Published in final edited form as:

Mol Immunol. 2008 August ; 45(14): 3797–3803. doi:10.1016/j.molimm.2008.05.020.

Complement activation by PEGylated single-walled carbon nanotubes is independent of C1q and alternative pathway turnover

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Abstract

We have investigated the interaction between long circulating poly(ethylene glycol)-stabilized single-walled carbon nanotubes (SWNTs) and the complement system. Aminopoly(ethylene glycol)₅₀₀₀-distearoylphosphatidylethanolamine (aminoPEG₅₀₀₀-DSPE) and methoxyPEG₅₀₀₀-DSPE coated as-grown HIPco SWNTs activated complement in undiluted normal human serum as reflected in significant rises in C4d and SC5b-9 levels, but not the alternative pathway split-product Bb, thus indicating activation exclusively through C4 cleavage. Studies in C2-depleted serum confirmed that PEGylated nanotube-mediated elevation of SC5b-9 was C4b2a convertase-dependent. With the aid of monoclonal antibodies against C1s and human serum depleted from C1q, nanotube-mediated complement activation in C1q-depleted serum was also shown to be independent of classical pathway. Nanotube-mediated C4d elevation in C1q-depleted serum, however, was inhibited by *N*-acetylglucosamine, Futhan (a broad-spectrum serine protease inhibitor capable of preventing complement activation through all three pathways) and anti-MASP-2 antibodies; this strongly suggests a role for activation of MASP-2 in subsequent C4 cleavage and assembly of C4b2a convertases. Intravenous injection of PEGylated nanotubes in some rats was associated with a significant rise in plasma thromboxane B2 levels, indicative of in vivo nanotube-mediated complement activation. The clinical implications of these observations are discussed.

Keywords

Biomaterials; Carbon nanotubes; Complement system; C4; Drug delivery system; Nanomedicine; SC5b-9

1. Introduction

Carbon nanotubes have received considerable attention as promising materials for a wide range of experimental diagnostic and therapeutic applications following intravenous injection, particularly in cancer scenarios (Cherukuri et al., 2006; Liu et al., 2007, 2008). Recently,

nanotube stability in the blood was enhanced by surface functionalization with poly(ethylene glycol)-phospholipid (PEG-PL) conjugates (Liu et al., 2007). Such surface modification procedures has further conferred longevity to SWNTs in the systemic circulation; this property most likely arises from the steric hindrance of the projected long PEG chains to nanotube–macrophage interaction, which is similar to what has been reported with PEGylated liposomes (Moghimi et al., 2001, 2006; Moghimi and Szebeni, 2003; Dos Santos et al., 2007). PEGylated SWNTs, through prolonged circulation times in the blood, can ultimately target elements of tumour vasculature following conjugation of targeting ligands to the distal end of the projected PEG chains bearing a reactive functional group and therefore act as experimental cancer nanomedicines (Liu et al., 2007).

The interaction between long circulating PEGylated carbon nanotubes and the complement system, which is an important effector arm of both innate and acquired immunity (Lambris et al., 2008), however, has not received previous attention. One serious consequence of complement activation is generation of anaphylatoxins and chemoattractants C3a and C5a; these split complement products can induce anaphylaxis in sensitive individuals (Szebeni, 2005). Apart from anaphylatoxin release, the terminal half of the complement pathway generates multiprotein C5b-9 complexes (Lambris et al., 2008) and these have the capacity to elicit non-lytic stimulatory responses from vascular endothelial cells (Hattori et al., 1989), and modulate endothelial regulation of haemostasis and inflammatory cell recruitment. Non-functionalized high pressure carbon monoxide single-walled carbon nanotubes (HIPco SWNTs) and double-walled carbon nanotubes (DWNTs) are capable of triggering complement (Salvador-Morales et al., 2006), but it is generally perceived that surface functionalization with PEG can suppress or inhibit such immunological processes. On the contrary, recent biochemical and immunological studies with clinically approved formulations of PEGylated liposomes (e.g., Doxil®) and other PEGylated nanoparticles have demonstrated that the steric hindrance of PEG may not necessarily prevent complement activation and fixation (Gbadamosi et al., 2002; Moghimi, 2002; Chanan-Khan et al., 2003; Moghimi and Szebeni, 2003). Indeed, infusion of Doxil® into a substantial percentage of human subjects has triggered immediate acute pseudoallergic reactions with symptoms of cardiopulmonary distress, which are strongly linked to generation of anaphylatoxins C3a and C5a, leading to the subsequent release of thromboxane A2 (TXA2) and other inflammatory mediators from immune cells (Szebeni, 2005; Chanan-Khan et al., 2003). These reactions are also reproducible in pigs where minute quantities of Doxil® injection induces cardiac anaphylaxis with C5a playing a causal role (Szebeni et al., 2006). In light of these observations, we sought to investigate the interaction of long circulating amino-PEG₅₀₀₀–distearoylphosphatidylethanolamine (amino-PEG₅₀₀₀–DSPE) and methoxy(MeO)PEG₅₀₀₀–DSPE functionalized SWNTs with the complement system both in vitro and in vivo. Here, for the first time we demonstrate that such entities can, indeed, trigger complement, in spite of their protective PEG armor. We have elucidated the likely pathways of complement activation by PEGylated SWNTs and our results may have important bearings for the future directions in nanoparticle surface functionalization with possible applications in experimental immunology and clinical medicine.

2. Materials and methods

2.1. Preparation of PEGylated SWNTs

As-grown SWNTs were sonicated in the presence of either 1 mg/mL amino-poly(ethylene glycol)₅₀₀₀–(1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine) (amino-PEG₅₀₀₀–DSPE) or methoxy (MeO)-PEG₅₀₀₀–DSPE (NOF Corporation, Japan) for 1 h followed by centrifugation at $24,000 \times g$ for 6 h to remove large bundles, aggregates and impurities as described earlier (Liu et al., 2007). Excess PEG-phospholipid conjugates were removed by filtration through 100-kDa Millipore filters. The amount of adsorbed PEG-DSPE was estimated as described

previously (Liu et al., 2007, 2008). Nanotube length was determined by atomic force microscopy and was taken from over 100 images. The equivalent spherical size distribution of nanotubes was measured by photon correlation spectroscopy (Moghimi et al., 2006). The concentration of SWNT was estimated by optical absorbance at 808 nm with a weight-concentration-based extinction coefficient of $46 \text{ L g}^{-1} \text{ cm}^{-1}$ (Liu et al., 2007, 2008).

2.2. PEGylated liposomes

Liposomes were composed of dipalmitoylphosphatidylcholine (DPPC) and MeOPEG₂₀₀₀-DPPC (mole ratio 9.5:0.5) and prepared by hydrating the dried lipid film with 10 mM phosphate-buffered saline (pH 7.2) and then extruded through polycarbonate Nuclepore filters of appropriate pore diameters using a high-pressure extruder. Prepared liposomes were $118 \pm 12 \text{ nm}$ in size (polydispersity index = 0.07) as determined by laser light scattering (Moghimi et al., 2006).

2.3. Preparation of human serum

Blood was drawn from healthy Caucasian male volunteers (aged 25–35 years) according to approved local protocols. Blood was allowed to clot at room temperature and serum was prepared, aliquoted and stored at -80°C . Serum samples were thawed and kept at 4°C before incubation with test reagents. Commercially available human C1q-depleted and C2-depleted sera were obtained from Quidel (distributed by Technoclone, UK).

2.4. Assays of in vitro complement activation

To measure complement activation in vitro, we determined SWNT-induced rise of serum complement activation product SC5b-9, Bb and C4d, using respective Quidel's ELISA kits according to the manufacturer's protocols as described previously (Moghimi et al., 2006). As a result of substantial biological variation in serum levels of complement proteins and the large number of positive and negative feedback interactions, we monitored generation of complement activation products in sera of 4 healthy individuals separately (Moghimi et al., 2006). The concentration of mannose binding lectin (MBL) in healthy, C1q-depleted and C2-depleted sera was determined by using the MBL-C4 complex ELISA kit (HyCult Biotechnology, The Netherlands). Only sera with physiological concentrations of MBL, in the range of 3000–5000 ng/mL, were selected for subsequent complement activation assays. The complement haemolytic activity of C1q-depleted and C2-depleted sera was restored following the addition of C1q (180 $\mu\text{g/mL}$) and C2 (650 $\mu\text{g/mL}$), respectively, as assessed by the haemolytic test using sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody (Szebeni et al., 1994; Gbadamosi et al., 2002). The functional activity of classical, lectin and the alternative pathways of complement were confirmed in all sera with Wielisa®-Total Complement Screen kit (Lund, Sweden).

For measurement of complement activation, the reaction was started by adding the required quantity of SWNTs to undiluted serum (SWNT:serum volume ratio; 1:4) in Eppendorf tubes (in triplicate) in a shaking water bath at 37°C for 30 min, unless stated otherwise. Reactions were terminated by addition of "sample diluent" provided with assay kit. SWNT-induced rises of serum SC5b-9, Bb and C4d was then measured following nanotube removal (to minimize interference in ELISA tests) by carefully layering 200 μL of Sephacryl S-200-HR gel (Sigma-Aldrich, UK) and subsequent centrifugation. The extent of nanotube-trapping in the gel was followed by measuring the absorbance of the supernatant at 808 nm (e.g., ~70–75% of amino-PEG₅₀₀₀-DSPE coated nanotubes were trapped by these procedures). Subsequently, appropriate controls were also made by adding the sufficient quantities of SWNTs to saline for background correction in ELISA experiments. The gel-trapping procedures had no effect in removing fluid phase complement activation products. Control serum incubations contained saline (the same volume as nanotubes and other additions) for assessing background levels of

SC5b-9, Bb and C4d. Zymosan was used as a positive control for complement activation (Moghimi et al., 2006). To monitor the possible binding of complement activation products to the nanotube surface, we incubated SWNT with standard samples of activation products. The level of the standard activation products in the supernatant was then measured by the respective ELISA test and compared with control incubations in the absence of nanotubes. In some experiments, SWNT-induced complement activation was monitored following pretreatment of serum with EDTA (20 mM final concentration), Futhan (150 µg/mL; Merck), *N*-acetylglucosamine (25 mM), *D*-galactose (25 mM) and monoclonal antibodies (against C1s, MBL, H- and L-ficolins, MASP-2 or an irrelevant antibody). Mouse monoclonal antibodies to human C1s (IgG1, clone M81), MBL, L-ficolin (clone GN5), H-ficolin (clone 4H5) and MASP-2 were from HyCult Biotechnology (The Netherlands). The anti-C1s monoclonal antibody, but not an irrelevant murine IgG antibody, blocked activation of the classical pathway in human serum as confirmed by complement activation experiments (C4d measurements) in the presence of cholesterol-rich liposomes (these liposomes activate complement through the classical pathway) (Szebeni et al., 1994, 1996).

For quantification of complement activation products, standard curves were constructed using the assigned concentration of each respective standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best-fit line for SC5b-9, Bb and C4d standard curves were within the manufacturer's specified range. The efficacy of SWNT treatments was established by comparison with baseline levels using paired *t*-test; correlations between two variables were analysed by linear regression, and differences between groups (when necessary) were examined using ANOVA followed by multiple comparisons with Student–Newmann–Keuls test. Similar patterns were observed in all tested sera; the result of a typical experiment is presented.

2.5. Determination of thromboxane B2 (TXB2) level in rat blood

Prior to intravenous injection of SWNT, PEG-phospholipids and liposome, 1.0 mL blood was taken from the tail vein of male Wistar rats (250–280 g) for plasma preparation to obtain the required baseline parameters. All procedures were in accordance with local regulations and approved protocols. For plasma preparations, blood was collected in EDTA/0.25 mM indomethacin-containing tubes to prevent prostaglandin metabolism. Bolus intravenous injections (SWNT, 1.2 mg/kg, PEG-PL conjugates, up to 10 mg/kg; PEGylated liposomes, 80 mg/kg) were made through the opposite tail vein. Further blood samples were taken at 6 and 60 min post-injection to obtain plasma. Plasma TXB2 levels were determined in triplicate samples as described previously (Szebeni et al., 1994; Moghimi et al., 2006) by following the procedures supplied with the ELISA kit.

3. Results and discussion

3.1. Nanotube characteristics

The diameters and lengths of functionalized nanotubes were 1–5 nm and 50–300 nm, respectively, as determined by AFM (Fig. 1). We have estimated the binding of ~50 PEG-PL conjugates (irrespective of the PEG terminal end group) to a typical nanotube with a mean diameter and length of 2 and 100 nm, respectively (Liu et al., 2007). For a surface monolayer arrangement on a cylindrical nanotube structure, this represents a minimum available surface area of 12 nm² per adsorbed PEG-PL, where the projected PEG chains are expected to assume a 'brush-like' or laterally compressed elongated random coil configuration, which is perceived as a necessary prerequisite for minimizing and/or combating protein adsorption (Moghimi, 2002; Gbadamosi et al., 2002; Moghimi and Szebeni, 2003). The prolonged stability of PEGylated SWNTs in both buffer and fresh serum was confirmed earlier (Liu et al., 2007).

3.2. PEGylated SWNTs activate the whole pathway of complement

During the terminal half of complement activation, C5b-9 complexes formed in the absence of a target membrane bind to the regulatory S protein; the generated SC5b-9 complex is the soluble, non-lytic form of the terminal attack complex (Lambris et al., 2008). We therefore monitored nanotube-mediated complement activation in sera of healthy subjects by measuring SC5b-9 generation, which is a sensitive and established measure of the activation of the whole complement cascade (Szebeni et al., 1998; Moghimi et al., 2006). PEGylated nanotubes caused complement activation, irrespective of the terminal end moiety of the projected PEG chains, as evident with significant rises of serum SC5b-9 levels over baseline at a final nanotube concentration of 40 $\mu\text{g/mL}$ (Fig. 2a), with a trend of reaching maximal efficacy at 60–80 $\mu\text{g/mL}$. Nanotube-mediated SC5b-9 generation also proceeded on a time scale of minutes and reached plateau at around 10 min (not shown). In contrast to PEGylated SWNTs, PEG-PL conjugates, even at concentrations as high as 2 mg/mL (twice above the equilibrium concentration of PEG-PL conjugates initially used for SWNT stabilization), did not elevate serum SC5b-9 levels; this was also in accordance with the previous studies (Moghimi et al., 2006).

3.3. The role of alternative pathway turnover

The alternative pathway is stimulated by the spontaneous cleavage of the thioester bond in C3 (“C3-tickover”) or when the internal thioester bond in the α -chain of nascent C3b undergoes nucleophilic attack in the presence of a foreign surface structure rich in nucleophilic groups (particularly hydroxyl- and amino-rich surfaces) (Szebeni et al., 1998; Lambris et al., 2008; Toda et al., 2008). We therefore monitored serum levels of the split-product Bb, a specific marker of alternative pathway turnover (Szebeni et al., 1998), following nanotube challenge. PEGylated SWNTs had no effect on serum Bb levels even at concentrations as high as 80 $\mu\text{g/mL}$ (Fig. 2b); this was rather surprising since the projected surfaces are rich in nucleophilic groups arising from PEG termini (Toda et al., 2008). Similar observations were also made in C2-depleted sera, where no Bb elevation could be detected. In all cases zymosan served as positive control. Control experiments, with standard Bb samples, further confirmed that Bb has no affinity for the nanotube surface. The lack of factor B cleavage also excludes a role for direct C3 adsorption to SWNTs with subsequent formation of the C3Bb convertase through altered C3 conformational changes that resembles C3b; such mode of activation was recently reported for the polystyrene surface (Andersson et al., 2002). Nevertheless, our results are in-line with the recent suggestion of Salvador-Morales et al. (2006) where non-functionalized HIPco SWNTs also failed to enhance alternative pathway turnover in human serum.

3.4. Nanotube-mediated complement activation is C4-dependent

Activation of classical pathway and lectin pathway by an activator liberates activated forms of serine protease C1s and mannose binding lectin (MBL)-associated serine proteases-2 (MASP-2), respectively (Matsushita et al., 2000; Vorup-Jensen et al., 2000; Fujita, 2002; Lambris et al., 2008). These proteases cleave C4; one of the final fluid phase degradation products of C4 cleavage is the C4d fragment, which is an established marker of both classical pathway and lectin pathway activation (Szebeni et al., 1997; Moghimi et al., 2006). In our hands, SWNTs elevated fluid phase C4d levels in all tested human sera by 2–3-fold above the background level (Fig. 2c). In agreement with SC5b-9 measurements, PEG-PL conjugates did not elevate serum C4d levels (Moghimi et al., 2006). Next, we used a C2-depleted human serum to confirm that the observed SWNT (80 $\mu\text{g/mL}$)-mediated elevation of SC5b-9 (as in Fig. 2a) was dependent on the generation of classical and lectin pathway C4b2a convertases, as these convertases trigger downstream reactions. Indeed, in C2-depleted serum, SWNTs failed to dramatically elevate SC5b-9 levels (872 ± 54 ng/mL serum) above the background

(811 ± 28 ng/mL serum). Following C2 restoration (650 μ g/mL), SWNT-mediated rises of SC5b-9 level were 2.2-fold above background.

To further distinguish between the calcium-sensitive C1q-dependent classical pathway and the lectin pathway mode of activation, we used a C1q-depleted human serum (with physiological MBL and C3 levels). Remarkably, nanotubes induced significant elevation of C4d levels in C1q-depleted serum both in the absence and the presence of EDTA (Fig. 3a); this is in contrast to non-functionalized carbon nanotubes where complement activation was reported to occur directly via C1q binding (Salvador-Morales et al., 2006). To further eliminate a direct role for the serine protease C1s in PEGylated SWNT-mediated complement activation, we pre-treated C1q-depleted serum with a monoclonal antibody against C1s (the antibody recognizes the binding site of C1s for C4 and reacts with both active and inactive C1s). Again, nanotubes significantly elevated C4d and SC5b-9 levels in serum above the respective baselines (incubations in the presence of an irrelevant antibody), thus eliminating the role of the classical pathway in SWNT-mediated complement activation (data not shown, see also Materials and Methods for respective functional controls involving liposomes).

Therefore, SWNT-mediated complement activation most likely proceeds through the lectin pathway. To check this hypothesis, we first measured SWNT-mediated C4d generation in C1q-depleted serum in the presence of *N*-acetylglucosamine (25 mM), since both MBL and ficolins bind to this monosaccharide in the mM range. Remarkably, *N*-acetylglucosamine, but not the nonantagonist β -galactose (25 mM), suppressed nanotube-mediated C4d elevation and the results were comparable to background levels (Fig. 3b), thus indicating a possible role for MASP-2 activation in subsequent C4 cleavage. Next we showed that PEGylated nanotube-mediated C4d elevation in C1q-depleted serum could be inhibited by both anti-MASP-2 antibodies and Futhan (a broad-spectrum serine protease inhibitor and capable of preventing complement activation through all three pathways) (Pfeifer et al., 1999) (Fig. 3b). On the basis of these observations we further exclude the involvement of a speculative complement triggering mechanism solely based on direct C4 binding to PEGylated nanotubes with subsequent formation of C4b2a convertase through altered C4 conformational changes.

3.5. Nanotube-mediated rise of thromboxane B2 levels in rats

To establish whether PEGylated SWNTs are also capable of inducing complement activation *in vivo*, we monitored plasma thromboxane B2 (TXB2) levels following intravenous nanotube injection into rats. TXB2 is a direct marker for TXA2. When complement is activated *in vivo*, then generated anaphylatoxins C3a and C5a usually induce TXA2 release from blood cells, but due to its short half-life (~ 30 s) TXA2 is hydrolyzed rapidly to TXB2. However, TXA2 formation can be monitored by quantifying TXB2 and demonstration of increased serum TXB2 levels provides evidence for *in vivo* SWNT-mediated complement activation (Szebeni et al., 1994; Moghimi et al., 2006). Erratic responses were observed in rats; while in some animals nanotube administration (1.2 mg/kg) was associated with a significant rise in plasma TXB2 level on a timescale of minutes with return to background levels at 1 h (a feature consistent with complement activation), other rats showed no response (Fig. 4). In contrast to nanotubes, intravenous administration of PEG-PL conjugates (1.2 mg/kg) did not alter plasma TXB2 levels, which was also in agreement with previous studies (Moghimi et al., 2006). As a positive control, PEGylated liposome administration induced rises in plasma TXB2 levels in all tested rats. The lack of response to SWNT injection in some rats could be due to generation of insufficient quantities of anaphylatoxins C3a and C5a necessary for initiating immune cell degranulation either directly or through the release of a secondary co-stimulus, such as those arising from the kallikrein-kinin system (Szebeni, 2005; Hamad and Moghimi, 2008). Therefore, higher doses of carbon nanotubes are presumably necessary to initiate positive responses more uniformly, however, we could not proceed with such dosing schedules because

of large injection volumes and viscosity problems associated with more concentrated samples. It is also plausible that, in contrast to human serum, SWNT-mediated complement activation could proceed through other pathways in the rat model. However, if nanotube-mediated complement activation in rats is through the lectin pathway, then the observed variations may arise as a result of different levels of plasma MBL- or ficolin-/MASP-2 and/or MASP-2 activity among individual animals. Liposome-mediated complement activation, however, occurs through both antibody-mediated C1q-dependent and alternative pathways in the described model, which presumably raises sufficient quantities of anaphylatoxins necessary for subsequent immune cell degranulation (Szebeni et al., 1994; Moghimi et al., 2006).

4. Hypothesis

The possible involvement of the lectin pathway in PEGylated SWNT-mediated complement activation in human serum seems remarkable as both MBL and ficolins express specificity for sugars with *N*-acetylated groups (Fujita, 2002). In addition, ficolins can also bind to acetylated compounds relatively independent of the structure of the acetylated molecule (Krarup et al., 2004). However, these structures are absent in our SWNT preparations and there was no sample contamination with endotoxins (confirmed by Lymulus lysate assay). Our further studies with monoclonal antibodies against MBL as well as H and L-ficolins failed to establish a direct role for either MBL or ficolins in SWNT-mediated complement activation, since antibody binding to MBL/ficolins activated MASPs as observed through enhanced C4 cleavage. As to the molecular basis of PEGylated SWNT-mediated complement activation via the presumed lectin pathway, at present we can only speculate a possible role for MBL and/or ficolin binding to some structural determinants of SWNTs, leading to subsequent MASP-2 activation, C4 cleavage and formation of C4b2a convertases. The structural features required for MBL and/or ficolin binding are presumably expressed in a simultaneous manner by both PEG chains and the nanotube surface (PEG-PLs do not activate complement by themselves; Fig. 2). It is highly plausible that a part of the recognition domain for MBL and ficolin binding on the nanotube surface is provided via some other adsorbed serum components, such as various apolipoproteins. Indeed, Salvador-Morales et al. (2006) demonstrated the binding of a select number of plasma and serum proteins, notably apolipoproteins A-I, A-IV and C-III to non-functionalized nanotubes. Therefore, complement activation by PEGylated SWNTs may have been triggered from inadequate surface protection of a certain population of SWNTs by PEG₅₀₀₀-PL conjugates against glycoprotein adsorption. Surface heterogeneity (with some populations poorly protected by PEG chains) may also explain the observed rapid deposition of a significant fraction of intravenously injected PEGylated SWNT in liver and spleen macrophages through opsonization events involving C3b and iC3b (Liu et al., 2007). Therefore, for better protection against complement activation and fixation, PEG-PL conjugates of longer PEG chains or those displaying branched PEG chains may be used. Indeed, such surface engineered SWNTs have recently provided improved blood pharmacokinetics (Liu et al., 2008). Also, earlier work with PEGylated liposomes has identified a role for the anionic charge localized on the oxygen-phosphate moiety of the PEG-PL conjugate, which in concert with specific structural moiety of the lipid bilayer orchestrate vesicular-mediated complement activation through antibody binding (Moghimi et al., 2006). A similar mode of action could also operate with PEGylated SWNTs, where the surface may activate MASP-2 directly or through electrostatic interaction with MBP and/or ficolins. These possibilities, therefore, warrant further investigations.

Finally, as to the inconclusive nature of the rat model in response to SWNTs, experiments in pigs and dogs would be prudent to establish whether PEGylated SWNT can induce complement activation-related pseudoallergy. The pig and dog are established models for assessing such acute reactions to nanomedicines, although the nature of responses is different (Szebeni, 2005; Hamad and Moghimi, 2008). For example, minute amounts of liposome administration

into the pigs induces rapid haemodynamic changes such as a massive rise in pulmonary arterial pressure, and a decline in systemic arterial pressure, cardiac output and left ventricular end-diastolic pressure. In dogs, haemodynamic responses are less dramatic, but remarkably dogs display considerable vegetative neural dysfunction, presumably indicating a set of unique interactions between the immune and the neural systems in this species (Szebeni, 2005). Nevertheless, a clear understanding of causal chemical and biological interactive factors behind PEGylated SWNT-induced complement activation may provide a platform for rational design of safer nanotubes for medical applications.

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Fig. 1.

An atomic force microscope image of amino-PEG₅₀₀₀-DSPE functionalized SWNTs over a SiO₂ substrate (a) and a photograph of MeO-PEG₅₀₀₀-DSPE stabilized SWNT suspension (b). Similar AFM images were also obtained with MeO-PEG₅₀₀₀-DSPE stabilized SWNTs (not shown). Scale bar = 250 nm. Photon correlation spectroscopy further revealed a mean particle size (equivalent spherical size) of 259 nm and a modal particle size of 214 nm for amino-PEG₅₀₀₀-DSPE functionalized SWNTs. The corresponding values for MeO-PEG₅₀₀₀-DSPE functionalized SWNTs were 175 and 117 nm, respectively.

Fig. 2.

PEGylated SWNT-mediated complement activation in a healthy human serum. SC5b-9, Bb and C4d levels in human serum at 30 min after nanotube treatment. In (a) SC5b-9 levels reached maximum at a final nanotube concentration of 60 $\mu\text{g/mL}$ of serum (not shown); the results were similar to those at 80 $\mu\text{g/mL}$. Zymosan was used as a positive control. The coating materials (PEG-PLs) themselves had no effect on serum levels of complement split-products Bb and C4d as well as the terminal complex, even at concentrations as high as 2 mg/mL (which is well above their critical micelle concentration) in serum and the results were similar to control (saline) (see also Moghimi et al., 2006). Significant difference with respect to control (saline): * $p < 0.05$, ** $p < 0.01$.

Fig. 3.

PEGylated SWNT-mediated rises of the fluid phase split-product C4d in C1q-depleted human serum. In both (a) and (b) final nanotube concentration was 40 μg/mL serum. Zymosan was used as a positive control for monitoring activation of the lectin pathway. In (b) addition of Futhan (150 μg/mL) or monosaccharides (25 mM final concentration) had no significant effect on C1q-depleted serum C4d background levels; all values were within $\pm 7\%$ of the background level (C1q-depleted serum C4d background level was 8.6 ± 0.54 μg/mL). In (a) significant difference is compared with the respective control incubation: * $p < 0.05$, ** $p < 0.01$.

Fig. 4.

Plasma TXB2 levels in rats following bolus intravenous injection of PEGylated SWNTs, PEG-PL conjugates and PEGylated liposomes. Data from each individual animal is shown. For MeOPEG-PL, 4 rats were also used and no responses were observed (only two representative examples are shown); even at higher concentrations of MeOPEG-PL, (tested up to 10 mg/kg), TXB2 levels were comparable to the background (not shown). Significant difference is compared with the 0 min (baseline, TXB2 level in plasma prior to nanotube, MeOPEG-PL and liposome injection): * $p < 0.05$, ** $p < 0.01$.