Effect of surface concentration on secondary and tertiary conformational changes of lysozyme adsorbed on silica nanoparticles

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ABSTRACT

Kinetics of tertiary conformation of lysozyme adsorbed on 90 nm silica nanoparticles was inferred using tryptophan fluorescence for different surface concentrations (0.24 to 0.92 mg/m²), pH (4, 7 and 9), ionic strength (10 and 100 mM), 2,2,2-trifluoroethanol (TFE) (5, 15 and 30%) and Dithiothreitol (DTT) (0.5 mg/ml) concentrations. A rapid initial unfolding, followed by a much slower refolding and subsequent unfolding, were observed with the extent of unfolding being higher at lower surface concentration, higher ionic strengths, higher TFE and DTT concentrations and at pH 9. The rate of unfolding was found to be higher at lower surface concentrations, pH 4, higher ionic strengths, higher TFE and DTT concentrations. In contrast, earlier results showed that β-lactoglobulin unfolded slower and exhibited only an initial rapid and a subsequent slow unfolding phase. Circular Dichroism spectra showed that α-helix content was lower for adsorbed lysozyme compared to bulk with a corresponding increase in β sheet and random coil. This decrease in α helix was found to be more pronounced at lower surface concentrations. DTT decreased α helix with a corresponding increase in random coil while TFE was found to have negligible effect on secondary structure.

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1. Introduction

High throughput approaches have been developed for profiling of proteins using mass spectrometry, tagging and subcellular localization and protein microarray. Protein biochip is a collection of miniaturized spots that are arranged on a solid substrate for high throughput analysis. Immobilization of enzymes in biochip can lead to partial or complete loss of enzyme activity due to random orientation and conformational changes. Nanoparticles possess unique properties due to the extremely large surface area per unit volume and therefore can be used as biosensors [1–3]. The nanoparticle surface can be functionalized through immobilization of enzymes for sensor applications in food pathogen detection, pesticide detection and food quality control. Among the different methods of immobilization, adsorption of enzymes onto the nanoparticle surface is the simplest. There has been increasing interest in the development of biosensors for detection of food pathogens and food quality. Biosensors obtain their specificity from a biological binding reaction which may be derived from any of a wide range of interactions, specificity, and affinities including antigen/antibody, enzyme/substrate, receptor/ligand, energy transducer systems. Enzyme/substrate reactions currently lack the spectrum of antigen/antibody systems, yet they possess inherent advantage in that they rapidly eliminate the target, release the product, and auto-regenerate the binding site with minimal loss of affinity and specificity over a large number of cycles. It is believed that immobilization of various enzymes onto nanoparticles will result in a much faster and accurate assay. Immobilization of enzymes by adsorption, however, may result in partial loss of enzymatic activity. It is important, therefore, to characterize the unfolding of proteins/enzymes at surfaces in order to arrive at rational methodology for the development of protein microarrays and nanoparticle-based sensors.

Physical immobilization of proteins/enzymes occurs via intermolecular forces, mainly ionic bonds and hydrophobic and polar interactions. Which intermolecular forces dominate in the interaction will depend on the type of protein and surface. The resulting adsorbed protein layer is likely to be heterogeneous and randomly oriented, since each protein molecule can form many contacts in different orientations in order to minimize repulsive interactions with the substrate and previously adsorbed proteins. The geometric size of the immobilized proteins will determine the adsorption capacity on the surface. High-density packing may sterically block active sites of proteins thereby interfering with functional properties. Drawbacks of the adsorption mechanism are random orientation and weak attachment, since proteins/enzymes may be removed by some buffers or detergents when performing the assays.

Lysozyme is an enzyme which hydrolyzes the polysaccharides found in many bacterial cell walls [4]. Lysozyme is a compact globular protein with 129 residues, consisting of five α helices, a three-stranded antiparallel β sheet, and a large amount of random coil and β turns [5–7]. Also its structure is stabilized by four disulfide bonds [8],
with most of the cysteins located in the α helices. The enzyme has an approximately ellipsoidal shape, with a large cleft in one side forming the active site which can bind six carbohydrates to execute its effective catalyst function.

Recent reviews describe the folding and unfolding pathways of lysozyme in solution [9–11]. The folding pathway for lysozyme has been well characterized and shown to consist of two folding phases. In the first fast folding phase, major parts of the α-domain and the β-domain are formed. The second slow folding phase results in the complete formation of secondary structure and native tertiary structure in less than 1 s. In addition, four disulphide bonds are also formed. However, unfolding of lysozyme on surfaces is a much slower process lasting several hours. Previous investigations have employed tryptophan fluorescence [12] to characterize the change in the tertiary conformation of proteins using the extent of blue shift of the emission spectrum. The secondary structure of proteins were monitored using Fourier Transform Infrared Spectroscopy (FTIR) [13–15] and Circular Dichroism (CD) [16,17]. Hydrophobic [14] and electrostatic interactions [13] between the protein and adsorbing surface have been shown to influence the changes in secondary structure. The extent of unfolding of seven structural intermediates of bovine serum albumin [18] and mutants of T4 lysozyme [19] at the interface has been shown to depend on the conformational stability of protein. Adsorption onto silica nanoparticle was shown to result in a greater loss of α helix content of lysozyme for larger surface coverage [17]. The change in secondary structure of lysozyme upon adsorption onto PTFE was found to exhibit an initial fast conversion of α helix to β sheet within a few minutes followed by a much slower conversion [15]. We have investigated the kinetics of tertiary conformational changes as well as the extent of secondary conformational changes of β lactoglobulin on silica nanoparticle surfaces using tryptophan fluorescence, FTIR and CD [20]. The tertiary conformational changes were shown to depend on surface packing density, pH and ionic strength and were influenced by protein–protein interactions on the surface. β lactoglobulin exhibited an initial rapid unfolding followed by a much slower unfolding with a surface packing dependent lag time. In this communication, we report the kinetics of tertiary conformational changes and the extent of secondary conformational changes of lysozyme adsorbed on silica nanoparticle using tryptophan fluorescence and CD. Interestingly, lysozyme was found to exhibit three distinct phases of conformational changes, an initial rapid unfolding phase, a slower refolding phase followed by a slower unfolding phase leading to equilibrium.

2. Materials and methods

2.1. Materials

Lysozyme was purchased from Sigma Co. Silica nanoparticles were purchased from Interfacial Dynamics Corporation, Japan. Silica nanoparticle is 90 nm in diameter and is stored at pH 11 with a zeta potential of around –70 mV. The nanoparticle suspension is extremely stable (electrostatic stabilization). The nanoparticles are monodispersed with very small standard deviation (10 nm) in the particle size. Silica nanoparticle is chosen as a model system since it is extensively used in other investigations.

Ultra GdmCl, 2,2,2-trifluoroethanol (TFE), Dithiothreitol (DTT), hydrogen chloride (HCl), and other chemicals were purchased from Sigma Co.

2.2. Methods

2.2.1. Protein unfolding in the bulk

Lysozyme solution in 10 mM phosphate buffer at pH 7 was subjected to unfolding by the addition of different amounts of GdmCl and allowing the reaction to occur for 2 h. Phosphate buffer was used for pH 7. pH 4 was adjusted by the addition of HCl and pH 9 was adjusted by the addition of NaOH. Unfolded fraction was inferred by comparing the fluorescence intensities with the fluorescence intensity of samples subjected to GdmCl induced unfolding.

2.2.2. Fluorescence spectra

Tertiary structural changes of lysozyme on the surfaces of nanoparticles were measured using Tryptophan fluorescence emission technique. Measurements were carried out on a Fluor Station II fluorescence spectrophotometer with a standard 96 well plate. The excitation wavelength at 288 nm was chosen because almost all the fluorescence emission signal excited at this wavelength is derived from tryptophan. The tryptophan emission fluorescence spectra were collected in the wavelength range of 360 nm to 500 nm. The fluorescence intensity at 380 nm, the wavelength for maximum intensity, was monitored as a function of time to characterize unfolding kinetics because it gave the biggest difference between the native and unfolded state of proteins. All data were collected at 25 °C.

2.2.3. Circular Dichroism (CD) spectra

CD spectra were measured from 190 nm to 300 nm at room temperature on a Jasco J-810 spectrometer (Jasco Spectroscopic Co., Hachioji, Japan) using a cell with a path length of 0.1 mm. Data were collected every 0.2 nm with 2 nm bandwidth, at a scan speed of 50 nm/min, by averages of three scans. Molar ellipticity (deg cm$^{-1}$ dmol$^{-1}$) is expressed on a mean residue concentration basis in the far UV. Spectra were analyzed for secondary structure content by using the program CONTIN [21].

2.2.4. Surface concentration of lysozyme

The amount of lysozyme adsorbed on the silica nanoparticle surface under different conditions for different exposure times was measured. 0.5 mg/ml protein solution was mixed with nanoparticles of different particles concentrations (0.75 to 3.75 wt.%). After exposure times of 30 min, 1 h and 4 h, this mixture was centrifuged at 4500 rpm (centrifuge) in a centrifuge tube equipped with membrane filter of 30,000 Da molecular weight cutoff, so as to exclude nanoparticles from the supernatant. The concentration of protein in the supernatant was inferred from UV absorbance at 290 nm using Cole Palmer UV2100 Spectrometer.

By material balance, the surface concentration was estimated from,

$$c_t + \Gamma(c_t) \frac{d}{d} = c_i$$

(1)

where $c_i$ and $c_t$ are the initial protein concentration and final protein concentration after equilibration with nanoparticles respectively, $a$ is the total surface area of nanoparticles and $V$ is the volume of protein solution.

In Eq. (1), the total area $a$ of nanoparticles is given by,

$$a = \frac{3w\phi}{\rho R}$$

(2)

where $w$ is the mass of the sample, $\phi$ is the mass fraction of nanoparticle of radius $R$ and density $\rho$.

2.2.5. Evaluation of unfolded fraction of adsorbed lysozyme from fluorescence measurements

The normalized fluorescence intensity $I^*$ from the adsorbed protein on nanoparticle surface was obtained by subtracting the fluorescence intensity $I_b(c_b)$ for the bulk of protein concentration $c_b$ as given by,

$$I^* = \frac{I_b(c_b)}{I_a} = \frac{I_b(c_b)}{(c_i - c_b)}$$

(3)

where $c_i$ and $c_b$ refer to the initial and final bulk protein concentrations respectively. The unfolded fraction of protein on nanoparticle surface
was inferred from $F/\left(\Gamma_0 (c_N-c_D) + \Gamma_0 (c_D-c_N)\right)$ (D and N refer to fully denatured and native protein solutions respectively) using the calibration of fluorescence intensities of protein solution with different extents of denaturation as obtained from GdmCl induced denaturation (see results below).

3. Results

3.1. Adsorption kinetics of lysozyme on nanoparticles surfaces under different conditions

Surface concentration of lysozyme adsorbed on nanoparticles under different conditions for three different mixing times (30 min, 60 min, and 240 min) are shown in Table 1. The results indicate that the surface concentration did not increase significantly from 30 min to 240 min thereby suggesting that protein adsorption approached equilibrium around 30 min. In addition, pH, ionic strength, TFE and DTT concentrations did not influence the surface concentration of lysozyme. As shown later, the evolution of tertiary structures of adsorbed protein was much slower than the rate of protein adsorption.

Assuming the protein molecule to be a point charge (may be a reasonable assumption since the size of the protein molecule is much smaller than that of nanoparticle), the electrostatic interaction force $F$ of an adsorbing protein molecule onto the adsorbed protein layer on the nanoparticle surface is given by

$$F = q\varepsilon_0 \frac{kT n_e^2}{\Gamma_0 (c_N-c_D) + \Gamma_0 (c_D-c_N)} \sinh \left(\frac{2z_0 e}{kT}\right)$$

where $q$ is the net charge of protein molecule, $e$ is the elementary charge, $k$ is Boltzmann constant, $T$ is the temperature, $n_e$ is the number concentration of ions, is related to ionic strength, $\varepsilon_0$ is permittivity of vacuum, $z_0$ is the dielectric constant and $\zeta$ is the zeta potential. The net charge of lysozyme at different pH was obtained from the potentiometric titration as reported by Ruxby and Tanford [22]. The $\zeta$ potential at different pH were measured. The ionic strength of the buffer was calculated using the dissociation constants of mono and dibasic phosphates as well as the concentration of charged protein molecules. The calculated interaction force at three different pH (4.5, 7 and 9) did not vary significantly (2.7 × 10$^{-11}$ to 3.2 × 10$^{-11}$ N) as can be seen from Table 2. This is consistent with the experimental surface concentrations at different pH (see Table 1).

Table 2

<table>
<thead>
<tr>
<th>pH</th>
<th>q (lysozyme molecule)</th>
<th>Ionic strength</th>
<th>$f_s$ (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>6.5</td>
<td>0.039</td>
<td>3.187 × 10$^{-11}$</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>0.035</td>
<td>2.702 × 10$^{-11}$</td>
</tr>
<tr>
<td>4.5</td>
<td>11</td>
<td>0.036</td>
<td>2.925 × 10$^{-11}$</td>
</tr>
</tbody>
</table>

The force is calculated assuming the protein molecule to be a point charge. The ionic strength accounts for the dissociation constants of mono and disodium phosphates and concentration of charged protein molecules. The net charge of lysozyme at different pH is obtained from potentiometric titrations.

3.2. Effect of surface concentration, pH and ionic strength, on the unfolding kinetics of lysozyme on nanoparticles surfaces

Fig. 1a shows the typical tryptophan fluorescence spectra of lysozyme solution (0.5 mg/ml) at different GdmCl concentrations at pH 7. Understandably, the fluorescence intensity was higher at higher GdmCl concentrations because of exposure of more tryptophan residues as a result of unfolding. Lysozyme retained its native conformation at lower GdmCl concentrations as indicated by no significant change in the spectra up to a GdmCl concentration of 3 M. Also, lysozyme was fully denatured at GdmCl concentration above 6 M as evidenced by no further change in the fluorescence spectra at higher concentrations (Fig. 1a). Fig. 1b shows the normalized fluorescence intensity at 380 nm for different GdmCl concentrations following the procedure of Sontoro and Bolen [23]. For a two state model, this fractional increase can be interpreted as the unfolded fraction. As shown in Fig. 1b, the unfolded fraction was zero for GdmCl concentrations below 3 M and was one for concentrations above 6 M. The variation of unfolded fraction with GdmCl in the intermediate concentrations is linear, consistent with results reported in the.

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**Table 1**

Surface concentration of lysozyme on nanoparticles surfaces under different conditions

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pH 8.9</th>
<th>pH 7</th>
<th>pH 4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The surface concentrations were calculated using Eq. (1) from the measurements of initial and final protein concentrations upon equilibration with nanoparticles respectively as explained in Methods section.
that addition of nanoparticles of different concentrations to the buffer did not result in significant increase in fluorescence intensity as a result of complete denaturation of protein. It was shown that addition of nanoparticles of different concentrations (in the range of 0.75 to 3.75 wt.%) to the buffer did not result in significant increase in fluorescence intensity because lysozyme contains 6 tryptophan amino acids (results not shown). As expected, addition of 8 M GdmCl to lysozyme solution further increased the fluorescence intensity as a result of complete denaturation of protein. It was shown that addition of nanoparticles of different concentrations to protein solution did not interfere with tryptophan fluorescence. The fluorescence intensity of adsorbed lysozyme on nanoparticle surface was found to fall in between the values for native and fully denatured proteins. The extent of unfolding of adsorbed lysozyme on nanoparticle surface was inferred from the normalized fluorescence intensity at 380 nm using the calibration in Fig. 1b as explained in methods section.

The kinetics of tertiary conformational changes for lysozyme adsorbed on silica nanoparticle surface is shown in Fig. 2 for different nanoparticle concentrations. The surface concentration of lysozyme on silica nanoparticle surface was calculated from the initial protein bulk concentration and the nanoparticle surface area as explained in the Methods section. As can be seen from Fig. 2, lysozyme at low surface concentration was unfolded to a greater extent in equilibrium state. This finding indicates the existence of a high energy barrier for unfolding in a crowded surface environment. The shape of the kinetics curves for different surface concentrations was quite similar.

It is also evident from Fig. 2 that there was a rapid increase in the unfolded fraction at short times followed by a period (around 60 min) during which there was a much slower renaturation of lysozyme possibly due to reorientation of adsorbed molecules at the nanoparticle surface. This was followed by a slow unfolding of lysozyme at longer times eventually reaching equilibrium. The equilibration time was found to be smaller for lower surface concentration. Therefore, one can attribute three phases for protein unfolding on the surface of nanoparticles. The initial phase occurs within minutes, which is believed to involve rapid unfolding of protein molecule on the surface after its adsorption. Because of the lower energy barrier due to the larger surface area available to the protein molecule at lower surface concentration, this process takes a very short time as indicated by an initial rapid increase in the unfolded fraction. Since the initial timescale was of the order of minutes, experimental measurements of unfolding kinetics within that timescale could not be made. Therefore, the reported experimental data observed an initial rapid jump in the unfolded fraction. This initial jump was found to be larger for smaller protein surface concentration. Fig. 2 also shows that as the surface concentration increased, the extent of the initial jump decreased. The extent of renaturation in the second phase was found to be higher at lower surface concentration. Also, the unfolding rate in the third phase became smaller with increasing surface concentration. At higher surface concentrations (crowded environment), protein–protein interactions become important at the surface because of smaller distance between neighboring protein molecules. However, at lower surface concentrations, protein–protein interaction would become significant only after the distance between neighboring molecules becomes sufficiently small as a result of unfolding. A possible explanation is that at low surface concentration, the predominant molecular interaction is between proteins and the hydrophobic surface of silica nanoparticle, which induce the unfolding of protein molecule without energy barriers due to the freely available space. However, at higher surface concentration (i.e., in a crowded environment), the unfolding behavior of protein molecules is strictly limited by the interaction between protein molecules, i.e., in such a crowded environment, the extension of protein structure is limited by the physical space and the energy barriers that arise from the interaction with other protein molecules close by. Apparently, this energy barrier not only decreases the extent of unfolding but also decreases the unfolding rate at high surface concentration.

The kinetics of unfolding of lysozyme on silica nanoparticle surface at three different pH values (pH 4, pH 7 and pH 9) is shown in Fig. 3. Since the net charge of protein molecule at pH 9, near pI, is much smaller than that at pH 7, the electrostatic interactions between adsorbed protein molecules were more pronounced at pH 7, which may result in inhibition of the unfolding of protein molecules. This is supported by the data in Fig. 3: the extent of unfolding was higher at pH 7 than at pH 9. However, at pH 4, the extent of unfolding is found to lie in between the two values for pH 7 and pH 9 even though the net positive charge is much higher at this pH. Such a behavior may be due to the fact that the protein, being highly positively charged, may be unstable [24]. In the pH range of 4 to 9, lysozyme is positively charged. Since the silica nanoparticle is negatively charged, the electrostatic interaction between the protein and nanoparticle surface should promote unfolding. This is evident from much faster rates of unfolding (as indicated by much smaller time of around 300 min to reach equilibrium) compared to our earlier results [20] of unfolding of β-lactoglobulin (of negative charge) on silica nanoparticle surface. However, the extent of unfolding for lysozyme is comparable to the values for β-lactoglobulin [20] in spite of favorable electrostatic interaction with the silica surface. This may be due to the presence of disulfide bonds. In addition, our earlier results [20] indicated that β-lactoglobulin exhibited only two phases, namely, an initial rapid unfolding (similar to the first phase for lysozyme) followed by a slower
unfolding phase. Typical comparison of the kinetics of tertiary conformational changes of lysozyme and β-lactoglobulin (Fig. 4) indicates that (i) the initial jump in unfolded fraction was larger for lysozyme (ii) there was refolding second phase for lysozyme which was absent for β-lactoglobulin and (iii) the rate of unfolding was faster for lysozyme.

Fig. 5 shows the kinetics of unfolding of lysozyme on silica nanoparticle surface for two different ionic strengths at pH 7 at three different nanoparticle concentrations. Higher extent of unfolding was observed for higher ionic strength at higher surface concentration. Higher ionic strength compresses the electrical double layer in the vicinity of adsorbed protein molecule thereby reducing the electrostatic interaction between adsorbed protein molecules which in turn leads to more unfolding. This effect was found to be less pronounced at lower surface concentrations. Since the average distance between adsorbed protein molecules is far greater than the interaction range of the double layers of neighboring protein molecules at lower surface concentration, a further compression of the double layer caused by increasing the ionic strength will not reduce the interaction of protein molecules. The diameter of the sphere of influence of adsorbed proteins can be estimated as $1/\Gamma$, where $\Gamma$ is the surface number concentration of protein on nanoparticle surface. Assuming the radius of adsorbed protein to be that of native $r_{\text{native}}$ (2 nm), one can estimate the surface to surface distance between neighboring adsorbed protein molecules as $s=(4/\pi \Gamma)^{1/2} - 2r_{\text{native}}$. For example, for high surface concentration of 0.92 mg/m², $s=1.8$ nm which is comparable to double layer thickness of 0.78 nm. However, for low surface concentration of 0.24 mg/m², $s=8$ nm. The average distance between adsorbed protein molecules decreased as adsorbed protein molecules unfold with time. This resulted in stronger protein–protein interactions. Therefore, the effect of ionic strength was more pronounced for smaller distance between adsorbed protein molecules at longer times. However, at higher ionic strengths, the rates of refolding and subsequent unfolding were smaller even though the initial jump in extent of unfolding was higher.

3.3. Effect of TFE and DTT on the tertiary structure of lysozyme on the surface

2,2,2-trifluoroethanol (TFE) induces the change of tertiary structure by destabilizing hydrophobic interactions while stabilizing hydrogen bonding. Dithiothreitol (DTT) is an unusually strong reducing agent. It is therefore used to reduce the disulfide bonds of proteins and thus leads to a loss of tertiary structure.

The kinetics of unfolding of lysozyme on silica nanoparticle surface at different TFE concentrations is shown for three different surface concentrations in Fig. 6. At higher surface concentration (0.92 mg/m²), the extent of unfolding as well as the unfolding rate increased as TFE concentration increased. However, at lower surface concentration (0.22 mg/m²), increasing TFE concentration had no significant effect on the extent of unfolding. This is because at low surface concentration, protein molecule almost fully extends its tertiary structure, thus leading to exposure of its hydrophobic groups to binding surface. As a result, addition of TFE had no significant effect on the extent of unfolding. TFE also decreased the rates of refolding and subsequent unfolding with the initial jump in extent of unfolding being higher. This difference in the initial jump was insignificant at low surface concentration.

The kinetics of unfolding of lysozyme on silica nanoparticle surface with or without DTT is shown in Fig. 7 at three different surface concentrations. At higher surface concentration, the extent of unfolding increased in the presence of DTT. Similar to the effect of TFE, the presence of DTT increased the initial jump in unfolding though it decreased the rates of refolding and subsequent unfolding. The effect of DTT was smaller at lower surface concentrations.

3.4. Changes in secondary structure of lysozyme on the surface

Comparison of CD spectra of lysozyme in solution with those of protein adsorbed on the nanoparticles surface at different surface concentrations, is shown in Fig. 8a which reveals that lysozyme lost its secondary structure as it unfolded more at lower surface concentrations as is evident from its loss of $\alpha$ helix with a corresponding
increase in random coil (see Table 3 and Fig. 8b). CD spectra of lysozyme in different TFE concentrations (0%, 5%, 15%, and 30%) without any nanoparticles are shown in Fig. 8c. These spectra show that the secondary structure of lysozyme is unaffected by increasing TFE in the solution. The secondary structure content of the protein as shown in Fig. 8d and Table 3, indicate an insignificant change with TFE concentration. Table 3 shows the effects of TFE and surface concentration on the secondary structure of adsorbed lysozyme on nanoparticle surface. At a fixed surface concentration, changing TFE concentration did not alter the secondary structure of lysozyme in the TFE concentration range of 0% to 30%. In contrast, TFE was found to increase the α-helix content for β-lactoglobulin [20].

Fig. 9 shows the effect of DTT on secondary structure of lysozyme. The CD spectra (Fig. 9a) show that the addition of DTT into the solution slightly increased β-sheet and decreased α-helix of lysozyme in solution. However, in the presence of nanoparticles, the α-helix content decreased while the β-sheet content increased. In addition, the decrease of surface concentration (more unfolding) of lysozyme on nanoparticles did not significantly affect β-sheet though it

### Table 3

Secondary structure content of lysozyme adsorbed onto nanoparticle surface at different TFE concentrations

<table>
<thead>
<tr>
<th>Surface Concentration (mg/m²)</th>
<th>TFE% (v/v)</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random coil and other</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.402</td>
<td>0.114</td>
<td>0.484</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.401</td>
<td>0.128</td>
<td>0.471</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.410</td>
<td>0.116</td>
<td>0.474</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.436</td>
<td>0.102</td>
<td>0.462</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.416</td>
<td>0.103</td>
<td>0.481</td>
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</tr>
<tr>
<td>5</td>
<td>0.414</td>
<td>0.120</td>
<td>0.466</td>
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</tr>
<tr>
<td>15</td>
<td>0.426</td>
<td>0.093</td>
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<td>0.166</td>
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<td>0.136</td>
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<tr>
<td>30</td>
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<td>0.140</td>
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<td></td>
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<tr>
<td>0</td>
<td>0.308</td>
<td>0.148</td>
<td>0.544</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.286</td>
<td>0.162</td>
<td>0.552</td>
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<tr>
<td>15</td>
<td>0.278</td>
<td>0.151</td>
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<td></td>
</tr>
<tr>
<td>30</td>
<td>0.294</td>
<td>0.159</td>
<td>0.547</td>
<td></td>
</tr>
</tbody>
</table>

The proportions of α-helix, β-sheet and random coil were determined from the CD spectra using CONTIN program.
Therefore DTT had a significant effect on the secondary structure of lysozyme at different surface concentrations in 0.5 µg/ml DTT; No Np No DTT refers to the solution without nanoparticle and DTT; No Np DTT refers to the solution without nanoparticle in the presence of DTT. The other conditions are at different surface concentrations in the presence of DTT.

decreased the α helix content. DTT mainly broke down the disulfide bonds and therefore changed the secondary structure. The enhanced effect of DTT at lower surface concentration also suggests a strong relationship between disulfide bonds and secondary structure: after the disulfide bonds are broken, the lysozyme molecule becomes more flexible thus making its secondary structure prone to be affected by the interaction with the surface. Such a behavior is similar to that with respect to the tertiary structure, i.e., more change at lower surface concentration. The secondary structure analysis (Fig. 9b) shows that there is a loss of α helix content at higher DTT concentration and lower protein surface concentration with a corresponding increase in the content of random coils and turns, whereas β sheet content stays almost the same. Such an exchange of secondary structure is mostly because one of the two cystein amino acids is located in α helix. Therefore DTT had a significant effect on α helix. Table 4 shows the secondary structure content of lysozyme at different surface concentrations in the presence or absence of DTT. In contrast, DTT was found to decrease β sheet for β lactoglobulin [20].

4. Discussion

The secondary conformational changes of lysozyme upon adsorption that we report here are consistent with earlier observations [15,17,25] in that adsorption leads to a loss of α helical content with a corresponding increase in β sheet and random coil. In addition, our observation of decrease in α helical content of adsorbed lysozyme at pH 7 with an increase in area per molecule (within the range of 25 to 100 nm²/molecule) are found to be consistent with the reported trend of Vertegel et. al. [17]. However, we observe a decrease in α helical content from 0.4 (in solution) to 0.308 (for area per molecule of 100 nm²) which is higher than the reported values of 0.1 to 0.075 by Vertegel et. al. [17]. This difference may be due to the fact that their results were reported for 20 nm diameter silica nanoparticles whereas our results are for 90 nm silica nanoparticles. The change in secondary structure of adsorbed lysozyme with surface concentration (area per molecule) can be attributed to the lateral protein–protein interactions on the silica surface [15]. Noinville et. al. [14] observed greater loss of enzymatic activity of α chymotrypsin upon adsorption onto a hydrophobic surface compared to a hydrophilic surface because of greater driving force for unfolding. We also report a faster rate of tertiary conformational changes (unfolding) for lysozyme (positively charged) adsorbed on negatively charged silica surface compared to our earlier results [20] of much slower rates of unfolding for β lactoglobulin (negatively charged) adsorbed on silica surface. This can be attributed to greater electrostatic driving force for unfolding in the former compared to latter.

The rapid unfolding of lysozyme upon adsorption onto nanoparticle surface (initial jump in fluorescence) is believed to be due to the rapid decrease in the free energy of adsorbed protein molecule. Lysozyme has two domains, namely, α and β domains. A rapid decoupling of these two domains upon adsorption would lead to a strong disruption of the tertiary structure with an immediate loss of α helix in α domain with a corresponding increase in β turn and random coil [15]. This is quite unstable since it would result in the exposure of most of the hydrophobic amino acid residues from the interior of the molecule to the solvent in a very short time. Therefore, a ‘molten globule-like’ structure is formed in which α helix is transformed to β sheet, thereby resulting in a refolding phase [26]. However, this intermediate structure is not stable on the silica surface because a collapse of these two domains may lead to a change in the surface charge distribution and therefore a further reorientation of the molecule leading to further unfolding such that there is largest contact of the positively charged patch to the negative surface. The molecular dynamics calculation of radius of gyration of lysozyme in solution [26] in which water molecules were introduced into the hydrophobic core to simulate denaturation also shows an initial rapid increase in the radius of gyration (unfolding) followed by a very short duration of decrease in the radius (refolding) and a subsequent slower increase (unfolding). These results are qualitatively similar to our experimental results in which we observed three phases of conformational changes, a first fast unfolding, a second shorter refolding and a third slow unfolding.

5. Conclusions

The unfolding kinetics of lysozyme on the surface of silica nanoparticle was measured using tryptophan fluorescence and CD. Experimental measurements of kinetics of adsorption indicated that the timescale of protein adsorption was much smaller than that of changes in tertiary structure of adsorbed protein. The surface concentration of lysozyme was found to be insensitive to variations in pH, ionic strength and TFE and DTT concentrations. Three phases could be identified for the unfolding of lysozyme on silica nanoparticle surface. The first phase led to a very rapid initial unfolding which was observed as an initial jump because of inability to make measurements at short

Table 4

<table>
<thead>
<tr>
<th>Surface Concentration (mg/m²)</th>
<th>DTT (µg/ml)</th>
<th>α-helix (%)</th>
<th>β-sheet (%)</th>
<th>Random coil and other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.402</td>
<td>0.114</td>
<td>0.484</td>
</tr>
<tr>
<td>0.01</td>
<td>0.5</td>
<td>0.356</td>
<td>0.13</td>
<td>0.514</td>
</tr>
<tr>
<td>0.51</td>
<td>0.5</td>
<td>0.258</td>
<td>0.280</td>
<td>0.462</td>
</tr>
<tr>
<td>0.23</td>
<td>0.5</td>
<td>0.187</td>
<td>0.304</td>
<td>0.509</td>
</tr>
</tbody>
</table>

The proportions of α-helix, β-sheet and random coil were determined from the CD spectra using CONTIN program.
times. This was followed by a second phase in which lysozyme renatures possibly due to its reorientation on the surface. In the third phase, lysozyme was found to denature eventually reaching an equilibrium unfolded state. The extent of unfolding was higher at lower surface concentrations. The extent of unfolding was found to be higher at higher ionic strength which is believed to be due to suppression of protein–protein electrostatic interactions on the nanoparticle surface. The extent of unfolding was highest at pH 9 and was lower at pH 4 and 7. In contrast, earlier results showed that β-lactoglobulin unfolded slower and exhibited only an initial rapid and a subsequent slow unfolding phases [20]. Circular Dichroism (CD) spectra were analyzed using CONTIN program to infer different fractions of secondary structure. The results of CD spectra of adsorbed lysozyme on the surface of silica nanoparticle showed that altering the surface concentration did change the secondary structure of lysozyme on the surface. TFE did not have any significant effect on the secondary structure of lysozyme. DTT, on the other hand, was found to result in a decrease in α helix fraction with a corresponding increase in random coil. The results of this investigation indicate that it is preferable to immobilize lysozyme onto silica nanoparticle at high packing densities in order to retain its activity. Also, the kinetic study of conformational change indicate the existence of a renaturation period up to around 60 min followed by denaturation. Consequently, it may be of interest to use lysozyme immobilized on silica nanoparticle as biosensor within an hour after immobilization for maximum efficiency.

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References