# Nanoparticles Prepared by Self-assembly of Chitosan and Poly-γ-glutamic Acid

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## ABSTRACT

The present investigation reports the formation of stable polyelectrolyte complexes of poly-gamma-glutamic acid (γPGA) and chitosan (Chit) by self-assembly in aqueous media at room temperature. 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, concentration, order of addition and the ratio of compound polyelectrolytes have been studied.

It was found, that the size and size distribution of the nanosystems depends on the concentrations of γPGA and Chit solutions and their ratio as well as on the pH of the mixture and the order of addition. The diameter of individual particles was in the range of 40-285 nm measured by TEM, and the average hydrodynamic diameters were between 80-200 nm. These biodegradable, self-assembled stable nanocomplexes might be useful for several biomedical applications.

***Keywords***: self-assembly, polyelectrolyte, nanoparticles, chitosan, poly-γ-glutamic acid

## 1 INTRODUCTION

The technique of self-assembly of polyelectrolytes opens many new opportunities to achieve idea models as delivery system. The oppositely charged biopolymers can self-assemble by the attractive interaction between the functional groups. The electrostatic interactions between charged macromolecules can result stable self-assembled nanosystems [1, 2], films [3] or hydrogels [4, 5]. A variety of studies have focused on preparation and characterization of these polyelectrolyte complexes [6, 7].

The present paper describes the preparation and characterization of novel biodegradable polyelectrolyte complexes based on self-assembly of poly-gamma-glutamic acid (γPGA) and chitosan (Chit). Self-assembly of these polyelectrolytes was evolved by ion-ion interaction between the carboxylic groups of linear γPGA chains and the amino groups of Chit linear chains formed stable nanoparticles in aqueous media at room temperature. Complexation was observed and separated spherical particles or aggregates were obtained.

In acidic media, γPGA is a poorly charged macromolecule, and this may lead to smaller sizes of the γPGA chains; the amino groups of Chit are positively charged resulting in a highly charged polyelectrolyte polysaccharide. Attractive interactions can be performed between the polyelectrolyte segments. In this case the carboxyl and amino groups are bound by ion-ion interaction and residual free functional groups are available, which can be stabilized the self-assembled nanosystems.

In our research work stable colloid particulate systems were performed by self-assembly of γPGA and Chit polyelectrolytes. The solubility and size of these nanoparticles in the dried and swollen states were investigated. Solubility of the systems has been surveyed by turbidity; the surface and the presumed structure were analyze by mobility experiments. The sizes of swelled complexes in aqueous solutions have been determined by means of dynamic light scattering (DLS). TEM micrographs made the visual observation of the dried nanoparticles possible. It was studied the correlation of size of particles, pH of the solutions, concentration, order of addition and the ratio of compound polyelectrolytes.

## 2 EXPERIMENTAL SECTION

### 2.1 Materials

Poly-γ-glutamic acid ($M_W$ = 1.2 x $10^6$) was prepared in our laboratory by using the biosynthetic methods described earlier [8, 9]. Briefly, poly-γ-glutamic acid was produced from *Bacillus licheniformis*, strain ATCC 9945a, which was maintained on 1.5% (w/v) Bouillon-agar slants to produce appropriate cultivation conditions. γPGA was precipitated by addition of acetone and filtered. The γPGA was re-dissolved in water, dialyzed against distilled water and freeze-dried.

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.

## 2.2 Characterization

*Turbidimetry.* The transmittances of γPGA-Chit mixtures of different composition and pH were measured by using Unicam SP 1800 Ultraviolet Spectrophotometer at an operating wavelength of λ = 500 nm in optically homogeneous quartz cuvettes. Turbidity (τ) of the samples can be determined from the following relationship: $\tau = (-1/L)\ln(I_t/I_0)$ where $L$ is the light path length in the cell (1 cm), $I_t$ is the transmitted light intensity, and $I_0$ is the incident light intensity.

*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 γPGA-Chit 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 $\lambda_o$ = 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.

*Electrokinetic Measurements.* Electrophoretic mobility of the nanoparticles was measured in the presence of 1 mM KCl at 25 °C in AQ-517 cell with ZetaPALS (Brookhaven) apparatus. Samples were prepared from the colloid dispersion containing the nanoparticles. Each sample was measured three times and average serial data were calculated.

## 2.3 Formation

*Synthesis of γPGA-Chit nanoparticles.* Solutions of γPGA and Chit were used for preparation of γPGA-Chit nanoparticles. The preparation technique, based on the ion-ion interaction process, involved the mixture of aqueous phases of both polymers at ambient temperature. The pH of the mixture was adjusted to the desired pH value with 0.1 M sodium hydroxide solution. Formation of complexes between these two biopolymers at various ratios, concentrations and orders of addition were performed.

# 3 RESULTS AND DISCUSSION

## 3.1 Formation of self-assembled nanoparticles

Stable self-assembled polyelectrolytes were developed by ion-ion interaction between the functional groups of γPGA and Chit linear chains. Due to the polyelectrolyte complexation, individual spherical particles or aggregates were obtained.

It can be concluded that the initial concentrations, solution ratios and the order of addition of the γPGA and Chit solutions have a strong effect on the stability of the nanocomplexes. By decreasing the initial concentration of biopolymers, more stable nanocomplexes were formed and the formation of smaller individual nanoparticles was favored. If the amount of the Chit is equal to or greater than the γPGA in the reaction mixture and if the γPGA solution were added to the Chit solution, swollen nanocomplexes were formed, and the residual protonated amino groups of Chit chains can stabilize the particles.

| Sample | Concentration of the solutions | | |
|---|---|---|---|
| | 1.50 mg/ml | 0.750 mg/ml | 0.375 mg/ml |
| γPGA-Chit 1:2 | precipitate | stable | stable |
| Chit-γPGA 2:1 | stable | stable | stable |
| γPGA-Chit 1:1 | precipitate | stable | stable |
| Chit-γPGA 1:1 | stable | stable | stable |
| γPGA-Chit 2:1 | precipitate | precipitate | stable |
| Chit-γPGA 1:2 | precipitate | precipitate | stable |

Table 1. Reaction conditions for the formation of γPGA-Chit nanoparticles. The pH of the polyelectrolyte solutions was 3.0.

On the strength of the results, it can be concluded that the residual free amino groups of low soluble Chit play an important role in the stability of nanosystems. When free amino groups are available after ion-ion interaction, then stable nanosystems do result, otherwise Chit and the nanosystem precipitates.

## 3.2 Turbidimetry

If the amount of the Chit was equal to or more than the γPGA in the reaction mixture, and if the γPGA solution were added to the Chit solution, a swollen nanocomplex was formed in a clear solution. All of the amino groups on the Chit chains are in the protonated forms at pH = 3. The chains of the Chit are expanded in this condition. The

decrease of the positive charges on the Chit chains due to the complex formation with the γPGA chains is negligible, and thus the positive charge of the Chit chains retain the swollen state of the nanocomplex. If the Chit solution were added to the γPGA solution regardless of their ratio the turbidity is more than 1, the solution is opaque which means that the nanocomplex is in a collapsed state. At pH= 3 most of the carboxyl groups on the γPGA chains are in their protonated forms. During the complex formation with the amino groups of the Chit chains, the number of the deprotonated carboxyl groups of γPGA decreased. This decrease caused the collapse of the γPGA chains in the nanocomplex increasing the turbidity of the solution.

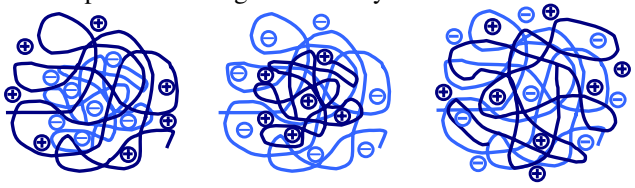

Figure 1. Schematic illustration of likely formation of self-assembled nanosystems.

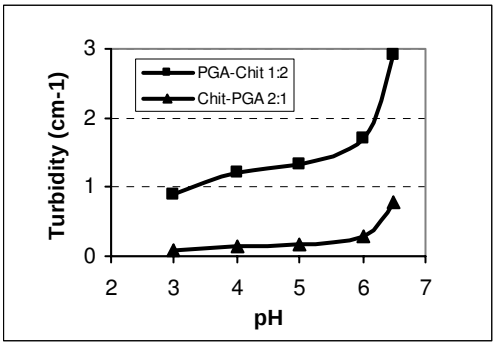


Figure 2. Effects of pH on the turbidity at the mixture conditions indicated. The concentration of the solutions was 0.315 mg/ml.

The turbidity was low when the γPGA was added to the Chit solution and the nanocomplex was in the swollen state. The turbidity increased abruptly when the pH was increase above 6, which can be caused by the deprotonation of the amino groups of the Chit chains. Above this pH the Chit chains are in a collapsed state. The deprotonation and the expansion of the γPGA chains cannot convert the globular particle to coil transition of the Chit polymer chains. However, by adding the Chit solution to the γ-PGA solution an increase in turbidity occurred and the formed nanoparticles had partially collapsed. The turbidity increased linearly as the pH increased up to pH 6.0. This increment is due to the deprotonation of the carboxyl groups of the γPGA chains. Above this pH, the turbidity increased significantly. If the pH is higher than 6, the Chit polymer chains undergo a globule→coil transition. This change can be explained by the deprotonation of the amino groups of the Chit chains, which is the decisive process in turbidity change. This fact can support the proposed mechanism for the complex formation.

## 3.3 Particle size by TEM

TEM micrographs provide visual evidence of the morphology and the size as well as the size distribution of the dried γPGA-Chit nanosystems. Figure 3 represent the self-assembled nanoparticles formed from γPGA-Chit nanosystems at pH 3.

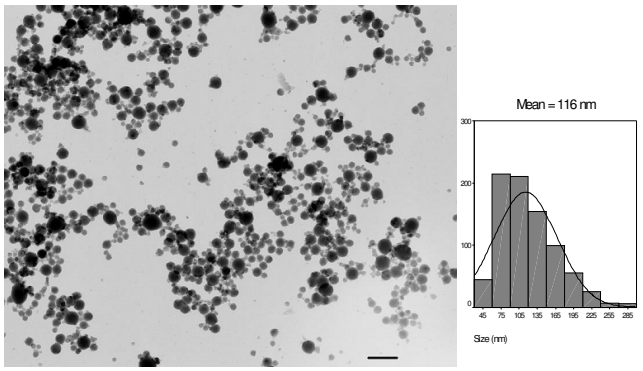


Figure 3. TEM image and size distribution of self-assembled nanoparticles formed in sample γPGA-Chit 1:1. The bar in the Figure is 500 nm.

The self-assembled nanoparticles separated into spherical particles in an aqueous environment and in dried state. TEM micrographs confirmed the nano-size of the dried particles. The diameter of the dried particles varied in the range of 40 – 285 nm.

## 3.4 Particle size by DLS

The order of addition and the proportion of biopolymers affect the hydrodynamic sizes of self-assembled nanosystems. At fixed pH, Chit plays a key role in the hydrodynamic sizes of swelled nanosystems. If the proportion of Chit is smaller, smaller self-assembled nanoparticles can arise independently of the order of addition. At low pH values, the highly charged Chit has an extended coil conformation, and the poorly charged γPGA biopolymer collapses in a compact globule. The hydrodynamic dimensions of these biopolymers were consistent with their conformations. Thus, the size of swelled self-assembled nanosystems was determined by the larger biopolymer, which is the Chit at low pH values.

The order of addition affects the hydrodynamic sizes of nanoparticles. When Chit was added to the γPGA (γPGA-Chit samples), the Chit oriented itself inside the globule as a core and the γPGA enfolded the Chit as a shell. Chit has a swelled coil conformation at acidic media, so it gives rise to the formation of larger nanosystems having a broad distribution of sizes.

The general trend is that the size distributions became increasingly broader as the proportion of Chit was increased. This effect became stronger when the Chit was added to the γPGA solution. In case of Chit-γPGA samples, the hydrodynamic radii of the nanosystems ranged from

50 nm to 280 nm and in case of γPGA-Chit samples, the hydrodynamic radii ranged from 70 nm to 255 nm.

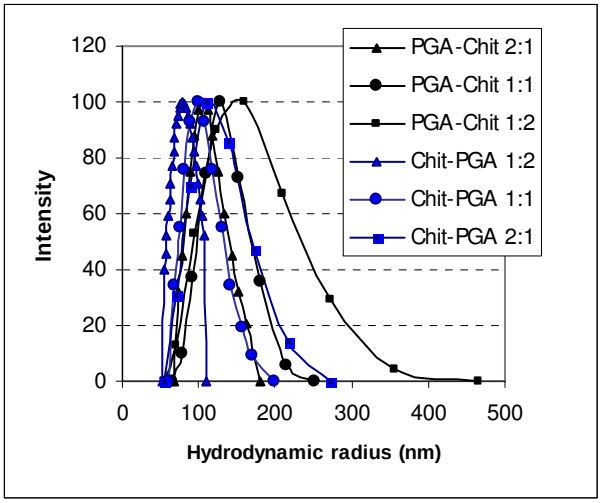


Figure 4. The size distribution by intensity of self-assembled nanoparticles. (pH = 3.0, c = 0.375 mg/ml)

## 3.5 Electrokinetic measurements

The nanoparticles have a positive surface charge if the pH is 6.0 or lower. This fact supports the proposed mechanism for the complexation between the Chit and γPGA chains. In the range of pH 3-5 the electrophoretic mobility decreased slightly. This decrease is caused by the deprotonation of the amino groups of the Chit polymer chains as well as the deprotonation of the carboxyl groups of the γPGA chains which results in a coil to globule transition of the γPGA macromolecules. If the pH is above 5 the electrophoretic mobility decreased drastically and the surface charge of the nanoparticles changed from positive to negative above pH 6.

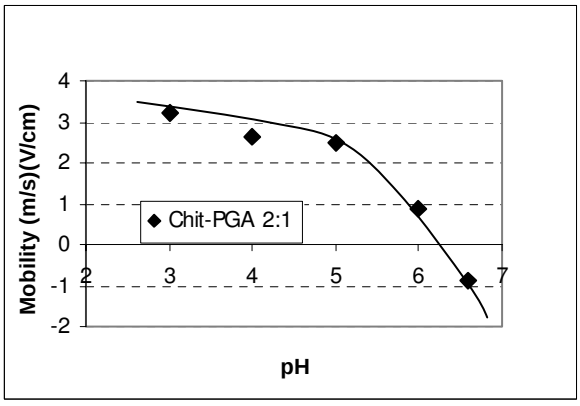


Figure 5. Effect of pH on the electrokinetic mobility of the Chit-γPGA 2:1 nanoparticles. The concentration of the solutions c = 0.315 mg/ml.

The deprotonation of the carboxyl groups increased the negative charges on the γPGA polymer chains in weak acidic condition while the Chit chains started to collapse with the strong deprotonation of the amino groups. These combined effects resulted in a change in the surface charge of the nanocomplex. The amount of the fixed charges on the polymer chains does not change with the pH, only the surface charge density changed with pH which affected on the particle size. The increased surface charge density increased the electrophoretic mobility. The electrophoretic mobility increased by 3.5 fold in the pH range of 3-6.

## 4 CONCLUSION

In this work, nano-sized particles have been successfully assembled from the γPGA and Chit without employing covalent linkages between these biopolymers. Several experimental methods were used to determine the mechanism of the complex formation. The nanocomplex was characterized by exploring the relationship between the size of particles, pH environment, concentration of the biopolymer solutions, their order of addition and the ratio of the biopolymers.

Depending on the initial concentrations of the biopolymers, stable colloid systems can be fabricated in aqueous medium at ambient temperature. We have pointed out that separated spherical nanoparticles were formed at pH 3.0 in aqueous medium. All of the experiments have supported the hypothesis that Chit chains, their protonated and deprotonated forms play an important role in the character of the self-assembled nanosystems. In the range of pH 3-6 due to the protonation of the amino groups of the Chit chains the nanocomplex is in a swollen state; above this pH the Chit chains undergo a globule-to-coil transition.

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