



Novel synthetic method for the preparation of amphiphilic hyaluronan by means of aliphatic aromatic anhydrides



Gloria Huerta-Angeles*, Martin Bobek, Eva Příkopová,
Daniela Šmejkalová, Vladimír Velebný

Contipro Pharma a.s., Dolní Dobrouč 401, 561 02 Dolní Dobrouč, Czech Republic

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ABSTRACT

The present work describes a novel and efficient method of synthesis of amphiphilic hyaluronan (HA) by esterification with alkyl fatty acids. These derivatives were synthesized under mild aqueous and well controlled conditions using mixed aliphatic aromatic anhydrides. These anhydrides characterized by the general formula $\text{RCOOCOC}_6\text{H}_2\text{Cl}_3$ can be easily prepared by the reaction of the corresponding fatty acid (R) with 2,4,6-trichlorobenzoyl chloride (TCBC) in the presence of triethylamine. The aliphatic aromatic anhydrides $\text{RCOOCOC}_6\text{H}_2\text{Cl}_3$ then react with the polysaccharide and enable the synthesis of aliphatic acid esters of HA in good yields. No hydrolytic degradation of hyaluronic acid could be observed. Parameters controlling the degree of esterification were systematically studied. Fatty acids with different chain lengths can be introduced applying this methodology. The degree of substitution was decreasing with increasing length of hydrophobic chain. The reaction products were fully characterized by Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), SEC-MALLS and chromatographic analyses. Although the esterified HA products exhibited aggregation in solution as demonstrated by NMR, microscopy and rheology, they were still water-soluble.

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1. Introduction

Amphiphilic oligo- and polysaccharides also known as hydrophobized polymers are able to self-aggregate due to intra and/or intermolecular hydrophobic interactions when dissolved in water while forming hydrophobic domains able to dissolve hydrophobic drugs. For this reason, amphiphilic polymers are very popular in drug delivery science and technology.

From the biomedical application point of view, polysaccharide hydrophobization is more interesting in case of biodegradable and biocompatible polymers, such as hyaluronan. Hyaluronan (HA) also known as hyaluronic acid is a naturally occurring polymer consisting of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) units linked by alternating β -(1→4)- and β -(1→3)-glycosidic bonds, respectively. Among the various reactions used for the hydrophobic modification of HA, esterification of either the carboxyl or hydroxyl groups has been one of the most studied reactions.

Esterification and amidation of carboxyl HA moiety are mostly mediated by carbodiimides (Schanté, Zuber, Herlin, & Vandamme, 2011). This methodology was used by some authors for grafting long alkyl chain amines (Finelli, Chiessi, Galeoso, Renier, & Paradossi, 2009) or poly(lactic acid) onto the polysaccharide using anhydrous conditions (Pravata et al., 2007). Nevertheless, in that methodology HA had to be converted to tetrabutylammonium salt so that it became soluble in organic media. This conversion increases the risk of chain fragmentation and the use of anhydrous solvents makes the process expensive and inconvenient. The same reaction conditions were used for the covalent attachment of ceramides (Jin et al., 2012), conjugation of 5 β -cholanolic acid (Choi et al., 2009, 2010) or bonding of glycerol- α -monostearate (Kong, Chen, & Park, 2011). The usage of carbodiimide as coupling reagent in hydrophobization reactions led to formation of side products, because the activation mechanism is not straightforward and a number of unwanted chemical reactions proceed. Additional issues associated with carbodiimides chemistry may include intramolecular cross-linking of the polysaccharide which decreases solubility of the final product.

Some esterification methodologies target the primary hydroxyl of HA and involve the use of symmetric fatty acid anhydrides or fatty acid chlorides for the conjugation of short, medium, and

* 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).

long aliphatic-chain fatty acids to HA. Both linear (Hu, Nie, & Xie, 2013; Šmejkalová et al., 2012; van den Broek & Boeriu, 2013) and cyclic (Eenschooten, Guillaumie, Kontogeorgis, Stenby, & Schwach-Abdellaoui, 2010) acid anhydrides were used. In case of cyclic anhydrides, high molar excess of the reagent had to be used in order to reach desired chemical modification. Fatty acid chlorides are usually used for high degree of functionalization of polysaccharides such as starch (Grote & Heinze, 2005) or cellulose (Crepy, Miri, Joly, Martin, & Lefebvre, 2011) in organic solvents. Even though fatty acid anhydrides and chlorides are described as useful intermediates, they are extremely sensitive to hydrolysis. Therefore, although some of these anhydrides and chlorides are commercially available, their high instability together with their high cost limits their use on industrial scale. Another disadvantage of described reactions is the fact that the medium and long chain fatty acids (both chlorides and anhydrides) are poorly soluble in water and for this reason polysaccharide modification is mostly carried out in anhydrous organic solvents such as *N,N*-dimethyl acetamide (DMAC), formamide or dimethylsulfoxide (DMSO). For modification of HA this means that the polysaccharide must be converted into its acidic form for solubilization in organic solvents. However, such conversion may lead to significant HA chain fragmentation, polydispersity increase and loss of material, resulting in poor reproducibility and low yields. Current trends are directed toward more efficient methods in order to avoid the use of harmful and toxic solvents.

Except for acid anhydrides and chlorides, direct activation of fatty acids can be carried out by acyl imidazoles, as it was demonstrated for esterification of cassava starch with *n*-alkyl fatty acids (C-12 to C-22) in DMSO at 90 °C (Barrios, Giammanco, Contreras, Laredo, & Lopez-Carrasquero, 2013). Unfortunately, high temperatures or harsh reaction conditions are not applicable for modification of HA due to its susceptibility to chain degradation under such conditions (Kapusniak & Siemion, 2007; Kshirsagar & Singhal, 2007; Namazi, Fathi, & Dadkhah, 2011).

Because of the above mentioned disadvantages of reported methods, alternative ways of fatty acid activation are needed to be developed. A mild synthesis of highly functionalized esters was reported by Yamaguchi (Inanaga, Hirata, Saeki, Katsuki, & Yamaguchi, 1979). The modified Yamaguchi reaction was used for the preparation of macrolactones (Shiina, Fukui, & Sasaki, 2007). However, according to the best of our knowledge the Yamaguchi reaction has never been probed for the preparation of amphiphilic polysaccharides.

For this reason, the aim of the present study is to apply Yamaguchi reaction and find out whether mixed aliphatic aromatic anhydrides are useful intermediates for the chemical modification (hydrophobization) of HA. 2,4,6-Trichlorobenzoyl chloride (TCBC) will be used as activating agent of short, medium and long fatty acids. The effect of the length of fatty acid chain and reaction parameters will be studied regarding the degree of substitution and reaction yield. Structural characterization of the obtained HA derivatives will be investigated by FT-IR, NMR, rheology and microscopy.

2. Materials and methods

2.1. Materials

Hyaluronic acid characterized with different mass average molecular weights was provided by Contipro Pharma a.s., Dolní Dobrouč, Czech Republic. The average molecular weight of the starting HA was determined by SEC-MALLS before the chemical modification. 4-Dimethylaminopyridine (DMAP) was obtained from Merck. Tetrahydrofuran (THF), isopropanol (IPA), triethylamine (TEA), *cis*-oleic acid (OA) and sodium chloride were

obtained from Lach-ner (Czech Republic). Stearic (C18:0), linoleic (C18:2), alpha-linolenic (C18:3), arachidic (C-20) and behenic (C-22) acids were commercially available products from TCI-Europe. Hexanoic (C-6), octanoic (C-8), decanoic (C-10) acids and 2,4,6-trichlorobenzoyl chloride (TCBC) were obtained from Sigma-Aldrich and used as received.

2.2. Methods

2.2.1. Synthesis of hydrophobized sodium hyaluronate

Hyaluronic acid (5 g, 12.5 mmol) was dissolved overnight in 100 ml of distilled water. To that solution 50 ml of THF were slowly added. After the solution was homogeneous, triethylamine (3.5 ml, 25 mmol) and DMAP (0.015 g, 0.125 mmol) were added and the mixture was stirred until a clear solution was obtained.

At the same time and in a second reaction flask, the fatty acid was activated using the molar ratio described in Tables 1 and 2. The molar equivalents of the fatty acid as resumed in Tables 1 and 2 were dissolved in tetrahydrofuran (10 ml). After that, TEA (5 ml, 25 mmol) were added, followed by one molar equivalent of 2,4,6-trichlorobenzoyl chloride (TCBC). The formation of the aliphatic aromatic anhydride was carried out for 30 min at room temperature (25 °C). Then, the solution containing the mixed anhydride was added to the solution containing the polysaccharide. The mixture is allowed to react for 3 h at room temperature under vigorous stirring to ensure a good homogenization of the components. The crude product was isolated by precipitation with the addition of 100 ml of distilled water and a super-saturated solution of sodium chloride. After that the product was washed with an excess of anhydrous isopropanol (250 ml). The product was washed again with solutions of isopropanol: water (85%, v/v, 4 × 250 ml). Finally, the precipitate was washed two more times with absolute isopropanol. The white precipitate was decanted and dried in an oven at 40 °C for at least 24 h. Yields of the reaction are resumed in Tables 1 and 2.

The yield (*Y*) was determined experimentally as an average of at least five independent batches using the following equation (Moura Neto, Maciel, Cunha, de Paula, & Feitosa, 2011):

$$Y = 100 \frac{m_{\text{product}}}{[1 + (DS M_{\text{FA}}/400)]m_{\text{starting}}} \quad (1)$$

where m_{starting} is the amount of HA used in the reaction feed (in grams), m_{product} is the weight of HA purified-derivative and isolated after reaction (in grams), M_{FA} is the molecular weight of the corresponding fatty acid and 400 represents the molecular weight (in g/mol) of HA–Na dimer.

The formation of the mixed aliphatic aromatic anhydride was confirmed by spectroscopic analyses (see Fig. 1a): ^1H NMR (CDCl_3): 0.9 (3H, t, $J = 7.05$), 1.29 (CH_2 , m), 1.68 (2H), 2.4 (2H), 2.46 (2H), 5.36 (2H, dd), 7.27 (1H), 7.46 (1H). The structure of the ester of hyaluronan was confirmed by FT-IR and NMR and described in detail in Section 3.6.

2.2.2. 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.

2.2.3. Nuclear magnetic resonance (NMR)

^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. Low molecular weight HA samples were analyzed by dissolving the derivatives (10 mg/mL) in D_2O . High molecular weight HA samples were dissolved in mixture of D_2O and $\text{IPA}-d_6$

Table 1

Degree of substitution (DS) and yields of HA esterified products prepared using fatty acids (FA) with varying length of the alkyl chain (C6–C22), including residual FA (unbound FA) impurity in esterified products.

Entry	Anhydride ^a	Molar ratio MA:HA ^b monomer	DS (%) ^c	Yield (%)	Unbound FA (wt.%)
1	C6-TCBA	1:1	23	98.0 ± 4.8	<0.05
2	C6-TCBA	2:1	35	98.0 ± 3.5	<0.05
3	C8-TCBA	1:1	16	91.2 ± 5.8	<0.05
4	C8-TCBA	2:1	34	67.6 ± 5.5	<0.05
5	C10-TCBA	1:1	14	94.3 ± 4.3	<0.05
6	C10-TCBA	2:1	36	62.0 ± 2.2	<0.05
7	C18-TCBA	0.5:1	4	84.0 ± 12.1	0.34
8	C18-TCBA	1:1	9	85.6 ± 4.9	<0.05
9	C18(symmetrical)	1:1	1	41.3 ± 11.6	<0.05
10	C18:1-TCBA	0.5:1	6	60.0 ± 5.6	0.15
11	C18:1-TCBA	0.75:1	9	89.9 ± 5.8	<0.05
12	C18:1-TCBA	1:1	12	88 ± 2.4	<0.05
13	C18:1-TCBA	2:1	18	94.6 ± 3.5	0.06
14	C18:1-TCBA	3:1	7 ^d	133 ± 9.2 ^d	17.25
15	C18:1-TCBA	4:1	3.5 ^d	124 ± 8.7 ^d	15.30
16	C18:2-TCBA	0.5:1	3	58.0 ± 4.5	0.07
17	C18:2-TCBA	1:1	10	52.0 ± 6.1	<0.05
18	C18:2-TCBA	2:1	13	65.0 ± 4.4	0.01
19	C18:3-TCBA	0.5:1	2	40.0 ± 3.8	<0.05
20	C18:3-TCBA	1:1	12	30.5 ± 15.4	0.02
21	C20:0-TCBA	1:1	0	–	–
22	C22:0-TCBA	1:1	0	–	–

^a Fatty acid and anhydride used for the reaction, examples: Hexanoic 2,4,6-trichlorobenzoic anhydride = C6-TCBA, stearic anhydride C18(symmetrical).

^b Mole reagent of corresponding anhydride per mole HA unit. The reaction is carried out at 25 °C for 2 h, HA (Mw = 15 000 g/mol, polydispersity 1.5) was used for the optimization.

^c degree of substitution (DS) determined by ¹H NMR spectroscopy.

^d large amount of free fatty acid impurity in product caused DS and yield overestimation.

(2:1). 2D HSQC NMR spectra were acquired using edited gradient pulse sequence and 1k data points, 3 kHz spectral width in f2, 80 scans per increment, 256 increments, and heteronuclear scalar coupling C–H set at 145 Hz. DOSY NMR experiments were performed using a stimulated echo pulse sequence with bipolar gradients (2.5 ms) and watergate 3–9–19 pulse train with gradients, diffusion time 0.8 s, and 2.0 ms sine-shaped pulses with 32.030 G cm^{−1}.

The degree of substitution (in mol.%, i.e. moles of fatty acid to moles of HA dimer) was obtained from ¹H NMR by normalizing the integral of the anomeric proton HA signals from 4.6 to 4.3 ppm to 67 (dimers) and reading the integral value at δ = 0.9 ppm corresponding to the terminal methyl group of fatty acid and thus to the degree of substitution.

2.2.4. SEC-MALLS

The chromatographic system consisted of an Agilent degasser Model G 1379A, an Agilent HPLC pump Model G 1310A, a Rheodyne manual injector Model 7125i, two 7.8 mm Ultrahydrogel Linear columns (Waters), chromatographic detectors included a DAWN EOS device, a ViscoStar differential viscometer, and an Optilab rEX differential diffractometer in series (all from Wyatt Technology, Santa Barbara, CA). Injection volume was 100 µl of 0.015–1% (w/v) HA solutions. The mobile phase was aqueous 50 mM phosphate buffered saline and 0.02% sodium azide solution at the flow rate

of 0.5 ml/min. A refractive index increment (dn/dc) of 0.155 mL/g was used for calculation of molecular weight and polydispersity (Mw/Mn) of HA and derivatives according to the methodology described before (Podzimek, Hermannova, Bilerova, Bezakova, & Velebny, 2010). The samples were measured three independent times and the reported value is an average of these determinations. Data acquisition and processing were performed using the Wyatt Technology Corporation ASTRA software, Version 5.3.1.4.

2.2.5. Rheological analyses

A TA Instruments AR-G2 rheometer was used to measure the dynamic viscosities of the HA solutions in the range of shear rate 0.001–10000 s^{−1}. Stainless steel double concentric cylinder geometry was used under steady state mode. The sample temperature was maintained at 25 °C using a temperature control system. Solutions of the derivatives and HA were prepared at concentrations of 10% (w/v) and transferred to the rheometer. The shear rate viscosity was recorded as a function of the frequency.

2.2.6. Cryo SEM analysis (micellar size and shape)

To detect self-assembled HA derivative 20 mg of hydrophobized sample dissolved in 1 ml of water was mixed with hydrophobic dye (oil red O) and filtered through 1.0 µm glass syringe filter. 2–3 µL of the prepared solution were transferred in an Al target plate,

Table 2

Effect of the molecular weight (Mw) of HA observed on degree of substitution and reaction yield of sodium oleyl hyaluronate. Reaction was carried out using TCBA for 2 h at 25 °C. The amount of free oleic acid in entries 23–30 was <0.05 wt.%.

Entry	Mw × 10 ³ g/mol (P) ^a	Molar ratio MA:HA	DS (%)	Yield (%)
23	4.8 (1.3)	0.75	25	80.1 ± 2
24	15 (1.5)	1.0	9	94.0 ± 3.19
25	38 (1.6)	1.0	8	92.7 ± 3.5
26	122 (1.8)	1.0	6	75.0 ± 2.3
27	202 (2.0)	1.0	5	81.2 ± 6.8
28	500 (2.0)	1.0	5	79.9 ± 5.4
29	1000 (1.8)	1.0	5	75.8 ± 3.8
30	1800 (1.8)	1.0	1	87.0 ± 3.2

^a Mw of starting HA used in the reaction feed, P = polydispersity.

Fig. 1. (A) Activation of the fatty acid with 2,4,6-trichlorobenzoylchloride (TCBC) and (B) Esterification of hyaluronic acid, wherein R = C₅H₁₁, C₇H₁₅, C₉H₁₉, C₁₇H₃₅, C₁₇H₃₃, C₁₇H₃₁ or C₁₇H₂₉.

3.2. Influence of fatty acid chain length on the degree of substitution and reaction yield

Table 1 indicates that the reaction can be applied for activation of short (C6–C8), medium (C-10) and long (C-18) fatty acids. Table 1 shows that higher DS is obtained for fatty acids with shorter alkyl chain length (entries 1, 5, 8, 12, 17 and 20 in Table 1). To ensure reproducibility of the reaction, each synthesis was repeated at least five times. In general, the procedure resulted in at least 5 g scale. In all the cases, products with identical structural characteristics, consistent degree of substitution (DS), yield and purity were obtained.

The increased reactivity for shorter aliphatic acids may be explained by their physical–chemical properties, such as better solubility in the reaction media that enables easier mixing and incorporation of these components in the reaction feed. Additionally, there are also steric factors and increasing hydrophobicity which cause a decrease of reaction efficiency with increasing alkyl chain length. Table 1 also shows that the highest DS was reached when two molar equivalents of hexanoic 2,4,6-trichlorobenzoic anhydride were used in the reaction feed. In this case maximum DS for hexanoic, octanoic, and decanoic acid was about 35%, while 18% and 13% of modified HA dimers were obtained for oleic and linoleic acid, respectively (entries 2, 4, 6, 13 and 18 in Table 1). With higher molar amounts of the mixed anhydride than two HA equivalents, the reaction mixture was becoming turbid and an increase in DS was not observed. In this latter case, large amounts of unbound fatty acid (more than 15 wt.%) (Table 1) remained in the reaction product, artificially increasing the reaction yields above 100% (entries 14 and 15). The presence of high amount of unbound fatty acid could have caused some DS overestimation, because free fatty acid signal overlaps in ^1H NMR spectrum with the integrated signal of bound fatty acid (terminal $-\text{CH}_3$ at 0.8 ppm). Such overestimation did not occur in other cases (entries 1–13 and 16–20), because there the amount of free fatty acid was negligible (between 0.01 and 0.5 wt.%, Table 1).

Table 1 further shows that the reaction conditions can be applicable for the covalent bonding of both saturated (C-18:0) and unsaturated (C18:1, C18:2 and C-18:3) fatty acids. However, in the case of unsaturated long fatty acids, a significant decrease of the yield was observed with increasing number of unsaturated bonds in the chain (entries 10–20, Table 1). The lowest yield (about 31%) was observed for α -linolenic acid (entry 20). This observation can be explained by possible liability of the double bond toward oxidation.

It was our intention to extend the presented methodology for the covalent attachment of very long fatty acids such as arachidic (C-20:0) or behenic acids (C-22:0) (entries 21 and 22). Unfortunately, the results were unfruitful for all the tested reaction conditions. Analyzing the reaction product by FTIR, we have observed that symmetric anhydride was formed during activation of the fatty acid with TCBC (data not shown). However, the symmetric anhydride did not react with the polysaccharide. In agreement with literature data, similar reaction intermediate was detected during the reaction mechanism of Yamaguchi esterification (Dhimitruka & SantaLucia, 2005).

To compare the influence of symmetric and asymmetric anhydrides on DS, these anhydrides were applied under similar reaction conditions and the final products were compared. There was no effect on DS in the case of short and medium fatty acids (data not shown). However, significant differences were noted for fatty acids with long chain. As it is shown in Table 1, entries 8 and 9, the DS was much higher when asymmetric aromatic carboxylic anhydride was used for the activation.

It should be noted here that in case of short aliphatic acids such as hexanoic, heptanoic and octanoic acid the presented activation methodology involving mixed aliphatic aromatic anhydrides

is more expensive when compared to a direct use of the commercially available symmetric anhydrides. The opposite is true in the case of medium and long fatty acids, where commercial symmetric anhydrides are very expensive and often unavailable. From the application point of view, medium and long fatty acid conjugates are more interesting due to their potential antineoplastic properties (Ali, Alazani, Mumtaz, Arun, & Sabir, 2013; Hassani, Hendra, & Bouchemal, 2012; Khan, Husain, Jabeen, Mustafa, & Owais, 2012; Wang, Luo, Li, & Zhao, 2012). The reaction was aimed to be scale up and attain similar products even though the scale factors were up to 50. In addition, the proposed reaction pathway overcomes the problem of instability of all commercially available anhydrides and thus represents an elegant alternative for scale up production of esterified polysaccharides.

3.3. Influence of Mw of HA on degree of substitution

The molecular weight of starting HA was found to be an important parameter affecting the success of the presented modification pathway. The effect of Mw of starting HA material on DS of the product is summarized in Table 2. It was observed that low molecular weight HA (4800 g/mol) is more reactive than HA with larger Mw. For low molecular weight HA high degree of substitution was obtained even when low molar equivalents of mixed aliphatic aromatic anhydride were used (Table 2, entry 23). However, the recovery of the reaction was only about 80%. This is unlikely to be explained by degradation of the polysaccharide during the reaction. More likely, the low molecular weight esters of HA were washed out during the purification process. An improved reaction yield was reached in case of 15 000 and 38 000 g/mol with DS 9 and 8%, respectively. Otherwise decreasing tendency of DS and reaction yields were observed with increasing Mw of HA (Table 2). This was probably caused by an inefficient reaction progress due an increased solution viscosity driven by HA molecular weight. There was almost no apparent substitution in the case of high molecular weight HA (1 800 000 g/mol).

3.4. Influence of DMAP on the reaction success

The use of DMAP as nucleophilic organocatalyst in the proposed pathway of esterification was found to be essential. The organocatalyst increases the nucleophilic acyl transfer of the long aliphatic fatty acids. This reagent presents a high catalytic activity derived from the electron-donating capability of the dialkylamino group (Baidya et al., 2007). Despite the high toxicity of the chemical, at least a catalytic amount of DMAP is required in order to successfully modify HA in the proposed way. There was no substitution observed in the absence of DMAP.

3.5. Estimation of residual reagents in reaction products

The purity of the derivatives was determined by quantifying possible entrapped residues of DMAP and TCBC in the product. The exact DMAP and TCBC contents in final products were estimated by HPLC methods and were found to be below 0.01% (w/w) for all products. Additionally, non-covalent bonded fatty acids were analyzed as well. Residual free fatty acids were determined by GC analyses and were found to be between 0.01 and 0.5% (w/w), except for the samples described in entries 14 and 15 in Table 1. Therefore, the experimental data revealed that impurities were present in negligible amounts.

3.6. Analytical characterization of amphiphilic derivatives

SEC-MALLS analyses showed an insignificant change of the weight average molecular weight of the polysaccharide before and

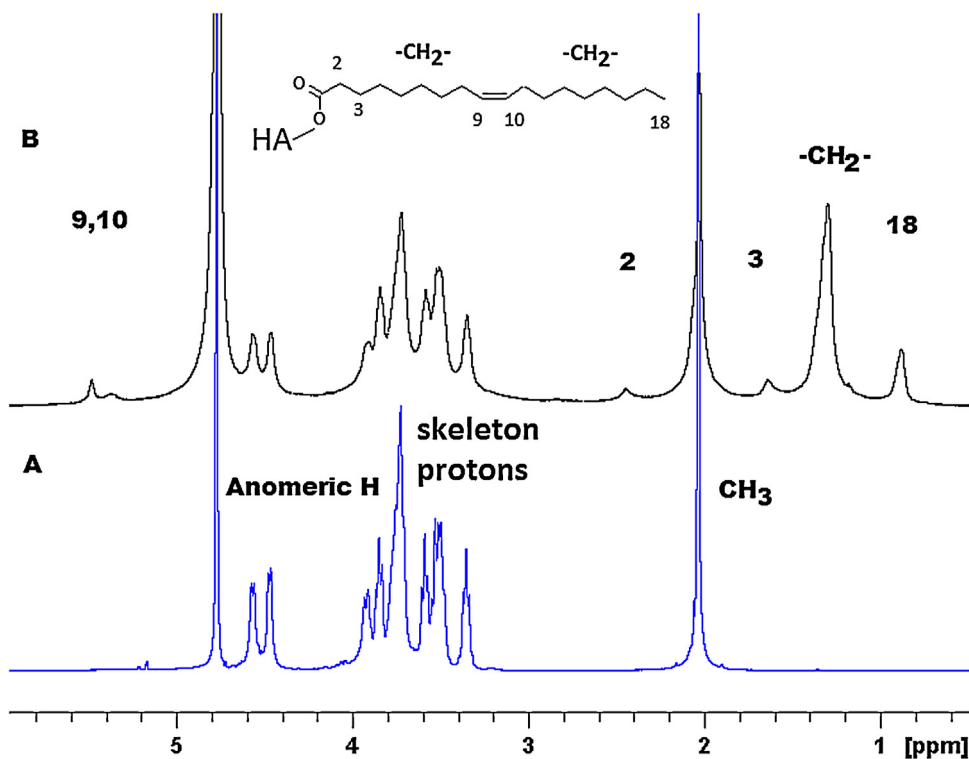


Fig. 2. ^1H NMR spectrum of (a) native HA and (b) esterified HA (sodium oleyl hyaluronate, DS = 12% and Mw = 15 000 g/mol) recorded in D_2O .

after chemical modification. For example, when HA with starting Mw = 15 000 g/mol was modified, an esterified product with Mw = 15 500 g/mol was repeatedly observed. That means that the reaction conditions did not cause significant degradation of HA

during the modification procedure. Unfortunately, the molecular weight could be only determined for samples modified with short fatty acid chains and having low degree of substitution. In agreement with literature, derivatives with higher DS than 10% or

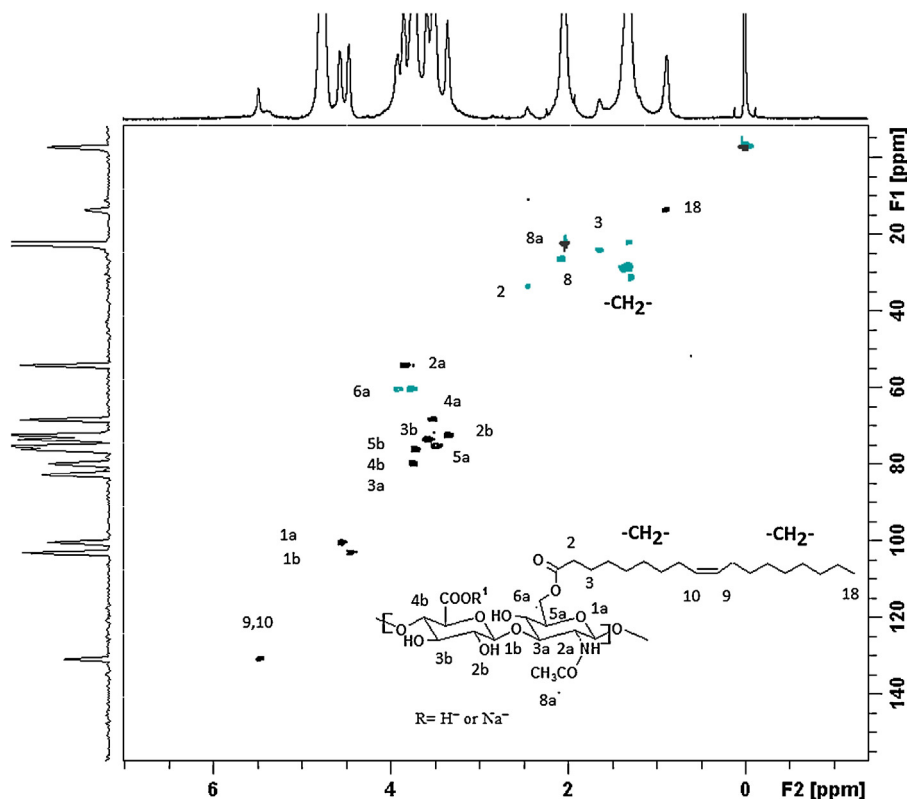


Fig. 3. Edited HSQC spectrum of sodium oleyl hyaluronate (DS = 12 and Mw = 15 000 g/mol).

derivatives modified with longer acyl chains were found to strongly interact with the column stationary phase, impairing a meaningful analysis (Tommeraa, Møllergaard, Mølle, & Skagerlind, 2011). Still, this is a good indication of the fact that the physical chemical properties of the modified HA have changed.

High-resolution two-dimensional NMR spectroscopy was used to determine the structure of HA esters. Moreover, ^1H NMR spectroscopy was applied for determination of degree of substitution (DS). Fig. 2 shows the ^1H NMR spectrum of sodium oleyl hyaluronate with a DS of 12% recorded in D_2O . This derivative will be used as an example to demonstrate the grafting of fatty acids to HA. All the spectra show typical proton chemical shifts of HA involving signal at 2.0 ppm belonging to COCH_3 group, skeletal signals at 3.4–3.9 and anomeric resonances at 4.4–4.6 ppm. Remaining signals detected in the spectrum of modified HA (upper spectrum in Fig. 2) at 0.8; 1.3, 1.6, 2.4; and 5.4, 5.5 ppm were attributed to CH_3 ; CH_2 ; and vinyl functional groups of acyl chain, respectively.

Fig. 3 shows an example of edited heteronuclear single quantum coherence (HSQC) spectrum, which was used to assign all the proton resonances of the esterified derivatives. The edited mode allowed recognition of CH_3 and CH from CH_2 groups in the structure. A complete assignment of signals of sodium oleyl hyaluronate is depicted directly in Fig. 3.

As it was expected, FT-IR spectra of modified HA were similar and independent of fatty acid used for modification. This is mainly typical for a series of derivatives where the only difference is the alkyl side chain length. Example of FT-IR spectra of native HA and sodium oleyl hyaluronate are shown in Fig. 4. The spectra of the derivatives contained many characteristic bands of HA and then stretch peak at 1733 cm^{-1} corresponding to ester carbonyl groups, which confirmed that fatty acid was forming covalent ester bonds with HA. Next, there was a decrease of the O–H stretching band at about 3600 cm^{-1} , a significant increase of C–H signal at 2925 cm^{-1} and formation of new band at 2854 cm^{-1} resulting from asymmetric and anti-symmetric vibrations of $-\text{CH}_2-$. There were not observed any signals typical for the free fatty acid (Fig. 4), and any reagents used for modification and thus confirming high product purity.

Formation of the linkage between fatty acid chain and HA was also established for all the derivatives by diffusion ordered NMR spectroscopy (DOSY) experiment. Because of the marked difference between the diffusion coefficients of the low molecular weight fatty acid and HA, the DOSY map can easily establish the presence of non-attached fatty acid groups to HA, which obviously diffuse much faster than the bound acyl groups. A typical DOSY NMR of esterified HA (oleyl derivative) is shown in Fig. 5. As compared to free oleic acid, an obviously slow diffusion of the bound oleyl chains confirms the formation of linkage between fatty acid and

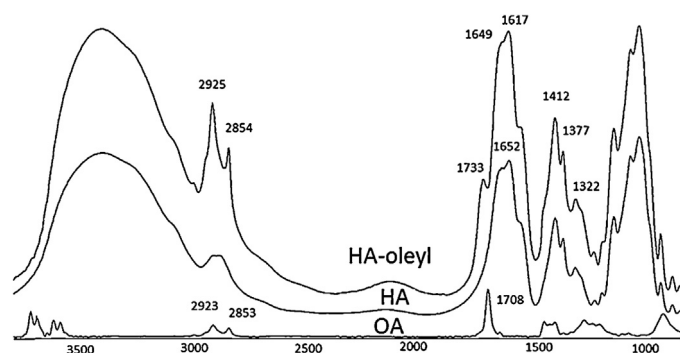


Fig. 4. Infrared spectra of oleic acid (OA), hyaluronan (HA) and sodium oleyl hyaluronate (SO-HA) DS = 12%, Mw = 15 000 g/mol.

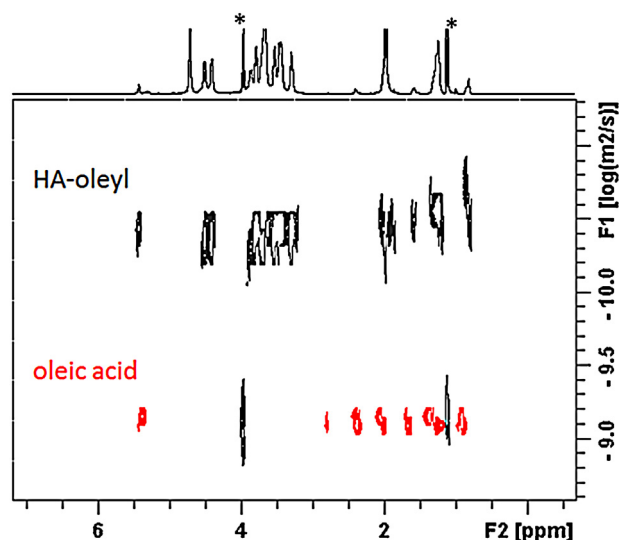


Fig. 5. DOSY NMR spectra of sodium oleyl hyaluronate in D_2O (Mw = 15 000 g/mol, DS = 12%, $c = 10\text{ mg/ml}$) and oleic acid in CDCl_3 .

HA. DOSY experiment showed similar diffusion behavior for all signals (except for isopropanol signal*), and thus indicated that all of the proton resonances in this region belonged to one structural complex (sodium oleyl hyaluronate). In addition, it can be well recognized that the diffusion of acyl chains is even slower than the diffusion behavior of HA (i.e. signals corresponding to fatty acid are placed at larger $-\log D$ values along y axis). This means that there is aggregation between the hydrophobic acyl chains leading to a formation of hydrophobic domains with more restricted motion and thus slower diffusion. The possible aggregation was further confirmed during rheology analyses, where the dynamic viscosity of modified HA was much higher than starting HA used for the modification (Fig. 6). In addition, the aggregation of acyl chains was also detected by Cryo-SEM analyses indicating formation of spherically shaped vehicles with average diameter size about 60 nm (Fig. 7). This figure also depicts possible self-aggregation of the hydrophobized HA derivative. As it was expected, the aggregated derivatives contained hydrophobic domains because they were able to dissolve non-polar dyes (data not shown). Similarly to other hydrophobized HA (Eenschooten et al., 2010), the aggregation of acyl chains was detected from very low concentrations ($<0.01\text{ mg/mL}$) of the hydrophobized derivatives and was influenced by DS, Mw and length of acyl chain of the derivative. A detailed study about the HA self-assembled aggregates is currently under investigation.

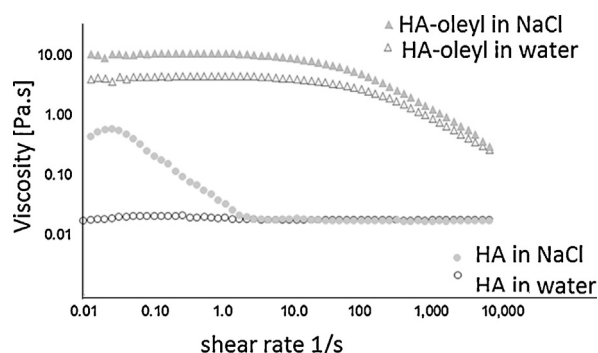


Fig. 6. Flow curve for HA (14 700 g/mol, 1%, w/v solution) and sodium oleyl hyaluronate (DS = 12%, 15 000 g/mol, 1%, w/v solution) at 25°C in H_2O and 0.9% NaCl.

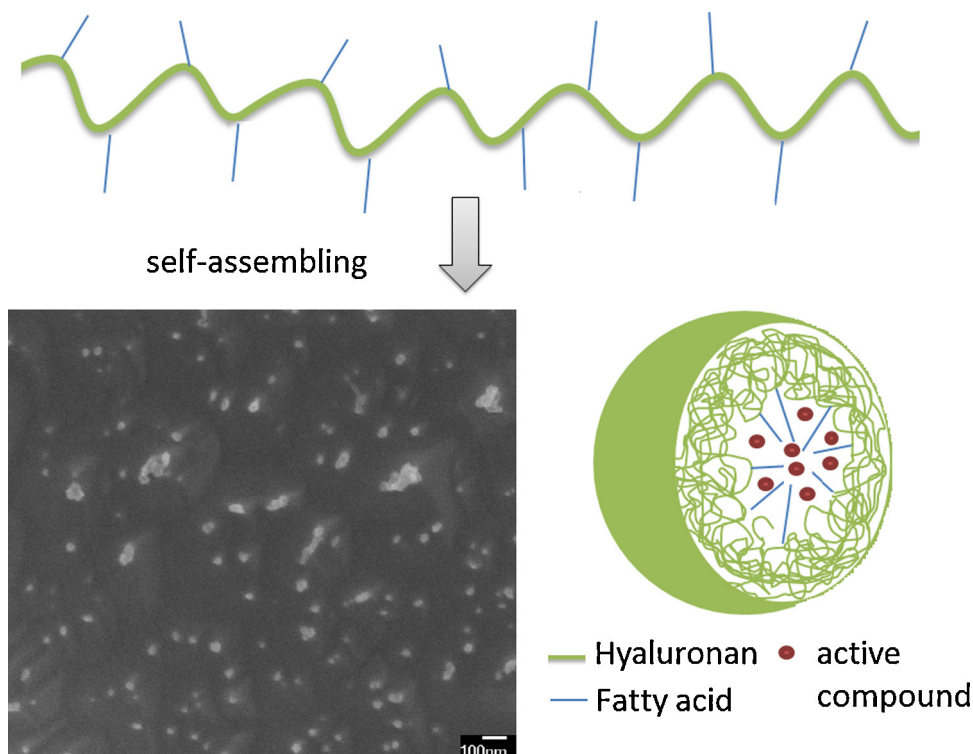


Fig. 7. Cryo-SEM image of micelles made in base of sodium oleyl hyaluronate (DS = 12 and Mw = 15 000 g/mol) and the schematic representation of self-assembling of this derivative.

4. Conclusions

The use of TCBC proved to be a good alternative for the chemical modification of HA in position C(6) of the polysaccharide (primary alcohol). TCBC had been effectively applied for the activation of short (C6–C8), medium (C10–C14) and long (C16–C18) fatty acids. In fact, TCBC failed to activate very long fatty acids (C20 and longer). In situ activation of the fatty acids with TCBC yielded products with degree of substitution up to 36%. The main advantages of the proposed reaction pathway are (i) the mild reaction conditions during which the native HA does not undergo any significant degradation, (ii) short reaction time and (iii) in situ formation of mixed anhydride. Unfortunately, it was demonstrated that the most restricting parameter for the reaction is the length of the fatty acid. In addition, the maximum degree of substitution which can be achieved was found to be decreasing with increasing length of acyl chain. The amphiphilic HA derivatives self-aggregate in aqueous solution while forming spherical vehicles with hydrophobic domains in which non-polar drugs could be encapsulated.

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