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Preparation and structural characterisation of novel and versatile amphiphilic octenyl succinic anhydride–modified hyaluronic acid derivatives

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ABSTRACT

The purpose of the present study was to prepare amphiphilic hyaluronic acid (HA) derivatives and to study the influence of a selection of reaction parameters on the degree of substitution (DS) of the derivatives. Octenyl succinic anhydride (OSA)–modified HA (OSA–HA) derivatives were prepared and structurally characterised by Fourier transform-infrared spectroscopy and proton nuclear magnetic resonance spectroscopy (^{1}H NMR). The influence of four reaction parameters on the DS of the derivatives was studied by means of an experimental design. The results showed that the OSA/HA molar ratio, the buffer ($NaHCO_3$) concentration and their interaction had the largest influence while the HA concentration and the reaction time only had a negligible effect. According to ^{1}H NMR the maximum DS achieved within the experimental conditions tested was 43% per disaccharide unit. Moreover optimal reaction conditions were identified for the preparation of versatile OSA–HA derivatives with a DS between 1.5% and 43%.

1. Introduction

Hyaluronic acid (HA) is a natural linear polysaccharide consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine residues linked through β-1,3 glycosidic bonds while the consecutive disaccharide repeating units are linked through β-1,4 bonds (Weissman & Meyer, 1954). While HA belongs to the class of amino sugar-containing polysaccharides known as the glycosaminoglycans (GAG) it is the only non-sulphated GAG. In vertebrates HA is ubiquitous in all organs and fluids and in the extracellular matrix (ECM) of soft connective tissues (Baier Leach & Schmidt, 2004). In humans HA is most abundant in the skin where it amounts to approximately 5 g or a third of the total body's HA content (Brown & Jones, 2005). As biomacromolecule HA possesses a large number of structural and biological functions. For example, in the ECM, HA provides a backbone for the distribution and organisation of important components such as proteoglycans, fibrin, fibronectin, collagen and elastin (Chen & Abatangelo, 1999). HA also triggers fundamental processes by binding with cells through specific interactions with hyaladherins. It is among others involved in complex signalling events such as cell proliferation, differentiation and migration (Baier Leach & Schmidt, 2004).

Due to its natural biocompatibility, resorbability, biological functions and due to the ease of its chemical functionalisation HA currently represents one of the most attractive building blocks for the design of advanced biomaterials with applications in the

Abbreviations: as, asymmetric; bd, bending; *C*(HA), HA concentration; *C*($NaHCO_3$), $NaHCO_3$ concentration; d, doublet; DMSO, dimethyl sulfoxide; DS, degree of substitution; ECM, extracellular matrix; FT-IR, Fourier transform-infrared spectroscopy; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcNAc, *N*-acetyl glucosamine; HA, hyaluronic acid; HA21, hyaluronic acid characterised by a weight average molecular weight of 21,000 Da; HA750, hyaluronic acid characterised by a weight average molecular weight of 750,000 Da; H-m, methyl proton; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; HPLC, high performance liquid chromatography; m, multiplet; MWCO, molecular weight cut-off; MV, model validity; MW_w, weight average molecular weight; OS, octenyl succinate; OSA, octenyl succinic anhydride; OSA–HA, octenyl succinic anhydride–modified hyaluronic acid; OSA/HA MR, OSA/HA molar ratio; Q^2, prediction coefficient; R^2, regression coefficient; RC, reproducibility coefficient; Rt, room temperature; s, singlet; SEC–MALLS, size exclusion chromatography–multi angle laser light scattering; st, stretching; sy, symmetric; *t*, reaction time.

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pharmaceutical and medical fields (Kogan, Soltes, Stern, & Gemeiner, 2007; Liao, Jones, Forbes, Martin, & Brown, 2005). However the properties of native HA can be incompatible with innovative applications such as, for instance, the design of HA-based nanocarriers towards the encapsulation and controlled/sustained delivery of hydrophobic compounds. Indeed, due to its high hydrophilicity, HA does not spontaneously assemble into segregated particles in aqueous solvents nor possesses the ability to stabilise hydrophobic substances. To overcome these limitations the physico-chemical properties of HA can be modified by the introduction of hydrophobic groups onto its backbone using the carboxyl and/or the hydroxyl functions of the polysaccharide. Indeed the resulting amphiphilic HA derivatives potentially constitute a more favourable starting material for the design of nanostructures and the durable encapsulation of hydrophobic active ingredients.

Historically, Jeanloz and Forchielli (1950) were the first to report the modification of HA's carboxyl groups with diazomethane, acetic anhydride and triphenylchloromethane towards the preparation of methylated, acetylated and triphenylchloromethylated HA, respectively. In the late 1980s, Della Valle and Romeo (1989) patented the modification of HA's carboxyl groups with alkyliodides in dimethyl sulfoxide (DMSO) using the tetrabutylammonium salt of HA. Other more recent modification methods non-exhaustively include the modification of HA's carboxyl groups with: (i) alkylaldehydes in water/ethanol mixtures in the presence of sodium cyanoborohydride using an adipic dihydrazide–modified HA intermediate obtained in the presence of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (Creuzet, Kadi, Rinaudo, & Auzely-Velty, 2006), (ii) alkylbromides in DMSO (Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000) and (iii) alkylsilyldiazomethanes in methanol/diethyl ether mixtures using the protonated form of HA (Kumar, Longin, Schwach-Abdellaoui, & Gross, 2008). HA's hydroxyl groups have also been functionalised with: (i) acylchlorides in *N*,*N*-dimethylformamide in the presence of pyridine (Kawaguchi, Matsukawa, Gama, & Ishigami, 1991), (ii) alkyloxymethyloxiranes in water/DMSO mixtures (Mlochova et al., 2007), (iii) alkylalcohols in DMSO in the presence of potassium hydroxide using a *p*-toluene sulfonyl–modified HA intermediate (Benesova, Pekar, Lapcik, & Kucerik, 2006), (iv) aryl/alkyl vinyl sulfones in water/acetone mixtures in the presence of sodium hydroxide (Eenschooten & Christensen, 2007) and (v) poly(lactic acid) mono-acyl chloride in DMSO using the cetyltrimethyl ammonium salt of HA (Schwach-Abdellaoui, Vert, & Pravata, 2006). Some of the above-cited derivatives have been shown to possess an altered solubility in aqueous media compared to native HA and, in some instances, self-associative properties which can be used to prepare spontaneously-segregating structures in aqueous media. These potentially represent attractive starting materials for the design of nanoparticulate carriers.

However the modification methods currently used for the preparation of amphiphilic HA present some drawbacks. Firstly the modification reactions are often conducted in organic solvents or mixtures of organic solvents and water. In addition to raise occupational and environmental issues and limit the upscalability of the preparation methods, the use of organic solvents or mixtures of these with water often requires converting HA into salts soluble in organic media or preparing reactive intermediates. This not only adds complex steps to the preparation methods but also often results in HA degradation (Bergman, Elvingson, Hilborn, Svensk, & Bowden, 2007; Pelletier et al., 2000). Secondly the modification reactions involving HA's carboxyl groups neutralise negative charges along the polymer backbone at physiological pH. Yet in the perspective of exploiting the self-assembling properties of the derivatives towards designing particulate systems it is desirable to maintain some charge distribution in order to allow for electrostatically-induced particle stability. Besides this Benesova et al. (2006) have underlined that HA's carboxylate groups are biologically and physiologically relevant chemical functions which integrity is important to maintain. For all these reasons simple modification reactions in aqueous media under mild conditions and exclusively involving HA's hydroxyl groups could represent advantageous alternatives for the preparation of amphiphilic HA derivatives.

With this in mind a new modification method for the preparation of amphiphilic HA was developed in our laboratories. The latter is based on the reaction between HA and octenyl succinic anhydride (OSA) (Tømmeraas & Eenschooten, 2007). The objective of the present study was to optimise this method and prepare versatile HA derivatives with tailored and extended degrees of substitution (DS). To achieve this, an experimental design was implemented in which four different reaction parameters were simultaneously varied and their influence on the DS of the OSA–modified HA (OSA–HA) derivatives was studied in detail. The chemical structure of the derivatives was characterised by Fourier transform-infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR), a method which was also quantitatively used to determine the DS of the derivatives.

2. Materials and methods

2.1. Materials

HA (HyaCare®) with a weight average molecular weight (MW_w) of 750,000 Da (HA750) was provided by Novozymes Biopharma DK A/S (Bagsvaerd, Denmark). 21,000 Da-HA (HA21) was prepared from HA750 by acid degradation using the procedure described hereafter. OSA (210.27 Da, purity ⩾97%, mixture of *cis* and *trans*) was obtained from Sigma–Aldrich (Saint Louis, Missouri, United States). All salts were used as purchased without further purification. The water used for the sample preparation and dialysis was distilled and purified to a resistivity of 18.2 MΩ cm in a milli-Q apparatus (Millipore, Billerica, Massachusetts, United States).

2.2. Preparation of HA21

HA21 was prepared from HA750 by acid degradation using H_3PO_4 at 60 °C. HA750 (70 g) was dissolved in milli-Q water (7 L) at 4 °C for 16 h (overnight). H_3PO_4 (85%, 250 mL) was added to the HA750 solution and the mixture was stirred at room temperature (Rt) for 10 min. The resulting acidic HA solution was incubated in a water bath at 60 °C for 24 h. After degradation the HA solution was cooled to Rt. The pH of this solution was measured and adjusted to 7.0 with NaOH (4 M). The resulting product was purified by filtration through glass microfiber filters (GF/F 1825 110, porosity 0.7 μm; Whatman, Maidstone, United Kingdom). The permeate was purified by ultrafiltration (Centramate™ Tangential Flow Systems; Pall, East Hills, New York, United States) through a membrane cassette (Omega™ Centramate™; Pall, East Hills, New York, United States) at Rt. The membrane cassette had a molecular weight cut-off (MWCO) of 3,000 Da. The purification was monitored by conductivity measurements of the permeate and was stopped when the conductivity had reached a value inferior to 5 μS/cm. The purified retentate containing HA21 was finally freeze-dried. Approximately 56 g of HA21 (i.e. 80%) of the starting HA750 were recovered. The MW_w of HA21 was determined by size exclusion chromatography–multi angle laser light scattering (SEC–MALLS) and amounted to 20,500 Da.

The structure of HA21 was investigated by FT-IR. The assignment of HA21's vibrational bands gave the following results: 3431 nm, O–H and N–H stretching (st); 2931 nm, C–H st; 1700–1600 nm, COO^- asymmetric (as) st and N–H bending (bd); 1423 nm, COO^- symmetric (sy) st; 1392 nm, C–O–H bd;

1154 nm, C–O–C as st; 1077 and 1038 nm, C–OH st; 954 and 900 nm, COO^- bd; 808 nm, C–O–C sy st.

The structure of HA21 was also studied by ^{1}H NMR. The assignment of HA21's chemical shifts gave the following results: 2.0 ppm, s, 3 H, H-m; 3.2–4.0 ppm, m, 10 H, H-2, H-5′, H-4′, H-3, H-3′, H-5, H-6′b, H-4, H-6′a, H-2; 4.3–4.7 ppm, d, 2 H, H-1, H-1′.

2.3. Preparation of the OSA–HA derivatives

HA21 was dissolved in milli-Q water (50 mL) at Rt for 6 h. $NaHCO_3$ was added to the HA solution and mixed at Rt for 1 h. The pH of the HA solution was measured and adjusted to 8.5 with NaOH (0.5 M). OSA was added drop-wise to the alkaline HA solution under vigorous stirring. The reaction medium was mixed at Rt for the desired time. The resulting crude product was dialysed against milli-Q water (7.5 L) using molecularporous membrane tubing (Spectra/Por®4, MWCO 12,000–14,000 Da; Spectrum Laboratories, Rancho Dominguez, California, United States) at 4 °C. The surrounding milli-Q water was changed every third-hour three times then once after 16 h (overnight) until its conductivity had reached a value inferior to 5 μS/cm. The purified OSA–HA was finally freeze-dried.

The reaction parameters studied in the experimental design and their ranges are reported in Table 1. The number of moles of HA was expressed in terms of the number of moles of disaccharide repeating units. For the calculations the molecular weight of one disaccharide unit was approximated to 400 Da. The combinations of reaction conditions tested were determined using an experimental design software (MODDE 8; Umetrics, Umeaa, Sweden) and a two-level interaction full factorial design. The experimental DS values were fitted with a *linear plus interaction model* using a *partial least square* regression analysis. The accuracy of the model was determined by separating the residual sum of squares into pure error (experimental error) and lack of fit.

The structure of the OSA–HA derivatives was investigated by FT-IR. The assignment of the derivatives' vibrational bands gave the following results: 3431 nm, O–H (HA), N–H (HA) st and C–H st (vinyl, OSA–HA); 2931 nm, C–H st; 1700–1600 nm, COO^- as st (HA), N–H bd (HA), C=C st (OSA–HA) and C=O st (ester, OSA–HA); 1423 nm, COO^- sy st (HA); 1392 nm, C–O–H bd; 1154 nm, C–O–C as st; 1077 and 1038 nm, C–OH st; 954 and 900 nm, COO^- bd; 808 nm, C–O–C sy st.

The structure of the OSA–HA derivatives was also studied by ^{1}H NMR. The assignment of OSA–HA's chemical shifts gave the following results: 0.9 ppm (s, 3 H, H-10″), 1.3 ppm (m, 6 H, H-7″–H-9″), 1.4–1.8 ppm (m, 2 H, H-6″), 2.0 ppm (s, 3 H, H-m), 2.2–3.0 ppm (m, 5 H, H-1″–H-3″), 3.4–4.2 ppm (m, 10 H, H-2, H-5′, H-4′, H-3, H-3′, H-5, H-6′b, H-4, H-6′a, H-2), 4.6–4.8 ppm (d, 2 H, H-1, H-1′), 4.9–5.7 ppm (m, 2 H, H-4″, H-5″).

2.4. Characterisation methods

2.4.1. SEC–MALLS

Each sample (10 mg) was dissolved at Rt in the mobile phase (10 mL) which was prepared as followed: NaCl (45.0 g), NaH_2PO_4 (7.8 g) and NaN_3 (1.25 g) on the one hand and NaCl (45.0 g), Na_2HPO_4 (22.4 g) and NaN_3 (1.25 g) on the other hand were dissolved in milli-Q water (5.0 L) at Rt for 1 h in two volumetric flasks to constitute two distinct solutions. These solutions were mixed at Rt for 1 h. The pH of the mixture was measured and adjusted to 7.0 with either HCl (4 M) or NaOH (4 M). The resulting mobile phase was filtered through ultra thin membrane filters (Anodisc 47, 6809–5012, porosity 0.1 μm, 47 mm; Whatman, Maidstone, United Kingdom). Prior to injection in the SEC–MALLS system each sample was filtered though a flow syringe filter (MiniSart, porosity 0.20 μm; Sartorius AG, Goettingen, Germany). Each sample was first fractionated by high performance liquid chromatography (HPLC, Alliance Waters 2695; Waters, Milford, Massachusetts, United States) through a HPLC column (7.8 × 300 mm TSK-GEL®, G5000PW$_{XL}$; Tosoh Corporation, Tokyo, Japan). The various sample fractions were then analysed by the MALLS (DAWN EOS; Wyatt Technology Corporation, Santa Barbara, California, United States) and refractive index (Optilab Rex; Wyatt Technology Corporation, Santa Barbara, California, United States) detection instruments. The flow rate was kept constant at 0.5 mL/min. The values of the osmotic second virial coefficient and of the variation of the polymer solution refractive index n as a function of the polymer concentration C (dn/dC) were 0.0023 mol mL/g^2 and 0.153 mL/g, respectively. The HPLC on the one hand and the MALLS and RI instruments on the other hand were piloted by the softwares Empower 1154 (Waters, Milford, Massachusetts, United States) and ASTRA 5.3.1.4 (Wyatt Technology Corporation, Santa Barbara, California, United States), respectively.

Table 1
Reaction parameters and ranges used in the experimental design.

Reaction parameter	Range
C(HA)[a]	5–25 mg/mL
C($NaHCO_3$)[b]	0.2–1.0 M
OSA/HA MR[c]	1–20
t[d]	6–24 h

[a] HA concentration.
[b] $NaHCO_3$ concentration.
[c] OSA/HA molar ratio.
[d] Reaction time.

2.4.2. FT-IR

Each sample (1 mg) was ground in KBr (~25 mg) and a pellet was prepared using a Qwik Handi-Press (P/N 0016–125; Thermo Spectra-Tech, Shelton, Connecticut, United States) and a 3-mm Die Set (P/N 0016–115; Thermo Spectra-Tech, Shelton, Connecticut, United States). The spectra were acquired at Rt in the wavelength range 4000–500 cm^{-1} (Nicolet Nexus 470 FT-IR, equipped with a Smart ARK™ system; Thermoelectron, Copenhagen, Denmark). Each spectrum was an overlap of 64 scans. The spectra of native HA and of the OSA–HA derivatives were assigned based on Pretsch, Buhlmann, and Affolter (2000), Alkrad, Mrestani, Stroehl, Wartewig, and Neubert (2003) and Haxaire, Marechal, Milas, and Rinaudo (2003).

2.4.3. ^{1}H NMR

Each sample (10 mg) was first dissolved in D_2O or DMSO, D6 (0.8 mL) at Rt for 16 h (overnight). It was then transferred into an NMR glass tube. The spectra were acquired at 80 °C (25 °C when DMSO, D6 was used as solvent) and 400 MHz (Varian Mercury TX 400 MHz; Varian, Palo Alto, California, United States). The DS of the derivatives which corresponds to the number of grafted reagent molecules per 100 disaccharide units was calculated by comparing the intensity per proton of the terminal methyl protons on the grafted octenyl succinate (OS) groups (0.9 ppm) to that of the methyl protons on native HA (2.0 ppm). The spectra of native HA and of the OSA–HA derivatives were assigned based on Pretsch et al. (2000), Welti, Rees, and Welsh (1979) and Pavia, Lampman, and Kriz (2001).

3. Results and discussion

3.1. Preparation of the OSA–HA derivatives

OSA–HA was prepared by reacting HA and OSA in aqueous mild alkaline media at Rt (Tømmeraas & Eenschooten, 2007) as illustrated in Fig. 1. Due to the linear structure of HA the products ob-

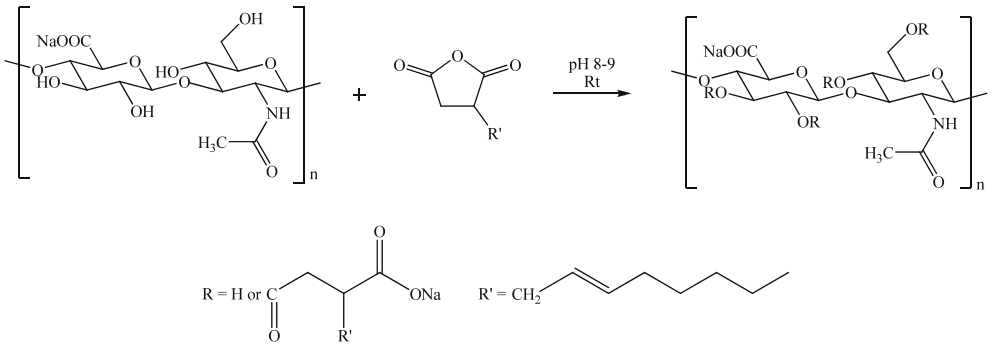

Fig. 1. Modification of HA with OSA. Reactions were conducted in $NaHCO_3$-buffered solutions (0.2–1.0 M), at pH 8–9, at room temperature and for between 6 and 24 h.

tained can be regarded as fishbone-like polymers consisting of a hydrophiphilic HA backbone and a number of hydrophobic octenyl succinate groups which reflects the degree of substitution of the derivative.

In order to identify the reaction parameters mostly influencing the DS of the OSA–HA derivatives an experimental design (2-level interaction full factorial design) was implemented in which four different reaction parameters were simultaneously varied. While the MW_w of HA, reaction pH and temperature were set to 21,000 Da, 8–9 and Rt, respectively, the influence of the HA concentration, buffer ($NaHCO_3$) concentration, OSA/HA molar ratio and reaction time were studied. These were varied between 5 and 25 mg/mL, 0.2 and 1.0 M, 1 and 20 and 6 and 24 h, respectively (as presented earlier in Table 1). The low, medium and high levels of these four reaction parameters were associated into 17 different combinations including a centre point which was replicated four times in order to assess the reproducibility of the experiments and calculate the experimental error. This brought the total number of samples for this experimental design to 20.

An additional OSA–HA sample was subsequently prepared using the following reaction conditions: HA concentration = 25 mg/mL, $NaHCO_3$ concentration = 2.0 M, OSA/HA molar ratio = 50, reaction time = 24 h.

3.2. Characterisation of the OSA–HA derivatives

The chemical structure of the OSA–HA derivatives was qualitatively studied by FT-IR by comparing the spectra of native HA21

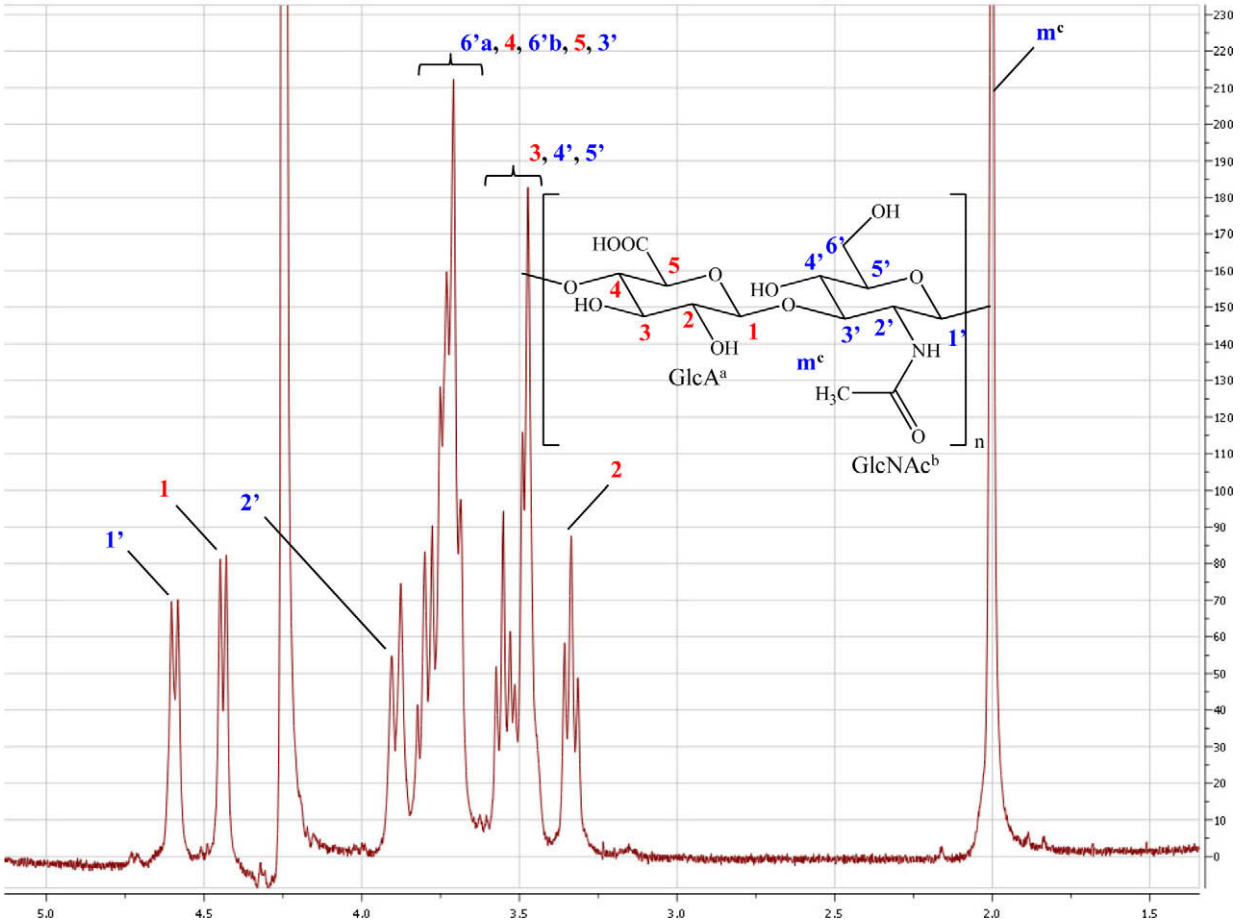

Fig. 2. ^1H NMR spectrum of HA21. This spectrum was acquired in D_2O at 80 °C and 400 MHz at a HA concentration of 12.5 mg/mL. [a] Glucuronic acid; [b] *N*-acetyl glucose amine; [c] Methyl protons.

and those of the derivatives. However these spectra did not show any significant differences compared to that of the native HA, independently of the DS of the derivatives. This lack of difference was probably due to overlapping of the characteristic bands of HA21 and OSA–HA. Indeed vinyl C–H stretching from the OS groups was most likely indiscernible from HA21's O–H stretching. Likewise C=C stretching and C=O (ester) stretching from the OS groups were certainly hidden by HA21's C=O stretching. As a result FT-IR could not confirm the modification of HA with OSA.

^{1}H NMR, however, proved efficient in demonstrating the grafting of OSA onto HA and was quantitatively used to calculate the DS of the derivatives. The spectrum of native HA21 is presented in Fig. 2 while that of OSA–HA (8) is shown as an example in Fig. 3. On Fig. 2, due to peak overlapping in the region 3.2–4.0 ppm, it was not possible to clearly assign the shifts of protons 3–5 and 3′–6′. Compared to HA21 the spectrum of OSA–HA (8) clearly showed an additional singlet at 0.9 ppm and a broad multiplet at 1.3 ppm. The former corresponds to the terminal methyl protons 10″ and the latter to the methylene protons 7″–9″ on the octenyl chain of the grafted OS groups. Due to the low signal intensity in the regions 1.4–1.8, 2.2–3.0 and 4.9–5.7 ppm it was not possible to clearly assign the chemical shifts of protons 1″–6″. This could be due to stacking of the OS groups via the various pi systems in presence resulting in fast relaxation of the neighbouring protons. However in the group of protons 1″–3″ and 6″, the methylene protons 6″ are expected to occur most upfield, possibly in the region 1.4–1.8 ppm, due their farthermost position with respect to the anisotropic fields of the carboxylate and ester groups. Conversely the methylene protons 1″ and the methine proton 2″ are expected to occur most downfield, potentially in the region 2.2–3.0 ppm, due to their closest position with respect to these fields. This leaves the methylene protons 3″ in between the resonances of protons 6″ and 1″–2″. Finally, the vinyl proton 4″ should appear downfield of the vinyl proton 5″ for a similar reason. By comparing the intensity per proton of protons *m* and protons 10″ the DS of OSA–HA (8) was estimated to 18% per disaccharide unit.

Similar spectra were observed for the other OSA–HA derivatives. However the relative intensity of the singlet at 0.9 ppm and, as a consequence, the DS of the derivatives varied as a function of the reaction conditions. Table 2 presents the 21 combinations of reaction conditions tested and the DS of the resulting OSA–HA derivatives as determined by ^{1}H NMR in D_2O.

In order to validate the DS values presented in Table 2 ^{1}H NMR spectra of the derivatives were also acquired in DMSO, D6. Indeed the introduction of hydrophobic groups onto HA is expected to alter the physico-chemical properties of the native polymer. In particular interactions between the OS groups of OSA–HA can potentially result into intra-/intermolecular association between polymeric chains in aqueous media through the formation of segregated hydrophobic domains. This is due to the fact water is a "good" solvent for the hydrophilic unmodified HA segments and supposedly a "bad" solvent for the hydrophobic OS groups. When ^{1}H NMR spectra are acquired in D_2O this aggregation phenomenon can possibly lead to perturbed and weakened resonances of the protons found along the OS groups. Contrary to D_2O DMSO, D6 is a known "bad" solvent for unmodified HA and is expected to be a "good" solvent for the OS groups. Indeed we observed in a preliminary study that DMSO, D6 was a good solvent both for OSA and

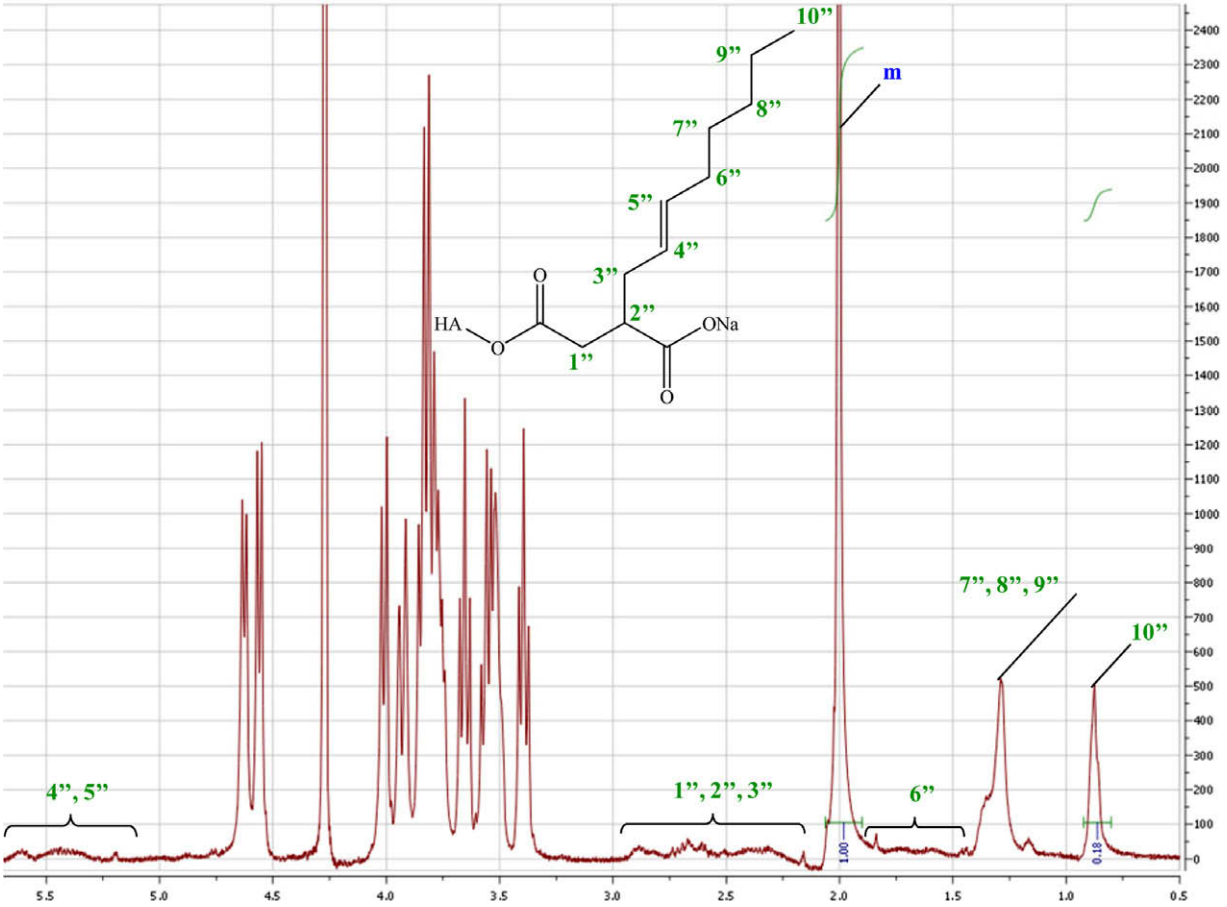

Fig. 3. ^{1}H NMR spectrum of OSA–HA (8). This spectrum was acquired in D_2O at 80 °C and 400 MHz and at an OSA–HA concentration of 12.5 mg/mL. OSA–HA (8) was obtained using the following conditions: $C(NaHCO_3)$ = 1.0 M, C(HA) = 25 mg/mL, OSA/HA MR = 20 and t = 6 h.

Table 2
Reaction conditions and DS of the OSA–HA derivatives as determined by ^{1}H NMR in D_2O.

Sample name	$C(NaHCO_3)$[a] (M)	$C(HA)$[b] (mg/mL)	OSA/HA MR[c]	t[d] (h)	DS
OSA–HA (1)	0.2	5	1	6	3.5
OSA–HA (2)	0.2	25	1	6	2.5
OSA–HA (3)	0.2	5	20	6	10
OSA–HA (4)	0.2	25	20	6	7
OSA–HA (5)	1.0	5	1	6	1.5
OSA–HA (6)	1.0	25	1	6	2
OSA–HA (7)	1.0	5	20	6	16
OSA–HA (8)	1.0	25	20	6	18
OSA–HA (9)	0.2	5	1	24	3
OSA–HA (10)	0.2	25	1	24	4
OSA–HA (11)	0.2	5	20	24	13
OSA–HA (12)	0.2	25	20	24	8
OSA–HA (13)	1.0	5	1	24	4
OSA–HA (14)	1.0	25	1	24	2
OSA–HA (15)	1.0	5	20	24	18
OSA–HA (16)	1.0	25	20	24	17
OSA–HA (17)	0.6	15	10.5	15	6
OSA–HA (18)	0.6	15	10.5	15	7
OSA–HA (19)	0.6	15	10.5	15	6
OSA–HA (20)	0.6	15	10.5	15	7.5
OSA–HA (21)	2.0	25	50	24	43

[a] $NaHCO_3$ concentration.
[b] HA concentration.
[c] OSA/HA molar ratio.
[d] Reaction time.

it hydrolysed diacid form obtained by reaction of OSA with water under alkaline conditions (data not shown). When dissolved in DMSO, D6 the OS groups of OSA–HA are therefore expected to be exposed to the solvent while the unmodified HA segments are

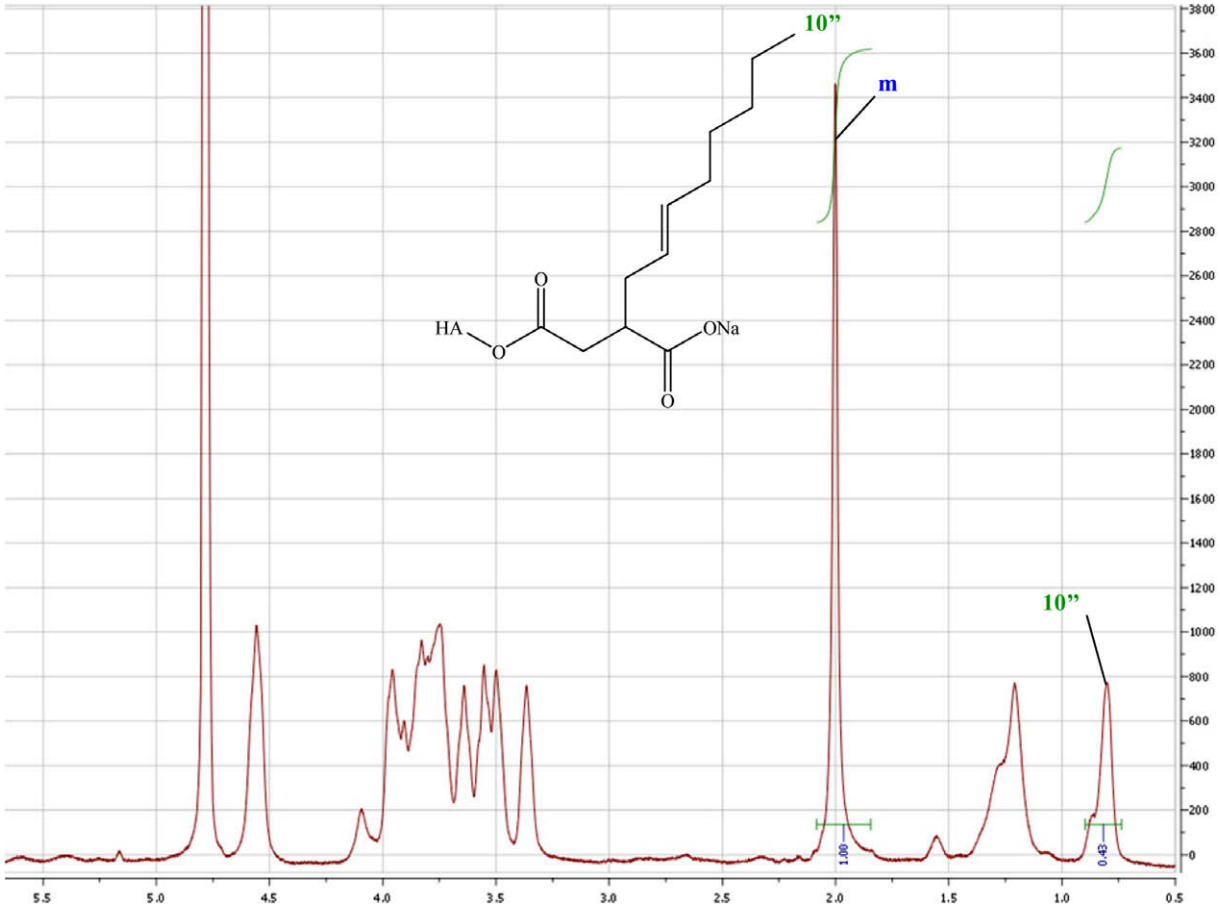

Fig. 4. ^{1}H NMR spectrum of OSA–HA (21). This spectrum was acquired in D_2O at 80 °C and 400 MHz and at an OSA–HA concentration of 12.5 mg/mL. OSA–HA (21) was obtained using the following conditions: $C(NaHCO_3)$ = 2.0 M, $C(HA)$ = 25 mg/mL, OSA/HA MR = 50 and t = 24 h.

awaited to be withheld in segregated hydrophilic domains possibly leading this time to perturbed and weakened resonances of unmodified HA protons. When determined from the spectra acquired in DMSO, D6 the DS values of the derivatives were very close to the ones calculated from the spectra acquired in D_2O. For instance the spectra of OSA–HA (21) in D_2O and in DMSO, D6 are presented in Figs. 4 and 5, respectively. It should be noted that OSA–HA (21) was chosen as an example because it contains the highest density of hydrophobic groups. Indeed this makes it most susceptible to aggregate in aqueous media. In Fig. 4 the characteristics peaks of OSA–HA were easily identifiable and the DS of OSA–HA (21) amounted to 43%. The characteristic pattern of OSA–HA could also be observed in Fig. 5 (although shifted by approximately 0.2 ppm upfield) and peaks at 2.5 and 3.35 ppm corresponded to the chemical shift of pure DMSO, D6 and water in DMSO, D6, respectively. In addition the DS of OSA–HA (21) was very similar to the one calculated from Fig. 4, namely 49%. The results of DS values calculated from spectra acquired both in D_2O and DMSO, D6 show that in the case of the present chemistry ^{1}H NRM is a reliable technique to determine the DS of the OSA–HA derivatives since it gives nearly identical results in solvents that are alternatively "good" or "bad" solvents for the hydrophilic and hydrophobic parts of the derivatives. This also means that if aggregation took place during the analysis it did not induce resonance perturbations.

3.3. Influence of the reaction conditions on the DS of the OSA–HA derivatives

The evaluation of the experimental design data (OSA–HA (1) to OSA–HA (20) in Table 2) shows that the DS of the four replicates OSA–HA (17), (18), (19) and (20), which were prepared on different days, were very similar. This indicates a general good reproducibility of the experiments. The DS of the derivatives was distributed between 1.5% and 18% per disaccharide unit and the two combinations of reaction conditions allowing the maximum DS (18%) were those of OSA–HA (8) and OSA–HA (15), i.e. when the OSA/HA MR and $NaHCO_3$ concentration were the highest.

The initial coefficient plot (data not shown) showed that only a few parameters and combinations of parameters had a significant influence on the DS of the OSA–HA derivatives, namely the OSA/HA molar ratio, the $NaHCO_3$ concentration and their interaction. This was due to the fact the OSA/HA molar ratio is directly related to the number of available reagent molecules and the $NaHCO_3$ concentration is associated to the buffering capacity of the reaction medium which is necessary to maintain an alkaline pH (8–9) for the activation of the polymer's hydroxyl groups. The HA concentration and reaction time had little influence on the DS of the derivatives (see for example OSA–HA (7) and OSA–HA (8) on the one hand and OSA–HA (8) and OSA–HA (16) on the other hand). This suggested that the viscosity of the 25-mg/mL HA21 solutions did not hinder mixing and diffusion of the OSA molecules nor limit the competitive hydrolysis of OSA more than the 5-mg/mL HA solutions and means that OSA–HA can be prepared from relatively concentrated low molecular weight HA solutions without compromising the extent of modification. Finally, increasing the reaction time from 6 to 24 h did not result in higher DS values which suggested that reactions between HA and OSA were already completed after 6 h.

After elimination of the parameters and combinations of parameters insignificantly influencing the DS of the OSA–HA derivatives, the experimental DS values were fitted with a *linear plus interaction model* using a *partial least square* regression analysis.

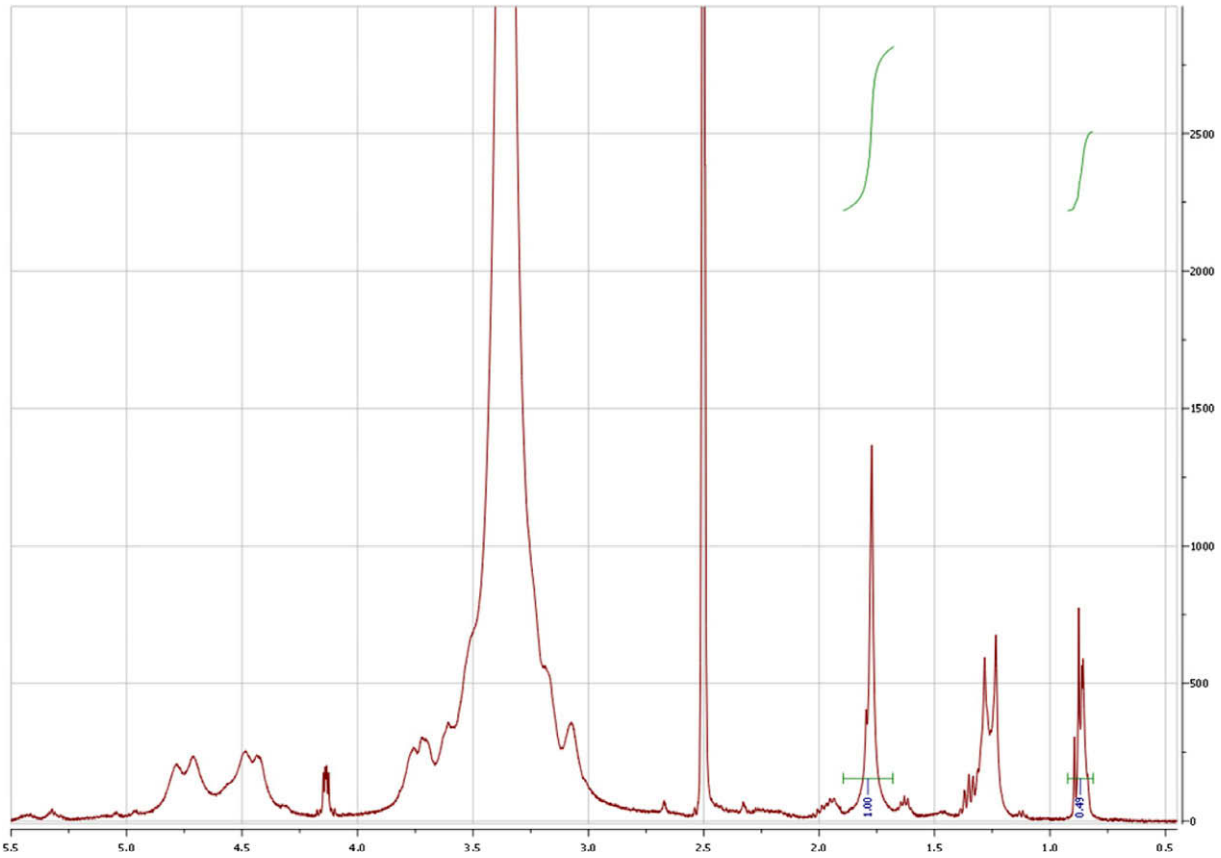

Fig. 5. ^{1}H NMR spectrum of OSA–HA (21). This spectrum was acquired in DMSO, D6 at 25 °C and 400 MHz and at an OSA–HA concentration of 12.5 mg/mL. OSA–HA (21) was obtained using the following conditions: $C(NaHCO_3)$ = 2.0 M, C(HA) = 25 mg/mL, OSA/HA MR = 50 and t = 24 h.

Table 3
Evaluation of the model used to fit the DS of the OSA–HA derivatives.

Coefficient	Value
R^{2a}	0.96
Q^{2b}	0.82
MV[c]	0.52
RC[d]	0.98

[a] Regression coefficient.
[b] Prediction coefficient.
[c] Model validity.
[d] Reproducibility coefficient.

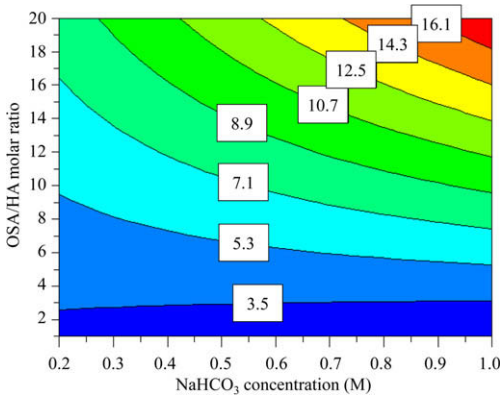

Fig. 6. Contour-plot of the DS of the OSA–HA derivatives, C(HA) = 15 mg/mL.

The model was evaluated by means of computing four different coefficients: the regression coefficient (R^2), the prediction coefficient (Q^2), the model validity (MV) and the reproducibility coefficient (RC) (Table 3). The high values of R^2, Q^2 and RC suggested that the choice of the mathematical model used to fit the influence of the reaction conditions on the DS of the OSA–HA derivatives was justified and suitable. In addition MV was larger than 0.25 which means that the model error was in the same range as the experimental error. Therefore a contour-plot of the DS of the OSA–HA derivatives as a function of the OSA/HA molar ratio and $NaHCO_3$ concentration was computed and is presented in Fig. 6. As already observed, the contour-plot predicts that the highest DS values are obtained for the highest OSA/HA molar ratio and buffer concentration, typically 20 and 1.0 M, respectively. Indeed, the higher the OSA/HA molar ratio, the larger the number of OSA molecules available for reaction. Likewise, the higher the $NaHCO_3$ concentration, the greater the buffering capacity and consequently the longer the activation of HA's hydroxyl groups.

Interestingly, when a higher OSA/HA molar ratio (50) and $NaHCO_3$ concentration (2.0 M) were used (the HA concentration and reaction time were 25 mg/mL and 24 h, respectively) for preparing OSA–HA (21), the DS could be increased to 43% (Figs. 4 and 5). This demonstrated that the OSA modification technology was very versatile despite a relatively poor reaction efficiency due to the competitive hydrolysis of OSA in the presence of water.

4. Conclusion

Amphiphilic hyaluronic acid derivatives were prepared using a simple water-based modification method targeting HA's hydroxyl groups thus preserving the biologically and physiologically important carboxyl groups of the polymer. HA was alkenylated with octenyl succinic anhydride to yield fishbone-like OSA–HA polymers and the influence of reaction parameters such as the HA concentration, buffer ($NaHCO_3$) concentration, OSA/HA molar ratio and reaction time on the degree of substitution of the OSA–HA derivatives was studied by means of an experimental design. The results showed that the OSA/HA molar ratio, $NaHCO_3$ concentration and their interaction had the largest influence on the DS of the derivatives while the HA concentration and reaction time only had a negligible effect. Combinations of reaction conditions allowing the preparation of OSA–HA derivatives with a DS between 1.5% and 43% per disaccharide unit according to ^{1}H NMR were identified. Moreover, it was demonstrated that the reactions were fast (6 h) and could be conducted in relatively concentrated low molecular weight HA solutions (up to 25 mg/mL). These aspects represent advantages in terms of volume and through-put in the perspective of upscaling the method. The introduction of octenyl chains onto HA is expected to alter the physico-chemical properties of the native polymer. Their investigation will be subject of a forthcoming paper.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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