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Hemotoxicity of carbon nanotubes ☆

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ABSTRACT

Carbon nanotubes may enter into the bloodstream and interact with blood components indirectly *via* translocation following unintended exposure or directly after an intended administration for biomedical purposes. Once introduced into systemic circulation, nanotubes will encounter various proteins, biomolecules or cells which have specific roles in the homeostasis of the circulatory system. It is therefore essential to determine whether those interactions will lead to adverse effects or not. Advances in the understanding of how carbon nanotubes interact with blood proteins, the complement system, red blood cells and the hemostatic system are reviewed in this article. While many studies on carbon nanotube health risk assessment and their biomedical applications have appeared in the last few years, reports on the hemocompatibility of these nanomaterials remain surprisingly limited. Yet, defining the hemotoxicological profile is a mandatory step toward the development of clinically-relevant medications or contrast agents based on carbon nanotubes.

Contents

1. Carbon nanotubes in the bloodstream

Since their atomic characterization by Iijima in 1991 [1], carbon nanotubes (CNTs) have been one of the most promising and thoroughly studied carbon-based nanomaterials. CNTs are classified as single-walled CNTs (SWCNTs) when their tubular structure is constituted of one layer of sp^2 bound carbon, double-walled (DWCNTs) when made of two concentric tubes, and multi-walled (MWCNTs) for more than two layers. Over the years, their impressive intrinsic physical properties have been revealed (*i.e.* mechanical, optical, electrical and thermal conductivity, electronic, and high surface area) [2], which combined with a chemically tunable surface (*via* chemical functionalization or interactions with aromatic or hydrophobic regions of molecules) [3,4] make them appealing for a wide range of applications [5]. CNTs have been proposed to offer new opportunities in various areas of biological and biomedical research [6], such as biosensors [7], substrates for cell growth [8], photodynamic therapy [9,10], molecular imaging [11,12], optical imaging [13,14], ultrasound contrast agents [15] and delivery systems (for vaccines, genes or drugs) [16,17].

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Along with the development of nanomaterials with the potential for biomedical applications, the question of their possible toxicity has increasingly gained attention. Given that nanotubes could be classified as high aspect ratio nanoparticles (HARNs), which may induce similar adverse health effects to other long, persistent fibers [18,19], the issue of pulmonary risk hazard posed by nanotubes in the context of air pollution in occupational health settings was the first to be investigated [20]. The effects of CNTs on the pulmonary cavity have been reviewed previously [21,22] and will be reviewed more extensively in this special issue by other authors, with the prevailing current understanding that airborne dispersions of long CNT powders should be handled with precaution to minimize inhalation and risks. Accidental exposure by inhalation or ingestion, *via* skin or mucosa, can potentially lead to the translocation of CNTs in the bloodstream. On the other hand, many of their proposed biomedical applications will require direct injection into the body *via* different routes of administration, that will most probably also lead to direct contact with blood components. The evaluation of nanotube blood compatibility is therefore considered of great importance given their interactions with blood components and the cardiovascular system. Over the last few years, major advances have been made toward a better understanding of the safety profile of carbon nanotubes; however the determination of potential adverse effects arising from their interactions with blood cells and proteins has been relatively poorly studied.

In this article, we offer a perspective on the research data produced today related to the hemotoxicity and hemocompatibility of CNTs. Different types of nanotube material have been studied, ranging from nanotubes specifically modified for potential biomedical applications, to nanotubes with no particular features designed toward an application but which can potentially enter into the bloodstream by unintended exposure. For clarity, only studies using CNTs as suspensions of materials have been considered here. Other technologies incorporating nanotubes as part of a scaffold, device or a substrate-embedded template that may also come in contact with blood components as a whole were not considered.

2. Brief introduction to hemotoxicity

The terms hemotoxicity and hemocompatibility are commonly used interchangeably, without any clear etymological difference between the two terms. Hemotoxic profiling of a chemical can be considered the identification of the possible adverse effects resulting from its interaction with blood components, including cells and proteins. Another interpretation of the term hemotoxicity can be through the different ways by which blood compatibility is evaluated for new biomaterials (*i.e.* materials that will be in contact with biological milieu or tissues) prior to being commercialized [23]. Activation of the complement system, blood cell changes, and interaction with processes related to the coagulation system (*i.e.* platelet (thrombocyte) activation, coagulation and thrombosis potential) are the key parameters assessed for the hemocompatibility assessment of biomaterials (also according to international standard ISO 10993-4).

2.1. Complement system

The complement system is part of the innate immune system, the frontline of the immune defense, and consists of small proteins and factors circulating in the bloodstream. It acts fast, in a non-specific manner. Upon its activation, the main role of the complement is to remove microorganisms and clear of modified cells such as apoptotic cells [24,25]. However, the over-activation of the complement system has been linked to pseudo-allergy syndromes observed with nanoparticles used as nanomedicines (*e.g.* liposomes [26]), it is therefore vital to investigate the way a nanomaterial may interact with the components of the complement system and eventually whether this interaction leads to over-activation or not.

2.2. Blood cell alterations

These can be changes in leukocyte (white blood cells) and reticulocyte (immature red blood cells) cell counts, as well as leukocyte activation (initiation of inflammatory processes) or hemolysis (lysis of erythrocytes — mature red blood cells). Leukocytes are components of the blood cell system and are therefore part of the common hemocompatibility panels. However, strictly hematologically, leukocyte cell number or activation of leukocytes is more likely considered as parameters related to inflammation, and is therefore not directly related to the evaluation of the hemocompatibility of nanomaterials but to their inflammatory profiling.

2.3. Hemostatic system

Thrombosis is the physiological process for the formation of a blood clot (thrombus) in response to blood vessel injury to attenuate blood loss [27]. Thrombi are the end-results of the blood coagulation pathway which involves blood cells (activated platelets) and proteins (coagulation factors). A material will be considered as thrombogenic when it tends to produce blood clots which may ultimately cause deprivation of the blood supply to vital organs (obstruction of the blood flow) and therefore lead to functional impairment of tissues [23].

In order for a nanomaterial to be considered as hemocompatible, it is essential that it does not affect significantly any of the above three key parameters (complement, blood cells, and hemostatic system). Currently, there are no special hemotoxicity study requirements for nanotechnology products. Current safety testing requirements are believed to be rigorous and robust to determine the blood compatibility of different materials, irrespective of their dimensions. If nanomaterial-specific adverse reactions are identified, it can potentially educate for the need of new testing requirements [28].

3. CNT interactions with blood proteins and complement system components

An important parameter that governs the overall hemocompatibility of any biomaterial making contact with the bloodstream is its potential to adsorb blood proteins (*e.g.* fibrin, fibrinogen) and to be opsonized by blood components, such as complement proteins (*e.g.* C1q, C3b proteins). When an engineered nanomaterial comes into contact with blood, plasma or serum proteins are likely to be adsorbed onto its surface rapidly, unless its surface has been specifically engineered to minimize such interactions [29,30]. The nature and composition of the layer of protein adsorbed, termed 'protein corona', will differ between materials depending on their chemical composition and surface features [31]. Moreover, it will influence the interactions with other biological components as the presence of the corona can alter the recognition patterns of that nanomaterial and therefore impact on the way in which the material interacts with cells, especially those of the immune system [32,33]. Consequently, the protein corona has been recognized as having a significant role in the determination of downstream biological effects of nanomaterials in systemic blood circulation.

CNTs in their pristine form are highly hydrophobic nanomaterials due to their aromatic rings of sp^2 bound carbons. Once functionalized [3,34–36], the original hydrophobic surface will be converted into a hydrophilic and/or charged surface which will interact with the plasma proteins in a completely different manner (Table 1). Surface features of functionalized CNTs (area, charge, hydrophilicity, and chemical features of appending groups) are therefore believed to significantly influence the protein binding and its eventual selectivity [37]. Fig. 1 summarizes the effects that can potentially result from the interaction of CNTs with blood proteins.

Table 1
Interaction of CNTs with blood proteins and complement components.

Type of CNT	Type of functionalization	Findings	Refs
SWCNT and DWCNT	Pristine purified	Selective blood protein binding: fibrinogen, apolipoproteins, and C1q Complement activation *via* classical and alternative pathways	Salvador-Morales et al. (2006)
MWCNT	Pristine or caprolactam/L-alanine coated	Blood protein (C1q proteins/factor H) binding influences alternative pathway activation Chemical modification modifies blood protein binding Complement activation *via* classical pathway is reduced with functionalization	Salvador-Morales et al. (2008)
SWCNT	Pristine, or fibrinogen/immunoglobulin/serum albumin/transferrin coated	π–π stacking and aromatic residues influence selective blood protein binding Blood protein coated SWCNT is less cytotoxic than non-coated SWCNT	Ge et al. (2011)
SWCNT	Covalent or non-covalent PEG-2000 functionalization	PEG conformation more important than charge for blood protein binding/non-covalent PEG SWCNT adsorbs preferentially immunoglobulin/covalent PEG adsorbs preferentially coagulation factor (*e.g.* fibrinogen)	Sacchetti et al. (2013)
SWCNT, DWCNT and MWCNT		No complement activation for any CNT, despite binding to C1q and C1s–C1r–C1r–C1s for MWCNT or to C1s–C1r–C1r–C1s for DWCNT	Ling et al. (2011)
SWCNT	Amino-PEG Methoxy PEG	Activation of complement: increase in C4d and SC5b-9, probably *via* lectin pathway/elevation of thromboxane in rats	Hamad et al. (2008)
SWCNT	Serum albumin coated	Activation of classical (C1q) and alternative (C3b) complement pathways	Andersen et al. (2013)
	Linear methoxy PEG	Activation of lectin complement pathway (L-ficolin and mannose)	
	Branched methoxy PEG	Activation of lectin complement pathway (L-ficolin only)	
MWCNT	PEG-1000/1500/2000 Further methoxy PEG-DSPE	Activation of complement Lower activation of complement with added PEG-DSPE coating	Andersen et al. (2013)

3.1. Blood protein adsorption onto CNTs

In one of the first studies dedicated to the assessment of nanotube blood compatibility, Salvador-Morales et al. demonstrated by using western blot and SDS-PAGE techniques that the CNT surface interacted selectively with some proteins constituting of the human plasma and serum. Among the few proteins from plasma or serum that were found to bind to non-functionalized nanotubes, fibrinogen and apolipoproteins – but not albumin – were reported to have greater affinity [38]. In another study, albumin, fibrinogen, and apolipoproteins were the proteins that bound in greater quantities to either pristine or chemically modified MWCNTs [39].

The binding mechanism of four major blood proteins (bovine fibrinogen, gamma globulin, bovine serum albumin and transferrin) on SWCNTs was also studied experimentally and by molecular modeling [40]. The abundance of hydrophobic aromatic residues (total number of tryptophan, tyrosine and phenylalanine residues) in the above mentioned proteins was correlated with the amount of protein binding. Interestingly, this study also reported that SWCNT coated with one of these proteins showed reduced cytotoxicity than uncoated SWCNT to human monocyte/macrophage cells (THP1) or to human umbilical vein endothelial cells (HUVEC).

Using human plasma and a set of SWCNTs non-covalently or covalently functionalized with polyethylene glycol (PEG) chains (PEG-2000), Sacchetti et al. have demonstrated that the PEG spatial conformation was more important than the overall surface charge of the coated material to influence the nature of human plasma proteins that competitively absorbed onto the CNT surface [41]. Overall, immunoglobulins had better affinity for the non-covalently functionalized PEG-SWCNTs than fibrinogen or complement proteins, whereas fibrinogen (and other coagulation-related proteins) adsorbed onto covalently functionalized PEG-SWCNTs to a higher extent than immunoglobulins or complement proteins. The quantity of immunoglobulins or complement proteins adsorbed on covalently functionalized PEG-SWCNTs was also lower compared to non-covalently coated PEG-SWCNTs. Adsorption of albumin and transport-related proteins was remarkably low for both types of PEG-SWCNTs. In the same study, the pattern of tissue distribution of the two types of SWCNTs following intravenous administration in mice was also studied. The non-covalently functionalized PEG-SWCNTs were found to accumulate in the spleen and liver to the same extent, while covalently functionalized PEG-SWCNTs showed higher accumulation in the spleen. These changes in biodistribution were attributed to the amount of apolipoprotein (ApoH) adsorbed onto PEG-2000-SWCNTs and the extent of cellular uptake by the liver cells. Such studies demonstrate the combinatory importance of both the adsorbed protein corona and the specifications of surface functionalization of carbon nanotubes to determine their overall patterns of bloodstream circulation and biokinetic profile.

3.2. Complement system activation and CNTs

The adsorption of proteins onto CNTs may trigger complement activation; therefore it is important to interrogate in more detail the effect of protein binding to the complement system. Even though limited information exists for carbon nanotubes, this topic of complement activation has been thoroughly reviewed previously [42]. Ling et al. studied the possible activation of the complement system by CNT *via* the classical pathway which is triggered by the formation of the C1 complex [43]. TEM observations were used to show that MWCNTs bound both complement component C1q (recognition protein) and the C1s–C1r–C1r–C1s complex (catalytic subunit), while neither DWCNT nor SWCNTs bound C1q (even though C1s–C1r–C1r–C1s also bound to DWCNT but not SWCNTs). Despite binding, C1-dependent complement activation was not reported for any of the nanotubes tested. In contrast, Salvador-Morales and colleagues demonstrated that both SWCNTs and DWCNTs could activate the complement system *via* the classical pathway [38]. Thousands of proteins present in the serum samples used were tested but very few of them showed any affinity for the carbon nanotubes. C1q proteins were found on the surface of DWCNTs which suggested highly selective binding that may be responsible for the observed complement activation *via* the classical pathway, however a clear mechanism to explain C1q selective binding to CNTs has yet to be described.

In a follow-up study, Salvador-Morales et al. investigated the effects of chemical modification of MWCNT on their complement activation potential [39]. They either used pristine MWCNTs modified with 1,8

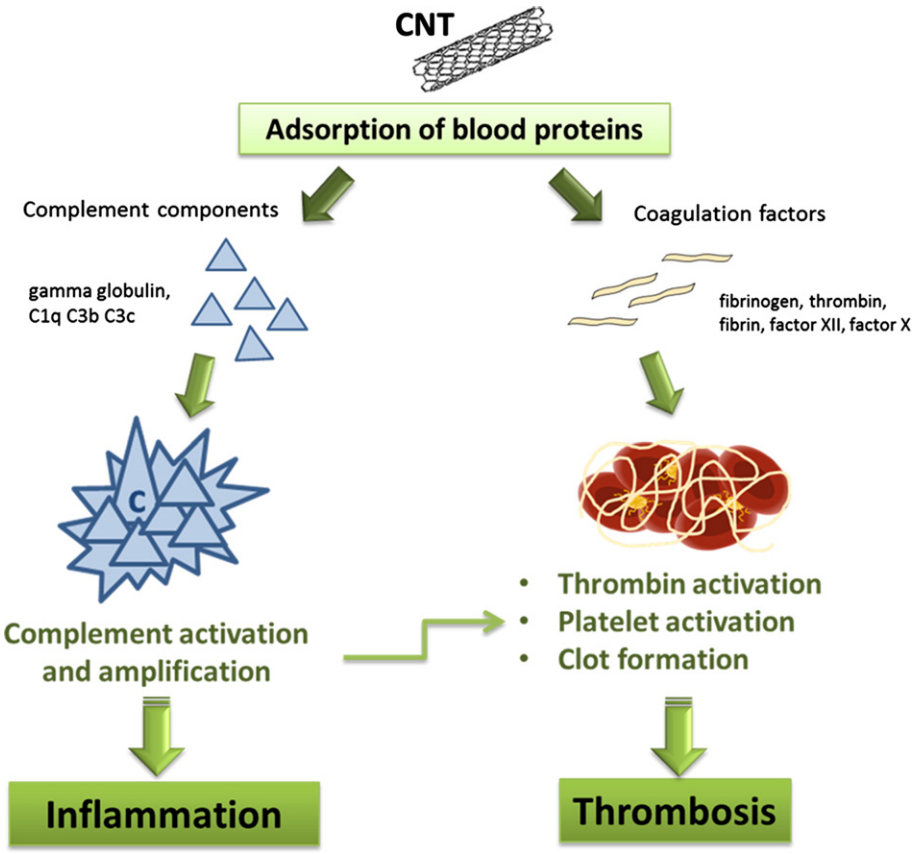

Fig. 1. Interaction of CNTs with blood proteins and possible adverse effects.

diamino-octane followed by the covalent attachment of ε-caprolactam or L-alanine, or pristine MWCNTs directly modified by reaction of 1-octadecylamine or 1,6 hexadithiol. It was reported that modification of the surface characteristics greatly changed the level of activation of the complement system *via* both its classical and alternative pathways. Chemically modified MWCNT induced less complement activation than non-modified MWCNTs and this was directly correlated with reduced levels of C1q protein binding and a higher level of factor H (complement activation regulator protein) binding.

Later, other studies examined the effect of nanotube surface coating by PEG-lipids using non-covalent methodologies [44–46]. While not directly investigating the molecular mechanism of activation (*e.g.* binding of complement component), PEGylation has been proposed to limit opsonization by proteins but not able to completely abolish the complement activation. The extent of activation was dependent on PEG conformation, PEG density and CNT surface coverage by PEG (full or partial coverage), similar to other nanoparticles. These studies used pristine SWCNT coated with PEGylated phospholipid molecules, either amino-PEG-5000-DSPE or methoxy-PEG-5000-DSPE [44]. Regardless of the terminal end moiety (amine or methoxy) of the PEG chains, activation of the complement in human serum was reported *in vitro*. Complement activation was shown to occur *via* the lectin pathway rather than the classical pathway, and depended on the complement component C4. In a subsequent study, SWCNTs coated with different macromolecules were compared [45]. Human serum albumin (HSA)-coated SWCNTs were able to activate the complement *via* both classical and alternative pathways, while methoxy-PEG coated SWCNTs activated the complement but to a lesser extent than HSA coated CNTs, and *via* the lectin pathway. In a recent study by the same group, various carboxylated MWCNTs covalently functionalized with PEG of different chain lengths [46] demonstrated activation of the complement in human serum in a concentration-dependent manner, irrespective of the PEG chain length (PEG-1000, PEG-1500 or PEG-4000). It has to be stressed that all of the above work on PEGylated carbon nanotubes has been carried out *in vitro* and the conclusions reached are in accord with other PEGylated nanoparticles (*e.g.* PEGylated liposomes). In general, the possibility of PEG-driven complement activation suggests caution and monitoring for oversensitive patients when clinically translating PEGylated nanoparticles.

4. Interactions with red blood cells

While it is vital to assess the overall CNT hemocompatibility upon intravenous injection, investigations of the hemolytic activity of CNTs are scarce (Table 2). The first study to do so reported that when low concentration of carboxylated SWCNTs (10 or 30 nmol/L) was put into contact with human erythrocytes, they did not induce any hemolysis and were

Table 2
Interaction of CNTs with red blood cells.

Type of CNT	Type of functionalization	Findings	Refs
SWCNT	Carboxylated	Neither hemolysis nor internalization in red blood cells	Donkor et al. (2009)
SWCNT	Pristine and carboxylated	Carboxylated SWCNT induces dose and time dependent hemolysis,	Sachar and Saxena (2011)
MWCNT	Aminated and carboxylated	No internalization in RBC but morphological changes and hemolysis. Damage level: short-aminated > long aminated > short carboxylated > long carboxylated	Meng et al. (2012)

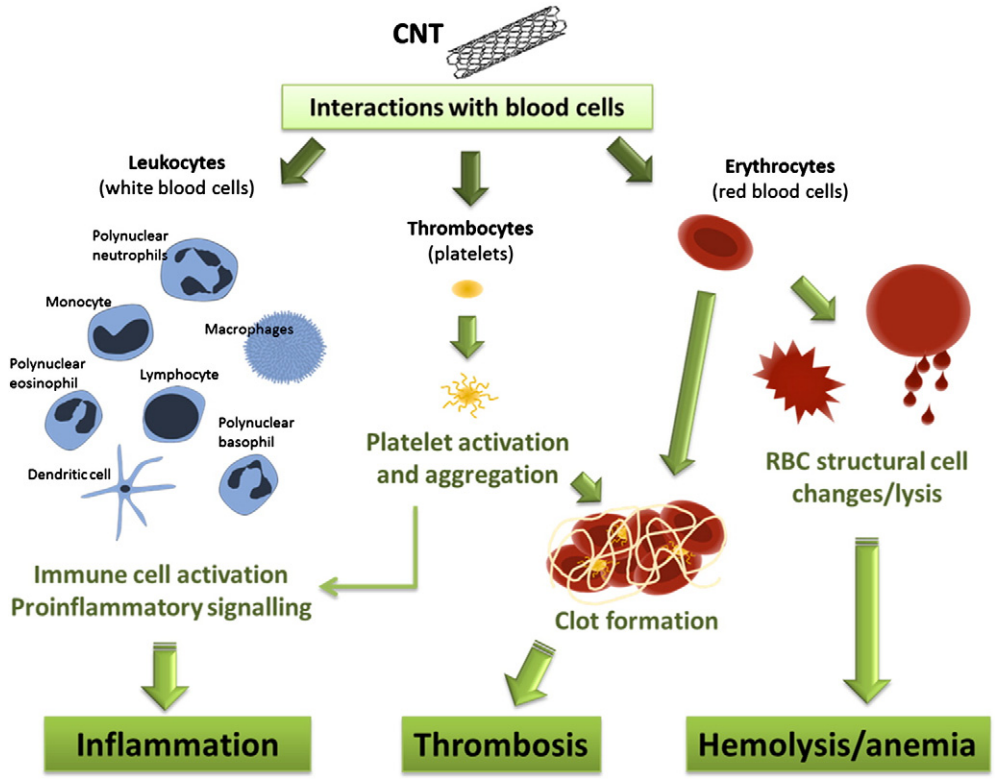

Fig. 2. Interaction of CNTs with blood cells and possible adverse effects.

not internalized by red blood cells (RBC) [47]. In contrast, Sachar and Saxena studied the impact of two types of SWCNTs (pristine and acid-treated) on mouse RBC and found that the carboxylated SWCNTs could induce hemolysis in a dose- and time-dependent manner, whereas the pristine did not [48]. In that study, the carboxylated SWCNTs were found to bind to the erythrocyte membrane and confocal microscopy imaging also suggested internalization by the RBC. An increase of phosphatidylserine externalization (early marker of apoptosis) on the membrane of RBC exposed to carboxylated SWCNTs was also reported, while no similar effect was observed for pristine SWCNTs. In the first *in vivo* study on such interactions, a transient anemic effect (low erythrocyte cell counts and hemoglobin levels) peaking within 24 h from intravenous injection of carboxylated SWCNTs was reported. Meng et al. investigated also the impact of four different MWCNTs (either long ones (50 μm): long-aminated and long-carboxylated; or short ones (0.5–2 μm): short-aminated and short-carboxylated) on human erythrocytes [49]. No MWCNTs were found inside the RBC (by TEM) and very few were observed on the RBC surface (by SEM). The interaction of human RBC with the MWCNTs resulted in cell morphology distortions, loss of membrane smoothness, and shrinkage that in cases led to membrane disruption. Overall, this TEM analysis showed that aminated MWCNTs had more severe effects than carboxylated (when comparing MWCNT of the same length) and short-MWCNTs were more damaging than long-MWCNTs when comparing nanotubes of the same chemistry. Interestingly, the conventional hemolytic activity assays to evaluate cell lysis and release of hemoglobin (Hb) were not used in this study because binding of Hb onto the CNT surface contributed to false negative results. In the most recent report on this topic, Guo et al. showed that functionalization *via* amination reduced the hemolytic activity of MWCNT [50]. Fig. 2 presents the possible adverse effects that CNTs can have on the different blood cells.[2]

[2] Interactions of CNTs with leukocytes are reviewed in greater detail by Battacharya et al. [http://dx.doi.org/10.1016/j.addr.2013.05.012] and Dumortier [http://dx.doi.org/10.1016/j.addr.2013.09.005] as part of this Special Issue.

5. Interactions with the hemostatic system

The possibility to induce blood clot formation is an essential risk factor to consider when developing nanomaterials with the intention to interact or transport within the circulatory system (Table 3). The adverse effects of prothrombotic nanomaterials could be severe and could lead to tissues being deprived of blood supply, with severe consequences as in pulmonary embolism. Therefore, the nanomaterial should not activate platelets, initiate the coagulation cascade, or promote thrombosis.

Radomski et al. were one of the first to investigate the thrombogenic activity of CNTs [51]. Comparing SWCNTs and MWCNTs, they demonstrated that SWCNTs induce a higher degree of human platelet aggregation than MWCNTs. Moreover, while it appeared that platelet degranulation did not occur, formation of pseudopodia and shape change upon nanotube exposure did suggest platelet activation, which was further confirmed by flow cytometry for the glycoprotein integrin receptor GPIIb/IIIa. Using a rat ferric chloride model of carotid artery thrombosis, they also showed that infusion of nanotubes accelerated the rate of arterial thrombosis development. That was consistent with the *in vitro* assay, confirming that SWCNTs were more prothrombogenic than MWCNTs. SWCNTs were also investigated in another study to assess the interaction of nanotubes with platelets [52]. *In vitro*, a significant increase in platelet P-selectin expression, platelet aggregation and formation of platelet-granulocyte complexes was reported. In that latter study, *in vivo* prothrombotic effects were also reported in small arteries and microvasculature. It was hypothesized that thrombus formation was mainly due to the high aspect ratio of SWCNT and direct interaction of CNTs with platelets. In another study, moderate platelet activation was also observed with four different types of MWCNTs (long-amidated, long-carboxylated, short-amidated, and short-carboxylated). It was found that long MWCNTs (50 μm) activated platelets more than short MWCNTs (0.5–2 μm), irrespective of functionalization [49]. Moreover, long-aminated MWCNTs were found to be cytotoxic to platelets, as shown by a reduction in cell count upon exposure. All MWCNTs were reported to affect clotting kinetics (earlier fibrin formation).

Table 3
Interaction of CNTs with the coagulation system.

Type of CNT	Type of functionalization	Main results	Refs
MWCNT	Aminated and carboxylated	Moderate platelet activation/long MWCNT activates more than short MWCNT/long aminated MWCNT is cytotoxic for platelet/all MWCNT is prothrombogenic	Meng et al. (2012)
SWCNT and MWCNT	Pristine	Platelet aggregation and activation: SWCNT > MWCNT SWCNT more thrombogenic than MWCNT in animal model	Radomski et al. (2005)
SWCNT	Pristine	*In vitro* platelet aggregation and activation *in vivo* Formation of thrombus: direct CNT–platelet interaction	Bihari et al. (2010)
SWCNT and MWCNT	Pristine	Platelet aggregation is dependent on extracellular Ca^{2+} influx	Semberova et al. (2009)
SWCNT and MWCNT	Pristine	CNT internalization in platelets/interaction with the dense tubular system that controls intracellular calcium stores,	Lacerda et al. (2011)
MWCNT	Pristine	CNT mediated calcium dependent platelet aggregation is related to intracellular sigalling pathways involving phospholipase C and protein kinase C	Guidetti et al. (2012)
MWCNT	Pristine/aminated/carboxylated/coated further with F127 pluronic or PEG	*In vitro* all MWCNT has pro-coagulant effects with PEG coated > pluronic coated and carboxylated > aminated/platelet activation: PEG coated < pluronic coated and carboxylated < aminated/*in vivo*, functionalization mitigates thrombogenic potential observed with pristine	Burke et al. (2011)

Simak et al. investigated the mechanism responsible for carbon nanotube-driven platelet activation [53]. Comparing two types of pristine SWCNTs and two types of pristine MWCNTs, all were found to induce platelet aggregation, dependent on extracellular Ca^{2+} concentration. Focusing on MWCNTs, it was further demonstrated that the nanotubes induced extracellular Ca^{2+} influx in platelets *via* activation of the Ca^{2+} entry complex. In a subsequent study, the same group reported that these MWCNTs were internalized by platelets and interacted with the dense tubular system (calcium storage complex within platelets) causing depletion of the intracellular calcium stores, without any observation of cellular membrane damage [54]. Morphologically, markers of activation such as pseudopodia formation and release of membrane microparticles were observed. Another group of researchers has investigated the mechanism that triggers platelet aggregation upon MWCNT exposure [55]. They demonstrated that Ca^{2+}- and integrin $\alpha_{IIb}\beta_3$-dependent platelet aggregation was mediated by stimulation of intracellular signaling pathways and extracellular second messengers. More specifically, platelet aggregation, which results from platelet activation and fibrinogen binding, was found to occur *via* stimulation of intracellular phospholipase C, protein kinase C, and GTPase Rap1b, that regulate integrin $\alpha_{IIb}\beta_3$ activation. Overall these studies demonstrate that CNTs induce platelet activation by modulating the intracellular Ca^{2+} concentration, which plays an important role in cellular signal transduction pathway.

Following a different strategy, Burke et al. studied the interaction of MWCNTs with components of the hemostatic system and the influence of surface modification (covalent chemical functionalization either carboxylation or amidation) followed or not by coating (either pluronic F127 or PEG phospholipids) on the MWCNT thrombotic activity [56]. *In vitro*, both amidated and carboxylated MWCNTs were reported to have procoagulant effects, irrespective of the presence of a coating or not. When considering the same surface chemical groups, PEG-coated nanotubes promoted thrombosis compared to pluronic-coated nanotubes, while carboxylated nanotubes had a more pronounced pro-thrombotic effect than amidated nanotubes. Moreover, PEG-coated pristine MWCNTs activated the intrinsic coagulation cascade whereas pluronic-coated pristine MWCNTs did not. With regard to the mechanistic interpretations of thrombogenicity that were proposed for functionalized MWCNTs, two mechanisms depended on clotting factors (factor IX and factor XII), while a third mechanism involved platelet activation only. Stimulation of the intrinsic pathway of the coagulation cascade was attributed to direct binding of nanotubes to factor IXa. Lastly, in terms of *in vivo* effects, covalent functionalization (both covalent and non-covalent) seemed to alleviate the procoagulant activity observed with pristine materials, regardless of surface modification.

Vakhrusheva et al. also studied the importance of surface characteristics on the thrombogenicity of SWCNTs [57]. Using nanotubes functionalized *via* carboxylation or covalent PEGylation, or nanotubes

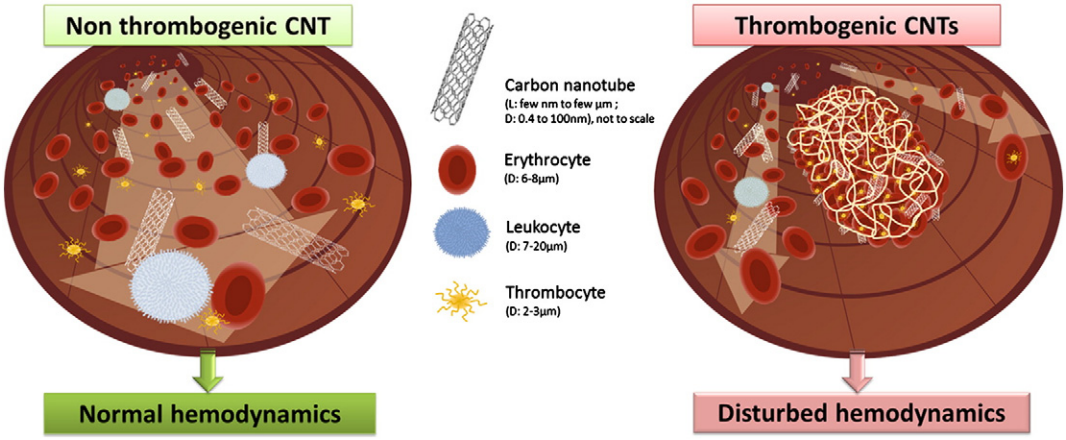

Fig. 3. Thrombogenic potential of CNTs and impact on blood flow. This scheme represents the possible effects of CNTs on blood cells, depending on their thrombogenic properties. When CNTs are thrombogenic they can lead to the formation of blot clots and disturb the normal hemodynamics.

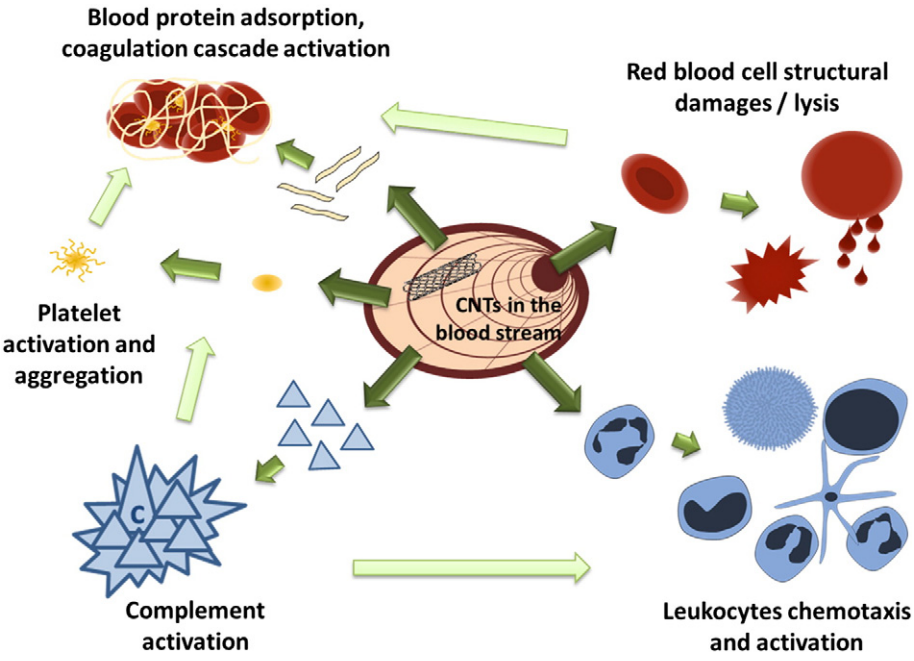

Fig. 4. Summary of potential hemotoxic effects of CNTs. This scheme recapitulates the effects of CNTs on both protein and cellular components of the blood that can range from damages to red blood cells and interaction with proteins to the activation of platelets, leukocytes, the complement or the coagulation cascade.

coated with human serum albumin, they demonstrated in whole blood that both carboxylated and PEGylated SWCNTs exerted thrombogenic effects, whereas pre-treatment of the nanotubes with albumin has a beneficial effect (*i.e.* reduction in platelet aggregation). Fig. 3 presents the effect of two types of CNTs on hemodynamics, depending on their thrombogenic action.

6. Conclusions

We aimed to provide a concise review of the most updated understanding of hemocompatibility or hemotoxicity of CNTs (Fig. 4). Overall, the limited amount of studies published necessitates more systematic and thorough investigations to elucidate CNT blood compatibility. Such knowledge will allow determination of a more rounded safety profile and is mandatory toward the development of nanomaterial-based injectable for therapeutic or diagnostic purposes, not only for CNTs but also for any kind of nanomaterials [58–61].

It seems that the common critical parameters that determine CNT hemocompatibility remain the chemical nature of surface modifications, surface charge, nanotube structure, and nanotube surface area available for interactions. Surface characteristics are the major parameter influencing the interactions with blood components and therefore the overall biological impact. Indeed, the addition of functional groups or coating on the surface has been proven to dramatically change the CNT reactivity [62,63]. In general, pristine CNTs have a highly hydrophobic surface that may cause aggregation and interactions with cells inducing apoptosis or inflammation, while functionalization helps significantly overcome these effects by making nanotubes more hydrophilic and water soluble, therefore more biocompatible [64,65]. However, improved biocompatibility does not necessarily mean improved hemocompatibility, as the bloodstream appears to be one of the most sensitive and reactive compartments of the body to foreign materials. As the biomedical applications of CNT are maturing, improvements in their hemocompatibility, their interactions with the complement system, red blood cells, and the hemostatic system, may pose one of the greatest challenges to clinical translation if not careful with the design characteristics to be avoided.

Acknowledgments

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