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Exact determination of the degree of substitution of high molar mass hyaluronan by controlling the conformation in solution

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ABSTRACT

The purpose of the present study was the development of an accurate method to determine the degree of substitution (DS) of modified hyaluronic acid (HA) by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. The influence of the effect of ionic strength and pH on ^{1}H NMR spectra of HA was studied. The results showed a correlation between the conformation of HA in solution and the quality of the ^{1}H NMR spectra. The best spectra with full proton mobility are obtained when HA is dissolved in D_2O with 2 M NaCl or D_2O with 0.1 M NaOD with a maximum concentration of 5 mg/ml. Under those conditions the size of the polymer coils is reduced below the concentration of chain overlap point, all the protons show the same response and a correct degree of substitution can be determined.

1. Introduction

Hyaluronic acid (also referred as HA or hyaluronan) is a linear polysaccharide made of repeating disaccharide units of β-1,4 glucuronic acid and β-1,3 N-acetyl glucosamine (Weissmann, Meyer, Sampson, & Linker, 1954). HA belongs to the class of glucosaminoglycans and is an essential component of the extracellular matrix of all animal tissues and some bacteria. In nature, the molecular weight can vary from 10^3 Da up to 10^7 Da and the biological function of HA depends strongly on its size. HA currently represents one of the most attractive building blocks for design of biomaterials with application in the pharmaceutical and medical fields (Eenschooten, Guillaumie, Kontogeorgis, Stenby, & Schwach-Abdellaoui, 2010). It finds application in eye surgery, viscosupplementation therapy for osteoarthritis, dermal filling, tissue augmentation, scaffolds for tissue engineering and many others. To improve the chemical and physical properties in biological systems, e.g. to prolong the half-life after internal administration, contemporary research is focused on chemical modification of HA. Since the properties of HA derivatives are strongly influenced by the degree of substitution (DS), the knowledge of exact DS values is greatly needed.

A very effective, reliable and widespread method for determination of DS of polysaccharides is ^{1}H NMR spectroscopy. The area below the signals in ^{1}H NMR spectra is proportional to the amount of protons responsible for the signal and can be used to obtain quantitative information about the chemical structure of the material. DS of modified HA is usually calculated from the ratio of signal integrals of methyl protons of the N-acetyl residue of HA as backbone reference peak and specific signals of protons of the grafted moiety (Oudshoorn, Rissmann, Bouwstra, & Hennink, 2007).

Low-molecular-weight HA derivatives can be easily characterized by NMR spectroscopy due to the very low viscosity of the solutions (Hardingam, 2004), but the accuracy of DS determination by conventional 1D ^{1}H NMR is strongly limited in highly viscous environment, where interactions between polymer chains are very strong and aggregation phenomena may occur. This leads to low resolution spectra with broad signals and reduced signal intensity and to uncertainty of up to 15% in the value of DS obtained from integrating the peaks (Chmelar et al., 2017).

A standard approach to overcome the problem of high viscosity of polyelectrolytes such as HA, chitosan or alginates is a decrease of molecular weight. For chitosanes and alginates ASTM standards were established to determine the degree of deacetylation and the chemical composition, respectively, by 1D ^{1}H NMR analysis. In order to obtain well resolved NMR spectra, depolymerization to a number average degree of polymerization of 15–50 and measurements at elevated temperature (80–90 °C) are required. For HA degradation by enzymatic or acid hydrolysis were reported as suitable methods for a higher resolution in NMR analysis (D'Este, Eglin, & Alini, 2014; Tømmeraas, Mellergaard, Malle, & Skagerlind, 2011). Drawbacks of these methods are the time-consuming sample preparation and the risk of a loss of pH-sensitive grafted moieties. Thus, a direct and correct analysis of high

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molecular weight HA (derivatives) would be advantageous.

NMR signals of specific groups are quantitatively only when they are mobile, i.e., not implied in an ordered structure such as helical structures which can be stabilized by hydrogen bonds in stereoregular polymers or by specific interactions. It was reported that in the case of high molecular weight HA spectra acquired at room temperature in D_2O, proton mobility of acetyl groups, backbone protons and anomeric protons varies between 41% and 56.4%. Increasing the temperature resulted in an increase of proton mobility to 74–83%, but it was observed that interactions between different chains were still present at 85 °C. In all cases, the protons of the N-acetyl groups were more affected than those of the sugar units and it was concluded that in the case of high molecular weight HA ^{1}H NMR spectroscopy cannot be used to test the acetyl content in the HA structure (Milas & Rinaudo, 2004).

It is well known, that viscosity of solutions of polyelectrolytes such as HA can be reduced by increasing the ionic strength of the solution, because the repulsion between negatively charged disaccharide units decreases and the polymer changes from a rod-like-structure toward the flexible conformation of the random coil (Nystrom et al., 2010). This should not only lead to a higher mobility of the macromolecules, but also to a higher proton mobility. However, until now this aspect was not considered in NMR analysis of HA and HA derivatives.

In the present study, the influence of ionic force, pH and additives such as urea on the conformation and proton mobility of HA was investigated and a correlation between proton mobility and conformation in solution was established. A method for accurate determination of DS of high molecular weight HA derivatives by ensuring full proton mobility of the N-acetyl-protons using disodium succinate as standard is presented.

2. Materials and methods

2.1. Materials

Native HA with a weight average molecular weight (Mw) of 1.20 MDa (87.1% of dry substance) was provided by Croma Pharma GmbH, Leobendorf Austria. Mw was determined by size exclusion chromatography with LS calibration. Data delivered by Croma Pharma were in good agreement with data obtained by static light scattering measurements. NaOD (40% in D_2O), DCl (20% in D_2O), D_2SO_4 (96% in D_2O) were purchased from Sigma-Aldrich. D_2O (99.9% deuteration) was purchased from Euriso-top. Hyaluronidase from bovine testes type IV, disodium succinate (anhydrous 99.99%), 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) and all salts and solvents were purchased from Sigma-Aldrich.

2.2. Preparation of HA benzylamide derivatives with differents DS

HA solution in phosphate buffer 1 mM at pH 6 with a concentration of 8.5 mg/ml is prepared. The solution is heated to 70 °C. 2 eq of benzylamine and varying amounts of DMTMM (1.35, 1.0, 0.75, 0.5 eq) are added to the solution. After 2 h of stirring the products are precipitated in 10 times its volume of 2-propanol. The precipitates are separated from the solution by centrifugation and are freeze dried until constant weight (Rydergren, 2013).

2.3. Measurements

2.3.1. Nuclear magnetic resonance spectroscopy

^{1}H NMR spectroscopy was carried out at 25 °C on a Bruker Avance™ 400 MHz. The proton spectra were recorded with 256 scans, 2.56 s acquisition time, 1.0 s relaxation delay and with 90° pulse angle. Chemical shifts, which were referenced to the signal of D_2O (4.79 ppm), are displayed in ppm. Peaks are integrated as shown in supplementary materials Table S1.

A stock solution of HA with 5 mg/ml (repeating unit molecular weight 402 g/mol) dissolved in deuterium oxide with disodium succinate (6.0 mM) was used for all the experiments. The stock solution was divided in different aliquots and different concentrations of NaOD (0.0036, 0.071, 0.125, 0.25, 0.5, 1, 2 M), urea (0.25, 1, 4 M), DCl (0.5 M), D_2SO_4 (0.5 M) and NaCl (0.15, 0.5, 1, 2 M) were added immediately before the measurements and the spectra were recorded within few minutes. Control experiments with HCl (0.5 M) confirmed that there is only a small loss of Mw after one hour (Supplementary Material Fig. S6) and therefore Mw reduction during the measurements can be excluded.

The percentage of mobile protons (H%) is determined with formula (1), where mol_{std} are the moles of the standard, mol_s are the mole of the sample, A_s is the area of the peak of interest of the sample, A_{std} is the area of the standard peak, N $prot_{std}$ are the number of proton of the standard (4 for disodium succinate) and N $prot_s$ are the number of proton of the peak of interest (3 for acetyl, 2 for anomeric protons at C1 and C1´, 10 for backbone protons at C2-C5 and C2´-C6´).

$$H\% = \frac{mol_{std.}}{mol_s}\frac{A_s}{A_{std.}}\frac{N\ prot_{std.}}{N\ prot_s} \bullet 100 \quad (1)$$

Degree of substitution (DS) of HA benzylamide (HAB) is determined by applying equation (2).

$$DS = \frac{A/5}{B/3} \quad (2)$$

A is the proton integral corresponding to the aromatic protons (generally between 7 and 7.5 ppm) and B is the proton integral corresponding to the acetyl protons (generally between 1.8 and 2.1 ppm). Chemical structure of HA and HA benzylamide is depicted in Fig. 1.

For comparison also NMR spectra of modified HA after enzymatic degradation were recorded. Modified HA was dissolved in D_2O with NaCl 1 M (concentration of HA: 5 mg/ml). 1 mg of hyaluronidase was

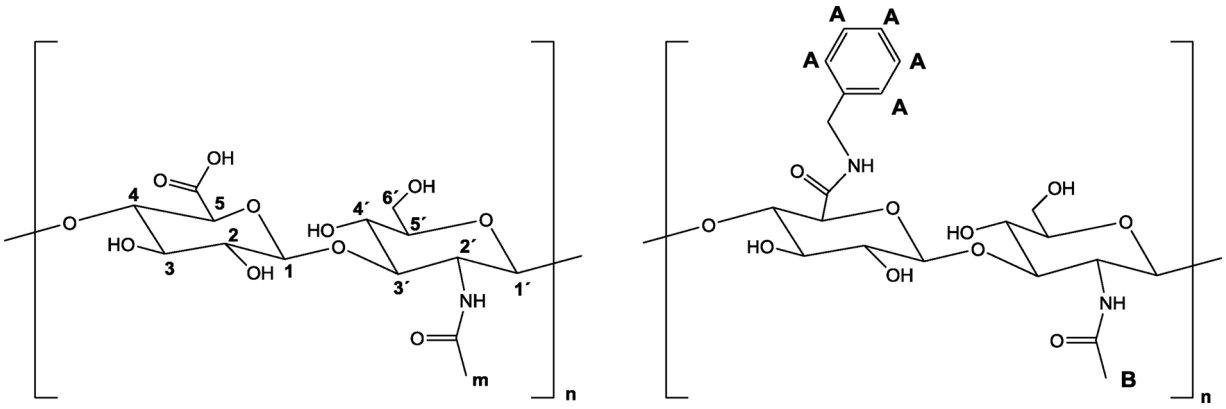

Fig. 1. Chemical structure of HA and HAB.