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Preparation and investigation of a cross-linked hyaluronan nanoparticles system

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ABSTRACT

The present paper describes the preparation and characterization of hydrophilic hyaluronan nanoparticles formed by cross-linking of hyaluronic acid (HA) with 2,2′(ethylenedioxy)bis(ethylamine) in the presence of water-soluble carbodiimide (CDI) in aqueous media. The particle size of cross-linked nanosystems was measured by dynamic light scattering (DLS) and transmission electron microscopy (TEM), transmittance by UV–VIS spectrophotometry, gel permeation chromatography (GPC) and rheology to characterize the physico-chemical properties.

The aqueous nanosystems prepared were stable, transparent or mildly opalescent with values of transmittance above 91%. It was observed, that a fraction of particles with size less than 20 nm, has been released during the purification by diafiltration. It was established that the hydrodynamic size of cross-linked HA nanoparticles can be controlled by variation of the reaction conditions such as concentration of HA, salt concentration of media, ratio of cross-linker and the final pH of the reaction mixture.

1. Introduction

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan that is an unbranched polysaccharide consisting of repetitive disaccharide units with reactive carboxyl groups. It is a biodegradable, biocompatible, non-toxic, non-immunogenic and non-inflammatory biomaterial; therefore, it has been used for several medical applications.

HA can be found in all tissues and body fluids of living creatures and most abundantly in the soft connective tissues. The total amount of HA in the adult human has been estimated to be 11–17 g (Laurent & Reed, 1991). The HA nanofibrous scaffold was successfully fabricated to mimic the architecture of natural extracellular matrix (Almond, DeAngelis, & Blundell, 2006; Ji et al., 2006).

The excellent water-binding capacity of HA is responsible for retaining moisture in eyes, joints, and skin tissues (Robert, Robert, & Renard, 2010). The solution of HA is highly viscous with unique viscoelastic properties which enables its use for orthopedy (Witteveen, Sierevelt, Blankevoort, Kerkhoffs, & van Dijk, in press). Many studies have been performed to create HA as an injectable form (Salk, Chang, D'Costa, Soomekh, & Grogan, 2006), which is used to treat osteoarthritis of the knee. (Strand et al., 2006).

HA can be used as an eye-treating solution (Nakamura, Sato, Chikama, Hasegawa, & Nishida, 1997). Oral application of HA has been lately suggested, although its effectiveness needs to be demonstrated.

Several HA derivatives have been developed for drug delivery. HA has potential as a biodegradable carrier for transdermal drug delivery (Avila et al., 2008). HA has also been used as a novel depot system (Oh et al., 2010). HA in the forms of physically and chemically cross-linked hydrogels (Kim & Park, 2002; Leach & Schmidt, 2005; Li, Liu, Shu, Gray, & Prestwich, 2004) has been developed as nano- and micro particulate systems (Choi et al., 2008; Segura, Chung, & Shea, 2005) for various protein, drug (He, Zhao, Yin, Tang, & Yin, 2009), peptide (Moriyama, Ooya, & Yui, 1999) or gene (Lee, Mok, Lee, Oh, & Park, 2007; Luten, van Nostrum, De Smedt, & Hennink, 2008) delivery.

Various methods have been developed to produce cross-linked hyaluronic acid, as hydrogels (Crescenzi, Francescangeli, Taglienti, Capitani, & Mannina, 2003; Masters, Shah, Leinwand, & Anseth, 2005), films (Liu, Shu, & Prestwich, 2005), or particulate systems (Dulong et al., 2004; Pitarresi, Craparo, Palumbo, Carlisi, & Giammona, 2007). Particulate systems are usually formed in emulsion, in which the size of droplets can control the size of particles. Solvent evaporation (Lim, Forbes, Berry, Martin, & Brown, 2002), spray-drying (Esposito, Menegatti, & Cortesi, 2005) and coacervation (Vasiliu, Popa, & Rinaudo, 2005) are also well-known techniques to produce micro- or nano-sized particulate systems.

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Table 1
Reaction conditions of matrix products prepared in pure water.

Name	Medium	HA (mg)	Concentration of HA (mg/ml)	Stoichiometric ratio of cross-linking (%)	Quantity of diamin (1.0%, v/v) (μl)	Quantity of CDI (mg)
A_1_25	Water	50	1	25	228	9.3
A_2_25	Water	100	2	25	456	18.5
A_3_25	Water	150	3	25	684	27.8
A_3_12	Water	150	3	12	328	13.3
A_3_7	Water	150	3	7	191	7.8

In this work, preparation of stable cross-linked HA nanoparticles is described. The particulate systems were obtained by covalently cross-linking of carboxyl groups of HA linear chain with a 2,2′(ethylenedioxy)bis (ethylamine) in the presence of water-soluble carbodiimide (CDI) in aqueous media, as described earlier (Bodnár et al., 2009). The purpose of the present study was to investigate the effect of the reaction conditions on the formation of HA nanoparticles. It was observed that the salt and HA concentrations in the reaction mixture have significant effect on the size of particles formed.

The results based on TEM, DLS and rheology experiments reveal that well-dispersed HA nanoparticles systems with spherical shape were obtained. It was found that the particle sizes and size distribution can be influenced by the concentration of HA and the salt concentration of the media.

Intra and intermolecular cross-linking processes were formed and nanosystems with broad size distribution were produced, however, the smaller particles were lost during the dialysis.

2. Experimental

2.1. *Materials*

The HA sodium salt (M_W = 4350 kDa) was obtained from Gedeon Richter Ltd., Hungary. Quality of the sodium hyaluronate met the European Pharmacopoeia (Ph. Eur.) requirements. 2,2′(Ethylenedioxy)bis(ethylamine) and 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide methiodide (CDI) were purchased from Sigma–Aldrich, Co. The pH was adjusted with NaOH and HCl solutions as required. All other chemicals were analytical grade. Millipore-filtered water was used throughout the study.

2.2. Reaction conditions

2.2.1. Preparation of cross-linked hyaluronan nanoparticles

Cross-linked HA nanoparticles were prepared according to the procedure first reported by Bodnár et al. (2009). Briefly, HA (M_W = 4350 kDa) was dissolved in aqueous media to produce a clear solution, and then adjusted to pH 5.5. The diamine solution (1.0 v/v% in water, pH = 5.5) was added to the HA solution and mixed for 30 min at room temperature. The water-soluble CDI solution was added dropwise, and the reaction was stirred for 24 h. The solution containing hyaluronan nanoparticles was purified by dialysis and freeze-dried. The yield of HA nanoparticles was between 76% and 94%. The reaction conditions of cross-linked HA nanoparticles are summarized in Table 1, and synthesis scheme of cross-linking reaction is represented in Fig. 1.

2.2.2. Media

Aqueous media were used for the preparation of nanoparticles. The cross-linking reaction of HA was carried out in three different media such as pure water (designated with letter “A”), NaCl (c = 0.09%, w/w, and c = 0.9%, w/w) solutions (designated with letter “B” and “C”, respectively). The water was purified by deionization, and reverse osmosis (Milli-Q-Plus instrument).

2.2.3. Matrix and matrix products

Matrix products are the end-products of the reactions which were synthesised under different reaction conditions, as medium applied, concentration of HA and the ratio of cross-linking. The nanoparticles obtained are identified by a three notation system as follows: the first letter means the media, the second notation is a number representing the concentration of HA (mg/ml), and the third notation stands for the stoichiometric ratio of cross-linking. For instance, A_1_25 notation means: the media is pure water (designated with letter “A”), concentration of HA is 1 mg/ml and the ratio of cross-linking is 25%.

A summary of the HA nanoparticles that have been prepared with their notations is shown in Table 2.

2.3. Characterization of the nanoparticles

2.3.1. Transmittance

Transmittances of cross-linked hyaluronan nanosystems were measured using an HP-8453 UV–VIS spectrophotometer at an operating wavelength of λ = 500 nm in optically homogeneous quartz cuvettes at 25 °C.

2.3.2. Dynamic light scattering (DLS)

Hydrodynamic diameter and size distribution of cross-linked hyaluronan nanosystems were measured using a Zetasizer Nano ZS instruments (Malvern Instruments Ltd., Worcestershire, UK), at an operating wavelength of λ_o = 532 nm. Measurements of size distribution and the Z-average size of nanoparticles were performed at 25 °C with an angle detection of 173° in optically homogeneous polystyrene cuvettes.

The samples were taken from the reaction mixture. Each sample was measured five times and average serial data were calculated.

The pH of the matrix products was adjusted by the addition of NaOH (c = 0.1 M) or HCl (c = 0.1 M) solutions.

2.3.3. Transmission electron microscopy (TEM)

Size and morphology of the dried hyaluronan nanoparticles were gauged by JEOL2000 FX-II transmission electron microscope.

For TEM observation, the hyaluronan nanoparticles were prepared from the reaction mixture at a concentration of 0.2 mg/ml. The pH of diluted colloid systems containing nanoparticles was adjusted to pH 10. The sample for TEM analysis was obtained by placing a drop (V = 10 μl) of the colloid dispersion onto a carbon-coated copper grid. The samples were dried at room temperature and then examined using a TEM without any further modification or coating.

The particle size distribution was obtained from measured particles visualized by TEM images and then analyzed using the Microsoft Office Excel 2007 program file.

Table 2
Matrix products of parallel reactions prepared in pure water.

A_1_25	A_2_25	A_3_25
A_1_12	A_2_12	A_3_12
A_1_7	A_2_7	A_3_7

2.3.4. Gel permeation chromatography (GPC)

GPC analysis of HA and that of cross-linked particles was performed on a Waters HPLC system using BioSuite 450 HR column. The effluent was monitored at 210 nm. The mobile phase was a mixture of 0.05 M NaOAc, 0.2 M NaCl and water–methanol with a ratio of 8:2 and the flow rate was 0.7 ml/min. Samples were dissolved in water, and filtered with a 5 μm pre-column sieve.

2.3.5. Rheology

Rheological measurements were carried out with a Physica MCR101 rheometer (Anton Paar, Austria). A cone-plate measuring device was used in which the cone angle was 1°, and the thickness of the sample was 0.046 mm in the middle of the cone. The measurements were performed at 25 °C. The pH was adjusted to 6.3 ± 0.2. Flow curves of the different samples were also determined. The

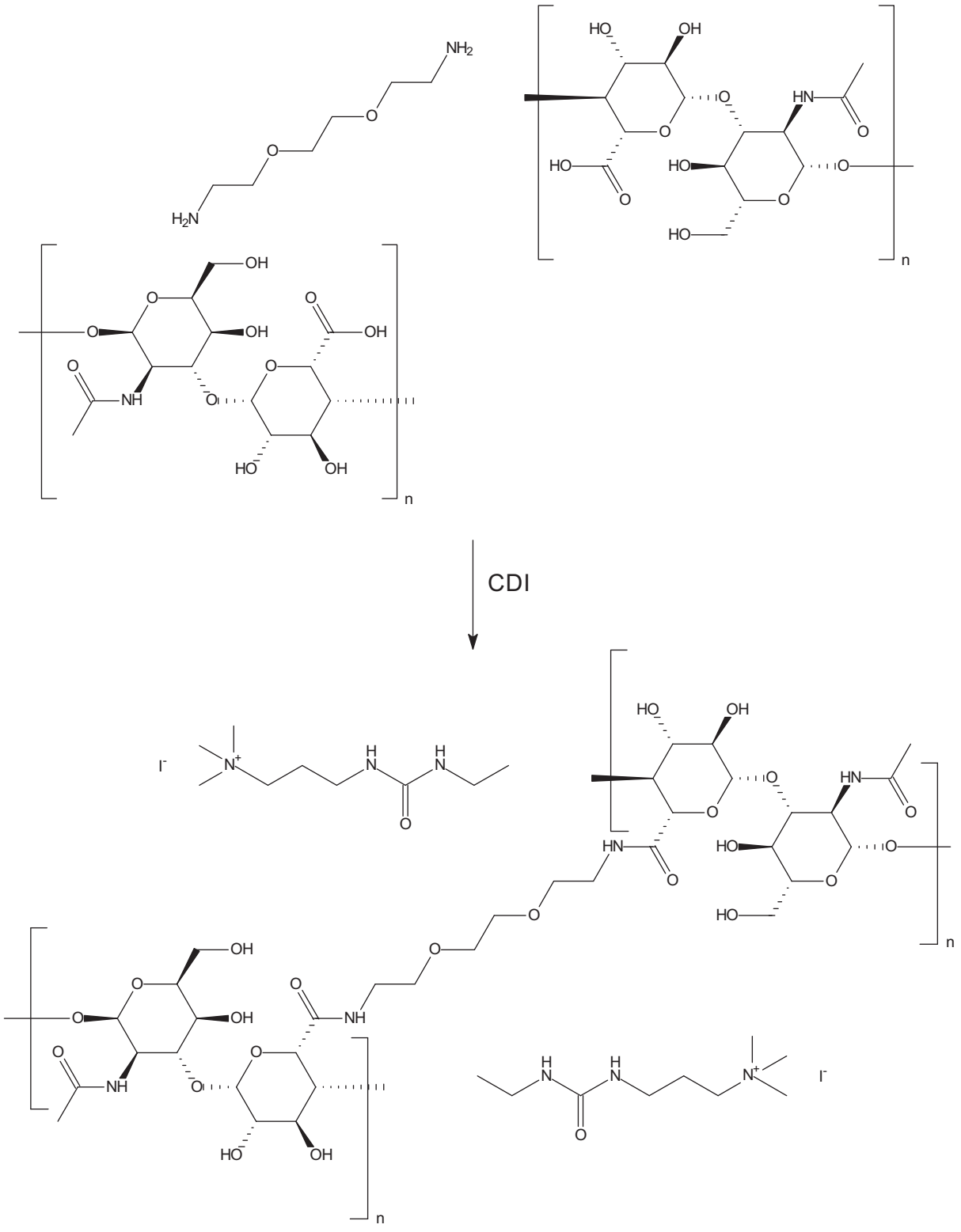

Fig. 1. Schematic representation of the synthesis of cross-linked HA derivatives.

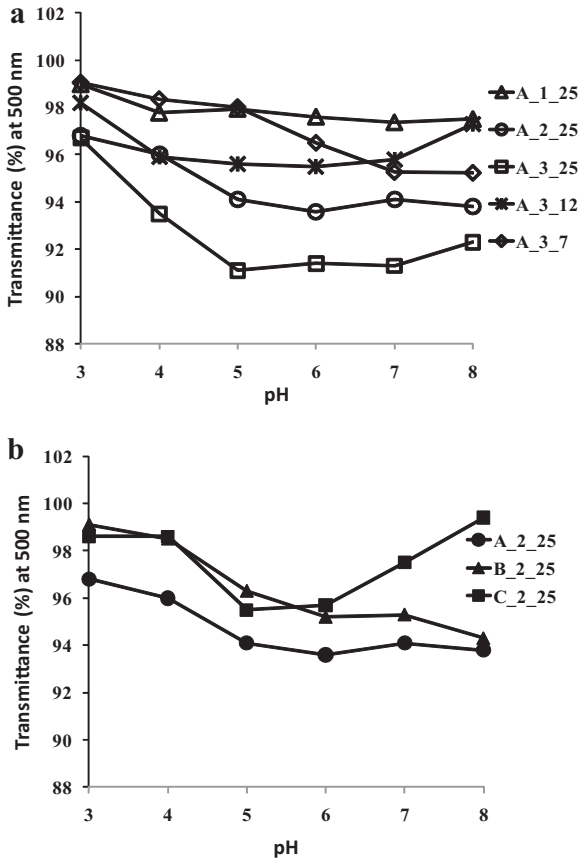

Fig. 2. (a) Effect of concentration of HA, the ratio of cross-linking and the pH of the environment on the transmittance of cross-linked hyaluronan nanoparticles. (b) Effect of pH and salt concentration of the media on the transmittance values of hyaluronan nanoparticles

shear rate was increased from 0.1 to 150 1/s (up curve), and then decreased from 150 to 0.1 1/s (down curve) in the CR mode. The shearing time was 300 s in case of both segments.

3. Results and discussion

3.1. Transmittance results

The transmittance values were measured from the reaction mixtures containing different cross-linked hyaluronan nanoparticles. These colloid dispersions were transparent or mildly opalescent systems in aqueous media. The transmittance values were between 91% and 99%.

The general trend that appears in Fig. 2a is in accordance with the Bouguer–Lambert–Beer law that transmittance values decrease due to the increasing concentrations of HA. The transmittance values were very high in wide pH range because of solvation of the cross-linked HA nanoparticles in aqueous media.

It was observed that the pH was not a factor for transmittance values of the aqueous systems. Stable colloid particles were formed over a wide pH range independently of the media (Fig. 2b) and ratio of cross-linking (from 7% to 25%, Fig. 2a).

3.2. DLS results

Samples were taken from the reaction mixture before and after dialysis. The pH of the samples was adjusted by sodium-hydroxide or hydrochloric acid solutions.

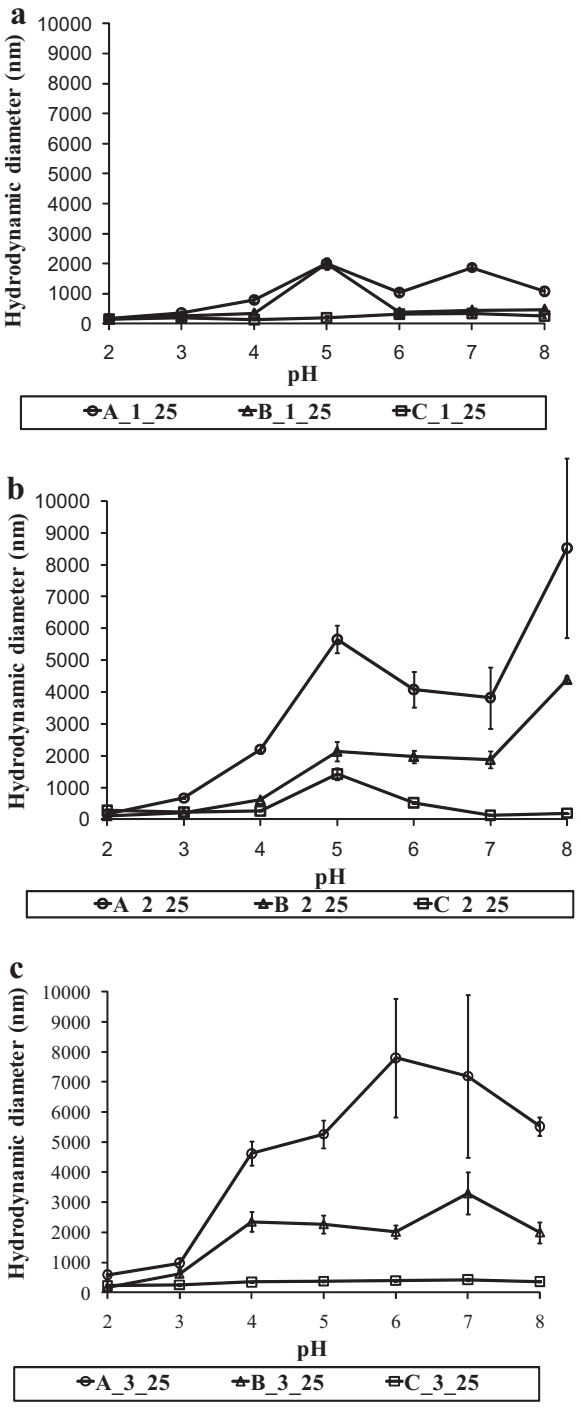

Fig. 3. Effect of pH and salt concentration of the medium on the *Z*-average hydrodynamic diameter of hyaluronan nanoparticles cross-linked at 25%, at the indicated HA concentration. (a) c_{HA} = 1 mg/ml; (b) c_{HA} = 2 mg/ml; and (c) c_{HA} = 3 mg/ml.

3.2.1. Hydrodynamic diameter

The cross-linking process of HA can result in intramolecular and intermolecular cross-linking. One part of cross-linked hyaluronan nanoparticles was formed as small, individual particles; however, large particles were also produced. The large particles can be aggregates, associations caused by secondary interactions or intermolecular cross-linked particles. Therefore, the hydrodynamic diameter of nanoparticles from reaction mixtures was not monomodal, so the *Z*-average size was used to compare hydrodynamic size.

Analysis of the *Z*-average size of nanoparticles (Fig. 3) revealed that the hydrodynamic diameter of nanoparticles decreases when the salt concentration of the media was increased.

Hydrodynamic size of swelled particles was calculated from the translational diffusion coefficient using the Stokes–Einstein equation: $d(H) = kT/3\pi\eta D$, where: $d(H)$ = hydrodynamic diameter, D = translational diffusion coefficient, k = Boltzmann's constant, T = absolute temperature, and η = viscosity. The translational diffusion coefficient depends both on the size of the particle and on the surface structure, as well as the salt concentration of the medium.

Low conductivity of the medium eventuates an extended double layer of ions around the particle, reducing the diffusion speed and

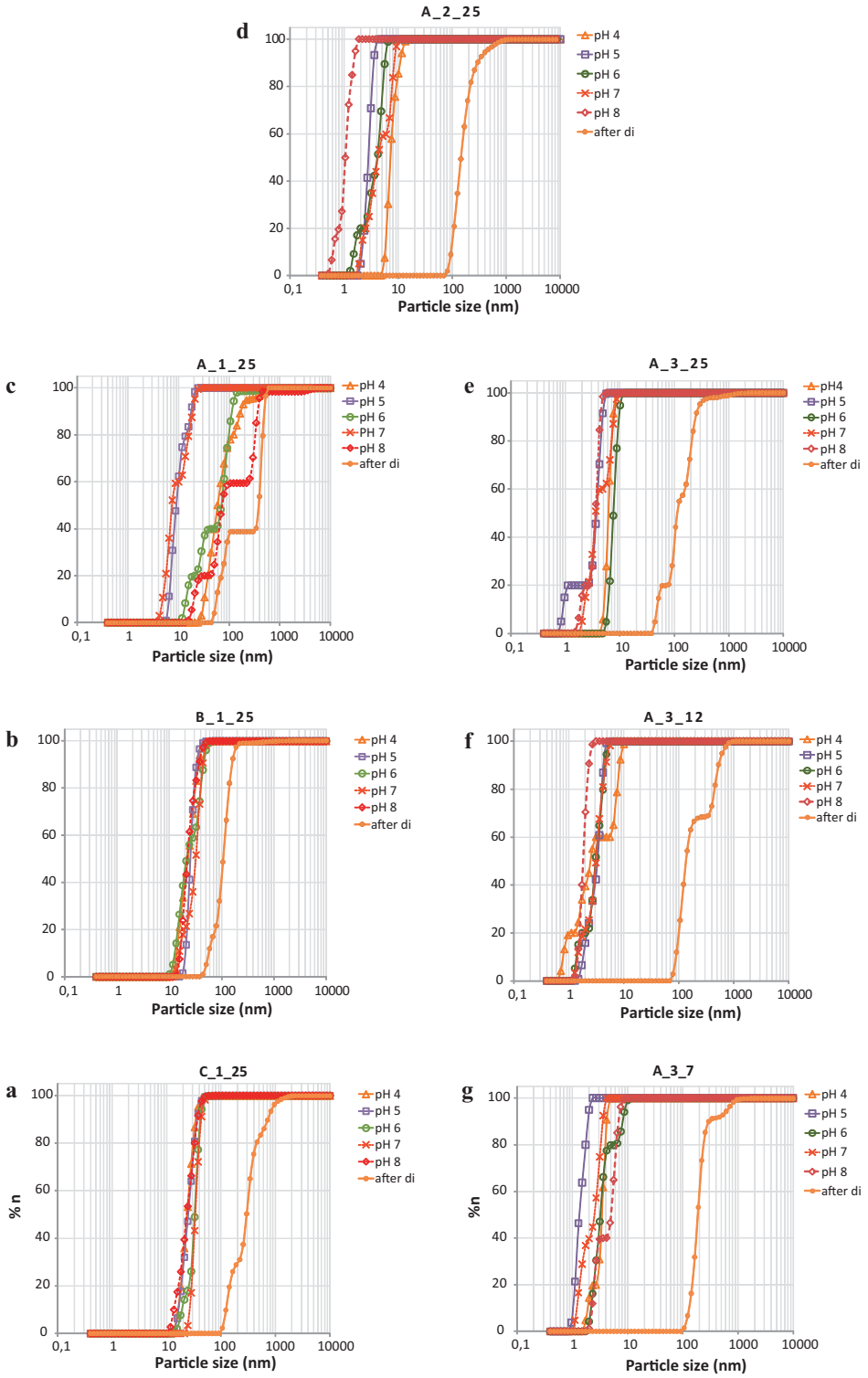

Fig. 4. Effect of pH and purification on the particle size distribution of cross-linked hyaluronan particles at the indicated reaction conditions: (a) c_{HA} = 1 mg/ml, ratio of cross-linking: 25%, c_{NaCl} = 0.9 m/m%; (b) c_{HA} = 1 mg/ml, ratio of cross-linking: 25%, c_{NaCl} = 0.09 m/m%; (c) c_{HA} = 1 mg/ml, ratio of cross-linking: 25%, prepared in water; (d) c_{HA} = 2 mg/ml, ratio of cross-linking: 25%, prepared in water; (e) c_{HA} = 3 mg/ml, ratio of cross-linking: 25%, prepared in pure water; (f) c_{HA} = 3 mg/ml, ratio of cross-linking: 12%, prepared in pure water; and (g) c_{HA} = 3 mg/ml, ratio of cross-linking: 7%, prepared in pure water.

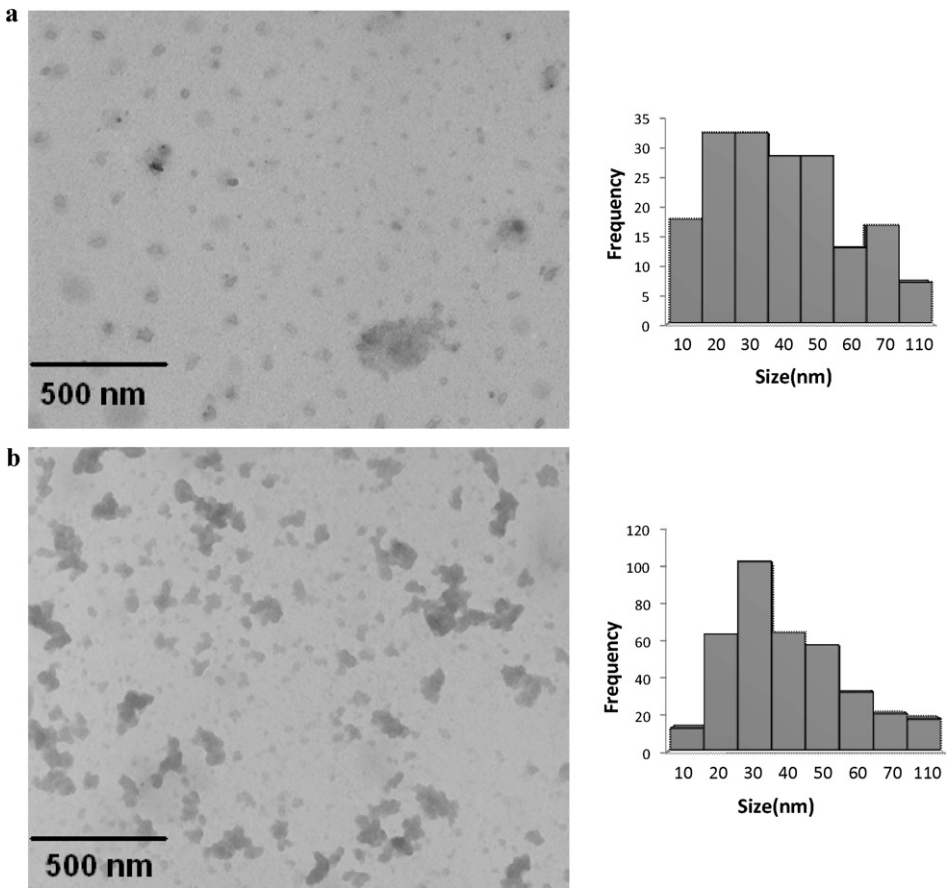

Fig. 5. TEM images and size distributions of cross-linked hyaluronan particles. (a) A_1_25 (c_{HA} = 1 mg/ml, at a cross-linking stoichiometric ratio of 25%, prepared in pure water) and (b) A_3_7 (c_{HA} = 3 mg/ml, at a cross-linking stoichiometric ratio of 7%, prepared in pure water).

resulting in a larger, apparent hydrodynamic diameter than using other media of higher conductivity.

Fig. 3 shows the effect of pH on the hydrodynamic size. It can be seen that by increasing the pH, the average size increased in all cases. This tendency increased as the initial concentration of HA increased and the salt concentration of medium decreased. The residual carboxyl groups of HA can be deprotonated, and the repulsive interactions between the negatively charged functional groups increase the size of particles.

To all appearances, the cross-linked hyaluronan particles can swell in aqueous media, the hydrodynamic size of particles depends on the pH and the salt concentration of the environment.

3.2.2. Particle size distribution (PSD)

Fig. 4 shows the particle size distribution of aqueous colloid systems containing cross-linked hyaluronan particles. Fig. 4c–e shows that the end point of number distribution profiles of the reaction mixtures shifts to the smaller particles by increasing the concentration of HA at the same 25% ratio of cross-linking. Namely, the HA formed smaller nanoparticles by increasing the concentration of HA a result independent of the pH.

The effect of cross-linking ratio on the hydrodynamic size was also studied.

Based on Fig. 4e–g it can be established that nano-sized particles with narrow size distribution can be obtained using lower (7% or 12%) cross-linking ratios. The size of nanoparticles in these systems was below 10 nm, and the pH was not a factor.

The effect of salt concentration on the size of the particles was also determined. Fig. 4a–c represents that by increasing the salinity of reaction mixtures, cross-linked particles with narrower size distribution were produced. Change in pH was without effect. Nevertheless, no significant correlations were established between the hydrodynamic diameters of swelled particles and the physicochemical parameters of the reactions; the size and size distribution of all hyaluronan particles were below 20 nm.

However, significant differences were observed between the PSD values of reaction mixtures and PSDs adopted for the dialyzed samples. The later mentioned profiles were indicated as 'after dial' text shown in Fig. 4. Considering that PSD for all of tested dialyzed matrix products showed a shift to the larger particle sizes, it became apparent that dialysis caused a loss of smaller particles. This was confirmed by GPC and rheology. The tested reaction mixtures contain larger particles, which make up all of the particles after dialysis.

In summary, the size and size distribution measurements indicate that intra- and intermolecular cross-linking processes did occur and nanosystems with broad size distribution were produced.

3.3. TEM results

TEM micrographs showed (Fig. 5) that the cross-linked nanoparticles were separated into spherical particles. TEM micrographs confirmed the nano-size of dried hyaluronan particles and showed the distribution of these derivatives. These results support the existence of the small nanoparticles ($d \sim 10$ nm) not only in the A_1_25 reaction mixture but also in A_3_7 where the HA concentration was higher and the ratio of cross-linking was lower. Based on the size distribution of histograms, it can be established that the sizes of the dried particles did not exceed 110 nm.

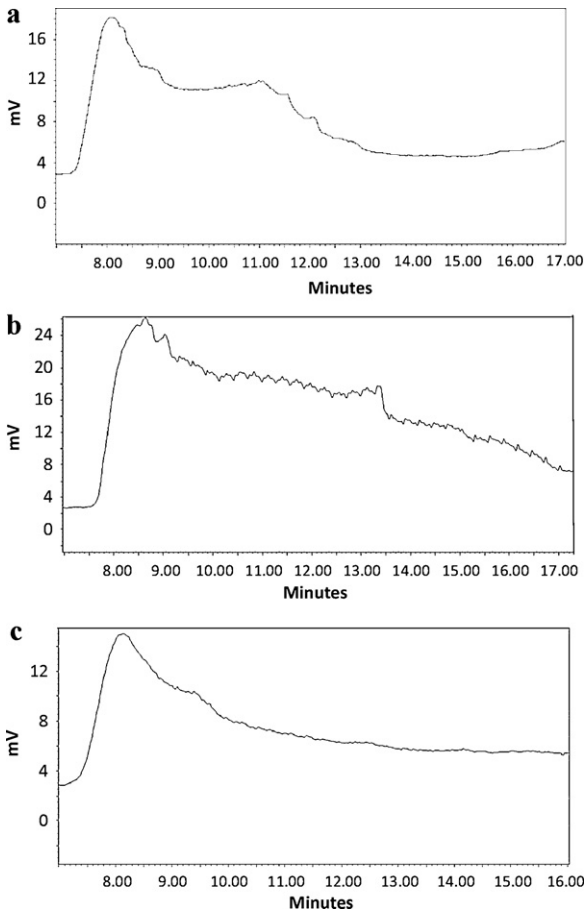

Fig. 6. GPC chromatograms of (a) HA (c_{HA} = 3 mg/ml); (b) the reaction mixture of the A_3_7 (c_{HA} = 3 mg/ml, at a cross-linking stoichiometric ratio of 25%, prepared in pure water); and (c) the purified A_3_7.

3.4. GPC results

Fig. 6a shows the GPC trace of HA. It implies the broad size distribution of linear biopolymer. Cross-linking modification of HA produced nanoparticles. Fig. 6b and c represents the GPC chromatograms of HA nanoparticles before and after dialysis. Reaction mixture containing HA nanoparticles (Fig. 6b) demonstrates that a unimodal nanoparticulate system was obtained with broad size distribution. Nevertheless, the retention time values for HA nanoparticles increased, smaller particles were formed during the cross-linking reaction.

Reaction mixture was purified by dialysis using cellulose dialysis tubes (MWCO = 12,000 Da). Fig. 6c shows the GPC chromatogram of the purified reaction mixture. This result suggests that the smaller particles were formed during the cross-linking reaction, and these particles diffuse through the membrane tube therefore they were lost during the dialysis.

3.5. Rheology

Viscosity is an important property of colloid systems, which is related to the nature, the extent of intermolecular interactions, and entanglements of polymer chains. Colloid systems containing cross-linked hyaluronan nanoparticles are colloid dispersions. The shear stress vs. shear rate graphs ("flow curves") indicate shear thinning, referring to a fact that the macromolecular systems behave as pseudoplastic materials. HA is a linear biopolymer, but the cross-linking reactions result in smaller particles. Fig. 7 shows

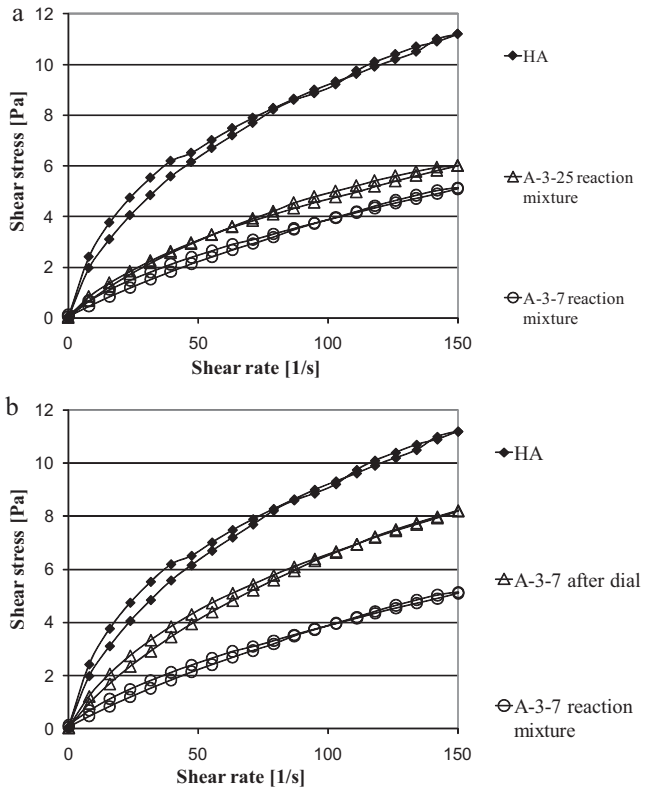

Fig. 7. (a) Shear rate dependence of the shear stress for the systems of HA and its cross-linked derivatives at the indicated cross-linking ratios. (b) Effect of purification on the rheological properties of cross-linked hyaluronan nanoparticles (c_{HA} = 3 mg/ml, pH = 6.3 ± 0.2).

the dependence of the shear rate on the shear stress of HA and hyaluronan nanoparticles at different ratios of cross-linking.

The flow curves of the nano-particulate systems were lower than flow curve of the linear HA, which is consistent with successful cross-linking and the formation of smaller particles.

Fig. 7a shows the effect of cross-linking ratio of HA on the rheological properties. It can be concluded that the curves of nanoparticles in this figure with no significant difference produced by the degree of cross-linking. In contrast, Fig. 7b shows the effects of dialysis on rheological measurements. The data demonstrates that dialysis increases shear stress.

These measurements are in agreement with the DLS and GPS results indicating that due to cross-linking small particles are formed and these diffuse through the membrane during dialysis. Therefore, HA nanoparticles possess a higher molecular weight and their spherical shape results in a lower viscosity than that exhibited by linear HA.

4. Conclusions

In this paper, we have shown that nano-sized particles based on HA have been successfully prepared by amidation with a bifunctional amine as a cross-linking agent in the presence of carbodiimide. Transparent or mildly opalescent colloid systems were fabricated in aqueous media at room temperature.

Physico-chemical properties, including transmittance of aqueous system containing HA nanoparticles, hydrodynamic size and size distribution of hyaluronan nanoparticles were controlled by varying the ratio of cross-linking, concentration of HA and parameters of media.

TEM and rheology results proved the existence of the well-dispersed HA nanoparticles systems with spherical shape.

Comparison of the particle sizes and size distribution at different phases of the technology leads to recognition of loss of the smaller particles during the dialysis.

DLS results indicate that the concentration of HA and the salt concentration of the media affect the hydrodynamic size of cross-linked nanoparticles, but the ratio of cross-linking did not.

The stability of these nanoparticles was not dependent on the media and feed ratio (from 7 to 25%), and no aggregation was found after several weeks.

The rheological measurements support the DLS and GPS results such as one part of cross-linked nanoparticles is formed with size less than 20 nm, but these particles can be lost during the purification.

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