J Incl Phenom Macrocycl Chem (2012) 72:241–246
DOI 10.1007/s10847-011-9961-2

SHORT COMMUNICATION

Protein stabilization through supramolecular host–guest interactions with cyclodextrin-modified nanoparticles

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Received: 19 January 2011 / Accepted: 11 April 2011 / Published online: 5 May 2011

Abstract This study demonstrates the protein stabilization of gelatin through supramolecular interactions of silica nanoparticles and the influence on the point of denaturation. The phenomenon was studied in diluted solutions by dynamic light scattering, viscosity measurements, and differential scanning calorimetry. Native gelatin is stabilized by cyclodextrin functionalized SiO_2 nanoparticles. After heating, increased supramolecular interactions of the nanoparticles with the denaturated gelatin coils are observed by progressive agglomeration. The described observation also resulted in a melting temperature shift from 30 °C, for native gelatin, to about 47 °C for the gelatin/CD-SiO_2, which indicates the supramolecular stabilization of the gelatin chain structure. It was found that the gelatin is supramolecularly immobilized on the nanoparticle up to a certain temperature through complexation by cyclodextrin. The described results, also confirmed by DSC and viscosity measurements, show the prospect of using cyclodextrin-modified surfaces for the immobilization of the proteins.

Keywords Denaturation · Gelatin · Melting point · Nanoparticles · Supramolecular interactions

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Introduction

Recently, much work was dedicated to the preparation of nanoscale hybrid systems containing biological macromolecules [1–3]. These bio-compatible nanodevices have multiple relevant applications in sensing and imaging nanotechnology, in drug delivery systems, and are of high interest for future nanorobots [4, 5]. In general, colloidal structures have been coated with protein monolayers through covalent immobilization methods. However, to the best of our knowledge, no attention has been paid to the use of supramolecular interactions for preparing multilayer protein nanomaterials on gelatin base.

In particular, the construction of silicon oxide/polymer hybrid systems attracted much interest due to their applications ranging from drug release to catalysis [6–8]. Most of these hybrid systems are designed as polymer-core/oxide-shell or oxide-core/polymer shells; bi-continuous structures were also described [9–11]. Several gelatin/silica hybrid systems have already been prepared [12]; however, the synthesis of supramolecular interacting gelatin/silica systems has never been reported. Recently, the gelatin–silicate interactions have been studied by Coradin et al., indicating decreased stability of gelatin gels by inducing gelatin depletion in solution through the addition of silica [13]. Taking this into account, the influence of supramolecular interactions on the gelatin denaturation was studied in highly diluted solution. This method to study the size, shape, and denaturation behavior of a protein is frequently used in bioscience to get a complete understanding of protein conformation and its role in protein folding [14–16]. The dimensions of a protein in the different unfolded conformations give important information about the structural changes of a protein during denaturation. Dynamic light scattering (DLS) and the resulting

hydrodynamic radius (R_h), although less direct, can easily be measured and give useful information about the agglomeration behavior and structural changes of a protein. The instantaneous intensity fluctuation resulting from the Brownian motion of the particles in a small volume is measured by DLS. This fluctuation is then analyzed to get the diffusion coefficient of the particles. R_h is then determined through the Stoke's–Einstein relationship. The obtained R_h represents the size of a spherical particle. Since most proteins are not spherical, the hydrodynamic radii vary in shape and size. Additionally, bound water molecules influence diffusion of the protein, and therefore also the R_h of the protein. Since proteins undergo significant changes in the hydrodynamic radii, observation of these changes in R_h may be a good indicator of protein unfolding. Special interest was also dedicated towards changes in the agglomeration behavior in the diluted systems with respect to temperature. A study of the protein denaturation of fish gelatin by DLS is presented in this paper.

The unfolding, and therefore denaturation, of a protein can also easily be studied by viscosimetry, since it is very sensitive to the overall dimensions of the protein [17]. Residual structure in the gelatin protein beyond the helix–coil transition is denatured to a random coil with free rotation. This phenomenon can be monitored by studying the relationship between complex viscosity and increasing temperature. Beyond the denaturation point, an apparent change in the slope of the viscosity is observed. Viscosimetric behavior of fish gelatin was analyzed to support the observations from DLS measurements.

Gelatin is the hydrolysis product of collagen, in this case fish gelatin was used to study protein–cyclodextrin interactions. During hydrolysis, the triple helical structure of collagen is broken down due to the rupturing of covalent bonds. Cooling produces cross-links or junction zones by the partial formation of ordered triple helices as well as disordered regions. The properties of gelatin, molecular weight, number of each kind of amino acid residues, and number of polypeptide chains depend on the position of the breaks. The typical helix–coil transition for fish gelatin is observed at a low temperature of about 8 °C. Above this point, the gelatin exhibits a random coil structure still including folded parts. When the hydrophobic moieties of the gelatin are exposed due to the denaturation process, severe agglomeration occurs. This agglomeration can easily be monitored by temperature trend measurement with DLS.

The present paper therefore concentrates on the temperature-dependent structural changes of supramolecular functionalized gelatin and silica nanoparticles studied by DLS. Viscosity measurements and differential scanning calorimetry (DSC) measurements were additionally employed to verify the results obtained by DLS.

Experimental part

Synthesis of thiol-modified SiO_2 nanoparticles

Cyclodextrin modified nanoparticles were prepared according to literature [17]. Briefly, TEOS (5.500 g, 26.4 mmol) was added to an aqueous solution of ammonium hydroxide (25%) and ethanol (30 mL). The mixture was stirred for 30 min and MPTMS (1.000 g, 5.1 mmol), dissolved in the above-described basic hydrolysis solution, was added. The mixture was stirred at 50 °C for another 2 h and kept at room temperature overnight. The SH-terminated particles were isolated by centrifugation and washed three times with ethanol.

IR (ATR): ν = 2935(w), 2859(w), 2560(s, SH), 1256–924(w, Si–O–Si); Elemental analysis: C: 8.65%; H: 2.28%.

Preparation of CD-functionalized nanoparticles

MPTMS-modified SiO_2 particles (0.521 g) and mono-6-*para*-toluenesulfonyl-β-cyclodextrin (5.046 g, 3.85 mmol) were added to an aqueous solution of potassium hydroxide (pH 11) and the reaction was performed under microwave conditions (100 W, 20 min). Unreacted mono-6-*para*-toluenesulfonyl-β-cyclodextrin was filtrated to obtain a clear, slightly yellow solution. The product was precipitated in acetone, decanted and dried under vacuum (12 mbar, 40 °C). IR (ATR): ν = 3600–3150(w), 2920(w), 1446(s), 1363(w), 1125(w), 1082(w, Si–O–Si), 1026(s), 1011(s, Si–C); Elemental analysis: C: 22.07%; H: 3.48%.

Preparation of samples for dynamic light scattering, differential scanning calorimetry and viscosity measurements

Dynamic light scattering measurements were performed with 1 mg/mL solutions of gelatin, gelatin/SiO_2, and gelatin/CD-SiO_2. Viscosity measurements were conducted on these polymer/particle combinations bearing concentrations of 5 wt%. DSC measurements were performed on the freeze dried solutions of gelatin, gelatin/SiO_2, and gelatin/CD-SiO_2. A solution of the gelatin 0.5 mg/mL in Millipore water was prepared to record a circular dichroism spectrum. In general, solutions bearing cyclodextrin modified nanoparticles and gelatin or bearing SiO_2 with gelatin were stirred for 24 h to ensure equilibration and afterwards subjected to the respective measurements.

Instrumentation

Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet 5 SXB FTIR spectrometer equipped with an ATR unit. The measurements were performed in the range

of 4000–300 cm^{-1} at room temperature. DLS experiments were carried out with a Malvern HPPS-ET. The particle size distribution was derived from a deconvolution of the measured intensity autocorrelation function of the sample by the general purpose mode algorithm included in the DTS software. All solutions had final polymer concentrations of 2 wt% and particle concentrations of 5 wt%. DSC measurements were performed on a Mettler DSC-30 instrument in a temperature range of −40 to 150 °C at the heating rate of 10 °C min^{-1}. For calibration, standard tin, indium, and zinc samples were used. The Tg values are reported as the average of three measurements using the midpoint method. The structure of the fish gelatin was further characterized by circular dichroism on a JASCO J-600 polarimeter.

Results and discussion

Preparation and analysis of cyclodextrin modified SiO_2 nanoparticles

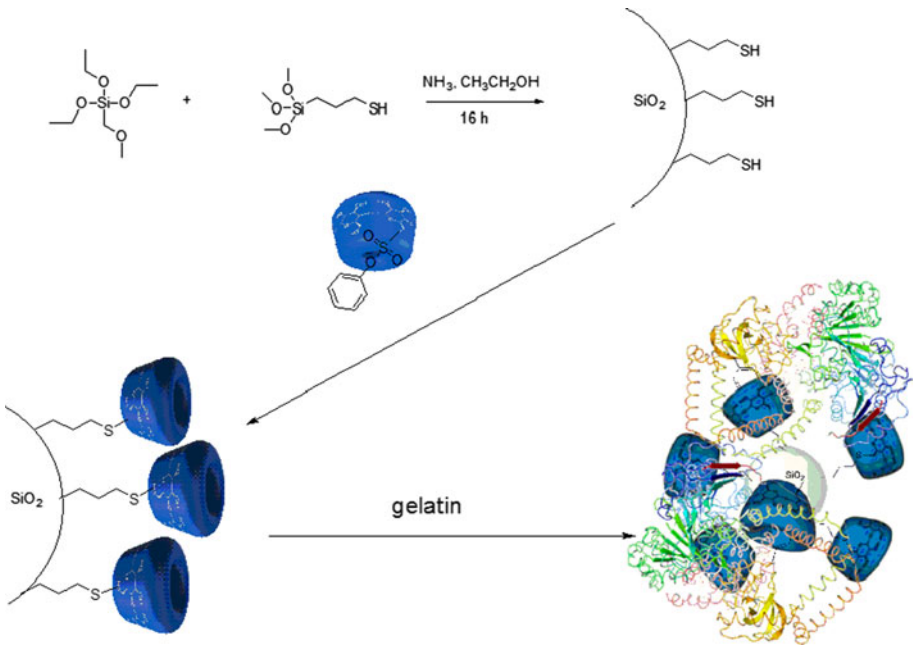
Schematic representation of the preparation of cyclodextrin modified structures is given in Scheme 1.

The preparation of cyclodextrin-functionalized nanoparticles was accomplished in a two-step procedures shown in Scheme 1. Silica particles with silanol surface groups were synthesized according to a modified Stöber process [18]. The particles were then grafted with (3-mercaptopropyl)trimethoxysilane (MPTMS), which resulted in thiol-modified nanoparticles. β-Cyclodextrin functionalized particles were prepared via a base-catalyzed reaction of the thiol-particles with mono-6-*para*-toluenesulfonyl-β-cyclodextrin in basic solution (pH 11) under microwave-assisted reaction conditions. The successful reaction of TEOS with 3-mercaptopropyltrimethoxysilane was qualitatively examined by FT-IR spectroscopy. The spectrum indicated a strong peak at 2553 cm^{-1}, representing the SH stretch. The successful reaction of MPTMS-SiO_2 with mono-6-*para*-toluenesulfonyl-β-cyclodextrin was also shown by FT-IR spectroscopy, which indicated the absence of the SH stretch. The hydrodynamic diameter of the CD-modified nanoparticles was determined by DLS to be 30 nm. The isolated particles were later combined with gelatin in an aqueous solution to study CD–protein interactions.

DLS studies on the temperature dependent denaturation of gelatin and the influence of supramolecular effects

Using the cyclodextrin modified silica nanoparticles, the supramolecular interactions and their influence on the denaturation of fish gelatin were studied. In order to characterize the conformation of the gelatin, circular dichroism measurements were carried out. The CD spectrum shown in Fig. 1 confirms the existence of the random coil structure of the gelatins with one negative minimum at $\lambda = 226$ nm in both cases.

Supramolecular self-assembled nanostructures comprising of cyclodextrin modified silica nanoparticles and gelatin were prepared in aqueous solution with concentrations of 2.5×10^{-5} mol/L. To study the temperature dependent agglomeration behavior and the melting point of gelatin, DLS was applied. DLS is frequently used as a convenient tool to detect the onset of protein denaturation and



Scheme 1 Preparation of cyclodextrin modified nanoparticles interacting with gelatin

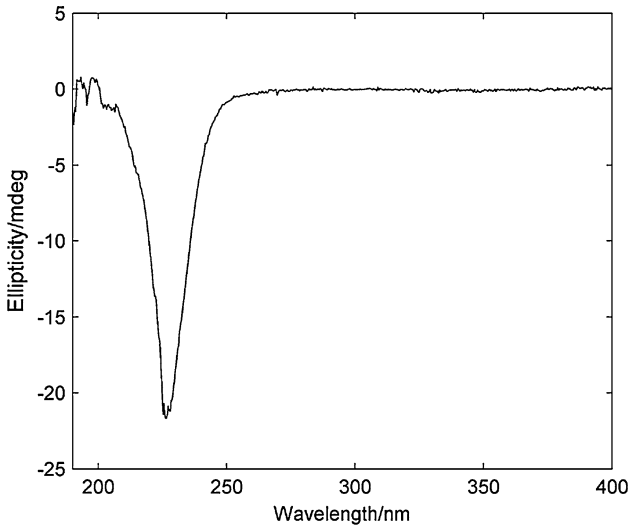


Fig. 1 Circular dichroism (CD) spectra of gelatin (0.5 mg × mL^{-1})

agglomeration [14–16]. The point where a protein starts to denaturate upon heating is defined as the melting point. For gelatin, the melting point was previously determined to be about 30 °C [19]. When gelatin is heated above its melting point, unfolding and denaturation of the gelatin occurs, and the exposed hydrophobic chains cause severe agglomeration (Fig. 2).

To study the influence of the CD-nanoparticles on the denaturation process of gelatin, aqueous solutions of the combination of these components were prepared. As apparent from the plot in Fig. 3, the melting temperature of the gelatin is shifted to higher values from 30 to 47 °C. This shift can be explained by the stabilization of the gelatin structure through the complexation of the cyclodextrin modified nanoparticles by the exterior phenyl groups of the gelatin chains. The particles are easily capable of stabilizing the structure of gelatin, since they do not undergo conformational changes during the heating process. Additionally, the slope of the curve beyond the melting-point differs from the above-described temperature behavior of native gelatin. Before the denaturation takes place, most of the spatial ordered hydrophobic phenylalanine groups along the protein chain are not easily accessible to the relatively bulky nanoparticles covered with CD's. However, after denaturation of the polymer, the hydrophobic moieties are exposed and therefore allow for complexation with cyclodextrin rings located on the surface of the nanoparticles (Fig. 3), which results in a progressive shift from a weakly- to the fully-agglomerated state. In the absence of the modified nanoparticles, a sharp transition of the two stages is observed. Additionally, the agglomeration is intensified for the CD-nanoparticle/gelatin system, as indicated by the higher value of 160 nm of the *Z*-average.

Viscosity and DSC measurements

To further confirm the results found by DLS, viscosity measurements of the gelatin solutions were conducted. Figure 4 shows the temperature dependent viscosity of gelatin and the hybrid system of gelatin/bare SiO_2. As shown in the first curve, a significant change in the slope at 32 °C takes place. This change can be explained by the denaturation of the gelatin structure at about 32 °C, which was already found by DLS measurements (see Fig. 3). The second curve for the hybrid system of gelatin/CD-SiO_2, shows a shift in the melting temperature to 44 °C, also supporting the DLS results (see Fig. 3).

To study the influence of supramolecular interactions in the dried materials the samples were freeze dried and the resulting powders analyzed by DSC. As evaluated from the DSC curves, gelatin exhibits a melting point of 62 °C in the dry state, while the mixture of gelatin and pure SiO_2 shows about the same values (Fig. 5).

Thus it was concluded that the unfunctionalized nanoparticles with analogous size as the modified particles do not have any influence on the denaturation point of gelatin. In comparison, the mixture of CD-SiO_2 and gelatin (see Fig. 6) shows an increased melting point of +12 °C up to 75 °C in solid state. This increase can again be explained by the supramolecular stabilization of the gelatin structure through the modified nanoparticles, even in the condensed phase.

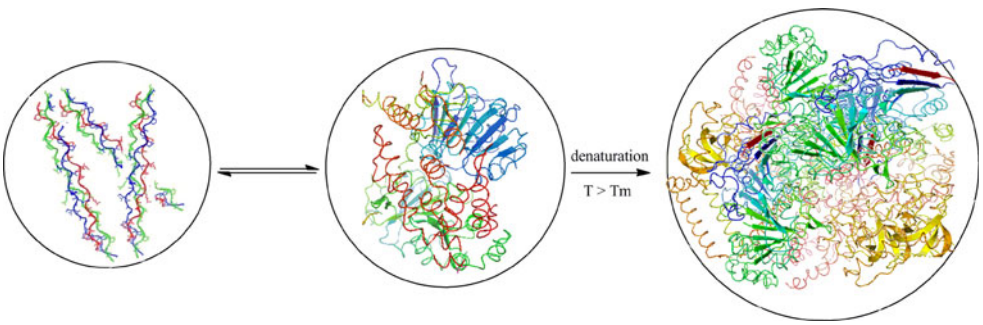


Fig. 2 Schematic representation of gelatin denaturation and agglomeration above the melting point. The larger circumference of the rightmost particle indicates the increase in hydrodynamic diameter

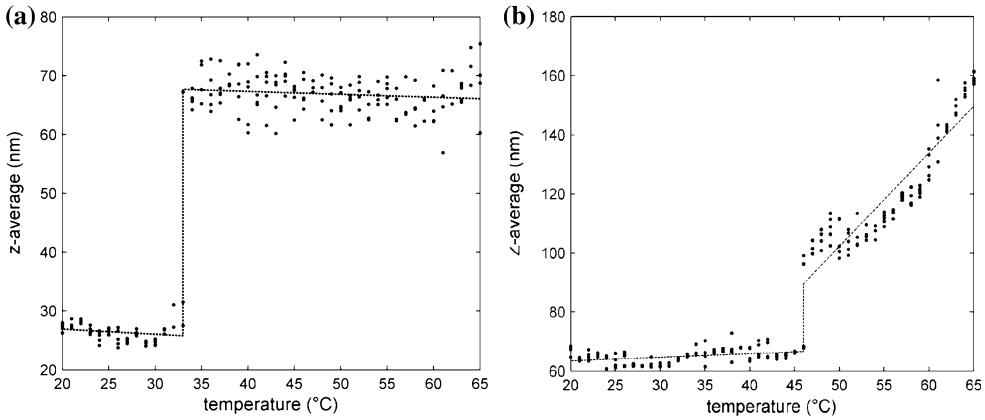


Fig. 3 Temperature trend curve for **a** gelatin/bare SiO_2 and **b** CD-SiO_2/gelatin complex

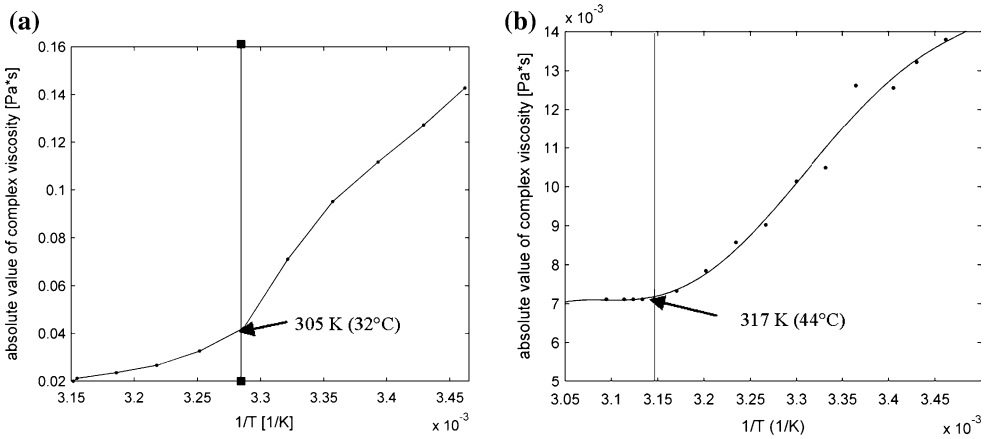


Fig. 4 Temperature dependent viscosity of gelatin/bare SiO_2 (*left*) and gelatin/CD-SiO_2 (*right*)

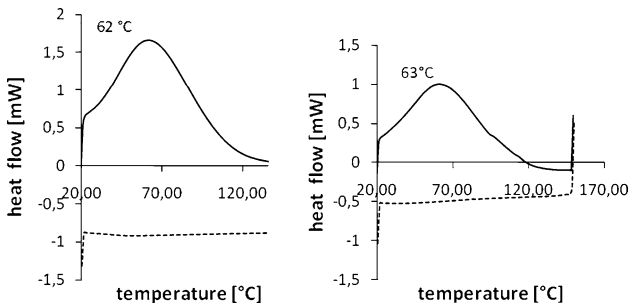


Fig. 5 Heating (*solid line*) and cooling (*dashed line*) curves of gelatin and gelatin/SiO_2 measured by differential scanning calorimetry

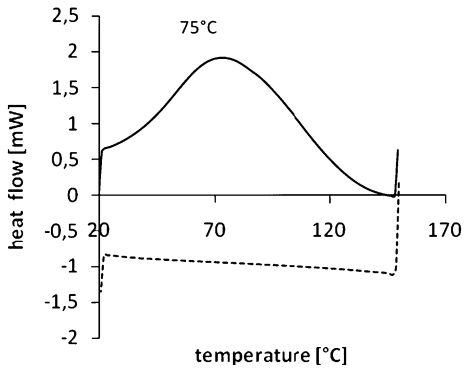


Fig. 6 Heating (*solid line*) and cooling (*dashed line*) curves of gelatin and CD-SiO_2 measured by differential scanning calorimetry

Conclusion

In summary, the results presented here demonstrate that supramolecular interactions of CD-modified SiO_2-nanoparticles with native gelatin have a significant influence on the chain stability of gelatin. The key phenomenon of an increase in the melting point for the hybrid composites was observed in all cases by DLS, DSC, and viscosity measurements. The models are particularly interesting due to the combination of organic and inorganic structures by supramolecular interactions. The ability to enhance protein stability through supramolecularly-functionalized nanoparticles can be used as medically applied hybrid materials. Additionally, the phenomenon of protein absorption to cyclodextrin modified surfaces can be a promising tool in biomedical applications.

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