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New amphiphilic hyaluronan derivatives based on modification with alkenyl and aryl succinic anhydrides

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ABSTRACT

Hyaluronic acid (HA) of various molecular weights (4000, 14,000, 30,000, 100,000 and 850,000 g/mol) was modified with aryl and alkenyl succinic anhydrides (ASA). The modifications were conducted at pH 8.0–8.5 for 16 h at 25 or 60 °C. The ^{1}H NMR spectrum of octenyl succinic anhydride modified HA (OSA-HA) was elucidated using 2D NMR (^{1}H,^{1}H g-COSY) spectroscopy. The modification was confirmed and the degree of substitution (DS) of 1.5–19% was determined using ^{1}H NMR spectroscopy. Light scattering (SEC-MALLS-RI) showed that negligible degradation of HA had occurred during the modification procedure. Further, dynamic measurements of surface tension (γ) was done on 0.01–1% OSA-HA (M_w = 30,000 g/mol, DS = 19%) showing γ < 50 Nm/m. Titration of the surface tension was done with OSA-HA (DS = 19%, M_w = 30,000) in 0.1 M NaCl, giving a critical aggregation concentration (CAC) of 25 g/L, showing that ASA-HAs form micelles/aggregates above a certain concentration. The emulsifying properties of the ASA-HA derivatives were evaluated in a test formulation of 30% oil phase and 70% water phase (containing 0.1 M NaCl and 0.2% ASA-HA). The stability of the emulsions was evaluated by visual inspection after 24 h and 3 weeks after storage dark at ambient temperature. The ASA-HA stabilized emulsions better than non-modified HA due to their surface activity.

1. Introduction

Hyaluronan, also known as hyaluronic acid (HA), is a linear biopolymer naturally abundant in mammalian tissues. A human adult contain approximately 15 g hyaluronic acid. Hyaluronic acid occurs in the eyes, skin (in both epidermis and dermis) and synovial fluid (Lepperdinger, Fehrer, & Reitinger, 2004). Further, hyaluronic acid makes up the backbone of the proteoglycan aggregates being a main component of sinew and cartilage (Heinegård, Björnsson, Mörgelin, & Sommarin, 1998). The polysaccharide has a high turnover in the body, 7 g/day, i.e. half of all HA in the body is exchanged every day (Lepperdinger et al., 2004). The molecular weight strongly depends on the biological origin (Lapcik, Lapcik, De Smedt, & Demeester, 1998). When produced by microbial fermentation the molecular weight ranges from just below 1×10^6 up to 4×10^6 g/mol (Armstrong & Johns, 1997). When prepared by extraction, molecular weights up to 6×10^6 have been reported (Lee & Cowman, 1994). Today, two microbial sources are exploited for hyaluronic acid production, *Streptococcus* strains (Sutherland, 1990) and recombinant *Bacillus subtilis* (Widner et al., 2005). In *Streptococcus* sp., hyaluronic acid is produced as part of the capsule bound to the cell membrane, making it challenging to isolate. Hyaluronic acid from *Bacillus*, on the other hand, is excreted extracellularly and can therefore conveniently be isolated as a high purity product.

The chemical structure consists of two alternating monosaccharide units, D-*N*-acetyl glucosamine (GlcNAc) and D-glucuronic acid (GlcA), connected by β-(1 → 3) and β-(1 → 4) glycosidic bonds, respectively. The negative charge on the repeating disaccharide causes many of the unique properties of this poly-electrolyte in solution (Tømmeraas & Wahlund, 2009). HA produces highly viscous solution in water (Gibbs, Merrill, Smith, & Chabrecek, 1968; Lapcik et al., 1998). Due to the alternating β-backbone, the viscosity of HA is strongly dependent on ionic strength (Hayashi, Tsutsumi, Norisuye, & Teramoto, 1996; Smidsrød & Haug, 1971). As the concentrations of salts increase, the repulsion between negatively charged disaccharide-units decrease and the polymer change from a rod-like towards the flexible confirmation of the random coil (Furlan, La Penna, Perico, & Cesàro, 2005).

The use of alkenyl succinic anhydrides (ASA) for making polysaccharides hydrophobic is well established in industry. Dispersions consisting of mixtures of long alkenyl chain succinic anhydrides (*n*-hexadecenyl and *n*-octadecenyl succinic anhydride) is used in paper industry for making the paper-surface more hydrophobic and water resistant (Chen & Woodward, 1986; Jansson & Järnström, 2005; Takihara, Yoshida, & Isogai, 2007). In the food industry, *n*-octenyl succinic anhydride (OSA) modified starches with degrees of substitution (DS) of up to 3% have been approved for more than

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30 years as an ingredient in sauces, puddings, infant formulas and low-fat food margarines and mayonnaises for stabilization of O/W emulsions (Trubiano, 1986; Wurzburg, 1995). Several clinical studies have been performed on OSA starch and it has been found that the OSA is and its metabolites are excreted in the urine (Jarowenko, 1986; Heacock, Hertzler, & Wolf, 2004). Another benefit of the OSA modified starches is the significant change in rheological properties when compared to non-modified starches (Bao, Xing, Phillips, and Corke (2003); Biswas, Shogren, Kim, and Willett (2006); Kelley, 1991; Lawal, 2004). In cosmetics, 'free-flowing starch' is starch granules that have been surface modified by OSA, followed by cross-linking with Al^{3+} ions. One of the major advantages of this modification chemistry compared to others previously developed for modification of HA (Chytil, Strand, Christensen, & Pekar, 2010; Park, Chung, & Yoo, 2004; Tölg, Hamilton, & Turley, 2004), is that the by-products has been shown to be non-toxic and the modification chemicals and procedure is both easy and in-expensive requiring only one step (Heacock et al., 2004; Takihara et al., 2007; Trubiano, 1986).

Hyaluronic acid has many potential applications in biomaterials due to its biocompatibility and biodegradability (Park et al., 2004). Further, there are several possible applications in drug delivery due to the number of receptors in the body that have specific affinity for HA that can be targeted, e.g. RHAMM and CD44 (Knudson & Peterson, 2004; Shu & Prestwich, 2004). One major challenge is to modify and customize the biodegradation and solution properties of the HA. Therefore, a number of chemical modification technologies have been developed (Park et al., 2004; Tölg et al., 2004). In this work, we present a new family of hyaluronic acid derivatives that make the HA molecules more amphiphilic without the need of harsh organic chemicals or solvents giving the benefit of minimal degradation or side-reactions occurring on the HA. Not only is the chemical structure elucidated, but also the solution properties of this new family of HA-derivatives are studied. Some of the individual derivatives have been further characterized in other works (Eenschooten, Guillaumie, Kontogeorgis, Stenby, & Schwach-Abdellaoui, 2010; Neves-Petersen et al., 2010).

2. Experimental

2.1. Materials

2.1.1. Hyaluronan

Seven experimental samples of hyaluronan (HA) with various molecular weights were obtained from Novozymes Biopolymer A/S as sodium salts. All samples were produced by fermentation of *B. subtilis* (Widner et al., 2005). Molecular weight was adjusted by acid hydrolysis (Tømmeraas & Melander, 2008) and were purified by dialysis (regenerated cellulose tubes from SpectraPor 4, MWCO 12–14 kDa) against de-ionized water (Milli-Q) until the conductivity was below 5 μSi/cm before freezing at −20 °C followed by lyophilization. The weight average molecular weight (M_W) for the samples was determined using SEC-MALLS-RI (Melander & Tømmeraas, 2010; Tømmeraas & Melander, 2008) to; 4000, 14,000, 20,000, 30,000, 100,000, 320,000 and 850,000 g/mol.

2.1.2. Alkenyl-/aryl succinic anhydrides

n-Octenyl succinic anhydride (OSA) (d.: 1.000, M_W 220.27, 97% purity) were obtained from Sigma–Aldrich. All other alkenyl and aryl succinic anhydrides (ASA) were kindly provided by Pentagon Chemical Specialties Ltd. (Workington, UK); phenyl succinic anhydride (PhSA), *n*-nonenyl succinic anhydride (NSA), *n*-dodecenyl succinic anhydride (DDSA), *n*-hexadecenyl succinic anhydride (HDSA) and *n*-octadecenyl succinic anhydride (ODSA). All other chemicals used were obtained from Sigma–Aldrich and were of p.a. quality if not otherwise stated.

2.2. Preparation of aryl and alkenyl succinic anhydride hemiesters of hyaluronan

2.2.1. Preparation of low molecular weight (LMW) hyaluronan derivatives

LMW HA (4000, 14,000, 23,000, 30,000 or 100,000 g/mol, 2.50 g, 12.5 mmol HA-monomer) was dissolved overnight at room temperature in deionized water (50 mL) before adjusting pH to 8.5. ASA was added under strong agitation of either equimolar (12.5 mmol; HA-monomer:ASA ratio 1:1) or 1/10 (1.25 mmol; HA:OSA ratio 10:1) of the molar concentration of HA monomers. OSA, NSA, DDSA were added as oil and the reaction proceeded at room temperature (25 °C). PhSA was added as powder a powder gradually during the reaction (portions of three; 1/3 was added at the beginning, 1/3 was added after 3 h and the last portion was added after 6 h) and the reaction was kept at room temperature. HDSA and ODSA were warmed up above their melting point (approx. 50 °C) and added as oil and the reaction solution was kept at 60 °C during the reaction. The reactions proceeded for 16 h with strong agitation (approx. 600 rpm, magnetic stirrer). During the reaction, the pH was maintained at 8.5–9.0 by use of a pH stat (adding 0.5 M NaOH). The products were dialyzed against de-ionized water (25 °C, 7 L, MWCO 12–14 kDa). The water external to the dialysis tube was changed every 3 h (or overnight) until the conductivity of the external solution had reached the same level as de-ionized water (approx. <5 μSi/cm).

2.2.2. Preparation of high molecular weight hyaluronan derivatives

High molecular weight (HMW) hyaluronan (850,000 g/mol, 1.42 g, 7.1 mmol) was dissolved overnight at room temperature in de-ionized water (200 mL) before adjusting pH to 8.5 with 4 M NaOH. ASA was added under strong agitation of either equimolar (7.1 mmol; HA-monomer:ASA ratio 1:1) or 1/10 (0.71 mmol; HA:OSA ratio 10:1) of the molar concentration of HA monomers. The solution was left on strong agitation (approx. 600 rpm) for 16 h at room temperature or 60 °C. During the reaction, the pH was maintained above 8.5 by use of pH-stat. At the end of the reaction, the pH was adjusted to 6.8 with 1.0 M HCl. The product was purified by dialysis as described in Section 2.2.1, frozen and lyophilized.

2.3. Polymer characterization (SEC-MALLS-RI)

The HA samples were analyzed using SEC-MALLS-RI system consisting of a Waters Alliance HPLC with Wyatt Optilab rex RI detector and Wyatt EON MALLS detector. Four TSK columns (2500, 4000, 5000, 6000 PW_{XL}) were eluted with a buffer of 50 mM HaH_2PO_4 and 150 mM NaCl at a flow rate of 0.5 mL/min and 30 °C. Samples were prepared of approximately 0.1% w/v for high molecular weight samples (above 100,000 g/mol), smaller molecular weight samples were analyzed at higher concentrations (up to 1%) due to weaker light-scattering signal for smaller polymers. An injection loop of 500 μL was used. Refractive index increment (dn/dc) of 0.153 and second virial coefficient (A_2) of 2.3×10^{-3} were used in the processing of the data (Tømmeraas & Melander, 2008). All data were calculated in Astra software v.5.1.3.0 and Microsoft Excel.

2.4. Structural characterization of hyaluronan derivatives using 1D and 2D ^{1}H NMR spectroscopy

The LMW ASA-HA samples were studied with ^{1}H NMR spectroscopy. Samples were prepared by dissolving ASA-HA (10 mg/mL)

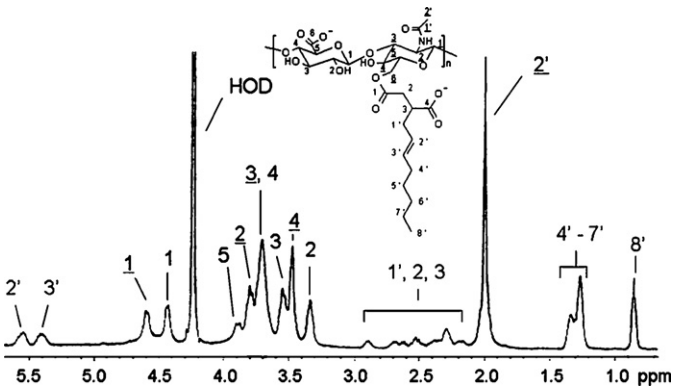


Fig. 1. The partially assigned ^{1}H NMR spectrum of the OSA-HA 4.

in D_2O with 5 μL from a 1% stock solution in D_2O of 3-(trimethylsilyl)-propionate-d_4 (TSP-d_4) added as internal standard. The solution (0.7 mL) was transferred to a 5-mm NMR tube before analysis. Spectra were acquired at a Varian Mercury 400 MHz at 30 and 80 °C. Conditions for 1D: Spectra were acquired using a 45° pulse angle, 256 scans, SW 6389.8 Hz, 2 s acquisition time and 1 s relaxation delay. The HMW ASA-HA samples for ^{1}H NMR spectroscopy were dissolving in D_2O (10 mg/mL). DCl (0.1 M total concentration) was added before the samples were heated to 60 °C for 48 h. The partially hydrolyzed samples were cooled to room temperature before the pD was adjusted to neutrality with 1 M NaOD. TSP-d_4 (5 μL from a 1% stock solution in D_2O) was added as internal standard before the solution (0.7 mL) was transferred to a 5-mm NMR tube before analysis as described for the LMW ASA-HA samples. One of the LMW OSA-HA samples were also analyzed by 2D NMR spectroscopy (^{1}H,^{1}H g-COSY) at 80 °C.

2.5. Preparation of emulsions containing ASA HA

Aqueous solutions (1% w/v) of ASA-HA products (OSA-HA 8, ODSA-HA 3, ODSA-HA 4, HDSA-HA 1, DDSA-HA 5 and DDSA-HA 6) and the HA starting material (200 mL) were prepared (freeze dried purified product was dissolved with stirring overnight at room temperature) in 0.1 M NaCl. Test formulations were prepared by the following general procedure: HA/ASA-HA (4 mL, 1%) was mixed with 0.1 M NaCl (10 mL) and pH adjusted to 7 before adding 6 mL mineral oil and high shear mixing (Ultraturrax, 25 s, 24,000 min^{-1}). The formulations were stored dark at room temperature for 8 weeks. Visual inspections were done after 24 h and 8 weeks.

3. Results and discussion

3.1. Preparation of alkenyl succinic acid anhydride derivatives of hyaluronic acid

The modification of hyaluronic acid by esterification with alkyl/aryl succinate anhydrides (ASA) to form hemi-esters is illustrated in Scheme 1. To determine the degree of substitution of the ASA-HA derivatives, ^{1}H NMR spectroscopy was used. The ^{1}H NMR spectrum of OSA-HA (100,000 g/mol) is shown in Fig. 1 with partial assignment of the peaks. The spectrum was elucidated using 2D NMR spectroscopy together with the literature values for hyaluronic acid (Siciński, Adams, & Lerner, 1993; Tawada et al., 2002). The ^{1}H,^{1}H g-COSY spectrum of OSA-HA (30,000 g/mol) is displayed in Fig. 2. The degree of substitution (DS) was determined by comparing the intensity (I) of the peak from the $-CH_3$ (at δ = 0.7 ppm) group from terminal end of the alkenyl chains with that of the acetyl $-CH_3$ (at δ = 2.04 ppm) of the GlcNAc unit of HA by the relationship: $DS = [1/3 \times I_{(ASA,-CH_3)}]/[2/3 \times I_{(GlcNAc,-CH_3)}] \times 100\%$.

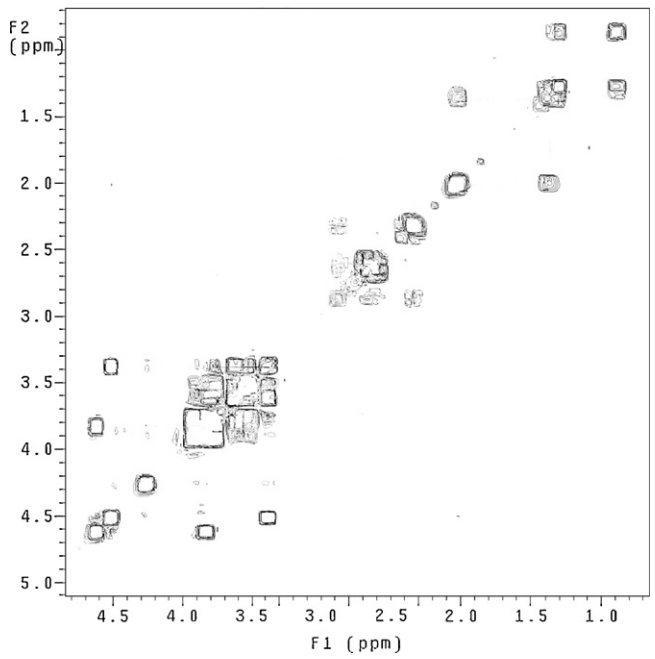
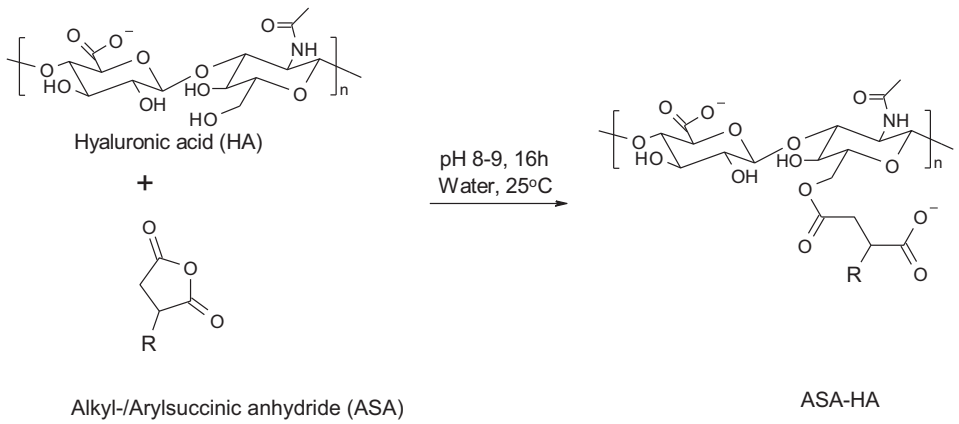


Fig. 2. ^{1}H,^{1}H g-COSY spectrum of OSA-HA 4.

Alternatively, the DS was calculated by comparing the intensity of the vinyl protons of the ASAs (5.4 and 5.6 ppm) with that of the peak acetyl protons in GlcNAc (2.04 ppm). Table 1 lists the various ASA-HA derivatives prepared with their determined DS. For the high molecular weight derivatives, the viscosity and solubility became limiting (turbid solutions were observed for some of them at higher concentrations). Therefore, a partial hydrolysis in DCl was performed to achieve higher resolution in the NMR analysis (48 h, 60 °C, 0.1 M DCl in D_2O). Some of the higher DS samples precipitated during the hydrolysis and did not re-dissolve upon neutralization. This is probably due to a combination of strong hydrophobic interaction between the modified parts of the polymer in addition to the effect of the increased ionic strength, enhancing the aggregation tendencies of the derivatives. It was not possible to determine in which hydroxyl moieties the ASAs reacted, or how evenly distributed the modification was along the polymer chain. According to earlier studies on starches (Shogren, Viswanathan, Felker, & Gross, 2000) both primary and secondary hydroxyl groups of the glucose repeating unit have been observed to react with the ASAs. Therefore, it should be expected that the same is the case for HA and most other polysaccharides. When it comes to distribution of the substituents along the HA backbone, it would be expected that this would depend on the molecular weight of the HA being modified and the concentration and solubility of the ASA in the reaction media. In general, most ASAs have a rather low solubility in water, e.g. OSA has a solubility <1% v/v. The solubility decreases with increasing number of $-CH_2-$ groups in the alkenyl chain. Therefore, the dissolution of the ASA in water is a rate limiting step for the reaction with HA, and becomes more important as ASA with longer alkenyl chains are used. During all experiments on high MW HA it was observed that the ASA formed amber-colored oil droplets that gradually divided into smaller droplets because of the agitation and the formation of anionic surfactants when ASAs reacts with water. At the end of the reaction, the solution was opaque which can be interpreted as formation of micelles or micro-scale aggregates/droplets above 500 nm in size. Even after purification by precipitation or dialysis for removal of excess OSA, this phenomenon was still observed to different extents depending on the MW of the starting HA and the length of the alkenyl side chain in the ASA.



Scheme 1. Schematic representation of ASA modification of hyaluronic acid. R: phenyl ($-C_6H_6$), n-octenyl ($-CH_2CH{=}CH(CH_2)_4CH_3$), n-dodecenyl ($-CH_2CH{=}CH(CH_2)_8CH_3$), n-hexadecenyl ($-CH_2CH{=}CH(CH_2)_{12}CH_3$), n-nonenyl ($-CH_2CH{=}CH(CH_2)_5CH_3$) or n-octadecenyl ($-CH_2CH{=}CH(CH_2)_{14}CH_3$).

An initial observation during initial experiments was that the pH fell gradually during the reaction due to the opening of the succinic anhydride ring, producing a weak carboxylic acid moiety. Therefore, it was necessary to either buffer the system with e.g., $NaCO_3$ or by continuous pH adjustment with base using a pH stat. It has previously been seen that it is important to keep the pH above 8.0 for the reaction to proceed efficiently and at the same time below 9.0 to avoid release of the ASA groups by hydrolysis (Bhosale & Singhal, 2006; Jeon, Viswanathan, & Gross, 1999; Trubiano, 1986). A statistical analysis of the prepared ASA-HAs presented in Table 1 was performed using SAS jmp. It was found that there was a statistically significant (with 95% confidence) inverse correlation between the DS obtained and the HA starting molecular weight. Equally, a negative correlation was found with the length of the alkyl side chain (i.e., the number of $-CH_2-$). The results of the analysis are summarized in the bivariate fits in Fig. 3A, B and C, D for reactions using 1:1 and 1:10 ratio of HA: ASA, respectively. This is in agreement with studies by Jeon et al. (1999) that found that the DS on starch decrease with increasing chain-length of the ASA.

3.2. Polymer characterization of the hyaluronan derivatives

Both the native HA samples and the prepared ASA-HAs were analyzed using SEC-MALLS-RI. Unfortunately, ASA-HAs of higher molecular weight interacted strongly with the column material, impairing analysis of polymer conformation. Still, this is a good indication in itself that the solution properties of the prepared ASA-HA material were changed compared to the native biopolymer. Meaningful analysis was only possible of the lower molecular weight ASA-HAs. In general, only a negligible change in M_w is seen after modification. No degradation of the polymer has happened during the modification procedure. For some samples, a slight increase of M_w was seen, which is probably due to a higher presence of aggregates in the samples due to the hydrophobic interactions between polymer chains.

3.3. Characterization of the surface activity of OSA-HA

HA is a very hydrophilic polymer that easily dissolves in water and that is without any surface activity. Already during the purifi-

Table 1
Results from the preparation of ASA-HA.

ASA-HA	Succinic anhydride side chain length	M_w (starting material) $\times 10^3$ g/mol	Molar ratio ASA:HA-monomer	DS[a] %
OSA-HA 1	C_8	4.8	1:1	13
OSA-HA 2	C_8	14	1:10	3.5
OSA-HA 3	C_8	14	1:1	9.7
OSA-HA 4	C_8	30	1:1	12
OSA-HA 5	C_8	30	1:10	2.6
OSA-HA 6	C_8	30	1:1	12
OSA-HA 7	C_8	850	1:10	0.30
OSA-HA 8	C_8	850	1:1	2.5
PhSA-HA 1	C_6	14	1:10	3.4
PhSA-HA 2	C_6	14	1:1	15
PhSA-HA 3	C_6	100	1:10	2.1
PhSA-HA 4	C_6	100	1:1	19
NSA-HA 1	C_9	14	1:1	11
NSA-HA 2	C_9	14	1:10	2.1
DDSA-HA 1	C_{12}	14	1:1	2.2
DDSA-HA 2	C_{12}	14	1:10	1.7
DDSA-HA 3	C_{12}	23	1:1	2.1
DDSA-HA 4	C_{12}	23	1:10	n.d.[b]
DDSA-HA 5	C_{12}	850	1:10	0.10
DDSA-HA 6	C_{12}	850	1:1	n.d.[b]
HDSA-HA 1	C_{16}	850	1:1	n.d.[b]
ODSA-HA 1	C_{18}	23	1:1	13
ODSA-HA 2	C_{18}	23	1:10	0.80
ODSA-HA 3	C_{18}	850	1:10	0.16
ODSA-HA 4	C_{18}	850	1:1	n.d.[b]

[a] DS was determined by ^{1}H NMR spectroscopy per monosaccharide unit of HA.
[b] Spectrum resolution too low to determine DS.

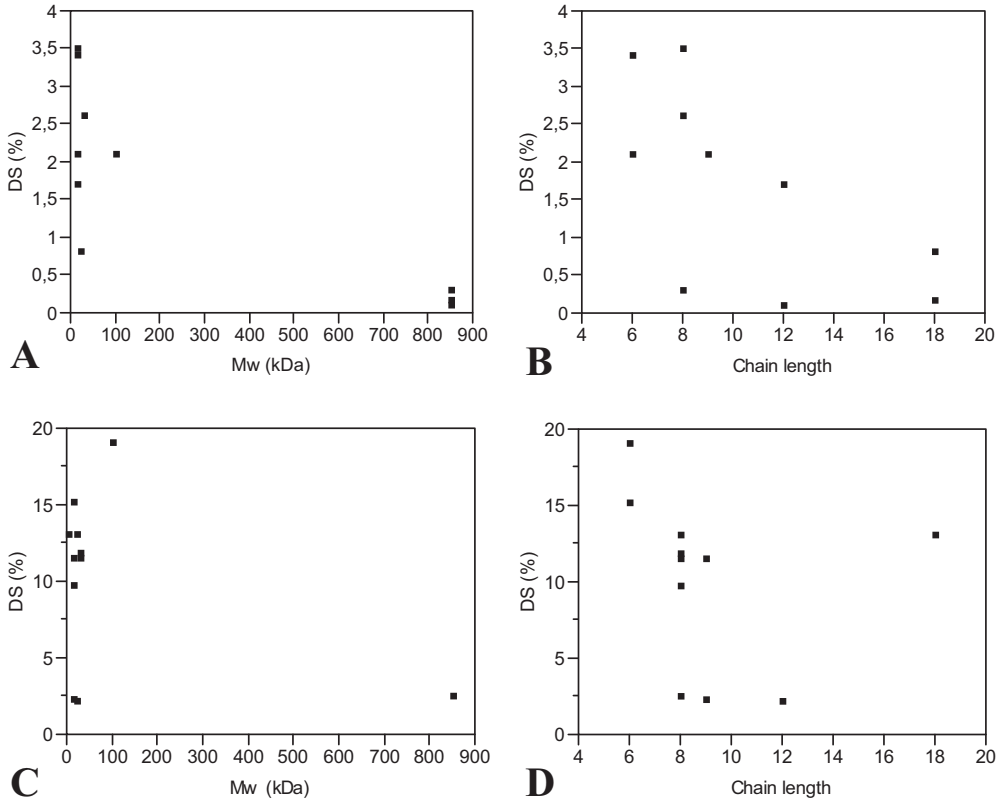


Fig. 3. (A) Bivariate fit of DS (%) by M_w (kDa) (ASA:HA molar ratio 1:10). (B) Bivariate fit of DS (%) by chain length (ASA:HA molar ratio 1:10). (C) Bivariate fit of DS (%) by M_w ($\times 10^3$ g/mol) (ASA:HA molar ratio 1:1). (D) Bivariate fit of DS (%) by succinic anhydride side chain length, i.e. number of C (ASA:HA molar ratio 1:1).

cation of the ASA-modified HAs it was observed that aqueous solutions of some of the ASA-HAs could stabilize foam for several days. A study of the surface activity of the OSA-HA was conducted. Three solutions containing 0.01, 0.1 and 1% w/v of OSA-HA 4 in 0.1 M NaCl was studied as function of time with the Wilhelmy plate method. For comparison, also a solution of unmodified HA of the same molecular weight was included in the study. The results are presented in Fig. 4. As can be seen, there is a clear tendency that the OSA-HA has a lower surface tension then both water and the reference non-modified HA solution. Further, the surface tension decreased markedly with increasing concentrations of OSA-HA. Another observation was that the surface tension for the solutions containing OSA-HA continues to decrease during the duration of the measurement (>1 h). This is explained by that the OSA-HA molecules are gradually diffusing to the air/liquid interface. Since they are large molecules, the diffusion constant can be expected to be low and therefore the process takes a long time compared to what is expected for smaller surfactants. Further, a 4% w/v of OSA-HA 4 (MW 30,000 g/mol, DS 19%) was titrated into a solution of constant ionic strength (0.1 M NaCl) during measurement with the Wilhelmy plate method (see Fig. 5), and show that the ASA-HAs form micelles or aggregates above a certain critical aggregation concentration (CAC). From Fig. 5, the CAC was determined to 25 g/L (equals to 8.5×10^{-4} mol/L when using the determined weight average molecular weight of 30,000 g/mol) with a surface

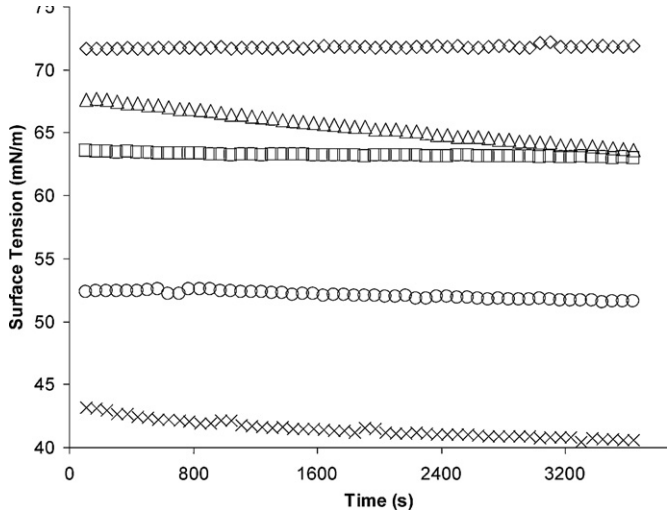


Fig. 4. Dynamic surface tension measurements on HA and LMW OSA-HA using Wilhelmy plate. Legend: (◊) reference de-ionized water, (□) 0.1% (w/w) non-modified HA (30×10^3 g/mol), (△) 0.01% (w/w) OSA-HA 6 (30×10^3 g/mol), (○) 0.1% (w/w) OSA-HA 6 (30×10^3 g/mol), (×) 1% (w/w) OSA-HA 6 (30×10^3 g/mol).

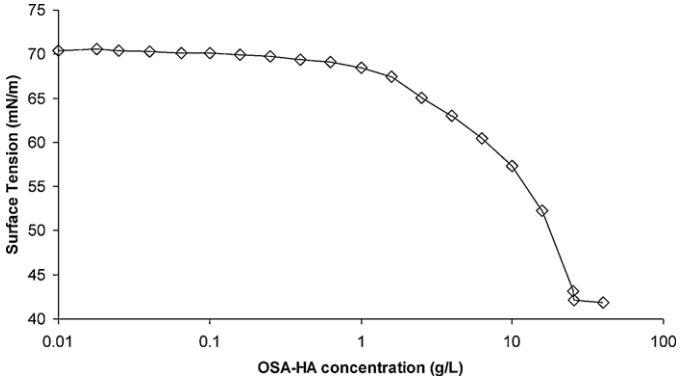


Fig. 5. Surface tension titration of OSA-HA 4 in 0.1 M NaCl.

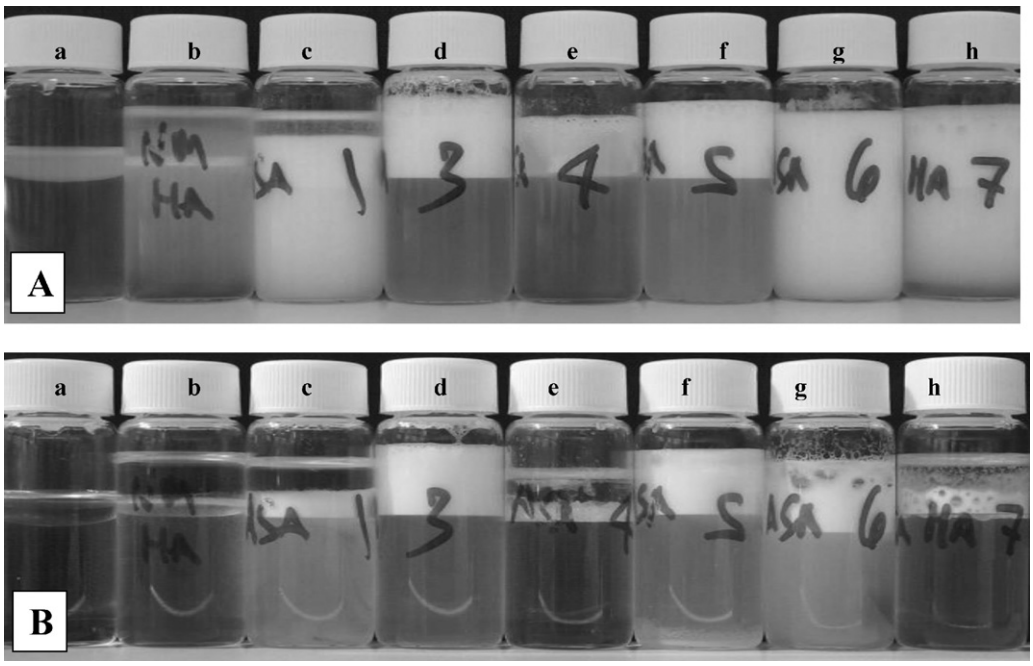


Fig. 6. (A) Mineral oil formulations after 24 h at room temperature. 0.2% HA/ASA-HA, 30% oil phase, 70% aqueous phase, 0.1 M NaCl. From the left: (a) water/oil without HA, (b) non-modified HA (850,000 g/mol), (c) OSA-HA 8 (d) ODSA-HA 4, (e) ODSA-HA 3, (f) HDSA-HA 1, (g) DDSA-HA 6, (h) DDSA-HA 5. (B) Mineral oil formulations after 8 weeks at room temperature. 0.2% HA/ASA-HA, 30% oil phase, 70% aqueous phase, 0.1 M NaCl. From the left: (a) water/oil without HA, (b) non-modified HA (850,000 g/mol), (c) OSA-HA 8 (d) ODSA-HA 4, (e) ODSA-HA 3, (f) HDSA-HA 1, (g) DDSA-HA 6, (h) DDSA-HA 5.

tension of 42.1 mN/m. For comparison, a HA of 840,000 g/mol modified with C_8 hydrophobic chain (DS 18.8%), linked by a carbamate bond, was determined to have a CAC of 6.5×10^{-7} mol/L and surface tension of 31.8 mN/m in 0.1 M NaCl (Chytil & Pekař, 2009). The reason for OSA-HA having a higher CAC is probably that the MW is only 1/28 part of the MW compared to the HA modified by the C_8-carbamate and therefore had a much higher critical overlap concentration. In addition, the OSA modification also introduces an additional carboxylic acid moiety that will reduce the hydrophobic character of the side chain and the hydrophobic chain has an unsaturated bond giving a different packing then a straight alkyl-chain. The CAC is the concentration when the water surface is saturated with polymer and further addition leads to formation of micelles (Chytil & Pekař, 2009). The property that micelles are formed is an important characteristic that can be exploited in drug-delivery systems for therapeutical agents that are poorly soluble in water. Probably, the hydrophobic character of the ASA-HA can be tailor-made by a combination of modifying the chain length of the ASAs, the degree of substitution and the HA molecular weight since all of these parameters have been shown for other hydrophobically modified HAs to have an effect on the CAC (Chytil & Pekař, 2009; Chytil et al., 2010; Duval-Terrié, Huguet, & Miller, 2003; Gang-Biao, Daping, Kairong, & Haihua, 2006; Jeong, Kang, Yang, Park, & Kim, 2005).

3.4. Emulsifying properties of ASA-HA

To evaluate the potential applications of ASA-HA in cosmetic or pharmaceutical formulations, their emulsifying properties were tested by a simple experiment: Three oils with different properties were mixed in water with or without the addition of HA or ASA-HA and subjected to emulsification using a homogenizer. All emulsions were made at the same ionic strength (0.2 M NaCl). The emulsions were then stored at room temperature in the dark for 8 weeks. After 24 h and 8 weeks, the solutions were visually inspected (see Fig. 6A and B, respectively) and the stability of the emulsions was evaluated (see Table 2). As can be seen, all the ASA-HAs stabilized emulsions better then the unmodified HA, telling us that the ASA groups give additional stabilization beyond only the effect of the viscosity increase in the aqueous phase caused by polymer addition. Starch modified with OSA stabilizes efficiently emulsions at very low concentrations even with DS at 2% or below which is the maxi-

Table 2
Stability of test formulations using various ASA-HA products (see Table 1 and cosmetic oils ('no' means two phases without any emulsion phase, '+', '++', '+++' means increasingly good emulsification, '(rev.)' means reversed phases).

Sample ID	Formulation stability					
	Mineral oil		Diethylhexyl carbonate		Ethylhexyl palmitate	
	24 h	8 weeks	24 h	8 weeks	24 h	8 weeks
No HA	No	No	No	No	No	No
HA (850,000 g/mol)	No	No	No	No	No	No
OSA-HA 8	+++	+	+++	+	+++	++
ODSA-HA 4	++	++	++	++	++	++
ODSA-HA 3	+	+	+	+	+	+
HDSA-HA 1	++	++	+++	+++	++	++
DDSA-HA 6	+++	++	+++	++	+++	++
DDSA-HA 5	+++	+	++ (rev.)	+ (rev.)	+ (rev.)	+ (rev.)

mum DS allowed for starch in food products (Nilsson & Bergenståhl, 2006; Nilsson, Leeman, Wahlund, & Bergenståhl, 2006). Starch contains amylopectin which is very large branched polysaccharides. If modified only to a low extent, the molecules can adsorb to the surface of oil droplets and thereby making emulsions more stable by steric stabilization (Nilsson & Bergenståhl, 2006). Since HA is a very hydrophilic molecule, the addition of hydrophobic moieties enable the polymer to adsorb to the oil/water interface and thereby stabilize the emulsion probably in very much the same way as has been seen for OSA-starch.

4. Conclusion

Both high and low MW hyaluronic acid was successfully modified with 2-alken-1-yl and phenyl succinic anhydrides (ASA). It was found that the ASA-HAs had a critical aggregation concentration (CAC) above which they form micelles. In addition, they were shown to stabilize emulsions efficiently, properties making these new biocompatible HA-derivatives interesting for potential applications in drug delivery systems of poorly soluble active pharmaceutical ingredients.

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