Chapter 1

Spectroscopic Insights into the Nano-Bio Interface

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Additional information is available at the end of the chapter

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Abstract

Engineered nanomaterials (ENMs) strongly interact with biomolecules due to their unique physicochemical properties. From the standpoint of nanotoxicity, it is imperative to achieve a comprehensive understanding of various nano-bio interactions to ultimately design benign ENMs that do not elicit adverse physiological responses. Spectroscopic tools are ideal for elucidating the underlying biophysical mechanisms of nano-bio interactions. In this chapter, we review spectroscopy techniques, such as Raman, infrared, circular dichroism (CD), and hyperspectral imaging, to illuminate the nano-bio interface. Particularly, we discuss the role of spectroscopic tools in gaining a fundamental understanding of the formation and influence of protein corona on ENM physiological responses.

Keywords: protein corona, spectroscopy, nano-bio interface, silver

1. Introduction

The advancement of nanotechnology over the past two decades has spurred the fields of health care, information technology, energy, homeland security, food safety, and transportation; and the global market for nanotechnology-related products reached more than $200 billion in 2009 with a projected $1 trillion per annum by 2015 (US Senate Committee, 2011) [1]. Despite this enormous global market, there remain several concerns regarding the impact of engineered nanomaterials (ENMs) on biological responses in living organisms and the environment at large [1–7]. A comprehensive knowledge of the ENM-biomolecular interactions is central to applications in nanomedicine, consumer goods, and other unintentional exposures. Currently, there are more than 40 nanopharmaceuticals in routine clinical use, and the patents and publications on

nanomedicine have been exponentially increasing [8]. Considering that nanomedicine efforts are a sudden convergence of contrasting scientific disciplines (e.g., materials science, bioengineering, pharmacology), the advancement and acceptance of nanotechnology rely heavily on a holistic interdisciplinary understanding of the impact of fundamental properties of ENMs (such as their morphology, size, defects, and chemical stability) on physiological and environmental systems.

Upon introduction into a biological system, ENMs rapidly associate a variety of macromolecules including proteins, peptides, amino acids, fatty acids, lipids, and other organic matter forming protein biocorona [9–11]. The formation of the corona is dictated not only by the physicochemical properties of the ENM but also by the composition of the physiological environment [12]. The addition of the corona on the surface of the ENM imparts a new distinctive interactive surface, which influences activity, deposition, clearance, and cytotoxicity [13–15]. The biocorona has also been shown to compromise the targeting capacity of functionalized ENMs and subsequently hinder delivery therapy [11, 16, 17]. In addition, the inconsistencies observed between *in vitro* and *in vivo* extrapolation of ENM toxicity are likely contributable to differences in ENM-biocorona formation [9, 18]. Spectroscopic tools are ideal for exploring the biological interactions of ENMs with proteins [19–21]. As discussed in this chapter, combining spectroscopic tools with traditional toxicological studies can provide unique insights into the nano-bio interface that could be used to ultimately design benign ENMs.

2. Spectroscopic insights into protein corona

2.1. Hyperspectral imaging

Gold and silver nanoparticles display a collective and resonant oscillation of surface electrons, known as plasmons, upon light excitation in the visible region (400–700 nm). The surface plasmon resonance is highly sensitive to both nanoparticles aggregation and the dielectric constant of the environment surrounding them. In a biological medium, the presence of protein corona around nanoparticles alters the SPR peak by modifying the dielectric constant [22]. Similarly, their cellular uptake could lead to aggregation, which is known to red shift the SPR peak [23]. Hyperspectral imaging is an excellent tool that combines microscopy and spectroscopy in real time by accumulating reflectance spectrum for each pixel in a micrograph. Thus, the state of nanoparticles and protein corona upon cellular uptake can be gleaned from the hyperspectral micrographs [24–26]. Recently, we explored the cellular uptake of silver nanoparticles (Ag NPs) with and without protein corona using hyperspectral imaging [23]. Our studies revealed intracellular modifications resulting from protein corona formation as shown in **Figure 1**. Changes in the microenvironment of AgNPs were evidently reflected in the shift of plasmon energies allowing us to differentiate between intra- and extra-cellular nanoparticles. Hyperspectral imaging presents an alternative to traditional electron microscopy methods for the identification of nanoparticles and protein corona inside the cell [27–30]. The exhaustive sample preparation needed for electron microscopy, such as encapsulation in a polymer followed by microtoming, often leads to artifacts. For example, the large agglomerates formed during the electron microscopy sample preparation are indistinguishable from agglomerates resulting from the loss of nanoparticles surface coating upon entering biological media.

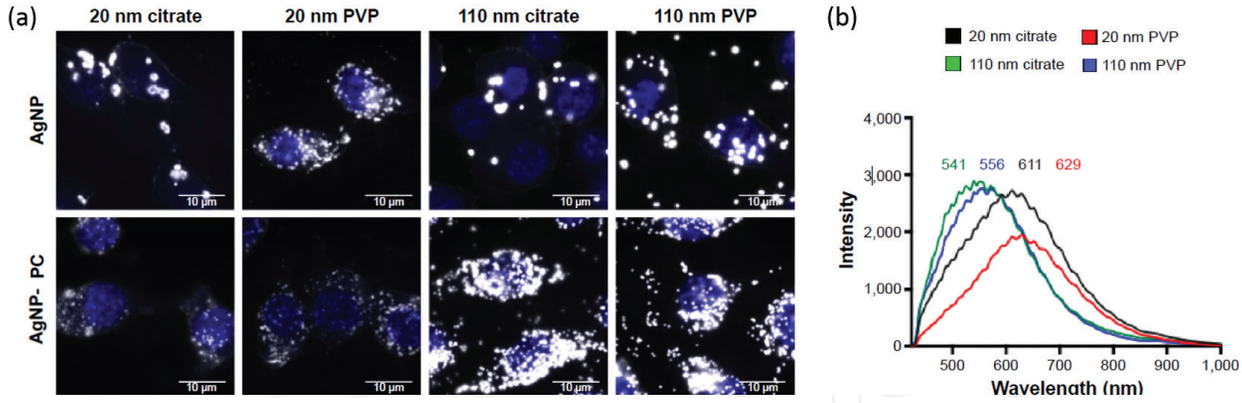

Figure 1. (a) Hyperspectral-enhanced darkfield images of macrophages exposed to AgNPs and AgNPs with protein corona for 2 hours, at a concentration of 25 µg/mL. Macrophage nucleus appears blue in the images due to DAPI stain. (b) Differences in mean spectra for AgNPs with different sizes (20 and 110 nm) and coatings (PVP and citrate). Based on such shifts in plasmon energies, it is possible to study the uptake and modification of AgNPs using hyperspectral imaging.

2.2. Circular dichroism (CD) spectroscopy

ENM-protein complex formation poses concerns on potential denaturation of proteins, which can alter protein binding to receptors and induce inflammatory responses. Lysozyme, for example, adsorbed on gold nanoparticles (AuNPs) was observed to induce misfolded proteins which likely catalyze the formation of aggregates [31]. Similarly, fibrinogen was found to unfold on the surface of stabilized (negatively charged with polyacrylic acid) AuNPs and bind to integrin receptor (MAC-I) leading to inflammatory response [32]. Circular dichroism (CD) spectroscopy has been extensively used to study conformational changes in biomolecules [33–35]. CD studies on ENM protein corona can elucidate the changes in protein secondary structures ensuing from their adsorption to ENMs surface [36]. For example, apolipoprotein (ApoA-I) protein, which is known to be abundant in the protein corona of metal nanoparticles, exhibited secondary structural changes dependent on the surface coating (**Figure 2**). ApoA-I is the major lipoprotein component of high-density lipoprotein (HDL). It adopts a shape similar to a horseshoe of dimensions 12.5 x 8 x 4 nm with high α-helix content [37–39]. The helices in ApoA-I are predicted to be amphipathic, with the hydrophobic (/hydrophilic) face mediating lipid (/aqueous) interactions. The thermodynamic drive to minimize the aqueous exposure of the hydrophobic residues is one of the major factors in ApoA-I adsorption on AgNPs [40, 41]. We studied the interactions between ApoA-I and 100-nm AgNPs with four different coatings, viz., citrate, polyvinylpyrrolidone (PVP), branched polyethylenimine (bPEI), and lipoic acid [42]. These coatings were chosen to provide both negative (citrate, PVP, lipoic acid) and positive charged surfaces (bPEI) with different affinities for AgNPs. While lipoic acid interacts strongly through Ag-S bonds, other coatings (citrate, PVP, bPEI) are considerably weaker. As shown in **Figure 2**, CD studies showed a significant decrease in α-helical content for all surface coatings with the complete disappearance of α-helices for AgNP-bPEI and AgNP-lipoic acid. From a physiological standpoint, we found a significant increase in the ability of ApoA-I-coated AgNP-bPEI and AgNP-lipoic acid to generate reactive oxygen species due to its unfolding. The conformational changes observed in CD can provide more information on ENM-protein corona complex and may be even used as a predictor for adverse immune responses.

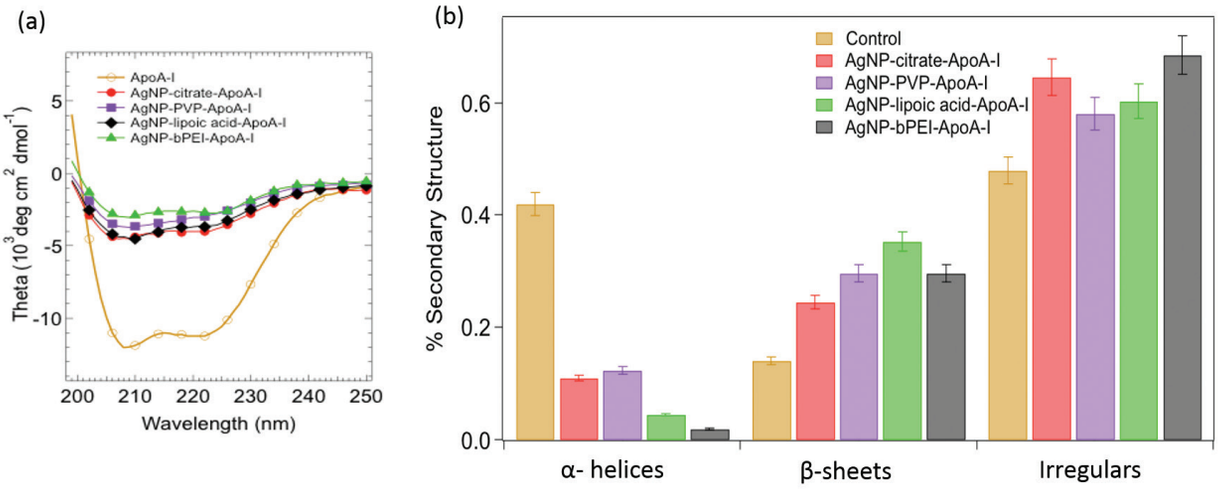

Figure 2. (a) Circular dichroism (CD) spectra for ApoA-I incubated with AgNPs of different surface coatings (citrate, PVP, bPEI, and lipoic acid) show (b) marked decrease in α-helix content with corresponding increase in β-sheets and irregular structures. Spectra were analyzed using CAPITO (a CD Analysis & Plotting Tool), and secondary structure content was estimated. CD spectroscopy provides secondary and tertiary protein structure content estimation of biomolecules, which is critical for analyzing conformational changes during ENM-protein interactions.

2.3. Infrared spectroscopy

Unlike metal nanoparticles, carbon-based ENMs exhibit strong optical absorption <240 nm due to their π-electron system, which can interfere with protein CD spectra collected in 200–300 nm range, precluding the use of CD to study nanocarbon-protein corona. Alternatively, we used ATR-FTIR (attenuated total internal reflection-Fourier transform infrared) spectroscopy to elucidate the adsorption-induced structural changes in proteins (bovine serum albumin or BSA and fibrinogen) on carbon ENMs such as multi-walled carbon nanotubes (MWNTs), graphene, and graphene oxide nanoribbons (GNRs and GONRs). The FTIR spectrum of proteins displays two main bands, Amide I (1600–1700 cm^{-1}) and Amide II (1500–1580 cm^{-1}), arising from the amide bonds that link the amino acids in proteins. While Amide I band is mainly associated with the C═O stretching vibration, Amide II band results primarily from bending vibrations of the N─H bond. Given that both the C═O and the N─H bonds are involved in the hydrogen bonding between the different peptide units, their spectral position and intensity can be used to determine the secondary structure content of a protein. For example, as shown in **Figure 3**, the α-helical content in BSA leads to strong adsorption of ~1640–1660 cm^{-1} (dashed lines), while the lower frequency component at ~1620–1640 cm^{-1} and the peak ~1555 cm^{-1} arise from β-sheets. Clearly, the rich secondary structure of BSA (particularly, the peak relating to α-helical content) significantly disappears upon its adsorption on to all carbon-based NMs, as expected from its low internal stability. Indeed, the changes in secondary structure are higher in the case of MWNTs (i.e., complete disappearance of secondary structure), suggesting that BSA unfolds much more, relative to GNRs and GONRs, in order to adhere to the tubular MWNTs. GNR and GONR retain BSA secondary structure to certain extent, as shown by the presence of ~1555 cm^{-1} for β-sheets. In the case of fibrinogen, the secondary structural changes are found to be higher for GONRs compared to MWNTs and GNRs plausibly due to the formation of hydrogen bonds. The α-helix peak was found to partially disappear for fibrinogen adsorbed on MWNTs and GNRs. Lastly, the

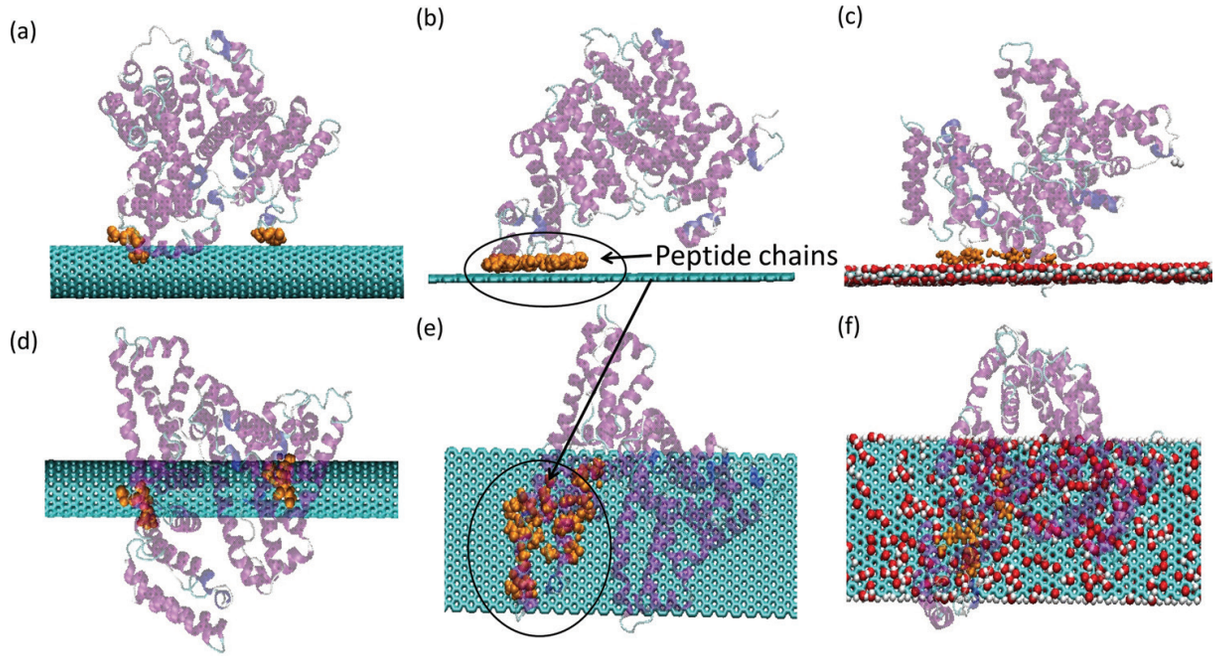

Figure 3. (a–c) Snapshots of MD simulations showing BSA on SWNT, GNR, and GONR 10 ns after adsorption. (d–f) show the top view. The adsorption is accompanied by disruption of α-helices into random peptide chains.

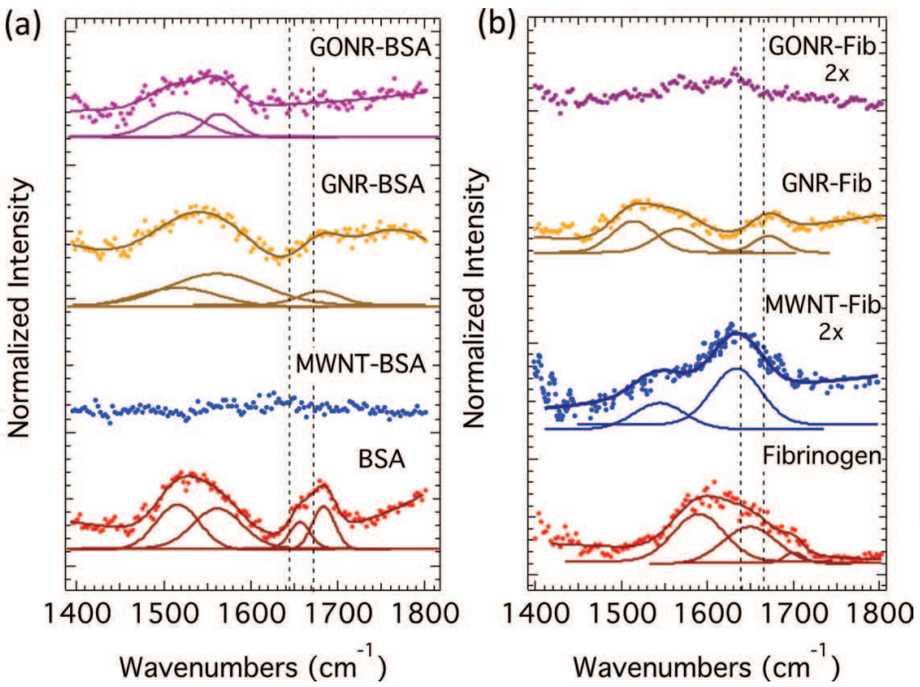

Figure 4. (a) FTIR spectra for native BSA and BSA adsorbed on MWNTs, GNRs, and GONRs show that the CNMs significantly affected the secondary structures of the proteins. The absorption peak for α-helix in native BSA, in the range from 1640 to 1660 cm^{-1} (indicated by the vertical dashed lines), showed a significant reduction (disappeared for MWNTs) for all CNMs, suggesting that the secondary structure of BSA adsorbed on CNMs was less compact. (b) In the case of fibrinogen, the loss of secondary structure was highest for GONRs possibly due to the formation of hydrogen bonds. While both MWNTs and GNRs exhibited a loss of α-helical content, the appearance of new peak ~1500 cm^{-1} (corresponding to random motifs) in GNRs suggested a lesser degree of relaxation for fibrinogen, compared to MWNTs.

structural changes for fibrinogen on GNRs seemed to be less pronounced than MWNTs possibly due to its shape. It could be rationalized that fibrinogen must unfold more to adhere to MWNTs due to their higher curvature than GNRs. To further understand NM-BSA interactions, we also performed large-scale molecular dynamics (MD) simulations of BSA-MWNT, GNR/GONR-water systems (detailed in [43]). As shown in **Figure 3**, we observed that proteins undergo conformational changes after initial contact (in accordance with our experimental observations, discussed in **Figure 4**), leading to some protein regions collapsing on to the ENM surface rather than gradually spreading from the initial region of contact.

3. Probing electronic interactions at the nano-bio interface

Charge transfer is known to play an important role in several many physiological processes including blood clotting, vitamin absorption, and oxidative stress [44–46]. Differences in electronegativity between proteins and the ENM surface may induce charge transfer which, along with other perturbations in the media (e.g., pH, thermodynamic fluctuations), plays a critical role in protein denaturation, protein-protein interactions, and alterations to the cellular and extracellular redox status. Such interactions are not necessarily specific, with conformational changes possibly resulting in newly exposed charged regions and hydrophobic domains attracting or repelling other surrounding proteins or nanostructures. Electronic properties of ENMs, including their band structure and density of states, have been shown to regulate protein adsorption dynamics via charge transfer, which are evident as shifts in absorption and emission spectra [47, 48].

3.1. Charge transfer during corona formation

The chemisorption of proteins on bulk material surfaces has been known to occur through charge transfer processes. For example, the aromatic amino acids present in BSA (tryptophan, phenylalanine, histidine, and tyrosine) could interact with the unhybridized p_z orbitals of the carbon-based ENMs, via providing a weak acceptor level in the electronic density of states (DOS) to allow partial charge transfer. It may be expected that a surface facilitating higher charge transfer at the nanoscale may lead to stronger surface-protein interactions and a subsequent increase in protein adsorption. Spectroscopic and electrochemical techniques such as micro-Raman, CD, and cyclic voltammetry can be used to analyze and elucidate the influence of charge transfer on protein affinity for ENMs and the alterations in secondary and tertiary structures that occur with adsorption. Among variety of ENMs, nanocarbons possess strong affinity for proteins through hydrophobic and aromatic π-π stacking interactions [49]. Previously, we elucidated the charge transfer interactions between nanocarbons and proteins using micro-Raman spectroscopy. Our results show that strong interaction of proteins (albumin and fibrinogen) with nanocarbons is strongly influenced by charge transfer between them, inducing protein unfolding which enhances conformational entropy and higher protein adsorption. For instance, the UV-visible absorption spectrum of single-wall carbon nanotubes (SWNTs) coated with BSA were found to blue shift significantly, while BSA-coated graphene sheets (both exfoliated and synthesized via chemical vapor deposition) exhibited no such changes [50]. Additionally, micro-Raman spectroscopy revealed alterations

in the structure of the G-band (or graphitic band) of BSA-coated SWNTs. The G-band, which is highly sensitive to charge transfer, was found to be upshifted with a Breit-Wigner-Fano lineshape resulting from electron transfer between BSA and SWNTs. No changes were observed for graphene upon protein coating, indicating that ENMs-BSA charge transfer is unique to SWNTs. Concomitantly, FTIR spectroscopy revealed subsequent conformational changes in BSA (**Figure 4**) in the form of uncompacting of α-helices, suggesting hard corona formation on SWNTs as opposed to graphene. These results insinuate that disruption in electrostatics due to ENM-protein charge transfer leads to the breaking of peripheral H-bonds in the α-helices and permanent denaturation of BSA on SWNTs [50, 51].

3.2. Two-dimensional (2D) materials

Unique properties of two-dimensional (2D) nanomaterials, including boron nitride (BN), graphene, and graphene oxide, relate to their electronic interactions with biomolecules. The 2D structure of these ENMs gives rise to π-electron clouds which can interact strongly with other π-electrons in proteins or amino acids [52]. While we predominantly focused on proteins, it is important to note that individual amino acid interactions with ENMs are equally significant indicators of corona formation and cytotoxicity modeling. In particular, aromatic amino acids offer π-electrons similar to 2D ENMs, and therefore exhibit this strong affinity for certain 2D materials through this π-π electron stacking. We studied interactions between aromatic amino acids (tryptophan, tyrosine, and phenylalanine) and 2D nanomaterials (graphene, graphene oxide or GO, and BN) using micro-Raman and photoluminescence (PL) spectroscopy combined with electrochemical characterization. Perturbation in the electronic structure was evident through changes in Raman spectra as shown in **Figure 5**, and results were quantified using CV measurements (**Figure 6**). Downshift in characteristic G and 2D bands in the spectra

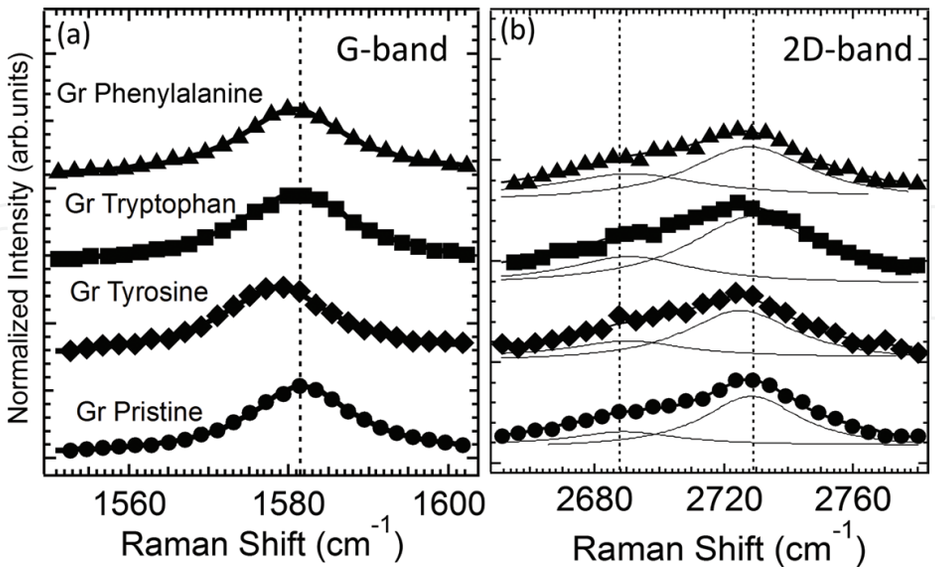

Figure 5. Raman spectra of graphene (Gr)-amino acid complexes show significant shifts in carbon characteristic (a) G band and (b) 2D band indicating charge transfer between amino acid and graphene. Solid line indicates fits to experimental data. Panel b shows deconvoluted peaks of 2D band. Downshift in G and 2D bands for graphene-tyrosine complex indicates upshift in fermi level (E_f) induced by electron transfer from tyrosine.

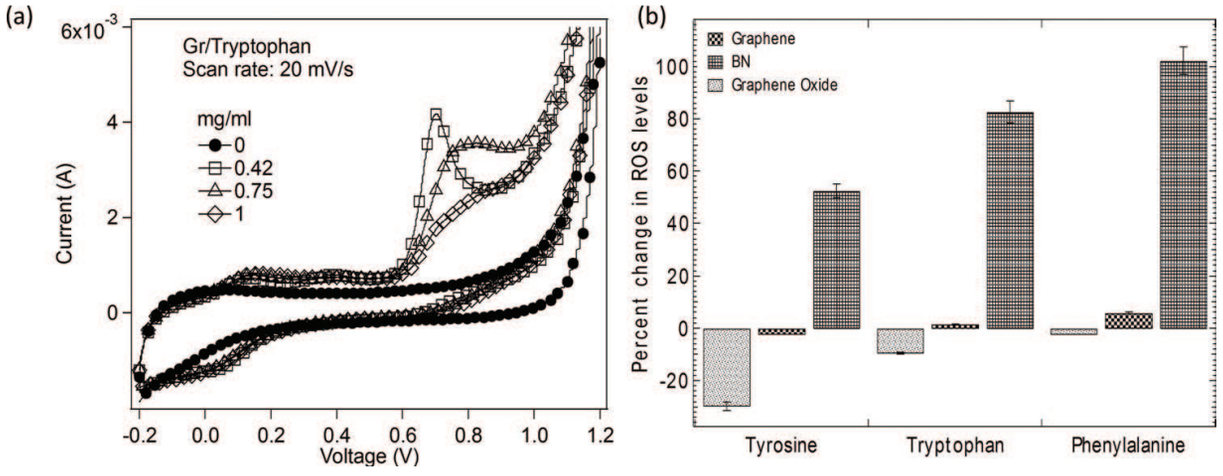

Figure 6. (a) Cyclic voltammetry (CV) curves for graphene and tryptophan show appearance of new peak ~0.6–0.8 V indicating irreversible charge transfer. (b) Percent change in reactive oxygen species (ROS) by macrophages when exposed different 2D materials like graphene (Gr), boron nitride (BN), and graphene oxide (GO) to amino acid complexes (tyrosine, tryptophan, and phenylalanine). Sensitivity to charge transfer makes electrochemical impedance spectroscopy a valuable tool in probing nano-bio interactions.

indicated upshift in Fermi level induced from electron transfer from the amino acid. In CV characterization, the application of gate voltage on the working electrode (i.e., ENMs) modulates its electronic energy levels, which when above (/below) the LUMO (/HOMO) levels of the protein can result in a charge transfer. While reversible charge transfer in redox couples appears as two peaks in the CV curve (one for oxidation and the other for reduction), only a single peak is often observed for irreversible charge transfer. The irreversibility of these charge transfer between 2D ENMs and amino acids was confirmed with appearance of new peak (~0.6–0.8 V) in our CV curves. Further findings suggested variance in π-electron cloud structure in graphene and BN determined electron stacking orientation and associated amino acid affinity, while functional groups mediated affinity through H-bonding in GO. As shown in **Figure 6b**, indeed adsorption of amino acids on 2D materials significantly alters their ability to generate reactive oxygen species [52].

4. Conclusions

Understanding the biological interactions of ENMs with a biocorona and its influence on cellular uptake, generation of reactive oxygen species, and cytotoxicity is critical to implement the safe use of ENMs. Spectroscopic tools offer critical insights into the nano-bio interface by providing information regarding the protein adsorption affinity, the influence of charge transfer, and protein unfolding on ENMs. Hyperspectral imaging is an excellent complementary technique to electron microscopy. The changes in the micro-environment of ENMs are reflected in their hyperspectra allowing one to identify intra-cellular ENMs. Circular dichroism and infrared spectroscopy are sensitive to the changes in protein structure, while Raman and cyclic voltammetry provide information about the charge transfer between ENMs and proteins. A comprehensive characterization of ENM-protein corona with spectroscopic tools

is necessary for establishing relationships between ENM physicochemical properties and their biological responses.

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