The Interaction of Protein-Modified Nanodiamond with Bacterial Cells

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ABSTRACT

The nanometer-sized diamond functionalizing and optimization of this process are studied. To create the functional groups on the nanodiamond surface the carboxylation/oxidization of 100 nm nanodiamonds was applied and its IR spectra were analyzed. The surface functionalizing was followed by conjugating with protein (lysozyme) via physical adsorption. Nanodiamond-protein interaction was analyzed using FTIR spectroscopy. The interaction of lysozyme-nanodiamond conjugates with *E. Coli* bacteria was observed by analyzing the adsorbed lysozyme antibacterial activity. Nanodiamond-lysozyme conjugates displayed high activity, equivalent to activity of lysozyme in solution. The results demonstrated that the lysozyme preserved its functionality in conjugates with nanodiamond, while in the same time nanodiamonds can serve as probe using spectroscopic methods.

Keywords: bioprobing, nanodiamond-protein conjugation, antibacterial activity

1 INTRODUCTION

Functionalizing of nanodiamond allows using diamond nanoparticles for immobilization of organic molecules and application for bioprobing. Such using is a promising subject of interest for bio-applications, due to nanodiamond availability in various nano-size, chemical stability, and biocompatibility [1, 2]. The nanoparticles functionalizing consists of creating surface functional molecular/ionic groups providing chemical/physical interaction of nanodiamond with organic molecules. The developed surface of diamond ultrafine particles can contain a large number of surface ionogenic groups like ether- C–O–C, peroxide- C–O–O–, carbonyl- C=O, and hydroxyl-type C–O–H bonding; as well as hydrocarbon fragments created directly during nanodimond production [3-6]; however different methods of controlled functionalizing are developed: hydrogenation of nanodiamond surface with H-plasma with following oxidation and photochemical conjugation with bio-active molecules [7-11]; or chemical-mechanical modification in stirring mill [12]. We are concentrated on such well-developed method as functionalizing with strong acids treatment, described initially by Ando et al., [3] and developed in many following works [14-17]. The described method includes many hours (24-72 h) treatment in H_2SO_4 and H_2NO_3 mixture, usually coupled with high temperature (75-90^oC) heating; at that many carbonyl- and hydroxyl- groups are formed.

In the presented work it is shown that the time of acid mixture treatment can be reduced significantly without losing the amount of surface functional groups as well as high temperature also is not determinative. Thus, the procedure of nanodiamond functionalizing can be simplified and accelerated.

Next step of bioprobe preparation is surface modifying with bioactive organic molecules for specific or non-specific interaction with cell structures. The organic molecules can be immobilized on the nanodiamond surface by physical adsorption [12, 16, 18, 19], by non-covalent or covalent chemical conjugation through linker molecules [13, 14, 20]. Carboxylated/oxidized diamond has positive electron affinity [7] and the functional groups on the particles surface can electrostatic interact with protein molecules, in particular, through hydrophobic and ionic interactions as well as hydrogen bonding and Van der Waals forces [16, 18]. In our work the physical adsorption of lysozyme on nanodiamond is used to analyze the interaction of protein with carboxylated nanodiamond. The surface bonded protein can change its conformation upon adsorption to a substrate [21] and such surface-induced conformational changes can also affect the protein function. The interaction of obtained lysozyme-nanodiamond conjugates with bacteria *E. Coli* has been observed and analyzed to estimate the adsorbed lysozyme antibacterial activity. These conjugates label the bacteria, with possibility to observe the nanodimond-bacteria complexes with fluorescent microscope, e.g. due to nanodiamond luminescent properties [22]. Nanodiamond-lysozyme conjugates display high antibacterial activity, equivalent to activity of lysozyme in solution.

2 MATERIALS AND METHODS

2.1 Nanodiamond carboxylation/oxidization. Synthetic diamond powder with size 100 nm (General Electric Company GE, USA) was carboxylated/oxidized in according with procedure described in [13, 14], consisting of the sample heating in a 9:1 mixture of concentrated H_2SO_4 and HNO_3 at 75°C for 3 days, then 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 diamond was extensively rinsed with deionized water and separated by sedimentation with a centrifuge at 12000 rpm and dried. To optimize the carboxylation/oxidization conditions the time of strong acids mixture treatment was varied in range of 3–72 h; temperature was varied in range 4-75^{o}C, H_2SO_4: HNO_3 was 9:1 and 3:1 (v/v). The nanodiamond surface functional groups were analyzed with FTIR spectroscopy. The FTIR-interferometer (Bomem MB154) was used with MCT detector in vacuum chamber at 10^{-5} torr; IR spectra of carboxylated nanodiamond were measured from dried samples on silicon substrate

2.2 Lysozyme adsorption. The lysozyme (AMRESCO, USA) in concentration 180-200 μM was dissolved in phosphate buffer saline (PBS), at pH=6.5-7. The protein concentration was checked with UV/Vis spectrometer (Jasco V-550) by the solution adsorption at 280 nm. The initial concentration of lysozyme in solution was measured before adsorption; then carboxylated nanodiamond was added to the solution in concentration 4-10 mg/ml. To ensure equilibration of the adsorption, the protein solution and the diamond powder were thoroughly mixed together with a shaker for 2 h, after that the mixture was several times centrifuged and washed with deionized water. After first separation of nanodiamond with adsorbed lysozyme, the residual concentration of protein in supernatant was measured. The amount of lysozyme on nanodiamond surface was estimated by difference between initial and residual protein concentrations in solution. FTIR spectra were measured from dried samples of nanodiamond with adsorbed lysozyme on silicon substrate in vacuum chamber at ~10^{-6} torr pressure.

2.3 Nanodiamond-lysozyme interaction with *Escherichia Coli* *(E. coli).* 25 μl *E. coli* suspension in PBS medium was mixed with 50 μl suspension of nanodiamond-lysozyme complexes in PBS. Concentration of nanodiamond was about 1 mg/ml; corresponding average concentration of lysozyme was estimated in accordance with UV/Vis absorption spectroscopic measurements and was 20~70 μM. For control measurements *E. coli* suspension was mixed with PBS and 20~70 μM lysozyme solution in PBS. The mixtures were incubated for 60-90 min at 37 ^{o}C. Then the bacterial suspensions were $10^4\times$ diluted, thoroughly stirred and were in 20 μl spread on Agar gel in Petri dishes. Petri dishes were incubated at 37^{o}C for 12-16 h, then the bacterial colonies in every dish were counted to estimate bacteria surviving and division after terminated treatment with lysozyme adsorbed on nanodiamond and to compare the result with estimate bacteria surviving and division after lysozyme solution treatment as well as with control.

3 RESULTS AND DISCUSSION

The IR spectra of nanodiamond carboxylated/oxidized at different conditions are plotted in Fig.1. The acid washing of nanodiamond creates the functional groups on the diamond surface, at that the IR bands at 1650-1850 cm^{-1} of diamond surfaces can be assigned to C=O stretching modes. The band near 1625 cm^{-1} can be assigned as C=O stretching for the carboxylated anion. The mode of carboxylic acid usually appears around 1775 cm^{-1} and that of carboxylic anhydrite at about 1800 cm^{-1}. In addition O-H stretching of surface –COOH group is appearing near 3710 cm^{-1} and near 3560 cm^{-1}; the band near 3710 can also be attributed as free-OH stretches of physically adsorbed water and the associated hydrogen-bonded-OH stretches are responsible to the broad absorption bands observed at 3000-3600 cm^{-1}. The bands in range 700-1450 cm^{-1} have been ascribed to ether-like groups on the diamond powders [3, 4, 16, 17, 23].

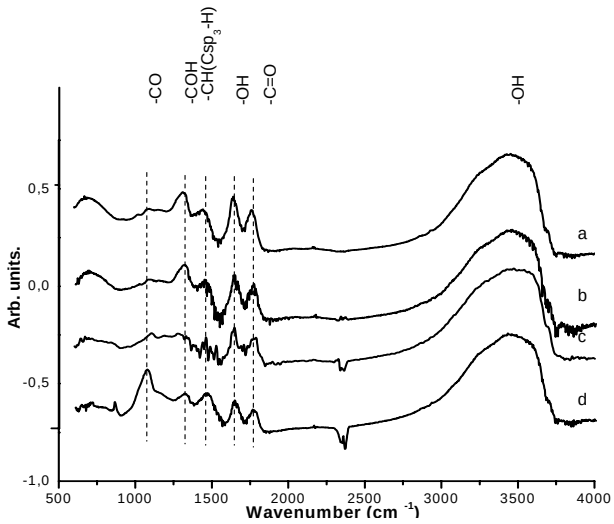

Fig.1 IR spectra of 100 nm nanodiamond prepared with different conditions of carboxylation: at acids ratio: H_2SO_4:HNO_3=9:1 and T_r (a); H_2SO_4:HNO_3=3:1 and T_r (b); H_2SO_4:HNO_3=9:1 and T=75^{o}C (c); H_2SO_4:HNO_3=3:1 and T=75^{o}C (d). Attribution of the peaks – in agreement with [25]

From Fig.1 one can see that varying temperature in wide range and strong acids ratio does not affect peaks positions as well as their relative intensities, as well as varying acid treatment time in range 3-72 h. The carboxylated/oxidized surface of nanodiamond in water solution (like PBS etc.) is negatively charged at least due to dissociation of -COOH and forming -COO^- ion group. That allows modification of the nanodiamond surface by biomolecules via adsorption.

Next we analyze the influence of different carboxylating conditions on the interaction of nanodiamond with protein. Lysozyme was used as a test protein in this analyzis. The IR spectra of lysozyme and carboxylated nanodiamond with lysozyme on the Si wafer are shown in Fig.2. The most characteristic IR bands of lysozyme, like other protenes, are connected with amide modes [24]; the bands arising first of all from the vibrations of the peptide backbones of the adsorbed protein can be identified at 1600-1700 cm^{-1} for the C=O, C-N stretching bonds (Amide I), at 1510-1580 cm^{-1} for the N-H deformation and also C-N stretching bonds (Amide II), as well as the band with maximum near 3250 cm^{-1} can be also attributed as N-H stretch bond [25]. Note also, that the spectral band of free water lie in the same spectral ranges that complicates the attribution – 1350-1750 cm^{-1}; 3600-3800 cm^{-1}; and of O-H stretching (including stretches of bonded water) – 3000-3400 cm^{-1}. All amide frequencies are conformational-sensitive. So, it's interesting that the wide peak with maximum at 3250 cm^{-1}, observed for pure lysozyme shifts and slightly splits for nanodiamond-adsorbed lysozyme to peaks 3240 and 3360 cm^{-1}. The surface bonded protein can change its conformation upon adsorption to a substrate [21]. So, the observed splitting can be connected with some conformational alignment of protein at adsorption on some structured matrix like nanocrystal diamond and conformational changes can also affect the protein function.

The amount of lysozyme adsorbed on nanodiamond surface was estimated by the UV/Vis spectra of lysozyme solutions before and after reaction with nanodiamond. Only limited amount of protein can be adsorbed on nanodiamond surface [14, 26]. Maximum amount of lysozyme which can be adsorbed by 1 mg of 100 nm carboxylated nanodiamond is about 80 ± 10 μg [26]. In Fig 3 the diagram is presented characterizing the maximal adsorption of lysozyme on nanodiamond carboxylated at different conditions in the carboxylation processes. The difference in adsorption lies within the data precision; therefore the varying of nanodiamond carboxylation conditions does not affect also the lysozyme adsorption. It means that in wide ranges these conditions can be chosen in accordance with other conditions of the carried experiment. It also tells us th at the processes of carboxylation as well as adsorption are not affected with some occasion fluctuations of the conditions.

To a nalyze how the interaction with nanodiamond

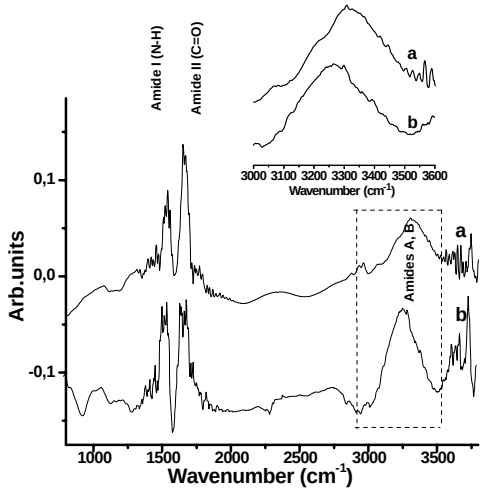

Fig.2 IR spectra of 100 nm carboxylated nanodiamond with adsorbed lysozyme (a) and lysozyme (b).

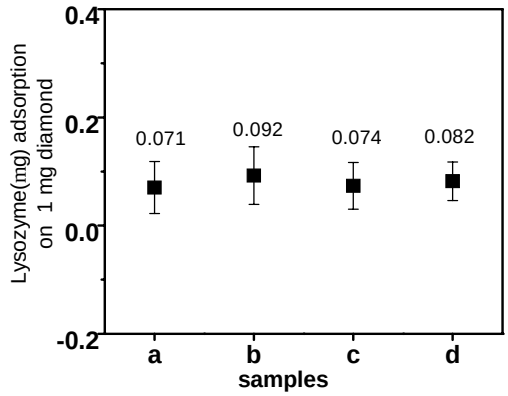

Fig.3 The lysozyme adsorption with 100 nm diamond prepared with different conditions of carboxilation: acids ratios are: H_2SO_4:HNO_3=9:1 and t_r (a); H_2SO_4:HNO_3=3:1 and t_r (b); H_2SO_4:HNO_3=9:1 and t=75°C (c); H_2SO_4:HNO_3=3:1 and t=75°C (d).

surface affects the lysozyme functional activity, the bacteria *E. coli* was treated with nanodiamond with adsorbed lysozyme. The diagram in Fig.4 demonstr ates survival rate of the colonies growth of *E. coli* after 60 min treatment and following incubation. The diagram averages the data of 3 separated experiments.

Nanodiamond was acids treated (H_2SO_4:HNO_3=3:1) at room temperature for 24 h. One can see that after the treatment with nanodiamond-lysozyme conjugates the colonies number decreases significantly relatively to control, so nanodiamond-lysozyme conjugates display antibacterial activity, equivalent to high activity of pure

lysozyme in solution within experimental error. This also demonstrates that the adsorption of lysozyme on nanodiamond significantly alter the original functionality of the protein, for the case of lysozyme.

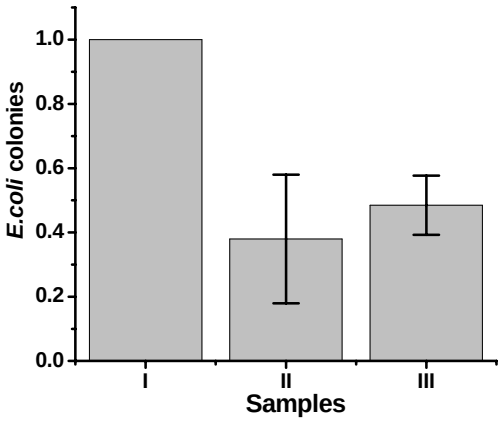

Fig.4. Standardized number of *E. coli* colonies after 60 min treatment with: (I) PBS – control; (II) lysozyme solution; (III) suspension of lysozyme-carboxylated nanodiamond conjugates.

4 CONCLUSION

Strong acids treatment modifies nanodiamond surface with functional molecular groups. The spectral properties of the surface are not varying too easily at various conditions of nanodiamond carboxylation with the acids treatment, such as temperature and time of treatment in wide range, as well as strong acids ratio. Following protein adsorption also does not depend on these conditions varying. It's significant because the adsorbing surface can influence protein conformation [14, 21, 26] and the conformation in essential degree determines the protein functions. However, decreasing antibacterial activity of diamond-lysozyme complexes relatively lysozyme solution is not observed. So, from that, as well as from the stability of the nanodiamond surface properties to the varying of conditions of its treatment, we confirm that diamond nanoparticles are convenient substrate for protein immobilization with preserving proteine's functionality, for development of nanobioprobes.

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