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Controlled Dispersion and Purification of Protein–Carbon Nanotube Conjugates Using Guanidine Hydrochloride

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Abstract: For the development of biofunctional carbon nanotubes for biosensors, drug carriers, and nanobiocatalysts, their aggregation and biofouling in aqueous solutions are crucial problems because this behavior leads to a reduction of their excellent optical and electrical properties and nanoscale size effects. This paper presents a new method for enhancing the dispersibility of protein–carbon nanotube conjugates and for exfoliating the protein from the carbon nanotube sidewalls through controlling the concentration of guanidine hydrochloride (Gdn·HCl) in the solution. In medium concentrations (2–3 M) of Gdn·HCl, the dispersibility of protein–carbon nanotube conjugates was found to be substantially increased without denaturation or aggregation of the proteins. At higher concentrations (>6 M) of Gdn·HCl, pristine carbon nanotubes were precipitated instantly as a result of dissociation of the protein. These phenomena indicate that Gdn·HCl functions not only as a dispersion adjuvant for biofunctional protein–carbon nanotube conjugates, but also as a cleaning agent for the purification of biofouled carbon nanotubes. The dissociation concentrations of Gdn·HCl were higher than the midpoint of protein denaturation, suggesting that protein adsorption on carbon nanotubes is more stable than protein folding toward Gdn·HCl.

Keywords: dispersion · guanidine hydrochloride · hybrid materials · nanotubes · proteins

Introduction

Biofunctional carbon nanotubes bound by proteins have attracted much attention as prominent biomaterials because of their optical and electrical properties and nanoscale size effects[1–6] ever since the discovery and development of carbon nanotubes.[7] However, their low dispersibility in aqueous solutions has limited their applications, since it leads to a reduction in the optical and electrical properties of carbon nanotubes.[8,9] Among the many methods that have been developed for the dispersal of carbon nanotubes, noncovalent modification using surfactants, organic compounds, or macromolecules is considered as the technique of choice to preserve their electronic and optical properties.[10–28] Recently, dispersion of carbon nanotubes using proteins has been reported.[29–33] Conjugation of protein and carbon nanotubes is anticipated not only as a simple dispersion technique but also as a means of fabricating biomaterials for use as biosensors,[1] drug carriers,[34] and nanobiocatalysts.[35] However, their degree of dispersibility has hitherto been insufficient for industrial applications because the interaction between the sidewalls of carbon nanotubes is stronger than the binding between protein and carbon nanotubes. More recently, we have reported that low concentra-

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tions of some alcohols improve the dispersibility of single-walled carbon nanotubes (SWNTs) because of the lowered hydrophobic interaction.[36] Although this method is ideal for proteins that are stable toward alcohols, in some cases, such as with alcohol-labile proteins or if high concentrations of alcohols are required, protein denaturation or aggregation might be induced. Therefore, a novel additive that enhances dispersibility without causing protein denaturation or aggregation in aqueous solutions is required. Another problem is biofouling of carbon nanotubes, that is, contamination starting with the nonspecific adsorption of proteins and other biomolecules onto the surfaces, which also represents a significant problem for application of carbon nanotubes because of the resulting impairment of function of the materials.[5] Consequently, not only have antifouling agents such as polymers and proteases[37–40] been proposed, but also exfoliating agents.

Guanidine hydrochloride (Gdn·HCl) and urea in high concentrations are well known as protein denaturants, designated as "chaotropes" because of these properties, which are in sharp contrast to those of "kosmotropes" (e.g., ammonium sulfate).[41,42] Chaotropes have been widely used for the solubilization of inclusion bodies, refolding, and thermodynamic analyses of proteins. Additionally, previous reports have indicated that Gdn·HCl and urea can solubilize hydrophobic low molecular weight compounds even at low concentrations.[43–45] They can then stabilize hydrophobic moieties of the compounds. Although the mechanism of this effect is usually explained in terms of preferential interaction or surface tension, it has not been fully elucidated.[45,46]

In this study, Gdn·HCl has been used as an additive for the dispersion of SWNTs in aqueous solution. The dispersibility of the conjugates was assessed by measuring their absorbance with a spectrophotometer. Medium concentrations of Gdn·HCl were found to enhance the dispersibility of the protein–SWNT conjugates without denaturation or dissociation of the protein on the SWNTs because of a decreased hydrophobic interaction between the SWNT sidewalls. The structure of the proteins on the SWNTs was measured using a circular dichroism spectropolarimeter. Structural changes of the protein through adsorption onto the SWNTs were observed, suggesting an impact of the SWNTs at the protein molecular level. On the other hand, high concentrations of Gdn·HCl led to dissociation of the protein from the SWNT surfaces because of a strong solubilizing effect, indicating that concentrated Gdn·HCl might be used as a cleaning agent for the purification of biofouled carbon nanotubes.

Results and Discussion

Gdn·HCl is well known to induce protein denaturation and to solubilize hydrophobic compounds in aqueous solutions.[44,45] Solubilization by Gdn·HCl is generally attributed to stabilization of hydrophobic moieties of the substances in the solution. Association of carbon nanotubes caused by hydrophobic interactions results in low dispersibility in aqueous solutions. Therefore, it can be inferred that Gdn·HCl might also be applicable for stabilizing carbon nanotubes in solution. In this study, we have attempted to enhance the dispersibility of protein–carbon nanotube conjugates by using Gdn·HCl to test the above hypothesis. Lysozyme–carbon nanotube conjugate solutions were adjusted to pH 3.4, at which the conjugates were well-dispersed by their positive net charges, as described previously.[32,33,36]

The dispersibility of the SWNTs after ultrasonication and centrifugation ($40\,000\times g$) processes was slightly reduced at low concentrations (≈ 1 M) of Gdn·HCl, although the dispersibility was enhanced at medium concentrations (2–3 M) of Gdn·HCl (Figure 1A). At high concentrations (>6 M), the dispersibility was markedly lower, indicating that the SWNTs were aggregated and precipitated by the Gdn·HCl. Notably, the SWNTs were not dispersed at every concentration of Gdn·HCl in the absence of protein (data not shown), indicating that the presence of proteins was necessary for the dispersion. The CD spectra of lysozyme in the solutions showed it to be denatured by Gdn·HCl at concentrations higher than 4 M (Figure 1B). Comparing the dispersibility and the ellipticity of lysozyme in various concentrations of Gdn·HCl, the dispersibility was enhanced without protein denaturation at around 2–3 M Gdn·HCl (Figure 1C), indicating that a medium concentration of Gdn·HCl is applicable as a dispersion adjuvant of protein–carbon nanotube conjugates. Important information can be inferred from Figure 1C. The dispersibility of lysozyme–SWNT conjugates is unrelated to the conformation of lysozyme in the bulk solution. Briefly, at 3 M Gdn·HCl, native lysozyme was adsorbed onto the SWNTs, but at 4 M Gdn·HCl, unfolded lysozyme was adsorbed onto them. However, SWNTs were not dispersed at 6 M Gdn·HCl. These results show that aggregation of SWNTs at high concentrations (>6 M) arises from a mechanism other than protein denaturation. Similar profiles were observed for hemoglobin (pH 3.4) and pepsin (pH 6.5) (Figure 2), with the proteins being substantially charged at the indicated pH values.[36] Therefore, it would seem that Gdn·HCl is universally applicable for the control of carbon nanotube dispersion using proteins.

Here, it is of interest to delineate how the dispersion is controlled by Gdn·HCl. The following mechanism is proposed for the dispersion using Gdn·HCl.

Mechanism of controlled dispersion of SWNTs by Gdn·HCl

Reduction of SWNT dispersibility at low concentrations of Gdn·HCl: As indicated in Figure 1, the dispersibility of SWNTs was low at low concentrations (≈ 1 M) of Gdn·HCl. Generally, electrostatic screening becomes effective at low salt concentrations. Because of the positive charge of lysozyme at pH 3.4, the electrostatic effect might be screened out by chloride ions. To demonstrate the screening effect, protein–SWNT conjugates were dispersed in the presence or absence of NaCl (Table 1). As expected, the dispersibility of lysozyme–SWNTs was decreased by 0.5 M NaCl. Similarly, the dispersibility of hemoglobin–SWNTs, which are positive-

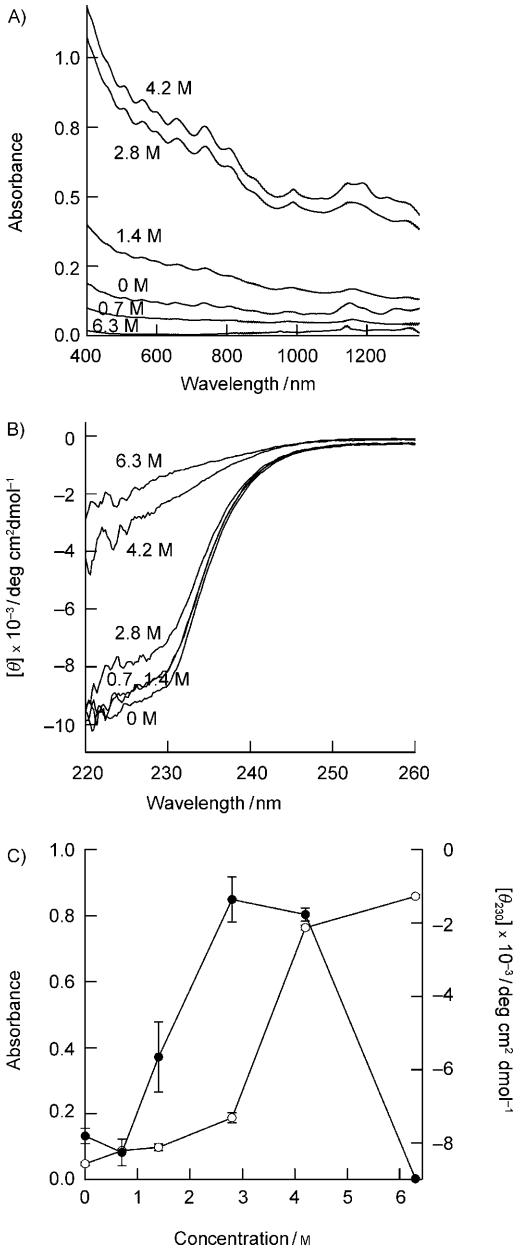

Figure 1. A) Absorption spectra of lysozyme–SWNT conjugates in the presence of Gdn·HCl. B) CD spectra of lysozyme in lysozyme–SWNT solutions. C) Dependences of the absorbance at 600 nm (closed circles) and the ellipticity at 230 nm (open circles) of the lysozyme–SWNT conjugates on Gdn·HCl concentration.

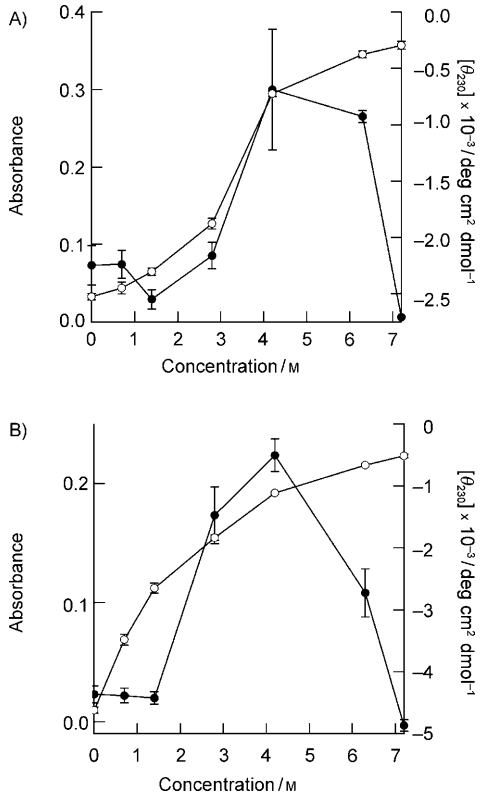

Figure 2. Dependences of the absorbances (closed circles) and the ellipticities (open circles) of A) pepsin–SWNT conjugates and B) hemoglobin–SWNT conjugates on Gdn·HCl concentration. The absorbances of pepsin–SWNT and hemoglobin–SWNT were measured at 600 nm and 900 nm, respectively.

Table 1. Effect of NaCl on dispersibility.

Proteins	NaCl [M]	Absorbance
lysozyme	0	0.0226 ± 0.0050[a]
	0.5	0.0001 ± 0.0004[a]
hemoglobin	0	0.0097 ± 0.0009[b]
	0.25	0.0047 ± 0.0021[b]
	0.5	aggregation[c]
pepsin	0	0.0220 ± 0.0023[a]
	0.5	0.0278 ± 0.0031[a]
	1	0.0255 ± 0.0051[a]
	1.5	0.0275 ± 0.0014[a]

[a] At 600 nm. [b] At 900 nm. [c] Data not obtained because of protein aggregation.

ly charged at pH 3.4, was reduced by 0.25 M NaCl and they were aggregated by 0.5 M NaCl. These results indicate that the reduced dispersibility of the conjugates resulted from the electrostatic screening effect of the chloride ion. On the other hand, the dispersibility of pepsin–SWNTs (pH 6.5), which have a negative net charge, was not decreased even at 1.5 M NaCl. This exception might be explained in terms of a weaker screening effect of sodium ion compared to guanidine.[47,48] These results are consistent with the fact that the dispersibility of protein–SWNT conjugates depends strongly on the net charge of the protein.[32,33,36]

Enhancement of SWNT dispersibility at medium concentrations of Gdn·HCl: The dispersibility of the protein–SWNT conjugates was enhanced at medium concentrations (2–3 M) of Gdn·HCl without protein denaturation (Figure 1). The data show that the concentrations of Gdn·HCl affect not the protein structure but the surface of the SWNTs. Actually, Gdn·HCl, designated as a "chaotrope", stabilizes hydrophobic compounds in aqueous solutions.[44,45] Therefore, the enhancing effect of Gdn·HCl on the dispersibility can be rationalized in terms of stabilization of the hydrophobic interface of the SWNTs. To verify this assumption, another cha-

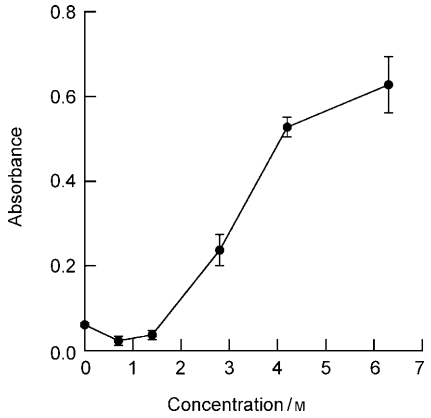

Figure 3. Dependence of the absorbance at 600 nm for lysozyme–SWNT conjugates on urea concentration.

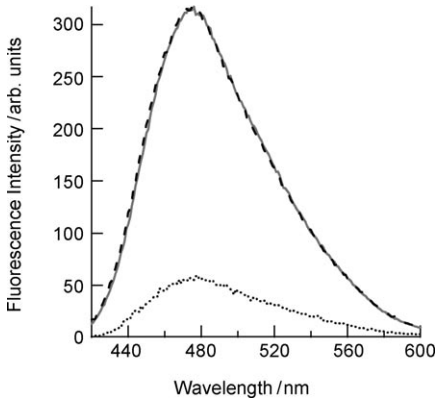

Figure 4. Dissociation of lysozyme from SWNT sidewalls in the presence or absence of 6 M Gdn·HCl (broken line) or 0.5 M NaCl (dotted line) assayed using fluorescamine. The solid grey line shows the results for a control solution.

otrope, urea, was also used as a dispersion adjuvant. Unlike Gdn·HCl, urea was found to enhance the dispersibility of lysozyme–SWNTs even at high concentrations (>6 M) (Figure 3). The effect of urea on dispersion is weaker than that of Gdn·HCl, which is consistent with the difference in their solubilization effects for hydrophobic compounds.[43,44] The data suggest that the enhancement of dispersibility at medium concentrations of Gdn·HCl is attributable to the stabilization of the hydrophobic interface of SWNT sidewalls, as our previous study also revealed.[36]

Aggregation of SWNTs and exfoliation of protein at high concentrations of Gdn·HCl: High concentrations of Gdn·HCl substantially solubilize low molecular weight organic compounds, as well as inclusion bodies of proteins because of a weakening of the interactions between molecules, including hydrophobic interactions and hydrogen bonds.[44] Based on these effects, it was hypothesized that high concentrations (>6 M) of Gdn·HCl would dissociate protein molecules from SWNT sidewalls. To confirm this hypothesis, lysozyme in the supernatants of solutions in which SWNTs had been aggregated by 6 M Gdn·HCl and 0.5 M NaCl was detected by fluorescence measurements using fluorescamine. The fluorescence intensity of the supernatant collected from aggregated SWNT solutions in the presence of 6 M Gdn·HCl was comparable to that of the supernatant collected from non-aggregated SWNT solutions in the absence of additives (Figure 4). The results indicated that pristine SWNTs formed aggregates in 6 M Gdn·HCl because of the dissociation of lysozyme from the SWNT sidewalls. On the contrary, the fluorescence intensity of the supernatant collected from aggregated SWNT solutions in the presence of 0.5 M NaCl was markedly weaker than that of the control solution (Figure 4), indicating that the aggregates contained lysozyme molecules because of the electrostatic screening of protein–SWNT conjugates using 0.5 M NaCl as described above. The remaining weaker fluorescence in the supernatant might be caused by some molecules that are prone to dissociation at some time during the course of the experiment.

To confirm the dissociation of lyzosyme from SWNT sidewalls in the presence of high concentrations of Gdn·HCl, TEM images of the samples before and after the addition of 6 M Gdn·HCl or 0.5 M NaCl and the subsequent dialysis were acquired, and these are compared in Figure 5. SWNTs after the treatment with 6 M Gdn·HCl were bundled together (Figure 5A). In high-resolution and cross-sectional TEM images, closely bundled SWNTs without lysozyme were ob-

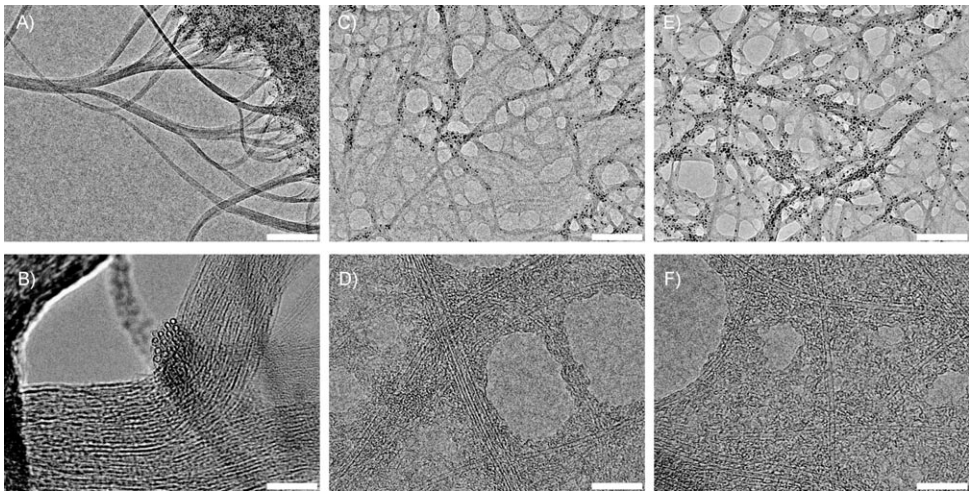

Figure 5. TEM images of samples treated with 6 M Gdn·HCl (A,B) or 0.5 M NaCl (C,D) and untreated samples (E,F). Scale bars are 100 nm (A,C,E) and 10 nm (B,D,F).

served (Figure 5B). On the other hand, SWNTs after treatment with 0.5 M NaCl were seen to be dispersed on the Cu mesh TEM grid (Figure 5C). In high-resolution TEM images, debundled SWNTs with lysozyme around their sidewalls were observed (Figure 5D). The images of the sample treated with 0.5 M NaCl are similar to those of the control (Figure 5E and F). Therefore, 0.5 M NaCl has no effect on the dissociation of protein from the SWNT sidewalls. Consequently, the results support the view that high concentrations of Gdn·HCl dissociate protein from SWNT sidewalls. This effect of Gdn·HCl might be exploited for the purification of protein-adsorbed materials, such as biosensors and scaffolds for tissue engineering.

Table 2. Comparison of the dispersibility of the conjugates, secondary structure of lysozyme, ζ-potential, and adsorption area of lysozyme on SWNTs in 0 and 2 M Gdn·HCl.

Gdn·HCl [M]	Dispersibility[a]	Ellipticity at 206 nm, $-[\theta]\times10^{-3}$ [deg cm^2 dmol^{-1}]	ζ-potential[b] [mV]	Mass ratio of protein to SWNT	Protein concentration on SWNT[c] [m^2 mg^{-1}]
0	0.10 ± 0.02	15.3 ± 1.6	27.5 ± 0.8	0.98 ± 0.13	0.52 ± 0.07
2	0.51 ± 0.07	19.3 ± 2.1	27.9 ± 0.8	1.28 ± 0.20	0.68 ± 0.11

[a] Absorbance at 600 nm. [b] Data obtained in the presence of 5 mM citrate–phosphate buffer. [c] Estimated using an approximation; adsorption area per lysozyme molecule is 4π nm^2.

Secondary structure, ζ-potential, and amount of protein adsorbed on SWNTs: Structural changes of proteins through their adsorption onto SWNT sidewalls, which lead to their inactivation, have commonly been observed, although retention of activity even upon adsorption has also been reported.[29,49] Therefore, practical information on proteins, such as their secondary structure, stability, and amount adsorbed on SWNTs, is important to delineate the impact of SWNTs at the protein molecular level and to apply them to biomaterials. Lysozyme–SWNT solutions were prepared by removal of the non-adsorbed lysozyme through dialysis. The secondary structure of lysozyme on the SWNTs differed from that of the non-adsorbed protein (Figure 6). This secondary structure was retained for more than a week (data not shown), indicating that the changed structure of lysozyme was more stable. Table 2 shows the dispersibilities of the lysozyme–SWNT conjugates, the ellipticities of lysozyme on the SWNT surfaces, the ζ-potentials of the lysozyme–SWNT conjugates, and the amount of lysozyme adsorbed on the SWNTs. These samples were prepared by ultrasonication in the presence of 0 and 2 M Gdn·HCl, with subsequent dialysis and centrifugation. Areas of adsorbed lysozyme per unit mass of SWNT were estimated from the 2 nm radius of lysozyme;[50,51] thereby, the adsorbed area of lysozyme per molecule is 4π nm^2. Although the two samples showed a fivefold difference in dispersibilities based on the absorbance at 600 nm, they were roughly identical in terms of ellipticity, ζ-potential, and concentration per unit mass of SWNT. These results support the hypothesis presented above that medium concentrations of Gdn·HCl stabilize the hydrophobic interface of SWNTs without having substantial effects on proteins. More importantly, the adsorbed areas of lysozyme per unit mass of SWNT were of the same order of magnitude as the specific surface area of the SWNT (Table 2).[52] Therefore, SWNTs can be densely covered with lysozyme molecules. The results show that Gdn·HCl stabilizes not only the protein–SWNT conjugates in the solutions because of the stabilization of the remaining hydrophobic areas, but also the debundled state of the SWNTs before protein adsorption. In other words, the debundled state was also kinetically stabilized in the ultrasonication process because the hydrophobic areas of pristine SWNTs before protein adsorption are significantly larger than those in the protein-adsorbed state.

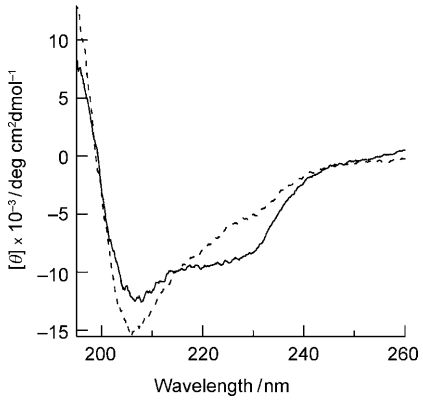

Figure 6. CD spectra of lysozyme on SWNTs. Solid line, non-adsorbed lysozyme; broken line, lysozyme–SWNT conjugates.

Mechanism of the dissociation of protein from SWNTs by Gdn·HCl: Consideration of the effect of Gdn·HCl on the dissociation and denaturation of proteins on SWNTs could be worthwhile for elucidating the physical properties of a protein on a nanostructure. As shown in Figure 4, 6 M Gdn·HCl dissociated unfolded proteins from the sidewalls of SWNTs, leading to the aggregation of pristine SWNTs. However, 4 M Gdn·HCl did not induce the dissociation of protein, even in an unfolded state (Figure 1C). Therefore, these results raise the question of the precise origin of the difference in the dispersibilities of SWNTs considering that the protein was in an unfolded state in both experiments. A schematic diagram of the proposed mechanism for dispersion induced by Gdn·HCl is shown in Figure 7. The critical point of Gdn·HCl for the dissociation is higher than that for the protein unfolding. The difference in the critical points results from the fact that stability of the adsorption on SWNTs is qualitatively higher than that of protein folding toward Gdn·HCl, which suggests that unfolded proteins can adsorb on SWNTs. Here, it is also noteworthy that heat treatment of the dispersed solutions, even at 98 °C, did not

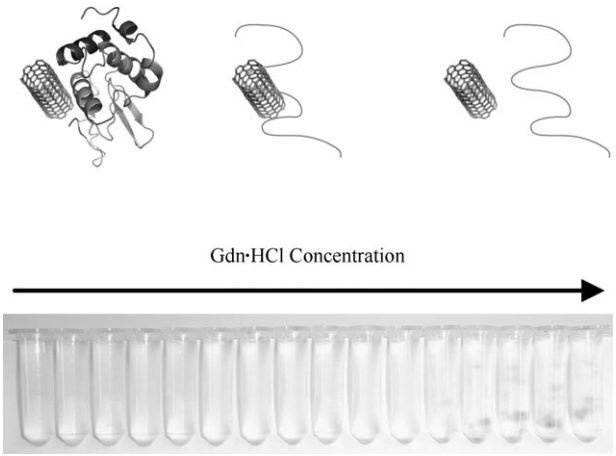

Figure 7. Schematic diagram showing the dissociation of protein from SWNTs in Gdn·HCl. In concentrated Gdn·HCl, unfolded protein is dissociated from the sidewalls of SWNTs, leading to aggregation of pristine SWNTs.

induce SWNT aggregation (data not shown). This result indicates that the thermally unfolded state of lysozyme is also capable of adsorbing on SWNTs. Consequently, these results support the suggestion made above that protein adsorption onto SWNTs is not necessarily accounted for by protein structure. Therefore, every protein is potentially capable of binding to SWNTs.

However, peptides do not necessarily adsorb onto SWNTs; the adsorption of peptides depends on their sequence.[11,22,53,55] These facts raise the question as to why unfolded proteins are capable of adsorbing onto SWNTs, as shown in this study. One answer is the hydrophobic interaction between protein and SWNTs. Proteins generally have a tertiary structure because of intramolecular hydrophobic interactions, whereas peptides usually have a disordered structure. The hydrophobic interaction of a protein is accounted for by its internal hydrophobic core composed of hydrophobic amino acid residues; this is in contrast to peptides, which lack such a core. Therefore, unfolded proteins, with their longer sequences than peptides, have higher hydrophobicity, resulting in the induction of intermolecular hydrophobic interactions, such as the protein aggregation described in our previous report.[55] The hydrophobicity induced by protein unfolding is also expected to increase the interaction with SWNTs, leading to the adsorption of unfolded protein onto SWNTs, as shown in this study. This suggestion is supported by the result that the adsorption of thermally unfolded protein on SWNTs was also retained, by which the protein exposes the hydrophobic domain. Another result, that protein was dissociated by concentrated Gdn·HCl, also supports the suggestion made above because Gdn·HCl stabilizes and solubilizes hydrophobic moieties.[44,45] Taking the results together, we surmise that proteins interact with SWNTs through a nonspecific hydrophobic interaction, in contrast to peptides, leading to the observations that both the unfolded protein in the presence of moderate concentrations of Gdn·HCl and the thermally unfolded protein adsorbed stably onto SWNTs.

The nonspecific adsorbability of proteins onto SWNTs, irrespective of their structure, causes protein denaturation, including partial unfolding, as shown in Figure 6, and subsequent inactivation beyond expectations when these materials are used as drug carriers or biosensors. This property might also engender false positives as drug aggregates.[56] Consequently, nonspecific adsorption is expected to be effectively reduced through surface modification of SWNTs.

Conclusion

In the development of protein–carbon nanotube conjugates, the role of co-solvents or additives in the solutions must be fully investigated. In this study, we have specifically examined the effects of Gdn·HCl on the dispersibility and adsorption of proteins on SWNT surfaces. Low concentrations ($\approx$1 M) of Gdn·HCl decreased the dispersibility of protein–SWNT conjugates because of electrostatic screening. A medium concentration (2–3 M) of Gdn·HCl enhanced the dispersibility without protein denaturation, which may be attributed to the interfacial stabilization of SWNTs, as seen with low concentrations of alcohols.[36] High concentrations ($>$6 M) of Gdn·HCl dissociated protein molecules from SWNT sidewalls, leading to aggregation and precipitation of pristine SWNTs. These results suggest that Gdn·HCl might be used not only as a dispersion adjuvant for protein–carbon nanotube conjugates, but also as an antibiofouling or exfoliating agent for purging their surfaces. The dispersibility was retained up to around 4 M Gdn·HCl, which is higher than the midpoint of denaturation, suggesting that the protein adsorption on SWNTs is more stable than the protein folding in the presence of Gdn·HCl. This system provides a wide view of the protein–nanoparticle interaction, which accounts not only for the activity of biomaterials but also for their nanotoxicity.

Experimental Section

Chemicals: For this study, Gdn·HCl, urea, NaOH, trisodium citrate dehydrate, and fluorescamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). They were used with chicken egg white lysozyme, bovine blood hemoglobin, porcine stomach mucosa pepsin (Sigma–Aldrich, St. Louis, MO, USA), and sodium dihydrogenphosphate dehydrate (Nacalai Tesque, Kyoto, Japan). Single-walled carbon nanotubes (SWNTs), produced by the high-pressure catalytic CO decomposition (HiPCO) process, were purchased from Unidym (Menlo Park, CA, USA). All compounds were of the highest commercially available grade. Dialysis membranes (Spectra/Por cellulose ester (CE) dialysis membrane) of MWCO 100000 were purchased from Spectrum Laboratories (Rancho Dominguez, CA).

Procedure for dispersion of SWNTs with protein: Highly dispersed SWNTs in aqueous solutions containing proteins were prepared as follows. Solutions containing 1 mg mL^{-1} of lysozyme, pepsin, or hemoglobin, citrate–phosphate buffer (50 mM, pH 3.4 or 6.5), and Gdn·HCl (0–7.2 M) were mixed with SWNT powder. The SWNTs were dispersed in the solutions by ultrasonication for 30 min at 20°C (including stirring initially

and after 15 min) using an ultrasonic processor (UT-250S; Sharp, Osaka, Japan). Highly dispersed SWNT solutions were obtained from the supernatants of these solutions after centrifugation (40000×*g* for 30 min at 25°C) using a high-speed refrigerated centrifuge (SRX-201; Tomy Seiko, Tokyo, Japan). The amounts of SWNTs in aqueous solutions were assessed by measuring the absorbance at 600 or 900 nm, which corresponded to the S22 transition after subtraction of the absorbance of the control protein solutions. The VIS-NIR absorption spectra of the products were measured from solutions in 1 cm path length quartz cells on a UV/VIS-NIR spectrophotometer (UV-3150; Shimadzu, Kyoto, Japan).

Circular dichroism spectra of protein–SWNT solutions: Circular dichroism (CD) spectra are generally measured to assess the secondary or tertiary structures of proteins. The secondary structures are typically detected as negative peaks in the CD spectra at 208 nm and 222 nm for the α-helix and at 218 nm for the β-sheet. The CD spectra of protein–SWNT solutions prepared using the process described above were measured using a CD spectropolarimeter (J-720W; Jasco, Tokyo, Japan) with a 1 mm path length cuvette. Far-UV CD spectra were obtained as a function of Gdn·HCl or urea concentration.

Detection of lysozyme dissociated from SWNT sidewalls by additives: Lysozyme–SWNT conjugates were dispersed by ultrasonication for 30 min at 20°C (including stirring initially and after 15 min) using an ultrasonic processor in the presence of 4 M Gdn·HCl solution. The dispersed lysozyme–SWNT solutions were then dialyzed against large volumes of distilled water for 34 h at room temperature to remove the non-adsorbed lysozyme and Gdn·HCl using cellulose ester dialysis membranes (MWCO 100000). The dialyzed solutions were then centrifuged (16800×*g* for 20 min at 25°C) to remove insoluble materials using a high-speed microcentrifuge (Himac CF 15RX; Hitachi Koki, Tokyo, Japan). Highly dispersed SWNTs solutions obtained from the supernatants were diluted to a final concentration of 6 M Gdn·HCl or 0.5 M NaCl and 40 mM citrate–phosphate buffer (pH 3.4), resulting in precipitation of the SWNTs. The samples were again centrifuged (16800×*g* for 20 min at 25°C) to remove SWNTs using a high-speed microcentrifuge. A control solution was obtained by dilution of the highly dispersed SWNT solution with a final concentration of 40 mM citrate–phosphate buffer (50 mM, pH 3.4) without subsequent centrifugation. Lysozyme contained in the supernatants after the centrifugation process or in the control solution was detected using the measurements described below.

Detection of lysozyme in solutions using fluorescamine: For the sensitive determination of lysozyme in solutions, fluorescamine was used as follows. Sample solutions (100 μL) were diluted with sodium phosphate buffer (1500 μL, pH 8). After rapid mixing of the resulting solution with 0.3 mg mL^{-1} of fluorescamine in dioxane (500 μL), the fluorescence intensity of the solutions was detected using a spectrofluorimeter (FP6500; Jasco, Tokyo, Japan). The concentration of lysozyme was estimated by comparison with the standard curve of native lysozyme (see the Supporting Information, Figure S1).

Transmission electron microscopy images of lysozyme–SWNT conjugate: To confirm the dissociation of lysozyme from SWNT sidewalls in the presence of a high concentration of Gdn·HCl, TEM (JEM2100, JEOL) was used at an accelerating voltage of 200 kV. One drop of the sample solution was placed on a Cu mesh TEM grid. The TEM grid was completely dried in air before the TEM measurements.

Samples for TEM measurements were obtained as follows. Highly dispersed lysozyme–SWNT conjugates, which were prepared through the addition of Gdn·HCl and subsequent dialysis as shown above, were precipitated by their dilution to a final concentration of 6 M Gdn·HCl or 0.5 M NaCl. Finally, the solutions were dialyzed against large volumes of distilled water for 34 h at room temperature to remove the dissociated lysozyme from SWNT sidewalls and the coexisting salts using cellulose ester dialysis membranes (MWCO 100000). A control solution was obtained without the addition of any salt and the subsequent dialysis, that is, a solution of highly dispersed lysozyme–SWNT conjugates.

Secondary structure, ζ-potential, and adsorption areas of lysozyme on SWNTs: Lysozyme–SWNT solutions were obtained through ultrasonication, dialysis, and centrifugation processes in the presence of 0 and 2 M Gdn·HCl, as described above. The CD spectra of the dispersed samples mixed with a final concentration of 10 mM sodium acetate buffer (pH 3.4) were measured. The ζ-potential of the conjugates in the dispersed sample, which were mixed with a final concentration of 5 mM citrate–phosphate buffer (pH 3.4), was measured using a zeta potential analyzer (He–Ne laser, Zetasizer Nano ZA; Malvern Instruments, UK). The respective concentrations of the adsorbed lysozyme and SWNTs in the dispersed solutions were quantified using the fluorescence of fluorescamine as described above and the absorbance at 600 nm (see the Supporting Information, Figures S1 and S2). Based on the quantified concentrations of lysozyme and SWNT, the amount of adsorbed lysozyme per unit mass of SWNT was determined. Adsorbed areas of lysozyme per unit mass of SWNT were estimated under the assumption that the radius of lysozyme is 2 nm, such that the adsorbed area of lysozyme per molecule is 4π nm^2.[50,51]

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