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SCIENCE DIRECT
Diamond & Related Materials 15 (2006) 622 – 625
DIAMOND AND RELATED MATERIALS
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Spectroscopic study of bio-functionalized nanodiamonds

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Available online 28 December 2005

Abstract

Nano-scale materials with attached biomolecules on the surface can be used as bioprobes in cell and tissue analysis. The conjugation of these nanoparticles with biomolecules is one of the key steps in the development of bioprobes. In this work nanometer-sized diamonds (5 and 100 nm) are functionalized and conjugated with protein lysozyme via physical adsorption. The process of creating functional groups on the nanodiamond surface is studied using Fourier Transform Infrared-spectroscopy (FTIR). The conjugation of nanodiamonds with biomolecules and nanodiamond–biomolecule interaction are analyzed with UV/VIS and FTIR spectroscopy.

Keywords: Nanodiamonds; Biomedical applications; Sensors; Surface characterization

1. Introduction

Nanocrystals with attached biomolecules on the surface are used as bioprobes in cell and tissue analysis for visualization of micro- and nano-objects and for observation of the biological processes in nano-scale [1]. The first reason for such application is that nanoparticles exist in the same size domain as many intercellular structures and large biomolecules. Initially the nanoparticles were used as carriers for bioactive molecules, but recently intrinsic physical and chemical properties of nanostructures make them promising candidates for biosensors [2]. The conjugation of nanoparticles and biomolecules is one of the key steps in the development of bioprobes. These nanoparticle–biomolecules complexes can interact specifically or non-specifically with components of investigated bio-object and hence many interactions can be studied in detail. Biomolecules can be immobilized on nanoparticles' surface through a variety of mechanisms such as physical adsorption, electrostatic binding, specific recognition and covalent coupling. The biomolecules on the nano-bio-probe allow to visualize and to distinguish some functional processes and structures of the investigated object, and to control physical and chemical treatments. Also, the attached biomolecules can prevent non-controlled adsorption of the investigated sample components. The most developed methods for applications using nanoparticles are: semiconductor nanocrystals (quantum dots) encapsulated by biomolecules to decrease toxicity and increase bio-compatibility are used for applications that utilizing their unique fluorescent properties [3–7]; metal nanoparticles are used for surface plasmon resonance microscopy and surface enhancement Raman spectroscopy [7–10]; magnetic nanoparticles are used for cell targeting both in nanobioprobing [11] and in drug delivery [12], etc.

Nanometer-sized diamond recently becomes an interesting material for the nano- and biotechnology integration. Nanodiamond–biomoleules complexes are thus studied for the purpose of using them as bioprobes [13,14] taking advantage of their chemical stability and biocompatibility. The nanodiamond particle surface can be functionalized with a large number of surface ionogenic groups (ether — C–O–C, peroxide — C–O–O–, carbonyl — C═O, and hydroxyl-type C–O–H bonding, etc.) as well as hydrocarbon fragments. The surface can also be modified with biologically active molecules by adsorption, covalent or non-covalent chemical immobilization [15,16]. The functional groups on the particle surface can interact electrostatically or chemically with bimolecular appurtenant to the investigated sample for cell specific interaction and targeting. Therefore the nanodiamond was used first of all as carrier for active molecules [2,17], but the electronic and optical properties of different nanodiamond structures render

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doi:10.1016/j.diamond.2005.11.019

them attractive candidates as drug delivery agent or biosensors [12,18]. For these applications, biological molecules, such as DNA [17,19–21], different proteins [16,22], fluorescent dyes [22], etc., were immobilized on the nanodiamond.

In this work nanodiamonds (5 and 100 nm) are functionalized and conjugated with biomolecules via physical adsorption. To create the functional groups the processes of carboxylation and oxidization nanodiamond were used. The creation of functional groups on the nanodiamond surface was studied with Fourier Transform Infrared (FTIR) spectroscopy. The attachment of biomolecules via physical adsorption and nanodiamond–biomolecule interaction were analyzed with UV/VIS and FTIR spectroscopy.

2. Experimental

Synthetic diamond powders with diameters 100 nm (Kay Industrial Diamond, USA) and 5 nm (UltraFine Diamond, Russia) were carboxylated/oxidized in according with standard procedure [16,19]. The sample (0.5 g) was subsequently heated in a 9:1 (v/v) mixture of concentrated H_2SO_4 and HNO_3 at 75 °C for 3 days, in 0.1 M NaOH aqueous solution at 90 °C for 2 h, and in 0.1 M HCl aqueous solution at 90 °C for 2 h. The resulting carboxylated/oxidized diamonds were extensively rinsed with deionized water, separated by sedimentation with a centrifuge at 12000 rpm and dried. The IR spectra of carboxylated nanodiamond were measured using an FTIR-interferometer (Bomem MB154) with the sample in vacuum chamber or flowing nitrogen to avoid water absorption.

The lysozyme (AMRESCO, USA), concentration 180–200 μM, was dissolved in double-distilled deionized water (pH 4.4–4.5) or in phosphate buffer saline (PBS), pH=6.5. The sample preparation by both media followed by spectral analysis was estimated. The initial protein concentration was checked with UV/VIS spectrometer (Jasco V-550) by the solution absorption at 280 nm. A molar absorbance of lysozyme at 280 nm (3.7547×10^4 M^{-1} cm^{-1}) served to calibrate the protein concentration by the measured absorbance at the Soret band maximum. The concentration of lysozyme in solution was measured before adsorption; then carboxylated nanodiamond, concentration 4–10 mg/ml, was added to the solution. To ensure maximum adsorption, the protein solution and the diamond powder were thoroughly mixed using a shaker for 2 h, after that the mixture was centrifuged and washed several times with deionized water. After first separation of nanodiamond with adsorbed protein, the residual concentration of protein in supernatant was measured. The quantity of protein adsorbed by nanodiamond was estimated by the difference between initial and residual protein concentrations. FTIR spectra were measured in ambient condition and in vacuum chamber at $\sim10^{-6}$ Torr pressure.

3. Results and discussions

Infrared spectra of carboxylated/oxidized nanodiamonds are plotted in Fig. 1. The acid treatment of nanodiamond creates the functional groups on the diamond surface and can be observed

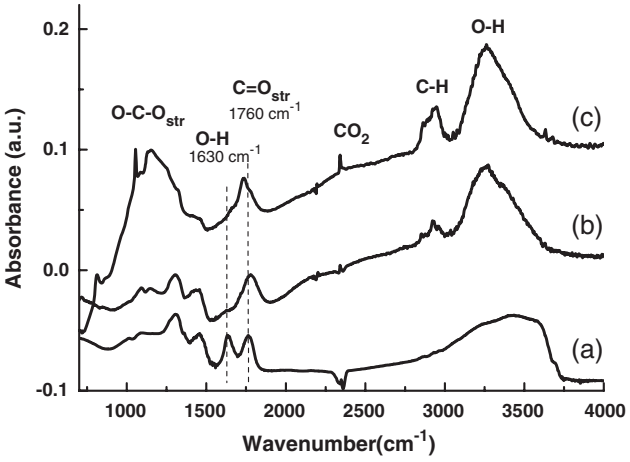

Fig. 1. IR spectra of carboxylated/oxidized nanodiamonds: (a) 100 nm; initial concentration in suspension 20 mg/60 μl, spectrum taken in ambient air; (b) concentration 1.4 mg/200 μl, spectrum taken in vacuum; (c) 5 nm; concentration 1 mg/200 μl, spectrum taken in vacuum.

using FTIR spectroscopy. Fig. 1(a) and (b) are from the same carboxylated 100 nm diamonds, except Fig. 1(a) was measured in air with higher diamonds concentration (20 mg/60 μl) while (b) was taken in vacuum chamber at lower concentration (1.4 mg/60 μl). The band near 1630 cm^{-1} can be attributed to the O–H bending of physically adsorbed water and the hydrogen bonding, which arise between neighboring carboxylated nanoparticles at high concentration [16]. The assignment is evidenced from the disappearing of this band when the spectrum was taken in the vacuum ($\sim10^{-6}$ Torr) as shown in Fig 1(b). For smaller 5 nm diamonds, this band shifted to higher wavenumbers that presumably arise from the intermolecular interaction of the neighboring bonding due to small particle size. The C═O bond of carboxylic acid usually appears around 1775 cm^{-1} and that of carboxylic anhydrite at about 1800 cm^{-1}. In Fig. 1, the IR bands at 1750–1760 cm^{-1} can be assigned as C═O stretching mode for the carboxylic acid. The shoulder, especially visible in the 5 nm carboxylated diamonds, in the ~1800 cm^{-1} may associate with the carboxylic anhydrite. In addition, O–H stretching from surface –COOH group appears near 3710 cm^{-1}; the broad band at 3000–3600 cm^{-1} can be attributed to the hydrogen-bonded-OH of physisorbed water on the surface, and when in vacuum it may desorb from the surface which results in the narrowing of the band as seen in Fig. 1(b), (c); the broad band near 3000 cm^{-1} can be caused by the CH stretching on the sample surfaces. The bands in the range 700–1450 cm^{-1} also (in particular, 1275 cm^{-1}) have been ascribed to ether-like groups on the diamond powders [23–25].

The carboxylated/oxidized surface of nanodiamond in water solution (including PBS etc.) is negatively charged due to dissociation of –COOH and the forming of $-COO^-$ ion groups. This is essential for modification of the nanodiamond surfaces with biomolecules. We analyze here physical adsorption of protein lysozyme. It is used as test protein for its well-known properties and structure. Lysozyme is a small globular protein widely used for researches in biophysics, molecular biology, etc. Particularly, lysozyme has been used for diamond film–lysozyme biochip construction [26]. In Fig. 2, typical

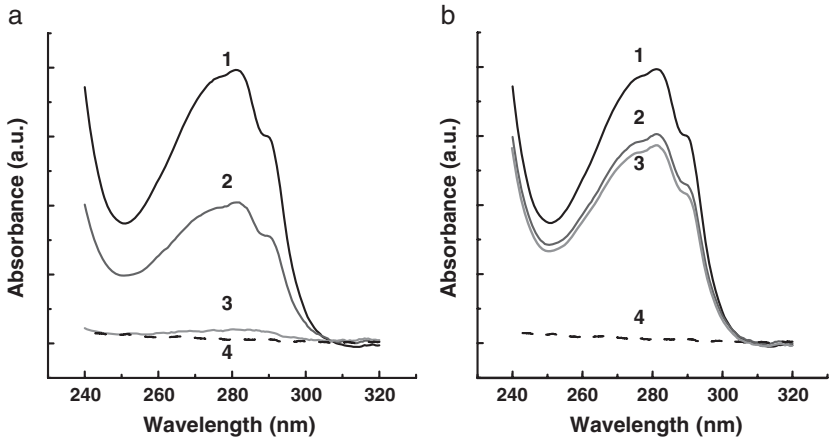

Fig. 2. UV/VIS spectra of lysozyme solutions at conjugation with (a) 5 nm nanodiamond: (1) before reaction; (2) after reaction in water; (3) after reaction in PBS; (4) after 1st washing in water; (b) 100 nm nanodiamond: (1) before reaction; (2) after reaction in water; (3) after reaction with in PBS; (4) after 1st washing in water.

UV–VIS spectra are shown for lysozyme in water and PBS before and after interaction with nanodiamonds. The spectra measured after diamond addition and thorough mixing show quite dramatic removal of the proteins from the solution as a result of physical adsorption of protein by carboxylated nanodiamond surface. For 5 nm nanodiamond the adsorption is extremely effective as indicated in the spectra in Fig. 2(a). The protein–nanodiamond interaction, determining the adsorption, depends on the environment as well as on nanodiamond surface properties. As one can see in Fig. 3, the saturation of nanodiamond surface with protein molecules is observed. Fig. 3 plots the adsorption isotherms of lysozyme on 5 nm diamonds in PBS (pH 6.5) and in bidistilled water (pH 4.5). Saturated adsorption of lysozyme is observed for 5 nm nanodiamond in water and in PBS. We estimate that 1 mg of 100 nm nanodiamond adsorbs on their surfaces up to 45 μg of lysozyme from water solution and 80 μg from PBS; 1 mg of 5 nm nanodiamond adsorbs up to 200 μg of lysozyme from water solution and 500 μg from PBS. Note also, negligible absorption at 280 nm was observed after second washing of supernatant fraction of the sample; it means that adsorption is strong and stable.

The adsorption of protein on carboxylated/oxidized nanodiamond is established through electrostatic attraction between the surface-terminated anionic groups ($-COO^-$) and the positively charged amino groups ($-NH_3^+$) in the protein. In addition to the charge–charge interactions, ionic hydrogen bonds can also form between $-NH_3^+$ and any CO-containing surface groups [16]. Although one can't see the difference in IR spectra of carboxylated/oxidized nanodiamond of sizes 5 and 100 nm, the applications of these two nanoparticles can be different. The difference arises from the crystal alignment, as well as the size. The size determinates the surface curvature and the proportion between the number of atoms on the surface and in volume. Jones and d'Hendecourt [27] had estimated for a nanodiamond of 4.2 nm in diameter, there are total of ~6700 carbon atoms and among them 16% are surface carbons. The total surface atoms decrease as the size of the diamond particle increases. The number of surface atoms contributes to the attaching of biomolecules on the nanodiamond surface and the quantitative estimation of adsorbance. Surface properties of

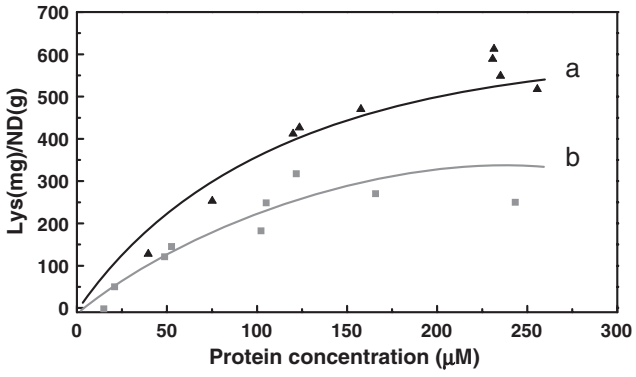

Fig. 3. Adsorption isotherm of lysozyme on 5 nm nanodiamond. (a) In PBS (pH 6.5) and (b) in bidistilled water (pH 4.5).

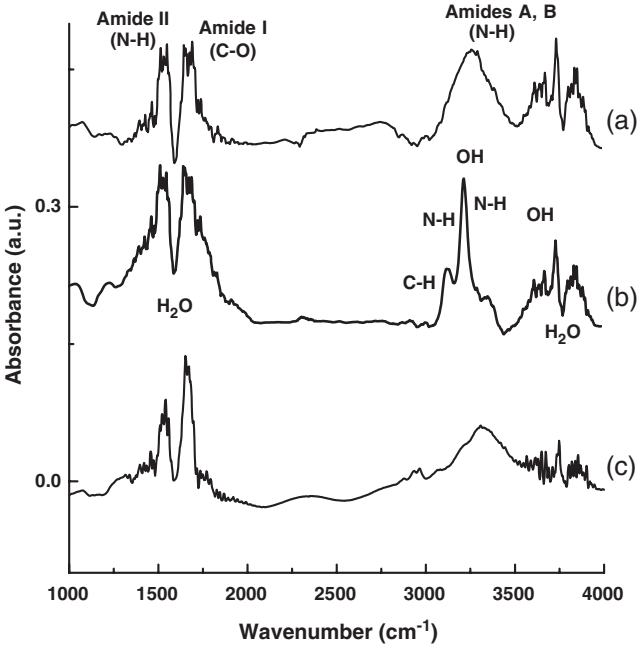

Fig. 4. The IR spectra of lysozyme (a); the IR spectra of nanodiamond–lysozyme conjugates: (b) 5 nm; (c) 100 nm.

diamond can be also adjusted by appropriate oxygen and hydrogen variations on its surface [15]. The crystal structure of nanodiamond particles surface and the existence of surface defects (like pores, dislocations) also play important roles in the protein adsorption by nanodiamond.

IR spectroscopy [16,19,21] has been used to study the adsorption of biomolecules on nanodiamond surface. The IR spectra of nanodiamond with adsorbed lysozyme were measured and compared with IR spectra of lysozyme (Fig. 4). The most characteristic IR bands of lysozyme, like other proteins, are the amide modes [28]. These bands arises from the vibrations of the peptide backbones of the protein located at 1600–1700 cm^{-1} from the C═O, C–N stretching (amide I), 1510–1580 cm^{-1} from N–H deformation and C–N stretching (amide II). In addition, a maximum near 3250 cm^{-1} can also be attributed to N–H stretching (amides A, B) [29]. The spectral band of free water lying in the same spectral ranges, 1350–1750 and 3600–3800 cm^{-1}, can complicate the attribution. All amide frequencies are conformational-sensitive. The wide peak with maximum at 3250 cm^{-1}, observed for pure lysozyme, splits to 3 peaks for lysozyme adsorbed on 5 nm nanodiamond with maximums at 3125, 3245, 3360 cm^{-1}, Fig. 4(b) and, essentially in less degree, to peaks 3245 and 3360 cm^{-1} on 100 nm nanodiamond, Fig. 4(c) . The surface bonded protein can change its conformation upon adsorption to a substrate [16]. So, the observed splitting can be connected with some conformational alignment of protein at adsorption on some structured matrix like nanocrystal diamond and needs in future study.

4. Conclusions

In this study, we presented the diamond–protein complex using the method of physical adsorption of lysozyme on carboxylated 5 and 100 nm diamonds. The results indicated the as-prepared nanodiamond–biomolecules conjugates can be stable, biocompatible and useful for biomedical applications. Our results indicated 5 nm nanodiamond can adsorb biomolecules more effectively due to its high surface to bulk carbon atoms available for immobilizing biomolecules. The prepared nanoparticle–biomolecule complex is applicable as an immobilizer for biologically active substances or low molecular weight compounds to be probed by optical microscopy and mass spectrometry. Further study on the physical characteristics of the nanodiamond surface functionalization and modification will allow the developing of this method for different kinds of biologically active molecules and the use for biosensor detection.

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