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Nanostructure of hyaluronan acyl-derivatives in the solid state

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ABSTRACT

Acyl derivatives of hyaluronan (acyl-HA) are promising materials for biomedical applications. Depending on the acyl length and the degree of substitution, these derivatives range from self-assembling water-soluble polymers to materials insoluble in aqueous environments. The behaviour of acyl-HA was studied in solution, but little attention was paid to the solid state, despite its importance for applications such as medical device fabrication. We thus used X-ray scattering and electron microscopy to explore the solid-state nano-structure of acyl-HA. The set of samples included various substituents, substitution degrees and molecular weights. The obtained data showed that all studied acyl-HA materials contained structures with dimensions on the order of nanometres that were not present in unmodified HA. The size of the nanostructures increased with the acyl length, while the degree of substitution and molecular weight had negligible effects. We suggest that the observed nanostructure corresponds to a distribution of hydrophobic domains in a hydrophilic matrix of unmodified HA segments.

1. Introduction

Hyaluronan (HA) is a naturally occurring anionic linear polysaccharide with the repeating unit consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by alternating $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ glycosidic bonds. HA is present, for example, in the extracellular matrix, skin, synovial fluid, vitreous humour, or various connective tissues. It plays an important role in processes such as tissue hydration, lubrication, or wound healing (Dicker et al., 2014). As an important constituent of our body, HA is biocompatible, biodegradable and non-toxic (Garg & Hales, 2004), making it a promising material for applications in medicine.

Due to its hydrophilic character, native HA is soluble in water. However, insolubility or limited solubility is required for applications such as medical device fabrication or drug delivery. A common approach for overcoming HA solubility is covalent cross-linking. The published methods include processes based on enzymatic reactions (Dvorakova et al., 2014), photochemistry (Bobula et al., 2015), or chemical cross-linking agents such as polyvalent hydrazides (Vercruysse, Marecak, Marecek, & Prestwich, 1997), glutaraldehyde, divinyl sulfone and carbodiimides (Collins & Birkinshaw, 2007, 2008). Another option is to chemically modify HA with hydrophobic side groups such as long acyl chains (Creuzet, Kadi, Rinaudo, & Auzély-Velty, 2006; Finelli et al., 2014; Huerta-Angeles, Bobek, Příkopová, Šmejkalová, & Velebný, 2014; Šmejkalová et al., 2012) or octenyl succinic anhydride (Eenschooten et al., 2012). These hydrophobized derivatives can be used in drug delivery applications (Eenschooten et al., 2012; Choi et al., 2010; Šmejkalová et al., 2017) or for the preparation of polymeric films (Foglarová et al., 2016) and fibres (Zápotocký et al., 2016).

The nanostructure of biomaterials can have a significant impact on their performance in applications (Tang et al., 2016). In the case of native HA, there is a number of studies concerned with the nanostructure and chain conformations both in the solid state (Cowman & Matsuoka, 2005; Haxaire, Braccini, Milas, Rinaudo, & Perez, 2000) and in aqueous solutions (Buhler & Boué, 2004; Cowman & Matsuoka, 2005; Matteini et al., 2009). On the contrary, the nano-scale behaviour of hydrophobized HA was only studied in solution with emphasis on drug delivery applications (Eenschooten et al., 2012; Šmejkalová et al., 2014). The solid-state structure of these derivatives was not yet explored, despite the intention to use them also in solid products. Apart from its practical importance, this topic is also interesting in primary research, since the nanostructure of polyelectrolytes such as HA is a complex problem (Svergun & Koch, 2003) that is still not completely understood.

Small-angle X-ray scattering (SAXS) is commonly used for studying

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the nanostructure of polymers (Chu & Hsiao, 2001). However, papers describing the use of SAXS for HA based systems are relatively scarce. As examples, we would mention studies concerned with highly viscous solutions of cross-linked HA (Gamini et al., 2002) and HA-chitosan polyelectrolyte complexes (Lalevée et al., 2016). In the case of hydrophobically modified polysaccharides, SAXS was used, for example, to study the nanostructure of palmitoyl-chitosan hydrogels (Chiu et al., 2009) or the assembly of hydrophobically modified alginate in aqueous solution, sol and hydrogel (Choudhary & Bhatia, 2012). However, to the best of our knowledge, SAXS was not yet used to study hydrophobized HA in the solid state. Other methods used for studying the nanostructure of polysaccharides are small-angle neutron scattering and various light scattering techniques (Buhler & Boué, 2004; Maki, Furusawa, Dobashi, Sugimoto, & Wakabayashi, 2017). For visualization, atomic force microscopy (Moffat, Morris, Al-Assaf, & Gunning, 2016), transmission electron microscopy (Chiu et al., 2009) or high-resolution scanning electron microscopy (Hussain & Jaisankar, 2017) were used.

This paper aims to elucidate the solid-state nanostructure of HA modified with acyl side chains (acyl-HA). For this purpose, a large set of acyl-HA derivatives was prepared that covered a broad range of acyl chain lengths, degrees of substitution and HA molecular weights. Most experiments were carried out with powder samples. Furthermore, selected derivatives were also measured in the form of a film to show whether the nanostructure is influenced by the sample form and the method of its preparation (powders by precipitation, films by slow drying). For characterization, wide- and small-angle X-ray scattering, transmission electron microscopy and extra-high resolution scanning electron microscopy were used.

2. Material and methods

2.1. Material

Commercial sodium hyaluronate (HA) with various molecular weights was provided by Contipro a.s. The molecular weight data were obtained using size exclusion chromatography with multiangle laser light scattering detection (SEC-MALLS), as described elsewhere (Podzimek, Hermannova, Bilerova, Bezakova, & Velebny, 2010). Sodium chloride, sodium hydroxide, trichloromethane, 2-propanol, hexane, triethylamine and tetrahydrofuran were obtained from Lachner. Hydrochloric acid, formic acid, undecanoic acid, and HPLC grade 2-propanol were obtained from Sigma-Aldrich. Fatty acids (hexanoic, decanoic, lauric, palmitic and *cis*-oleic), fatty acid anhydrides and *N,N*-dimethylaminopyridine were obtained from TCI Europe. Benzoyl chloride was obtained from Merck.

The acyl-HA derivatives were prepared by the esterification of OH groups on the HA backbone (Fig. 1). Advantageously, the used modification does not involve the carboxyl groups that remain available for receptor interactions. Furthermore, the molecular weight of HA is not expected to change significantly during the reaction (Huerta-Angeles et al., 2014). The exact reaction procedure for each derivative was chosen based on our previous experience. The palmitoyl (HA-C16) and lauroyl (HA-C12) derivatives were prepared by the acylation of HA with a symmetric fatty acid anhydride (Šmejkalová et al., 2012). Briefly, HA was first dissolved in demineralized water with a subsequent addition of triethylamine and the catalyst *N,N*-dimethylaminopyridine. After one hour of mixing, the solution was diluted with tetrahydrofuran and further homogenized. Next, the symmetric anhydride was added. At the end of the reaction, the mixture was diluted with 50% 2-propanol, followed by the addition of a saturated sodium chloride solution. The product was precipitated with absolute 2-propanol, washed, decanted and dried for 48 h at 40 °C. The hexyl (HA-C6), decanoyl (HA-C10) and oleyl (HA-C18:1) derivatives were prepared by a similar method that differs in the use of a mixed fatty acid anhydride instead of the symmetric one (Huerta-Angeles et al., 2014).

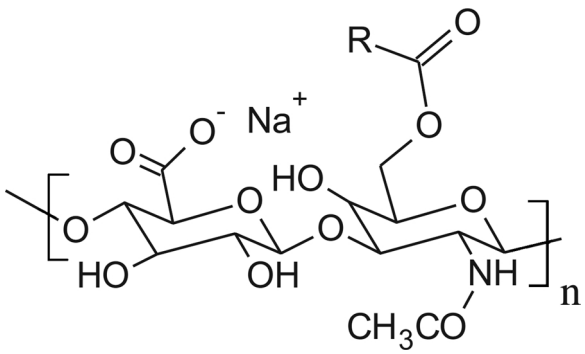

Fig. 1. Scheme of acyl-HA structure denoting a substituted disaccharide unit, where R is the aliphatic chain of the acyl group and n is the number of repeating disaccharide units.

The degree of HA substitution (DS) was determined by gas chromatography, as described elsewhere (Chmelař et al., 2017). Briefly, the method is based on the alkaline hydrolysis of the samples, extraction of the hydrolyzed fatty acids and their quantification by gas chromatography. Note that we express DS as the number of acyl chains per 100 HA repeating disaccharide units. For example, a DS of 20% means that there are in average 20 acyl chains on each 100 disaccharide units.

2.2. Preparation of acyl-HA films

The acyl-HA films were prepared by solution casting in a custom built apparatus (Foglarová et al., 2016), where the solution is evaporated between two plates with controlled temperature. This design enables precise process control, ensuring good reproducibility. First, the acyl-HA solution was prepared by dissolving the polymer in 50 wt.% aqueous 2-propanol to a concentration of 10 mg/ml. To ensure complete dissolution, the solution was vigorously stirred at 25 °C for 18 h (Collins & Birkinshaw, 2013). The acyl-HA solution (20 ml) was applied into the drying apparatus on a hydrophobized glass substrate. The apparatus was then closed, the lower and upper plate temperature set to 50 and 20 °C, respectively, and the solution was left to dry for 6 h. Subsequently, the apparatus was opened and the dry film removed from the substrate. The used film preparation conditions were chosen based on previous experiments (Foglarová et al., 2016).

2.3. X-ray scattering

The small- and wide-angle X-ray scattering (SAXS/WAXS) experiments were performed on a SAXSess mc^2 instrument (Anton Paar). All samples (powders and films) were measured in point collimation geometry using a GeniX Microfocus X-ray point source with a Cu anode (50 kV and 1 mA) and single-bounce focusing X-ray optics. Image plates together with a CyclonePlus® Reader (PerkinElmer, Inc.) were used to record 2D X-ray scattering patterns. During measurement, the whole chamber of the instrument was evacuated to a pressure below 1 mbar to minimize undesirable parasitic absorption and scattering by air. The range of the scattering vector magnitude q was $0.2 - 28\,\mathrm{nm}^{-1}$. Both the SAXS and WAXS range were thus covered. Note that q is defined as

$$q = \frac{4\pi}{\lambda}\sin\theta \quad (1)$$

where λ is the X-ray wavelength (0.15418 nm for CuK_α) and θ is the half-scattering angle.

The film samples were fixed directly in the sample holder and the powder samples were glued in between two pieces of scotch tape. Since all 2D patterns were circularly symmetric, the 1D radial scattering-intensity profiles were obtained from them by azimuthal averaging using the SAXSquant software (Anton Paar). The scattering intensities were corrected with respect to background scattering. Finally, incoherent-