



## Viscoelastic and mechanical properties of hyaluronan films and hydrogels modified by carbodiimide



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### ABSTRACT

This study investigated an effect of different ways of the preparation of insoluble hyaluronan material on its mechanical and viscoelastic properties. Hyaluronan (NaHy) of molecular weight  $M_w = 500,000 \text{ g mol}^{-1}$  was modified with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), to be able absorb liquid without changing its mechanical properties. The modified, water insoluble NaHy materials were prepared in different geometry; as modified films and modified cylinders with exact dimensions. The occurrence of modification was confirmed by FT-IR (Fourier transform infrared spectroscopy) and <sup>1</sup>H NMR (proton nuclear magnetic resonance) spectroscopy and swelling test. The determined mechanical and viscoelastic properties of unmodified and modified hyaluronan revealed the high dependency of elasticity changes depending on the gel processing method. Moreover, NaHy gels in the cylindrical form with the sponge-like structure predominant them as a convenient geometry for application in a humid environment.

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### 1. Introduction

Hyaluronic acid (HA) is a linear biopolymer with a high molecular weight, naturally presented in vertebrate organism. The highest amount of hyaluronic acid was discovered in the extracellular matrix of connective tissues. Furthermore, it can be found as a constituent in all body fluids and in the vitreous humour of the eye and the synovial fluid in the joints (Manasa, Sridevi, Chandana Lakshmi, & Dedeepya, 2012; Fraser, Laurent, & Laurent, 1997). Its structural unit consists of *N*-acetyl-D-glucosamine and D-glucuronic acid connecting with alternating β-1,3 and β-1,4 glycosidic bond (Laurent & Fraser, 1992). HA chain may expand to thousand fold of its original volume and form a loose hydrated network because of hydrophilic nature. The molecular weight of NaHy can reach  $10^6$  to  $10^8 \text{ g mol}^{-1}$  according to different sources and methods used to its determination (Cowman & Matsuoka, 2005). Due to

such high molecular weight and its viscoelastic and rheological properties predestine hyaluronic acid to play important roles in living organisms (Kogen, Šoltés, Stern, & Gemeiner, 2007; Price, Berry, & Navsaria, 2007; Brown & Jones, 2005; Guillaumie et al., 2009). The above mentioned, unmodified hyaluronic acid has many important applications in drug delivery and surgery as well as in the field of ophthalmology, tissue engineering and wound healing (Saettone, Monti, & Torracca, 1994; Moore & Willoughby, 1995; Davidson, Nanney, Broadley, Whitsett, & Aquino, 1991; Vercruyse & Prestwich, 1998; Prestwich, Marecak, Marecak, Vercruyse, & Ziebell, 1998; Collins & Birkinshaw, 2013). Many authors have reported the important influence of molecular weight and degree of substitution of NaHy on the final film formation (Minařík, Smolka, & Lapčík, 2011; Mlčochová et al., 2006; Li et al., 2007; Obadait et al., 2010). Chemical modification of sodium salt hyaluronan (NaHy) allows modifying its solubility, viscosity, amphiphilicity, rate of degradation and *in vivo* residence time. Schanté, Zuber, Herlin, and Vandamme (2011) reviewed a number of chemical modification methods relevant for NaHy. The most common modification of hyaluronic acid is a chemical cross-linking, which can fix its structure (Lu, Lai, Ma, & Hsiue, 2008). The intensive research

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was devoted to study of NaHy cross-linking mechanism (Collins & Birkinshaw, 2007). The functional groups enabling cross-linking of HA molecules are carboxyl and hydroxyl groups, whereby carboxyl groups can be easily cross-linked generating an ether bond, and hydroxyl groups form ester bond. Moreover, other reactive groups such as amino groups can be also introduced in NaHy. Water-insoluble NaHy films can be prepared by an esterification of hyaluronic acid with mono-functional organic halides whereas highly swollen gels or insoluble plastic materials by cross-linking with divinylsulfone, bisepoxides, formaldehyde, glutaraldehyde, bishalides and carbodiimide (Balazs & Leshchiner, 1986; Balazs & Leshchiner, 1987; De Belder & Mälson, 1986; Malson & Lindqvist, 1986; Valle & Romeo, 1988; Valle & Romeo, 1987; Hu, Sabelman, Tsai, Tan, & Hentz, 2000; Zhang et al., 2011; Perng Ch, Wang, Tsi Ch, & Ma, 2011). Hamilton, Fox, Acharya, and Walts (1970) patented in 1970 cross-linking of HA by carbodiimide. Further experiments concerning modification of NaHy by carbodiimides were performed by many other authors (Xuejun, Netti, Ambrosio, Nicolais, & Sannino, 2004; Tomihata & Ikada, 1997; Kuo, Swann, & Prestwich, 1991; Sannino et al., 2005). Generally, carbodiimide is considered to be a preferable cross-linking agent with low cytotoxicity and water solubility.

Despite of considerable research concerning of physico-chemical properties of cross-linked or modified NaHy (Haxaire, Braccini, Milas, Rinaudo, & Pérez, 2000; Hamilton et al., 1970) rather less attention has been paid to the investigation of viscoelastic properties of modified NaHy materials depending on its geometry. We hypothesize that cross-linking and modification of NaHy change not only its solubility but especially viscoelastic properties. Moreover, the geometric form of the final modified NaHy can significantly influence its mechanical and viscoelastic properties. In the literature it is evident, that the most common forms of modified NaHy are hydrogels in the form of films. We have supposed that modified NaHy in the form of cylindrical hydrogel (NaHy.B.cylinder) could promote rather intermolecular than intramolecular modification, contributing to the overall stabilization and probably more superior mechanical properties in comparison with flat hydrogel (NaHy.B.film). In addition to this, cylindrical hydrogel (NaHy.B.cylinder) may find a vacancy in applications such as wound healing, drug delivery substrates, scaffolds for tissue engineering or absorbent medications. Moreover, NaHy.B.cylinder due to its geometry may be applicable to remove heavy metal ions from aqueous solutions. In this work the water-insoluble hyaluronan hydrogels in the form of films and cylinders were prepared and characterized by nuclear magnetic resonance ( $^1\text{H}$ NMR), FT-IR spectroscopy, tensile testing and dynamic mechanical analysis (DMA).

## 2. Materials and methods

The sodium hyaluronate sample (NaHy) of molecular weight  $M_w = 500,000 \text{ g mol}^{-1}$  was provided by Contipro, (Czech Republic). Cross-linking agents *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (HMS) were purchased from Sigma-Aldrich. All of the chemicals were of analytical grade and used without further purification.

### 2.1. Preparation of unmodified hyaluronan films

Stock solutions of NaHy in concentration of 1% (w/w) were prepared by dissolving the polymer in Milli-Q water using gentle stirring for 24 h at 50 °C. The solution of hyaluronan was then poured into a polystyrene mould with dimensions of  $100 \times 80 \times 0.05 \text{ mm}$  and dried to constant weight at 50 °C.

### 2.2. Preparation of modified hyaluronan films

Cross-linking agent EDC works as the activating ingredient of the carboxylic groups (100 mM) and HMS as the proton exchanger in a weight ratio 2:1 were added to already prepared 1% (w/w) hyaluronan and stirred for 1 h at 25 °C. This solution was then titrated with 0.1 M HCl to adjusting pH in the range of 4.5–4.75 under continuous stirring 1 h. Resulting solution was then poured into a polystyrene mould with dimensions of  $100 \times 80 \times 0.05 \text{ mm}$  and dried to constant weight at 50 °C.

### 2.3. Preparation of modified hyaluronan cylinders

The NaHy cylindrical hydrogels with a sponge-like solid structure were prepared as follows: 100 mM EDC was added to 1% (w/w) hyaluronan solution and stirred for 1 h at 25 °C. A 0.1 M HCl was then added to adjust pH to 4.5–4.75 under continuous stirring during 1 h. Subsequently, this solution was casted in polyethylene bins and kept at  $-18 \text{ °C}$  for 72 h to prepare hyaluronan hydrogels in the cylindrical form with the diameter of 2.8 cm and the height of 0.7 cm.

### 2.4. Swelling test

The swelling test was used to determine an extent of modification of hyaluronan materials. Prepared hyaluronan samples were kept in a desiccator for one week. Afterwards were cylinders cut to round shape samples of diameter 11 mm, and dried to constant weight at 50 °C. The modified hyaluronan samples were then swelled in Milli-Q water and reweighed immediately at predetermined time intervals. The water content ( $W_{\text{H}_2\text{O}}(\%)$ ) in samples was calculated according to the following equation:

$$W_{\text{H}_2\text{O}}(\%) = \frac{(w_i - w_0)}{w_i} \times 100 \quad (1)$$

where  $w_i$  is the weight of the swollen sample and  $w_0$  is the weight of the dry sample before swelling. The presented results are average values of 4 measurements.

### 2.5. FT-IR analysis

The FT-IR spectra of dried NaHy film, modified NaHy film and cylinder were recorded with Nicolet 6700 FT-IR spectrometer in ATR arrangement with diamond crystal. All spectra were recorded in the absorbance mode in the wave number range  $4000\text{--}400 \text{ cm}^{-1}$  and 64 accumulations at the resolutions of  $4 \text{ cm}^{-1}$ .

### 2.6. $^1\text{H}$ NMR

The NaHy samples and EDC were dissolved in  $\text{D}_2\text{O}$  (Sigma-Aldrich, 99.9 atom% D, with no internal standard), and the spectra were calibrated to 4.70 ppm for residual HDO signal. Samples investigated by NMR were modified using deuterized water and 0.1 M deuterium chloride acid solution (prepared from 35 wt% DCl in  $\text{D}_2\text{O}$  (Sigma-Aldrich, 99 atom% D) and  $\text{D}_2\text{O}$ ) as described in Section 2.3 and transferred into NMR cuvette under an inert atmosphere. For quantitative analysis was taken  $0.5 \text{ cm}^3$  of liquid that was gently compressed from hydrogel, and  $0.01 \text{ cm}^3$  of DMSO (used as an internal standard). Concentrations of ingredients were calculated according Eq. (2) where  $I$  is integral intensity of  $^1\text{H}$  NMR signal,  $N$  is the number of H-atoms providing signal under consideration. The volume change related to the mixing of  $0.5 \text{ cm}^3$  of sample with  $0.01 \text{ cm}^3$  of DMSO was ignored.

$$c_i = \frac{I_i}{N_i} \times \frac{N_{\text{DMSO}}}{I_{\text{DMSO}}} \times c_{\text{DMSO}} \quad (2)$$

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