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Evidences For Charge Transfer-Induced Conformational Changes In Carbon Nanostructure-Protein Corona

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Abstract

The binding of proteins to a nanostructure often alters protein secondary and tertiary structures. However, the main physical mechanisms that elicit protein conformational changes in the presence of the nanostructure have not yet been fully established. Here we performed a comprehensive spectroscopic study to probe the interactions between bovine serum albumin (BSA) and carbon-based nanostructures of graphene and single-walled carbon nanotubes (SWNTs). Our results showed that the BSA “corona” acted as a weak acceptor to facilitate charge transfer from the carbon nanostructures. Notably, we observed that charge transfer occurred only in the case of SWNTs but not in graphene, resulting from the sharp and discrete electronic density of states of the former. Furthermore, the relaxation of external α -helices in BSA secondary structure increased concomitantly with the charge transfer. These results may help guide controlled nanostructure-biomolecular interactions and prove beneficial for developing novel drug delivery systems, biomedical devices and engineering of safe nanomaterials.

Keywords

Protein corona; charge transfer; graphene; carbon nanotubes; Raman spectroscopy; FTIR spectroscopy

I. INTRODUCTION

It is well known that proteins adsorb to the surface of nanostructures (particles, tubes, wires and sheets) that are entrained in a biological medium to form a soft nanostructure-protein corona (NS-PC).^{1–4} Subsequently, the soft NS-PC exchanges the adsorbed proteins with other free proteins through equilibration to form a hard corona. For example, human serum albumin and fibrinogen may be predominantly adsorbed onto a nanostructure surface for short periods of time due to their abundance in biological systems, however these proteins may be displaced by lower-abundant proteins such as apolipoprotein which possess a higher affinity for the nanostructure.^{1–3} The dynamics of protein association/dissociation depends on the physicochemical properties of the nanostructure such as surface properties, charge, shape and size. Importantly, these dynamic processes play a vital role in determining the

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interactions between NS-PC and biological receptors.^{3–6} Thus, it is essential to understand the physical nature of the interaction between proteins and nanostructures, which determine the fate of nanostructures in any biological environment.

The non-specific binding of proteins to a nanostructure is one of the fundamental problems in nanotoxicology and cancer nanomedicine.⁷ Such non-specific binding and subsequent corona formation can mediate the uptake of the nanomaterial via receptor-mediated endocytosis.⁷ Thus, same nanostructures may exhibit different toxic response in different media. In case of nanomedicine applications, nanostructures conjugated with therapeutic agents can be opsonized due to non-specific binding of some plasma proteins (especially the proteins involved in the complement system such as C3). The main blood plasma proteins involved (both directly and indirectly) in opsonization are albumins, fibronectins, complement proteins, fibrinogen, immunoglobulins and apolipoprotein. This opsonization process leads to phagocytosis of the nanostructure by monocytes/macrophages and ultimately promoting an immune response and inflammation. Although, many nanostructures (such as carbon nanotubes, metal nanoparticles) may only be partially degraded by lysozyme or perforins, phagocytosis is detrimental to the conjugated therapeutic payload.⁷

The binding of proteins to a nanostructure during the corona formation can also alter protein secondary structures and, in the extreme case, lead to protein unfolding.^{3, 4, 6, 8} Any such changes in the protein (present in NS-PC) conformation are often irreversible and permanent.⁹ It is important to note that multi-layer protein adsorption can also occur via protein NS-PC interactions.^{6, 10} On the other hand, the NS-PC induced conformational changes can expose charged or hydrophobic domains in the adsorbed protein resulting in non-specific (adsorbed) protein-(free) protein interactions. Such unnatural protein-protein interactions mediated by the NS-PC may further cause an adverse immune response.

The physical causes that elicit protein structural changes are not fully understood. Yet it is plausible that the difference in electronegativity between the protein and the nanostructure may induce charge transfer. Any perturbation generated by such charge transfer, along with hydrogen bonding breakage, thermodynamic fluctuations, changes in pH and salt strength, as well as crowding may cause protein unfolding and denaturation. It is understood that charge transfer between a protein and its substrate plays an important role in several physiological processes such as blood clotting, and is often exploited for designing improved sensors for biomolecular detection.^{11–15} Biomedical implants such as coronary stents often face problems of blood clotting in their vicinity due to the charge transfer between fibrinogen and the stent material. Thus, a fundamental understanding of the charge transfer mechanism and ensuing protein conformation changes in NS-PC are of crucial importance for developing novel nanomaterials with minimal adverse physiological responses.

Here, we investigated the relationships between charge transfer, secondary structure changes in protein bovine serum albumin (BSA), and nanostructured carbon morphologies (graphene and single-walled carbon nanotubes or SWNTs) using micro-Raman spectroscopy, photoluminescence, UV-Vis spectrophotometry, infrared absorption and electron microscopy. We show that charge transfer occurs only when BSA binds to SWNTs but not graphene sheets, which is attributed to the unique electronic density of states (EDOS) of the SWNTs. Further, the charge transfer between BSA and SWNTs leads to enhanced relaxation of α -helices in BSA secondary structure. Finally, our photoluminescence study indicates that the BSA adsorption onto the carbon nanostructures follows the multi-layer Freundlich type isotherms.^{16–20}

II. RESULTS AND DISCUSSION

Electron microscopy and SDS-PAGE

The chemical vapor deposition (CVD) method yielded polycrystalline graphene that mimicked Ni crystal grains, as shown in Fig. 1a. After incubating in BSA solution, graphene grains were not as clearly visible as in the pristine sample due to significant coverage of the proteins on the substrate (Fig. 1b). Figures 1c & d show transmission electron micrographs of BSA-coated SWNTs and exfoliated graphene, respectively. As seen in Fig. 1d, the adsorbed proteins formed islands on the flat surface of exfoliated graphene nanosheets. In contrast, large BSA ($4 \times 4 \times 14 \text{ nm}^3$) domains encompassed SWNTs (diameter $\sim 1.4 \text{ nm}$) completely covering the SWNT surface (Fig. 1c). Our microscopy result suggests that all SWNT samples were well coated with the proteins. Furthermore, SDS-PAGE confirmed the presence of hard corona in our samples. Specifically, we employed rigorous centrifugation and several subsequent wash steps to remove any unbound proteins and soft corona (see materials and methods). Figure 2 shows lanes from coomassie-stained gels for SWNTs and exfoliated graphene incubated in BSA. Clearly, the last two columns exhibit bands of $\sim 65 \text{ kDa}$ confirming that SWNTs and exfoliated graphene samples contained BSA hard corona.

UV-Vis spectroscopy

It is well known that SWNTs strongly absorb in the near-infrared region.²¹ Their absorption spectrum exhibits several peaks related to the electronic transitions connecting the i^{th} van Hove singularities (vHS) in valence and conduction bands (or E_{ij}). Any subtle changes in the surrounding environment of SWNTs are clearly reflected in the absorption spectrum due to these sharp features in the SWNT density of states. For instance, O'Connell *et al.*²² showed that the SWNT optical absorption peaks upshifted in energy when bundled SWNTs were processed into sodium dodecyl sulfate (SDS)-coated isolated SWNTs. Figure 3 shows the UV-Vis absorption spectrum for pristine and BSA-coated SWNTs. It is evident that all SWNTs E_{ij} blue shifted upon protein coating, akin to SDS micellar coated, isolated SWNTs.²² As shown in Fig. 3b, the UV-Vis absorption spectrum of graphene exhibited a pronounced resonance (known as the *pi-plasmon* peak) at $\sim 4.7 \text{ eV}$ ($\sim 270 \text{ nm}$) which arises from the electronic transitions near the *M*-point (from *pi* to *pi*^{*} orbital) in the Brillouin zone of graphene.^{23, 24} In addition to this *pi-plasmon* peak, the locally flat energy dispersion of graphene (saddle point) near the *M*-point led to strong absorption at $\sim 5.7 \text{ eV}$ ($\sim 215 \text{ nm}$). Unlike the SWNTs spectrum, the UV-Vis spectrum of graphene did not change significantly upon protein coating. This result is expected, as the interactions between graphene layers are known to have little or no effect on their *pi-plasmon* feature in the optical absorption spectra.^{23, 24}

Photoluminescence and adsorption kinetics

The dynamic nature of protein corona formation warrants a detailed kinetic study of protein adsorption on nanostructures. Previously, Langmuir and Freundlich isotherms have been extensively used to describe the formation of protein corona on noble metal and polymer nanoparticles.^{16–20, 25, 26} In our case, Langmuir adsorption is insufficient to describe the NS-PC formation since it limits the adsorption to a single unit of adsorbate per adsorption site. In contrast, Freundlich isotherm allows multi-particle adsorption and is therefore more suitable for describing the NS-PC formation.¹⁶ The Freundlich isotherm is often expressed in the following logarithmic form.¹⁷

$$\log(\alpha) = \frac{1}{n} \log(C) + \log(k_f) \quad (1)$$

Here, α is the amount of adsorbed protein, C is the initial concentration of adsorbed BSA, and n , k_F are fitting parameters. As shown in Fig. 4a, we used photoluminescence (PL) spectroscopy to study the adsorption kinetics of BSA on SWNTs/graphene. The aromatic amino acids (mainly, tryptophan) in BSA absorb at ~280 nm and emit strongly at ~338 nm. Thus, the PL intensity of the emission peak (~338 nm) can be used to determine the concentration of BSA using the calibration curve shown in Fig. 4a. It is important to note that all the PL spectra (in Fig. 4) were normalized with respect to the excitation intensity. This calibration curve was obtained by fitting the PL intensity (~338 nm) of native BSA alone at various concentrations. Figures 4b and c show the PL spectra for SWNTs/graphene incubated in different BSA concentrations. We obtained the amount of BSA adsorbed per mg of SWNTs/graphene by the combined analysis of the calibration curve and (cf. Fig. 4a) Figs. 4b, c. As shown in Fig. 4d, the Freundlich model fits our data very well with moderately high R^2 values, confirming that the BSA adsorption was indeed multi-layered in both cases of SWNTs and graphene.

In the Freundlich model, the NS-PC formation proceeds via dynamic absorption and desorption of proteins from the nanostructure surface. Thus, the rate of change in protein concentration may be expressed as

$$\frac{dC}{dt} = -k_1 C^i + k_2 \alpha^j. \quad (2)$$

In eqn. 2, k_1 (k_2) is adsorption (desorption) rate constant and i (j) is the order of the reaction. At equilibrium, eqns. 1 and 2 may be re-written as

$$\log \alpha = \log K + \left(\frac{1}{n}\right) \log C$$

$$n = \frac{j}{i}, K = \left(\frac{k_1}{k_2}\right)^{1/j} \quad (3)$$

Our fits show $1/n > 1$ suggesting that the adsorption (and desorption) proceeded in pseudo-first order. Furthermore, the values of $\log K$ (~1.1 for SWNTs and ~0.85 for graphene) suggest that desorption of BSA occurred faster than adsorption for both SWNTs and graphene. Such observations are in accordance with Milani *et al.*, who reported two different time scales for multi-layered protein adsorption. According to their fluorescence correlation spectroscopy studies, soft corona exchanges proteins at a much faster scale (~1 min) compared to the hard corona (few hours), in agreement with the higher desorption rates observed in our experiments.

Micro-Raman spectroscopy

SWNTs and graphene are known to exhibit strong Raman features due to resonance effects. Notably, the tangential band (*G-band*) of SWNTs that arises from the planar vibration of carbon atoms (longitudinal and transverse vibrations in SWNTs) was previously found to be highly sensitive to charge transfer (see Fig. 5).^{27, 28} The *G-band* frequency is known to upshift (downshift) when any acceptor (donor) species interacts with SWNTs or graphene via hole (electron) transfer. Importantly, the lineshape of the *G-band* deviates from a symmetric Lorentzian to an asymmetric Breit–Wigner–Fano (BWF) lineshape due to any charge transfer.^{27, 28} The BWF lineshape is described as:

$$I(\omega) = I_0 \frac{\left(1 + \frac{\omega - \omega_0}{q\Gamma}\right)^2}{\left(1 + \left(\frac{\omega - \omega_0}{\Gamma}\right)^2\right)} \quad (4)$$

Here I is the peak intensity, ω is the frequency, $1/q$ is the interaction parameter and Γ is peak width. Figure 5 shows the Raman spectra of SWNTs before and after the protein coating. Pristine SWNTs exhibited 2 peaks ~ 1565 (G^-) and 1589 cm^{-1} (G^+), corresponding to the longitudinal and transverse vibrations of carbon atoms. In our pristine samples, both G^- and G^+ features can be fit using a Lorentzian lineshape (as shown by deconvoluted fits in Fig. 4). Interestingly, BSA-coated SWNTs exhibit a BWF lineshape unlike pristine SWNTs, indicating a charge transfer between the BSA and the SWNT substrates. The BWF fit (eqn. 4) to BSA-coated SWNT Raman spectrum yielded a renormalized frequency of 1593 cm^{-1} with $1/q \sim 0.61$. This upshift of G -band was a result of the SWNT phonon frequency renormalization occurring due to electron transfer from the SWNTs to the BSA. Such upshift was also observed for CVD grown SWNTs confirming the validity of charge transfer mechanism (see supporting information). The aromatic amino acids present in BSA (tryptophan, phenylalanine, histidine, and tyrosine) could interact with the unhybridized p_z orbitals of the SWNTs, via providing a weak acceptor level in the SWNT density of states to allow partial charge transfer from the SWNT to BSA (Fig. 6a). It is worth noting that the introduction of a relatively weak acceptor level (from the BSA) could still affect the Raman spectrum of the SWNTs, which possess sharp and discrete EDOS. On the contrary, graphene (both CVD grown and exfoliated) do not contain such discrete features in their electron density of states. In fact, the only ν_{HS} in graphene occurs at the M-point in the Brillouin zone due to locally flat energy dispersion (see Fig. 6b). Figs. 5b & c show the Raman spectra of CVD and exfoliated graphene before and after protein coating. Unlike SWNTs, in the case of graphene, the G-band is a single peak present at ~ 1585 cm^{-1} since the longitudinal and transverse vibrations are degenerate in graphene plane. Remarkably, we did not observe any evident shifts or lineshape changes in the G-band of graphene compared to SWNTs upon protein coating. The interaction with BSA did not affect the Raman spectrum of graphene since the EDOS is continuous in graphene. Further, the presence of inter-layer van der Waal interaction (in exfoliated few-layer graphene) and the Ni substrate (in CVD grown graphene) may also inhibit charge transfer between graphene and BSA.

FTIR spectroscopy

FTIR spectroscopy has been widely used for determining the secondary structure of proteins. The differences in orientation of amide bonds in the protein peptide backbone can reveal the composition of its secondary structures.^{19, 29} As shown in Fig. 6c, native BSA exhibited a strong absorbance ~ 1651 cm^{-1} due to the presence of α -helices. The small shoulder seen at lower energy ~ 1626 cm^{-1} occurs due to extended chains and the β -sheets. Interestingly, the FTIR spectrum for BSA bound to SWNTs exhibited a much stronger absorption at 1626 cm^{-1} , rather than 1651 cm^{-1} , indicating that the α -helices became less compact. We attribute this change in the BSA secondary structures to the charge transfer between the SWNTs and the BSA, as shown in the micro-Raman spectrum (cf. Fig. 5). It is possible that the charge transfer led to disruption to the electrostatics and breakage of the peripheral H-bonds in the α -helices. In general, the downshift of IR absorbance occurs when the α -helices of proteins transform into more open and random chains. Such downshift in IR absorption was indeed observed for BSA-coated exfoliated graphene as well. However, in the case of graphene, the FTIR spectrum exhibited a strong peak at ~ 1632 cm^{-1} , suggesting that the α -helices of the BSA adsorbed on the graphene were more compact than that in SWNT-BSA. This difference concurs with the absence of charge transfer as seen in the

Raman spectra for graphene-BSA binding (cf. Fig. 5). Collectively, our FTIR, UV-Vis, and micro-Raman measurements all pointed to the direction that BSA formed a hard corona around SWNTs to enable a charge transfer that disrupted the secondary structures of the BSA. These secondary structure changes are of crucial importance since they may alter cell immune responses evoked by the introduction of nanomaterials.

CONCLUSIONS

In summary, we found that the morphology of nanostructures exerts a strong influence on their interactions with proteins. We observed that the binding of BSA led to a blue-shift in the optical absorption of SWNTs, and did not effect on the properties of graphene. Interestingly, the *G*-band of BSA-coated SWNTs exhibited a typical Breit-Wigner-Fano lineshape that is indicative of a charge transfer. In contrast, BSA-coated graphene (both CVD-grown and exfoliated) did not exhibit a notable change in its Raman spectra when compared to the corresponding spectrum of pristine graphene. Such spectroscopic differences exhibited by graphene and SWNTs is attributed to the presence of sharp, discrete vHS in the one-dimensional SWNTs. Importantly, the charge transfer between the BSA and the SWNTs triggered significant conformational changes in the secondary structures of the BSA by relaxing their external α -helices. Such structural changes have great implications for understanding the formation and dynamics of nanoparticle-protein corona and their impact on biological responses.

MATERIALS AND METHODS

Purified arc-discharge grown SWNTs (diameter $\sim 1.4 \pm 0.3$ nm) were purchased from Carboxlex, Inc., Lexington, KY. Bi-layer graphene was synthesized using a chemical vapor deposition (CVD) technique and few-layer graphene nanosheets were prepared via solvent-based chemical exfoliation procedure.³⁰ For CVD, 25 μ m Ni foils were placed away from the center of tube furnace (diameter: 24 mm), which was maintained at 900°C under a flow of Ar (230 sccm) and H₂ (120 sccm).³¹ After 60 min, Ni foils were moved to the center and graphene was synthesized by decomposing methane (10 sccm) for 10 min at a reduced temperature (850°C). Subsequently, methane flow was shut off and the samples were moved away from the center. The furnace temperature was ramped down to 400°C at 5°C/min and was maintained at 400°C for 90 min. The H₂ flow was shut off immediately upon reaching 400°C, and the samples were cooled down to room temperature under Ar flow. For solvent exfoliation of graphene, bulk graphite (~ 1 g) was dispersed in 100 ml of N-methyl-2-pyrrolidinone (NMP) and sonicated using 1/8" tip sonicator (Branson 250) at 100 W for 2 hrs. The resulting dispersion was filtered through 0.45 μ m nylon filter and re-suspended in 100 ml of fresh NMP. Subsequently, the solution was bath sonicated for 6 hrs and centrifuged at 500 rpm for 45 min. The supernatant was vacuum filtered using a 0.45 μ m nylon filter. Finally, the filtered powder was washed several times using deionized water to remove residual NMP.

For protein binding, SWNTs or exfoliated graphene were added to a BSA solution of 3 g/dL in PBS and bath sonicated for 5 min to achieve good dispersion. These dispersions were incubated at 37°C. After 1 h of incubation, the samples were centrifuged (at 1500 rpm for 15 min) and the obtained pellets were washed in deionized water and then re-suspended in PBS. Three such wash steps were employed in order to remove any soft corona and unadsorbed BSA. Finally, the pellets were washed and resuspended in PBS for UV-Vis absorption studies and gel electrophoresis. The aforementioned rigorous wash steps were necessary to remove any unbound albumin and confirm the hard corona of the protein. For Raman spectroscopy, the re-suspended pellets were vacuum filtered and air-dried overnight at room

temperature. For FTIR spectroscopy, 2 mg of each sample was mixed with 98 mg of KBr and cold pressed into a pellet.

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed using a 4–20% gel (Bio-Rad, USA). The gel was stained with Coomassie blue. A *Smart* Protein standard (Genscript, USA) was used to identify and analyze the presence of BSA bound to the samples. UV-Vis absorption measurements were performed using a Perkin-Elmer Lambda 950 spectrometer. Micro-Raman spectra were collected using a Dilor XY triple grating spectrometer equipped with TE-cooled CCD coupled to an Ar⁺ laser excitation at 514.5 nm. Photoluminescence measurements were performed using Horiba Jobin Yvon Nanolog spectrometer equipped with TRIAX 550 Liq. N₂ cooled CCD. The FTIR spectra of the samples were measured using a Bruker IFS v66 spectrometer. Hitachi S-3400N and H-7600 microscopes were used for scanning and transmission electron microscopy measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) SEM image of pristine graphene grown on Ni foils showing that the graphene grain structure (see dashed white lines) is similar to the underlying Ni crystal domains (scale bar: 1 μm). b) SEM image of BSA-coated graphene showing significant coverage of protein (white spots) on the graphene (shown in green) substrate (scale bar: 1 μm). c) TEM image clearly shows that the SWNTs (scale bar: 100 nm) are completely coated by BSA (shown in dashed white lines). The arrow points at the uncoated end of SWNT (see Fig. S1 for corresponding TEM image of pristine SWNTs). d) TEM image shows islands of BSA (black spots) on the flat surface of graphene (scale bar: 100 nm).

Figure 2.

Coomassie-stained gel lanes for different samples. The left column, “Ladder”, represents the molecular weight standard lane. The following lane, “BSA”, shows a strong band for BSA of ~65 kDa. The last two lanes, “Gr-BSA” and “SW-BSA”, show the protein coronas formed around exfoliated graphene and SWNTs incubated in BSA. The existence of the BSA bands in SWNTs and graphene suggests the presence of hard corona.

Figure 3.

The top panel shows the optical absorption spectra of pristine and BSA-coated SWNTs. Protein coating led to a downshift in the optical spectra of SWNTs. The bottom panel shows that the optical absorption spectrum of graphene did change upon protein adsorption.

Figure 4.

a) Standard photoluminescence curve of BSA at various concentrations (emission at 338 nm), b) and c) show the photoluminescence from the BSA-SWNT and BSA-graphene suspensions. d) The amount of adsorbed BSA per mg of SWNTs/graphene exhibits power-law dependence with the initial BSA concentration, suggesting Freundlich type multi-layer adsorption (see text for more detail).

Figure 5.

a) Raman spectra of pristine and BSA-coated SWNTs. Pristine SWNTs clearly exhibited a Lorentzian lineshape while the BSA-coated SWNT spectrum was not only upshifted but also exhibited a typical Breit-Wigner-Fano lineshape. BSA Raman spectrum is shown for completeness. b) & c) show that the Raman spectra of BSA-coated CVD and exfoliated graphene did not exhibit any changes upon protein coating.

Figure 6.

a) The EDOS of SWNTs exhibit sharp and discrete singularities. BSA provides a weak acceptor level above the valence band leading to an electron transfer from SWNT. b) The EDOS of graphene are continuous unlike SWNTs. The only singularity in graphene occurs at the M-point (shown by thicker lines). The continuous nature of graphene EDOS prevents any charge transfer to BSA. c) FTIR spectra for native BSA, BSA-coated SWNTs and graphene show that the nanostructures significantly affected the secondary structures of the proteins. The peak α -helix absorption in native BSA $\sim 1651\text{ cm}^{-1}$ decreased to 1626 cm^{-1} (1632 cm^{-1}) in SWNTs (graphene), suggesting that the secondary structures of the hard coronas were less compact.