Interaction of Carbon Nanotubes with Human Blood Platelets

S.H. Lacerda[*], J. Semberova[*], K. Holada[**], O. Simakova[***], M.P. Gelderman [*], and J. Simak[*]

[*] Center for Biologics Evaluation and Research, FDA,
1401 Rockville Pike, HFM-335, Rockville, MD 20851-1448, USA, jan.simak@fda.hhs.gov
[**]1st Faculty of Medicine, Charles University, Prague, Czech Republic
[***]NIH Clinical Center, Bethesda, MD, USA

ABSTRACT

We showed, by using light transmission platelet aggregometry that carboxylated multi-walled carbon nanotubes (MWCNTs) from NanoLab at 100 μg/mL induced marked aggregation of blood platelets in citrated plasma, similarly to non-functionalized MWCNTs. In contrast, fullerene (nC60) and polystyrene beads (20 nm and 200 nm) did not induce platelet aggregating activity. Due to the limited dispersability of commercial carboxylated MWCNTs, we prepared carboxylated MWCNTs in house. Our preparation of carboxylated MWCNTs was well dispersed in PBS, but unstable in citrated blood plasma, and formed large agglomerates. Field Emission Scanning Electron Microscopy (FESEM) showed that platelets in contact with carboxylated MWCNTs undergo morphological changes from the resting discoid state to activated state with pseudopodia. In conclusion, carboxylated MWCNTs activate blood platelets in a similar fashion like their pristine counterparts.

Keywords: carbon nanotubes, blood platelets, thrombogenicity, biocompatibility, blood toxicity

1 INTRODUCTION

Carbon nanotubes (CNTs) are tubular nanostructures of covalently bonded carbon atoms, formed by rolling up graphene sheets. Remarkable combination of very large aspect ratios, high mechanical strength, and versatile electronic properties make CNTs a promising new class of materials with many potential biomedical applications. CNTs have a profound impact on the development of diagnostic biosensors, drug delivery nanosystems, imaging nanoprobes for intravascular use or other devices that come in contact with blood. Therefore, the biocompatibility/ toxicity of CNTs in blood have to be assessed carefully.[1] It has been shown that some carbon nanoparticles activate platelets and enhance arterial thrombosis.[2] Therefore, an understanding of the effects of CNTs on platelets is a critical safety issue. Since the industrial production of CNTs is markedly increasing, the concerns about their potential toxicity rise accordingly. In addition, CNTs are released in the atmosphere during the combustion of wood, clean-burning gas sources, or diesel engine emission and it has been shown that inhaled non-functionalized CNTs are able to cross the endothelial barrier.[3]

We have previously shown that different agglomerates of non-functionalized CNTs activate human platelets by inducing extracellular Ca^{2+} influx.[4] Functionalization of CNTs with carboxylic groups is a procedure that not only reduces impurities of the pristine materials, but also provides water solubility to the CNTs. Moreover, carboxylated CNTs allow easy bioconjugation. It is likely, however, that biological effects of pristine CNTs are different from that of their chemically modified or functionalized derivatives. The type of functional groups on the surface of nanomaterials, along with size and shape, dictate their biological response.[5] Therefore, here we investigate the effects of carboxylated MWCNTs on human platelets *in vitro* in comparison to their pristine counterparts.

2 MATERIALS AND METHODS

2.1 Materials

Multi-walled carbon nanotubes with an outer diameter 60-100 nm, length 1-2 μm (M60) were purchased from SES Research, Houston, TX. Non-functionalized MWCNTs M30 and carboxylated MWCNTs M30(COOH) with an outer diameter of 30 ± 15 nm, length 1-5 μm were from NanoLab, Inc., Newton, MA. Fullerene nC60 was from MER Corp., Tuscon, AZ. Nanosphere size standard polystyrene nanobeads 20 nm and 200 nm were from Duke Scientific Corp., Fremont, CA. In our experiments materials were tested as polydisperse suspensions in phosphate buffered saline (PBS). Materials were resuspended to a concentration of 1 mg/mL and sonicated immediately prior

to use for 1 minute at 30W output frequency 20 kHz (Tekmar Sonic Disruptor, Cincinnati, OH). We confirmed purity and structure of all tested materials by TEM analysis.

2.2 Methods

Preparation of carboxylated MWCNTs

Carboxylated-MWCNTs M60(COOH) were prepared by refluxing pristine MWCNTs M60 in a concentrated 3:1 (v/v) H_2SO_4 and HNO_3 mixture at 70°C for 2 hours; followed by washing and dialysis in water. Characterization was performed by field emission scanning electron microscopy (FESEM).

Platelet Aggregation

To assess the effect of carbon nanotubes on platelet aggregation, we performed light transmission aggregometry (PAP-8E aggregometer, Bio/Data Corp., Horsham, PA). Platelet rich plasma (PRP) was prepared from blood of healthy donors (ACD anticoagulated, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, MD). Fifty mL of whole blood was centrifuged at 150 *g* for 10 minutes at room temperature and platelet rich plasma (PRP) was collected. The sediment was then centrifuged at 1000 *g* for 15 minutes at room temperature to obtain platelet poor plasma (PPP), used as a blank. The PRP platelet count was assayed (ABX Pentra 60, Horiba ABX, Inc., Irvine, CA) and diluted with PPP to 250 x 10^3 platelets/μL. Nanomaterials were tested at final concentration of 100 μg/mL in platelet rich plasma. Nanomaterial platelet–aggregating activity was expressed as maximum platelet aggregation (MA) in % (mean of 5 experiments ± SEM).

Field Emission Scanning Electron Microscopy (FESEM)

For FESEM measurements, 450 μL of platelets (250 x 10^3 platelets/μL) was incubated with 50 μL of carbon nanomaterial solution (1 mg/mL in PBS) at 37°C under agitation for 15 minutes. Platelets were then fixed with 1% paraformaldehyde for 15 minutes at room temperature. Fixed platelets were let to attach to glass slide for 15 minutes. The slides were washed with phosphate buffer solution and incubated with 2% gluteraldehyde for 30 minutes, washed with cacodylate buffer, dehydrated using ethanol series, and dried overnight under vacuum. Samples were coated with a 15 nm layer of gold prior to the FESEM analysis.

3 RESULTS AND CONCLUSION

In a pilot experiment using light transmission platelet aggregometry we showed that carboxylated MWCNTs M30(COOH) from NanoLab (>95% purity, outer diameter 15 ± 5 nm) at 100 μg/mL induced marked platelet aggregation similarly to non-functionalized MWCNTs M30 (Figure 1). In contrast, the fullerene (nC60) and polystyrene beads (20 nm and 200 nm) did not induce platelet aggregating activity. Due to the limited dispersability of commercial carboxylated MWCNTs, we prepared carboxylated MWCNTs M60(COOH) in house.

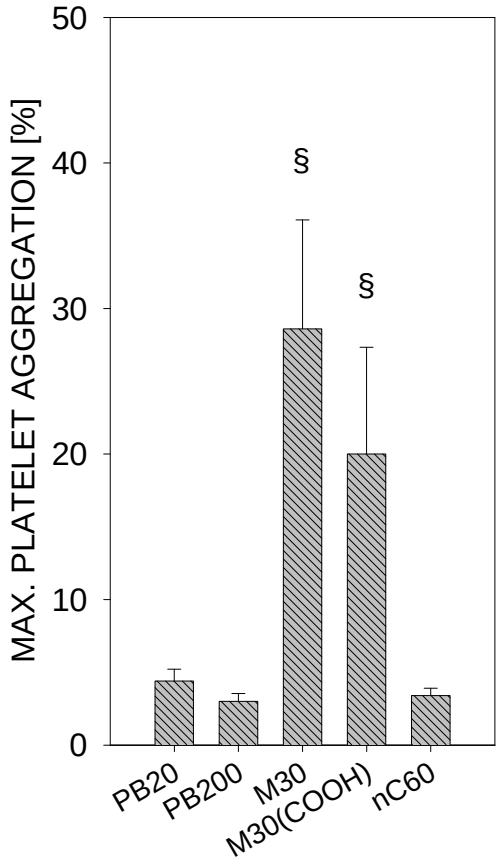

Figure 1: Comparison of platelet aggregating activity of MWCNTs M30 and carboxylated MWCNTs M30(COOH). Significant difference from fullerene (nC60), and standard polystyrene beads 20 nm (PB20) and 200 nm (PB200) (§) is shown ($p<0.05$, mean of 5 experiments ± SEM). Nanomaterials were tested at final concentration of 100 μg/mL in platelet rich plasma.

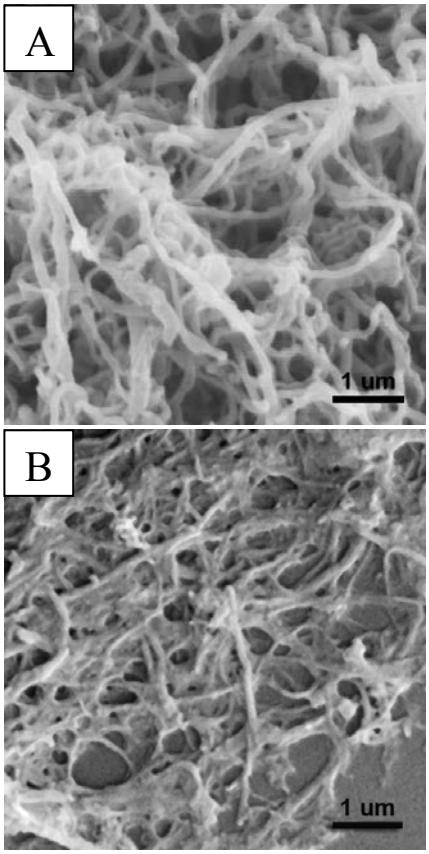

Figure 2: FESEM of non-functionalized M60 (A) and carboxylated M60 (B) showing surface topography and material aggregation in plasma.

Non-functionalized CNTs are highly hydrophobic and tend to form bundles and larger agglomerates due to the strong van der Waals interactions. However, after functionalization, the repulsion imposed by the negatively charged carboxylic groups does not completely prevent the CNTs to organize themselves into "ropes". Our carboxylated MWCNTs M60(COOH) were well dispersed in PBS, but unstable in citrated blood plasma, and formed larger visible agglomerates (Figure 2). The entanglements of nearby CNTs led to a rope-like structure formation with a size of about 10 μm. As shown in Figure 2, numerous protuberances exist on the top surface of M60 and M60(COOH) clusters, which were formed by the relatively long, dense net, highly tangled CNTs without specific orientation.

The state of the agglomeration of CNTs in solution is complex. The complexity of CNTs structure increases by plasma protein adsorption onto the carbon fibers. Proteins adsorbed on CNTs are expected to modulate the cellular response such as uptake kinetics and mechanism, subcellular localization, clearance and toxicity.

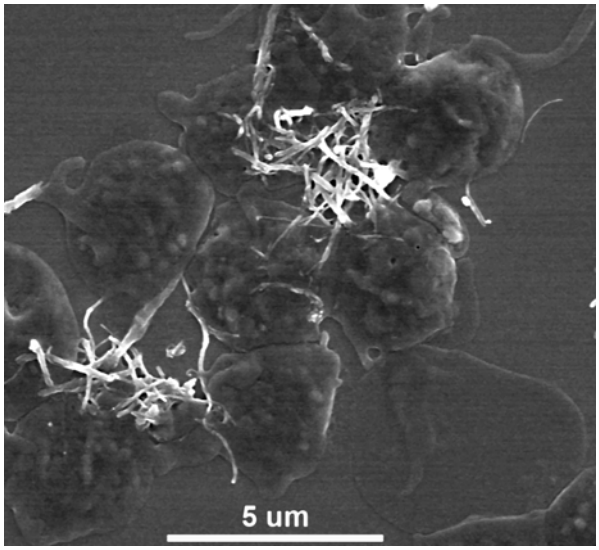

Figure 3: FESEM of carboxylated M60 agglomerates causing platelet activation and aggregation in plasma.

It has been shown that proteins drive nanoparticles to self-assemble. Protein-nanoparticle interactions are determined by the nature of proteins and nanoparticles, along with the size, shape and surface coating of the nanoparticles. We have recently shown that blood plasma proteins such as albumin, fibrinogen, gamma-globulin and insulin bind to nanoparticles, leading to protein conformational change and nanoparticle self-assembly.[6]

FESEM showed that platelets in contact with carboxylated MWCNTs undergo morphological changes from the resting discoid state to an activated state with pseudopodia (Figure 3). Platelets adhere to the protein coated surface of M60(COOH) agglomerates which is followed by their activation and aggregation. Hydrophobic surfaces are known to accumulate a stable layer of fibrinogen and other plasma proteins which may increase platelet binding and activation. The activation of platelets is induced not only by the hydrophobic M60 and M30 but also by the hydrophilic M60(COOH) and M30(COOH) where the protein adsorption characteristics are likely different.

In conclusion, carboxylated MWCNTs activate human blood platelets similarly like their pristine counterparts. The mechanism of this event is being investigated.

4 DISCLAIMER

The findings and conclusions in this study have not been formally disseminated by the Food and Drug

Administration and should not be construed to represent any Agency determination or policy.

5 ACKNOWLEDGEMENTS

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6 REFERENCES

[1] J. Simak, Nanotoxicity In Vivo and In Vitro Models to Health Risks (Sahu S., Casciano, D., eds.) , John Wiley & Sons, 191-225, 2009.

[2] A. Radomski *et al.*, Br. J. Pharmacol., 146, 882–93, 2005.

[3] A. Nemmar *et al.*, Circulation, 105, 411-414, 2002.

[4] J. Semberova *et al*., Nano Lett., 9, 3312-3317, 2009.

[5] M. L. Becker *et al.*, Advanced Materials, 19, 939-945**,** 2007.

[6] S.H. Lacerda *et al.*, *ACS Nano*, 4, 365-369, 2010.