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SHORT COMMUNICATION

Nanoparticles prepared by self-assembly of Chitosan and poly-γ-glutamic acid

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Abstract The present paper describes the preparation and characterization of novel biodegradable nanoparticles based on self-assembly of poly-gamma-glutamic acid (γ-PGA) and chitosan (CH). The nanosystems were stable in aqueous media at low pH conditions. Solubility of the systems was determined by turbidity measurements. Surface charge and mobility were measured electrophoretically. The particle size and the size distribution of the polyelectrolyte complexes were identified by dynamic light scattering and transmission electron microscopy (TEM). It was found that the size and size distribution of the nanosystems depends on the concentrations of γ-PGA and CH 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 20–285 nm measured by TEM, and the average hydrodynamic diameters were between 150 and 330 nm. These biodegradable, self-assembling stable nanocomplexes might be useful for several biomedical applications.

Keywords γ-PGA, Chitosan · Polyelectrolyte · Nanoparticles · Complexation · Self-assembly · Electrophoretic mobility

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Introduction

A number of novel drug delivery systems have been developed for biomedical applications [1–3]. Many recent attempts have been made to create particulate systems based on biopolymers to obtain small vehicles for controlled drug [3–5] or gene [6–8] delivery. Several ionic cross-linking methods were evolved to produce stable nanosystems [1, 3, 9] or hydrogels [10–12] from biopolymers. These methods are based on the interaction between biopolymers with various charged ions [13, 14] using ionotropic gelation process.

The self-assembly of polyelectrolytes opens many new opportunities to develop 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 particulate systems [1, 3, 8, 9, 15–18] films [5, 19] or hydrogels [10, 11, 20, 21]. A variety of studies have focused on preparation and characterization of these polyelectrolyte complexes [22–25].

Chitosan (CH) is a renewable, basic linear biomaterial containing β-[1→4]-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. 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 [10, 26–28], pharmaceuticals [2, 9, 22], metal chelation [14], food additives [4], and other industrial applications [29]. A limiting factor in its application is its low solubility in aqueous media. However, chitosan can be solubilized by the protonation of its amino groups in acidic media, resulting in a cationic polysaccharide with high charge density.

Many reviews on polyelectrolyte complexes containing chitosan have been published [8, 9, 21, 22]. In this study, poly-gamma-glutamic acid (γ-PGA) was utilized to create stable colloid particles by self-assembly of these two biopolymers in aqueous media.

Poly-γ-glutamic acid consists of repetitive glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid functional groups. The secondary structure of γ-PGA in aqueous solution has been described as an α-helix. γ-PGA is different from other proteins in that glutamate is polymerized via the non-peptide γ-amide linkages, and thus, is synthesized in a ribosome-independent manner [30].

γ-PGA is a water-soluble, biodegradable, edible and nontoxic polyanion. Therefore, γ-PGA and its derivatives have been employed extensively in a variety of commercial applications such as cosmetics, food [30], medicine [21, 31–33], and water treatment [34].

The present investigation reports the formation of polyelectrolyte complexes of poly-γ-glutamic acid and chitosan by self-assembly in aqueous media at room temperature. The PGA–CH complexes may form stable colloid particulate systems. The solubility and size of these nanoparticles in the dried and swelled states will be described and discussed. Solubility of the systems has been surveyed by turbidity; the surface and the preliminary structural conformation were analyzed by electrophoretic mobility experiments. Transmission electron microscopy (TEM) measurements made the visual observation of the nanoparticles possible. The sizes of the swelled complexes in aqueous solutions have been determined by means of dynamic light scattering (DLS). It was the study the correlation of size of particles, pH and concentration of the solutions, order of addition, and the ratio of compound polyelectrolytes.

Experimental section

Materials

Poly-γ-glutamic acid (M_W=1.2×10^6) was prepared in our laboratory using the biosynthetic methods described earlier [30, 35, 36]. 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. γ-PGA was precipitated by the addition of acetone and filtered. The γ-PGA was redissolved in water, dialyzed against distilled water, and freeze-dried. Chitosan [degree of deacetylation (DD)=88%, M_V=3.2×10^5] was purchased from Sigma-Aldrich, 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.

Formation

Synthesis of PGA-CH nanoparticles Solutions of γ-PGA and CH were used for preparation of PGA–CH 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.

Characterization

Turbidimetry The transmittances of PGA–CH mixtures of different composition and pH were measured 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. The results from the spectrophotometer are presented in terms of the turbidity.

Transmission electron microscopy A JEOL2000 FX-II transmission electron microscope was used to characterize the size and morphology of the dried γ-PGA 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 using SPSS 11.0 program file.

Dynamic light scattering Hydrodynamic radius of PGA–CH nanoparticles was gauged 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. 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.

Results and discussion

Formation of self-assembled PGA–CH nanoparticles Stable self-assembled polyelectrolytes were developed by ion–ion interaction between the carboxylic groups of linear γ-PGA chains and the amino groups of chitosan linear chains. Due to the polyelectrolyte complexation, individual spherical particles or aggregates were obtained. The size and the stability of the particles depended on the pH, the concentration and the ratio of γ-PGA and CH solution, as well as the order of addition. These variable had a strong influence on the size and the stability of the nanocomplexes. The conditions used for the preparation of the complexes are summarized in Table 1.

It can be concluded that the initial concentrations, solution ratios, and the order of addition of the γ-PGA and CH 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 CH is equal to or greater than the γ-PGA in the reaction mixture and if the γ-PGA solution were added to the CH solution, swollen nanocomplexes were formed, and the residual protonated amino groups of CH chains can stabilize the particles.

On the strength of the results of Table 1, it can be concluded that the residual free amino groups of low soluble CH 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, CH and the nanosystem precipitates.

Turbidimetry Turbidity measurements were performed on the PGA–CH nanoparticles in aqueous solution at pH 3.0 and at ambient temperature. Table 2 summarizes the turbidity of the investigated nanocomplexes. The ratio, as well as the order of the mixing of the solutions, was varied during the preparation of the nanoparticles.

It can be concluded from these measurements that if the amount of the CH is equal to or more than the γ-PGA in the reaction mixture and if the γ-PGA solution were added to the CH solution, a swollen nanocomplex is formed in a clear solution. All of the amino groups on the CH chains are in the protonated forms at pH=3. The chains of the CH are expanded in this condition. The decrease of the positive charges on the CH chains due to the complex formation with the γ-PGA chains is negligible, and thus, the positive charge of the CH chains retains the swollen state of the nanocomplex. If the CH solution were added to the γ-PGA solution regardless of their ratio, the turbidity is more than 1, and the solution is opaque, which means that the nanocomplex is in a collapsed state. At pH=3, most of the carboxyl groups on the γ-PGA chains are in their protonated forms. During the complex formation with the amino groups of the CH chains, the number of the deprotonated carboxyl groups of γ-PGA decreased. This decrease caused the collapse of the γ-PGA chains in the nanocomplex, increasing the turbidity of the solution. The proposed mechanism for the complex formation according to the turbidity measurements is seen in Fig. 1.

Table 1 Reaction conditions for the formation of PGA–CH nanoparticles

Sample	Concentration of the solutions (mg/ml)			
	1.50	0.750	0.375	0.188
PGA–CH 1:2	Precipitate	Stable	Stable	Stable
CH–PGA 2:1	Stable	Stable	Stable	Stable
PGA–CH 1:1	Precipitate	Stable	Stable	Stable
CH–PGA 1:1	Stable	Stable	Stable	Stable
PGA–CH 2:1	Precipitate	Precipitate	Stable	Stable
CH–PGA 1:2	Precipitate	Precipitate	Stable	Stable

The pH of the polyelectrolyte solutions was 3.0.

Table 2 Turbidity values of the solutions containing PGA–CH nanoparticles (pH=3.0, c=0.375 mg/ml)

Sample	PGA–CH 1:2	PGA–CH 1:1	PGA–CH 2:1	CH–PGA 2:1	CH–PGA 1:1	CH–PGA 1:2
Turbidity (cm^{-1})	0.896	1.221	1.470	0.088	0.105	0.511

The effect of pH on turbidity was investigated. The results can be seen in Fig. 2. Measurements were performed on two different samples. One of the samples (filled square) was prepared by the addition of twice the amount of CH solution to that of γ-PGA, while the other (filled triangle) was prepared by adding γ-PGA solution to twice the amount of CH.

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

Particle size by TEM TEM micrographs provide visual evidence of the morphology and the size as well as the size distribution of the dried PGA–CH nanosystems. Figure 3 represents the self-assembled nanoparticles formed from PGA–CH nanosystems at pH 3.

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 20–285 nm. The size of the dried nanoparticles is smaller, and their size range is narrower than the swollen particles obtained from DLS. These results support the conclusion that self-assembled nanosystems based on linear biopolymers can swell in aqueous media.

Results from the calculation of size distribution, as well as from the DLS experiments, show that the order of addition is one of the main factors which determine the size and the size distribution of the self-assembled nanosystems. When γ-PGA was added to the CH (CH–PGA system; Fig. 3a), smaller particles were obtained, and the size distribution of this system was narrower.

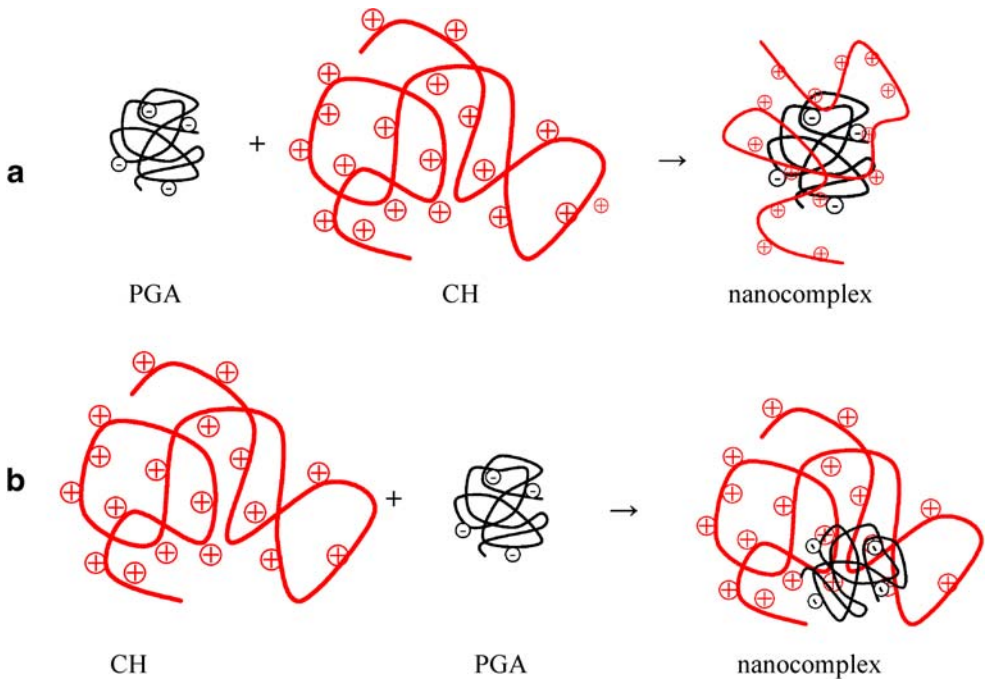

Fig. 1 Schematic picture of the complex formation between the γ-PGA and CH chains. **a** CH solution is added to the γ-PGA solution, **b** γ-PGA solution is added to the CH solution

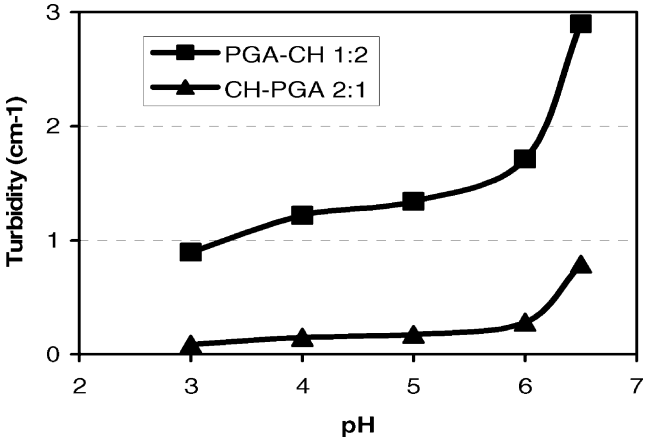

Fig. 2 Effects of pH on the turbidity at the mixture conditions indicated. The concentration of the solutions was 0.315 mg/ml

Dynamic light scattering Samples were taken from the reaction mixture. The concentration of the solutions containing self-assembled nanosystems was usually 0.375 mg/ml. The pH of the original samples was 3.0, and then it was adjusted by addition of sodium hydroxide solution when necessary.

In polydispersed systems, the interpretation of results depends on the method of fitting. The average hydrodynamic radii 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 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 numerous simultaneous measurements. Table 3 summarizes the average hydrodynamic radii of swelled self-assembled nanoparticles.

The results in Table 3 reveal that the order of addition and the proportion of biopolymers affect the hydrodynamic sizes of self-assembled nanosystems. At fixed pH, CH plays a key role in the hydrodynamic sizes of swelled nanosystems. If the proportion of CH is smaller, smaller self-assembled nanoparticles can arise independently of the order of addition. At low pH values, the highly charged CH has an extended coil conformation, and the poorly charged γ-PGA 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 CH at low pH values.

The order of addition affects the hydrodynamic sizes of nanoparticles. When CH was added to the γ-PGA (PGA–

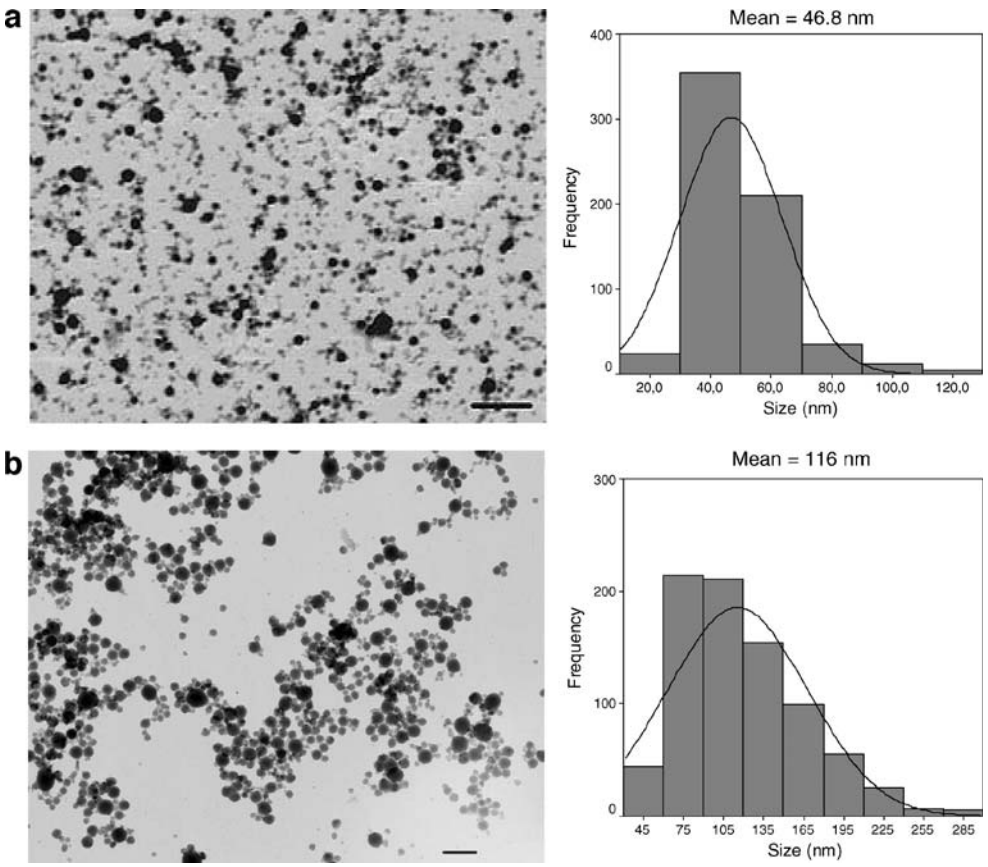

Fig. 3 **a** TEM image and size distribution of self-assembled nanoparticles formed in sample CH–PGA 1:1. The *bar* in the figure is 500 nm. **b** TEM image and size distribution of self-assembled nanoparticles formed in sample PGA–CH 1:1. The *bar* in the figure is 500 nm

Table 3 Average hydrodynamic radii of the self-assembled nanosystems determined by DLS (pH=3.0, c=0.375 mg/ml)

Sample	PGA–CH 1:2	PGA–CH 1:1	PGA–CH 2:1	CH–PGA 2:1	CH–PGA 1:1	CH–PGA 1:2
Hydrodynamic radius (nm)	160±20	135±10	105±10	115±5	110±5	80±5

CH samples), the CH oriented itself inside the globule as a core, and the γ-PGA enfolded the CH as a shell. CH has a swelled coil conformation at acidic media, so it gives rise to the formation of larger nanosystems having a broad distribution of sizes.

Figure 4 shows the size distribution by intensity of the self-assembled nanoparticles. The general trend is that the size distributions became increasingly broader as the proportion of CH was increased. This effect became stronger when the CH was added to the γ-PGA solution. In the case of CH–PGA samples, the hydrodynamic radii of the nanosystems ranged from 50 to 280 nm, and in the case of PGA–CH samples, the hydrodynamic radii ranged from 70 to 255 nm.

Figure 5 shows the effect of the pH and the concentration on the size of the nanocomplex. At constant ratio of biopolymers, the hydrodynamic radii increased as the concentration of the components were increased and occurred over a broad pH range. At higher concentrations of the components, small aggregates formed. As the concentration of biopolymers increased, formation of large nanosystems was favored.

In the case of CH–PGA 2:1 nanosystems, residual functional groups of biopolymers can be observed after self-assembly. The free amino groups of CH can be protonated in acidic media, and hydrodynamic sizes are determined by the repulsive interaction of segments of the linear chains. At higher pH, the free carboxylic groups of γ-PGA can be deprotonated, and repulsive interactions of segments of γ-PGA likely occur. The combination of the adhesive interactions between the different biopolymers and the repulsive interactions within segments of the biopolymers promotes the self-assembled nanosystems and determines the hydrodynamic sizes of these particles.

Figure 5 shows that the hydrodynamic sizes of nanosystems increased as the pH increased. This trend can be observed independently of the concentration of the components. This effect may be caused by the changing conformation of the biopolymers. Likely, there are dynamic and opposing forces operating in this nanosystem. These are: (1) the conformational stability of the polysaccharide rings, which establishes a stable framework, possibly discouraging the swelling of nanoparticles and (2) the greater flexibility of γ-PGA.

In summary, the self-assembled nanoparticles swell in aqueous media. The hydrodynamic radii and the size distributions of these systems depend on the ratio and the concentration of biopolymers, on the mixing order, and on the pH. Summarizing the values reported, the average hydrodynamic radii of swelled nanoparticles ranged between 75 and 165 nm.

Electrokinetic measurements To further understand the nature of complex formation of the biopolymers, electrokinetic measurements were performed in 1.0 mM KCl solution at 25 °C. The effect of the pH on the electrophoretic mobility of the nanoparticles which were formed by adding the γ-PGA to twice the amount of CH solution is seen in Fig. 6. The initial concentration of the solutions is 0.315 mg/ml.

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 CH and γ-PGA chains. In the range of pH 3–5, the electrophoretic

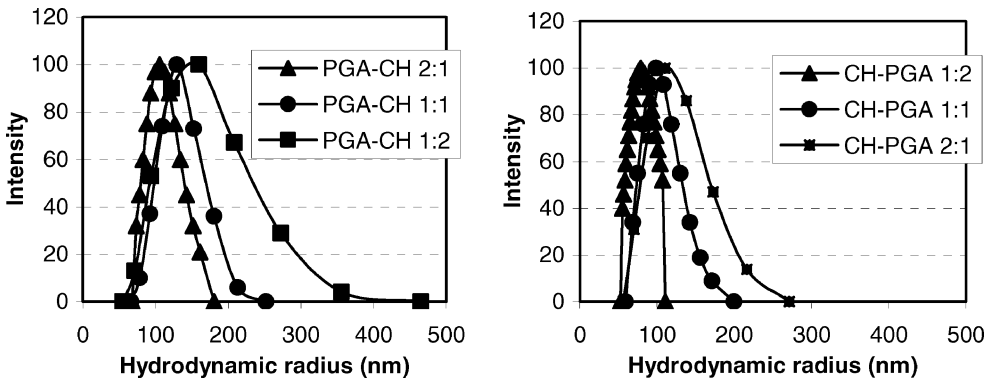

Fig. 4 The size distribution by intensity of self-assembled nanoparticles. (pH=3.0, *c*= 0.375 mg/ml)

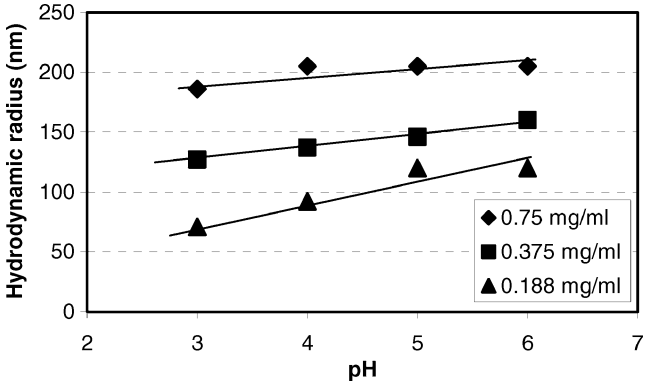

Fig. 5 Effect of pH and concentration on the hydrodynamic sizes of self-assembled CH–PGA 2:1 nanoparticles

mobility decreased slightly. This decrease is caused by the deprotonation of the amino groups of the CH polymer chains as well as the deprotonation of the carboxyl groups of the γ-PGA chains which results in a coil to globule transition of the γ-PGA 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. The deprotonation of the carboxyl groups increased the negative charges on the γ-PGA polymer chains in weak acidic condition, while the CH 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 (σ) changed with pH, which affected on the particle size. The increased surface charge density increased the electrophoretic mobility (u). The electrophoretic mobility increased by 3.5-fold in the pH range of 3–6. This result can be interpreted by the following equation:

$$\sigma = \frac{Q}{\pi a^2} = \varepsilon\kappa\phi_0\left(1 + \frac{1}{\kappa a}\right) \quad (1)$$

where Q is the electric charge, ε is the dielectric permittivity of the medium, κ is the inverse of the Debye–Huckel screening length, ϕ_0 is the surface potential, a is the radius of the particle.

The electrophoretic mobility of the particles:

$$u = \frac{2\varepsilon\zeta}{3\eta} f(\kappa a) \quad (2)$$

where η is the viscosity of the medium.

If the radius of the particle is substituted with the hydrodynamic radius ($a = R_H$) in Eq. 1, the potential (ϕ_0) is the electrokinetic potential (ζ). These equations demonstrate that the electrophoretic mobility is in inverse proportion to the square of the hydrodynamic radius of the particle.

In the case of the PGA–CH nanoparticles, not only the size of the particle has an effect on the electrophoretic mobility, but the deprotonation of the carboxyl as well as the amino groups affect mobility of the particles.

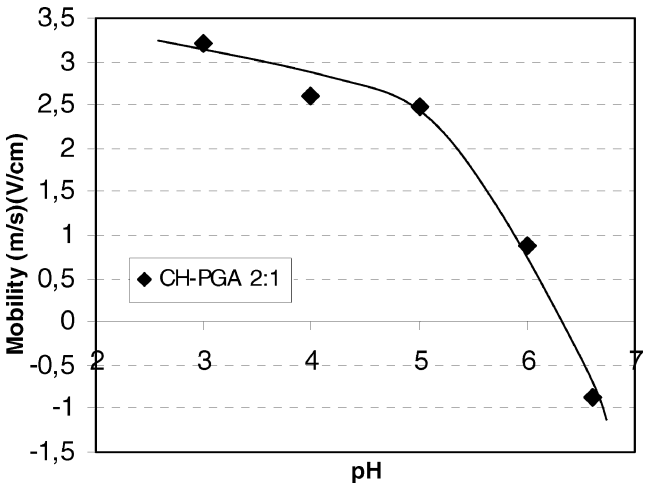

Fig. 6 Effect of pH on the electrokinetic mobility of the CH–PGA 2:1 nanoparticles. The concentration of the solutions $c = 0.315$ mg/ml

Conclusions

In this work, we have shown that nano-sized particles have been successfully assembled from the γ-PGA and chitosan 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 CH 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 CH chains, the nanocomplex is in a swollen state; above this pH, the CH chains undergo a globule-to-coil transition.

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