The injection volume was 50 µL. The instrument set-up consisted of HEMA BIO 1000 and HEMA BIO 300 (250 × 8 mm) columns in series (Tessek, Czech Republic) thermostated at 40 °C, combined with a miniDAWN MALLS detector (Wyatt Technology Corporation, three angles) followed by a RI (Shimadzu RID-10A) detector. The mobile phase was 0.1 M sodium phosphate buffer (pH adjusted to 7.5) and 0.05% NaN_3, the flow rate was 0.8 mL min^{-1}. Data acquisition and M_w calculations were performed using the ASTRA software, version 4.90.07 (Wyatt Technologies Corporation). Refractive index increment d*n*/d*c* of 0.155 mL g^{-1} was used for the SEC-MALS.

2.6. Solubility of derivatives

The results of HPLC analysis were used for determination of solubility of the prepared samples. Soluble portion was determined by comparison of values obtained by weighting of original samples and those values indicated by RID detector of HPLC after filtration through polyethersulfone filter (Puradisk 25 AS, porosity 0.2 µm).

2.7. Enzymatic degradation

The biological degradability of the tested samples was studied in acetate buffer (concentration 5 mg mL^{-1}). To this solution stirred at 35 °C, bovine testicular hyaluronidase (40 mg mL^{-1}) was added. The samples for measurements were taken in certain time intervals. Time effect on kine-

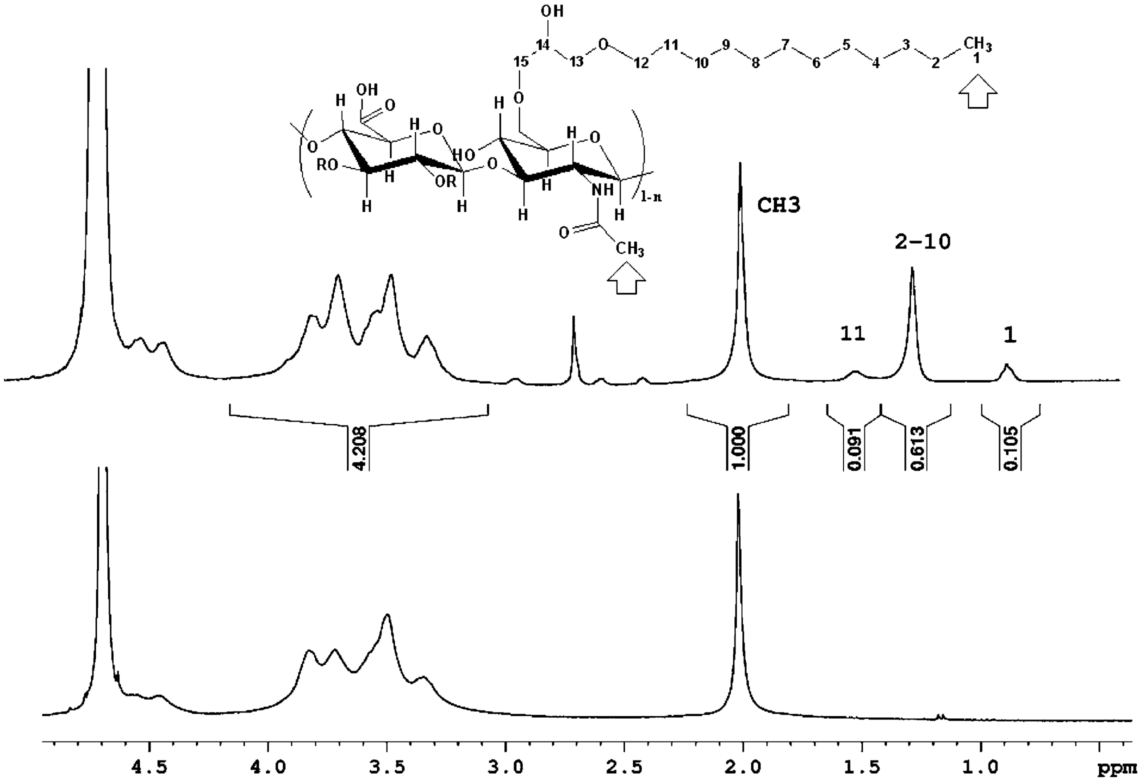


Fig. 2. ^{1}H NMR spectrum of HA (at bottom) and dodecyl derivative of HA (*DS* = 11%) in D_2O at 300 K.

matic viscosity (v) and M_w, determined by light scattering, was studied. Enzymatic hydrolysis was stopped by denaturation of enzyme by heating the sample in boiling water. Kinematic viscosity was measured at 25 °C in Ubbelohde viscosimeter with constant A = 0.00999 at 25 °C. The 0.5% (w/w) solution in acetate buffer (pH 5.1) was tempered 15 min at 25 °C before measurement.

2.8. Isolation of cells

The samples of cartilage were obtained from patients undergoing a surgery for a total knee replacement. Cartilage was cut into small pieces (2 mm^3) and digested overnight with collagenase II (1 mg mL^{-1}, Gibco). The cells were filtered using a cell strainer (40 μm, Becton Dickinson) and centrifuged at 1200 rpm for 15 min. Then, they were washed twice with Nutrient Mixture Ham's F-12 Medium (Gibco), the pellet was resuspended in this medium supplemented with 20% fetal bovine serum (Gibco), 1% antibiotic/antimycotic (Gibco) and 1% insulin–transferrin–selenium supplement (Gibco). The cells were cultured in monolayer at 37 °C in a humidified atmosphere of 5% CO_2 with medium exchange every second day. The primary chondrocytes were passaged once before the experiments. The cells were seeded at the density of 4×10^3 cells per well (96-well microtitration plates) for proliferation experiments, or 1×10^5 per well (6-well plates) for RNA isolation.

2.9. Covering the plastic surfaces with HA and hexyl-HA

Five milligrams of sample was dissolved in 1 mL of injection water. Culture plates (96-well microtitration plates and 6-well plates, TPP, Switzerland) were covered with the solution (1 mL per well for 96-well plates, 100 μL per well for 6-well plates) and incubated overnight at 37 °C. Wells were gently rinsed with 1 mL (300 μL) of phosphate buffer saline and incubated with 1% solution of bovine serum albumin (BSA, Sigma) in injection water for 2 h at 37 °C to block non specific binding sites. Wells were rinsed once with phosphate buffer saline and used immediately for cell seeding.

2.10. Chondrocyte proliferation

The chondrocyte viability and proliferation was monitored after 24, 48 and 72 h as a response of an addition of hyaluronan and his derivative into the culture medium in concentrations of 50 and 500 μg mL^{-1} using XTT Cell proliferation Kit II (Roche, Germany). This assay is based on the reduction of tetrazolium salt by viable cells to colourful formazan. Formazan product was measured at 492 nm by using spectrophotometer (VersaMax Microplate Reader, Molecular Devices). An increase in number of living cells correlates to the absorbance. Cells cultured in medium without HA and hexyl-HA were used as control. In the next experiment, chondrocytes were seeded on HA (hexyl-HA) coated surfaces and proliferation was evaluated by XTT Cell Proliferaon Kit as well. BSA attached to the bottom of culture plates was used as a control.

2.11. Detection of chondrocyte differentiation

To test the differentiation of chondrocytes adhered to HA and hexyl-HA on the molecular level, RT-PCR was proposed for monitoring the expression of differentiation markers (collagen I, II, aggrecan), CD44 and PCNA after 24 h. As a control, chondrocytes adhered to the surface covered with BSA was used. Total RNA was isolated using TRIzol Reagent (Invitrogene, USA) according to the manufacturer's instruction and subjected to reverse transcription. 2 μg of total RNA per 20 μL of reaction mixture was reverse-transcribed into cDNA. Reaction mixture contained Random Primer 3 μg (1 μL), diethylpyrocarbonate (DEPC), water 5.1 μL (0.1%), 1st Strand buffer 6 μL, dithiotreitol (DTT) 0.1 M (3 μL), Rnase OUT™ Ribonuclease Inhibitor Recombinant 5000U (1 μL), dNTP 2.4 μL, BSA 3.0 μL, Superscript™II RT 10000U (1.5 μL) (Invitrogene, USA). 2–5 μL aliquots of the resulting cDNA were amplified in a total 20 μL volume containing DEPC water (13.4 μL), PCR Buffer 10× (2.5 μL), $MgCl_2$ 50 mM (1.5 μL), BSA 1 mg mL^{-1} (1 μL), oligonucleotides 10 μM (0.5 μL), dNTP 0.5 μL, *Taq* DNA Polymerase Recombinant 500 U (0.1 μL) at appropriate annealing temperatures using Peltier Thermal Cycler PT200 (MJ Research, Inc., USA). All chemicals for PCR were obtained from Invitrogene (USA). Specifity of primers was checked by BLAST. The levels of cDNA amount needed for PCR were compared to the housekeeping gene β-actin. Intensity of expression was determined and quantified by gel documentation system (Syngene, UK).

3. Results and discussion

3.1. Effect of reaction conditions

Effect of concentration of DMSO in reaction mixture on product features is given in Table 1 (for decyl derivative, DEC). The solubility of HA in aprotic solvent is

Table 1
Effect of concentration of DMSO in reaction mixture on product features

Sample	c(DMSO) % (v/v)	M_w origin (g mol^{-1})	M_w result (g mol^{-1})	Soluble ratio (%)	DS (%)
DEC1	90	380,000	343,700	42	78
DEC2	80	380,000	356,300	52	33
DEC3	40	380,000	329,400	55	16
DEC4	20	380,000	341,450	53	17

limiting factor for the reaction. In contrary to 2-hexyloxymethyloxiran and analogs with shorter alkyl chain, the 2-decyloxymethyloxiran, 2-dodecyloxymethyloxiran and tetradecyloxymethyloxiran are already insoluble in water and therefore, reaction mixtures were not homogenous. The water soluble original hyaluronan has soluble portion of 88% and this value was not reached for alkyl derivatives with longer alkyl chains. It was found, that increasing amount of DMSO resulted in increase of *DS* but slight decrease of M_w. The hyaluronan of molecular weight about 369,700 g mol^{-1} has soluble portion of 88%.

The influence of reaction time on selected parameters of prepared derivatives is shown in Fig. 3 with hexyl derivative prepared without organic solvent.

Molecular weight increased with increasing degree of substitution and corresponded with kinematic viscosity. After reaction time of 36 h, disintegration of polymer chain may occur.

The influence of reactants ratio R-EP/HA (R = butyl) with two different M_w of HA in aqueous solution without DMSO at 45 °C and pH = 9.0–9.5 was also studied. Evidently, the increase of the ratio R-EP/HA caused increase of *DS* independently on M_w (Table 2).

Table 2
Effect of ratio R-EP/HA (R = butyl) on reactivity of HA with different M_w

M_w (g mol^{-1})	n(R-EP)/n(HA)	*DS* (%)
1,630,000	5.9	13.1
	4.2	8.5
	2.0	4.8
385,000	5.9	10.3
	4.1	8.3
	1.9	4.8

We have also tested the changes in M_w of products prepared in 56% aqueous DMSO at 30 °C, pH = 9.3 and decyl-EP/HA = 3.7 with three different original M_w of HA. In the case of original M_w = 50 kDa, slight increase of M_w was observed while evident decrease of M_w was registered for two higher original M_w. The *DS* did not change significantly.

As may be expected, these modifications result in reduced water solubility. Slight increase in structural rigidity can be caused by the interaction of hydrophobic chains linked to hyaluronan and by the decrease of stable hydrogen bond bridges. Hyaluronic acid has a considerably greater ability to trap water than other polyelectrolyte polysaccharides (Hatakeyama & Hatakeyama, 1998) but in

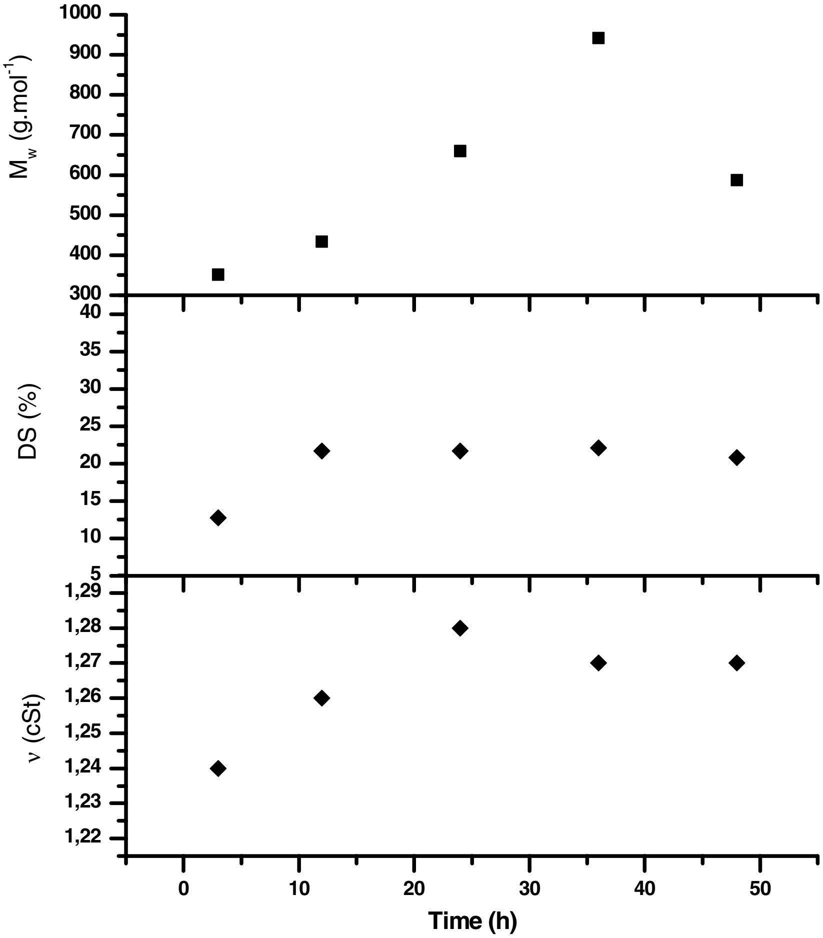


Fig. 3. Characteristics of hexyl derivative of HA as a dependence on reaction time.

Table 3
Effect of original M_w on characteristic of derivatives

M_w-origin (kDa)	M_w (kDa)	M_w/M_w-origin (%)	Gyration radius (nm)	Polydispersity M_w/M_n	DS \(%)
50	55	110	<15	1.42 ± 0.28	6.1
550	446	81	57	1.27 ± 0.14	7.0
1470	652	44	77	1.44 ± 0.20	5.4

the case of above described derivatives, this ability may be slightly changed. Hydrodynamic measurement indicates that radius of gyration is almost the same as the radius of the solved sphere (see Table 3).

The effect of the temperature and pH is very important. It was found that higher *DS* of products were obtained when the reactions were performed at higher temperature and higher amount of aqueous sodium hydroxide. Regarding the M_w, temperature upper 30 °C was inconvenient because of higher degradation of biopolymer chain. At this temperature, the initial M_w remained almost unchanged. The increasing pH caused increase of *DS*. For example, for initial $M_w = 365$ kDa, the resulting $M_w = 384.4$ kDa and $DS = 4.4\%$ were observed at pH = 9.0. On the other hand $M_w = 433.1$ kDa and $DS = 17.0\%$ were obtained at pH = 10.5.

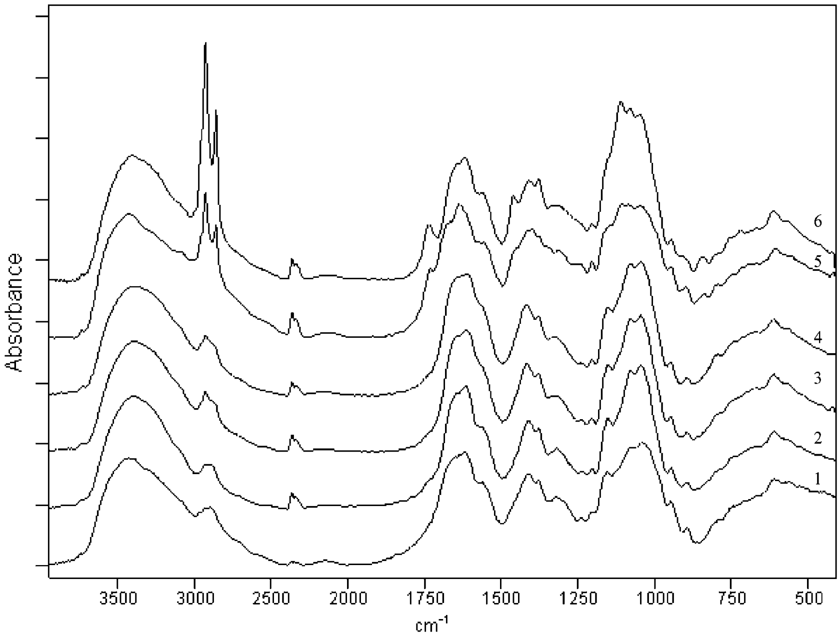


Fig. 4. FT-IR spectra of HA-decyl derivatives with various *DS*: (1) 0%; (2) 2%; (3) 8%; (4) 16%; (5) 23%; (6) 67%.

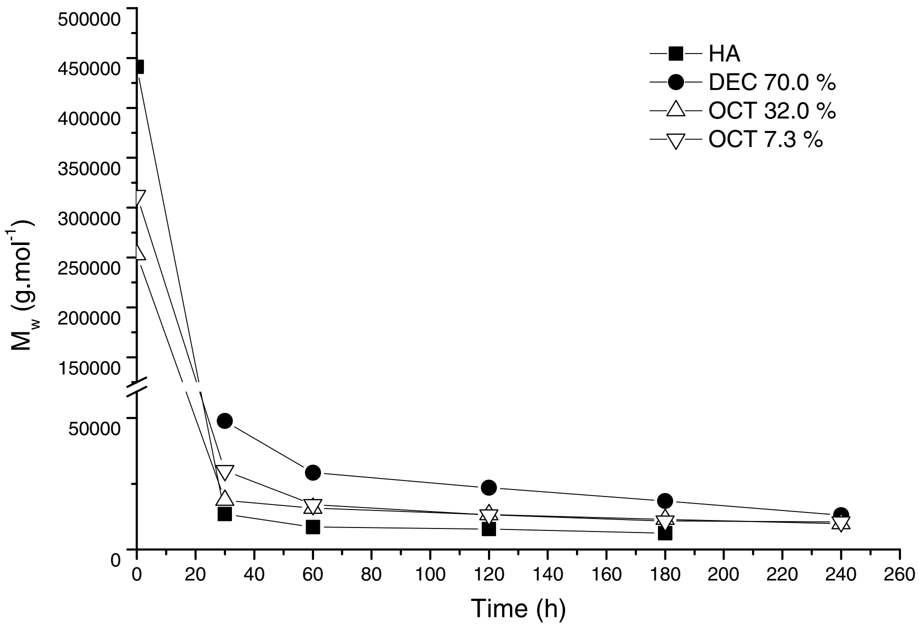


Fig. 5. Degradation of ether-linked HA-derivatives with various *DS* and alkyl length.

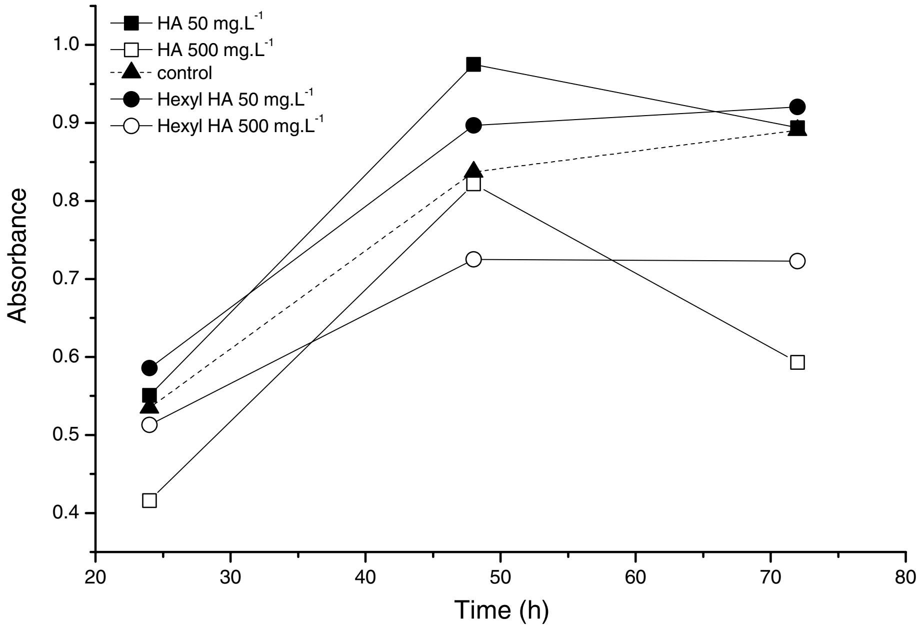


Fig. 6. Growing curve of chondrocytes cultured in medium supplemented with HA and hexyl-HA.

The reactions were monitored by infrared spectroscopy. The resulting HA-alkyl derivatives have specific vibration $v_s(CH_2)$ and $v_{as}(CH_2)$ in the range of 2920–2860 cm^{-1} (see Fig. 4). The differences between individual spectra are so significant, that they could be used for determination of *DS*. Characteristic peak of the ether bond was found in the range of 1150–1100 cm^{-1}. The other vibrations belonging to the HA correspond with data given in the literature (Gilli, Kačuráková, Mathlouthi, Navarini, & Paoletti, 1994).

In ^1H NMR spectra (see Fig. 2), the peaks at 0.85–1.5 ppm corresponding to alkyl chain were observed. The anomeric protons were registered at 4.4–4.6 ppm. The other protons of pyranose rings were found in the range of 3.2–4.0 ppm.

3.2. Biodegradability of derivatives

We have also studied the enzymatic degradability of HA-alkyl derivatives. All of the prepared samples were fully biodegradable by testicular hyaluronidase. Fig. 5 shows samples with different alkyl chain lengths (octyl derivative, OCT; decyl derivative, DEC) as well as various *DS*. Generally, the derivatives with longer alkyl chains and higher *DS* were degraded more slowly than original HA. The advantage of these derivatives is retained structure of original hyaluronan, with untouched carboxyl groups important for interaction with specific cell surface receptors.

3.3. Influence of the hydrophobized derivative of HA on chondrocytes

The interaction between biomaterials and cells may involve the direct interaction of cell receptors with specific molecules on the surface of the material. We described and compared the effect of low molecular weight hyaluronan and its hydrophobized derivate coming from the same batch on chondrocytes isolated from human articular cartilage to find the possibility of using this derivative as a scaffold for *in vitro* chondrocyte cultivation essential to autologous chondrocyte transplantation. The main point was to observe the ability of human articular chondrocytes to adhere, proliferate and keep the phenotype when growing in culture medium supplemented or on surfaces covered with HA or its hydrophobized derivative.

According to the XTT test (24–72 h), the optimal concentration for chondrocyte proliferation was established as 50 $\mu g\,mL^{-1}$ for both HA and hexyl-HA. Hexyl-HA supported cell proliferation also after 72 h (Fig. 6), whereas proliferation of chondrocytes treated with HA decreased after this period. The growing curve gave evidence of ability of chondrocytes to live in culture medium supplemented with hexyl-HA (HEX1).

To observe the effect of HA and hexyl-HA on chondrocytes during a longer time, proliferation was also monitored at regular intervals i.e. in the 3rd, 4th and 5th days using the XTT test. HA and hexyl-HA adhered to the

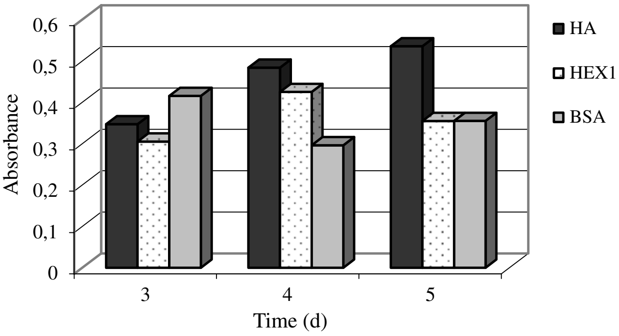


Fig. 7. Proliferation of chondrocytes adhered to HA and hexyl-HA.

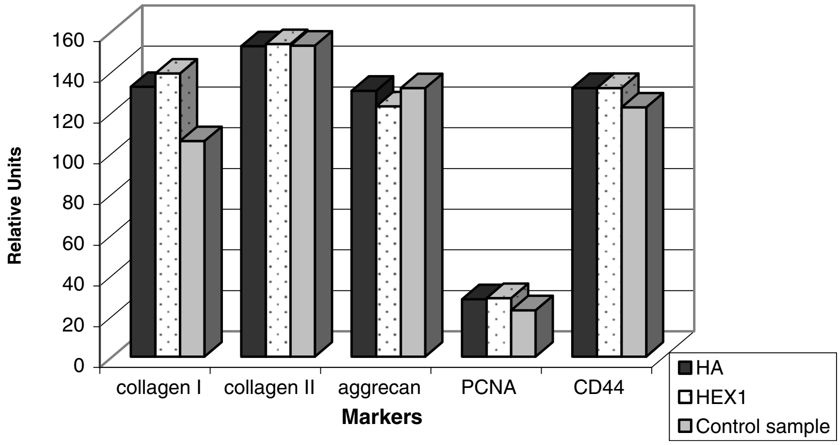


Fig. 8. Expression of differentiation markers on chondrocytes adhered to HA, hexyl-HA after 24 h.

bottom of microtitration plates at the concentration of 5 mg mL^{-1} significantly increased chondrocyte proliferation during five days, whereas hexyl derivative caused lower increment (Fig. 7) in comparison with control cells adhered to 1% BSA.

The ability of cells to maintain the chondrocyte phenotype after HA and hexyl-HA exposure was evaluated by measuring the expression of specific markers (collagen type I, collagen type II, aggrecan and PCNA) using RT-PCR. During endochondral ossification, chondrocytes in cartilage undergo a series of differentiation events, including proliferation, hyperthrophy and terminal differentiation. Each zone of differentiation is characterized by the expression of specific markers. Articular chondrocytes express PCNA, collagen type II and aggrecan. Culture of chondrocytes in monolayer leads to their differentiation into fibroblast-like cells and production of collagen type I instead of type II (Pfander, Swoboda, & Kirsch, 2001). We were also interested in the effect of both the HA and hexyl-HA on CD44 expression since CD44 as a specific receptor for HA plays a pivotal role in interactions between this molecule and chondrocytes. Chondrocytes, which were exposed to the effect of hydrophobized derivative of hyaluronan or hyaluronan itself, expressed nearly same or slightly increased levels of all the markers if compared with untreated cells (Fig. 8).

4. Conclusion

The preparation of selected alkyl ethers of HA is based on proper concentration of reagent, DMSO, pH and reaction time. The critical parameter for solubility of products is the length of alkyl chains, *DS* as well as M_w. The derivatives can be easily characterized by IR spectra.

Additionally, the aim of this paper was also to study the effect of HA and its derivative on chondrocyte proliferation and differentiation. Positive effect of presented HA derivatives comparable with HA was observed. The solution properties, as well as biocompatibility and complete biodegradability make this new biomacromolecules interesting material for general biological applications.

References

Ambrosio, L., Borzacchiello, P. A., Netti, P. A., & Nicolais, L. (1999). Properties of new materials. Rheological study on hyaluronic acid and its derivative solutions. *Journal of Macromolecular Science – Pure and Applied Chemistry, A36*(7–8), 991–1000.

Benedetti, L. M., Topp, E. M., & Stella, V. J. (1990). Microspheres of hyaluronic acid esters – fabrication methods and in vitro hydrocortisone release. *Journal of Controlled Release, 13*, 33–41.

Benešová, K., Pekař, M., Lapčík, L., & Kučerík, J. (2006). Stability evaluation of *n*-alkyl hyaluronic acid derivates by DSC and TG measurement. *Journal of Thermal Analysis and Calorimetry, 83*(2), 341–348.

Campoccia, D., Doherty, P., Radice, M., Brun, P., Abatangelo, G., & Williams, D. F. (1998). Semisynthetic resorbable materials from hyaluronan esterification. *Biomaterials, 19*, 2101–2127.

Chen, J. H., Tsai, B. H., Chang, H. T., Chen, M. L., Chen, Y. H., Jan, S. H, & Liu, M. J. (2003). Hyaluronic acid derivative with urethane linkage. G.B. Patent 2408741.

Creuzet, C., Kadi, S., Rinaudo, M., & Auzély-Velty, R. (2006). New associative systems based on alkylated hyaluronic acid. Synthesis and aqueous solution properties. *Polymer, 47*, 2706–2713.

Gilli, R., Kačuráková, M., Mathlouthi, M., Navarini, L., & Paoletti, S. (1994). FTIR studies of sodium hyaluronate and its oligomers in the amorphous solid phase and in aqueous solution. *Carbohydrate Research, 263*, 315–326.

Hariki, T., Matsuzaki, F., Yamaguchi, M., & Akima, K. (1994). Solvent-insoluble hyaluronic acid and its production. Japan Patent 6025306.

Hatakeyama, H., & Hatakeyama, T. (1998). Interaction between water and hydrophilic polymers. *Thermochimica Acta, 308*, 3–22.

Kawaguchi, Y., Matsukawa, K., & Ishigami, Y. (1992). Conformational changes of hyaluronates with partial palmitoylation and the adsorption structures on the surface of oil droplets. *Carbohydrate Polymers, 18*, 139–142.

Matsuda, T. (2000). Glycosaminoglycan derivatives and utilization thereof. WO 0112675.

Mlčochová, P., Bystrický, S., Steiner, B., Machová, E., Koóš, M., Velebný, V., et al. (2006). Synthesis and characterization of new biodegradable hyaluronan alkyl derivatives. *Biopolymers, 81*, 74–79.

Mouzin, G., Cousse, H., Rieu, J-P., & Duflos, A. (1983). A convenient one-step synthesis of glycidyl ethers. *Synthesis* (2), 117–118.

Oka, T., Yanaki, T., & Yamaguchi, M. (1996). Low-molecular-weight acetylated hyaluronic acid, emollient, and processes for producing and purifying the acid. WO 9605233.

Pelletier, S., Hubert, P., Lapicque, F., Payan, E., & Dellacherie, E. (2000). Amphiphilic derivatives of sodium alginate and hyaluronate: synthesis and physico-chemical properties of aqueous dilute solutions. *Carbohydrate Polymers, 43*, 343–349.

Pfander, D., Swoboda, B., & Kirsch, T. (2001). Expression of early and late differentiation markers by human osteoarethritic chondrocytes. *American Journal of Pathology, 159*, 1777–1783.

Vercruysse, K. P., & Prestwich, G. D. (1998). Hyaluronate derivatives in drug delivery. *Critical Reviews in Therapeutic Drug Carrier Systems, 15*(5), 513–555.

Yui, N., Otani, T., & Nakama, T. (2004). *n*-Alkanoylated hyaluronic acid or its salt, and its production method. Japan Patent 123785.