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Nanotechnology in the intensive care: Intravascular biocompatibility of carbon nanomaterials – effect of carbon nanotubes on blood platelets.

Nanotechnologie v intenzivní péči: Intravaskulární biokompatibilita uhlíkových nanomateriálů – uhlíkové nanotuby a krevní destičky

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Abstract

Carbon nanotubes (CNTs) are among the principal materials currently used in biomedical nanotechnologies. CNTs possess superior mechanical and chemical characteristics. As a result they are very popular for use in various biomedical applications, many of these may lead ultimately to contact of carbon nanomaterials and blood. Furthermore, CNTs may also be present intravascularly as a result of environmental or occupational exposure. Therefore, the investigation of intravascular biocompatibility of CNTs is an important safety issue.

We studied the effects of structurally different purified CNT materials on human platelets (PLT) and compared their effects to amorphous carbon black nanoparticles, fullerene C_{60}, fullerenol $C_{60}(OH)_{24}$ and standard polystyrene nanobeads. Using light transmission aggregometry, we found that various CNTs induce PLT aggregation and this occurs in a concentration dependent manner. Flow cytometry analysis showed that CNTs induce platelet activation, demonstrated by the detection of surface exposure of CD62P and CD63 and the release of $CD62P^+$ and $CD63^+$ platelet membrane microparticles. Field emission scanning electron microscopy and transmission electron microscopy confirmed that the platelets in contact with CNTs undergo morphologic changes consistent with activation, membrane budding and microparticle shedding. CNTs induce increase in intracellular Ca^{2+} concentration as detected by ratio fluorometry. CNT-induced extracellular Ca^{2+} influx could be inhibited by calcium channel blockers SKF 96365 and 2-APB. Investigating this phenomenon, we observed that CNTs penetrate the platelet plasma membrane without any discernible damage but then interact with the dense tubular system causing depletion of intracellular Ca^{2+} stores as shown by electron and immunofluorescent microscopy. This process is accompanied by clustering of stromal interaction molecule 1 (STIM1) co-localized with Orai1 protein, indicating activation of the store-operated Ca^{2+} entry (SOCE) mechanism.

To investigate the effect of size and charge of nanosized materials we used the PAMAM dendrimer model.

In conclusion, we were able to prove that CNTs induce platelet activation and aggregation and thus possess highly prothrombotic properties. Furthermore we revealed the underlying molecular mechanism of CNT-induced platelet activation. These findings are critical in the evaluation of the biocompatibility of carbon nanomaterials with blood.

Abstrakt

Uhlíkové nanotuby patří v současnosti mezi nejdůležitější nanomateriály. Vyznačují se unikátními mechanickými a fyzikálně-chemickými vlastnostmi, které z nich činí materiály velmi atraktivní pro použití v různých oblastech biomedicíny, z nichž mnohá zahrnují přímý kontakt s krví. V organismu se však mohou ocitnout i po průniku tkáněmi z kontaminovaného okolního prostředí. Výzkum intravaskulární biokompatibility těchto materiálů je klíčový pro zajištění bezpečnosti jejich dalšího praktického použití.

V naší studii jsme se věnovali vlivu uhlíkových nanotub na krevní destičky. Agregometrií jsme prokázali, že uhlíkové nanotuby způsobují agregaci krevních destiček v lidské plasmě. Analýza průtokovou cytometrií potvrdila, že kontakt s uhlíkovými nanotubami vede k zvýšení exprese aktivačních povrchových markerů CD62P a CD63 a uvolnění CD62P a CD63 pozitivních membránových mikropartikulí. Elektronová mikroskopie (rastrovací a transmisní) potvrdila, že u krevních destiček po kontaktu s uhlíkovými nanotubami dochází k morfologickým změnám typickým pro aktivaci. Fluorescenční mikroskopie za použití indikátoru FURA-2AM prokázala, že kontak destiček s uhlíkovými nanotubami vede k rychlému zvýšení hladiny nitrobuněčného vápníku. Pozorované zvýšení nitrobuněčné koncentrace Ca^{2+} bylo inhibovatelné blokátory kalciových kanálů SKF 96365 a 2-APB. Dokumentovali jsme průnik uhlíkových nanotub plasmatickou membránou destičky bez jejího zjevného poškození a dále jejich interakci se strukturou densního tubulárního systému. Elektronovou a imunoflourescenční mikroskopií jsme ozřejmili, že tato interakce vede k uvolnění nitrobuněčných zásob vápníku a následnému otevření membránových vápníkových kanálů. Proces byl doprovázen shlukováním molekul STIM1 s molekulami proteinu Orai1 v cytoplasmatické membráně, což dokazuje aktivaci mechanismu na zásobách závislého vstupu vápníku do buňky (store-operated Ca^{2+} entry, SOCE). Obecný efekt velikosti a náboje nanomateriálů na krevní destičky jsme dále zkoumali pomocí modelu polyamidoaminových dendrimerů, materiálů, kde jsou obě tyto veličiny lehce modifikovatelné.

V předložené práci dokazujeme, že uhlíkové nanotuby způsobují aktivaci a agregaci krevních destiček a jsou tedy značne protrombogenní. Podařilo se nám objasnit molekulární mechanismus tohoto jevu. Výsledky mají zásadní význam pro hodnocení biokompatibilty uhlíkových nanomateriálů s krví.

1. Introduction and overview

1.1. Introduction - Carbon nanotubes – friend or foe?

Nanotechnology has developed very rapidly over the past number of years. It seems like "nano" is ready to enter all aspects of everyday life. The evidence is all about us: we eat food wrapped in packaging enhanced by nanomaterials to help it to stay fresh longer; we treat our skin with cosmetic products containing nanosized liposomes hoping for better results; we routinely use communication and imaging technology containing nanomaterials at work and in our home; we drive cars with plastics filled with nanotubes to increase endurance; and, we enjoy sports equipment made partly of carbon nanomaterials to improve performance. Obviously, the field of health care is not left behind. Due to their unique characteristics, certain nanomaterials have emerged as important tools in modern medicine.

Carbon nanotubes are among the most promising nanomaterials for biomedical use. They possess qualities like superior mechanical strength, increased flexibility, large surface area, high aspect ratio and high absorbency, great thermal and electric conductivity. They have a profound impact on the development of targeted therapeutics and drug delivery carriers, diagnostic biosensors and imaging nanoprobes for intravascular use. Among its expected future applications are nano robots, artificial muscles and other advanced prosthetics, for example, bionic limbs.

However, new discoveries bring new hazards. Simultaneous with the growth and success of the whole nanotechnology business, the possibility of environmental and occupational exposure has increased substantially. Given the relatively low cost for the industrial production of carbon nanotubes, production levels are continuously rising, making them readily available. Furthermore, carbon nanotubes are released into the atmosphere as a part of combustion-related pollution, which, despite various international initiatives, is not decreasing at all. Nanoparticles pose unique environmental and societal challenges, particularly with regard to biocompatibility and toxicity. The pulmonary toxicity of carbon nanotubes was proven in various experiments, which showed, after intratracheal instillation of the material, the development of epithelioid granulomas, together with interstitial and peribronchial inflammation. Some nanomaterials have been shown to penetrate through the skin, pass across epithelia and ultimately present in different compartments. *In vitro* experiments showed multiple adverse effects on various cell cultures,

including pro inflammatory, pro apoptotic, increased oxidative stress, inhibition of cell proliferation and ultimately cell death. The question comes to mind: are we facing the “new asbestos“?

Although the interactions of nanomaterials with biological systems have been recently extensively studied, only a very few studies explore their effect in the intravascular environment. Particularly lacking are studies concerning blood platelets, cells playing vital role in hemostasis and inflammation. Nanomaterials may be administered directly into the intravascular compartment as a part of treatment or diagnostic procedure. A nanomaterial-containing device may be implanted into the circulation or extravascular tissue. Blood could come into contact with a nanomaterial-based device or device surface extracorporally, for example, as a part of various extracorporal circulation techniques, apheresis, filtration or dialysis or simply in the tubing or device containing nanoparticles-enhanced plastics. Nanomaterials may also materialize intravascularly as a result of environmental/occupational exposure, transferred through the different points of entry, including transcutaneously, after ingestion or inhalation. Intravascularly present nanoparticles may interact with all the crucial functions of endothelia, the plasma coagulation system and blood cells, including platelets. In the worst-case scenario, this interaction may lead to development of irreversible multiple organ failure and death.

Taking into account all the available information, the area of intensive care is logically one of the first dealing with nanomaterials in medicine. We are the ones seeking to maximize all the advantages of nanomaterial-containing devices to more effectively treat our patients. Therefore, we are also the ones who will be dealing with any potential adverse affects and complications.

For that reason, we are the ones who should be among the first to study carefully the effects and interactions of this new player in our field.

1.2. Nanotechnology

Nanotechnology could be defined as the design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nano scale. Nanotechnology deals with structures sized between 1 to 100 nanometers in at least one dimension. Materials reduced to the nanoscale - nanomaterials - show different properties compared to what they exhibit on a macroscale. These characteristics enable unique applications but also are posing new risks (www.nanowerk.com, www.nano.gov). The crucial features of nanotechnology are the size, man-made nature and having properties that only arise because of the nano dimensions (Feature. Nature Nanotechnol, 2006).

1.3. Carbon nanomaterials

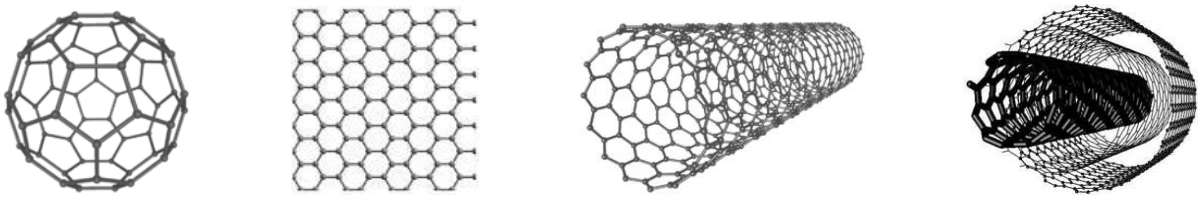
Carbon nanomaterials are among those with the largest number of potential or currently in use applications. The main types of carbon nanomaterials are carbon nanotubes (CNTs) and fullerenes.

1.3.1 Fullerenes

Fullerenes are allotropes of carbon of spherical shape. Originally, fullerene C60 (buckminsterfullerene) is a spherical molecule about 1nm in diameter, comprising 60 carbon atoms arranged as 20 hexagons and 12 pentagons - the configuration of a football (fig.1). There are now thirty or more forms of fullerenes, up to and beyond C120 (www.nanowerk.com).

1.3.2. Carbon nanotubes

Carbon nanotubes are extended family of cylindrical structure molecules related to graphene. The molecular structure of graphene is planar network of interconnected hexagonal rings of carbon atoms (fig.1). When graphene sheets are rolled into cylinders, they form CNTs. Two main types of nanotubes are single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) according to the number of layers (fig.1). CNTs in general have large length-to-diameter ratio and come in a variety of diameters, lengths, and functional group content (Kalbac et al., 2008, www.nanowerk.com).



Fullerene C60 Graphene sheet Single-walled CNT Multiwalled CNT

Figure 1. Schematic representation of selected carbon nanomaterials.

(www.nanowerk.com, www.physicstoday.org)

Carbon nanotubes (CNTs) attract a lot of attention for their electronic, mechanical, optical, and chemical characteristics. They are mechanically very strong (as stiff as diamond), amazingly flexible, have large surface area, great conductivity, high aspect ratio and high absorbency.

1.4. Nanomaterials in medicine

1.4.1 Applications

The current and future applications of nanotechnology in medicine are in the fields of treatment, diagnosis, monitoring, and control of biological systems. More specifically these are targeted therapeutics and carriers for drug and gene delivery targeting not only organs, but also individual cells, molecular imaging, biosensors, improved diagnostic and imaging devices, advanced tissue engineering.

1.4.2. Nanomaterials other than CNTs

Among the most frequently used nanomaterials are Quantum dots (QDs), nanoparticles composed of inorganic semiconductor molecules from a variety of different compounds. QDs can be targeted to the organs or tissues and allow specific precise and non-invasive optical imaging *in vitro* and *in vivo* (Moghimi et al., 2005, Murthy et al., 2007). Other nanomaterials used in imaging are superparamagnetic nanoparticles, which enable better, targeted visualization in MRI.

Undoubtedly, the most advanced nanomaterial application is drug delivery. This is large part due to the success of polymer- and liposome-based drug delivery systems, many of which are in clinical use today (Murthy et al., 2007, Goldberg et al., 2007). These include polymeric micelles, dendrimers, polymeric and ceramic nanoparticles, protein cage architectures, viral-derived capsids, nanospheres, polyplexes, and liposomes. Polymer and liposome nanocarriers are currently in use in various cancer treatment regimens (for example drugs Paclitaxel, Doxorubicin), HIV therapy, neurodegenerative diseases therapy, respiratory therapy and vaccines vehicles (Murthy et al., 2007).

1.4.3 Biomedical applications of carbon nanotubes (CNTs)

Carbon nanotubes certainly are among the nanomaterials with the largest amount of potential and in-use biomedical applications. Cells have been shown to grow on CNTs without excessive adhesion, potentially giving rise to applications such as tissue scaffolds, coatings for prosthetics and surgical implants, vascular stents and other devices for cardiovascular and bypass surgery, (Moghimi et al., 2005, Veetil et al., 2009) and neuron growth and regeneration (Moghimi et al., 2005, Massobrio et al., 2008).

Carbon nanotubes found enormous application in molecular electronics and bioelectronics. The electrical behaviour of CNTs promotes a large variety of biosensors for individual molecules sensing (Polizu et al., 2006), for example glucose, lactate and other small molecules, (Sun et al., 2010, Boero et al 2011). Carbon nanotubes have proven to be promising materials also in drug delivery, as part of targeted delivery systems assuring stability and possible visualization, mainly delivering anticancer therapeutics and DNA (Huang et al., 2011, Wu et al., 2008, Ruggiero et al., 2010).

Other applications are various bioseparators, biocatalysts, nanoporous carbon membranes which could find use in immunoisolation devices, pacemakers, kidney dialysis membranes, microdialysis systems, various bio-filters and other devices (Narayan et al., 2007). They found use also in optical imaging, magnetic resonance and as radiotracer contrast agents. Important are carbon nanotubes as fillers of plastic composites becoming parts of various medical devices. (Koyama et al., 2006)

1.5. Biocompatibility of carbon nanotubes

As the number of CNTs applications constantly increases, questioning and testing the biocompatibility and toxicology is becoming alarmingly important. Also, the number of industrial facilities producing the CNTs for a relatively low cost has raised and therefore, the chance of occupational and environmental exposure has increased as well (Colvin 2003).

Possible adverse effects of carbon nanomaterials have been shown in numerous *in vitro* assays. Gelderman and Simak showed pro-inflammatory and pro-apoptotic effect of fullerenol C60(OH)24 on human umbilical vein endothelial cells (HUVECs) (Gelderman et al., 2008). Magrez et al. tested the toxicity of multiwalled carbon nanotubes, carbon nanofibers, and carbon nanoparticles in vitro on lung tumor cells. They observed inhibition of cell proliferation and cell death. Cytotoxicity of the materials and the effects were proven to be size-dependent (Magrez et al., 2006). Manna et al. presented study of effect of single-walled carbon nanotubes on human keratinocyte cells, showing increased oxidative stress and inhibition of cell proliferation (Manna et al., 2005).

Unfavourable effects were shown also in *in vivo* studies targeting various systems. The pulmonary toxicity was proven in multiple experiments, for example Lam et al. demonstrated formation of epithelioid granulomas, interstitial and peribronchial inflammation after intratracheal instillation of single-walled CNTs. The effect was time and dose dependent (Lam et al., 2004). The mechanism behind these changes could be induction of oxidative stress, influence of different transcription factors with upregulation of proinflammatory protein synthesis. (Lam et al., 2004, Medina et al., 2007) Also skin and other epithelia proved to be surprisingly permeable to various nanomaterials (Shvedova et al., 2009, Rouse et al, 2007, Ryman-Rasmussen et al., 2006).

1.5.1. Intravascular biocompatibility of carbon nanotubes

Any foreign material present in the intravascular compartment, unless optimally biocompatible, induces a complex response. The material recognized as a pathogenic factor (insult) elicits the defense process at the tissue level known as the acute inflammatory response. Main components in the inflammatory process induced by intravascularly exposed nanomaterials would be endothelial cells of the vessel wall, platelets, white blood cells, red blood cells, plasma

coagulation system, and complement system. At a certain level of intensity, systemic inflammatory response progresses to multiple organ dysfunction (MODS). MODS is associated with a failure of adequate perfusion of vital organs due to microcirculation disorders including oedema, microthromboses, and other thrombohaemorrhagic complications, possibly leading to the irreversible impairment (Simak, 2009).

The interaction of a foreign material *in vivo* with blood and vessel wall is always complex, although the effect on particular components may be more apparently manifested (Simak, 2009). Focusing on interaction with platelets, the exposure to nanomaterial may possibly lead to the disorder in platelet count and functions and corresponding clinical manifestations. In the worst case scenario, platelet interaction with nanomaterial may result into development of multiple organ dysfunction and death (fig.2).

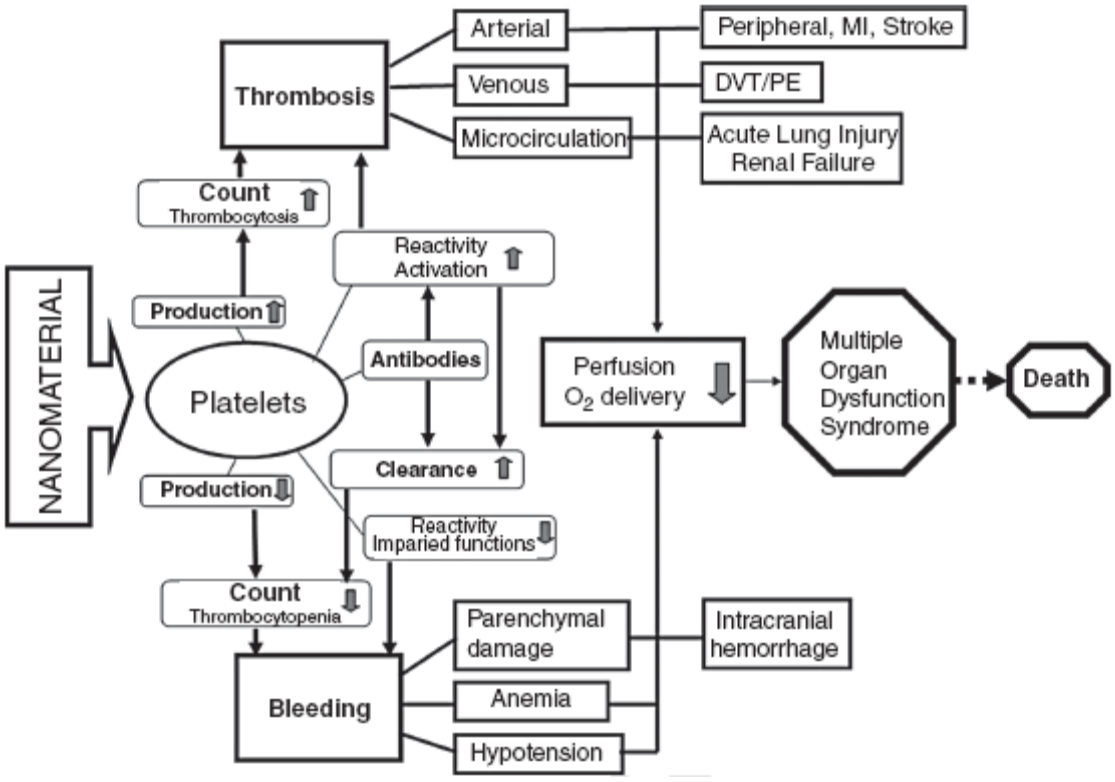


Fig.2. Possible in vivo serious adverse effects of nanomaterial interactions with platelets. MI, myocardial infarction; DVT, deep venous thrombosis; PE, pulmonary embolism. (Simak, 2009). Published with courtesy of the author.

1.6. Blood platelets

Platelets are small anucleated discoid cells, of size approximately 2–5 μm × 0.5 μm and volume of 6 –10fl. They are produced by megakaryocytes in the bone marrow by the extension of dynamic protrusions into microvessels, sheared into the microcirculation (Junt et al., 2007). Platelets circulate for about 10 days in the blood stream before they are cleared by macrophages in spleen and liver. Normal platelet count in peripherial blood is 150-450x10^9/L.

Platelets are the key elements of hemostasis. They are very reactive with large amount of surface receptors for various activation and inhibition agonists. After the activation via different pathways, ultimately leading to the increase in intracellular calcium, platelet shape changes from discoid to spherical, pseudopodia are formed and further spreading, adhesion and aggregation occurs. Activated platelets expose phosphatidylserine on the plasma membrane, providing procoagulant surface for activation of plasma coagulation system.

In addition, activated platelets release various soluble mediators from different types of granules and membrane microparticles. Membrane microparticles (MPs) are phospholipid vesicles of about 0.1-1 μm in size released from plasma membrane of stimulated platelets and other cell types (Simak et al., 2006). Platelet MPs also expose various platelet membrane antigens and a majority of platelet MPs also exposes phosphatidylserine.

Platelets play important roles in other processes beyond hemostasis and thrombosis. They are strongly involved in various stages of inflammatory response and participate in the host defense of pathogens, regulation of vascular tone and integrity, wounds healing and tissue regeneration. Platelets also contribute to tumor growth and metastasis (Simak, 2009, Smyth et al., 2009).

Engineered nanomaterials can potentially interfere with different steps of platelet activation, aggregation and overall haemostasis and also influence all the other platelet functions, as schematized in fig.2.

2. Hypothesis

Our aim was to test the hypothesis that carbon nanotubes activate platelets and cause membrane microparticle release, therefore poses prothrombogenic properties. We also aimed to investigate the underlying mechanisms on subcellular level.

3. Project goals

The overall goal of the project is to contribute to elucidation of the mechanism of prothrombotic effect of carbon nanotubes (CNT) by investigating their interaction with blood platelets.

The specific goals are as follows:

3.1. Evaluation of the effect of different types of carbon nanomaterials on blood platelets using light transmission aggregometry.

3.2. Flow cytometry assessment of CNT-induced platelet activation and microparticles release.

3.3. Electron microscopy characterization of carbon nanotubes interaction with platelets.

3.4. The role of calcium influx in CNT-induced platelet activation: evaluation of changes in intracellular calcium after platelet contact with carbon nanotubes using ratio fluorometry.

3.5. Investigation of the underlying molecular mechanism of CNT-induced platelet activation: Assessment of possible involvement of store operated calcium entry by direct visualization of its major components, STIM1 and ORAI1 proteins.

3.6. Evaluation of the effect of nanoparticle charge and size on platelets-nanoparticles interactions: PAMAM dendrimers model.

4. Materials and methods

4.1. Carbon nanotubes

We have studied the effects of structurally diverse purified CNTs on human platelets (PLTs) and compared their effects to amorphous carbon nanopowder (ACN), C60 fullerene (nC60), fullerenol (C60(OH)24), and standard polystyrene nanobeads (PBs). Carbon nanomaterials were purchased from various manufacturers and their purity ranged from 90-99%. All the tested materials are listed in Table1.

Nanomaterial	**Abbrev.**	**Manufacturer**	**Minimal Purity**	**Outer Diameter**	**Length**	**Aggreg. activity***
Amorphous carbon nanopowder	ACN	Sigma-Aldrich	>99%	~ 30nm	N/A	+
SWCNT	S15	SES	>90%	<2nm	5-15μm	+++
MWCNT	M60	SES	>95%	60-100nm	1-2μm	+++
Fullerene C60	nC60	MER	99.9%	~0.7nm	N/A	-
Fullerenol C60	C60(OH)24	MER	N/A	~1.3nm	N/A	-
MWCNT	M15	NanoLab	>95%	15 +/- 5nm	1-5μm	+
MWCNT-hollow structure	MH	NanoLab	>95%	30 +/- 15nm	1-5μm	+++
MWCNT-bamboo structure	MB	NanoLab	>95%	30 +/- 10nm	1-5μm	+
MWCNT-functionalized COOH	MF(COOH)	NanoLab	>95%	15 +/- 5nm	1-5μm	++
MWCNT-functionalized NH2	MF(NH2)	NanoLab	>95%	15 +/- 5nm	1-5μm	+++
SWCNT	S30	NanoAmor	>95%	1-2nm	5-30μm	+++
NIST standard polystyrene nanobeads	PB20 PB200	Duke Scientific	N/A	20nm 200nm	N/A N/A	- -

Table 1. List of tested materials. *Semiquantitative evaluation of platelet aggregating activity. Maximum platelet aggregation 0-5%, - ; 6-15%, + ; 16-25%, ++ ; >25%, +++. All materials were tested at concentration 100 μg/mL. MWCNT, multiwalled carbon nanotubes; SWCNT, single-walled carbon nanotubes; SES, SES Research, Houston, TX, USA; MER, Materials and Electrochemical Research Corporation, Tucson, AZ, USA; NanoLab, Nanolab Inc., Waltham, MA, USA; NanoAmor, Nanostructured and Amorphous Materials, Inc., Houston, TX, USA; Duke Scientific, Duke Scientific Corp., Fremont, CA, USA; N/A, not available

We confirmed the purity and the structure of all tested materials by transmission electron microscopy (TEM) analysis. In order to study interactions of pristine CNTs, amorphous carbon, and C60 fullerene with platelets, these materials were tested as polydisperse suspensions prepared by minimal sonication to allow platelet contacts with material surface but to avoid its chemical changes. In platelet activation experiments, the sonicated suspensions were immediately added to the platelet rich plasma (PRP) to final concentration of nanomaterial 100 μg/ mL. Macroscopic aggregates of CNTs readily formed after sonication. To characterize the size distribution and shape of these agglomerates under our experimental conditions, we performed flow particle image analysis of nanomaterial suspensions in plasma using FPIA 3000 (Sysmex, Kobe, Japan, provided by Malvern Instruments, Columbia, MD). The analysis showed that nanomaterials formed polydisperse agglomerates of a median size 0.7-2.7 μm, depending on the type of nanomaterial. For further experiments, we selected MWCNTs with a 60 nm diameter, M60, as a representative CNT material due to its purity as determined by inductively coupled plasma mass spectrometry (ICP-MS) and transmission electron microscopy and highly reproducible platelet aggregating performance.

4.2. Blood platelets

For all the platelet experiments, platelet rich plasma (PRP) was prepared from blood of healthy donors (ACD anticoagulated, Department of Transfusion Medicine, Clinical Center, NIH, Bethesda, MD). 50 mL of whole blood was centrifuged at 150 *g* for 10 minutes at room temperature and platelet rich plasma (PRP) was collected with Pasteur pipette. The sediment was then centrifuged at 1000 *g* for 15 minutes at room temperature to obtain platelet poor plasma (PPP). 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. All experiments performed with unfixed platelet were completed within 4 h after blood collection to ensure normal platelet responsiveness.

4.3. Light transmission aggregometry

To assess the effect of carbon nanotubes on platelet aggregation, we employed light transmission aggregometry (PAP-8E aggregometer, Bio/Data Corp., Horsham, PA). Thrombin Receptor

Activator Peptide (TRAP-6, SFLLRN), Collagen and ADP were used as positive controls. Each experiment was performed using at least 3 blood samples from different donors.
While investigating the mechanism of CNT-induced platelet aggregation, we questioned the role of calcium influx. For all Ca^{2+} influx experiments, whole blood was heparinized and subsequently re-calcified to reach a final Ca^{2+} concentration of 2.5 mM.

4.4. Analysis of platelet surface activation markers and platelet membrane microparticles (MPs) by flow cytometry

To further characterize CNT-induced PLT activation, we investigated PLT surface exposure of activation markers CD62P and CD63 using flow cytometry (Simak et al., 1999). CD62P (P-selectin) is expressed in resting platelets on the membrane of platelet α-granules and it is exposed on the platelet surface after α-granule secretion. CD63 is another degranulation dependent platelet surface marker that in resting platelets resides on the membranes of the lysosomes and dense granules (Israels et al., 2007). We also determined the release of these markers in the form of platelet MPs after CNTs stimulation of platelets.

Platelets were stimulated with CNTs, TRAP and PBS were used as positive and negative controls respectively. Platelet surface markers were labeled with fluorescent monoclonal antibodies, CD41a (FITC labeled) and CD62P or CD63 respectively (both PE labeled). Data were acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA) equipped with CELLQuest software and subsequently analyzed using FlowJo (Tree Star, Inc. Ashland, OR). Expression of platelet activation markers was evaluated as a percentage of CD62P+ and CD63+ platelets. Platelet MPs assays and platelet surface marker analysis were run in parallel. MPs were defined as particles ≤ 1 μm in size compared on forward scatter with the size standard polystyrene nanobeads of 1 μm in diameter. Counts of CD41a+CD62P+MPs and CD41a+CD63+MPs in the platelet supernatant were evaluated using double fluorescence plots acquired for 60 seconds at the standard flow rate. MPs count per microliter of PRP was calculated.

4.5. Platelet-CNT interaction assessed by Electron microscopy

Platelet morphology and intracellular structure after exposure to the nanomaterials were directly observed by transmission electron microscopy (TEM) and field-emission scanning electron microscopy (FESEM) (Philips EM 400T* microscope equipped with a Soft Imaging System CD camera (Cantega 2K).

To further explore the possible interaction of CNTs with individual organelles and thus elucidate the mechanism of CNTs effect on platelets, we were able to directly visualize Dense Tubular System by peroxidase labeling with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB)-osmium product (White, 2004).

4.6. Calcium influx in CNT-induced platelet activation

In order to confirm the CNT-induced extracellular Ca^{2+} influx in platelets, we investigated the acute effect of CNTs on intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ in individual platelets loaded with a Ca^{2+} -sensitive probe FURA-2 AM employing ratio fluorometry, method modified for platelets (Simakova and Arispe, 2007). Changes in fluorescence of individual platelets were monitored at 340 nm and 380 nm excitation using a Nikon inverted epi-fluorescence/phase microscope equipped with a low-light level integrating CCD camera with a microphotometer assembly (InCyt I/P-2 TM Imaging and Photometry System, Intracellular Imaging Inc., Cincinnati, OH). Real time $[Ca^{2+}]i$ was calculated from the ratio of emission detected at 510 nm and by comparison to a standard curve established for these settings using buffers of known free Ca^{2+}.

In further experiments, we measured the changes in the Ca^{2+} concentration in the platelet intracellular compartments using Fura FF/AM, a fluorescent probe that is used to measure Ca^{2+} from intracellular stores (Lopez et al., 2006, Wokosin et al, 2004). An increase in the fluorescence of Fura-FF/ AM indicated the depletion of Ca^{2+} from stores. Changes in the Ca^{2+} content in platelet stores were monitored by measuring the excitation at 380 nm and emission at 510 nm.

4.7. Underlying molecular mechanism of CNT-induced platelet activation: Store-Operated Calcium Entry (SOCE)

After experiments with calcium influx modification by Ca^{2+} pathway inhibitors suggesting the role of SOCE in CNT-induced platelet activation, we aimed to detect main parts of SOCE system – proteins STIM1 and Orai1 and their localization change after stimulation with CNTs.
We used TEM and confocal microscopy to visualize STIM and STIM1-Orai1 co-localization in CNTs activated platelets respectively.

We performed 5-nm gold immunolabeling of STIM1 for TEM of PLTs stimulated by CNTs or Thapsigargin (positive control).

STIM1 and Orai1 in platelets were immunolabeled for laser scanning confocal microscopy (LSCM), detected with Alexa Fluor 488-conjugated goat antimouse IgG and Alexa Fluor 633-conjugated donkey anti- goat IgG respectively. Zeiss 710 LSM 710 NLO microscope with a Plan Apochromat 100x/1.40 oil objective was employed, MatLab and Image J software were used for image deconvolution.

4.8. PAMAM dendrimers model.

Four generations (G3, G4, G5 and G6) of polyamidoamine (PAMAM) dendrimers from NIST (National Institute of Standards and Technology, Gaithersburg, MD) with etylenediamine core, functionalized with succinamic acid, amine and ethanolamine groups were used in this study. The aim was to evaluate the effect of nanoparticle charge and size on platelet-nanoparticle interaction. In dendrimers, both variable characteristics are easily modified.

We performed analysis of dendrimer-induced platelet aggregation by light transmission aggregometry. First we tested all G3 to G6 amine-terminated PAMAM dendrimers at concentration 100 µg/mL. G6 amine-terminated PAMAM dendrimers were then tested for dose-response effect. Further on we focused on comparison of “small” G3 and “large” G6. Flow cytometric analysis of platelets treated with G3 and G6 amine-terminated dendrimers was performed by assessing the expression of activation marker CD62P and the release of CD62+ platelet microparticles was also determined.

5. Results

Light transmission aggregometry demonstrated that all tested CNTs produced significant platelet aggregation activity in PRP. In contrast, fullerene nC60, fullerenol C60(OH)24, or polystyrene nanobeads (PBs) did not cause any significant PLT aggregation. We have also proved that CNTs induce platelet aggregation in a dose-dependent manner.

While investigating the mechanism of CNT-induced platelet aggregation, we questioned the role of calcium. Initially we focused on effects of CNTs on intracellular free Ca^{2+} concentration $[Ca^{2+}]i$ in platelets since Ca^{2+} is a key second messenger controlling critical steps in platelet activation. Performing the experiments in an environment with different Ca^{2+} content showed that the CNT-induced platelet aggregation response is proportional to the extracellular Ca^{2+} concentration. This finding led us to hypothesize that CNTs induce platelet activation by facilitating extracellular Ca^{2+} influx.

To characterize the CNT-induced Ca^{2+} influx, we employed inhibitors of platelet calcium signaling pathways. We found that M60-induced platelet aggregation was suppressed with calcium channel blockers SKF 96365 and 2-APB, indicating the possible involvement of store operated calcium entry (SOCE). While SKF 96365 has overlapping effects on SOCE and receptor-operated calcium entry, 2-APB has more specific activities, including direct extracellular inhibition of SOCE channels (Ben-Amor et al., 2006, Diver et al., 2001, Bird et al., 2008, Bootman et al., 2002, Marumo et al., 2005). In contrast, no effect on M60-induced PLT aggregation response was observed with DM-BAPTA AM (membrane permeant intracellular Ca2+ chelator), NF 449 (P2X1 receptor blocker), MRS 2500 (P2Y1 receptor blocker), or TBHQ (SERCA3 blocker).

To further assess CNT-induced PLT activation, we investigated PLT surface exposure of activation markers CD62P (P-Selectin) and CD63 using flow cytometry (Simak et al., 1999).

M60-induced PLT activation led to significantly higher surface exposure of CD62P and CD63 compared to amorphous carbon nanopowder (ACN) and untreated platelets. Also, CD62P expression after treatment with ACN differed significantly from untreated platelets. However, the increase in the surface exposure of CD62P and CD63 on M60-stimulated platelets was not as

high as expected. Therefore we investigated whether these antigens were released from platelet surface in membrane microparticles. In contrast to ACN, M60 induced marked release of CD41a+CD62P positive MPs and CD41a+CD63 positive MPs.

Platelet morphology and intracellular structure after exposure to different carbon nanomaterials were directly observed by TEM and FESEM. The morphologies of nontreated platelets and platelets treated with nC60 are typical of the nonactivated state, that is, the platelets have a smooth surface and discoid shape (Jackson, 2007). The maximum platelet activation (positive control), was achieved by treating platelets with thrombin receptor-activating peptide (TRAP) (Simak et al., 1999, Furman et al., 1998). M60 clearly induced similar platelet morphological changes, pseudopodia formation, membrane budding, and the release of membrane microparticles. TEM and FESEM show that M60 CNTs interact with the platelet plasma membrane (PM) and are translocated into the cytosol, as observed in other cell lines (Becker et al., 2007, Porter et al., 2007). Although nC60 is also internalized, it does not cause significant platelet activation.

In house prepared carboxylated carbon nanotubes M60-COOH, although well dispersed in PBS, formed agglomerates in plasma and clearly, as documented by FESEM, induced platelet activation to the same degree as their pristine counterparts.

As shown in flow cytometry and electron microscopy experiments, CNTs activate platelets and cause exposure of surface activation markers, shape change, membrane budding and microparticle release. An essential event during platelet activation is an increase in intracellular free calcium Ca^{2+}. In aggregation experiments, we have already questioned involvement of Ca^{2+} influx on the CNT-induced platelet activation by employing various calcium pathways inhibitors, suggesting the role of Store-operated calcium entry (SOCE).

In order to confirm the CNT-induced extracellular Ca^{2+} influx in platelets, we investigated the acute effect of CNTs on platelet intracellular free Ca^{2+} concentration. Using ratio fluorometry in individual platelets, we demonstrated that M60 induced a rapid concentration-dependent increase in platelet $[Ca^{2+}]i$ indicative of Ca^{2+} entry. The increase of $[Ca^{2+}]i$ above base level was 90±4 nM immediately upon addition of M60. In contrast, after administration of ACN or PBs no changes in $[Ca^{2+}]i$ were detected. Also, there was no response to M60 observed in experiments conducted

in a calcium free condition, confirming the extracellular origin of Ca^{2+}. Moreover, in agreement with the platelet aggregation experiment results, no Ca^{2+} influx was observed when platelets were pretreated with calcium entry blocker SKF 96365.

We have demonstrated that CNT-induced platelet activation follows the influx of extracellular Ca^{2+}. TEM and FESEM showed that M60 CNTs interact with the platelet plasma membrane (PM). Injury of the membrane could possibly cause passive Ca^{2+} efflux from the extracellular environment. However, the fact that we were able to block the CNT-induced calcium efflux with the blockers targeting SOCE mechanism does not support this purely mechanistic theory.

SOCE is the leading mechanism for Ca^{2+} entry in platelets, it is a process controlled by the Ca^{2+} concentrations in the intracellular Ca^{2+} stores (Redondo et al., 2008). The DTS (Dense Tubular System, similar to the endoplasmic reticulum in other cells) is the major source of Ca^{2+} in platelets (Jardin et al., 2008) and the depletion of Ca^{2+} from the DTS triggers the activation of SOCE channel in the plasma membrane to allow the influx of extracellular Ca^{2+} (Varga-Szabo et al., 2009).

Having entered the platelets, M60 could induce mechanical injury of DTS membranes or stimulate a local phase transition in the membrane phospholipid structure (Wallace et al., 2008). Both processes could lead to the depletion of Ca^{2+} from the DTS through leakage or passive efflux into the cytosol. To test this hypothesis, TEM visualization of the DTS was performed (White, 2004). We have observed M60 CNTs that are puncturing or in close proximity to the DTS membranes. Next, we measured the changes in the Ca^{2+} concentration in the platelet intracellular compartments (Lopez et al., 2006, Wokosin et al., 2004). An increase in the fluorescence of Fura-FF/ AM indicated the depletion of Ca^{2+} from intracellular stores. M60 and the Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) pump inhibitor Thapsigargin (TPG) induce a marked decrease in Ca^{2+} in the DTS. SERCA is responsible for refilling the Ca^{2+} stores (Redondo et al., 2008), but in the presence of TPG, SERCA activity is blocked and the luminal Ca^{2+} content is passively effluxed to the cytoplasm. Under the same conditions, nC60 did not induce the depletion of Ca^{2+} from the stores, similar to the untreated control platelets.

The depletion of Ca^{2+} from the DTS is usually not sufficient to activate platelets (Jardin et al., 2008). SOCE critically regulates platelet activation by allowing the much higher extracellular

concentration of Ca^{2+} to enter, which leads to the cytoskeletal reorganization required for the platelet activation. Two major factors in SOCE have been identified: the 4-transmembrane-spanning calcium release-activated channel moiety Orai1 and STIM1, a Ca^{2+} sensor expressed predominantly in the DTS (Deng et al., 2009, Wei et al., 2009). STIM1 molecules sense the depletion of Ca^{2+} from the DTS, oligomerize, translocate to junctions adjacent to the plasma membrane, and organize with Orai1 to form the elementary unit of SOCE activation.

To elucidate whether SOCE has a role in CNT-induced platelet activation, we evaluated the effect of M60 and nC60 on STIM1 aggregation by gold immunolabeling and laser scanning confocal microscopy (LSCM). TEM analysis of cross sections of platelets labeled with gold anti-STIM1 showed clusters of STIM1 in platelets exposed to TPG or M60, while STIM1 in platelets exposed to nC60 was homogeneously distributed in a manner similar to that of nonactivated control platelets. LSCM detection visualized the clustering of STIM1 and its colocalization with Orai1 at discrete, tightly coupled DTS-PM junctions after the treatment of platelets with TPG or M60. Thus, our results demonstrate that SOCE plays a key role in CNT- induced platelet activation.

To investigate the general effect of size and surface charge of nanomaterial, we used the polyamidoamine (PAMAM) dendrimer model. We tested G3 to G6 generations of amine terminated PAMAM dendrimers, since previous experiments shown no platelet aggregation activity of carboxy- and hydroxyl-terminated counterparts. On the other hand, in amine-terminated dendimers platelet aggregation response decreased gradually with decreasing amount of surface amine groups, proving the important role of surface charge. Comparing G3 and G6 generations by LTA showed that only G6 induced significant platelet aggregation. G6 was further tested in multiple concentrations proving the dose-response relationship. In addition, we performed flow cytometry analysis of expression of platelet activation marker CD62P after incubation of platelet with G3 and G6 dendrimers. Interstingly, beside platelet activation, both type of dendrimers caused profound morphological change and membrane derangement, observed on characteristic FCS/SSC plot, and shift in isotype control binding. The effect was much more pronounced in G6 dendrimers**.** Nor G3 neither G6 induced significant microparticle release. The reason is probably dendrimer-caused platelet membrane "damage" preceding possible microparticle formation, as this is highly organized process (Simak 2006, Ahn 2005).

6. Discussion

As the research to date suggests (Radomski et al., 2006, Bihari et al., 2010, Nemmar et al., 2007, Niwa and Iwai, 2007), CNTs activate platelets and therefore may contribute to the development of thrombotic and other complications. In our experiments we proved that CNTs induce platelet aggregation, activation and marked release of platelet membrane microparticles.

The increase in platelet aggregation activity induced by carbon nanotubes, and not by fullerene, is in agreement with the results published by Radomski *et al* (Radomski et al., 2006). Investigators reported that mixed carbon nanomaterials (MCN), carbon nanotubes (SWCNT and MWCNT) and standard urban particulate matter (SRM1648) caused activation and aggregation of human platelets, unlike fullerene. The findings also emphasize both the importance of detailed material characterization and, also, the role of different nanoparticle properties like size, shape and surface.

Regarding the shape, one attractive theory is that nanotubes may form interplatelet bridges and as a result promote aggregation, while spherical fullerenes do not. This could be true for single molecules, but is doubtful when considering the agglomerates formation. Bihari *et al*. (Bihari et al., 2010) did also mention the theory of interplatelet connection formed by nanotubes questioning the nature of platelet aggregation. Microscopy experiments performed both by Radomski et al. and our group clearly showed that the platelet morphology changes corresponded with platelet activation.

Radomski *et al*. also showed that the tested nanoparticles, except fullerene, induced the surface expression of various platelet activation markers (Radomski et al., 2006). Our results, together with Bihari *et al*. (Bihari et al., 2010) indicate the ability of CNTs to induce platelet activation manifested by significant expression of CD62P and also CD63.

We have also documented the release of platelet membranes microparticles positive for alpha granular and dense granular/lysosomal membrane proteins as evaluated by flow cytometry. In addition, we were able to visualize microparticles formation directly by electron microscopy (FESEM and TEM). So far our study has been the first to show microparticle release after platelet contact with CNTs.

Using gold immunolabeling for TEM and laser scanning confocal microscopy (LSCM), we were also able to show STIM1 aggregation and STIM1 redistribution and colocalization with Orai1 in DTS-PM junctions. This visualization of its crucial parts demonstrate, that MWCNTs are able to induce extracellular Ca^{2+} influx in platelets by activation of the store-operated Ca^{2+} entry (SOCE). Therefore it becomes clear that the CNT- induced platelet activation involves alterations in intracellular Ca^{2+} homeostasis rather than pure loss of plasma membrane integrity caused by perforation by CNT. Of course it would be intuitive to consider that CNTs could injure the PM and cause extracellular Ca^{2+} entry. However, together with the experiments with calcium entry inhibitors, our TEM and FESEM data did not reveal plasma membrane (PM) breakage, suggesting that the membrane potentially self-seals around the nanotubes immediately after penetration, as previously reported for other cell types (Wallace et al., 2008, Pogodin et al., 2011, Chen et al., 2007).

On the other hand, we were able to document the interaction of CNTs and Dense Tubular System (DTS) and intracellular Ca^{2+} increase accompanying this phenomenon. Recent studies have shown that CNTs can induce a membrane local phase transition (Wallace et al., 2008) which is known (Tsvetkova et al., 1999) to cause lipid bilayers to become leaky to Ca^{2+}. Interestingly, the cholesterol content in the DTS membrane is 5-fold lower (Fauvel et al., 1986, Menashi et al., 1981) than that in the PM. Therefore, compared to the PM, the DTS membrane seems to be more vulnerable to local phase transition (Koronkiewicz et al., 2004, Vlahakis et al., 2002, Corvera et al., 1992) and injury induced by CNTs. Such an injury may result in the increased passive permeability of Ca^{2+} from DTS which activates SOCE mechanism.

Based on these findings, we propose possible molecular mechanism of CNTs induced platelet activation. This consists of nanopenetration of carbon nanotubes without plasma membrane damage, interaction of CNTs with DTS membrane leading to the depletion of Ca^{2+} from DTS stores and thus activation of SOCE mechanism. (fig.3)

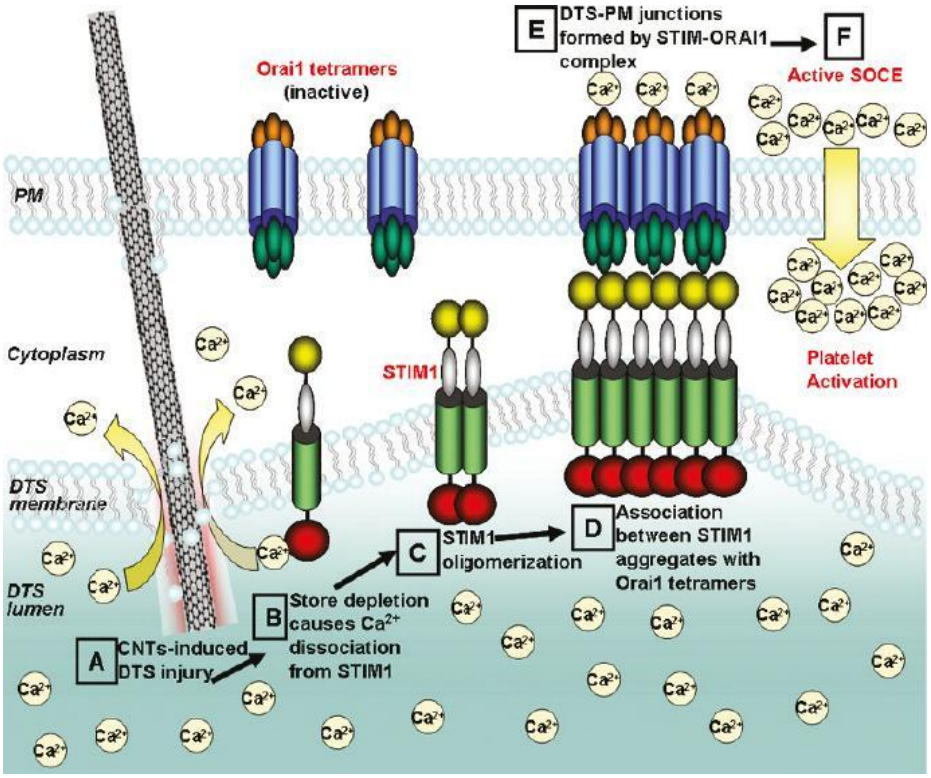


Figure 3. Mechanism of platelet activation by CNTs. (A) CNTs "nanopenetrate" platelets, causing injury to the DTS. (B) Ca^{2+} depletion from the DTS leads to Ca^{2+} dissociation from STIM1, causing (C) STIM1 oligomerization followed by the (D) diffusion, aggregation, and accumulation of STIM1 in DTS - plasma membrane junctions that (E) conformationally gates Orai1 to form a STIM1 - Orai1 complex that (F) activates SOCE for Ca^{2+} influx. (Deng et al., 2009)

Overall our findings could be seen as one "success story", from the observation of the effect through multiple experiments providing crucial information to the elucidation of the underlying mechanism. Thus, the described mechanism of PLT activation might be very unique to the used material – Carbon nanotubes M60. Other investigators described the interference of various nanomaterials with various platelet activation pathways, suggesting different possible activation processes (Radomski et al., 2006, Bihari et al., 2010, Nemmar et al., 2007, Niwa and Iwai, 2007).

The Dendrimer study, to which we contributed, revealed that large cationic PAMAM dendrimers induce platelet aggregation through disruption of membrane integrity, thus showing another possible mechanism of nanoparticle-platelet interaction. This study also confirmed that the size

and charge play crucial role in nanomaterial effect and that platelet activating mechanism may vary significantly, proving PAMAM dendrimers as a useful model.

All these findings emphasize that there are no general rules how nanoparticles "behave" in contact with blood. It means that there is a need to test ultra-carefully all the nanomaterials intended for clinical use. The complicating factor is the possible interactions and interference with conventional toxicology assays (Dobrovolskaia et al., 2008, Oberdorster et al., 2005, Dobrovolskaia et al., 2010). Also the reaction with the plasma protein may change nanoparticle reactivity significantly (Lacerda et al., 2010). The only way to overcome those difficulties to assess nanomaterial safety is the meticulous approach, employing carefully chosen battery of appropriate in vitro and in vivo testing methods. Their results should be confirmed in well designed clinical trials, ideally including also the "second hit" model, since the real patients, severely ill with already dysregulated haemostasis, may profit the most from new diagnostic and therapeutic methods using nanomaterials.

7. Summary and conclusions

In summary, we proved that CNTs induce platelet aggregation, activation and marked release of platelet membrane microparticles. In addition to this, we elucidated the underlying molecular mechanisms for the platelet-activating and potentially thrombogenic effect of pristine MWCNTs. We demonstrated the ability of MWCNTs to nanopenetrate the platelet plasma membrane and interfere with Ca^{2+} homeostasis. MWCNTs caused no discernible plasma membrane damage possibly due to the ability of the lipid bilayer to self-sealing around the nanotubes. However, the interaction of MWCNTs with the DTS caused depletion of Ca^{2+} from the intracellular store. This process was accompanied with STIM1 clustering and its colocalization with Orai1, indicating the activation of SOCE. Thus, SOCE plays a pivotal role in the CNT-induced platelet activation.
Understanding the nature of the interaction of CNTs with platelets will ultimately advance the development of general concepts for designing and testing carbon nanomaterials for optimal biocompatibility in blood.

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