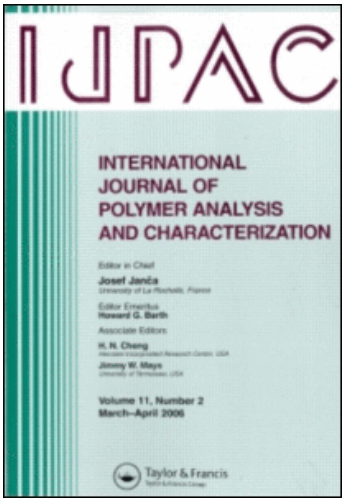

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CHEMICAL MODIFICATION OF HYALURONIC ACID: ALKYLATION

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Hydrophobically modified derivatives of hyaluronic acid (HA) are characterized by means of differential scaning calorimetry (DSC) measurements. Simultaneously, viscosity and elasticity are monitored by rheological measurements. Alkylation of HA macromolecule brings about a strong change in the rheological properties of the biopolymer, which leads to the succesful micelle formation process. The latter then create a highly organized supramolecular structure in the solution, which at the length of the alkyl chain of C15 and higher, is characterized by the creation of the isotropic liquid crystal phase transition.

Keywords: Alkyl derivatives; DSC; Hylauronic acid; Liquid crystal phase transition; Micelle formation; Rheology; Viscosity

INTRODUCTION

Hydrophobically modified (HM) water-soluble polymers. e.g.,. cellulose, starch, and guar, exhibit enhanced solution viscosity and unique rheological behavior. These properties are explained in terms of intermolecular hydrophobic associations.[1,2] The general solution viscosity behavior of HM polymers of different hydrophobic chain length (most frequently in the range of C_8 to C_{24}) is characterized by the peak viscosity region observed for a certain alkyl group content (between 1 and 5 wt.%). After reaching the maximum increase in viscosity (peak region) at a certain hydrophobic level, the viscosity dramatically decreases with increasing number of hydrophobic moieties on the polymer chain. HM polymer chains (e.g., HM (hydroxyethyl)cellulose (HEC)) in the peak region exhibit highly non-Newtonian rheological behavior at low shear rates, while nonmodified HEC behaves as a pure

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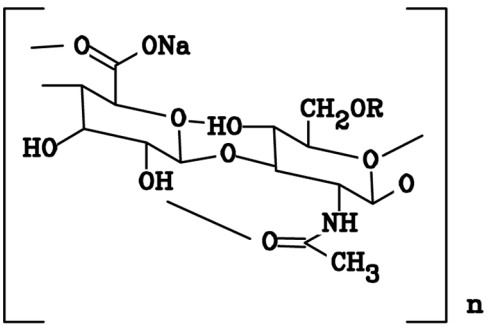
Newtonian liquid under the same experimental conditions. However, increase in elasticity is not observed under shearing conditions, when the association process is successfully inhibited due to the liquid motion.

A different situation is observed in dilute solutions (conc. $< c^*$), where the HM HEC molecules are separately present in an untangled configuration, thus suppressing the ability to form large associates. However, to minimize the disruption of the water structure in the vicinity of the polymer chain, the hydrophobic side chains tend to cluster within each other on the same chain. This process results in a much reduced hydrodynamic volume and is simultaneously reflected in the lowering of the viscosity.

Hydrophobically modified polysaccharides are used mainly as bioactive surfactants in medical pharmacological applications, for the development of micelle-forming polymeric drugs, especially those with superior anticancer activity, which has been done since the early 1990s.[3–5] The monomer unit of HA consists of N-acetyl-D-glucosamine and D-glucuronic acid bonded via ß(1-3) and ß(1-4) interglycosidic bonds (see Scheme 1, structure (1)).[6] In general, alkylation of cellulosic derivatives proceeds via the corresponding alcoholates. Dialkylsulfates are not suitable due to their toxicity and carcinogeneity. Preparation of alkyl-modified HA chains therefore is achieved upon the alkylation with alkyl halides. In both procedures the selectivity in the attachment of the alkyl chain is limited.

We are primarily interested in preserving the polymerization degree of the polymer, while maintaining the biologically and physiologically important side chain groups, i.e., the negatively charged carboxyl group and the acetylamide. For these reasons, a more selective method for modifying the primary alcohol group was preferred. In the first step, alkyl sulfate and the polymer alkoxide form an alkoxy-sulfonyloxy-complex. Under strict temperature control, this reaction is highly selective for primary alcohols.[7–10] In the second step, the complex and the



(1) R = H

(2) R = p–Toluenesulfonyl

(3) R = Pentadecyl

Scheme 1. (1) Sodium hyaluronate (HA), (2) HA-tosyl, and (3) pentadecyloxy-HA.

alcohol are added to powdered KOH and stirred in DMSO at room temperature for several hours.[11] Alkylation of hyaluronic acid was first described in a conference paper abstract in 1995,[10] while alkylation of cellulosic materials has been of scientific interest since the early 1970s.

In this article, new hydrophobically modified derivatives of hyaluronic acid are characterized by means of differential scaning calorimetry (DSC) measurements. Simultaneously, viscosity and elasticity are monitored by rheological measurements.

EXPERIMENTAL SECTION

Preparation of 1-Hexyloxy and 1-Pentadecyloxy Derivatives of HA

All reactions were carried out under nitrogen atmosphere. All chemicals were of analytical purity grade and were used without further purification.

In 40 mL of freshly distilled dry pyridine (dried with calcium hydride (Janssen Chimica, Belgium)) 0.5 g of sodium hyaluronate (Diosynth, The Netherlands) extracted from rooster combs was dissolved. The weight average molecular weight of HA was 1.15 MDa. The estimated protein concentration in the polysaccharide was less than 0.5 wt.% as determined by the Coomassie Brilliant Blue G (Sigma, USA) reaction. The reaction mixture was gently stirred at -5° to 0°C. After 15 min, the calculated molar equivalent (based on the number of primary alcohol groups in HA) of p-toluenesulfonyl chloride (TSC) (UCB, Belgium) was added. After 90 min, the solution was kept overnight at 4°C. Then HA-tosyl (Scheme 1, structure (2)) was precipitated by the addition of chilled ethanol (Vel, Belgium). After repeated precipitations, the product was freeze-dried. Tosylated HA was characterized by UV (λ_{max} 203 nm, 255 nm).

Next, 4 molar equivalents KOH (Merck, Darmstadt, Germany) was stirred at room temperature with 20 mL of dry dimethyl sulfoxide (DMSO; Alltech Associates, Inc., Applied Science Labs, Deerfield, Ill., USA). After 10 min, 2 molar equivalents of the appropriate alcohol were added: 1-hexyl alcohol (Vel, Belgium), 1-pentadecanol, and 1-hexadecanol (Aldrich, USA), respectively. The solution was then stirred for 17 h at room temperature, and the mixture was poured into water (10 mL) and precipitated by addition of chilled ethanol. The reaction product was purified by repeated precipitation and freeze-dried. Chemical yield of the reaction was 20%.

To ensure that no degradation of the main HA polymer chain occurs during the synthesis, a blank experiment was performed. The weight average molecular weight (Mw) as well as the molecular weight distribution functions were estimated by size exclusion chromatography (SEC). The Mw of the original sample (1.15 MDa) did not change (1.12 MDa).

Methods

UV-Vis spectroscopy. The measurements were carried out in 1 cm quartz cells on a Hewlett Packard 8452A Diode Array Spectrophotometer controlled by a an HP Vectra ES/12 computer (Palo Alto, Calif., USA).

Determination of the tosylation degree by UV spectroscopy. Tosylation degree of the studied samples was estimated by means of the standard calibration

method, which is based on measuring the absorbance at 255 nm wavelength, i.e., the wavelength that is characteristic for the absorption of the tosyl chromophore. This value was then compared with the standard calibration curve. All samples were allowed to stay at room temperature for 24 h prior to the measurement to avoid the scattering of the light, which is caused by insufficient homogeneity of the solution. To calculate the degree of tosylation, the spectrum of pure HA was subtracted from the spectrum of HA-TSC to get the absorbance attributed to the tosyl moiety. This value was then used in later calculations. For this reason, all UV spectra were measured at the same HA concentration. Estimated reaction yield was 20%. This means that instead of each, only every fifth primary alcohol group is tosylated after the addition of the theoretical amount sufficient for 100 mol.% tosylation.

DSC measurements. Standard aluminium sample pans (PerkinElmer, USA) for volatile liquids were used. The samples were weighed using a Mettler automatic electrobalance. Approximately 5–12 mg of polymer were weighed into an empty pan. In all experiments, samples were precipitated from absolute ethanol and then freeze-dried for 2 h.[13] Then the samples were stored prior to the measurement at 4°C for 24 h in a closed vessel. Thermograms of the samples were recorded against an empty pan as a reference on a Du Pont Instruments 9900 Computer/Thermal Analyzer 910 Differential Scanning Calorimeter (USA) in the temperature range of (−) 25°C to (+) 50°C at a scanning rate of 3°C/min. The samples were prepared by cooling from (+) 23°C to (−) 30°C at 5°C/min cooling rate.

Size exclusion chromatography. All experiments were carried out on coupled Ultrahydrogel 1000 and Ultrahydrogel 2000 columns (Millipore) connected with a Waters 510 Pump. For detection, the Waters 410 Differential Refractometer (USA) was used. A 300 μL amount of the sample was injected through a Waters U6 K Universal Injector (USA). As mobile phase 0.5 M NaCl was used. The flow rate of the mobile phase was 1 mL/min. The SEC column was calibrated by standard calibration method using the pullulan standards (Sigma).

Rheological measurements. For measuring the shear rate dependence of dynamic viscosity and for the oscillatory measurements, a HAAKE Rotovisco rotational viscometer (Hamburg, Germany) was used. All measurements were carried out at 25°C. For each measurement, the solutions were prepared by dissolving freeze-dried HA two days prior to the measurements.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry Measurements

The main characteristic of HA hydrogels is the ability to bind extremely high amounts of water in the swollen state. This phenomenon is very important in relation to the synergetic effects on diffusion phenomena in the tissue.

It is known that in the connective tissue matrix, the strength component of the system is due to the presence of the collageneous microfibrillar domains, which are interpenetrated by the highly elastic swollen system of glycosaminoglycans. At the present time, it is still not clear how this composite structure is built up in order to obtain a material of both high strength and high elasticity in the swollen state.

Another important parameter of this system is the ability of controlled release of various proteins having different sizes and shapes through such a matrix. The diffusion is strongly affected not only by the size of the pores, i.e., by the hydrodynamic conditions, but also by the electrostatic interactions. This model accounts for the data observed for the static and dynamic mechanical properties of the ethyl and benzyl esters of HA,[14] where the fundamental role of the substituents on the microstructure is to modulate the hydrophobic and hydrophillic microdomains.

Previous studies have shown that the transport properties of tightly bounded water differ from those of free water. The nature of the water present in the native and modified polymer influences drug release from such matrices.

Water molecules present in protein solutions were classified into three categories,[15–17] i.e., type I (free water), type II (freezing bound water), and type III (nonfreezing bound water). The presence of type II water in the structure is thought to be due to the equilibrium binding of water to the hydrophillic moieties of the polymer chain.

In completely dry HA, a glass transition temperature (T_g) was not observed because T_g overlapped with the decomposition temperature. T_g is strongly dependent on the water moisture content. With increasing water content, the glass transition temperature decreases dramatically.[18]

Endothermic free water peak at the temperature characteristic for the melting of the free water (T_m) of 2.3°C was observed on measuring the DSC heating curve of the rooster comb HA sample (Figure 1). The cold-crystallization temperature of amorphous ice (T_{cc}) was measured at (−) 0.84°C. The T_g was shifted to (−) 25.56°C. Such a low value of the glass transition temperature can be attributed to the presence of a certain amount of water in the sample, hence to the higher plasticity of the polymer network.

The DSC heating curve measured for hexyloxy-(100 mol.%) modified HA is shown in Figure 2. An endothermic peak temperature T_m at (+) 0.96°C is characteristic for free water, while the appearance of the broad exothermic peak temperature

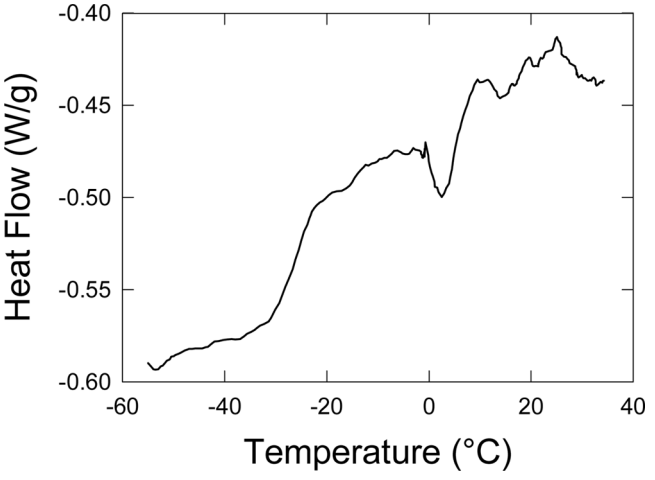


Figure 1. DSC heating curve of the native rooster comb sodium hyaluronate.

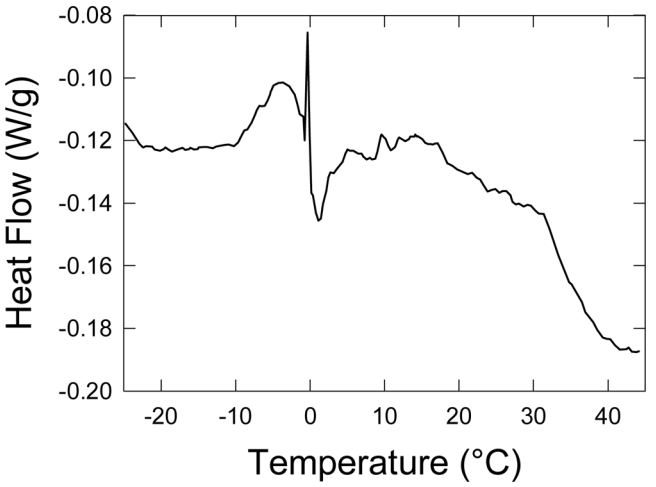


Figure 2. DSC heating curve of the 100 mol.%-modified hexyloxy-HA.

T_{cc2} at (−) 3.96°C reflects the presence of the water hexameric structure. The glass transition temperature was shifted to (+) 31.00°C and the cold-crystallization temperature to (−) 0.58°C.

A completely different DSC heating curve was measured for the 6.97 mol.% modified pentadecyloxy-HA (3) (Figure 3). The main DSC characteristics, i.e., T_{cc} ((−) 1.01°C), T_m ((−) 0.80°C), and T_g ((+) 30.29°C) were shifted to lower temperatures than those for hexyloxy modified HA. A new endothermic peak temperature at (+) 5.57°C is characteristic for the transition from the liquid crystalline state to the isotropic liquid state (T^*).[19–21] The lowering of the T_{cc} and the T_m peak temperatures in comparison to the original rooster comb HA can be due to the weaker binding of water molecules in the modified polymer. This interaction results from the

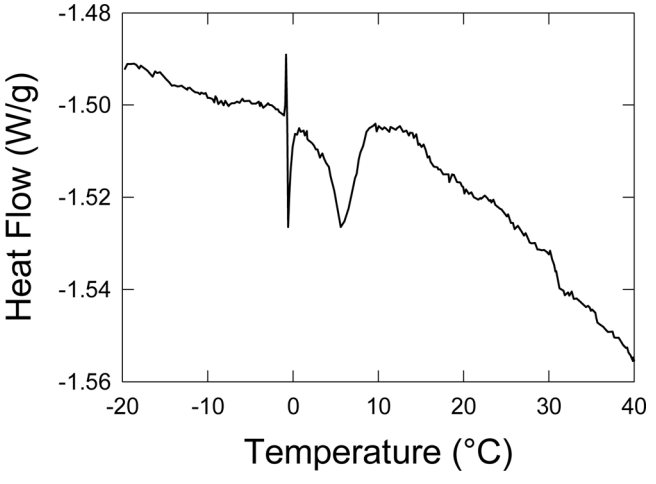


Figure 3. DSC heating curve of the 6.97 mol.%-modified pentadecyloxy-HA.

formation of somehow spatially oriented hydrophobic and hydrophylic moieties in the polymer backbone, thus leading to their self-association. This phenomenon is reflected in the creation of inter- and intra-molecular micelles, thus forming a colloidal suspension. This is also reflected in the increase of the scattered light in the modified sample. Because of the polyanion character of HA, i.e., due to the presence of the negatively charged regions in the bulk as well as on the surface of the HA micelles, the latter are highly hydrated and surrounded by a diffuse layer composed of the oriented water molecules and counterions. Thus, the lowering of T_m temperature suggests that in the pentadecyloxy-modified hyaluronate more water molecules are present in the form of freezing-bound water. Finally, in solution this complex colloidal system is governed by electrostatic, van der Waals, and hydrodynamic forces, which form a "crystal-like" structure in the solution of monodisperse polystyrene.[22] For this reason, the appearance of the T^* peak temperature in 6.97 mol.% modified pentadecyloxy-hyaluronate can be explained by the occurence of the liquid crystal phase transition induced by the micelle ordering, which proceeds in the system due to the electrostatically induced temporary lattice formation. More detailed characterization of these phenomena should be done by fluorescence and light-scattering measurements.

Rheological Measurements

The forming of micelles in the solutions of the alkyl-modified HA samples can be monitored by measuring the changes of the rheological characteristics. It is well known that the solutions of native HA are highly viscous and elastic.

A typical viscosity pattern is shown in Figure 4. The dynamic viscosity of native HA depends on the shear rate (γ) as for non-Newtonian liquids, showing decrease of dynamic viscosity with an increase of the shear rate. This reflects a

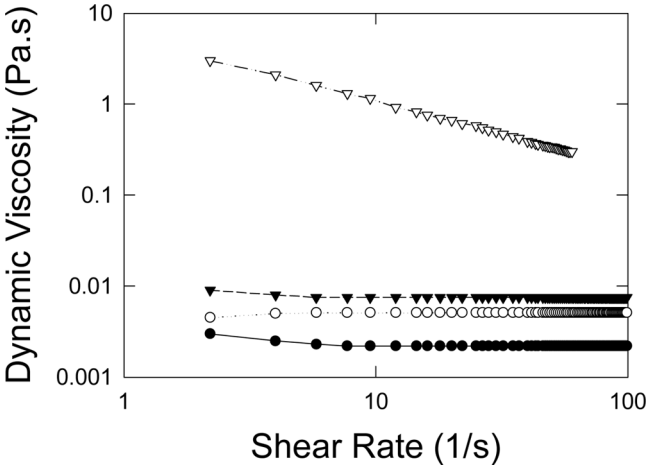


Figure 4. Dynamic viscosity dependencies on the applied shear rate of the native HA (c = 6.8 mg/mL) (empty triangle); 100 mol.%-modified hexyloxy-HA at concentrations of 6.1 mg/mL (filled circle), 7.4 mg/mL (empty circle), and 5.0 mg/mL (filled triangle).

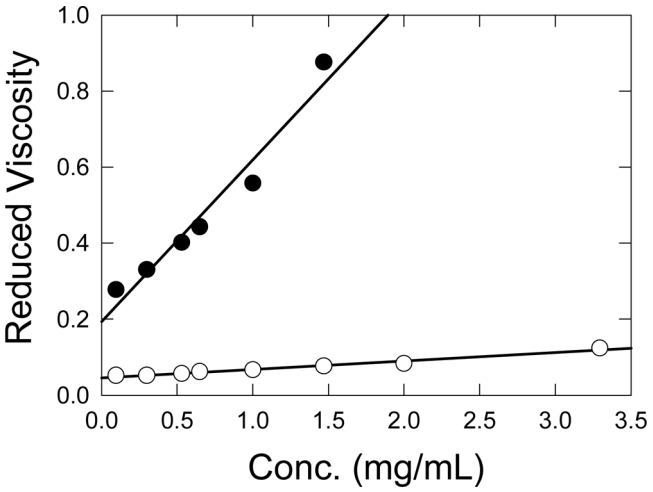


Figure 5. Huggins plots of the $\eta_{sp}/c = f(c)$ dependencies of the 100 mol.%-modified hexyloxy-HA (empty circle) and native HA (filled circle) as measured by the Ubbelohde capillary viscosimeter at 25°C.

disruption of the hydrogen-bonded system. Alkyl-modified HA has a typical Newtonian liquid viscosity behavior. The observed decrease in zero shear viscosity and the appearance of the viscosity independence of the applied shear rate can be attributed to the formation of intra-molecular hydrophobic associates, resulting in the collapse of the macromolecular coils. This phenomenon is clearly visible also from the decrease of the intrinsic viscosity after alkyl modification, as shown in Figure 5. The 100 mol.% modified hexyloxy-HA form spatially smaller macromolecular coils than the native HA chains. It seems that the latter are present in a denser configuration, which is reflected in the lower viscosity. The presence of the hydrophobic microdomains on the polymer chain is indicated by the decrease of the slope of the reduced viscosity dependencies, i.e., by the decrease of the Huggins constant.

Size Exclusion Chromatography

The above-mentioned phenomena show a strong change in the dimensions of the HA macromolecules as a result of the side chain alkyl modification. To confirm micelle formation, SEC experiments were performed (Figure 6). These show the change of the SEC pattern of the native HA from a narrow distribution of the molecular sizes (elution time of 13.77 min) to the typical bimodal pattern observed for 100 mol.%-modified hexyloxy-HA, reflecting the presence of the expanded molecules (14.53 min) and the contracted ones (21.04 min). The equivalent given in molecular weight units (based on pullulan standards) was 1.153 MDa for the native HA, 1.07 MDa for the high molecular weight (or expanded molecules) fraction, and 0.31 MDa for the low molecular weight (contracted molecular coils) fraction, respectively. Observed negative elution peak can be ascribed to the poor baseline recovery between the end of the polymer chromatogram and the beginning

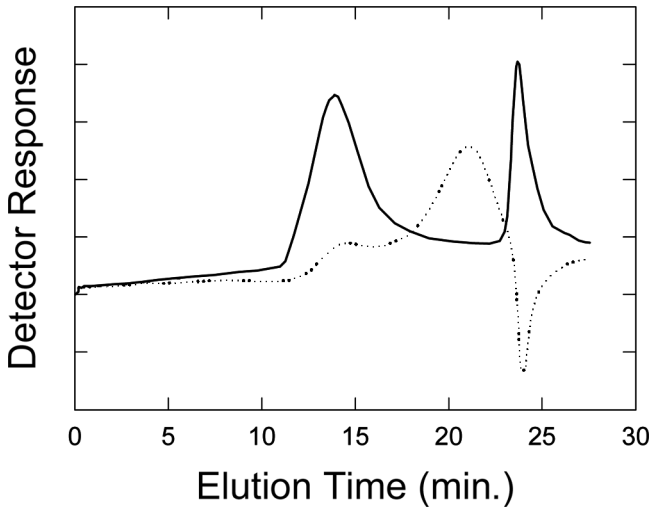


Figure 6. Size exclusion chromatography pattern of the native rooster comb HA (solid line) and of 100 mol.%-modified hexyloxy-HA (dotted line).

of the solvent-related impurity peaks. This phenomenon is described in detail in the Mori et al.[23]

CONCLUSIONS

Based on our measurements, it can be concluded that the alkylation of HA macromolecule brings about a strong change in the rheological properties of the biopolymer, which leads to the succesful micelle formation process. The latter then create highly organized supramolecular structure in the solution, which at the length of the alkyl chain of C15 and higher, is characteristic by the creation of the isotropic liquid crystal phase transition.

NOMENCLATURE

c^*	overlap concentration
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
HA	hyaluronic acid (or hyaluronate for salts)
HEC	(hydroxyethyl)cellulose
HM	hydrophobically modified
Mw	weight average molecular weight
T_{cc}	phase transition temperature of the cold-crystallization of amorphous ice
T_g	glass transition temperature, i.e., the second-order phase glass-rubber transition temperature
T_m	phase transition temperature of the melting of free water
T^*	liquid crystalline state to isotropic liquid state phase transition temperature
UV	ultraviolet

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