Functionalization of single-walled carbon nanotubes regulates their effect on hemostasis

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Abstract. Applications of single-walled carbon nanotubes (SWNTs) in medical field imply the use of drug-coupled carbon nanotubes as well as carbon nanotubes functionalized with different chemical groups that change nanotube surface properties and interactions between nanotubes and cells. Covalent attachment of polyethylene glycol (PEG) to carboxylated single-walled carbon nanotubes (c-SWNT) is known to prevent the nanotubes from interaction with macrophages. Here we characterized nanotube’s ability to stimulate coagulation processes in platelet-poor plasma (PPP), and evaluated the effect of SWNTs on platelet aggregation in platelet-rich plasma (PRP). Our study showed that PEG-SWNT did not affect the rate of clotting in PPP, while c-SWNT shortened the clot formation time five times compared to the control PPP. Since c-SWNT failed to accelerate coagulation in plasma lacking coagulation factor XI, it may be suggested that c-SWNT affects the contact activation pathway. In PRP, platelets responded to both SWNT types with irreversible aggregation, as evidenced by changes in the aggregate mean radius. However, the rate of aggregation induced by c-SWNT was two times higher than it was with PEG-SWNT. Cytological analysis also showed that c-SWNT was two times more efficient when compared to PEG-SWNT in aggregating platelets in PRP. Taken together, our results show that functionalization of nanoparticles can diminish their negative influence on blood cells. As seen from our data, modification of c-SWNT with PEG, when only a one percent of carbon atoms is bound to polymer (70 wt %), decreased the nanotube-induced coagulation in PRP and repelled the accelerating effect on the coagulation in PPP. Thus, when functionalized SWNTs are used for administration into bloodstream of laboratory animals, their possible pro-coagulant and pro-aggregating properties must be taken into account.

Prospects for using single-walled carbon nanotubes (SWNTs) in targeted drug delivery and tumor treatment have raised concerns about their biocompatibility and possible adverse effects on human health. In recent years, many studies have been devoted to in vitro investigation of the interaction between carbon nanotubes and blood proteins and cells [1]. In particular, it was shown that SWNTs added either to isolated platelets or platelet-rich plasma (PRP) stimulate platelets to aggregate [2, 3, 4]. However, SWNTs may interact simultaneously with many factors of hemostasis, and their effect on blood coagulation will not be limited to induced platelet aggregation. Therefore, in this study we focused on the effect of SWNTs on coagulation not only in human PRP, but also in platelet-poor plasma (PPP). Furthermore, applications of SWNTs in medical field imply the use of drug-coupled, functionalized carbon nanotubes as well as carbon nanotubes functionalized with different chemical groups, leading to a significant
change in nanotube surface properties and interaction between nanotubes and cells. In order to increase the dispersability of carbon nanoparticles, they are modified by the addition of charged groups on the surface. For example, the oxidation of SWNTs generates carboxyl groups on the nanotube surface. The further covalent attachment of polyethylene glycol (PEG) to the produced carboxylated single-walled carbon nanotubes (c-SWNTs) can prevent the interaction of the nanotubes with macrophages. Intravenously injected PEG functionalized SWNTs (PEG-SWNTs) conjugated with a cancer chemotherapy drug paclitaxel was shown to suppress the breast tumor growth in mice with a higher efficacy than clinical paclitaxel [5].

Here we evaluated the interaction of functionalized nanotubes c-SWNTs and PEG-SWNTs (Carbon Solutions Inc., USA, [6]) with blood plasma proteins in PPP, characterized nanotube’s ability to stimulate coagulation processes in PPP, and evaluated the effect of SWNTs on platelet aggregation in PRP.

The nanotube’s capacity to bind human plasma proteins appeared to be significantly different between c-SWNTs and PEG-SWNTs. c-SWNTs efficiently and non-selectively adsorbed proteins with a concomitant sedimentation. PEG-SWNTs adsorbed by an order lower amount of proteins and displayed no adsorption preference for major plasma protein albumin (Fig. 1).

The pro-coagulant property of nanotubes was assessed by their effect on the coagulation cascade. The latter involves circulating coagulation factors (which act as enzymes, which require activation, and cofactors), as well as calcium and platelets and a binding surface upon which the coagulation cascade proceeds. The coagulation cascade has the tissue factor pathway (or the extrinsic pathway) and the contact activation pathway (or the intrinsic pathway) that converge at the generation of thrombin from prothrombin, the cleavage of fibrinogen, by thrombin, and the formation of fibrin fibers which form blood clots. We characterized blood coagulation in PPP by (1) the clot formation time, i.e. the amount of time required for stable clot formation, and (2) the presence of fibrinopeptides and absence of prothrombin in serum (after clotting). The clot formation was stated if the meniscus at the top of plasma in a reaction tube remained parallel to the bottom of the tube even when we inclined the tube at 60 degrees. PEG-SWNTs did not affect the rate of clotting, while c-SWNTs shortened the clot formation time five times compared to the control PPP. Probably, the difference in the affinity of plasma proteins to different nanotubes underlies the latter discrepancy between the two types of nanotubes. In all samples after clotting, fibrinopeptides and no prothrombin were detected in serum. Since c-SWNTs failed to accelerate coagulation in plasma lacking coagulation factor XI, it may be suggested that c-SWNTs affects the contact activation pathway. Considering that factor XII autoactivation, which readily occurs in contact with a variety of negatively charged surfaces, is enough for initiation of the contact activation pathway, we may suggest that c-SWNTs provides a surface that is suitable for contact activation of blood coagulation.

At the same time, both SWNTs types nearly equally quicken the clotting in PRP. The addition of c-SWNTs or PEG-SWNTs to PRP shortened the clot formation time 4 and 3 times, respectively. Since platelet participation in blood coagulation hastens the process 15 times, it was necessarily to examine the effect of SWNTs on platelets. Platelet aggregation was assessed by measuring the aggregate mean radius [7]. The results of our experiments show that in PRP, platelets responded to both SWNTs types with irreversible aggregation. However, the rate of c-SWNTs induced aggregation was 2 times higher than it was for PEG-SWNTs (Fig. 2A). The addition of EDTA completely inhibited platelet aggregation, which indicates calcium ion (Ca\(^{2+}\)) participation in the SWNT-induced platelet aggregation. Calcium ions can act as a bridge between negatively charged groups on the cell surface and negatively charged groups (carboxyl or hydroxyl) on the nanotube surface. Calcium is also required to stabilize the binding of coagulation factors V and VIII to platelets. A slight accelerating effect, similar for both c-SWNTs and PEG-SWNTs, on ADP-induced platelet aggregation in PRP (Fig. 2B) can be attributed mainly to pre-activation of platelets by the nanotubes before the addition of ADP.
These phenomena have been also observed for non-functionalized carbon nanotubes [2, 3, 4]. Cytological analysis of PRP demonstrated as well that platelets are aggregated by c-SWNTs and PEG-SWNTs (Fig. 3). The number of small aggregates (2-4 platelets per aggregate) and the number of big aggregates (5-10 platelets per aggregate) was 4- and 11-times, respectively, higher than that in the control blood plasma sample. A similar effect was seen with PEG-SWNTs, though it was significantly less. The number of small aggregates increased 2.6-times, and the number of big ones increased 4-times. Thus, despite significant differences in the binding of plasma proteins to c-SWNT and PEG-SWNTs, these nanotube types are only slightly different in respect of the interaction with platelet membrane and induction of platelet aggregation.

Taken together, our results show that even functionalized carbon nanotubes can cause irreversible alterations in hemostasis: both SWNT types used in our work induce platelet aggregation and blood coagulation. However, the functionalization of nanoparticles can diminish their negative influence on blood cells. As seen from our data, modification of c-SWNTs with PEG, when only a one percent of carbon atoms is bound to polymer (70 wt % [6]), decreased the nanotube-induced coagulation in PRP and repelled the accelerating effect on the coagulation in PPP. Thus, when functionalized SWNTs are used for administration into bloodstream of laboratory animals, their possible pro-coagulant and pro-aggregating properties must be taken into account. The simple methods used in our work to evaluate the effect of SWNTs on blood coagulation can be useful for preliminary evaluation of the effect of functionalized nanoparticles on hemostasis. Prospects for the use of SWNTs in nanopharmacology call for additional approaches to nanotube functionalization to prevent thrombogenesis.

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Figure 1. SDS-PAGE of plasma proteins (middle line) and proteins adsorbed on c-SWNTs (P3, left line) and PEG-SWNTs (P7, right line). The molecular masses (kDa) are indicated on the left.

Figure 2. (A) SWNTs induce platelet aggregation in PRP. a) Aggregate mean radius in PRP as function of time: (1) c-SWNTs, (2) PEG-SWNTs, (3) PRP incubated 2 min with 100 μM EDTA before SWNTs addition. SWNTs = 20 μg/ml. (b) The rate of aggregate mean radius formation increases on nanotubes concentration.

(B) SWNTs activate ADP-induced platelet aggregation in PRP. a) Aggregate mean radius was measured as function of time after addition of 1 μM ADP to control plasma (1) or plasma pre-incubated with nanotubes: (2) PEG-SWNTs or (3) c-SWNTs. SWNTs = 20 μg/ml. b) Maximum radius of aggregates in PRP on nanotubes concentration.
Figure 3. Images of platelets in PRP. Smears were stained by Romanovsky-Giemsa method. The single platelets of various shape and size are present in control plasma, formation of platelet aggregates were detected after nanotubes addition (20 μg/ml, 10 min - incubation time).