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Adsorption of human blood plasma on nanodiamond and its influence on activated partial thromboplastin time☆,☆☆

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ABSTRACT

Ever increasing use of engineered nanodiamond (ND) into the human blood for various biomedical studies and applications has increased the demand to thoroughly understand the interaction of NDs with blood and its effect on blood coagulation. Here, we report on the study of adsorption of human blood plasma on various sized carboxylated nanodiamonds (cNDs) using UV/visible spectroscopy and Fourier transform infrared spectroscopy (FTIR). The adsorption of human blood plasma on 5 nm and 100 nm sized cNDs is confirmed from UV/visible spectra. FTIR shows minor change in the shape of amide I absorption peak (1600–1700 cm^{-1}) indicating that the protein secondary structure remains unaffected for human blood plasma. The influence of cNDs on the blood coagulation has been estimated using Activated Partial Thromboplastin Time (APTT) test. The APTT test is one of the standard tests used to investigate the efficacy of the intrinsic pathways of coagulation. The APTT test results indicate that 5 and 100 nm cNDs with various concentrations (10–500 μg/ml) do not show delay in time when coagulation was initiated through the intrinsic pathway.

1. Introduction

In recent years, research on the biomedical applications of nanodiamond (ND) has increased substantially, especially in the fields of drug delivery (i.e. cancer therapeutics) [1–4], photoluminescent/Raman biomarker and bioprobes [5–7], nanosurgery [1], prosthetic devices for retinal implants [1], analytical diagnostics [1], biosensors [8], and bio-chips [9], etc. These applications directly benefit from various interesting properties of NDs, such as optical and spectroscopic properties [7,10,11], high chemical stability, special structure/surface [1] as well as extremely low toxicity to various animal cells and living organisms [7,11–13]. In addition to these properties, ND's surface with relatively large number of surface atoms makes them amenable for functionalization/conjugation with various bio/medical molecules/drugs of interest [14,15]. ND's effect on the function of biological objects, such as whole organisms, individual organs, tissues and ND's interactions with blood and its components are important to understand.

In particular, for the research related to nanoparticle's application in biomedicine, it is mandatory to inject nanoparticles in blood. Recently, many investigations of nanoparticle interactions with blood and blood components have been performed. The extent of protein adsorption such as albumin, immunoglobulins, fibrinogen, histone, insulin, etc. from blood plasma, on different types of nanoparticles of various size and surface chemistry has been investigated [16–19]. Wasdo et al. identified 69 unique proteins that exhibited adsorption to various nanoparticles [20]. Uptake of blood electrolytes from simulated blood fluid (SBF) and the stability (by dispersion/aggregation) of nanoparticles in SBF have been examined [21]. Effects of various nanoparticles on red blood cell hemolysis and blood clotting parameters have been measured [9,21–23]. Human platelet aggregation in-vitro and rat vascular thrombosis in-vivo using engineered carbon nanoparticles have been studied [24]. Blood platelet activation with inducing extracellular Ca^{2+} influx and with a marked release of platelet membrane microparticles positive for the granular secretion markers along with platelet aggregating activity has been analyzed using carbon nanotubes [25].

As for ND, interaction of ultrafine detonated ND with whole blood affects blood's some biochemical characteristics by causing destruction of mammalian cells, but serious blood cell destruction was not observed [9]. Wasdo et al. studied the interactions of blood plasma proteins (albumin and γ-globulin) with nanoparticles including NDs [20]. They found low affinity of both albumin and γ-globulin to nanoparticles in in-vitro conditions. The influence of ND interaction on the oxygenation and de-oxygenation states of hemoglobin and micro rheological

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properties with human RBCs in-vitro suggested that the ND can be used effectively as bio-label and drug delivery tool in ambient conditions, without complicating the blood's physiological conditions [23].

Mostly, hemolytic properties are used as one of the common tests for understanding nanoparticle interactions. Hemolytic effect was quantitatively estimated for 5 and 100 nm carboxylated NDs (cNDs) and the hemolysis did not exceed 2 to 4% as compared with hemolysis created by Triton X-100 [23]. Another important test is blood coagulation. When the blood is exposed to exogenous factors (like tissue damage) blood coagulation is activated and this leads to generation of thrombin and fibrin clot that stops the bleeding. Thus, it is important to determine whether cNDs could affect the coagulation factors.

Carbon nanoparticle induced platelet aggregation and vascular thrombosis was studied by Radomski et al. [24]. It is important to mention that carbon single-wall and multi-wall nanotubes and amorphous carbon nanoparticles are clearly different when it comes to their platelet reactivity. Effects of CNT on prothrombotic mechanisms and vascular toxic effects have been discussed [25]. As for detonation diamond nanoparticles, cNDs evoked significant activation of human platelets and in vivo pulmonary thromboembolism [26]. However, rarely any research exists on ND effects on blood clotting. Studies concerning the blood components' interaction with diamond-like carbon (convenient sp^3/sp^2 ratio, hydrogenation state) and nano-crystalline diamond (good structure without defects) thin film surfaces demonstrated high level of resistance to blood platelet adhesion and thrombus formation [27–29]. This has been explained by significantly reduced plasma protein adhesion to such surface. In contrast to this observation, significantly large amount of blood plasma proteins on the diamond nanoparticles has been reported [16,30]. Morphology and coagulation of human blood cells attached to borosilicate glass and diamond substrates were compared [31]. It was shown that diamond surface is less thrombogenic than the glass surface, thus indicating that diamond could be an attractive material for future designing of prostheses and medical devices such as surgery instruments, blood pumps and artificial hearts.

As a consequence, it is absolutely essential for researchers to pay close attention to interactions of NDs with blood and its components and study its blood coagulation compatibilities. In the present work, we have studied the interaction of cND (5 and 100 nm) with human blood plasma. UV–visible absorption has been used to observe the loading of blood plasma on cND. FTIR spectroscopy and ζ-potential measurements were used to access the structural changes. In addition, the influence of ND on the blood coagulation is quantified using Activated Partial Thromboplastin Time (APTT) test, which is one of the standard tests used to investigate the efficacy of the intrinsic pathways of coagulation.

2. Experimental procedure

Synthetic diamond powders with nominal particle sizes of 5 and 100 nm were purchased from Microdiamont AG, Switzerland and Kay Diamond, USA respectively. Previously it was reported that after carboxylation of diamond the surface molecular and ionic groups facilitate the interaction of diamond with biomolecules and show better biocompatibility [32,33]. Therefore, the diamond powders were carboxylated (referred to as cND) using strong acid treatments to create carboxyl groups and to clean the surface admixtures, defects, and impurities according to the methods described earlier [32,33].

The experiments using human subjects were coherent to the national regulation of Human Subject Research (Taiwan) and the Declaration of Helsinki. To achieve the purpose, we had formally obtained written and signed consent from all the volunteers, the PI, as well as the students who performed the experiments and had taken lectures concerning ethical issues. The protocol is approved by the research ethics committee, Tzu Chi General Hospital, Hualien Taiwan. The committee is organized under, and operates in accordance with, the good clinical practice guidelines and governmental laws and regulations. 5 ml of whole blood was withdrawn from healthy volunteers with the use of EDTA as anticoagulant. The plasma fraction was separated by centrifugation at 1500 rpf for 5 min at 4 °C and the whole supernatant was defined as plasma which was platelet-poor plasma. For interaction of cND with plasma, 2 mg of cNDs were dispersed in 1 ml of plasma and were allowed interacting uniformly using vortex at 37 °C for 2 h. After 2 h through agitation, the mixture was centrifuged at 11000 rcf for 10 min to separate the unreacted plasma components present in supernatant. The supernatant was subject to three times washing to remove the non-interacted plasma. The supernatant was decanted and this suspension was used to measure the UV/vis absorption spectra which were recorded using a Jasco V550 UV/visible spectrophotometer (JASCO International Co., LTD., Tokyo, Japan). The sediment (cND interacted with plasma) was dispersed in 1 ml of standard phosphate buffer saline (PBS: NaCl 0.4 g; KCl 0.01 g, Na_2HPO_4 0.072 g, KH_2PO_4 0.021 g; H_20 50 ml; pH 7.5) by ultrasonication for 15 min followed by vortex for 10 min. The process of washing was done thrice to completely remove the un-reacted plasma. The cND–plasma complex was centrifuged at the rate of 3400 rpf for 1 min and the cND–plasma complex sediment was dropped on Si substrate and dried naturally. FTIR spectrum of dried cND–plasma complex on Si substrate was obtained using Bomem MB154 spectrometer equipped with MCT liquid nitrogen cooled detector. The spectrum was recorded in the scan range of 400–4000 cm^{-1}. The ζ-potential of plasma, 5 nm and 100 nm cNDs and cND–plasma was determined for pH 7.5 using the Zetasizer Nano-ZS, Malvern instruments, UK. The effect of NDs on plasma coagulation activity was performed using Activated Partial Thromboplastin Time (APTT) test. For APTT determination, various concentrations (10 μg/ml, 100 μg/ml, 500 μg/ml) of cND–plasma complexes were incubated at 37 °C for 60 s. Actin FSL activating reagent (Siemens, Germany) was added and the mixture was incubated for 180 s. The reaction was monitored for 190 s, after the addition of 50 μl of calcium chloride. Plasma coagulation experiments were performed using a Sysmex CA-50 plasma coagulation analyzer from Sysmex Corporation (Kobe, Japan).

3. Results and discussion

Fig. 1 shows the absorption spectra of plasma before and after interaction (supernatant of plasma) with 5 nm and 100 nm cNDs at pH ~ 7.5. The difference in the absorption before and after interaction exemplifies the amount of plasma components adsorbed on cND particles. It can be seen that 5 nm cND adsorbs more amount of components of blood plasma as compared to 100 nm cND. This is probably because, for 5 nm cND, the total surface area is larger and the surface structure is different than 100 nm cND. Additionally, the electrostatic interaction plays an important role for studying the interaction of plasma with the surface of cND. The charge state of cND has been characterized using ζ-potential measurements for plasma, 5 and 100 nm cNDs, and cND–plasma as shown in Fig. 2. Measurements were performed at pH ~ 7.5. The ζ-potentials are negative, for plasma it is ~−5 mV (average for the plasma components), for 5 nm and 100 nm before interaction they are ~−8 mV and ~−22 mV, respectively. After plasma absorption ζ-potentials shift to ~−5 mV for 5 nm cND and ~−20 mV for 100 nm cND. The ζ-potential becomes less negative because the adsorption of less electronegative components from plasma decreases the electronegativity of the complex on the surfaces, which results in the variation of the ζ-potential.

FTIR spectroscopy is sensitive to ND's surface molecular and ionic groups and usually used for analysis of ND's surface. Measured FTIR spectra for human blood plasma, 5 and 100 nm cNDs and cND–plasma complexes are displayed in Fig. 3. Fig. 3((a, b)-i) shows the spectra of pristine 5 and 100 nm cND particles. The most prominent absorption bands after carboxylation are related to C═O stretching