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ORIGINAL CONTRIBUTION

Preparation and characterization of cross-linked hyaluronan nanoparticles

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Abstract The present investigation describes the synthesis and characterization of novel biodegradable nanoparticles based on hyaluronic acid (HA). The diamine, 2,2′(ethylenedioxy)bis(ethylamine) was used for cross-linking of the HA linear chains. The condensation reaction of amino groups and pendant carboxyl groups of HA was performed in aqueous media at room temperature using water-soluble carbodiimide. The prepared nanosystems, aqueous solutions, or dispersions of nanoparticles were stable, transparent, or mildly opalescent systems depending on the ratio of cross-linking, findings consistent with values of transmittance above 77%. The structure of products was determined by nuclear magnetic resonance spectroscopy, and the particle size was identified by laser light scattering (DLS) and transmission electron microscopy (TEM) measurements. Particle size measured by TEM varied less than 130 nm; in the swollen state, the average size of the particles measured by DLS was in the range of 30–140 nm depending on the ratio of cross-linking and the molecular weight of HA. Formation of cross-linked nanoparticles results in a viscosity drop compared to the viscosity of the corresponding solution of the HA, and this trend becomes decreasingly appreciable as the molecular weight of HA decreases.

Keywords Hyaluronic acid · Nanoparticles · Cross-linking

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Introduction

Hyaluronic acid (HA) is a linear polysaccharide consisting of alternating units of β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine (Fig. 1). Chemically, it is a non-sulfated glycosaminoglycan and occurs primarily in vivo as sodium hyaluronate. It is one of the major components of the extracellular matrix of connective tissues. It is present in the synovial fluid of joints, in the vitreous body, in the umbilical cord, and in scaffolding that comprises cartilage. It plays an important role in many biological processes such as in tissue hydratation, in organization of the extracellular matrix, in lubrication, and wound healing.

HA is native to the body; it is a non-immunogenic, biocompatible, biodegradable, and bioactive polysaccharide. Furthermore, HA could be an ideal biomaterial for several biomedical applications, such as tissue engineering, drug- [1], or gene-delivery systems [2].

HA is soluble in water, independently of its molecular weight, which typically ranged from 1×10^4 to 2×10^7 Da. Several depolymerization methods have been developed [3–5], such as ultrasonic, oxidative degradation, or acid hydrolysis, for preparing low molecular weight macromolecules of HA. It was shown that the molecular weight of the HA depends on the pH, the initial concentration of

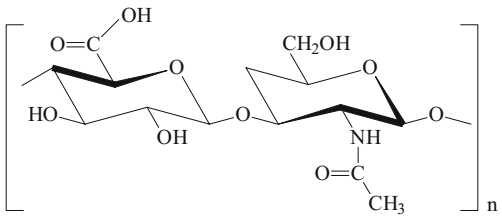

Fig. 1 Chemical structure of hyaluronic acid

HA, the temperature, and the reaction time. The molecular weight of HA decreased exponentially in the course of degradation. Most of the depolymerization of HA occurred within the first 3 h of the reaction and then slowed significantly [3].

Various methods have been developed for cross-linking HA, which commonly result in gel [6, 7] or film [8] formation. Cross-linkers are molecules with at least two reactive functional groups that allow the formation of bridges between polymeric chains. The most common cross-linkers of HA are aldehydes [9], thiols [10, 11], hydrazides [12], and other agents [13]. Hydrogels have been utilized in a wide range of biomedical application, such as, scaffolds and carriers for drugs [14–16] and gene [17], or implants for tissue engineering [18–20].

Many recent attempts have been made to create particulate systems based on HA [21, 22]. Hyaluronan nano- and microsystems can be prepared in a wide range of methods: coacervation [23], spray drying [24], or solvent evaporation [25] are well-known techniques to produce cross-linked suspension performed in emulsion. By emulsion methods, the size of the particles can be controlled by controlling the size of droplets; however, the emulsion systems contain at least two phases and surfactant.

Several studies report on the preparation of hyaluronan microparticulate systems using carbodiimide technique [26–29]. These results represent synthesis of hyaluronan hydro-, micro-, or nanogels with a range of 200 nm–30 μm diameter size. Hyaluronan particulate systems can be employed for several biomedical applications, such as drug- [30] or gene-delivery systems [31, 32].

The present investigation reports a method for the preparation of nano-sized particulate systems based on HA by covalently cross-linking via carboxyl groups of the HA chain with a diamine in aqueous media at room temperature using carbodiimide technique. Different molecular weight HAs were produced by degradation and were used to investigate the correlation of size, ratio of cross-linking, and the molecular weight of HA. The solubility, structure, and size of these nanoparticles in the dried and swollen states will be described and discussed. Cross-linked hyaluronan nanoparticles may dissolve or form stable colloid systems in aqueous media. They are nano-sized and can be attractive candidate delivery biosystems for a variety of biomedical applications.

Experimental

Materials

HA sodium salt (HA 0 h: M_w=4,350 kDa) was obtained from Gedeon Richter Ltd. It was a pharmaceutical product. 2,2′(ethylenedioxy)bis(ethylamine) was purchased from Sigma-Aldrich Co., Hungary and was used as received without further purification. Water-soluble 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide methiodide (CDI) was applied as a condensation agent.

Preparation of low molecular weight hyaluronic acid

HA sodium salt was dissolved in water to obtain a 1% (m/m) solution and then adjusted to pH 2.0 with 6.0 M hydrochloric acid solution. The degradation was carried out at 70°C stirring for 3, 6, and 9 h. The pH of the solution was then adjusted to 6.0 with 1.0 M sodium hydroxide solution, and a sodium chloride solution was added to obtain 4% (*v*/*v*) solution. The degraded HA sodium salt was precipitated with four volumes of absolute ethanol. The precipitated HA sodium salt was separated by centrifugation and washed with absolute ethanol. The degraded HA sodium salt was freeze-dried.

Determination of molecular weight

Molecular weights of HAs were measured by size exclusion chromatography [29, 33, 34]. Waters high performance liquid chromatography system was used with Ultrahydrogel linear (300×7.8 mm) aqueous column, and absorbance was measured at 210 nm. The flow rate was 1.0 mL/min, and the mobile phase was a mixture of 0.05 M acetate aqueous solution containing 0.2 M NaCl at pH 6.6 and methanol with a ratio of 8 to 2. The molecular weight calibration curve was prepared with pullulan (Sigma-Aldrich Co., Hungary) standards. Generally, apparent molecular weight corresponding to the elution peak apex was used to characterize the samples.

To determine the effect of molecular weight on the properties of nanoparticles, lower molecular weight HAs were prepared from commercial HA (0 h) with a molecular weight of 4,350 kDa by depolymerization and were used to produce nanoparticles by cross-linking. They are HA 3 h: M= 980 kDa, HA 6 h: M=660 kDa, and HA 9 h: M=350 kDa.

Modification

Cross-linked hyaluronan nanoparticles were prepared by the CDI technique. Diamine was used as cross-linking agents. The overall synthetic route for the modification of HA is summarized in Fig. 2.

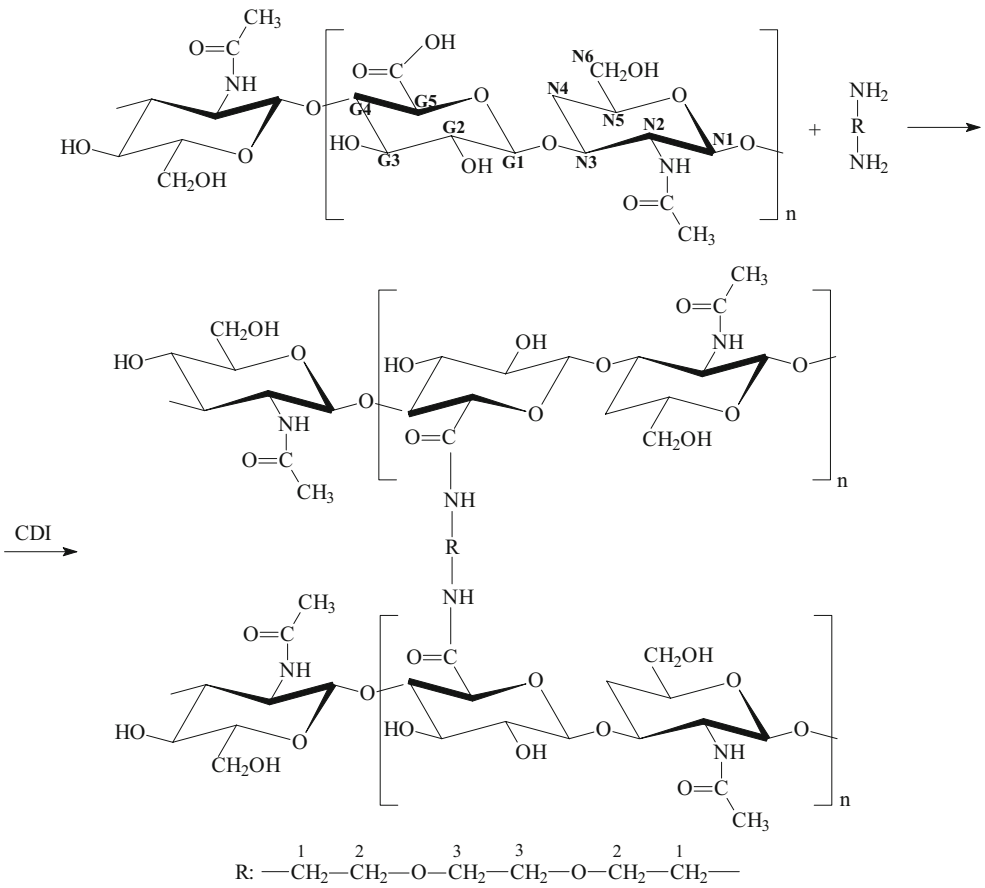

Fig. 2 Schematic representation of the synthesis and structure of cross-linked hyaluronan derivatives (The notation will be used for assignments of structure measured by NMR.)

Synthesis of cross-linked hyaluronan nanoparticles HA was dissolved in water to produce 1 mg/mL solution and then adjusted to pH 5.5 with 0.1 M sodium hydroxide solution. The diamine was dissolved in water to produce 1.0*v*/*v*% solution and pH-adjusted to 5.5 with 0.1 M sodium hydroxide solution. The diamine solution was added to the HA solution and mixed for 30 min at room temperature. After the addition of water-soluble carbodiimide solution droppwise, the reaction was stirred at room temperature for 24 h. The solution containing hyaluronan nanoparticles was purified by dialysis for 7 days against distilled water and freeze-dried.

Synthesis of cross-linked hyaluronan nanoparticles with 2,2′(ethylenedioxy)bis(ethylamine) at diverse stoichiometric cross-linking ratios was accomplished according to the described reaction conditions. The data of the synthesis are summarized in Table 1.

Characterization

Nuclear magnetic resonance spectroscopy The HA and the cross-linked hyaluronan nanosystems were analyzed structurally with nuclear magnetic resonance (NMR) spectroscopy. ^{1}H NMR, ^{13}C NMR, and ^{1}H-^{13}C HETCOR NMR spectra were obtained on a Bruker AM 500 MHz instrument. The samples were dissolved in D_2O. The chemical shifts were represented in parts per million (ppm), based on the signal for sodium 3-(trimethylsilyl)-propionate-d_4 as a reference.

Transmittance Transmittances of hyaluronan nanoparticle solutions were measured by using an HP-8453 ultraviolet spectrophotometer at an operating wavelength of λ= 500 nm in optically homogeneous quartz cuvettes. The samples were performed from the reaction mixture after

Table 1 Reaction condition of synthesis of cross-linked hyaluronan nanoparticles

Quantity of hyaluronic acid is independent of molecular weight

Hyaluronic acid (mg)	Stoichiometric ratio of cross-linking	Quantity of diamine (μl)	Quantity of CDI (mg)
100	25%	470	20
100	50%	940	40
100	100%	1,880	80

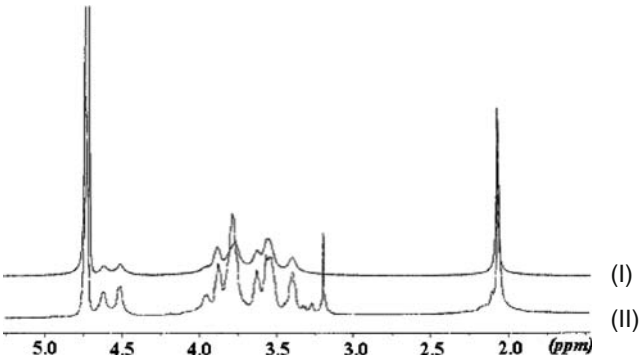

Fig. 3 500 MHz ^{1}H NMR spectra of hyaluronic acid (HA) 3 h (I) and hyaluronan nanoparticles based on HA 3 h cross-linked with diamine at 50% (II)

dialysis at 25°C. The concentration of the HA derivative solutions was 1 mg/mL.

Rheology Viscosity measurements of HAs and systems containing cross-linked hyaluronan nanoparticles were conducted in an AR 550 (TA Instruments Ltd.) advanced rheometer with a cone angle of 1° and a cone diameter of 60 mm. The rheometer is equipped with a temperature unit (Peltier plate) that provides an accurate temperature control over an extended time. The measurements were carried out at 25°C. Samples were dissolved in distilled water to obtain 5 mg/mL solutions; pH was adjusted to 5.5.

Laser light scattering (DLS) The hydrodynamic diameters of cross-linked hyaluronan nanoparticles were gauged by using a BI-200SM Brookhaven research laser light scattering (DLS) photometer equipped with a NdYAg solid state laser at an operating wavelength of λ_o=532 nm. Measurements of the average size of nanoparticles were performed at 25°C with an angle detection of 90° in optically homogeneous quartz cylinder cuvettes. The samples were prepared from the reaction mixture after dialysis. The concentration of the HA derivative solutions was 100 μg/ml and was sonicated for 1 min. Each sample was measured three times, and average serial data were calculated.

Transmission electron microscopy (TEM) A JEOL2000 FX-II transmission electron microscope was used to characterize the size and morphology of the dried hyaluronan nanoparticles. For transmission electron microscopy (TEM) observation, the hyaluronan nanoparticles were prepared from the reaction mixture after dialysis at a concentration of 100 μg/ml. The sample for TEM analysis was obtained by placing a drop of the colloid dispersion containing the nanoparticles onto a carbon-coated copper grid. It was dried at room temperature and then examined using a TEM without any further modification or coating.

Mean diameters and the size distribution of diameters were obtained from measured particles visualized by TEM images and then analyzed by using SPSS 11.0 program file.

Results and discussion

NMR results

The structure of HA used for the synthesis and the obtained cross-linked nanosystems (Fig. 2) were characterized by NMR spectroscopy. The assignments and chemical shifts of the ^{1}H and ^{13}C NMR signals of HA 3 h and cross-linked hyaluronan nanoparticles based on HA 3 h were determined. The chemical shift values are in accordance with results published [35, 36].

Proton chemical shift values are given in Fig. 3, which illustrates the difference between the signs of the original HA 3 h material (I) and the hyaluronan nanoparticles based on HA 3 h cross-linked at a stoichiometric ratio of 50% (II). The assignments and chemical shifts of the ^{1}H signals are given as follows: HA 3 h: ^{1}H NMR (D_2O): δ=4.51 (G1), δ=4.61 (N1), δ=3.40 (G2), δ=3.92 (N2), δ=3.63 (G3), δ=3.74–3.85 (N3, N6, G4, G5), δ=3.51–3.62 (N4, N5), and δ=2.07 ($NCOCH_3$); HA 3 h cross-linked with diamine at 50%: ^{1}H NMR (D_2O): δ=4.51 (G1), δ=4.62 (N1), δ=3.39 (G2), δ=3.96 (N2), δ=3.63 (G3), δ=3.70–3.84 (N3, N6, G4, G5, C1, C2), δ=3.49–3.60 (N4, N5), δ=2.07 ($NCOCH_3$), and δ=3.33 (C3).

The ^{13}C assignment was performed on the basis of ^{13}C projection. Figure 4 demonstrates the difference between the signs of the original HA 3 h material (III) and the hyaluronan nanoparticles based on HA 3 h cross-linked at a stoichiometric ratio of 50% (IV). The assignments and chemical shifts of the ^{13}C signals are given as follows: HA 3 h: ^{13}C NMR (D_2O): δ=103.71 (G1), δ=101.05 (N1), δ=73.08 (G2), δ=54.82 (N2), δ=74.17 (G3), δ=83.28 (N3), δ=80.48 (G4), δ=69.07 (N4), δ=76.88 (G5), δ=75.91 (N5), δ=61.10 (N6),δ=174.58 (COO^{-}), and δ=23.04

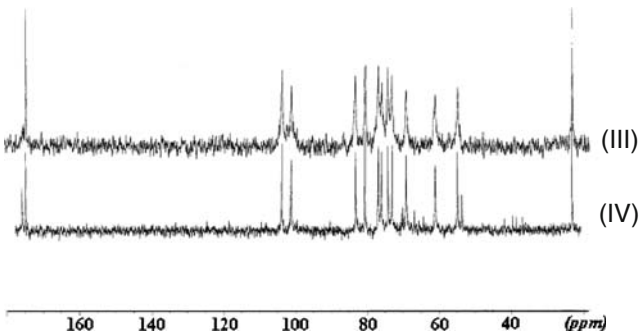

Fig. 4 500 MHz ^{13}C NMR spectra of hyaluronic acid (HA) 3 h (III) and hyaluronan nanoparticles based on HA 3 h cross-linked with diamine at 50% (IV)

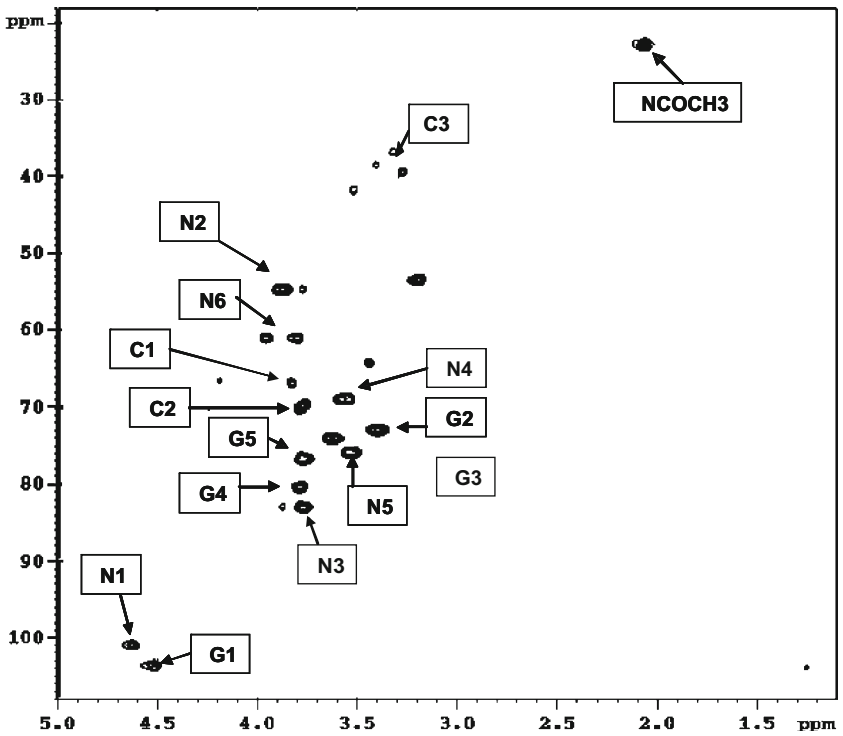

Fig. 5 ^{1}H-^{13}C hetero single quantum correlation spectrum map of hyaluronan nanoparticles based on hyaluronic acid 3 h cross-linked with diamine at 50%

($NCOCH_3$); HA 3 h cross-linked with diamine at 50%: ^{13}C NMR (D_2O): δ=103.63 (G1), δ=101.02 (N1), δ=73.01 (G2), δ=54.83 (N2), δ=74.14 (G3), δ=80.10 (N3), δ=80.47 (G4), δ=69.02 (N4), δ=76.77 (G5), δ=75.92 (N5), δ=61.10 (N6), δ=174.52 (COO^-), δ=23.03 ($NCOCH_3$), and the signs of cross-linker δ=66.93 (C1), δ=70.18 (C2), and δ=38.75 (C3).

Figure 5 shows the ^{1}H-^{13}C hetero single quantum correlation spectrum of hyaluronan nanoparticles. The assignments were performed on the basis of ^{1}H and ^{13}C projection.

The degree of cross-linking was evaluated from the integral intensity of signs by using ^{1}H NMR spectra of cross-linked hyaluronan nanoparticles. Peak at δ=2-2.1 ppm belongs to the NDCOCH3 group protons of HA, and peak at δ=3-3.2 ppm means four protons carbon atom of cross-linker signed C3. Ratio of cross-linking was calculated based on the integral intensity of these two peaks referred on unit protons. In case of 25% stoichiometric ratio, the degree of cross-linking varied between 15% and 25%. Increasing the stoichiometric ratio caused the degree of cross-linking to increase. In case of cross-linking at a stoichiometric ratio of 50%, it was between 30% and 45%, and it was 60–70% at a stoichiometric ratio of 100%.

Transmittance

The solubility of HA is independent of its molecular weight. The transmittance values of the aqueous solution of these polysaccharides were 98–99%; the solutions were transparent.

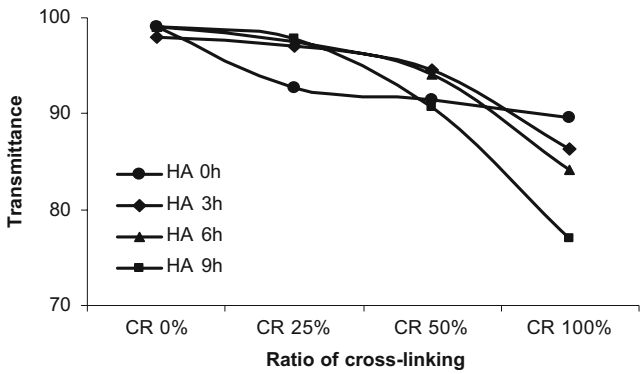

Fig. 6 Transmittance of cross-linked hyaluronan nanoparticles (c=1 mg/mL, pH=6.0)

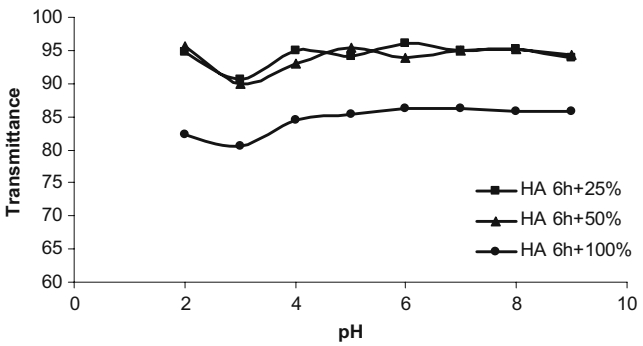

Fig. 7 Effect of the pH on the transmittance values of hyaluronan nanoparticles (c=1 mg/mL)

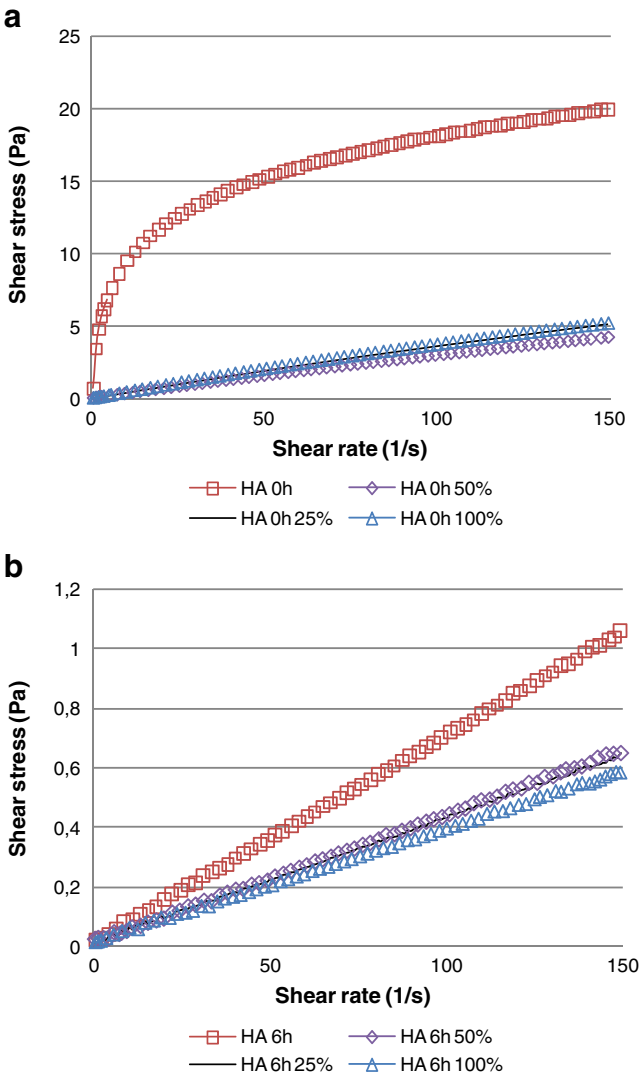

Fig. 8 Shear rate dependence of the shear stress for the systems of hyaluronic acid (HA) 0 h (**a**) and HA 6 h (**b**) and their cross-linked derivatives at the indicated cross-linker densities (c=5 mg/mL, pH=5.5)

The cross-linked hyaluronan nanoparticles form colloid dispersions in neutral condition. These colloid dispersions are transparent or mildly opalescent systems in aqueous media at pH 6.0 and stable at room temperature for several weeks. The transmittance was between 77% and 99%.

The general trend that appears in Fig. 6 shows that the value of the transmittance was reduced as the ratio of cross-linking increased, and this effect strengthened as the molecular weight of HA decreased. This is compatible with the idea that as the ratio of cross-linking increased, the particles became more compact.

It was observed that the pH was not a factor for transmittance values of the aqueous solutions independently of the molecular weight of HA (Fig. 7), but a minimum transmittance point was observed in all curves at pH approximately 3 close to the pKa (approximately 3.2) value of HA.

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 [37]. The shear rate dependence of the shear stress (Fig. 8) and of the viscosity (Figs. 9 and 10) are shown for HA and hyaluronan nanoparticles at different degrees of cross-linking.

The shear stress vs shear rate graphs ("flow curves") indicate virtually Newtonian behavior of aqueous colloid systems containing hyaluronan nanoparticles. In case of cross-linked particles, the relation between the shear stress and the strain rate looks like linear. These colloid systems containing hyaluronan nanoparticles are colloid dispersion. The small size of particles could be the reason of the linear relation between the shear stress and the shear rate, suggesting presence of individual particles principally as well as intramolecular cross-linking process. In case of HA 0 h, shear thinning can be seen, referring to a fact that

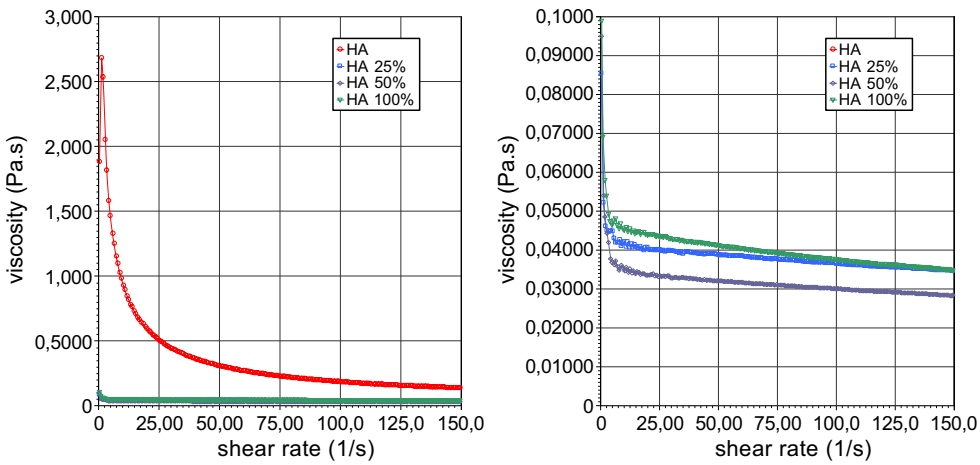

Fig. 9 Shear rate dependence of the viscosity for the systems of hyaluronic acid 0 h and its cross-linked derivatives at the indicated cross-linker densities (c=5 mg/mL, pH=5.5)

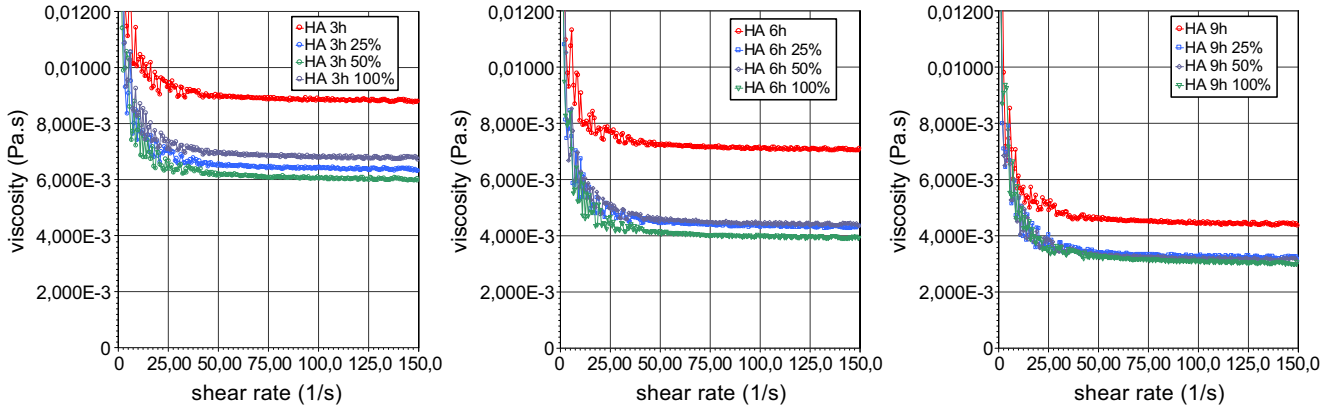

Fig. 10 Shear rate dependence of the viscosity for the systems of degraded hyaluronic acids and hyaluronan nanoparticles at the indicated cross-linker densities and molecular weight (c=5 mg/mL, pH=5.5)

the macromolecular solution behaves as a pseudoplastic material, but this property is not appreciable as the molecular weight decreases.

Decreasing of the viscosity, over the considered shear rate domain, is observed for the colloid systems containing nanoparticles at all levels of cross-linking and molecular weight and looks like constant at high rates of shear. This result and the low viscosities of the solutions indicate that no interconnected network structures are formed. The solutions of the linear HAs exhibit higher viscosity and a gradually more pronounced shear-thinning effect at higher shear rates, as the molecular weight of HA increases. This suggests polymer association and formation of entanglements in the solutions of HA as the molecular weight of polymer rises. The progressive decrease in viscosity as the shear rate rises is ascribed to the breakdown of the network junctions; that is, the rate of network disruption exceeds the rate at which associations are reformed. These features and the TEM results clearly support the hypothesis that the cross-linking process yields particles.

When a low amount of cross-linker was added to the polymer solution, loosely cross-linked nanoparticles were formed, and they result in higher viscosity values over all share rate range. At higher ratios of cross-linking, the process yields more compact particles and, in consequence, lower viscosity. This feature was gradually more representative as the molecular weight of degraded HA increased. The general trend that appears is that the viscosity exhibits a minimum at a cross-linking density of 100%, followed by a rise as the degree of cross-linking decreases.

In case of parent HA 0 h, in accordance with the DLS results, the intermolecular cross-linking process becomes more appreciable by increasing the cross-linking ratios. This trend reveals in the viscosity values, which exhibit a minimum at a cross-linked density of 50%. Small, individual particles can be formed by intramolecular cross-linking at low ratios, but interpolymer interactions and entanglement in polymer systems become more pronounced as the molecular weight and the ratio of cross-linking rose. Therefore, probably, they contribute to the interpolymer cross-linking, formed aggregates resulting in increase in the value of viscosity at a stoichiometric ratio of 100%.

Particle size by DLS

Solution samples were prepared from the reaction mixture after dialysis. The concentration of the polysaccharide solution was 100 μg/ml.

In polydispersed systems, the final results depended upon the method of fitting. The average hydrodynamic diameters were calculated by non-negative least squares (NNLS) method, which separated the different peaks at multimodal distribution and provided more exact results at multimodal systems than obtained with other methods. The

Table 2 Average hydrodynamic diameter of hyaluronan nanoparticles determined by DLS

Hyaluronic acid	Ratio of cross-linking	Peak I (nm)	Peak II (nm)
HA 0 h	25%	80±20	500±70
	50%	75±30	420±80
	100%	110±30	580±80
HA 3 h	25%	110±20	310±15
	50%	90±20	370±40
	100%	80±15	390±40
HA 6 h	25%	80±10	210±20
	50%	70±20	280±30
	100%	50±30	350±20
HA 9 h	25%	70±10	270±20
	50%	50±15	350±20
	100%	60±15	350±20

c=100 μg/mL, pH=6.5

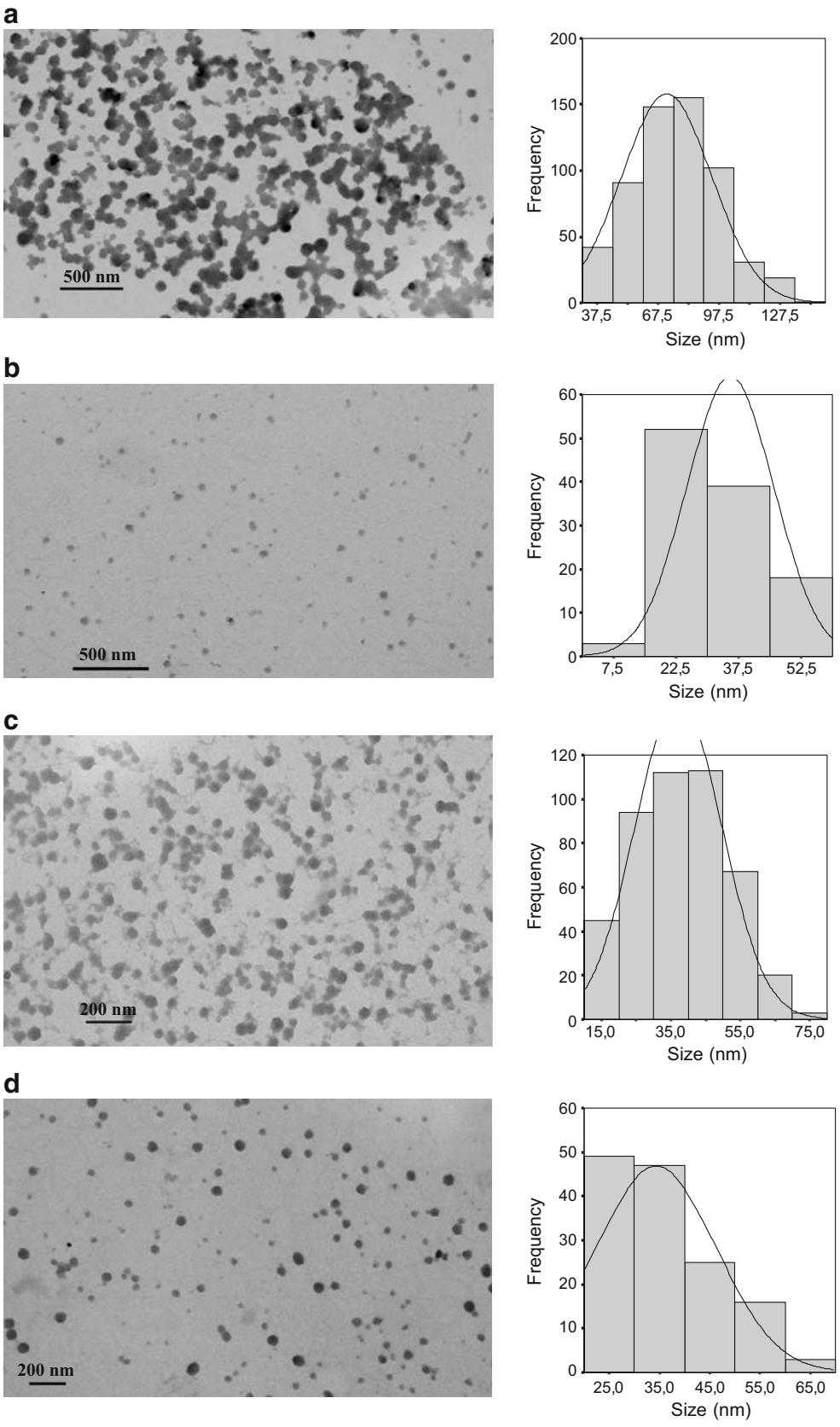

Fig. 11 TEM images and size distributions of cross-linked hyaluronan nanoparticles. **a** hyaluronic acid (HA) 6 h at 50%. **b** HA 6 h at 100%. **c** HA 3 h at 50%. **d** HA 3 h at 100%

intensity-delay time correlation function was evaluated by means of NNLS fit, called automatic routine was applied to determine the intensity diameter distribution. The effect of dust was cancelled by averaging of numerous simultaneous measurements. Table 2 summarizes the average hydrodynamic diameters of swelled hyaluronan nanoparticles.

HAs with different molecular weights were used for the cross-linking modification at different stoichiometric ratios. The cross-linked nanosystems were soluble in aqueous media and can swell. Depending on the ratio of cross-linking, the residual carboxyl groups of these macromolecules can be deprotonated, and the repulsive interaction can affect the hydrodynamic diameters.

Bimodal size distribution of hydrodynamic diameters of hyaluronan nanoparticles were measured in all cases (Table 2). Cross-linking process can consist of intramolecular and intermolecular cross-linking. Size distribution by number shown that majority of hyaluronan nanoparticles were formed as small, individual particles by intramolecular cross-linking, but large particles could develop also. The large particles (peak II) can be aggregates, association by secondary interactions between the individual particles or result of intermolecular cross-linking. TEM micrographs show that in dry state, no aggregates were found at a stoichiometric ratio of 100%, but aggregated particles were observed at a stoichiometric ratio of 50% independently of the molecular weight. Therefore, the TEM images could not confirm that intramolecular cross-linking of hyaluronic acid or secondary interactions caused large particles. Rheological curves and data can support our idea based on intra- and intermolecular process, therefore, bimodal size distribution.

To analyze the size of individual particles (peak I), it was found that by decreasing the molecular weight, the hydrodynamic diameters decreased. This correlation was direct, but no typical relationship was observed between the size of spherical particles and the molecular weight.

In accordance with the viscosity results, the size of cross-linked particles is similar and as good as independent of the cross-linking density. The size of cross-linked particles prepared from degraded HAs decreased as the ratio of cross-linking increased; intramolecular cross-linking was the dominant process. In case of HA 0 h, considerable interplay between the intermolecular and intramolecular cross-linking was observed as the cross-linking density increased caused by high molecular weight of the biopolymer.

In summary, the DLS measurements indicate that the intramolecular cross-linking process is dominant, and the average hydrodynamic diameter of swelled individual cross-linked hyaluronan nanoparticles was between 30 and 130 nm. To all appearances, the nanoparticles swell in aqueous media depending on the ratio of cross-linking. The molecular weight of HA influences the size of the spherical particles appreciably.

Particle size by TEM

The cross-linked hyaluronan nanoparticles separated into spherical particles in an aqueous environment and in dried states. TEM micrographs (Fig. 11) confirm the nano-size of dried cross-linked hyaluronan particles and show the distribution of these derivatives. Based on the results of histograms, the ratio of cross-linking affects the size of the nanoparticles. Increasing the ratio of cross-linking reduced the size of dried particles, while the molecular weight did not influence the size considerably. The size of the dried particles did not exceed 130 nm.

Conclusions

We have shown that nano-sized particles based on biocompatible HA has been successfully prepared by condensation reaction using 2,2′(ethylenedioxy)bis(ethylamine) as cross-linking agents. Transparent or opalescent stable colloid systems were fabricated in aqueous medium at room temperature. The results of characterization reveal that the size of particles in dried state (TEM) and in solution (DLS) as well as the rheological properties depend on the molecular weight of HA. Both TEM and DLS data suggest formation of particles. Average hydrodynamic diameters of individual particles ranged from 30 to 140 nm. The cross-linked nanoparticles have much lower viscosity than the parent linear biopolymer, because of contraction of linear chains in connection with intrachain cross-linking and the absence of entanglement coupling. At high molecular weight of biopolymer, interplay between inter- and intramolecular cross-linking were found.

The low viscosity and the nano size of cross-linked, separated hyaluronan nanoparticles could lead to the development of new methods or products in medicine, pharmaceutics, and cosmetics.

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