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Effects of solute–matrix interaction on monitoring the conformational changes of immobilized proteins by surface plasmon resonance sensor

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Abstract

A real-time and labeling-free surface plasmon resonance (SPR) sensor was used to monitor the conformational changes of immobilized globule proteins (RNase A and lysozyme) in chemical unfolding and refolding. The effects of chemical denaturants on the protein structures were investigated. The methodology in protein conformational study on the solid surface is refined through the theoretic calculations and the conformational information of native/denatured proteins in solution. Additionally, our observation illustrates that the ambient buffer solution is merit to influence the refractive index of immobilized protein films and directly be observed from the SPR resonance angle shifts.

Keywords: Protein conformation; Surface plasmon resonance; Refractive index; Denaturant

1. Introduction

The structural conformation of proteins in the asymmetric interface, such as the cell membrane and solid–liquid interface of immobilization, is of paramount importance to their biological activity and functions [1]. Thus, related knowledge is also important to the understanding of several pathologies associated with protein misfolding in vivo [2] and to the biotechnology industry in terms of protein engineering [3,4], drug design [5] and biosensor [6].

Although the traditional optical and spectroscopic techniques, like CD, fluorescence, NMR and X-ray scattering, are routinely used to investigate the conformational state of proteins in solution or crystal [7,8]. The spectroscopic measurements to proteins on solid surface are difficult without any modifications and suffer from several methodological deficiencies [9,10].

Surface plasmon resonance (SPR) was originally designed to measure the binding behavior of chemical and biological reactions on the interface, with the advantages of being highly sensitive, real-time and labeling-free [11–14]. Indirect observations of protein conformational changes using SPR have been reported. These works adapt intermolecular binding and kinetic studies to infer conformational changes [15–17]. Only a few works deal with the direct observation of conformational change using SPR, including electrochemically induced conformational transitions [18] and the work on renaturation of firefly luciferase [19]. The pH-induced structural transition of immobilized proteins is the first idea to investigate the correlation between the resonance angle shifts and conformational changes of immobilized protein [20]. But, this method is more appropriate to elucidate electric changes of protein than its conformational changes [21]. Recently, using SPR to directly measure the analyte-induced conformational changes of protein has also been reported [22–24]. However, interpretative and technical controversies still exist in the source of the signal and the relationship between signal and conformation. The materials used in immobilization techniques may interfere with protein conformation and the performing of SPR signals [25], thus, caused controversies in interpretation. Moreover, a stable protein conformation usually involves interaction of the ambient

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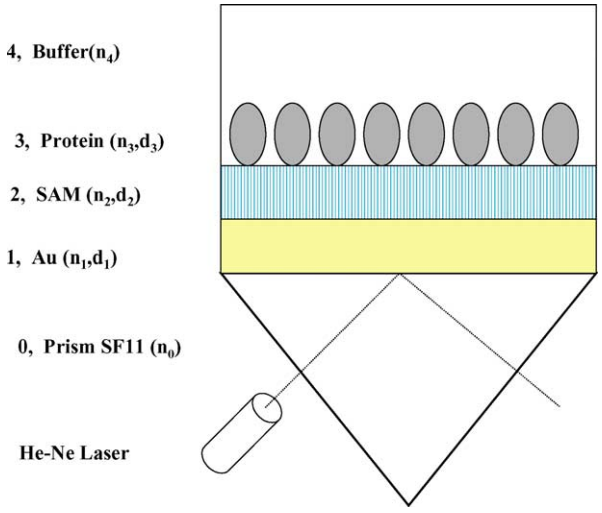

Fig. 1. The multilayers model of SPR device in Kretschman configuration.

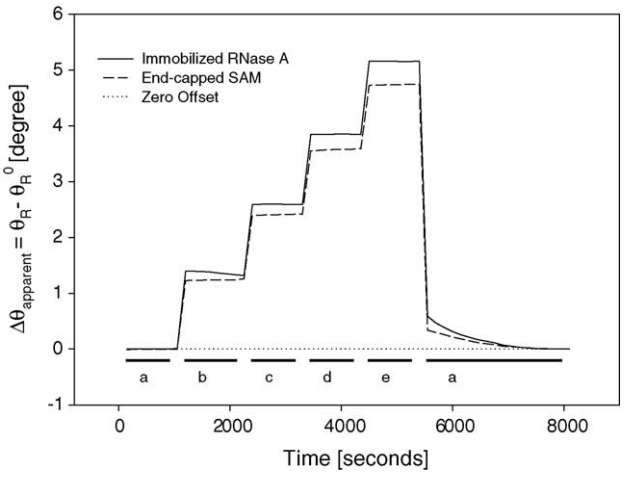

Fig. 2. SPR sensorgram of the apparent change of the resonance angle ($\Delta\theta_{apparent} = \theta_r - \theta_r^0$) in different denaturing solutions. Solid line represents protein-immobilized surface and dashed line is reference surface (end-blocking SAM). The mobile solutions (dashed bold bars), "a", phosphate buffer, "b–e", 2, 4, 6 and 8 M urea.

solvent (such as the hydrogen bonds of water molecules), while protein–solvent interaction makes discrimination of SPR signal from different conformation very complex and difficult.

In the early stages of development and application of SPR techniques, we can never be too careful to interpret the large volume of data generated, especially in the source of signal variation [26]. In this study, we tried more systematical experiments to trace the source of SPR signal changes and to answer a basic question: "What types of SPR signal were observed while the protein conformation changes by an exogenous analyte?"

Some simple principles about SPR device and experimental procedures are introduced for convenience in discussing our data. The major optical components of SPR are shown in Fig. 1. Medium 0, a 90° ATR prism optically couples to the glass slice in same material. Medium 1, a thin metallic film (Au) is coated on the slice. Medium 2 is the self-assembled monolayer (SAM) as a spacer to immobilize the target proteins, as the medium 3. The buffer solution is medium 4. Surface plasmon is collective oscillations of free electrons in a metallic film. Under appropriate conditions, the plasmon can be made to resonate with light, which results in adsorption of light. The measured reflectivity has a sharp minimum value θ_r (commonly referred to as the resonance angle) at this angle corresponding to the SPR spectrum [27]. Because the resonance is extremely sensitive to the refractive index and thickness of the medium next to the metallic film, adsorption of molecules on the metal-electrode surface or conformational changes in the adsorbed molecules can be accurately detected [28]. The apparent change of the resonance angle, $\Delta\theta_{apparent} = \theta_r - \theta_r^0$, synchronized measurements are shown in Fig. 2, where θ_r and θ_r^0 are resonance angles detected for the experimental and blank (phosphate buffer) solutions, respectively.

Two model proteins, the bovine pancreatic ribonuclease A (RNase A) and the hen egg white lysozyme were used in studies. Their conformations under various conditions have been described clearly and completely in uniform systems, including the interactions between protein and the denaturants [29]. The denaturants using in this study, such as urea and guanidinium chloride (GdmCl), are widely applied to solve and recover the inclusion body in biotechnology. Moreover, their conformational changes in kinetic transitions are also measured and characterized to investigate the protein unfolding or refolding pathways [30,31]. This abundant and detailed information served in this study as evidence to discriminate and evaluate the differences of protein conformations. Our understanding towards protein conformation on solid matrix surface is also enhanced by our results.

2. Experimental

2.1. Chemicals and reagents

Two globular proteins, RNase A (M.W. = 14.4 kDa) and lysozyme (M.W. = 13.7 kDa), were purchased from Sigma (U.S.A.) and used without purification. All of chemicals using in this study, including urea, GdmCl, 16-mercaptohexadecanoic acid (MHDA), *N*-ethyl-*N*-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) and 2-*N*-morpholino-ethanesulfonic acid (MES) are ultrapure or analytical grade and the water as a solvent is the ultrapure deionized water by Milli-Q system. A 10 mM, pH 7.4 phosphate buffer was used to prepare the denaturing buffers, each contain the urea (0, 2, 4, 6 and 8 M) or the GdmCl (0, 2, 4 and 6 M), respectively, and as the mobile phase of the flow system, referred to the blank solution in these experiments. The refractive index of each buffers were measured using a refractometer with temperature complement (RA-500N, KEM, Japan).

2.2. The SPR device

In our study, a homemade angle resolved SPR, in the Kretschmann configuration using attenuated total refection (ATR), combined with the flow analysis system is used. Matching oil with the same refractive index ($n = 1.778$ at 632.8 nm) is used to fill the gap between the slice (SF11) and the 90° ATR prism. The metallic film has a thickness of 47.5 nm gold and is coated on the one side of SF11 slice via a sputtering deposition process, exposed to the ambient solution in sensor cell. A p-polarized light wave from a 632.8 nm wavelength He–Ne laser, as a light source, is incident directly at an internal angle of θ at the gold-prism interface. This SPR metrology system has an angular accuracy (not resolution) of 0.0001° and is capable of yielding precise analytical data.

2.3. Surface modification of sensor chips

The bare gold surface of a conventional SPR sensor chip was treated with 1 mM MHDA in the ethanol to form a dense carboxyl-terminal self-assembled monolayer. After properly cleaning, the surface was immersed into the solution consisting of 1 mg/ml EDC with 40 mM MES for 4 h at room temperature. The activated surface was washed with pure water thoroughly and dried for the next immobilization of proteins.

2.4. Protein immobilization

The protein was immobilized covalently on the activated surface by immersing the surface into 1 mg/ml protein buffer solution for 4 h. The surface of the sensor chip was washed with water and treated with some drops of 1 M ethanolamine to block the unreacted activated sites for 2 h at room temperature. Then, the surface of the sensor chip was cleaned with pure water and dried by nitrogen before use. Thus, the chip with immobilized protein was obtained and the other chips without immobilized protein were treated with ethanolamine to block activated SAM surface as a controlled surface.

2.5. Monitoring by SPR

The flow rate is 30 μl/min and the measurement is taken 30 min after inducing new denaturing buffer to ensure the stableness of signal. After the introduction of 10 mM phosphate buffer (pH 7.0) to fill the sensor cell, the SPR resonance angle θ_r^0 reached a plateau and was defined as a zero baseline. The introduction of denaturing buffers resulted in a new resonance angle θ_r. Thus, the apparent change of the resonance angle can be measured by subtraction of θ_r^0 from θ_r. Using a temperature controller on the fluid and the sensor cell in a constant temperature 28.5 °C with an accuracy of ±0.1 °C to suppress the fluctuations of electronic signal by ambient environment.

3. Results and discussion

3.1. The SPR signals from immobilized protein films

With an incident light in given wavelength λ, the SPR resonance angle is sensitive to the thickness and optical property (refractive index) of multi-layered configuration. Thus, the SPR resonance angle would shift when the protein molecules undergo conformational changes via association or reaction with the denaturants from the solution [23]. As shown in the curves of Fig. 2, while the denaturants in the sensor cell are removed and replaced by the phosphate buffer (blank), SPR signal returns back to the original baseline ($\Delta\theta_{\text{apparent}} = 0$) and all resulted in good signal reproducibility. The denaturants did not result in permanent changes to the interface and these sensor chips can be reused in more denaturing experiments, which demonstrated good robustness and reproducibility.

With the appliance of Fresnel reflective model, the apparent change of the resonance angle, $\Delta\theta_{\text{apparent}}$ can be shown as the following formula, which discriminates the bulk effect of solution and the thickness and optical property of protein layer [28].

$$\Delta\theta_{\text{apparent}} \approx \Delta\theta_{\text{protein film}}(n_3, d_3) + \Delta\theta_{\text{bulk solution}}(n_4) \quad (1)$$

The term of solution effect, $\Delta\theta_{\text{bulk solution}}$, is the function of the solution refractive index, n_4. We deduced the following linear empirical equation from the measurements of various solutions on bared gold and modified surfaces with SAMs: $\Delta\theta_{\text{bulk solution}}(n_4) = \alpha_d \times C_d$, where C_d is the denaturant concentration of buffer solution and α_d is an experimental constant adjusted as solute, which measured from our experiment is 0.532 and 1.182 (degree/molar) for urea and GdmCl, respectively, as shown in Fig. 3A. Simple regression analysis resulted in good linear relationship ($R^2 > 0.9995$). This also demonstrates that the SPR resonance angle shifts can be used to evaluate the property of corresponding fluid (refractive index of solution) under proper conditions. The immobilization of lysozyme and RNase A caused 0.6614° and 0.6333° in SPR angular shift, respectively. The immobilized molecular densities on the sensor surface derived from the quantitative relationship (0.0001° of SPR angular shift corresponds to 1 pg/mm^2 of immobilized protein) are 0.4593 and 0.4623 pmol/mm^2, respectively.

3.2. Effect of chemical denaturants on protein conformational changes

The resonance angle shifts of the protein film, $\Delta\theta_{\text{protein film}}$ is the signal contribution from protein layer, correspondent to various denaturing buffers to unfold the immobilized proteins are shown in Fig. 3B. The relative extended uncertainty resulted from triplicate experiments is less than 3.5% of $\Delta\theta_{\text{protein film}}$, with confidence level >99%. Without the reducing agent, the $\Delta\theta_{\text{protein film}}$ is increased as the increasing concentrations of chaotropic agents (urea and GdmCl). According to the theoretical calculation [23], it is known that

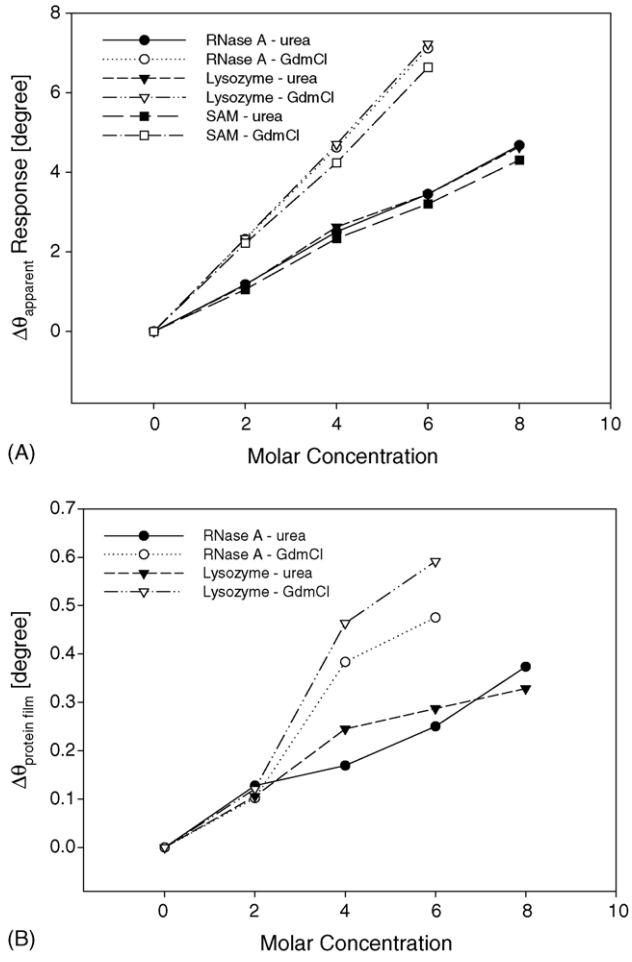

Fig. 3. (A) The $\Delta\theta_{\text{apparent}}$ changes curves of immobilized RNase A, lysozyme and the end-capping SAM induced by the various concentrations of chaotropic agents, urea (2, 4, 6 and 8 M) or GdmCl (2, 4 and 6 M) and (B) the $\Delta\theta_{\text{protein film}}$ unfolding curves of immobilized RNase A and lysozyme induced by chaotropic agents.

$\Delta\theta_{\text{protein film}}$ is in proportion to the change of average thickness (d_3) and refractive index (n_3) of protein layer, and can be simplified as shown by Eq. (2),

$$\Delta\theta_{\text{protein film}} \approx a_1\Delta d_3 + a_2\Delta n_3 = b\left(\frac{\Delta d_3}{d_3}\right) \quad (2)$$

where a_1 and a_2 are wavelength dependent constants; $b = a_1 d_3 - 2a_2\varepsilon_3\,(\varepsilon_3+2)\,(\varepsilon_3-1)/3$, ε_3 is the efficient dielectric function of the third layer and hence the refractive index, $n_3 = (\varepsilon_3)^{1/2}$. Boussaad et al. suggested that the increases in thickness of the protein layer yielded a decrease in SPR signal [18]. Thus, such an increase in $\Delta\theta_{\text{protein film}}$ for higher denaturant concentration implies the decrease in thickness of the protein layer for denaturation without reduction. However, the protein unfolding can be described as a transition from a predominantly rigid, folded structure to an ensemble of denatured states [32]. The suggestion derived from theoretical calculation would be in conflict with the volume expansion viewpoint of the proteins in denaturation by other experimental data [33].

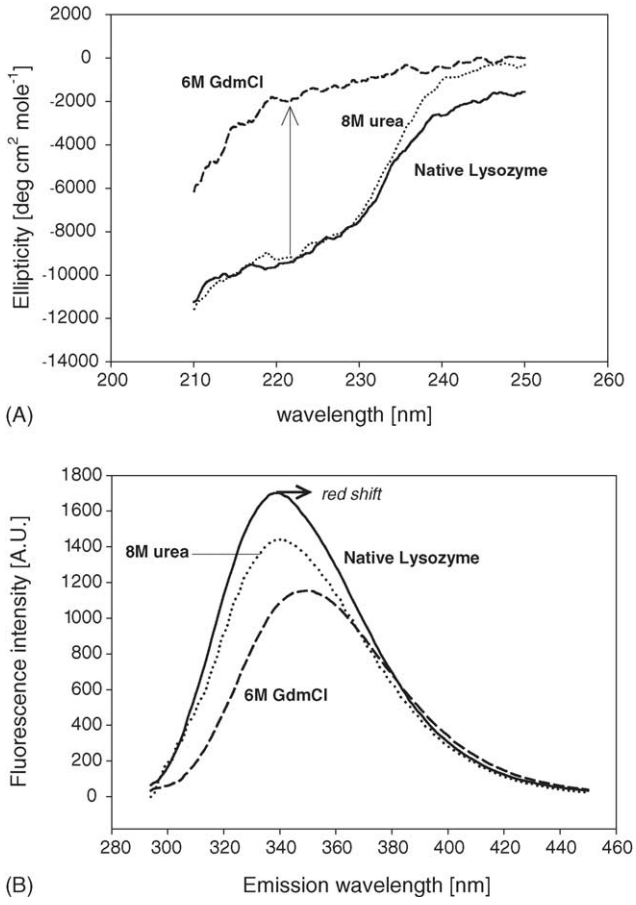

Fig. 4. (A) The far-UV CD spectra and (B) the fluorescence spectra for the native/denatured lysozyme in solution.

3.3. Protein conformational changes in solution

To compare this conformational change of proteins immobilized on the solid interface to that in solution, we measured the far-UV CD and fluorescence spectra of native/denatured-protein solutions. Fig. 4A shows the far-UV CD spectra for native and denatured lysozyme in the denaturing buffer used in our study. The signal intensity changes of CD at 222 nm (an indicator of α-helix) show that lysozyme was unfolded to a state that contained moderate α-helical structures in 8 M urea, but few in 6 M GdmCl. Additionally, the red shift and decreased intensity of maximal emission peak in fluorescence spectra of denatured lysozyme (in Fig. 4B) shows that the hydrophobic region buried within native protein was exposed to the buffer solution in unfolding by 6 M GdmCl disrupted the tertiary structure. The fluorescence spectrum of RNase A is not shown here because its fluorescence emission intensity is very small (absence of the intrinsic tryptophan) and the discrimination of changes in fluorescence spectra is difficult. The spectroscopic data shows that significant structural ruptures of the two non-reductive proteins were caused in high concentration of GdmCl but not urea, due to that the ionic GdmCl not only provides a hydrophobic environment in so-

lution but also further destroys the intra-molecular salt-bridge which stabilized protein structures [29,34].

3.4. The contribution of the interval ambient solution between immobilized proteins

Because of the stereo-hindrance, the immobilized proteins cannot occupy the full space in the protein layer, and both average thickness (d_3) and refractive index (n_3) of protein layer are related to the surface coverage, which can be considered as the fraction distribution of protein molecules in this film derived from Lorentz–Lorenz relation and an effective medium model [23]. Of course, the interval solution between the immobilized protein molecules would affect the efficient refractive index of protein film and the refractive index of interval solution is related to the buffer solution [35]. We compared the effects of different denaturing buffers on the non-reductive protein layer and inferred that GdmCl produce greater signal than urea in equal molarity, which is consistent with our SPR observation. Moreover, the changes of refractive index reflect the variances of the velocity of light in two different media, such as denaturing buffer and the phosphate buffer. Thus, we transform the unit of denaturing buffers from the molarity to the corresponding refractive index, as shown in Fig. 5, for the normalization of optical measurements. The variances of apparent resonance angular shifts for different denaturant types are not significant at either the two immobilized protein surfaces or SAM in Fig. 5A. The $\Delta\theta_{\text{apparent}}$ change at the two immobilized protein surfaces are greater than that at the SAM surface under the same denaturing conditions and thus highlights the effect of protein films in SPR measurements. Worth noting, the five points in Fig. 5B fall into the two categories. In the first category (α and β in Fig. 5B), the SPR signals for immobilized protein surfaces at the same denaturing condition are similar, indicating that at low denaturant concentration, the denatured-protein films have similar conformational changes. In the second category (γ, δ and λ in Fig. 5B), the SPR signals for the same immobilized protein surfaces at different unfolding curves are overlapping, indicating that at high denaturant concentration, the conformational changes of denatured-protein films are independent of the denaturant types. The results imply that the interval solution or denaturant binding may affect partially the SPR signal. We suppose that the non-reductive immobilized protein maintain a native-like or ordered-structural conformation in denaturation. Evidence to support our speculation has been presented through hydrogen exchange NMR and calorimetric studies [36,37]. The covalent disulfide bonds and the ionic bonds buried in the proteins restrict the flexibility of polypeptide chains [38,39].

3.5. Refolding of immobilized proteins

Changes of SPR signal occur under the different concentrations of denaturants. After multiple denaturing, the fresh phosphate buffer was induced to remove the chaotropic agents, the signal slowly returned to the original baseline

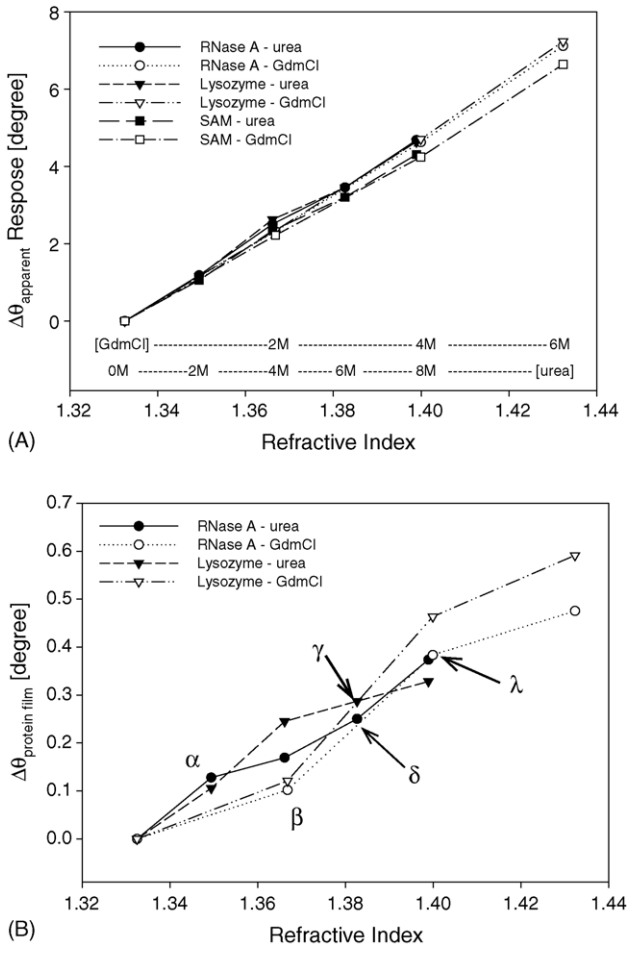

Fig. 5. The dimensional transformation in refractive index: (A) the $\Delta\theta_{\text{apparent}}$ changes curves of different surfaces and (B) the $\Delta\theta_{\text{protein film}}$ unfolding curves of immobilized protein surfaces induced by chaotropic agents.

($\Delta\theta_{\text{apparent}} = \Delta\theta_{\text{protein film}} = 0$) and the denatured protein refolded to the original (native-like) conformation. Therefore, the effect of denaturing buffers on the unfolding of immobilized proteins is reversible and the immobilization could stabilize the unfolding structures in denaturation. It can be speculated that the immobilization of protein restricts the flexibility of their side chains and therefore prevents intermolecular entangledness, which forms insoluble inclusion body and induces intra-molecular misfolding. The chaotropic agents not only disrupt the secondary structures but also stabilize the unfolded conformation [40]. Moreover, our results could apply to recover the enzymatic activity and stabilize conformation of proteins in interface or engineering.

3.6. Effects of the supported matrix in immobilization

The carboxymethyl-dextran (CM-dextran) is widely used as the material substrate of the spacer layer connecting protein molecule and metal layer. Zako et al., in the study of firefly luciferase immobilized onto CM-dextran, found that the signal does not return to the initial baseline after the removal of denaturing buffer [19]. Paynter and Russell suggest

that the CM-dextran matrix may interact with charged peptide and side chain of proteins via electrostatic interaction during the process of repetitively adjusting pH and the SPR signal is not regular and poor in reproducibility [25]. Moreover, literature has reported that the dextran as an artificial chaperonin assists in the refolding of protein and the stabilizing the structures of non-native conformation [41]. Our suggestion is that as spacer layer, the conformational changes of dextran directly or indirectly make some contribution to the SPR signal observed. To enhance the accuracy and precision of the experimental design, a thin thickness and functional SAM was used as a spacer layer to immobilize the protein in this study. It is also consistent with the result mentioned above that SPR signal changes are affected by solution property but unrelated with the surfaces.

4. Conclusions

We demonstrate a real-time and labeling-free method to monitor the conformational changes of immobilized proteins in unfolding process with denaturants and the advantage of immobilization techniques to stable the structures and assist refolding process. To summarize the conformational changes of immobilized proteins via SPR monitoring. The global conformation for denatured states is native-like and perturbed slightly by denaturants owing to the immobilization and the intrinsic disulfide bonds within proteins. Further, we refine the use of SPR in protein conformational study and reveal the influence of chaotropic agents and their contribution in SPR detection. According to the theoretic calculation, the SPR signal illustrates a high relativity with the compactness (rigidity or flexibility) of immobilized proteins in unfolding or refolding. Thus, our study will provide unique conformational information of proteins on the solid surface, even extend to elucidate and characterize structural changes of biomolecules, including protein, DNA and polysaccharide [42], in sensor and molecular recognition fields.

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