Single-Walled Carbon Nanotubes Probing the Denaturation of Lysozyme

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Resonance Raman spectroscopy measurements of lysozyme-bound single-walled carbon nanotubes have been made during different stages of the chemically and thermally induced misfolding and of the denaturation process of nanotube-bound lysozymes. Changes to the Raman intensity of single-walled carbon nanotubes (SWNTs) have been observed during the denaturation of lysozyme. The Raman intensity changes are attributed to excitonic transition energy ($E_\text{t}$) shifts of the SWNTs during the denaturation of lysozyme. The $E_\text{t}$ shift of SWNTs was confirmed by photoluminescence measurements.

Introduction

Irreversible degradation and aggregation of therapeutic proteins is a long-standing problem in the biopharmaceutical industry. To ensure product integrity at the time of administration, a high concentration protein product must remain stable for an extended period of time during transport and storage, often at elevated temperatures. The degradation process is generally described as a multistage pathway, starting with the unfolding and misfolding in protein monomers and the onset of protein degradation and aggregation, involving a complex set of competing misfolding and self-association reactions. In this manner, a fraction of the overall product may be degraded, and the small percentage of degradation can sometimes be laborious and time-consuming to detect and resolve using chromatography and light-scattering methods. Recent developments in self-interaction nanoparticle spectroscopy using gold nanoparticles suggest that the very sensitive optical transitions of nanoparticles could be exploited to develop a nanoparticle-based protein interaction assay to readily detect changes in the weak protein–protein versus protein–nanoparticle interactions in misfolded protein products. Aside from gold nanoparticles, one of the particularly interesting candidates for such nanoscaled probes is single-walled carbon nanotubes (SWNTs).

SWNTs have unique electronic properties and rich spectroscopic properties. The detailed geometric structure of SWNTs as denoted by their ($n,m$) indices determines their electronic properties as well as their spectroscopic properties, and these subjects have been studied extensively. Because the excitonic transition energy ($E_\text{t}$) of SWNTs is particularly sensitive to the dielectric environment, SWNTs have been used in a number of optical and electrical devices to directly probe conformational changes in biomacromolecules. The strong and photobleach-resistant photoluminescence (PL) and resonance Raman signals in the near-infrared region make SWNTs promising as labels in biosystems. For example, the shift of the PL emission energy of DNA-wrapped SWNTs due to the wrapping agent has been used to monitor conformational changes in DNA.

In this report, we use optical spectroscopy to qualitatively probe the interactions in a complex system involving a small percentage of misfolded/denatured proteins and nanotubes. Even though the weak interactions between a nanotube and a fraction of the degraded proteins can cause a small, yet definitive, shift in $E_\text{t}$, the shift in the PL signal could be difficult to resolve with high-energy resolution due to the wide resonance window of SWNTs in a biological environment. Thus, resonance Raman spectroscopy was used as an additional (yet possibly more sensitive) probe to detect subtle shifts in the position of the resonance window and the position of the $E_\text{t}$. As a result of the altered protein–nanotube and protein–protein interactions, when a small fraction of the proteins in the solution undergo denaturation. Many proteins can be attached to SWNTs through noncovalent binding. In this study, lysozyme was chosen as a model protein to study the protein–nanotube interaction. With 129 residues, lysozyme is a small and widely studied model protein whose folding kinetics is well-understood. In its native state, 83% of the surface of the lysozyme is hydrophobic, with a hydrophobic box defined by residues Trp 28, Trp 111, Tyr 23, Met 105, and Trp 108 and the stability and weak interactions are mostly determined by the colloidal stability of the proteins in solution. As we initiate misfolding and denaturation reactions, the secondary structure and the hydrophobic interior region of lysozyme are gradually exposed, and eventually, the protein is unfolded.

For the lysozyme-wrapped SWNTs (LYZ-SWNTs) without any denaturant, SWNTs may be bound to the hydrophobic...
Results and Discussion

CD spectra for LYZ-SWNT dispersions at different concentrations of GndHCl are presented in Figure 2a. The CD band at 200–260 nm can be used to calculate the percentage of denatured lysozyme. The calculated percentage of denatured lysozyme obtained from the CD intensity at 220 nm is plotted against the concentration of GndHCl (Figure 2b), which shows that lysozyme in LYZ-SWNT begins to irreversibly denature at ~4.5 M GndHCl. This concentration is higher than that for free-state lysozyme (2.5 M).

Figure 3a,b shows Raman spectra of the LYZ-SWNT dispersion at different GndHCl concentrations. At 532 nm excitation (Figure 3a), Raman bands are assigned to the (9,3) nanotube (metallic) according to the observed resonant excitation. At 647 nm excitation, the radial breathing modes (RBM) at 283 and 298 cm\(^{-1}\) can be assigned to two semiconducting nanotubes: (7,5) and (8,3), respectively. Frequencies of the G bands (~1590 cm\(^{-1}\)) and G’ bands (~2600 cm\(^{-1}\)) did not shift at either the 532 nm or the 647 nm excitation. This observation is consistent with the hypothesis of a pure hydrophobic interaction between the protein and nanotubes. Because lysozyme does not contain a metallic center, no doping level changes or charge transfer is expected between lysozyme and SWNTs during the lysozyme denaturation process.

The structural change of lysozyme during denaturation may change the conformation of the LYZ-SWNT complex, which results in changes of the surroundings of SWNTs and then changes the CD spectrum of SWNTs. The thermal intensity (M) changes for a certain Raman peak because the over-
peaks (Figure 3c). The RBM intensity ratio (for the (7,5) and (8,3) nanotubes were fit by two Lorentzian changes of the RBM intensity ratio, the observed RBM bands for (7,5) (red line) and (8,3) at 647 nm (1.916 eV) around 1150 nm. (f) Calculated normalized intensity of the Stokes RBM bands for (7,5) to (8,3) tubes is then plotted against the concentration of GdnHCl (Figure 3d), which shows that the ratio increases significantly for GdnHCl concentrations higher than 4 M. This was attributed to the dielectric constant after lysozyme denaturation. We also note that, even at GdnHCl concentrations lower than 4 M, the intensity ratio increases, while the CD spectrum shows an opposite change (Figure 3).

We explain the observed Raman intensity ratio changes based on resonance Raman theory. The resonance Raman intensity of SWNTs can be expressed by the following expression,

$$I_{\text{Stokes}} \propto \frac{1}{(E_{\text{ph}} - E_{\text{Stokes}} - i\Gamma)(E_{\text{laser}} - E_{\text{ph}} - E_{\text{Stokes}} - i\Gamma)},$$

where $E_{\text{laser}}$ is the laser energy, $E_{\text{ph}}$ is the phonon energy, $i$ is the imaginary unit, and $\Gamma$ is the width of the resonance window. In writing this expression for $I_{\text{Stokes}}$, the matrix elements for optical absorption, electron–phonon interaction, and optical emission are considered to be constant. For all mod $[(n - m), 3] = 2$ SWNTs, a red shift occurs for both $E_{22}$ and $E_{11}$ when the SWNTs are surrounded by materials having a dielectric constant larger than unity. Therefore, the $E_{22}$ level for (7,5) and (8,3) nanotubes is red shifted during the denaturation of lysozyme. The $E_{22}$ values for (7,5) and (8,3) nanotubes are around 1.92 and 1.88 eV, respectively, and $E_{11}$ should increase during the thermal denaturation of lysozyme. The $E_{22}$ transitions red shift, the RBM intensity for the (7,5) nanotubes should increase and the RBM intensity of the (8,3) nanotube should decrease (Figure 3f). As a result, the RBM intensity ratio for (7,5) to (8,3) should increase, which is in good agreement with the experimental results (Figure 3d).

To confirm our results and to eliminate any spectral artifacts that might be introduced by the chemical denaturant used in this experiment, thermal denaturation of LYZ-SWNT was carried out. The denaturation of lysozyme under elevated temperature has been studied extensively before. In this experiment, the denaturation of lysozyme was conducted at 80 °C and 90 °C. A similar change in the Raman results was observed: the G band and G' band peaks did not shift (not shown), but the RBM intensity ratio of (7,5) to (8,3) ($I_{7,5}/I_{8,3}$) increased during the thermal denaturation (Figure 4). At 90 °C, the RBM intensity ratio increased rapidly within the first 10 min and then approached a maximum value of $\sim 3.2$. The maximum value of
the RBM intensity ratio suggests the complete denaturation of lysozyme in the LYZ-SWNT dispersion. Assuming that the RBM intensity change is proportional to the denatured lysozyme, the first-order rate constant \( k \) for the thermal denaturation of lysozyme can be obtained by fitting the time-dependent RBM intensity ratio by an exponential function, \( I = A \exp(-kt) + B \), where \( I \) is the RBM intensity ratio, \( t \) is the denaturation time, \( k \) is the rate constant, and \( A \) and \( B \) are constants. The fit result gives a \( k \) value of \( 0.15 \pm 0.02 \) min\(^{-1}\) at 90 °C, which is lower than that for free lysozyme (~1.2 min\(^{-1}\) estimated for \( 3 \times 10^{-4} \) M lysozyme at pH = 6.2\(^{33}\)). The smaller rate constant of denaturation indicates that nanotube-bound lysozyme may be more thermally stable. At 80 °C, the RBM intensity ratio increases slowly, also indicating a lower rate constant. More systematic thermal studies will be required to elucidate the detailed thermally induced degradation kinetics for proteins in the protein-bound nanotube system.

Conclusion

In conclusion, Raman and PL measurements have been done on LYZ-SWNT dispersions during the denaturation of lysozyme. Intensity changes in the Raman peaks and a red shift of the PL on LYZ-SWNT dispersions during the denaturation of lysozyme. Assuming that the RBM intensity ratio suggests the complete denaturation of lysozyme, the first-order rate constant \( k \) for the thermal denaturation of lysozyme can be obtained by fitting the time-dependent RBM intensity ratio by an exponential function, \( I = A \exp(-kt) + B \), where \( I \) is the RBM intensity ratio, \( t \) is the denaturation time, \( k \) is the rate constant, and \( A \) and \( B \) are constants. The fit result gives a \( k \) value of \( 0.15 \pm 0.02 \) min\(^{-1}\) at 90 °C, which is lower than that for free lysozyme (~1.2 min\(^{-1}\) estimated for \( 3 \times 10^{-4} \) M lysozyme at pH = 6.2\(^{33}\)). The smaller rate constant of denaturation indicates that nanotube-bound lysozyme may be more thermally stable. At 80 °C, the RBM intensity ratio increases slowly, also indicating a lower rate constant. More systematic thermal studies will be required to elucidate the detailed thermally induced degradation kinetics for proteins in the protein-bound nanotube system.

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References and Notes