Fluorescent Nanoparticles Based on Chitosan

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ABSTRACT

The present investigation reports the formation and characterization of novel biocompatible chitosan nanoparticles based conjugated with fluorescent dye for biomedical applications. The solubility and size of these nanoparticles in the dried and swollen states will be described and discussed. The correlation of size of nanoparticles, pH of the solutions, ratio of cross-linking, and the dye-content of nanosystems have been studied.

It was found, that biocompatible fluorescent chitosan nanosystems have been successfully prepared. The size and the size distributions of the nanosystems depend on the pH, but at a given pH, it was independent of the ratio of cross-linking. The diameter of individual dried particles varied in the range of 100 – 300 nm, and the average hydrodynamic diameters were in the range of 270 – 370 nm depending on the pH. The biocompatible fluorescent chitosan nanoparticles in aqueous media, might be useful for various *in vitro* experiments related to biomedical applications.

Keywords: nanoparticles, chitosan, fluorescent,

1 INTRODUCTION

Chitosan is a renewable biomaterial, β-[1→4]-2-amino-2-deoxy-D-glucopyranose, a functional and basic linear polysaccharide. Currently, because of its special set of properties, which include low or non-toxicity, biocompatibility, biodegradability, low immunogenicity and antibacterial properties, chitosan has found wide application in a variety of areas, such as biomedicine [1,2] and pharmaceuticals [3,4].

Chitosan can be modified easily to create particulate systems or porous hydrogels, because of the presence of reactive amino groups. In covalent cross-linking reactions, cross-linkers are molecules with at least two reactive functional groups that allow the formation of bridges between polymeric chains [5].

Chitosan nano- and microsystems can be employed in a wide range of biomedical application, such as drug or gene-delivery systems [6,7]. For cytotoxicity *in vitro* and toxicity *in vivo* study, it is necessary to use dye-labeled biopolymers. Many recent dyes, such as Alexa Fluor, Cibacron Blue or FITC, have been made to create dye-labeled chitosan particulate systems [8-12].

In our research work stable colloid particulate systems were performed based on chitosan by covalently cross-linking, via amino groups of the chitosan chain with dicarboxylic acids in aqueous media at room temperature. The chitosan and its cross-linked derivatives were dyed by FITC molecules to make the nanosystems suitable for biological study. The solubility and size of these nanoparticles in the dried and swollen states will be described and discussed. The correlation of size of particles, pH of the solutions, and the dye-content of the nanosystems have been studied.

Cross-linked chitosan nanoparticles may dissolve or form stable colloid systems in aqueous media at acidic and neutral pH. Because they are nano-sized at neutral pH, they can be attractive candidate delivery biosystems for a variety of related biomedical applications, which require the dye-labeled systems.

2 EXPERIMENTAL SECTION

2.1 Materials

Chitosan (degree of deacetylation (DD) = 88%, M_v = 3.2 x 10^5) 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 powder of chitosan and used for further experiments.

Succinic acid, malic acid, tartaric acid and citric acid-1-hydrate were purchased from Sigma-Aldrich Co., Hungary, and were used as received without further purification. Water soluble 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (CDI) was applied as a condensation agent for crosslinking reactions.

Fluorescein isothiocyanate isomer 1 (FITC) was purchased from Sigma-Aldrich Co., Hungary. It was dissolved in dimethyl sulfoxide (anhydrous) to produce a dye concentration of 1 mg/ml.

2.2 Characterization

Transmittance. The transmittances of colloid dispersions containing chitosan nanoparticles were measured by using 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.

Transmission Electron Microscopy (TEM). A JEOL2000 FX-II transmission electron microscope was used to characterize the size and morphology of the dried nanoparticles. 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 by 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.

Dynamic Light Scattering (DLS). Hydrodynamic radius of nanoparticles was 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 ºC with an angle detection of 90º in optically homogeneous quartz cylinder cuvettes. Each sample was measured three times and average serial data were calculated.

Absorbance. Absorbance of the fluorescent nanoparticles was measured by using Unicam SP 1800 Ultraviolet Spectrophotometer in optically homogeneous quartz cuvettes. The samples were obtained from the reaction mixture after dialysis at 25 °C.

2.3 Formation

Synthesis of cross-linked nanoparticles. Synthesis of cross-linked chitosan nanoparticles with natural carboxylic acids at diverse stoichiometric cross-linking ratios were made by CDI technique. Di- and tricarboxylic acids (tartaric-, malic-, succinic-, citric acid) were used as cross-linking agents.

Chitosan was dissolved in 0.1 M hydrochloric acid, the cross-linker carboxylic acid was added to the solution, and then adjusted to pH 6.5 with 0.1 M sodium hydroxide solution. After the dropwise addition of water soluble carbodiimide solution, the reaction mixture was stirred at 4 ºC 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.

Synthesis of fluorescent nanoparticles. Chitosan and its cross-linked derivatives were dissolved in 0.1 M hydrochloric acid to produce a chitosan concentration of 1 mg/ml, and then adjusted to pH 6.5 with 0.1 M sodium hydroxide solution. FITC, dissolved in dimethyl sulfoxide (anhydrous), was added dropwise to the solution containing chitosan or its derivatives. The reaction mixture was stirred at ambient temperature for 24 h in the dark. The solution containing fluorescent chitosan nanoparticles was purified by dialysis for 7 days against distilled water.

3 RESULTS AND DISCUSSION

3.1 Formation of nanoparticles

Formation of cross-linked nanoparticles. Polycation, polyanion, polyampholyte or uncharged cross-linked nanoparticles can be prepared by chemical modification of chitosan linear chains using di- and trifunctional carboxylic acids as cross-linking agents at different stoichiometric ratios.

Polycation or uncharged cross-linked nanoparticles can be prepared by chemical modification of chitosan linear chains using dicarboxylic acid as cross-linking agents at different stoichiometric ratios of cross-linking. Chitosan cross-linked with tricarboxylic acid can result in polyanion or polyampholyte nanoparticles depending on the ratio of cross-linking, as well as the residual functional amino and carboxyl groups.

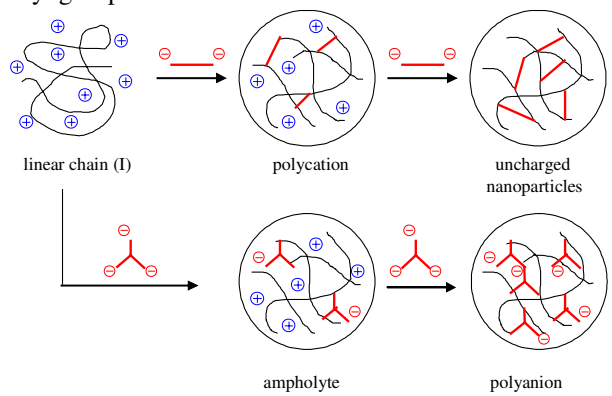

Figure 1. Schematic illustration of cross-linked polyelectrolytes based on chitosan.

Formation of fluorescent nanoparticles. Chitosan linear chain and cross-linked chitosan polycations can be carriers for FITC molecules. The residual free amino groups can be conjugated with FITC to obtain dyed nanoparticles. The dyeing reaction can produce novel biodegradable nanoparticles of chitosan for several biomedical applications.

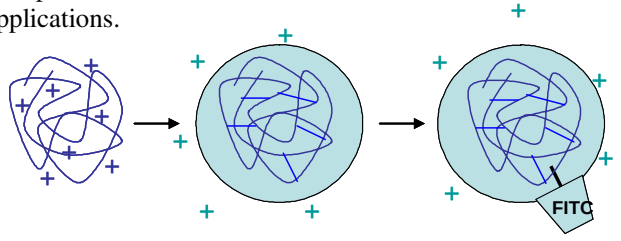

Figure 2. Schematic representation of structure of chitosan, cross-linked chitosan and FITC-labeled cross-linked chitosan.

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3.2 Transmittance

Transmittance of cross-linked chitosan derivatives was evaluated in deionized water at pH 6.5. Solutions were either clear or opaque aqueous colloid systems, and stable at room temperature for several weeks.

At lower concentrations of cross-linker, the transmittance of the colloid dispersion was greater. This was caused by the protonation of free amino groups of the chitosan chain. An increasing ratio of cross-linker corresponds to increasing opalescence of solutions. As the cross-linking increased, the particles became more compact. Transmittance of the chitosan nanoparticles is related to the hydrophilic character of the cross-linking agents and the ratio of free amino groups of the chitosan chain. Thus, the increase in hydrophilic character of carboxylic acids used, increased the transmittance values of the chitosan particles.

Chitosan nanoparticles cross-linked with citric acid at a stoichiometric ratio of 100% precipitated in aqueous media, despite the presence of residual free carboxyl groups. This result is probably related to the use of a tricarboxylic acid, which reacts as a difunctional cross-linking agent stoichiometrically.

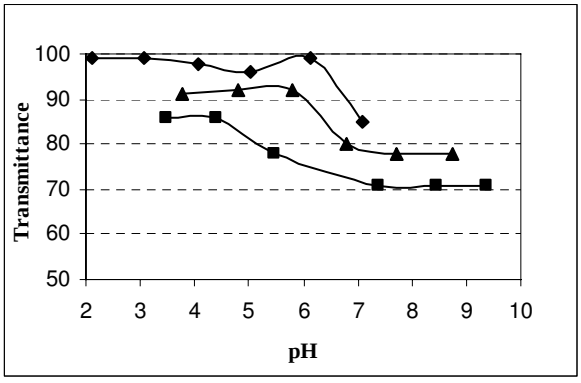

Figure 3. Transmittance of chitosan nanoparticles cross-linked with malic acid at a stoichiometric ratio of 25% (♦) 50% (▲)100% (■).

Chitosan nanoparticles cross-linked with malic acid form colloid dispersions in neutral condition. These colloid dispersions are opalescent systems, the transmittance is between 70% and 85%. It was observed, that by decreasing the pH, the transmittance of cross-linked particles increased, coused by the protonation of free amino groups of chitosan chains. It can be found that depending on the pH, cross-linked chitosan nanoparticles can become macromolecular solution.

3.3 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 provide visual evidence of the morphology and the size as well as the size distribution of the dried nanosystems. Figure 4 and 5 represent the chitosan nanoparticles formed by cross-linking modification.

The diameter of the dried particles varied in the range of 100 – 300 nm.

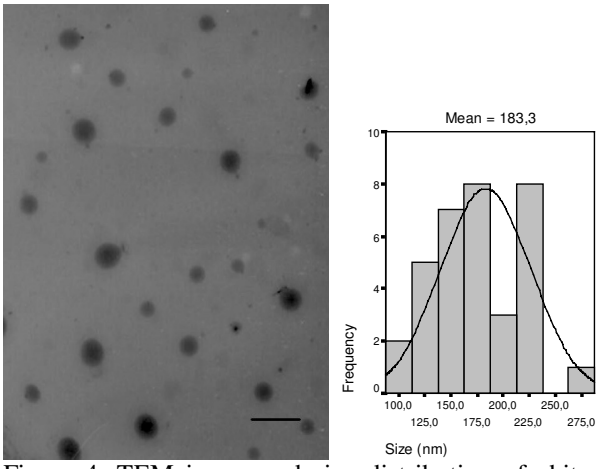

Figure 4. TEM image and size distribution of chitosan nanoparticles cross-linked with malic acid at 50%. The bar in the Figure is 500 nm.

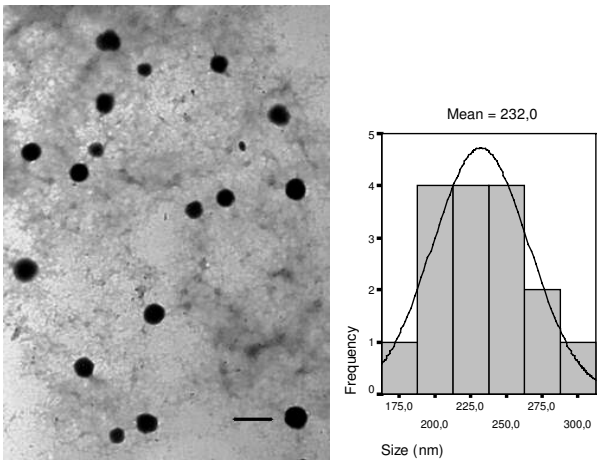

Figure 5. TEM image and size distribution of chitosan nanoparticles cross-linked with citric acid at 50%. The bar in the Figure is 500 nm.

3.4 Particle size by DLS

Figure 6 shows the hydrodynamic diameters of cross-linked chitosan nanoparticles measured at different pH. It can be seen, that by increasing the pH, average size decreased in case of tartaric acid, but no effect was found when the cross-linker was malic acid. These macromolecules are hydrophilic. The residual amino groups can be protonated, and the repulsive interaction of positive ions increase the size of macromolecules. Polyampholyte macromolecules were obtained from chitosan cross-linked with citric acid at a stoichiometric ratio of 50%. Depending on the pH, the residual amino groups of chitosan chain were protonated or the carboxylic groups of citric acid were

deprotonated. Charges of these macromolecules affect the size of nanoparticles. Summarizing the values reported, the average hydrodynamic diameters of swelled cross-linked chitosan nanoparticles were between 270 nm and 370 nm. To all appearances, the nanoparticles swell in aqueous media depending on the pH, but the polysaccharide rings established a stable framework, resulting in a conformation, which limits the swelling.

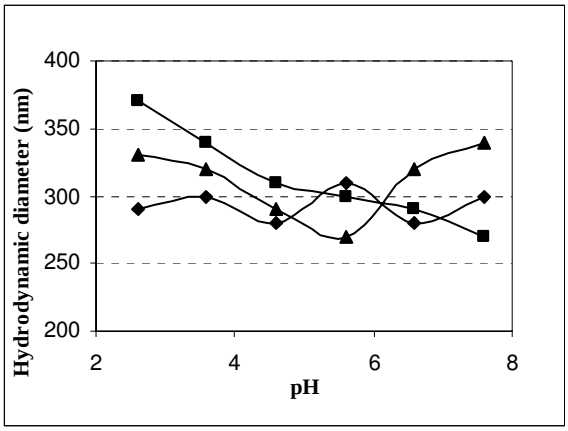

Figure 6. Average hydrodynamic diameter of chitosan nanoparticles cross-linked at a stoichiometric ratio of 50% with tartaric acid (■),malic acid (♦) and citric acid (▲).

3.5 Absorbance

Absorbance measurements support, that the chitosan and the cross-linked chitosan nanoparticles were successfully labeled with FITC molecules. Therefore, the dyed nanosystems might be useful for biological study, such as cytotoxicity in vitro and toxicity in vivo.

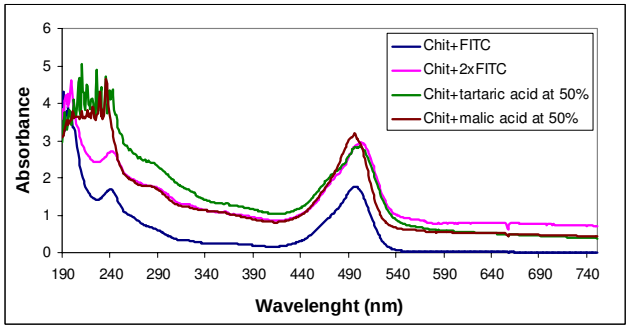

Figure 7. UV-VIS spectra of chitosan and its cross-linked derivatives labeled with FITC molecules.

There are no considerable differences between the FITC-labeled chitosan nanosystems. The main peaks of the dyed nanosystems can be found between 497 and 502 cm^{-1}. The chitosan linear chain and as well as the cross-linked nanoparticles were successfully conjugated with the FITC molecules.

4 CONCLUSION

In this work, nano-sized cross-linked particles have been successfully produced from chitosan by using CDI technique. Natural dicarboxylic acids were used as cross-linking agents. Chitosan linear biopolymer and its cross-linked derivatives were labeled with FITC dye for making them suitable for several biomedical applications. Several experimental methods were used to determine the cross-linking formation and dye-content of the nanosystems.

Clear or opalescent stable colloid systems based on chitosan were fabricated in aqueous medium at room temperature. Hydrodynamic diameter of individual particles was in the range of 270-370 nm, but aggregate were found in all cases, likely caused by secondary interactions. The size was independent of the hydrophilic character of the cross-linker used or stoichiometric ratio of cross-linking, but depended on the pH, exclusively.

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