Synthesis and Study of Cross-Linked Chitosan-*N*-Poly(ethylene glycol) Nanoparticles

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Received May 24, 2006; Revised Manuscript Received September 3, 2006

The present investigation describes the synthesis and characterization of novel biodegradable nanoparticles based on chitosan. Poly(ethylene glycol) dicarboxylic acid was used for intramolecular cross-linking of the chitosan linear chains. The condensation reaction of carboxylic groups and pendant amino groups of chitosan was performed by using water-soluble carbodiimide. The prepared nanosystems were stable in aqueous media. The structure of the products was determined by nuclear magnetic resonance (NMR) spectroscopy, and the particle size was identified by dynamic light scattering (DLS) and transmission electron microscopy (TEM) measurements. It was found that biodegradable cross-linked chitosan nanoparticles experienced considerable swelling because of the length and flexibility of the cross-linking agent. The aqueous solutions or dispersions of nanoparticles were stable and clear or mildly opalescent systems depending on the ratio of cross-linking and molecular weight of chitosan, findings consistent with values of transmittance above 75%. Particle size measured by TEM varied in the range of 4−24 nm. In the swollen state, the average size of the individual particles measured by DLS was in the range of 50−120 nm depending on the molecular weight of chitosan and the ratio of cross-linking.

Introduction

Chitosan is a basic linear polysaccharide containing β-[1→4]-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. Because of the presence of reactive amino groups, chitosan can be modified easily to create nano- and microparticles or porous hydrogels.

Various methods have been developed for the cross-linking of chitosan, which commonly result in gel formation. In this type of covalent cross-linking reaction, 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 chitosan are aldehydes,[1,2] epoxides,[3] cyanates,[4] and other agents.[5,6] Ionic cross-linking reactions with charged ions[7] or molecules[8−11] have also been employed by using ionotropic gelation methods to form hydrogels based on chitosan. Hydrogels have been utilized in a wide range of biomedical applications, such as enzyme immobilization,[12,13] as scaffolds,[14] and as carriers for drugs[15] or implants.[8,9,16,17]

Many recent attempts have been made to create chitosan particulate systems. Ionotropic gelation methods are based on the interaction between chitosan and various anions.[18] Examples of this method are the emulsion cross-linking method and the reverse micellar medium method, or coacervation.[19] Covalently cross-linked chitosan nanoparticles can be prepared by several different methods: emulsion cross-linking,[20] reverse micellar,[21] solvent evaporation,[22] or spray-drying.[23] Chitosan nano- and microsystems can be employed in a wide range of biomedical applications, such as drug-[15,18,21] or gene-delivery systems.[24,25]

Poly(ethylene glycol) (PEG) is one of the most popular oligomers for the chemical modification of biomaterials. Because of its unique physicochemical and biological properties, including hydrophilicity, solubility in both water and organic solvents, lack of toxicity, biocompatibility, biodegradability, and ease of chemical modification, PEG has been employed in a wide range of biomedical applications.[26,27] A variety of studies have focused on the modification of chitosan by using PEG. Porous membranes and biocompatible films were produced by blending chitosan with PEG[28−30] or a preparation of semi-interpenetrating polymer networks.[31] Graft copolymers were synthesized to enhance the water solubility of chitosan by employing water-soluble linkages like PEG.[32,33] These modifications can also[34] result in the formation of a hydrogel, which can be employed in tissue engineering[14] or drug release.[34]

In our previous work,[35] we discussed the preparation and characterization of nanoparticles based on chitosan with high molecular weight. Natural di- and tricarboxylic acids with short chains were used for the intramolecular cross-linking of chitosan linear chains. The condensation reaction of carboxylic groups and pendent amino groups of chitosan was performed by using water-soluble carbodiimide. The prepared colloid systems were stable in aqueous medium at room temperature. We found that particle size depended on the pH, but, at a given pH, size was independent of the ratio of cross-linking and the hydrophilic character of the cross-linking agent.

The present investigation reports a method for the preparation of nanoparticles based on chitosan by covalently cross-linking, via amino groups, the chitosan chain with PEG dicarboxylic acid in aqueous media at room temperature. Different molecular weight chitosans were produced by degrading high molecular weight chitosan, and were used to study the correlation of size, molecular weight, and the ratio of cross-linking. The solubility, structure, and size of these nanoparticles in the dried and swollen states will be described and discussed. Cross-linked chitosan nanoparticles may dissolve or form stable colloid systems in aqueous media at acidic and neutral pH. Because they are

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10.1021/bm0605053 CCC: $33.50
Published on Web 10/19/2006

Table 1. Reaction Conditions of the Synthesis of Cross-Linked Chitosan Nanoparticles[a]

chitosan (mg)	stoichiometric ratio of cross-linking agent	quantity of PEGDC (mg)	quantity of CDI (mg)
100	25%	46	46
100	50%	92	92
100	100%	184	184

[a] The quantity of chitosan is independent of molecular weight.

nanosized at neutral pH, they are attractive candidates as delivery biosystems for a variety of biomedical applications.

Experimental Section

Materials. Chitosan (CH; degree of deacetylation (DD) = 88%, M_v = 320 000) was purchased from Sigma-Aldrich Co., Hungary. It was dissolved in 2.0% aqueous acetic acid solution to give a polymer concentration of 1.0% w/w, and then filtered and dialyzed against distilled water until the pH was neutral. The product was dried by lyophilization to obtain a white chitosan powder and used for further experiments. Poly(ethylene glycol)bis(carboxymethyl)ether (M_n = 600) as an α,ω-dicarboxylic acid (PEGDC) 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.

Determination of DD and Molecular Weight. The DD of the chitosan material employed was determined from the integral intensities of all protons by its ^{1}H NMR spectrum.[36] The measurement was performed in D_2O containing a few drops of 20% w/w DCl/D_2O and trace DSS as a reference. The viscosity average molecular weight was determined by measuring the relative viscosity with an Ostwald viscometer. The solvent system used was 0.10 M CH_3COOH/0.20 M NaCl. Molecular weight was calculated from the intrinsic viscosity based on the Mark−Houwink equation. The values for the constants K and a were 1.81×10^{-3} and 0.93.[37]

Degradation of Chitosan. Low molecular weight chitosans were prepared by oxidative degradation with $NaNO_2$ performed at room temperature. Chitosan was dissolved in 1.0% (w/w) acetic acid solution to produce 1.0% (w/w) chitosan solution. When chitosan was completely dissolved, 0.10 M $NaNO_2$ solution was added to the mixture with magnetic stirring. The reaction mixture was neutralized with 1.0 M NaOH solution to pH 8.0 to precipitate chitosan. The precipitated chitosan was recovered by centrifugation, washed several times with deionized water, and dried by lyophilization.[38]

Chitosan Modification. Cross-linked chitosan nanoparticles were prepared by the CDI technique. PEGDC was used as the cross-linking agent.

Synthesis of Cross-Linked Chitosan Nanoparticles. Chitosan was dissolved in 0.10 M hydrochloric acid, PEGDC was added to the solution, and the solution was then adjusted to pH 6.5 with 0.10 M sodium hydroxide solution. After the dropwise addition of water-soluble carbodiimide solution, the reaction mixture was stirred at 4 °C for 4 h, then at room temperature for 20 h. The solution containing chitosan nanoparticles was purified by dialysis for 7 days against distilled water and freeze-dried[39]

The synthesis of cross-linked chitosan nanoparticles with PEGDC at different stoichiometric cross-linking ratios is summarized in Table 1.

Characterization. *NMR Spectroscopy*. The chitosan and the cross-linked systems were analyzed structurally with NMR spectroscopy. ^{1}H, ^{13}C, and ^{1}H−^{13}C HETCOR NMR spectra were obtained on a Bruker AM 500 MHz instrument. The samples were dissolved in D_2O containing a few drops of 20% w/w DCl/D_2O. The chemical shifts were represented in ppm, based on the signal for sodium 3-(trimethylsilyl)-propionate-d_4 as a reference.

Transmission Electron Microscopy (*TEM*). A JEOL2000 FX-II transmission electron microscope was used to characterize the size and morphology of the dried chitosan nanoparticles. For TEM observation, the chitosan 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 chitosan nanoparticles onto a carbon-coated copper grid. It was dried at room temperature and then examined using TEM without any further modification or coating.

Dynamic Laser Light Scattering (*DLS*). The hydrodynamic diameters of cross-linked chitosan nanoparticles were gauged by using a BI-200SM Brookhaven Research laser light scattering 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 chitosan derivative solutions was 100 μg/mL. Each sample was measured three times, and the average serial data were calculated.

Transmittance. Transmittances of chitosan nanoparticle solutions were measured by using a Unicam SP 1800 ultraviolet spectrophotometer at an operating wavelength of λ = 480 nm in optically homogeneous quartz cuvettes. The samples were obtained from the reaction mixture after dialysis at 25 °C. The concentration of the chitosan derivative solutions was 1 mg/mL.

Results and Discussion

Formation of Nanoparticles. Chitosan is a weak base and has low solubility at neutral and alkaline pH values. In acidic medium, the amino groups are positively charged, resulting in a highly charged polyelectrolyte polysaccharide. At low charge densities, polyelectrolyte chains collapse in a compact globule. At high charge densities, polyelectrolytes have an extended coil conformation. The coil−globule transition is influenced by the attraction and repulsion interaction between the polymer segments.[35]

Polycation or uncharged cross-linked nanoparticles can be prepared by the chemical modification of chitosan linear chains using PEGDC as cross-linking agents at different ratios. Polycations were obtained by reacting chitosan with PEGDC. The stoichiometric ratio of cross-linking was less than 100%. In this case, the carboxyl groups were bound covalently, and residual free amino groups of the chitosan chain were available, which can be protonated in acidic medium, forming polycations. Chitosan cross-linked with PEGDC at a stoichiometric ratio of 100% can result in uncharged nanoparticles in aqueous media because each of the functional amino and carboxyl groups are covalently bound.

Degradation of Chitosan. The depolymerization method used has been described elsewhere.[38] It was shown that the molecular weight of the chitosan depends on the chitosan/$NaNO_2$ molar ratio, the initial concentration of chitosan, and the reaction time. Keeping the molar ratio and chitosan concentration constant, we determined the effect of time on the degradation process (Figure 1). Commercial chitosan was used as the starting material. The intrinsic viscosity of each sample was determined, and the molecular weights were calculated according to the Mark−Houwink equation.

The molecular weight of chitosan decreased exponentially in the course of degradation. Most of the depolymerization of chitosan occurred within the first 15 min of the reaction, and then slowed significantly.

To determine the effect of molecular weight on the properties of nanoparticles, three low molecular weight chitosans were

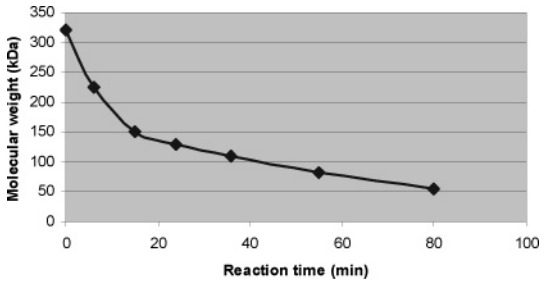

Figure 1. Effect of reaction time on the molecular weight of degraded chitosan.

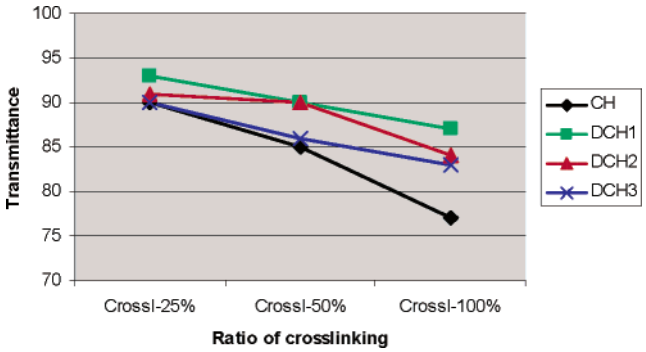

Figure 2. Transmittance of cross-linked chitosan materials.

prepared from commercial chitosan (CH) with a molecular weight of 320 kDa by depolymerization and were used to produce nanoparticles by cross-linking. They are DCH1 (M_v = 190 kDa), DCH2 (M_v = 100 kDa), and DCH3 (M_v = 55 kDa).

Solubility. The solubility of chitosan derivatives was evaluated in deionized water at pH 6.5. Solutions were clear and opaque aqueous colloid systems and were stable at room temperature for several days. Figure 2 summarizes the transmittance of the various nanoparticles composed of different molecular weight chitosans cross-linking with PEGDC at different cross-linking ratios. At lower concentrations of cross-linker, the solubility of the nanoparticles was greater, and the solutions were clear. This was caused by the protonation of the free amino groups of the chitosan chain. The molecular weight of the original chitosans did not influence the transmittance values. The difference between the values was negligible. As the cross-linking increased, the particles became more compact, and the solutions became opalescent. In the case of degraded chitosans, the values are similar at each cross-linking ratio. A considerable difference between the values of transmittance was found at a stoichiometric ratio of 100% only.

NMR Results. The structures of the chitosan used for the synthesis and the resulting cross-linked nanosystems (Figure 3) were characterized by NMR spectroscopy. The assignments and chemical shifts of the ^{1}H and ^{13}C signals of DCH3 chitosan and chitosan nanoparticles based on DCH3 cross-linked with PEGDC at 50% were determined. The chemical shift values are in accordance with results published for chitin and chitosan and their oligomers.[36,40−42]

Proton chemical shift values are given in Figure 4, which illustrates the difference between the signs of the original DCH3 chitosan material (I) and the chitosan nanoparticles based on DCH3 cross-linked with PEGDC at 50% (II). The assignments and chemical shifts of the ^{1}H signals are given as follows: DCH3 chitosan: ^{1}H NMR (DCl/D_2O): δ = 4.92 (1-H_D deacylated), δ = 4.63 (1-H_{Ac} acylated with acetic acid), δ = 3.21 (2-H), δ = 3.47−4.18 (3-H, 4-H, 5-H, 6-H), δ = 2.08 ($NCOCH_3$). DCH3 cross-linked with PEGDC at 50%: ^{1}H NMR (DCl/D_2O): δ = 4.90 (1-H_D, deacylated), δ = 4.61 (1-H_{Ac} residual $NCOCH_3$ and acylated with PEGDC), δ = 3.19 (2-H), δ = 3.45−4.15 (3-H, 4-H, 5-H, 6-H), δ = 2.06 ($NCOCH_3$), δ = 3.65−3.75 (c-CH_2 and b-CH_2 of PEGDC), δ = 4.24 (a-CH_2 of PEGDC).

The ^{13}C assignment was performed on the basis of ^{13}C projection. Figure 5 demonstrates the difference between the signs of the original DCH3 chitosan material (III) and the chitosan nanoparticles based on DCH3 cross-linked with PEGDC at 50% (VI). The assignments and chemical shifts of the ^{13}C signals are given as follows: DCH3 chitosan: ^{13}C NMR (DCl/D_2O): δ = 101.33 (1-C_{Ac}), δ = 97.70 (1-C_D), δ = 56.15 (2-C), δ = 70.28 (3-C), δ = 76.71 (4-C), δ = 75.06 (5-C), δ = 60.42 (6-C). DCH3 cross-linked with PEGDC at 50%: ^{13}C NMR (DCl/D_2O): δ = 97.67 (1-C_D deacylated), δ = 56.13 (2-C), δ = 70.26 (3-C), δ = 76.68 (4-C), δ = 75.04 (5-C), δ = 60.39 (6-C), and the signs of PEGDC δ = 69.83 (c-CH_2), δ = 70.42 (b-CH_2) and δ = 67.90 (a-CH_2).

The degree of cross-linking was evaluated from the integral intensity of signs by using the ^{1}H NMR spectra of cross-linked chitosan nanoparticles. In the case of a 25% stoichiometric ratio, the degree of cross-linking was between 15 and 25%. Increasing the stoichiometric ratio caused the degree of cross-linking to increase. In the case of cross-linking at a stoichiometric ratio of 50%, it was between 23−40%, and it was 31−60% at a stoichiometric ratio of 100%.

Figure 6 shows the ^{1}H−^{13}C hetero single quantum correlation (HSQC) spectrum of chitosan nanoparticles. The assignments were performed on the basis of ^{1}H and ^{13}C projection.

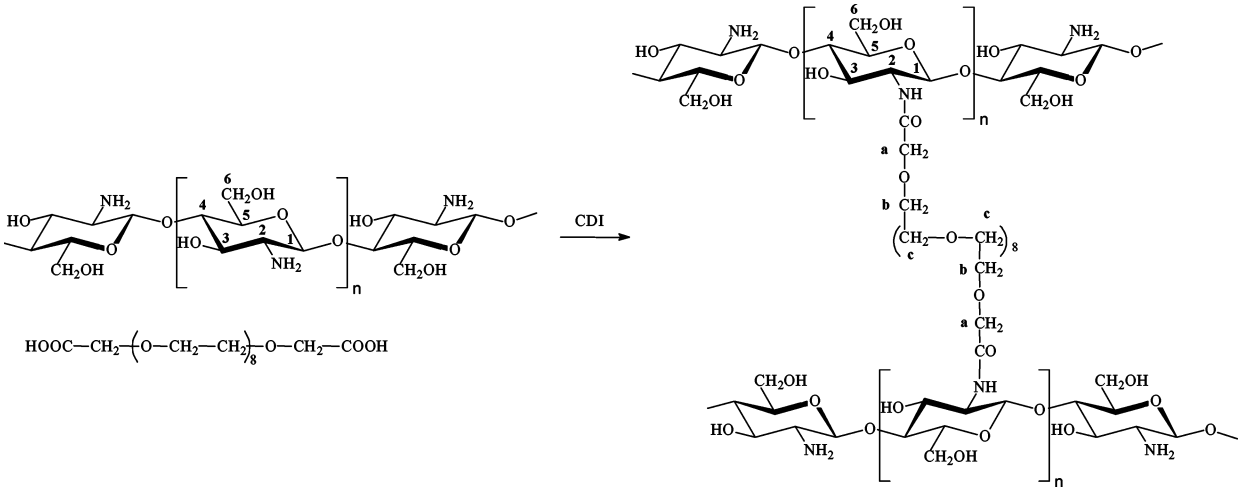

Figure 3. Schematic representation of the synthesis and structure of cross-linked chitosan derivatives.

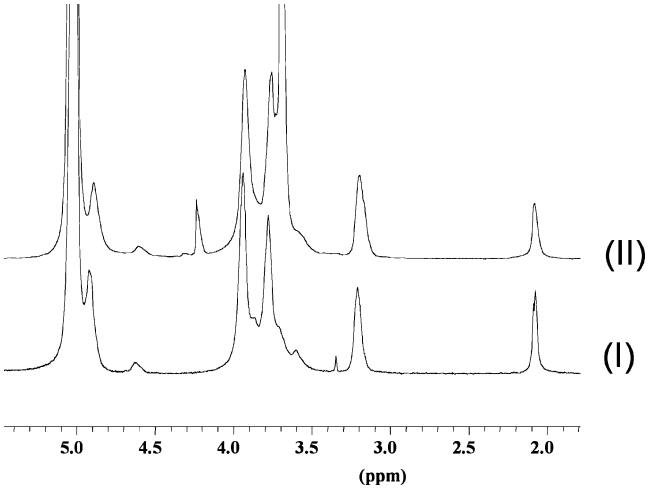

Figure 4. 500 MHz ^{1}H NMR spectra of DCH3 chitosan (I) and chitosan nanoparticles based on DCH3 cross-linked with PEGDC at 50% (II).

Particle Size by TEM. Chitosan material can be prepared as a film. However, the cross-linked chitosan nanoparticles separated into spherical particles in an aqueous environment and in dried states. TEM micrographs confirmed the nanosize of the dried, cross-linked chitosan particles. Figure 7 shows the distribution of these derivatives. The size of the dried particles varied in the range of 4−24 nm. The size of the dried cross-linked particles is smaller and their size range is narrower than the swollen particles obtained from DLS. Because PEGDC is a flexible hydrophilic oligomer, cross-linked chitosan can undergo significant swelling.

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 depend on the method of fitting. The average hydrodynamic diameters were calculated by the non-negative least-squares (NNLS) method, which separated the different peaks at multimodal distribution and provided more exact results at multimodal systems than those obtained with other methods. The intensity−delay time correlation function was evaluated by means of an NNLS fit, called an automatic routine, which was applied to determine the intensity diameter distribution. The effect of dust was cancelled by averaging numerous simultaneous measurements. Table 2 summarizes the average hydrodynamic diameters of swelled chitosan nanoparticles.

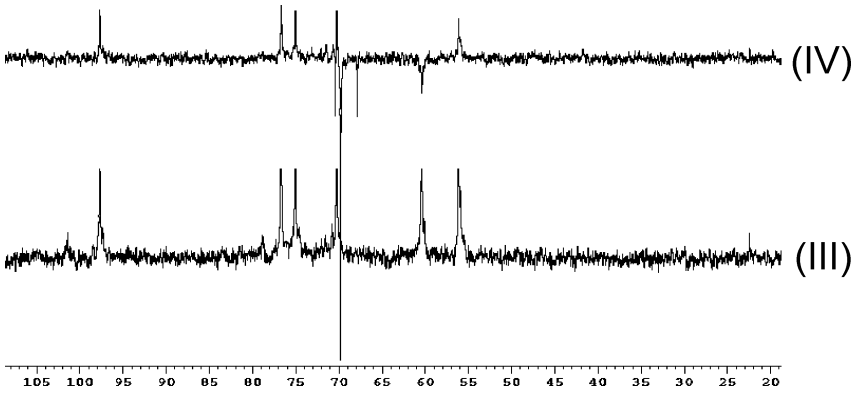

Figure 5. 500 MHz ^{13}C NMR spectra of DCH3 chitosan (III) and chitosan nanoparticles based on DCH3 cross-linked with PEGDC at 50% (VI).

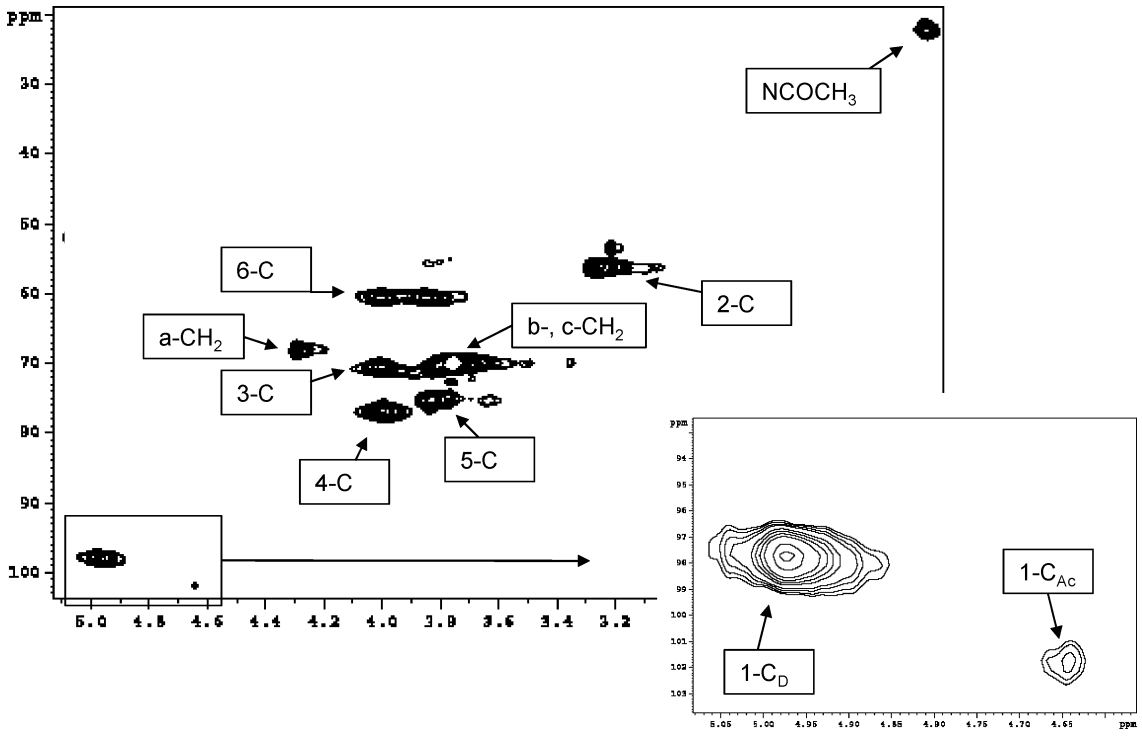

Figure 6. ^{1}H−^{13}C HSQC map of chitosan nanoparticles based on DCH3 cross-linked with PEGDC at 50%.

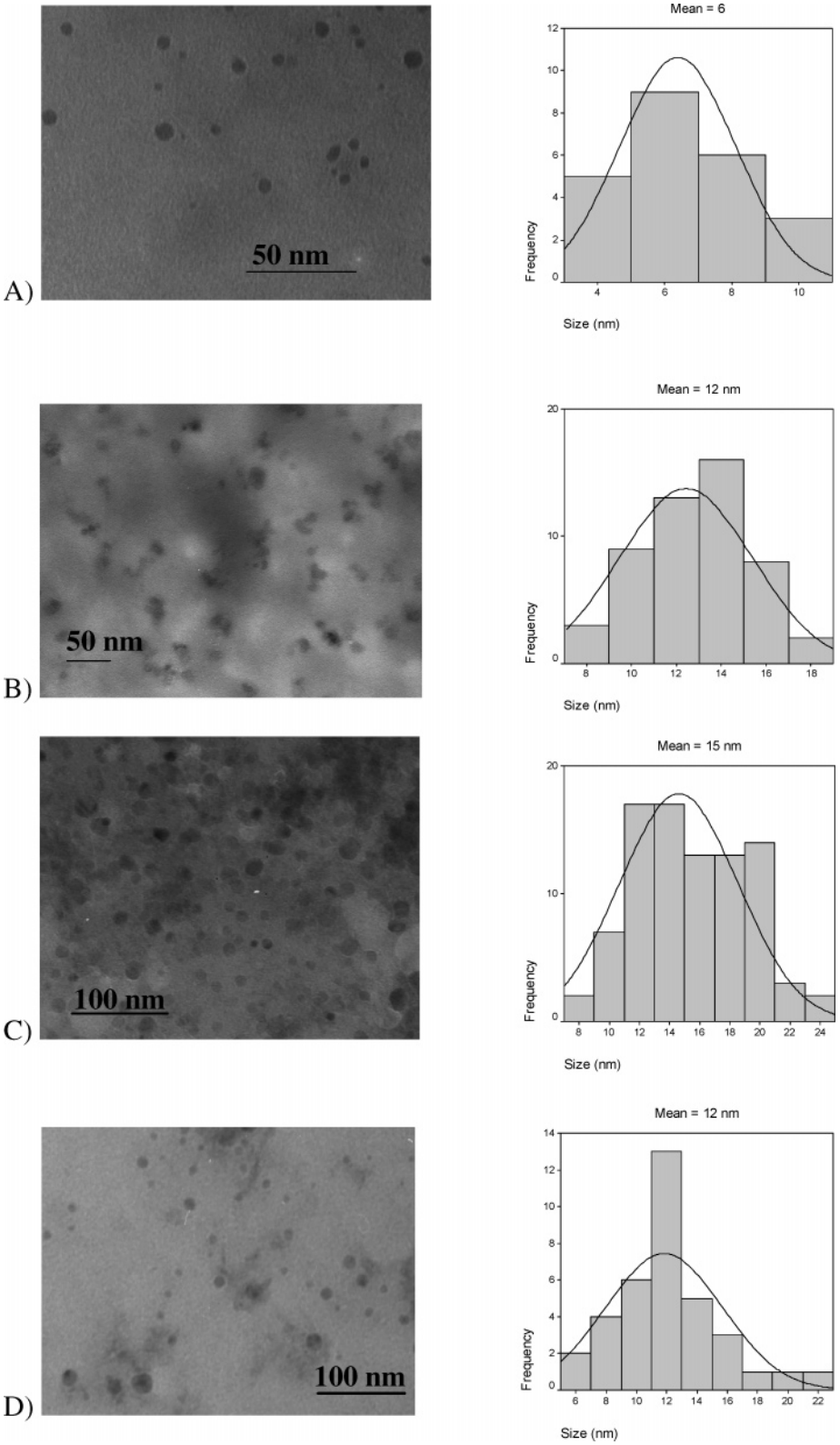

Figure 7. TEM images and size distribution of chitosan nanoparticles cross-linked with PEGDC based on DCH3 at 25% (A), DCH1 at 25% (B), DCH3 at 100% (C), and DCH2 at 100% (D).

Different molecular weight chitosans were cross-linked at different stoichiometric ratios. The cross-linking agent is a hydrophilic oligomer with a long flexible chain, resulting in a loosely cross-linked chitosan. The nanosystems produced are soluble in aqueous media and can swell. Depending on the cross-linking ratio, the residual amino groups of these macromolecules can be protonated, and the hydrodynamic diameters increase because of the repulsive interaction. The conformation of polysaccharide rings also influenced the swelling of cross-linked nanoparticles. Weak correlations were found between the hydrodynamic diameters of swelled particles and the stoichiometric ratio of cross-linking. The DLS measurements indicate that the average hydrodynamic diameter of individual nanoparticles is between 50 and 120 nm.

Chitosans with different molecular weights were used for the modification. It was found that by decreasing the molecular

Table 2. Average Hydrodynamic Diameter of Chitosan Nanoparticles Determined by DLS

chitosan	ratio of cross-linking agent	hydrodynamic diameter (nm)	aggregate size (nm)
CH	25%	108 ± 12	430 ± 40
	50%	105 ± 15	300 ± 20
	100%	100 ± 15	570 ± 60
DCH1	25%	92 ± 15	420 ± 20
	50%	92 ± 10	280 ± 20
	100%	60 ± 12	340 ± 20
DCH2	25%	98 ± 9	340 ± 20
	50%	89 ± 12	270 ± 20
	100%	80 ± 10	320 ± 20
DCH3	25%	73 ± 14	290 ± 30
	50%	78 ± 13	200 ± 20
	100%	80 ± 12	280 ± 30

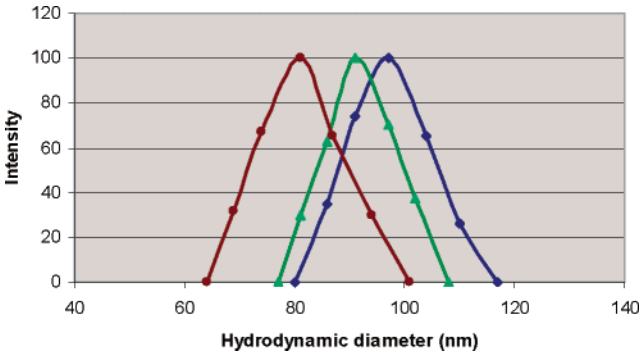

Figure 8. The size distribution, by intensity, of individual chitosan nanoparticles cross-linked with PEGDC based on DCH2 at stoichiometric ratios of 25% (◆), 50% (▲), and 100% (●). The size of the nanoparticles ranged from 65 to 115 nm, and the average size is between 80 and 100 nm.

weight, the hydrodynamic diameters decreased. This correlation was weak, and no typical relationship was observed between the size of spherical particles and the molecular weight.

Aggregates of nanoparticles were found in all cases. Aggregation can be caused by secondary interactions between the individual particles or intermolecular cross-linking reactions. TEM micrographs showed that, in the dry state, no aggregates were found. The TEM images demonstrated that intramolecular cross-linkings were obtained and that secondary interactions caused particle aggregation.

Figure 8 shows a size distribution by intensity of the cross-linked chitosan nanoparticles. It seems that, by increasing the ratio of cross-linking, the hydrodynamic diameter of cross-linked DCH2 chitosan decreases. The size distributions appear to be similarly independent of the ratio of cross-linking.

In summary, the average hydrodynamic diameters of swelled cross-linked chitosan nanoparticles were below 120 nm. To all appearances, the nanoparticles swell in aqueous media depending on the ratio of cross-linking. The swelling is limited by the framework of the polysaccharide ring structures. The molecular weight of chitosan did not influence the size of the spherical particles.

Conclusions

We have shown that a nanosized particle has been assembled from the biopolymer chitosan using a condensation reaction with PEGDC oligomer as the cross-linking agent. Clear or opalescent stable colloid systems based on chitosan were fabricated in aqueous medium at room temperature. The hydrodynamic diameters of individual particles were in the range of 50−120 nm, but aggregates were found in all cases, likely caused by secondary interactions. Weak correlations existed between the size of the nanoparticles and the stoichiometric ratio of cross-linking. Considerable decrease in particle size was observed for degraded chitosan. There was a direct but weak correlation between the particle size and the molecular weight of the chitosan used to make the particle.

Acknowledgment. This work was supported by RET (Grant of the Regional University Knowledge Center), contract number RET-06/432/2004, and by ElizaNor Polymer LLC, USA. The authors would like to thank Lajos Daroczi for TEM micrographs.

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BM0605053