Raman microspectroscopy of nanodiamond-induced structural changes in albumin

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Abstract. Nanodiamonds (NDs) are promising agents for theranostic applications due to reported low toxicity and high biocompatibility, which is still being extensively tested on cellular, tissue, and organism levels. It is presumed that for experimental and future clinical applications, NDs will be administered into the organism via the blood circulation system. In this regard, the interaction of NDs with blood components needs to be thoroughly studied. We studied the interaction of carboxylated NDs (cNDs) with albumin, one of the major proteins of blood plasma. After 2-h long in vitro incubation in an aqueous solution of the protein, 100-nm cNDs were dried and the dry samples were studied with the aid of Raman microspectroscopy. The spectroscopic data indicate significant conformational changes that can be due to cND–protein interaction. A possible decrease in the functional activity of albumin related to the conformational changes must be taken into account in the in vivo applications.

Keywords: Raman microspectroscopy; nanodiamonds; protein structure; albumin.

1 Introduction

Nanoparticles are promising for applications in biotechnology and theranostics since they can be used for addressed drug delivery and visualization of various processes in living organisms. Nanoparticles and their conjugates with drugs can be administered and spread via the blood circulation system. In this regard, it is expedient to study the interaction of nanoparticles with tissues and blood components within the framework of the problems of nanotoxicology and nanosafety. It is known that the nanoparticles are sometimes able to induce dramatic effects on protein interactions.1 The general use of nanoparticles in nanomedicine has been reviewed in multiple works (see, e.g., Refs. 2–4).

Diamond nanoparticles, nanodiamonds (NDs), exhibit high biocompatibility and relatively low toxicity,3 their properties can be characterized using fluorescence and Raman spectroscopy,6 and the corresponding signals can be used for ND visualization.5 The bioconjugation can be facilitated with the aid of functionalization of the ND surface (e.g., by means of carboxylation).15,19 The absence of toxicity of carboxylated NDs (cNDs) has been demonstrated in Refs. 8 and 13 for in vitro interaction with various cells of human organisms. However, several experiments with in vivo and in vitro incubations have shown that NDs are accumulated in internal organs of laboratory animals (rats)12 and can stick to the membranes and penetrate into erythrocytes.15 In the tested range of particle concentrations, such an interaction did not cause hemolysis or aggregation of the cells but potentially it may affect their microrheologic properties (reversible aggregation and deformability).15

It is known that the proteins of blood plasma are adsorbed on the ND surface.16–18 FTIR data show that the interaction of human serum albumin (HSA) and cNDs with linear sizes of 5 and 100 nm leads to the changes of the amide I band shape and variations in the relative intensities of amide I–III bands, which indicate variations in the protein secondary structure.19

The results of Ref. 20 show a minor increase in the relative contents of random coils and β-sheets upon the dominant contribution of α-helices to the secondary structure of bovine serum albumin (BSA) that forms a complex with 5-nm cNDs. In addition, the complex formation leads to a more blue shift of the UV-VIS absorption band of the protein and significant quenching of the protein fluorescence.

Lysozyme binding with 100-nm cND results in the splitting and low-frequency shifting of the band at 3250 cm−1 (N–H stretching) in the FTIR spectra.21,22 In this case, the enzymatic activity remains almost unchanged. The interaction with 5-nm cNDs also leads to the low-frequency shifting and splitting of the band into three narrow bands. The FTIR spectra of lysozyme9,23 show a high-frequency shift of the amide I band due to complex formation with cNDs. The shifts for 5- and 50-nm cNDs are greater than the shift for the 100-nm cNDs. The relative contents of random coils and β-sheets slightly increase in the predominantly α-helical protein. The enzymatic activity of lysozyme decreases by 80%, 80%, 25%, and 40% in complexes with 5-, 50-, 100-, and 500-nm cNDs, respectively.

However, the results of Ref. 24 show that the adsorption of blood plasma on NDs does not lead to variations in the secondary structure of the blood-plasma proteins and that incubation with NDs does not affect the dynamics of blood coagulation. Thus, the existing data indicate the interaction of blood-plasma proteins and NDs; however, the nature and result of such interaction remain unclear and a conclusion on variations...
in the protein structure due to adsorption on ND surface is not commonly accepted. Biofunctionalization of nanoparticles involves the functional activity of protein molecules bound to the surface of a nanoparticle. Hence, it is expedient to learn more about the effects of the interaction with ND on the structure and function of proteins.

In this work, we employed Raman microspectroscopy to study the conformational changes of albumin that were induced by the interaction with NDs. Raman microspectroscopy is an efficient tool in the study of the structure of various biological objects. Raman spectra of proteins exhibit several conformation-sensitive bands: amide I (∼1640 cm⁻¹), amide III (∼1270 cm⁻¹), tyrosine doublet (830 and 855 cm⁻¹), bands of the disulfide bridges (510, 525, and 540 cm⁻¹), and several bands assigned to the vibrations of tryptophan residues (e.g., 1361 cm⁻¹).

2 Samples and Methods

In the experiments, we used BSA from MP Biomedicals (CAS #9048-46-8) (M_BSA 66.3 kD), HSA from Sigma Aldrich (CAS #70024-90-7) (M_HSA 66.2 kD), and NDs from Kay Industrial Diamond. The NDs with a characteristic size of about 100 nm were carboxylated using a mixture of sulfuric and nitric acids (3:1). The characteristic size of a single ND is approximately 20 times greater than the characteristic linear size of an HSA molecule.

To prepare a buffer solution, we sequentially dissolved sodium chloride (4 g), potassium chloride (0.72 g), orthophosphoric acid (0.72 g), and potassium dihydrogen phosphate (0.12 g) in deionized distilled water (500 ml). The buffer pH (7.4) was equal to the mean pH of human blood. The buffer was sterilized and stored at a temperature of 4°C. Carboxylated NDs (cNDs) were added to the HSA solution in buffer: the concentrations were 2 and 7.6 mg/mL. The solution was processed in an ultrasonic bath over 30 min and stored at room temperature over 2 h. Then the sample was lyophilized. A reference HSA sample was prepared using the same procedure.

For the Raman measurements, we used a Thermo Scientific DXR Raman confocal microscope. The excitation wavelength was 532 nm, the mean power at the sample was no greater than 10 mW, and the spectral resolution was 5 cm⁻¹ (the dispersion was about 1 cm⁻¹/pixel). The diameters of the focal spots on the sample and the confocal resolution were 0.6 and 4 μm, respectively (100x objective and 25-μm pinhole). Raman bands were assigned using the results of Refs. 29 and 31.

3 Results and Discussion

Optical microscopy shows that the cND-HSA samples under study consist of irregularly shaped microstructures. Figure 1(a) presents a typical microphotograph of a dried cND-HSA sample. It is clearly seen that the sample consists of microstructures that apparently represent the aggregates of protein molecules and cNDs. To estimate the characteristic size of the microstructures, we used ImageJ software. Figure 1(b) shows the resulting size distribution with a mean linear size of about 3 μm. Note that most microstructures on the surface of the samples are in close contact with each other, and the thickness of the sample exceeds the size of the microstructures. Therefore, obtaining the real size distribution of microstructures is not feasible.

We measured the Raman spectra at 100 randomly located microstructures to assess their identity. Based on the spectral

![Fig. 1](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics) (a) Microphotograph of a surface fragment of cND-HSA sample dried on silicon substrate and (b) distribution of microstructures with respect to their size.
data, we conclude that most microstructures predominantly consist of cNDs [Fig. 2(a)]: an intense narrow band at 1332 cm$^{-1}$ is assigned to diamond (vibrations of carbon in sp$^3$ hybridization), overlapped broadbands at 1410 and 1560 cm$^{-1}$ are assigned to defect and ordered graphites, respectively (D and G graphite bands), and the band at 3130 cm$^{-1}$ corresponds to the C$\equiv$H stretching mode. Note the absence of Raman signatures of proteins in these spectra, which, however, does not exclude the presence of small amounts of protein molecules.

The measurements at two microstructures of 100 yielded the spectra that can be identified as the Raman spectra of protein with a contribution of the diamond band at 1332 cm$^{-1}$. The spectra were measured at five sites on each microstructure. In these 10 almost identical spectra [Fig. 2(b) shows three of them], we observe the protein bands at about 540 cm$^{-1}$ (vibrations of disulfide bridges), 830 and 850 cm$^{-1}$ (tyrosine doublet), 1006 cm$^{-1}$ (breathing mode of phenylalanine ring), and 1640 cm$^{-1}$ (amide I). The bands assigned to the C$\equiv$H and N$\equiv$H stretching modes are observed at $\sim$2900 and $\sim$3300 cm$^{-1}$, respectively.

Below, we consider the Raman spectrum of one of the cND-HSA samples that results from averaging the above 10 measurements.

Figure 3 shows the Raman spectra of the reference HSA and cND-HSA samples. Background signals were subtracted using the method described elsewhere, and the resulting spectra were normalized by the intensity of the band at 2930 cm$^{-1}$ (C$\equiv$H stretching mode).

It is seen that the intensities of the bands at 510 and 675 cm$^{-1}$ are significantly lower in the spectrum of the cND-HSA sample. The bands in the interval 500 to 550 cm$^{-1}$ are assigned to S=S stretching vibrations. C=S stretching vibrations are manifested at 660 to 670 and 700 cm$^{-1}$ in C=S=S=C and C=S=S configuration, respectively. The Protein Data Bank (PDB) data for HSA show that the protein structure contains 17 disulfide bridges, 34 C=S bonds in C=S=S=C configuration, 6 C=S bonds in C=S=C configuration, and 1 C=S bond in C=S=H configuration. Thus, a decrease in the intensities of the bands at 510 and 675 cm$^{-1}$ can be due to the cleavage of disulfide bonds and the corresponding decrease in the number of C=S bonds in C=S=S=C configuration. To prove such an interpretation, we additionally studied the mixture of HSA with TCEP. The inset to Fig. 3 compares the Raman spectra of lyophilized HSA and TCEP-HSA samples and shows that the cleavage of disulfide bonds leads to a decrease in the intensities of the bands at 510 and 670 cm$^{-1}$. Such spectral changes are similar to the spectral changes for cND-HSA samples.

For cND-HSA samples, we also observe an increase in the intensity ratio of tyrosine doublet $R = I_{850}/I_{830}$, which characterizes the H-bonding of tyrosine residues, from $R = 1.4$ for HSA to $R = 2.1$ for cND-HSA. Based on the existing data,

![Fig. 2](https://example.com/fig2.png) Raman spectrum of cND sample (dotted line). Solid lines show Raman spectra of cND-HSA sample measured at several sites on its surface that are classified as the spectra of (a) diamond and (b) protein. The dashed lines show the positions of the Raman bands at 1332, 1410, 1560, and 3130 cm$^{-1}$ (see text for details).

![Fig. 3](https://example.com/fig3.png) Raman spectra of reference HSA sample (solid line) and cND-HSA sample (dashed line). The inset shows Raman spectra of lyophilized HSA (solid line) and TCEP-HSA samples (dashed line).
we conclude that OH groups of tyrosine residues become stronger acceptors in H-bonds.

We additionally measured the Raman spectra of cND-protein samples in which BSA was used instead of HSA. The spectra of 100 microstructures were measured. The protein spectrum was observed for only one microstructure. The measurements at 10 points at this microstructure yielded a decrease in the intensities of the bands at 510 and 670 cm$^{-1}$ and a variation in $R$-ratio as well as the measurements with HSA did.

To compare the shapes of amide I bands, we preliminary subtracted linear backgrounds and normalized the resulting spectra by the integrals over the spectral interval 1640 to 1720 cm$^{-1}$ (curves 1 and 2, Fig. 4). We also used the double differentiation of the spectra (curves 1’ and 2’, Fig. 4). Note an increase in the intensity at 1650 cm$^{-1}$ and a decrease in the intensity at 1660 to 1670 cm$^{-1}$ in the spectrum of the cND-HSA sample. In accordance with PDB data, the relative contents of the main elements of the secondary structure (alpha-helix, beta-sheet, and random coil) are 78%, 0%, and 22%, respectively.35 Alpha-helices and random coils predominantly contribute to the spectral intensities in the intervals 1650 to 1657 and 1660 to 1665 cm$^{-1}$, respectively.29,36 Therefore, the experimental results show that the relative content of alpha-helices (random coils) increases (decreases) in the cND-HSA samples.

The absence of Raman signatures of protein in the spectra of most microstructures is an unexpected result. Estimations based on the initial concentrations of cNDs and HSA and the concentrations of protein in supernatant fluids after centrifuging (results of spectrophotometric measurements) show that about 10,000 protein molecules are bound to a single cND. The result of spectrophotometric measurements at several points on such a sample yielded identical concentrations of protein in supernatant fluids after centrifuging on the initial concentrations of cNDs and HSA and the concentrations of protein molecules bound to the surface of cNDs. In respect to the effect of the alteration of the functional activity of HSA on the biofunctionalization of cND for drug delivery, it should be noted that the altered structure and functional activity of albumin may impede the functionalization needed for attaching the drug to the nanoparticle.

4 Conclusion

The cND-HSA dried samples are structurally nonuniform and consist of microstructures. Few areas with relatively high concentrations of protein molecules are detected although the estimations show that the total amount of protein molecules in the sample under study is sufficient for reliable detection at any point of the sample under the conditions for uniform distribution.

The spectral data indicate the following conformational changes of protein molecules induced by the interaction with cNDs: cleavage of disulfide bridges, modifications of H-bonds of tyrosine residues, and a minor increase (decrease) in the relative content of $\alpha$-helices (random coils).

Such significant conformational changes of HSA in the cND-HSA complex may lead to a decrease in the functional activity of protein molecules bound to the surface of cNDs. In respect to the effect of the alteration of the functional activity of HSA on the biofunctionalization of cND for drug delivery, it should be noted that the altered structure and functional activity of albumin may impede the functionalization needed for attaching the drug to the nanoparticle. Thus, the addressed drug delivery with the aid of cNDs that are biofunctionalized using HSA molecules may be inefficient. However, the system under study was obtained under conditions that substantially differ from in vivo conditions. In native medium, the cND-HSA interaction can be different, so this interaction in aqueous medium should be additionally studied in more detail.

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References


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