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Synthesis, properties, and interaction with tween 80 in self-assembled beta-lactoglobulin/chondroitin sulfate nanoparticles

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1. Background-Aim

Background

Beta-Lactoglobulin (β-LG)

- Globular, lipocalin protein found in most mammal milks, except for human breast milk (lack of β-LG homologue).
- Capable of binding various hydrophobic molecules and ligands (antioxidants and vitamins) and increase their transport in biological systems.
- β-LG-based NPs produced as carriers of caffeine and as stabilizers and enhancers in insulin nanodelivery.

Chondroitin Sulfate (CS)

- Negatively charged polysaccharide of the family of sulfated glycosaminoglycans (GAGs).
- Important dietary supplement for therapy of osteoarthritis.
- Acquired from bovine, porcine or marine cartilage, where it acts as a structural component forming the side chains of aggrecan.

Tween 80 (T80)

- Non-ionic surfactant and emulsifier, used in vitamin D delivery, anticancer drug delivery and in skincare formulations.
- Cheap compound with low toxicity levels.
- Ability of protein stabilization against thermal aggregation, denaturation and surface adsorption.

Study aim

- Electrostatic complexation of β-LG and CS in acidic conditions to prepare well-defined biocompatible NPs.
- Stabilization of NPs by thermal treatment for aggregation of β-LG inside the complexes.
- Test in solutions of added salt and biological fluids for potential biocompatible delivery nanosystems.

2. Materials & Methods (I)

Materials and sample preparation

- Bovine β-LG and Tween 80 were purchased from Sigma-Aldrich, while porcine CS in the form of sodium salt (Na-CS) was purchased from Bioberica. All materials used without additional treatment.
- Stock solutions were prepared under constant stirring at concentrations of 1 mg/mL at pH 4 and kept at 4 ^{o}C for 20-24h to reach equilibrium.
- Solutions of β-LG/CS/T80 NPs (total volume at 1 mL) were prepared by mixing-stock solutions of β-LG, CS, T80 and water, while preserving the final concentration of β-LG at 0.1 mg/mL (unless stated differently).
- Order of addition: Water, CS, β-LG and, T80.
- Solutions of NaOH and HCl (1M each) were used to alter the pH of 4 to neutral (pH=7) or to strongly acidic (pH=1.5).
- Thermal treatment (TT) was performed according to thermal treatment protocol of 85 ^{o}C for 50 minutes at pH 4. Samples were thermally treated after their formation was achieved at pH 4.
- For transmission electron microscopy (TEM) and circular dichroism (CD) experiments, the samples were prepared at a β-LG concentration of 0.2 mg/mL to obtain a satisfactory signal.

2. Materials & Methods (II)

Experimental techniques

- **Static and Dynamic Light Scattering (SLS and DLS):** Light scattering measurements were performed by employing a classic ALV system (ALV GmbH) executing an analysis via an ALV-CG-3 goniometer and an ALV-5000/EPP multi-tau digital correlator with a He-Ne laser (λ=632.8 nm).
- **Fourier-Transform Infrared Spectroscopy (FTIR):** Measurements were performed using a Bruker Equinox 55 device with an attenuated total reflectance (ATR) diamond accessory.
- **Transmission Electron Microscopy (TEM):** TEM experiments were conducted utilizing a JEOL JEM-2100 device at a voltage of 200 keV, while the TEM images were obtained using an Erlangshen CCD camera.
- **Circular Dichroism (CD):** A CD Jasco J-815 model spectrometer was used to perform CD measurements by placing the sample solutions into quartz cells of 1 mm thickness. The quartz cells were carefully cleaned using Milli-Q water, ethanol and nitrogen gas.

Size distributions and Guinier plots of β-LG/CS/T80 NPs at different pH before and after TT

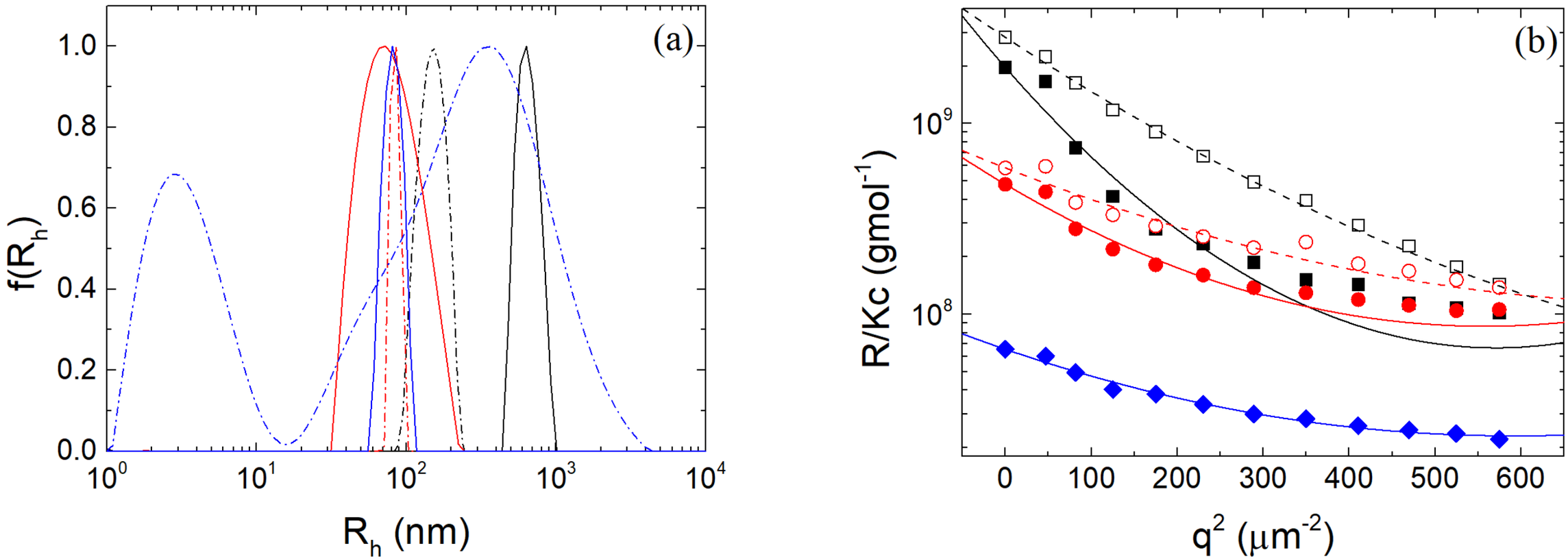

Figure 1. (**a**) CONTIN analysis at 90º and (**b**) Guinier plots of β-LG/CS complexes for 50% T80/β-LG mass ratio at pH 1.5 (black), 4 (red) and 7 (blue), before (dashed-dotted/dashed lines) and after (solid lines) TT (T80 added before TT).

Morphology and structure of β-LG/CS and β-LG/CS/T80 NPs before and after TT

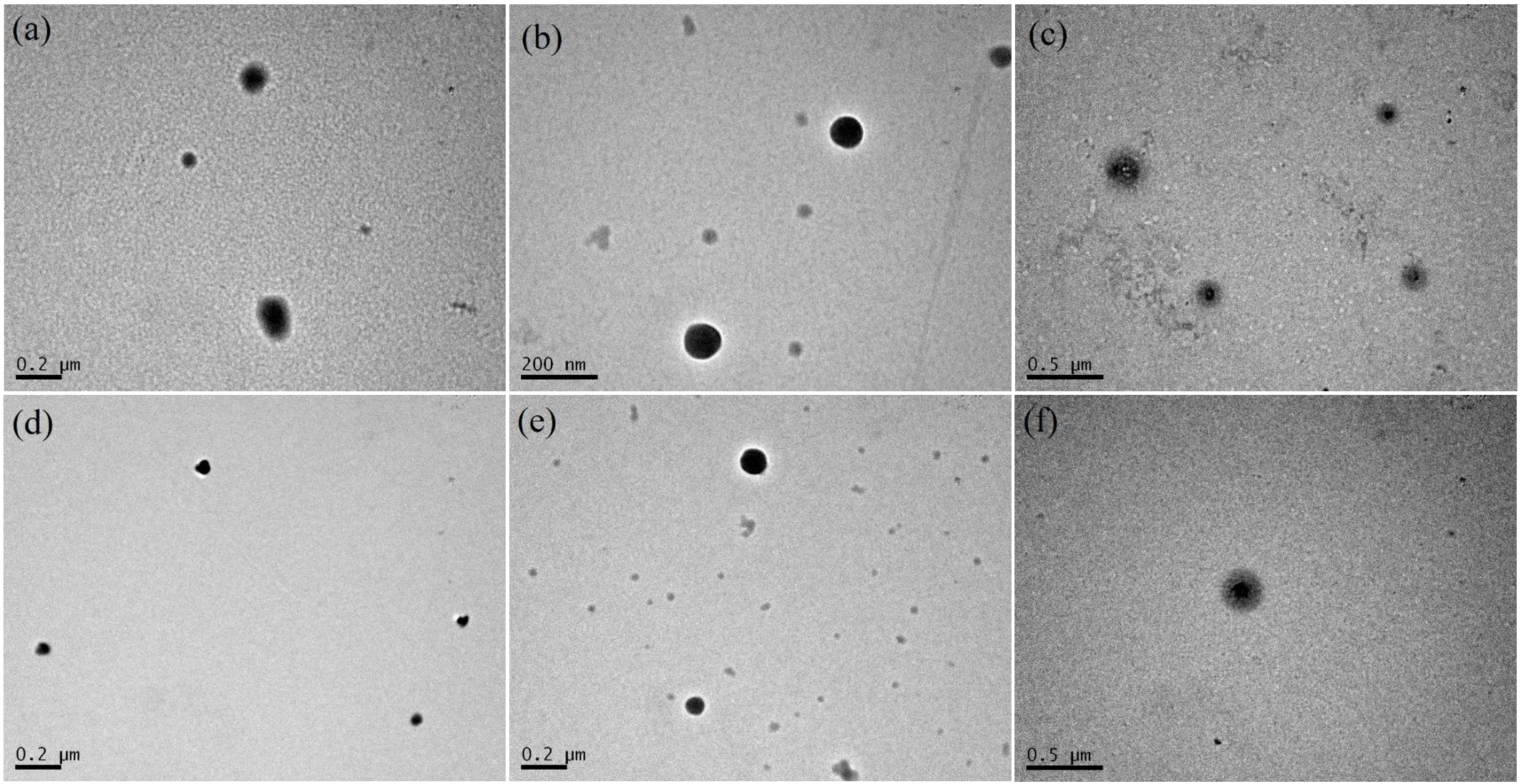

Figure 2. TEM images from β-LG/CS NPs with r_m 0.4 at 0.2 mg/mL β-LG: (**a**) before TT at pH 4; (**b**) after TT at pH 4; (**c**) after TT at pH 7. TEM images from β-LG/CS/T80 NPs at 0.2 mg/mL β-LG: (**d**) before TT at pH 4; (**e**) after TT at pH 4; (**f**) after TT at pH 7.

3. Results (III)

Secondary structure of β-LG inside the β-LG/CS and β-LG/CS/T80 NPs via FTIR deconvolution and CD

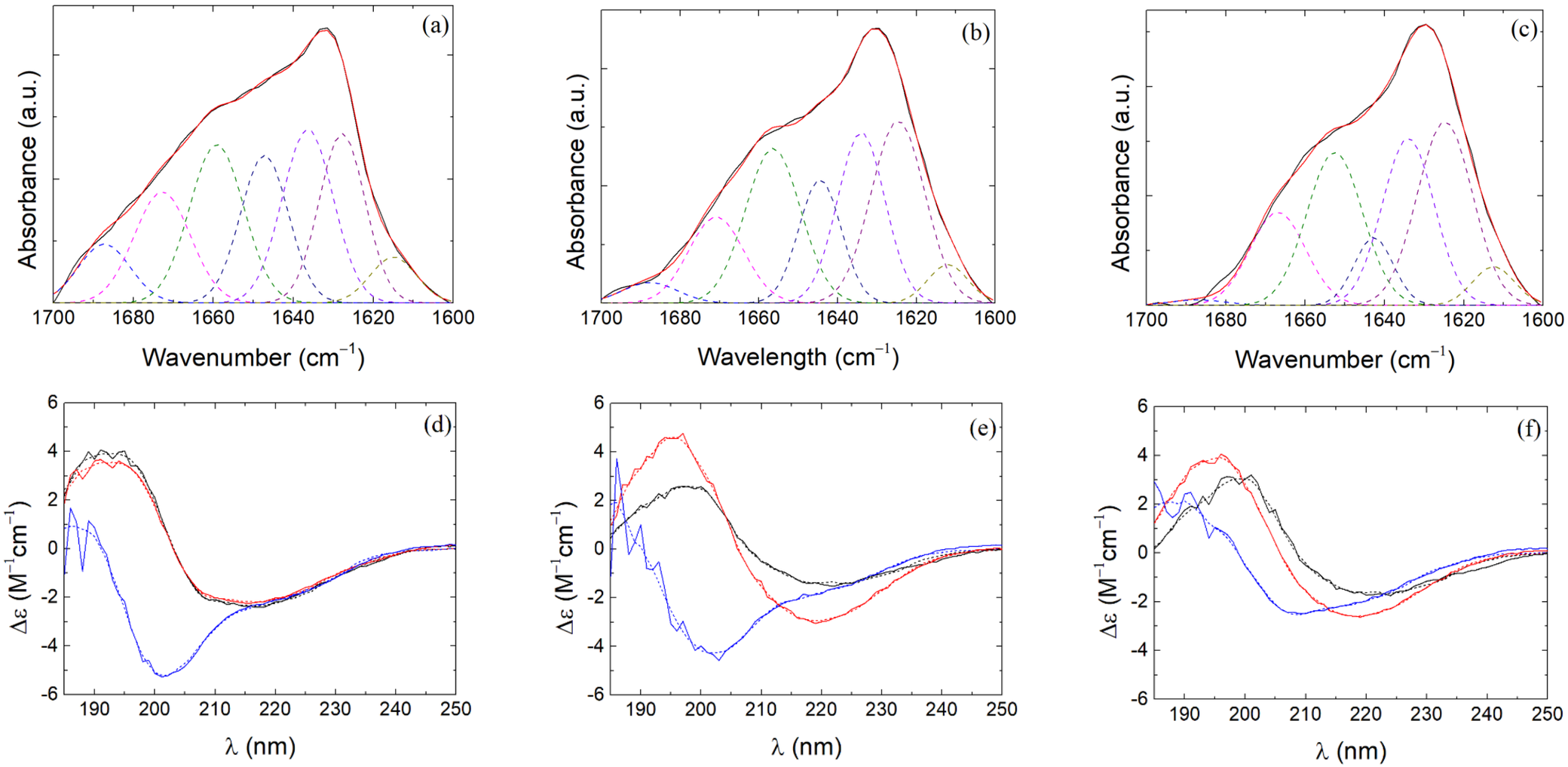

Figure 3. Deconvolution of the signal in the amide I region at pH 4 from: (**a**) pure β-LG before TT; (**b**) β-LG/CS NPs after TT; (**c**) β-LG/CS/T80 NPs after TT. Experimental (solid lines) and fitting profiles (short dash lines) of molar ellipticity of (**d**) pure β-LG, (**e**) β-LG/CS NPs and (**f**) β-LG/CS/T80 NPs before (black) and after (red) TT at pH 4 and after TT (blue) at pH 7.

Table 1. Secondary structure of β-LG determined from ATR-FTIR data. β-LG at 0.1 mg/mL. In β-LG/CS/T80 complexes, T80 at 50% T80/β-LG mass ratio and at pH 4 (r_m = 0.4) (error in estimation of percentages is approximately ±1.0%).

Assignment	B-Sheet/B-Turn	A-Helix	Random Coil	Intramolecular β-Sheet	Others
Wavenumbers (cm^{-1})	1685–1663	1655–1650	1648–1644	1632–1621	1639–1635 1616–1600
Pure β-LG	21.3%	19.6%	16.2%	17.8%	25.1%
β-LG/CS (NoTT)	15.4%	22.0%	13.9%	20.3%	28.5%
β-LG/CS (TT)	15.1%	21.8%	13.3%	25.0%	24.7%
β-LG/CS/T80 (NoTT)	18.5%	22.2%	14.1%	19.7%	25.5%
β-LG/CS/T80 (TT)	14.8%	23.0%	7.1%	27.3%	27.8%

Table 2. Secondary structure estimated by CD. Content of pure β-LG and β-LG in β-LG/CS and β-LG/CS/T80 NPs before and after TT at pH 4 and after TT at pH 7 (error in estimation of percentages is approximately ±1.0%).

Pure β-LG	A-Helix	B-Sheet	B-Turn	Irregular/Others
Untreated/pH 4	14.6%	35.0%	10.3%	40.1%
Treated/pH 4	13.1%	35.4%	10.9%	40.7%
Treated/pH 7	13.2%	22.3%	14.6%	49.8%
β-LG/CS NPs	**α-helix**	**β-sheet**	**β-turn**	**Irregular/Others**
Untreated/pH 4	3.1%	36.4%	15.1%	45.3%
Treated/pH 4	9.8%	30.0%	10.7%	49.6%
Treated/pH 7	9.2%	28.0%	17.5%	45.3%
β-LG/CS/T80 NPs	**α-helix**	**β-sheet**	**β-turn**	**Irregular/Others**
Untreated/pH 4	5.9%	37.4%	13.5%	43.2%
Treated/pH 4	10.4%	33.6%	12.4%	43.4%
Treated/pH 7	16.1%	26.7%	13.5%	43.7%

4. Conclusions

- The electrostatic complexation between β-LG and the negatively charged CS was fruitful and the formation of well-defined complexes was achieved.
- Thermal stabilization against disintegration at neutral pH was achieved. NP aggregation was present at pH 1.5 before and after thermal treatment.
- Thermal stabilization in the presence of T80 resulted to narrower size distributions upon the change for acidic to neutral pH. Microgel morphology was preserved.
- Morphology from TEM results corresponded to the shape factor extracted from LS results. NPs at pH 7 showed a core-shell morphology.
- Deconvolution of the FTIR signal exhibited variances from the CD results in the determination of the secondary structure of β-LG, attributed to different methods of measuring between the two techniques.
- Colloidal properties of the NPs showed remarkable stability upon the span of one month.
- Potential suitability for encapsulation and delivery of mainly hydrophobic compounds for food science and pharmaceutical applications.

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(Pispas, I.; Spiliopoulos, N.; Papagiannopoulos, A. Biocompatible Preparation of Beta-Lactoglobulin/Chondroitin Sulfate Carrier Nanoparticles and Modification of Their Colloidal and Hydropathic Properties by Tween 80. *Polymers* **2024**, *16*, 1995.)

5. References

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