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Encapsulation of Milk β-Lactoglobulin by Chitosan Nanoparticles

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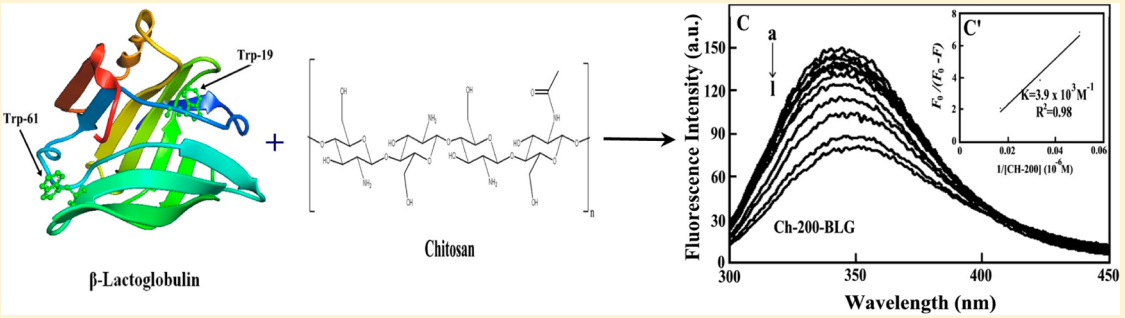

ABSTRACT: Naturally occurring polymers, such as chitosan, have been extensively studied as carriers for therapeutic protein and gene delivery systems. β-Lactoglobulin (β-LG) is a member of the lipocalin superfamily of transporters for small hydrophobic molecules. We examine the binding of milk β-lactoglobulin with chitosan of different sizes such as chitosan 15, 100, and 200 KD in aqueous solution at pH 5−6, using FTIR, CD, and fluorescence spectroscopic methods. Structural analysis showed that chitosan binds β-LG via both hydrophilic and hydrophobic contacts with overall binding constants of $K_{\beta\text{-LG-ch-15}}$ = 4.1 (±0.4) × 10^2 M^{-1}, $K_{\beta\text{-LG-ch-100}}$ = 7.2 (±0.6) × 10^4 M^{-1}, and $K_{\beta\text{-LG-ch-200}}$ = 3.9 (±0.5) × 10^3 M^{-1} with the number of bound protein per chitosan (n) 0.9 for ch-15, 0.6 for ch-100, and 1.6 for ch-200. Chitosan 100 KD forms stronger complexes with β-LG than chitosans 200 and 15 KD. Polymer binding did not alter protein conformation inducing structural stabilization. Chitosan 100 is a stronger protein transporter than chitosan 15 and 200 KD.

INTRODUCTION

In recent years, soluble and biodegradable carriers based on chitosan and its derivatives have received particular interest for the delivery of therapeutic proteins and antigens.[1,2] Chitosan (Scheme 1) is a natural polymer obtained by a partial deacetylation of chitin.[3] It is a nontoxic, biocompatible, and biodegradable polysaccharide. Chitosan nanoparticles have gained more attention as drug delivery carriers because of their better stability, low toxicity, simple and mild preparation method, and ability to providing versatile routes of administration.[3−6] The deacetylated chitosan backbone of glucosamine units has a high density of charged amine groups, permitting strong electrostatic interactions with proteins and genes that carry an overall negative charge at neutral pH conditions.[3,4] The fast expanding research of the useful physicochemical and biological properties of chitosan has led to the recognition of the cationic polysaccharide, as a natural polymer for drug delivery.[7−10] Therefore it is of major interest to study the encapsulation of protein with chitosan of different sizes, in order to evaluate the efficacy of chitosan nanoparticles in protein delivery.

β-Lactoglobulin (Scheme 1) contains several high affinity binding sites for fatty acids, lipids, aromatic compounds, vitamins, and polyamines.[11−19] There are two potential binding sites in the lipocalin β-lactoglobulin for small hydrophobic molecules. One is located in the interior cavity and the other on the surface cleft of the β-lactoglobulin structure.[20,21] The structure of this protein is well-known, and at neutral pH, β-LG exists as a mixture of monomers and dimers of which the equilibrium ratio depends on the association constant of the dimer and on the protein concentration.[22] Each monomer consists of 162 amino acid residues and has a molecular mass of 18 kDa.[22,23] As a member of the lipocalin family, β-LG is a small globular protein folded into a calyx formed by eight antiparallel β-strands and an α-helix located at the outer surface of the β-barrel.[24] Several spectroscopic and X-ray crystallographic reports examined the binding sites of hydrophobic ligands on β-LG.[11,12,23,24] Competitive binding studies based on fluorescence spectroscopy showed different binding sites for hydrophobic compounds with β-LG.[23,24] However, the exact binding sites of the hydrophobic ligands on β-LG are still the subject of much controversy. Therefore, it was of interest to determine the binding sites of chitosan with β-LG and the effects of polymer complexation on the protein stability and conformation.

Fluorescence quenching is considered as a useful and reliable technique for measuring binding affinities.[25] Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with a quencher molecule.[26] Therefore, it is

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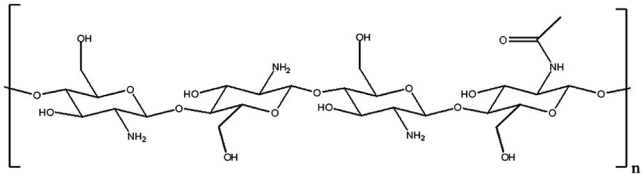
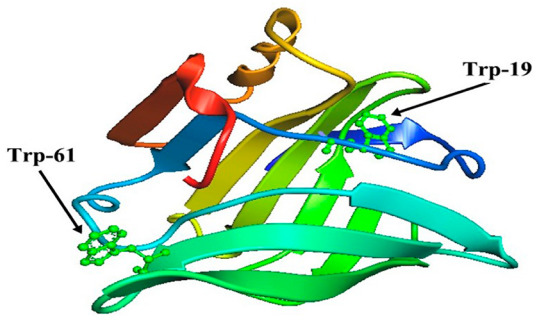
Scheme 1. Chemical Structures of Chitosan and β-Lactoglobulin with Tryptophan Residues in Green



Chitosan



β-Lactoglobulin

possible to use quenching of the intrinsic tryptophan fluorescence of Trp-61 and Trp-19 in β-LG[11,12] as a tool to study the interaction of chitosan with β-LG in an attempt to characterize the nature of polymer−protein interaction.

We report spectroscopic analysis of the interaction of β-LG with chitosan of different sizes in aqueous solution at pH 5−6, using constant protein concentration and various polymer contents. Structural analysis regarding protein binding sites and the effect of polymer−protein complexation on the β-LG stability and conformation is reported here.

MATERIALS AND METHODS

Materials. Purified chitosans 15, 100, and 200 KD (90% deacetylation) were from Polysciences Inc. (Warrington, USA) and used as supplied. β-Lactoglobulin (A variant, purity >90%) was purchased from Sigma-Aldrich Chemical Co (St-Louis, MO) and used as supplied. Other chemicals were of reagent grade and used as supplied.

Preparation of Stock Solutions. An appropriate amount of chitosan was dissolved in acetate solution (pH 5−6). The β-lactoglobulin was dissolved in aqueous solution (8 mg/mL to obtain 0.5 mM protein content) containing acetate buffer. The protein concentration was determined spectrophotometrically using the extinction coefficient of 17600 M^{-1} cm^{-1} at 280 nm.[27]

FTIR Spectroscopic Measurements. Infrared spectra were recorded on a FTIR spectrometer (Impact 420 model, Digilab), equipped with deuterated triglycine sulfate (DTGS) detector and KBr beam splitter, using AgBr windows. Solution of chitosan was added dropwise to the protein solution with constant stirring to ensure the formation of homogeneous solution and to reach the target polymer concentrations of 15, 30, and 60 μM with a final protein concentration of 0.25 mM. Spectra were collected after 2h incubation of β-lactoglobulin with polymer solution at room temperature, using hydrated films. Interferograms were accumulated over the spectral range 4000−600 cm^{-1} with a resolution of 2 cm^{-1} and 100 scans. The difference spectra [(protein solution + chitosan solution) − (protein solution)] were generated using water combination mode near 2300 cm^{-1}, as standard.[28] When producing difference spectra, this band was adjusted to the baseline level, in order to normalize the difference spectra.

Analysis of Protein Conformation. Analysis of the secondary structure of β-lactoglobulin and its chitosan complexes was carried out on the basis of the procedure already reported.[29] The protein secondary structure is determined from the shape of the amide I band, located at 1660−1650 cm^{-1}. The FTIR spectra were smoothed and their baselines were corrected automatically using the built-in software of the spectrophotometer (OMNIC ver. 7.3). Thus, the root-mean square (rms) noise of every spectrum was calculated. By means of the second derivative in the spectral region 1700−1600 cm^{-1}, five major peaks for β-lactoglobulin and the complexes were resolved. The above spectral region was deconvoluted by the curve-fitting method with the Levenberg-Marquadt algorithm and the peaks related to α-helix (1660−1650 cm^{-1}), β-sheet (1640−1610 cm^{-1}), turn (1680−1660 cm^{-1}), and β-antiparallel (1692−1680 cm^{-1}) were adjusted and the area were measured with the Gaussian function. The areas of all of the components of the bands assigned to a given conformation were then summed up and divided by the total area. The curve fitting analysis was performed using the GRAMS/AI Version 7.01 software of the Galactic Industries Corporation.

Circular Dichroism. CD Spectra of β-lactoglobulin and its chitosan complexes were recorded with a Jasco J-720 spectropolarimeter. For measurements in the far-UV region (178−260 nm), a quartz cell with a light path length of 0.01 cm was used in nitrogen atmosphere. Protein concentration was kept constant (12.5 μM), while varying each polymer concentration (15, 30, and 60 μM). An accumulation of five scans with a scan speed of 50 nm per minute was performed and data were collected for each nm from 260 to 180 nm. Sample temperature was maintained at 25 °C using a Neslab RTE-111 circulating water bath connected to the water-jacketed quartz cuvettes. Spectra were corrected for buffer signal and conversion to the Mol CD ($\Delta\varepsilon$) was performed with the Jasco Standard Analysis software. The protein secondary structure was calculated using CDSSTR, which calculates the different assignments of secondary structures by comparison with CD spectra, measured from different proteins for which high quality X-ray diffraction data are available.[30,31] The program CDSSTR is provided in CDPro software package which is available at the Web site: http://lamar.colostate.edu/~sreeram/CDPro.

Fluorescence Spectroscopy. Fluorimetric experiments were carried out on a Perkin-Elmer LS55 Spectrometer. Stock solution of polymer (200 μM) was prepared at room temperature in acetate buffer (24 ± 1 °C). Various solutions of chitosan (1 to 100 μM) were prepared from the above stock solutions by successive dilutions. Stock solution of β-lactoglobulin (10 μM) in acetate buffer was also prepared at 24 ± 1 °C. Samples containing 0.06 mL of the above protein solution and various polymer solutions were mixed to obtain final chitosan concentrations ranging from 1 to 100 μM with constant β-LG content (10 μM). The fluorescence spectra were recorded at λ_{exc} = 280 nm and λ_{emi} from 290 to 500 nm. The intensity at 340 nm (from tryptophan residues) was used to calculate the binding constant (K) according to previous reports.[32−38]

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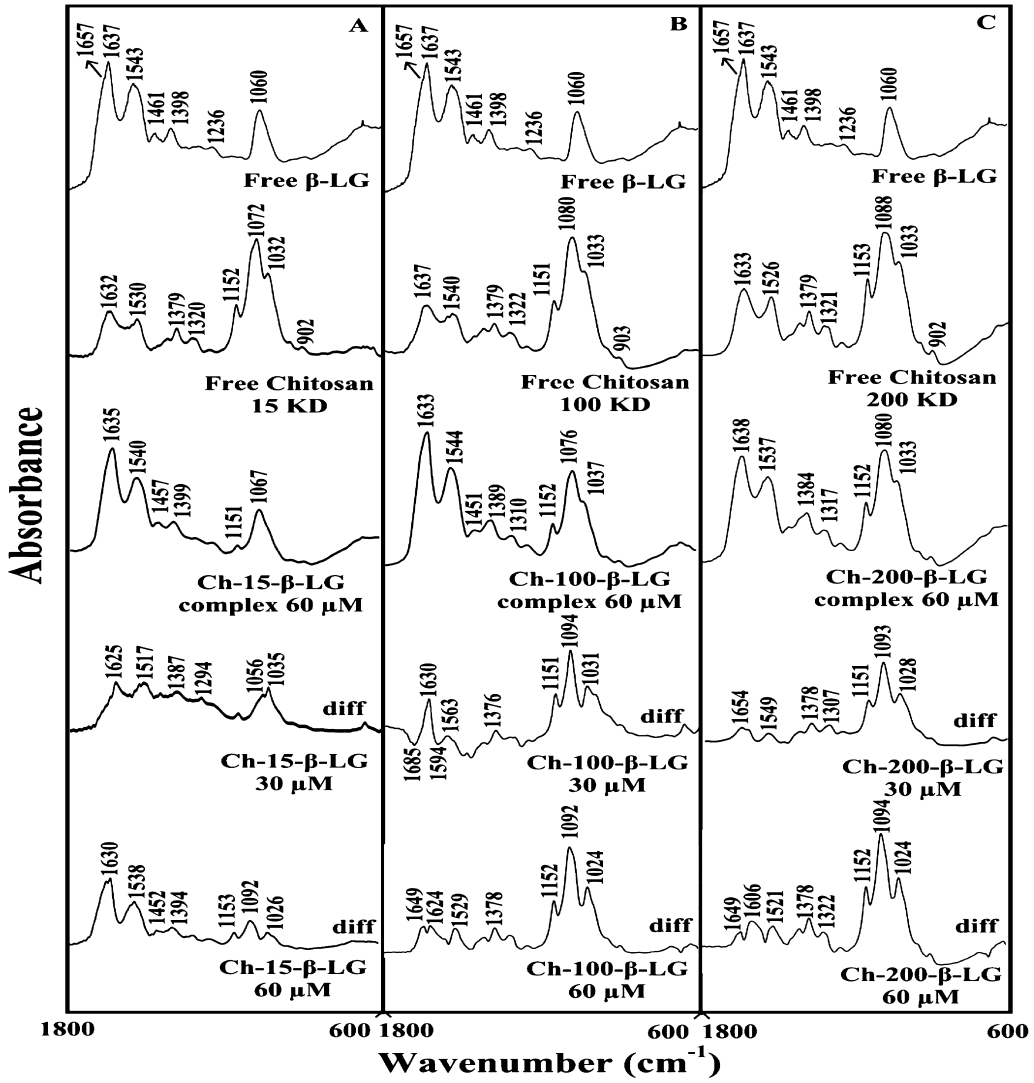

Figure 1. FTIR spectra in the region of 1800−600 cm^{-1} of hydrated films (pH 5−6) for free β-LG (0.25 mM) and its chitosan complexes for (A) chitosan-15, (B) chitosan-100, and (C) chitosan-200 KD with difference spectra (diff.) (bottom two curves) obtained at different polymer concentrations (indicated on the figure).

On the assumption that there are (n) substantive binding sites for quencher (Q) on protein (B), the quenching reaction can be shown as follows:

$$nQ + B \Leftrightarrow Q_nB \tag{1}$$

The binding constant (K_A) can be calculated as

$$K_A = [Q_nB]/[Q]^n[B] \tag{2}$$

where, $[Q]$ and $[B]$ are the quencher and protein concentration, respectively, $[Q_nB]$ is the concentration of non fluorescent fluorophore-quencher complex and $[B_0]$ gives total protein concentration

$$[Q_nB] = [B_0] - [B] \tag{3}$$

$$K_A = ([B_0] - [B])/[Q]^n[B] \tag{4}$$

The fluorescence intensity is proportional to the protein concentration as described

$$[B]/[B_0]\alpha F/F_0 \tag{5}$$

Results from fluorescence measurements can be used to estimate the binding constant of chitosan-protein complex. From eq 4

$$\log[(F_0 - F)/F] = \log K_A + n \log[Q] \tag{6}$$

The accessible fluorophore fraction (f) can be calculated by modified Stern−Volmer equation

$$F_0/(F_0 - F) = 1/fK[Q] + 1/f \tag{7}$$

where F_0 is the initial fluorescence intensity and F is the fluorescence intensities in the presence of quenching agent (or interacting molecule). K is the Stern−Volmer quenching constant, $[Q]$ is the molar concentration of quencher, and f is the fraction of accessible fluorophore to a polar quencher, which indicates the fractional fluorescence contribution of the total emission for an interaction with a hydrophobic quencher.[25,26] The K will be calculated from $F_0/F = K[Q] + 1$.

RESULTS AND DISCUSSION

FTIR Spectra of Chitosan-β-LG Complexes. The chitosan-β-lactoglobulin interactions were characterized by

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infrared spectroscopy and its derivative methods. The spectral shifting and intensity changes for the chitosan and protein amide I bands at 1657 and 1637 cm^{-1} (mainly C═O stretch) and amide II band at 1543 cm^{-1} (C−N stretching coupled with N−H bending modes)[29,39] were examined, upon polymer interaction. The difference spectra [(protein solution + polymer solution) − (protein solution)] were obtained, in order to monitor the intensity variations of these vibrations and the results are shown in Figure 1. Similarly, the infrared self-deconvolution with second derivative resolution enhancement and curve-fitting procedures[29] were used to determine the protein secondary structures in the presence of chitosan (Figure 2 and Table 1).

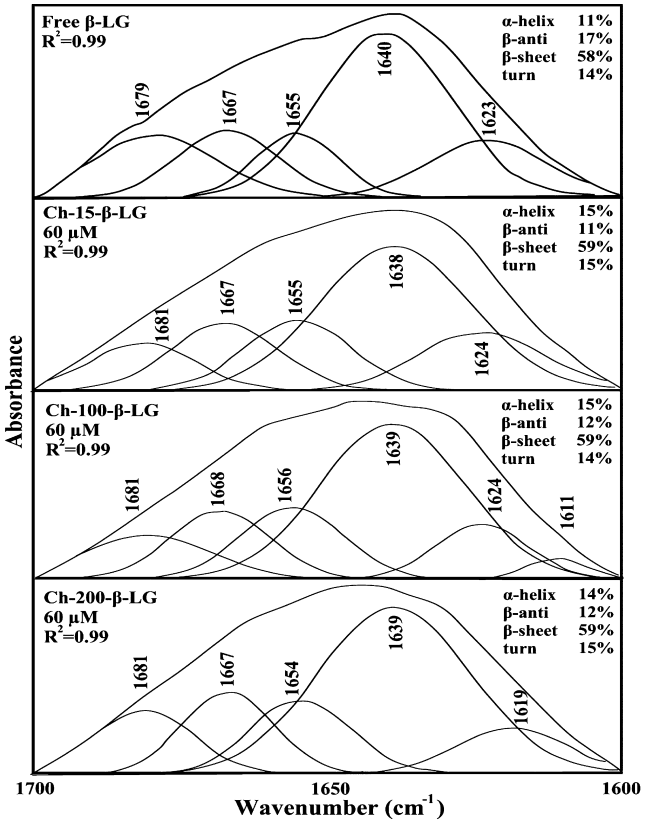

Figure 2. Second derivative resolution enhancement and curve-fitting of the amide I region (1700−1600 cm^{-1}) for free β-LG and its chitosan complexes with 60 μM polymer.

Table 1. Secondary Structure Analysis (Infrared Spectra) for the Free β-LG and Its Polymer Complexes in Hydrated Film at pH 5−6

amide I (cm^{-1}) components	free β-LG (%) 0.25 mM	Ch-15 (%) 60 μM	Ch-100 (%) 60 μM	Ch-200 (%) 60 μM
α-helix (±4) 1654−1660	11	15	15	14
β-sheet (±2) 1614−1637	58	59	59	59
turn (±2) 1670−1678	14	15	14	15
β-antiparallel (±1) 1680−1691	17	11	12	12

At low chitosan concentration (15−30 μM), an increase in the intensity was observed for the protein amide I at 1637 and amide II at 1537 cm^{-1}, in the difference spectra of the drug-β-LG complexes (Figure 1, diffs., 30 μM). The positive features are located at 1625 and 1517 (ch-15-β-LG), at 1630 and 1563 cm^{-1} (ch-100-β-LG), and at 1654 and 1549 cm^{-1} (ch-200-β-LG), in the difference spectra of polymer−protein complexes (Figure 1A−C, diff., 30 μM). These positive features are related to the increase in the intensity of the amide I and amide II bands upon chitosan-β-LG complexation. The increase in the intensity of the amide I and amide II bands is due to polymer bindings to protein C═O, C−N, and N−H groups (hydrophilic interaction). Additional evidence to support drug interaction with C−N and N−H groups comes from the shifting of the protein amide A band at 3300 cm^{-1} (N−H stretching mode) of the free β-LG to 3295−3290 cm^{-1}, upon polymer complexation (spectra not shown).

As polymer concentration increased to 60 μM, further increase in the intensity of the protein amide I and amide II bands was observed with positive features at 1630, 1538 (ch-15-β-LG), at 1649, 1624, and 1529 cm^{-1} (ch-100-β-LG) and at 1649, 1606, and 1521 cm^{-1} (ch-200-β-LG), in the difference spectra of polymer−protein complexes (Figure 1A−C, diff., 60 μM). The major increase in intensity of the protein amide I and amide II bands are due to further polymer−protein complexation. Similarly, The major shifting and intensity variations of the chitosan amide I and amide II bands in the spectra of the polymer−protein complexes are due to the interaction of protein with chitosan NH_2 groups.[40,41]

A quantitative analysis of the protein secondary structure for the free β-LG and its drug complexes in hydrated films has been carried out and the results are shown in Figure 2 and Table 1. The free β-LG has major β-sheet 58% (1640, 1623), α-helix 11% (1655 cm^{-1}), turn 14% (1667 cm^{-1}), and β-antiparallel 17% (1679) (Figure 2 and Table 1). These data are consistent with the spectroscopic studies of β-LG previously reported.[14,15,19] Upon chitosan interaction, no major alterations of protein secondary structure were observed (Table 1).

CD Spectra. CD spectroscopy was also used to analyze the protein conformation in the polymer-β-LG complexes and the results are shown in Figure 3 and Table 2. The CD results exhibit marked similarities with those of the infrared data (Figure 3 and Table 1). β-LG contains major β-sheet structure and minor α-helix, consistent with the literature report.[42] The protein conformational analysis based on CD data showed also no major changes of protein conformation upon chitosan complexation (Figure 3 and Table 2). This is indicative of a minor stabilization of protein secondary structure upon chitosan complexation.

Fluorescence Spectra and Stability of Chitosan-β-LG Complexes. β-lactoglobulin has two tryptophan residues Trp-19, and Trp-61. Trp-19 is located in an apolar environment and contributes to nearly 80% of the total fluorescence, while Trp-61 is partly exposed to aqueous solvent and has only minor contribution (about 20%) to the total tryptophan fluorescence.[11,12] When other molecules interact with β-LG, tryptophan fluorescence may change depending on the impact of such interactions on the protein conformation.[25,26] The decrease of fluorescence intensity of β-LG is monitored at 340 nm for chitosan-β-LG systems (Figure 4 A-B shows representative results for each system). The plot of $F_0/(F_0 - F)$ vs 1/[polymer] (Figure 4A′−C′) show representative plots). Assuming that the observed changes in fluorescence come from the interactions between polymer and protein, the quenching constant can be taken as the binding constant of the complex formation. The K values given here are averages of three-replicate runs for polymer/protein systems, each run involving several different concentrations of chitosan (Figure 4). The

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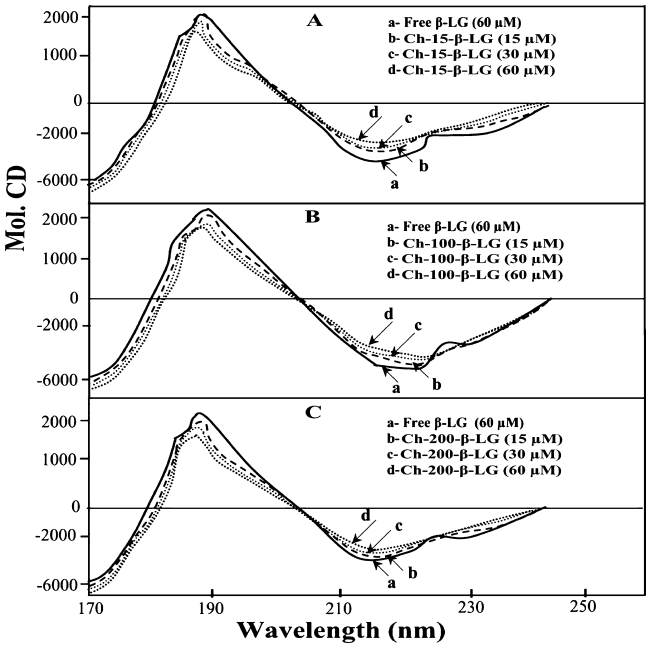

Figure 3. CD spectra of free β-lactoglobulin at 60 μM (a) and complexes with chitosan-15, chitosan-100, and chitosan-200 KD for chitosan-15 (A), chitosan-100 (B), and chitosan-200 KD (C) at different concentrations 15 μM (b), 30 μM (c), and 60 μM (d).

Table 2. Secondary Structure of β-LG and Its Polymer Complexes (pH 5-6) Calculated by CDSSTR Software (CD Spectra)

components conformation	free β-LG (%) 60 μM	Ch-15(%) 60 μM	Ch-100 (%) 60 μM	Ch-200 60 μM
α-helix (±3)	11	13	13	14
β-sheet (±2)	58	57	56	56
turn (±1)	13	12	16	13
random (±2)	18	18	15	17

overall binding constants were $K_{\beta\text{-LG-ch-15}}$ = 4.1 (±0.4) × 10^2 M^{-1}, $K_{\beta\text{-LG-ch-100}}$ = 7.2 (±0.6) × 10^4 M^{-1}, and $K_{\beta\text{-LG-ch-200}}$ = 3.9 (±0.5) × 10^3 M^{-1} (Figure 4A′−C′ and Table 3). Similar binding constants were also reported for several ligand-protein adducts using fluorescence spectroscopic methods.[32−38]

It is worth mentioning that chitosan-15 forms weaker protein complexes than chitosan-100 and chitosan-200 KD (Table 3). Chitosan-15 is smaller than ch-100 and ch-200, and as the polymer size gets larger the increases in overall polymer charges will result in stronger polymer−protein−polymer complexation. However, in the case of ch-200 KD aggregation of polymer occurs at pH near 6, which leads to lesser affinity of the aggregated chitosan toward protein interaction (self-aggregation is less observed for ch-15 and ch-100). Therefore, ch-100 forms more stable complexes than the ch-15 and ch-200 KD (Table 3). The f value calculated from eq 7 represents the mole fraction of the accessible population of fluorophore to quencher. The estimated f values were from 0.15 to 0.50 for these chitosan−protein complexes indicating a large portion of fluorophore was exposed to quencher (Table 3).

The number of chitosan molecules bound per protein (n) is calculated from $\log[(F_0 - F)/F] = \log KS + n \log [\text{chitosan}]$ for the static quenching.[43−46] The n values from the slope of the straight line plot showed 0.9 for ch-15 KD, 0.6 for ch-100 KD, and 1.2 molecules for ch-200 KD that are bound per β-LG

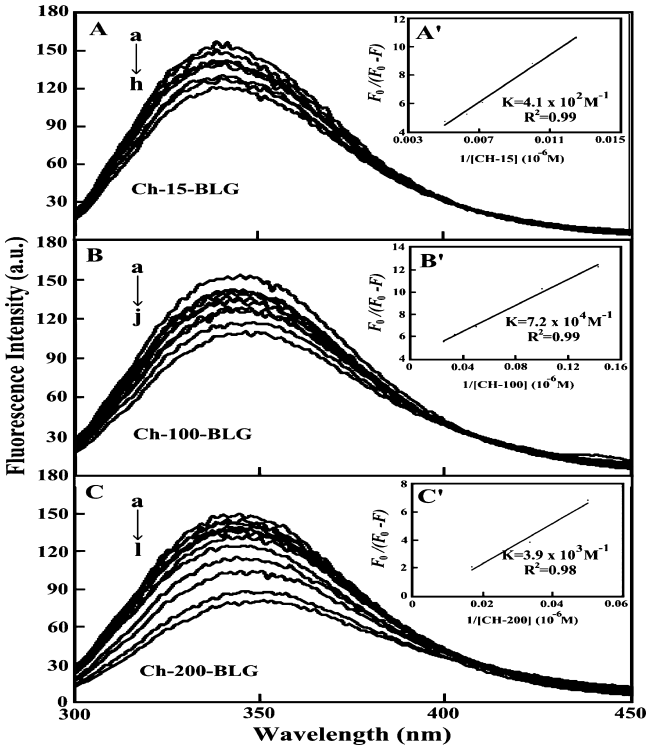

Figure 4. Fluorescence emission spectra of chitosan-β-LG systems in 10 mM acetate buffer pH 5−6 at 25 °C presented for (A) ch-15-LG: (a) free β-LG (10 μM), (b−h) with polymer 30, 40, 80, 100, 140, 160, and 200 μM; (B) ch-100−β-LG: (a) free β-LG (10 μM), (b−j) polymer at 1, 7, 10, 15, 20, 30, 40, 50, and 60 μM; and (C) ch-200−β-LG: (a) free β-LG (10 μM), (b−l) polymer at 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, and 60 μM. Inset: $F_0/(F_0 - F)$ vs 1/[polymer for A′ (ch-15-β-LG), B′ (ch-100-β-LG), and C′ (ch-200-β-LG).

Table 3. Binding Parameters for Chitosan-β-LG Complexes

complexes	K (M^{-1})	n	K_q (M^{-1} S^{1-})	f
Ch-15-LG	4.1 × 10^2	0.9	1.2 × 10^{12}	0.15
Ch-100-LG	7.2 × 10^4	0.6	4.3 × 10^{12}	0.25
Ch-200-LG	3.9 × 10^3	1.6	1.6 × 10^{13}	0.50

molecule (Figure 5A−C and Table 3). These data indicate some degree of cooperativity for polymer−protein complexes.

In order to verify the presence of static or dynamic quenching in drug-β-LG complexes we have plotted F_0/F against Q and the results are shown in Figure.6. The plot of F_0/F versus Q is straight line for chitosan-β-LG adducts indicating that the quenching is mainly static in these polymer−protein complexes (Figure 6). The K_Q was estimated according to the Stern−Volmer equation

$$F_0/F = 1 + k_Q t_0[Q] = 1 + K_{sv}[Q] \quad (8)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the quencher concentration and K_{sv} is the Stern−Volmer quenching constant,[38] which can be written as $K_{sv} = k_Q t_0$; where k_Q is the bimolecular quenching rate constant and t_0 is the lifetime of the fluorophore in the absence of quencher 1.2 ns for free β-LG at neutral pH.[11] The quenching constants (K_Q) are 1.2 × 10^{12} $M^{-1}s^{-1}$ for ch-15-β-LG, 4.3 × 10^{12} M^{-1} s^{-1} for ch-100-β-LG, and 1.6 × 10^{13} M^{-1} s^{-1} for ch-200-β-LG (Figure 6A-C and Table 3). Since these values are much greater than the maximum collisional quenching constant (2.0 × 10^{10} M^{-1}/s), thus it was believed

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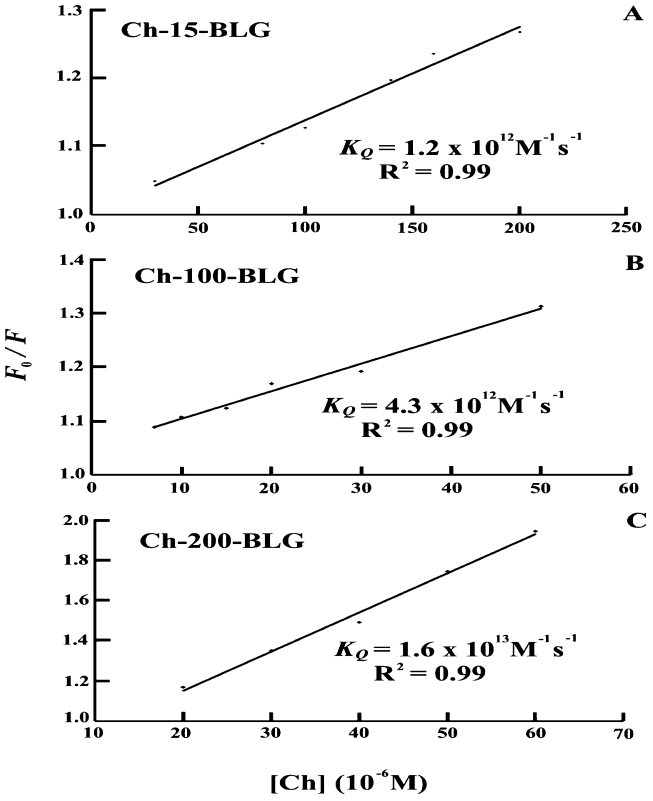

Figure 5. Plot of $\log(F_0 - F)/F$ as a function of log(chitosan concentration).

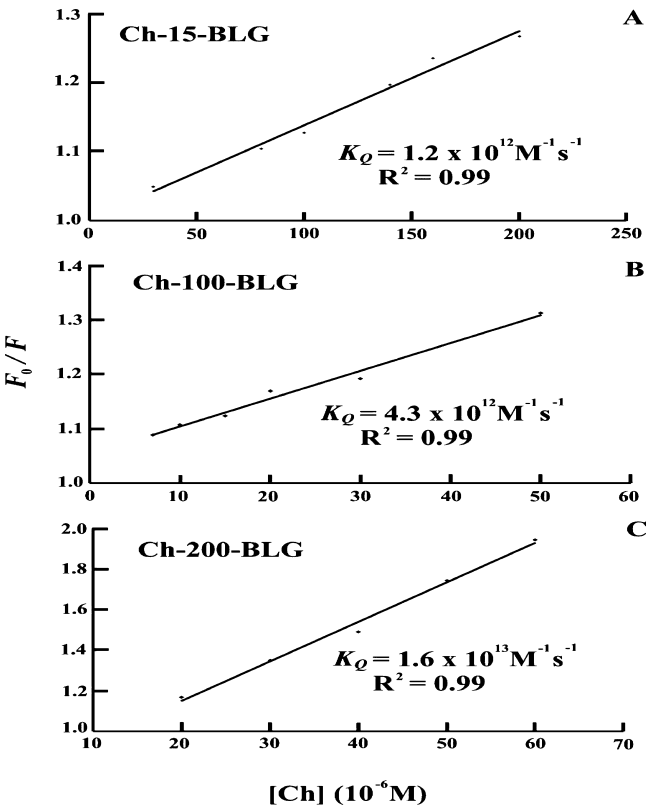

Figure 6. Stern–Volmer plots of fluorescence quenching constant (K_Q) for the β-LG and its polymer complexes at different chitosan concentrations (A) ch-15- β-LG, (B) ch-100- β-LG, and (C) ch-200-β-LG.

that static quenching is dominant in these polymer-β-LG complexes.[11]

CONCLUSIONS

Our study showed that chitosan-15 forms weaker complexes with protein than chitosan-100 and chitosan-200 KD. As chitosan size get larger protein self-aggregation occurs, which induces a major effect on polymer–protein interaction. Both hydrophobic and hydrophilic interactions are observed in the polymer–protein complexation. Chitosan binding stabilizes the protein secondary structure. Chitosan 100 is a stronger protein carrier than those of chitosan-15 and chitosan-200 KD

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

β-LG, beta-lactoglobulin; ch, chitosan; FTIR, Fourier transform infrared; CD, circular dichroism

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