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In vitro platelet activation, aggregation and platelet–granulocyte complex formation induced by surface modified single-walled carbon nanotubes

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ABSTRACT

Surface modification of single-walled carbon nanotubes (SWCNTs) such as carboxylation, amidation, hydroxylation and pegylation is used to reduce the nanotube toxicity and render them more suitable for biomedical applications than their pristine counterparts. Toxicity can be manifested in platelet activation as it has been shown for SWCNTs. However, the effect of various surface modifications on the platelet activating potential of SWCNTs has not been tested yet. *In vitro* platelet activation (CD62P) as well as the platelet–granulocyte complex formation (CD15/CD41 double positivity) in human whole blood were measured by flow cytometry in the presence of 0.1 mg/ml of pristine or various surface modified SWCNTs. The effect of various SWCNTs was tested by whole blood impedance aggregometry, too. All tested SWCNTs but the hydroxylated ones activate platelets and promote platelet–granulocyte complex formation *in vitro*. Carboxylated, pegylated and pristine SWCNTs induce whole blood aggregation as well. Although pegylation is preferred from biomedical point of view, among the samples tested by us pegylated SWCNTs induced far the most prominent activation and a well detectable aggregation of platelets in whole blood.

1. Introduction

The utilization of carbon nanotubes (CNTs) has been suggested for various fields of nanomedicine, such as specific delivery of bioactive molecules, drugs or contrast materials (Sakamoto et al., 2010; Zhang et al., 2012b). CNTs used for human therapy or diagnostics need to form a stable dispersion in physiological solutions and must not be toxic. Increased dispersibility can be achieved by attaching various functional groups to the surface of CNTs, like $CONH_2$, COOH, OH, or PEG-molecules (Imasaka et al., 2009; Heister et al., 2010; Wang et al., 2012). However, any kind of surface modification of CNTs may affect their pharmaco-kinetic properties (Lam et al., 2006; Zhang et al., 2012a) and thus their toxicity. Pegylation is a very effective tool in increasing the dispersibility of CNTs in aqueous media (Hadidi et al., 2011). Its effect has been studied the most among the many surface modification procedures as a mean to shield CNTs against phagocytosis and to lessen their toxicity (Bottini et al., 2011; Yang et al., 2012).

The effect of CNTs either pristine or surface modified has been studied using various cell lines (Albini et al., 2010; Gutierrez-Praena et al., 2011; Jos et al., 2009; Montes-Fonseca et al., 2012; Pichardo et al., 2012). Importantly, most biomedical applications (Bottini et al., 2011; Chen et al., 2008; Cheng et al., 2011; Kolosnjaj-Tabi et al., 2010; Lam et al., 2006; Perán et al., 2012; Peretz and Regev, 2012) result in a close contact between blood cells and CNTs. One of the consequences of the interaction between blood cells and nanotubes is that certain CNTs affect the hemostasis (Sokolov et al., 2011) especially the platelet function (Guidetti et al., 2012; Radomski et al., 2005). Indeed, the in vivo thrombogenic potential of pristine CNTs in animal models has been reported (Bihari et al., 2010; Radomski et al., 2005). The CNTs also

Abbreviations: CNT, carbon nanotube; SWCNT, single-walled carbon nanotube; p-SWCNT, pristine single-walled carbon nanotube; COOH–SWCNT, carboxylated single-walled carbon nanotube; OH–SWCNT, hydroxylated single-walled carbon nanotube; $CONH_2$–SWCNT, amidated single-walled carbon nanotube; PEG–SWCNT, pegylated single-walled carbon nanotube; MFI, mean fluorescence intensity; SE, standard error of mean; SEM, scanning electron microscope; TEM, transmission electron microscope; EDS, energy dispersive X-ray spectroscopy.

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induce formation of heterotypic platelet-leukocyte aggregates, mainly via platelet activation. This kind of interaction plays an important role in platelet dependent granulocyte activation and migration (Kornerup et al., 2010), thus initiating crosstalk with the innate immune system (Li et al., 2000; Speth et al., 2013).

Although functionalized CNTs have been shown to circulate longer in blood than their pristine counterpart (Hadidi et al., 2011; Heister et al., 2010), their effect on platelet function has been investigated less. Only a few data are available to date on functionalized CNTs in terms of their platelet activating potential (Burke et al., 2011; Meng et al., 2012; Sokolov et al., 2011). In addition, we could not reference any study dealing with the effect of the various functionalized carbon nanotubes exerted on the platelet–granulocyte complex formation. Thus the aim of our recent study was to judge the *in vitro* toxicity of several surface modified single walled CNTs through their potential to induce platelet activation, platelet aggregation and platelet–granulocyte complex formation.

2. Materials and methods

2.1. Single-walled carbon nanotubes studied

Single-walled carbon nanotubes (SWCNTs) and its various surface modified forms were purchased from the following companies with characteristics as indicated on the technical sheets attached: pristine SWCNTs (p-SWCNTs, cat.# 900-1351, Outer diameter < 2 nm, length: 1–5 μm, purity: >90% CNTs and >50% SWCNTs) – SES Research (Houston, USA); pegylated SWCNTs (PEG–SWCNTs, cat.# 652474, diameter 4–5 nm, Length 0.5–0.6 μm, Purity: >80% carbon basis, 4–5% trace metals) and amidated SWCNTs ($CONH_2$–SWCNTs, cat.# 685380, diameter 4–6 nm, length 0.7–1.0 μm, purity: >90% carbon basis, 5–8% trace metals) – Sigma–Aldrich; carboxylated SWCNTs (COOH–SWCNTs, cat.# 1288YJF, Average diameter 1–2 nm, length 5–30 μm, purity: >95% CNTs, >90% SWCNTs, –COOH: 2.59–2.87 wt%) – NanoAmor (Los Alamos, USA) and hydroxylated SWCNTs (OH–SWCNTs, cat.# SO-SL1, Outer diameter 1–2 nm, length: 3–8 μm, purity: >98 wt% SWCNTs, OH: 2–4 wt%) – NanoShel (Delaware, USA).

2.2. Characterization of nanotubes

The morphology of studied nanotubes was characterized both with Zeiss ULTRAplus scanning electron microscope (SEM) and with Jeol JEM 2010 transmission electron microscope (TEM). In the case of SEM, very low accelerating voltage (3 kV) was used in order to analyze samples without any coating procedure. In the case of TEM, the accelerating voltage was 120 kV. Energy-dispersive X-ray spectroscopic (EDS) analyses were carried out with Thermonoran Vantage EDS-analyzer attached on the TEM. Theoretical detection limit of EDS analyzer is $Z > 5$ (boron) and element content > 0.1 wt%. Nanotube samples were prepared from each SWCNTs powder by crushing them gently between glass slides and mixing the powder either to ethanol or to distilled water and applying a drop onto copper grid or copper–holey carbon layer grid sample holder for scanning and transmission electron microscopy, respectively.

2.3. Preparation and characterization of nanotube dispersions

Stock solutions of all the SWCNTs samples were prepared according to Bihari et al. (2008b) in distilled water using sonication with 4.2×10^5 kJ/m^3 specific energy in three consecutive steps for 2 min each. After the first sonication human serum albumin at final concentration of 1.5 mg/ml was added followed by a second sonication with the same parameters as in the first time. Before the third sonication step, the physiological ionic strength of the sample was attained by adding a proper volume of 10 × concentrated phosphate buffered saline solution (Sigma, cat.# P5493) to ensure a final phosphate buffer concentration of 0.01 M and a NaCl concentration of 0.154 M, pH 7.4. The final concentrations of stock nanotube dispersions were 0.2 mg/ml and 0.5 mg/ml for flow cytometry and for aggregometry measurements, respectively. Control sample was prepared in the same way as nanotube dispersions replacing nanotubes by distilled water.

The 0.2 mg/ml stock nanotube dispersions were characterized by their polydispersity indices (PdI) and zeta-potentials measured by dynamic light scattering and by laser Doppler velocimetry, respectively, using a Zetasiter Nano-ZS instrument (Malvern, UK). The main component and the Z-average of the equivalent hydrodynamic diameters of various nanotubes were obtained from the dynamic light scattering data as well.

2.4. Human blood samples

Human blood was collected from the cubital vein of healthy volunteers into Vacuette test tubes containing trisodium-citrate anticoagulant (Greiner, Austria, Kremsmünster) in accordance with the approval policy of the local Ethical Committee after the donor had given informed consent. Blood counts of samples involved in this study were in the normal range ($160–320 \times 10^9$/L).

2.5. Flow cytometry

100 μl of citrated blood was incubated with 100 μl of 0.2 mg/ml nanotube dispersion at room temperature for 10 min followed by another 5-fold dilution with Tyrode-HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, pH 7.4) containing 5.5 mM glucose and 0.35% bovine serum albumin. For control measurements nanotube dispersion was replaced by control solution. 1 μM ADP (Chrono-Log, USA) served as positive control for platelet activation.

Platelet activation was characterized by changes in the surface expression of CD62P (P-selectin), the platelet activation marker. Platelets in the whole blood were stained with PE-labeled anti-CD62P (Dako, Glostrup, Denmark) and FITC-labeled anti-CD41 (Immunotech, Marseilles, France) antibodies according to the manufacturer's instructions. Aspecific staining of samples was checked by using appropriate isotype controls. After staining samples were diluted with Tyrode-HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, pH 7.4) containing 5.5 mM glucose and 0.35% bovine serum albumin in such an extent that a 250-fold final dilution of blood was attained. To minimize the spontaneous activation of platelets, no washing steps were used. The CD41 platelet marker was used as a trigger signal for data collection, and platelets were gated on the forward-side scatter dot plot and the mean CD62P fluorescence intensity (MFI) was measured.

When the platelet–granulocyte complex formation was analyzed blood samples were treated in the same way as in the case of platelet activation experiments. Platelet–granulocyte complexes were measured as described previously (Bihari et al., 2008a; Fent et al., 2008). Briefly: whole blood was stained with FITC-labeled anti-CD41 (Immunotech, Marseilles, France) and Cy5-labeled anti-CD15 (BioLegend, San Diego, USA) antibodies. 500-fold dilution of blood was used in these measurements, thus coincidence of platelets and granulocytes did not result in false double positivity. The CD15 granulocyte marker was used as trigger signal for data collection. The amount of platelet–granulocyte complexes was determined as percentage of CD41 positive events in the