Recognition of Carbon Nanotubes by the Human Innate Immune System

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Abstract A major function of the human innate immune system is to recognize non-self: i.e., invading microorganisms or altered, damaged self macromolecules and cells. Various components of the human immune system recognize foreign synthetic materials, including carbon nanotubes (CNTs). The complement system proteins in blood, and the collectins, SP-A and SP-D in the lungs bind to carbon nanotubes, in competition with other plasma proteins, and may influence their subsequent adhesion to and uptake by cells and their localization in the body. Modification of the surface chemistry of carbon nanotubes alters their binding to complement proteins and collectins, and provides information on the mechanism by which binding of these proteins occurs.

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1 The Innate Immune System

Multicellular living organisms have developed very varied systems to protect themselves from attack by organisms of other species. One aspect of these protective mechanisms is an immune system, which has evolved to protect multicellular organisms from attack by small parasites or from infectious microorganisms, such as bacteria, viruses and fungi. Most multicellular organisms also have to solve a problem, which occurs as they grow and develop: this is to remove their own obsolete, dying or damaged cells. Parts of the immune system appear to have evolved to take on this role also, so that the immune system can recognize not only foreign microorganisms, but also altered or damaged host cells and macromolecules. Recognition of these foreign or altered particles is mediated by specific proteins, which bind to the foreign or altered particle (target); then trigger downstream reactions, which lead to the destruction of the target.

Vertebrate animals, including humans, have sophisticated immune systems, which can conveniently be divided into two parts: innate immunity and adaptive immunity. In innate immunity, recognition of the target is mediated by proteins which are encoded directly by genes of the host organism, and are usually present at all times in the blood, body fluids or tissues of the host, throughout its life-span. For adaptive immunity, however, new recognition proteins are created in response to a challenge by an invading microorganism, by rearrangement and mutation of genes in specialized cells (lymphocytes) of the host. This is called somatic mutation and creates antibodies (B cell receptors) and T cell receptors.

In this review, we will discuss only the innate immune system, which has evolved generalized mechanisms to recognize, on the surface of targets, repetitive patterns of chemical groupings, which are not present in healthy host cells [1]. In this way, the innate immune system can distinguish between "self" and foreign or altered-host materials. However, since many synthetic materials are polymer-based and have repetitive surface chemical groupings, the innate immune system can recognize these also. Carbon nanotubes, as discussed below, are one of many synthetic polymers recognized by vertebrate innate immune systems [2, 3].

Innate immune system components include:

1. Soluble proteins (in the blood or other body fluids). The complement system and collectins are the major protein systems involved.
2. Cells, of many types but mainly phagocytes (macrophages and neutrophils in the body fluids or in tissues), with their repertoire of surface receptors. The main receptor types involved in innate immune recognition are the toll-like receptors (TLRs), scavenger receptors, integrins, and sugar-binding receptors (lectins), such as the mannose receptor or dectin.

A common feature of recognition of targets by innate immune system components is "multiple low-affinity binding" [4]. In such binding, a site on the recognition protein binds weakly to (recognizes) a molecular motif, such as a small charge cluster (2 or 3 negative charges), a single neutral sugar (monosaccharide),

vicinal hydroxyl groups (vicinal diols), or single acetyl groups. This one-to-one weak interaction is not enough to hold the target and recognition protein together, nor is it enough to confer specificity (distinction of self from non-self). These problems are overcome by polymerization or multivalency, so that the recognition protein takes the form of a polymer, presenting multiple binding sites, which can recognize multiple similar or identical motifs on a target surface. The multiple binding gives high avidity, strong interaction, and the geometric spacing of the motifs recognized confers specificity. Alternatively, the recognition protein may not polymerize, but may be present in hundreds of copies on a cell surface, which can engage simultaneously hundreds of motifs on the target surface.

2 Complement

The complement system is a group of over 40 soluble and cell surface proteins which interact together to recognize and opsonize foreign and altered-self materials [4–6]. Complement system recognition proteins bind to the target and trigger activation of proteases (see Fig. 1) [7, 8]. Activation occurs by any of three different pathways (classical, alternative or lectin) each of which recognizes a different spectrum of targets**.** In the classical pathway, the recognition protein C1q binds to charge clusters or hydrophobic patches on targets. When it binds, two

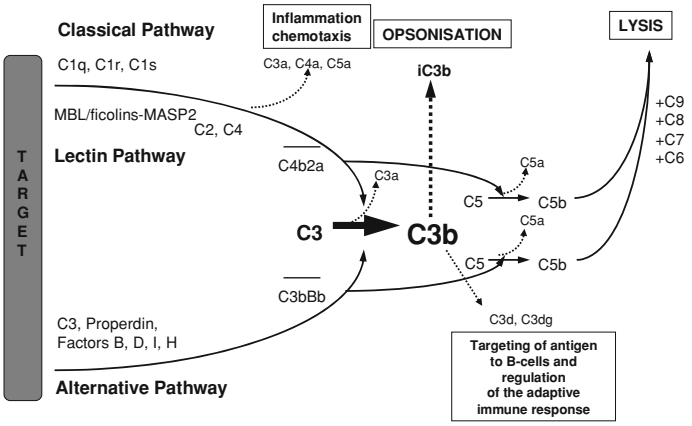

Fig. 1 The complement system. As discussed in the text, complement proteins C1q, MBL or ficolins recognize and bind to "foreign" materials, activate proteases including C1r, C1s and MASP2, which in turn activate the protease C4b2a, which cleaves C3. Many molecules of C3b bind covalently to the target, and mediate opsonization (mostly via a breakdown product iC3b) or interaction with the adaptive immune response (via the breakdown product C3d or C3dg). Fixation of C3b onto C4b2a also allows C5 to be activated, which leads to the formation of a C5b, 6, 7, 8, 9 complex [the membrane attack complex (MAC)] which can lyse cellular targets. The alternative pathway has a more complex initiation procedure, but goes through similar steps up to the activation of C3

proteases (C1r and C1s) which are bound to C1q are activated, and C1s in turn activates the complement proteins C4 and C2. They form a complex protease, called C4b2a, which activates C3, the most abundant complement protein. Activated C3 (called C3b) binds covalently to the surface of the target, and when hundreds of copies of C3b are bound, the target is opsonized. Phagocytic cells have surface receptors for C3b (and a further breakdown product iC3b) and so the C3b/iC3b-coated target binds strongly to the phagocyte and is ingested, then digested in the lysosomes of the cell. During C3 activation, one C3b will bind covalently onto C4b2a, forming C4b2a3b, which binds and activates the next complement protein, C5. Activated C5 (C5b) forms a large protein complex C5b–C6–C7–C8–C9 (C5b–9), the membrane attack complex (MAC), which can insert into lipid bilayers and will lyse targets which have a lipid bilayer.

In the lectin pathway, the recognition proteins are MBL (mannose-binding lectin) or the ficolins, of which there are three (L-, H- and M-ficolin) [9]. MBL binds to vicinal diols on sugars, such as mannose, fucose or glucosamine. The target recognition specificity of the ficolins is not well established, but L-ficolin will bind to many acetylated species, such as *N*-acetyl galactosamine or *N*-acetyl glucosamine, and some sialic acids [10]. L-Ficolin does bind several bacteria and parasites, such as *Mycobacterium tuberculosis* and *Trypanosoma cruzi* [11, 12].

When MBL or the ficolins bind to a target, a protease called MASP-2 (similar to C1s) is activated, and cleaves C4 and C2, starting off the same series of complement protein reactions as described for the classical pathway [8, 13].

C1q, MBL and the ficolins are all multimeric proteins [13–15], which bind to targets by multiple low-affinity binding. The general structure of these proteins is shown in Fig. 2. C1q is made up of three homologous polypeptide chains, A, B and C, each about 23 kDa. The N-terminal part of each chain has collagen-like amino acid sequence (repeating Gly-X-Yaa triplets, where X can be any amino

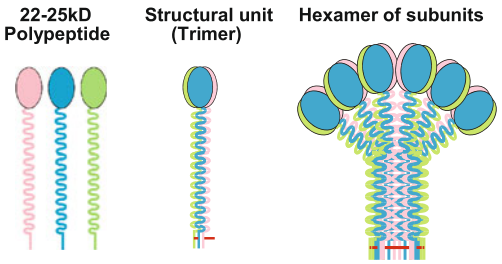

Fig. 2 The structure of C1q. Three homologous polypeptide chains, A, B and C combine to form a trimeric subunit, which then polymerizes to form a six-headed "bunch-of tulips" shape. Each of the six globular heads contains three lobes, derived from the homologous A, B, C chains. Each of these lobes recognizes a different spectrum of targets (with some overlap). The structures of MBL, ficolins and SP-D are similar except that each is a homopolymer, i.e. contains only 1 type of polypeptide. SP-A contains two types of polypeptide. MBL, the ficolins and SP-D assemble on average to a four-headed structure, not six-headed as for C1q and SP-A. In each protein, the polypeptides are made up of a globular region, and an extended collagen-like sequence. Figure provided by Mayumi Bradley

acid, and Yaa is often proline or hydroxyproline). The C-terminal part is a globular domain, which binds to targets mainly via charge interactions. These chains associate together to form a trimeric subunit, and the collagenous regions form a collagen triple helix. The subunits then polymerize, with some disulphide bridging between them, to form a hexamer of subunits.

Each globular region can bind to a target. The final hexameric form of C1q has 18 globular domains (three in each "head") so it can bind to a target by up to 18 interactions. MBL has a similar structure, but it has only one type of polypeptide chain, which assembles in trimeric subunits. The polymerization of MBL is variable, however, and it forms mostly tetramers of subunits, although dimers, trimers, pentamers and hexamers also occur. Each MBL polypeptide has one globular domain (called a C-type lectin domain) through which it can bind one sugar (e.g. Mannose), in a Ca^{++}-dependent interaction. Each subunit therefore has three C-type lectin domains, and the fully assembled molecule has 12–18 lectin domains.

Each ficolin, similar to MBL, has only one type of polypeptide, and assembles mainly to form tetramers of subunits: so each ficolin molecule can bind to a target by 12 sites. The binding domain of ficolins is called a fibrinogen (fbg) domain. The fbg domains of H, L and M ficolins are homologous, but not identical, and each recognizes a different spectrum of targets [10, 16].

The alternative pathway does not have any recognition molecule of the type represented by C1q, MBL or ficolins. A hypothesis which is both old (1950s) and new (2007) suggests that another type of multimeric protein, properdin, may initiate alternative pathway activation [17]. Properdin binds to charge clusters. Properdin has a single type of polypeptide chain, about 50 kDa, which is made up of six similar domains (called TSRs, or thrombospondin domains) [18]. These chains form variable oligomers, from dimers to tetramers. The tetramers may have four charge-cluster binding sites, or may have a multiple of four, since it is not known whether more than one of the TSRs in each chain can participate in binding. Once properdin binds to a target, C3b, formed either from classical or lectin pathway activation, or from another low rate spontaneous activation process, binds to the properdin. Factor B then binds to the C3b, and is cleaved by the protease Factor D to form C3bBb. This is homologous to the classical pathway C3-cleaving protease, C4b2a, and it activates more C3b, mediating the same C3, C5 turnover and MAC assembly as for the classical pathway (Fig. 1). The alternative pathway C3-activating enzyme is C3bBb: it contains C3b, the activation product of C3. This is a huge amplification mechanism. The enzyme makes C3b, which then binds Factor B, and makes more enzyme, C3bBb. Whenever some C3b is made by the classical or lectin pathways, it will trigger alternative pathway activation, and amplify turnover of C3. However the alternative pathway can be activated independently of the other two pathways.

C3 turnover is controlled by Factor H, an important down-regulator of complement activation: Factor H binds to C3b and competes out the binding of Factor B, preventing formation of C3bBb. Once a C3b–FH complex has formed, factor I, a protease cleaves C3b to a form called iC3b, which does not participate in forming C3bBb.

3 Collectins

The collectins are a small family of proteins which are collagenous lectins [19, 20]. MBL, discussed above, is a collectin. However there are several other collectins which do not activate the complement system. In humans, the other important collectins are two proteins found mainly in lung, called surfactant protein A (SP-A) and surfactant protein D (SP-D). SP-A and SP-D have a multimeric structure similar to C1q and MBL (Fig. 2) and are present in lung surfactant, the thin aqueous and lipid layer which forms the interface between inhaled air and the cells of the lungs. When we inhale, we take in enormous numbers of particles, including inorganic and organic dust, viruses, bacteria, fungal spores, allergenic particles, etc. Most of these particles become trapped in mucus and are moved back up the respiratory tract by the movement of cilia in the trachea. Once they have been moved back up from the lungs, they can be spat out or swallowed. Very small particles however, penetrate to the alveoli, and there they can cause damage, or establish infection, unless they are destroyed by alveolar macrophage. Although SP-A and SP-D have many roles, a major role is to bind to invading particles and then to promote their binding to alveolar macrophages. Their main mode of binding to targets is to recognize vicinal diols on sugar residues on bacterial, viral, fungal surfaces. This is a calcium ion-dependent interaction, in which a Ca^{++} ion, bound to the lectin domain of SP-A or SP-D, interacts with the diols. Once a particle has become coated with multiple SP-A or SP-D molecules, these will interact, via their collagenous regions, with receptors on alveolar macrophage.

Any small particle (including of course nanoparticles) can be inhaled, and so it is important to know whether these interact with the innate immune proteins of the lung.

4 Cells and their Receptors

The cells of the innate immune system include all of the white blood cell types (except most lymphocytes, which are part of the adaptive immune system), red blood cells, macrophages and dendritic cells in all the tissues, and several other groups of cells, such as mast cells. These cells have receptors through which they can recognize and bind many "foreign" organisms, such as bacteria, viruses and fungi. Interaction with these receptors may result in adhesion, phagocytosis and destruction of the foreign particle, or may signal to the cell to cause it to secrete cytokines or chemokines. Receptor types involved include TLRs, scavenger receptors, integrins, lectin-like receptors (which bind to sugars) such as mannose receptor or dectin. The cells also have receptors which allow them to recognize particles which already have complement proteins or antibodies bound to them (complement receptors or, for antibodies, Fc or immunoglobulin receptors).

When a particle has been recognized by the complement system, it will activate complement and will have molecules of C1q (or MBL or ficolins) and many molecules of C3b bound to it. Once C3b has bound, it is gradually broken down by proteases to forms called iC3b and C3d. Each of these interacts with cellular receptors. A C3b-coated particle in the blood will bind mainly to red blood cells, which have a receptor (complement receptor 1 (CR1) which binds C3b (see, e.g. [21]). As the particle circulates, bound to red blood cells, the C3b will gradually be converted to iC3b, which binds only weakly to CR1, but binds strongly to CR3 and CR4 (complement receptors 3 and 4) which are found on phagocytic cells. When the red blood cells pass through the liver and spleen, where there are many macrophages, the particles, now coated mainly with iC3b, will transfer to phagocytic cells and be ingested and destroyed. If the particle is in circulation for longer, iC3b will eventually be broken down to C3d, which binds to a receptor called CR2. CR2 is not present on phagocytic cells, but is most abundant on B lymphocytes.

Receptors also exist for C1q, MBL, the ficolins and the lung collectins, SP-A and SP-D, so that these proteins, when bound to a target, may also promote adhesion of the target to cells. Generally however, this effect would be weaker than for C3b or iC3b, because fewer molecules of C1q, MBL or ficolins are bound to targets. Adhesion requires interactions between hundreds of receptor-ligand pairs, so C3b or iC3b, which can be fixed to the target in clusters of hundreds of molecules [22], are more effective. A receptor which will bind all of these collagenous proteins, C1q, MBL, ficolins, SP-A and SP-D has been identified. It is calreticulin, bound to cell surfaces via CD91 [14, 15, 23, 24].

5 Interaction of CNTs with Plasma Proteins and Components of Innate Immunity

Plasma Proteins

If carbon nanotubes are to be considered as potential novel compatibile and non-toxic biomaterials, there is a need for more detailed investigation of the interaction between CNTs and the innate immune system. Contact with human blood or body fluids is inevitable in some steps of a drug delivery process, so a deeper understanding of the interactions between CNTs and blood proteins in general is also essential.

Among hundreds of proteins present in human plasma or serum, only a few bind spontaneously to unmodified (“pristine”) CNTs and so the binding is highly selective. This is not unexpected, as most homogeneous wettable surfaces, such as metal catalysts, silica-based materials and various organic polymers show highly selective binding of plasma proteins. The very abundant human plasma proteins, such as human serum albumin, fibrinogen, and high-density lipoprotein (HDL,

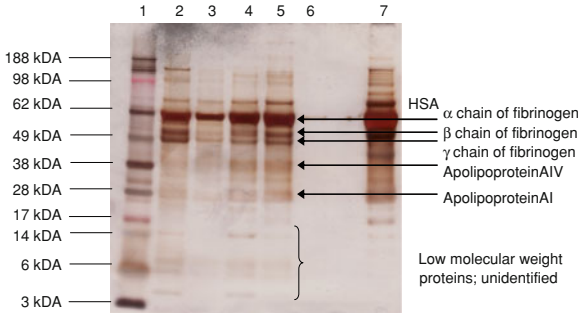

Fig. 3 Human plasma proteins bind selectively to DWCNTs. Samples of CNTs incubated with human plasma and washed were analyzed by SDS-PAGE in reduced conditions. *Lane 1* Molecular weight marker, *lane 2* human plasma proteins bound to oxidized DWCNTs, *lane 3* human plasma proteins bound to less oxidized DWCNTs, *lane 4* human plasma proteins bound to non-functionalized DWCNTs (1st batch), *lane 5* human plasma proteins bound to non-functionalized DWCNTs (2nd batch), *lane 6* control–human plasma proteins bound to Sepharose, used as a carrier for DWCNTs during incubation, *lane 7* human plasma. α chain of fibrinogen co-runs with HSA. Protein bands were stained using a BioRad Silverstain Kit. The method used is similar to that in [3]

which contains lipid and apolipoproteins AIV, AI, CIII) bind to CNT to the greatest extent [2, 3]. Other very abundant proteins, such as IgG, IgM, alpha-2 macroglobulin, bind negligibly or not at all (Fig. 3).

In various experimental procedures, CNTs are often pre-coated with proteins such as bovine serum albumin (BSA) in order to form stable dispersions in biological buffers. When such BSA-coated CNTs are added to human serum or plasma, the BSA is gradually displaced by human proteins, with a half-life of about 0.5–1 h at 37°C (K.M. Pondman, unpublished data). There are several different modes of binding of proteins to pristine CNTs, and these have not yet been very extensively explored. Serum albumin (human or bovine), for example, bind independently of fibrinogen, so the two types of protein are not competing for the same type of binding site. Precoating of CNTs with either serum albumin or fibrinogen before exposure to human serum or plasma does not prevent the binding of the complement protein C1q, and subsequent complement activation, and so C1q is recognizing a third type of binding site on CNTs (K.M. Pondman, unpublished data).

Complement

Our studies show that non-functionalized single-walled and double-walled carbon nanotubes (SWCNTs and DWCNTs), when placed in contact with human serum, activate complement via the classical pathway, or to a lesser extent through the alternative pathway [3]. Studies with four types of covalently modified multi-

Table 1 Biological activators of complement, which act by binding C1q

Gram-negative bacteria via lipid A of lipopolysaccharides
Gram-positive bacteria via lipoteichoic acids
Viruses, including Moloney, vesicular stomatitis, HTLV-1, HIV-1, DNA polyoma
Polyanions:
Heparin and chondroitin sulfate
Single- and double-stranded DNA and other polynucleotides
Anionic phospholipids in vesicles or on apoptotic cells
Other target-bound proteins:
Ligand-bound pentraxins (C-reactive protein and serum amyloid P-component)
Immunoglobulins: Fc portion of antigen-bound IgM, IgG
Altered-host proteins: amyloids, fibrin clots, prion aggregates

walled carbon nanotubes (MWCNTs) [25] showed that the extent of complement activation can be increased or decreased by altering the surface properties of the CNTs [26]. For example, MWCNTs functionalized with alanine or ε-caprolactam showed >75 and >95% decrease in classical pathway activation when compared with pristine MWCNTs [26]. Surface alterations, which diminish classical pathway activation, however, do not necessarily diminish alternative pathway activation, so both pathways must be studied separately. For classical pathway activation, the extent of activation was proportional to C1q binding, whereas for the alternative pathway, extent of activation was inversely correlated with binding of factor H, a protein which downregulates activation [26].

Direct binding of C1q and factor H to CNTs has been shown. These proteins bind in much lower quantity than do serum albumin, fibrinogen or HDL. For biological targets, C1q binds generally by ionic interactions (Table 1) with rearrangement to stronger hydrophobic interaction [27]. C1q also binds to many synthetic materials (Table 2).

Chemical modifications of polymers and other types of nanoparticles (mainly liposomes) show that coating with heparin or high-density polyethylene oxide (PEO) or polystyrene sulphonic acid or polyethylene glycol (PEG) tends to diminish activation. This type of modification has not yet been tested extensively for CNTs, but Moghimi and colleagues have shown that PEGylated SWCNTs do still activate complement, not via C1q, but possibly by the binding of L-ficolin [28, 29].

Table 2 Synthetics and non-biologicals, which activate complement via C1q (a small selection from recent papers)

SiO_2
Polyvinylpyrrolidone-coated nanoparticles
Polyvinylchloride
Polymethylmethacrylates
Polystyrene nanoparticles
Polyacrylonitrile
Carbon nanotubes

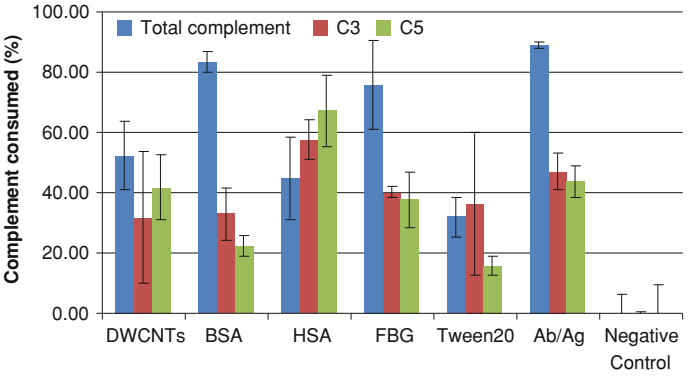

Fig. 4 Complement consumption by DWCNTs pre-coated with different proteins. Whole complement consumption and C3 and C5 consumption were determined in human serum incubated with CNTs. CNTs with no pre-coating (CNTs), and CNTs pre-coated with bovine serum albumin (BSA), human serum albumin (HSA), human fibrinogen (FBG), or the detergent Tween 20 were used. Ab/Ag is the positive control, representing complement activation by antibody-antigen complexes in the absence of CNTs. The negative control is serum incubated with no complement activator. The method used is similar to that in [3]

C1q binding alone does not guarantee that the whole complement system is activated, or that the nanotube becomes coated in C3b. Once C1q has bound, C1r and C1s are activated, followed by C4 and C2. For the next step, C4b2a has to be anchored to the surface of the nanotube. For biological targets which activate complement, the common mechanism for the binding of C4b (and also C3b) is covalent binding. This occurs by reaction of an internal thiolester in these proteins with surface OH, NH_2 or SH groups on the target. A much less common mechanism is hydrophobic adsorption. Pristine CNTs have no such reactive groups, but in serum, they adsorb other proteins, which could provide the necessary binding sites. Preliminary experiments (K.M. Pondman, unpublished) confirm that C4b and C3b do become bound to CNTs during complement activation, but there was no evidence that they bind covalently to other proteins, and so the binding is likely to be mostly by direct hydrophobic binding to the CNT surface. This is not entirely consistent with the finding mentioned above, that pre-coating CNTs with albumin or fibrinogen does not greatly alter total complement system activation (Fig. 4), but it does alter C3 and C5 consumption to a greater extent.

By whichever pathway CNTs activate complement, the MAC is the final product of the enzymatic and non-enzymatic protein cascade for each pathway. A significant rise in MAC levels after interaction between PEGylated SWCNT and undiluted human serum has been reported, indicating that complement activation proceeds through the whole complement cascade [28].

Nucleic acid wrapping or coating of the surface with surfactants, or other polymers can have a considerable impact on CNT interaction with human blood proteins, including complement. As shown in Fig. 4, pre-coating with Tween 20 diminishes overall complement activation. Ideally, the coating of CNT should be

stable in contact with blood or body fluids. Although it is very important to make CNT suspensions stable in conditions similar to physiological and make them more biocompatible by changing their surface properties, the non-covalent binding of proteins to DWCNTs is not stable over time and the proteins dissociate from the CNT surface to be replaced by other plasma proteins. Chemical modification of CNTs, either covalent or non-covalent, can significantly change the extent or perhaps the mechanisms by which CNTs interact with the innate immune system. Impurities present on the surface of CNTs, such as traces of Mo, Co or Fe, etc., may also influence the interaction of CNTs with blood proteins and lead to biological reactions, including reactive oxygen species (ROS) generation.

The chemical composition of CNT surface, size and the presence of catalytic impurities can affect the way complement is activated and in consequence cause possible harmful effect for cells or cell tissues. Activation of complement by both unmodified and chemically modified DWCNTs leads to the generation of peptides, C3a, C4a and C5a which can cause inflammation, which is strongly undesirable if the CNTs are to be used as biomaterials in the human body.

Regarding interaction between carbon nanotubes and complement in vivo, very little has been reported so far. To name one example, Hamad and co-workers have reported that PEGylated nanotubes can induce complement activation in rats, which is C4 dependent [28].

Although in vitro complement activation assays, including hemolytic assays and direct protein binding studies, are very valuable methods to determine whether CNTs interact with complement and in consequence cause complement activation in human serum or plasma, further investigation is needed to show whether the binding of complement proteins (mainly C3b, iC3b) does really influence the fate of CNT products placed in contact with the blood or other body fluids or influence the cellular internalization of CNTs by phagocytes and their cellular accumulation Further in vivo studies could provide more information about the interactions of CNTs with complement and the potential outcomes that may arise.

Collectins

Inhaled carbon nanotubes can be treated as ‘foreign’ material entering the lungs and therefore show potential pulmonary toxicity. The interaction of DWCNTs with collectins was recently investigated for the first time [30]. The lung proteins SP-A and SP-D selectively bind to DWCNTs and transmission electron microscopy revealed that the binding occurs through their heads [30]. This binding, unlike that of C1q or of serum albumin, fibrinogen or HDL, was found to be Ca^{++}-ion dependent. [30]. With biological targets, SP-A and SP-D bind to surface sugars by a Ca^{++}-dependent coordination of vicinal diols (adjacent hydroxyl groups). It was shown that SP-A and SP-D bind to oxidized CNT, and hypothesized that some binding to CNT which have not been deliberately oxidized is due to inadvertent oxidation during synthesis and purification. The presence of oxygen-containing

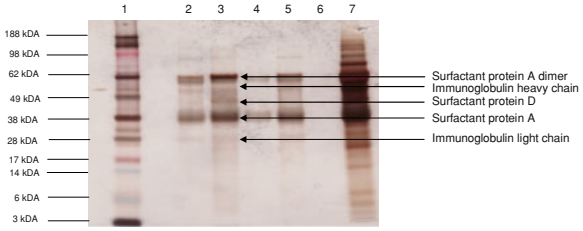

Fig. 5 Selective binding of BALF proteins to different DWCNTs. BALF (bronchoalveolar lavage fluid) was passed through Sepharose and Sepharose-CNT columns as in [26]. After exhaustive washing in the running buffer, samples were analyzed by SDS-PAGE in reduced conditions. *Lane 1* molecular weight marker, *lane 2* BALF proteins bound to less oxidized DWCNTs, *lane 3* BALF proteins bound to oxidized DWCNTs, *lane 4* BALF proteins bound to non-functionalized DWCNTs (1st batch), *lane 5* BALF proteins bound to non-functionalized DWCNTs (2nd batch), *lane 6* Control–BALF proteins bound to Sepharose, used as a carrier for DWCNTs during incubation, *lane* 7. BALF. Protein bands were stained using a BioRad Silverstain Kit

functional groups on the carbon nanotube surface is an important factor in this [31]. Figure 5 represents the selective binding of lung surfactant proteins to different chemically modified CNTs.

SP-A and especially SP-D are present in very low quantities, even in the lung, which is their major site of localization. If a relatively large quantity of well-dispersed CNT was inhaled, a proportion of these might escape entrapment in mucus, and reach the lower airways and alveoli. Here they might sequester a large proportion of the available SP-A and SP-D, leaving the lungs temporarily susceptible to infection. Chronic exposure to lower quantities of airborne CNTs would have similar effects. These events are relatively unlikely, but point to the need for good control of airborne particulates where CNTs are manufactured on industrial scale. Binding of these proteins to CNTs would be expected to promote their adhesion to alveolar macrophages, and their ingestion, if they are not too long or too large (aggregated). CNTs which cannot be ingested may cluster macrophages around them, with possible eventual formation of granuloma [30].

SP-A and SP-D have functions other than promoting adhesion to macrophage, and binding of these proteins may contribute to lung inflammation by other routes. C. Salvador-Morales (unpublished work) has shown that SP-A coated MWCNTs induced an inflammatory response in IFN γ-primed RAW cells (a mouse macrophage cell line), as assessed by MWCNT uptake and measurement of the generation of nitric oxide.

Cells and Cellular Receptors

There have not yet been any direct studies of the interaction of CNTs with the types of innate immune system receptors discussed above. However, in general

terms, using CNTs as potentially efficient drug or protein vehicles involves understanding of interaction between CNTs and cells and cell surfaces. The reported fates of CNTs in cells and their localization in cellular organelles varies widely. Determining their potential cytotoxicity and route of safe excretion from the cells is important. Drug-targeting involves the design of CNTs with specific ligand molecules present on the CNT surface which can be recognized by cellular receptors. Uptake of CNTs into cells is critically dependent on their length and degree of bundling (aggregation). Many studies of CNT uptake into cells do not give any information on the size distribution of the particles used in the experiments, and quite major differences in findings can often be attributed to the lack of characterization of the length distribution and dispersion of the CNTs. The uptake of DNA-wrapped SWCNTs, for example, is size and length dependent and endocytosis rate depends on tube bundle diameter [32].

CNTs can be taken up by many cells (primary and established lines), among them human monocyte derived macrophage (HMM) cells, which play a significant role in the body's immune response [33], T cells (Jurkat), Chinese hamster ovary cells (CHO), 3T3 fibroblasts [34], HeLa cells [35], H596, H446 and Calu-1 lung tumor cells [36], bacterial, fungal and yeast cells [37]. Unmodified CNTs can be toxic for many cell lines, including HEK 293 cells [38], Haca T cells [39] and alveolar macrophages [40] and induce cell death. SWCNTs reduce the amount of glial cells in both peripheral and central nervous system derived cultures [41], but the interaction of CNTs with the nervous system remains poorly understood. No uptake of SWCNTs was reported in A549 cells, BEAS-2B and RAW 264.7 (a mouse peritoneal macrophage cell line) [42]. CNTs, in contrast to graphite, can be highly adhesive to human osteoblast-like cells (Saos2) [43].

Certain functionalizations of CNT surfaces can not only reduce their toxicity, but also eliminate non-specific uptake by the cells and promote their prolonged circulation in the blood. PEG is frequently used for such surface modifications. The coating of SWCNTs by PEG adsorbed to their surface blocked their uptake by the ovarian cancer cell line SKOV-3 [44]. SWCNTs functionalized by PEG can be additionally functionalized for interaction with cell receptors. SWCNTs were functionalized by folate and EGF specifically to target FRα and EGFR receptors expressed on cancer cells [44]. Similar results were obtained with the ovarian cancer cell line OVCA 433 [44]. SWCNTs non-covalently functionalized with chitosan, linked with folate and fluorescently labeled were taken up by Hep G2 cells via a folate receptor-mediated pathway that is concentration-dependent [45]. When the folate receptors on the surface of the Hep G2 cell membrane were blocked, no internalization of the SWCNT was observed [45].

Modification of the surface chemistry of CNTs can change the interaction of CNTs with lipid bilayers and thereby influence the uptake into the cell. The compatibility of the CNT surface with biological components or proteins situated on the cell surface can play a key role in cell–CNT interactions. The CNT surface is intrinsically hydrophobic and this can lead to non-specific adsorption to the cell surface. To avoid this, the surface modification of CNTs can be considered as an effective method of eliminating unwanted non-specific cell adsorption. Mucins are

present on the surface of many cell lines and present epitopes for receptor-mediated cell–cell recognition. Glycosylated polymers on the CNT surface can mimic mucins. A coating which introduces α-GalNac residues onto the CNT surface, can be recognized by a specific receptor present on the cells [46].

SWCNTs have the ability to penetrate mammalian cells and serve as transporters for many different proteins. Among those which have been tested are streptavidin, protein A, bovine serum albumin and cytochrome *c* [47, 48]. Endocytosis was confirmed as the internalization mechanism for fluorescein-labeled proteins attached to SWCNTs. The SWCNT-protein conjugates were colocalized with endocytosis markers for endosomes, lysosomes and cytoplasm [48]. The mechanism of uptake of CNT can be either energy-dependent or energy-independent. In the majority of cases endocytosis is involved, but uptake is also possible under endocytosis-inhibiting conditions [37] or by incomplete phagocytosis [49]. After being internalized by cells, CNT can be located in different cellular organelles, such as cytoplasm, endosomes, lysosomes, Golgi apparatus or mitochondria. TEM studies by Kang et al. [45] revealed that functionalized SWCNTs are bound to the cellular membrane and then transfer to endosomes and lysosomes. The SWCNTs caused lysosomal damage and a concentration-dependent apoptosis. Generally CNTs are not localized in the nucleus, but functionalized SWCNT can be found in the perinuclear region [33, 37].

Macrophages play a central role in clearing particles from the alveolae of the lung. Currently little is known about the fate of MWCNTs in macrophages and the likelihood of being cleared from the lung via macrophages [49]. It might be expected that a proportion of CNTs ingested by lung macrophage will be moved up the airways by ciliary transport and then spat out or swallowed. Gold-labeled SWCNTs in the lungs can escape phagocytosis and migrate into the alveolar septae [42].

The interaction of CNTs with cells is very complex and dependent on many factors, such as the chemical composition of CNTs, their surface charge, length, dispersion, diameter, type of cells and the presence of cell receptors. Tip and side wall functionalization of CNTs may be important in cell or tissue engineering [50, 51]. Magnetically drivable CNTs can be used as efficient drug delivery vehicles. These magnetic CNTs can enter cells and tissues in the presence of an appropriately oriented magnetic field [52].

Being aware of the cellular uptake of CNTs can be very useful in designing new drug carriers. Additional information, like cytotoxicity, efficacy, cellular localization and the fate of CNTs inside the cell are also valuable.

6 Potential Toxicity and Immunotoxicity of CNT

In Sect. 5 the interaction of various CNTs with the complement system was described. Such interactions may or may not be desirable, depending on the intended function or targeting of the CNTs. Activation of complement may have

potential harmful effects (inflammation) on human tissues. Complement activation is not the only criterion in preclinical safety assessment of biocompatibility. Interaction of a potential drug candidate with blood cells, platelets and the coagulation system must also be considered [53]. For example, PEGylated liposomes were reported by Dobrovolskaia et al. [53] to activate complement, but were approved as a drug called 'Doxil', which can still be used in treatment of metastatic ovarian cancer. It is an aspiration that nanotechnology-based drugs will have an advantage over conventional drugs, as they can be designed to cause fewer adverse reactions [53]. However, supposedly CNTs can cause endothelial dysfunction, have effects on blood clotting and suppress immune responses and this may result in pathophysiological responses, like stroke, thrombosis, autoimmunity or artherogenesis. Nonetheless, the experimental evidence to support these potential side effects is still unsatisfactory [54].

Airborne CNTs can be simply inhaled into the lung and this organ is likely the most important target for potential CNT toxicity. Lungs are composed of more than 40 different cell types and therefore their interactions with CNTs can be quite complex. In vitro studies on triple cell cultures consisting of A549 human epithelial lung cells, human monocyte-derived macrophages and monocyte-derived dendritic cells were carried out by Müller et al. [55] and potential for oxidation stress and inflammation were observed. As shown by Simon et al. [56], MWCNTs were cytotoxic for A549, HepG2 and NRK-52E cell lines being models for lung, liver and kidney cells, respectively.

There are already many reviews discussing toxicity, safety concerns and potential dangers of CNT exposure to human lungs and other organs [57–62]. The impact of various types of CNTs on the lung has attracted substantial attention, but still relatively little is known about the potential toxicity of CNTs to other organs of the human body. Although CNTs can have asbestos-like structure and therefore be involved in lung toxicity, they probably do not exist in the air in the fibrous asbestos-like form [63].

When inhaled, non-aggregated CNTs can penetrate the aveoli and interact with lung surfactant proteins. As presented in Sect. 5, CNTs can bind SP-A and SP-D. Pulmonary surfactant proteins, SP-A and SP-D, are the main protein constituents of lung surfactant and are involved in innate immunity [19, 20]. The absence of SP-A and SP-D in knockout mice caused infection and emphysema in their lungs. SP-A and SP-D do not activate the complement system in contrast to mannan-binding lectin (MBL), another collectin involved in immune defence. SP-A can modify the inflammatory response in vivo by enhancing macrophage phagocytosis and clearance of bacteria. Clearance of apoptotic cells in lung inflammation is a principal role of SP-A and especially of SP-D [19, 20] .

Interactions of CNT with collectins can affect the physiological functions of these proteins and could potentially lead to bronchial inflammation or increased susceptibility to lung infection or allergy, therefore it is necessary to understand the mechanism involved in the interactions. As shown by Salvador-Morales et al. [30, 31], no binding of SP-A and SP-D from BALF to DWCNTs occurred in the presence of EDTA, a commonly used divalent cation chelator. The binding of

SP-A and SP-D to CNTs was dependent on the presence of ketone, aldehyde, ester or carboxylic acid functional groups on CNT surface.

In in vivo studies of aspiration of MWCNTs suspended in phosphate buffer into mouse lungs, increased secretion of mucous and of SP-D was observed, with indications of systemic oxidative stress. These MWCNTs were not oxidized so probably did not bind SP-D [64, 65].

In further in vivo studies, Mitchell et al. [66] showed that inhalation of MWCNTs by C57BL/6 male mice affected systemic immunity, but did not cause lung damage. Studies using a natural killer cell assay showed that the innate immune response was suppressed by inhalation of MWCNTs [66]. Increased expression of an indicator of oxidative stress, NQO1, and an indicator of altered immunity, IL-10 were not observed in lungs, but were seen in spleen. IL-10 is one of anti-inflammatory cytokines and plays an important role in maintaining homeostatic control of innate and immune responses mediated by cells. IL-10 is secreted by macrophages and T cells [66]. Its expression can suppress normal immune responses and increase receptiveness to infection. The immune function responses observed in the spleen have not been evaluated in other parts of the body like the lymph nodes or immune cells in the lungs. The immune response to inhaled MWCNTs was time- and dose-dependent and was consistently observed even in the presence of low concentrations of MWCNTs [66].

A mechanism by which inhaled MWCNTs induce systemic immune suppression was proposed by Mitchell et al. [67]. TGFβ secretion in the lung can be activated by MWCNTs and have an effect on prostaglandin production in spleen cells, leading to the immune suppression and affect the function of T cells [67].

CNTs can be internalized by macrophages and cleared by the lymphatic system if they are short and tangled [68]. However, typically they are long and their prolonged accumulation in the lung (months or even years) can arise from incomplete phagocytosis [68]. The internalization of SWCNTs in macrophages can be responsible for activation of various transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1) followed by induction of oxidative stress, release of pro-inflammatory cytokines, leukocytes, gene expression and activation of T cells [69]. This can cause innate and adaptive immune responses including chronic pulmonary inflammation and granuloma formation. Histopathological studies by Warheit [70] revealed that pulmonary exposure to SWCNTs produced multifocal granuloma. Poland et al. [71] showed that long MWCNTs caused inflammatory changes to mouse lungs, further developing granuloma. The uptake of SWCNTs into macrophages induces oxidative stress in mitochondria. A remarkable increase in SOD2 (superoxide dismutase) expression in SWCNT-treated macrophages was observed by Chou et al. [69]. SWCNTs induced the expression of protective and antiapoptotic genes [69].

As shown by Jacobsen et al. [72], apolipoprotein E knockout (Apo $E^{-/-}$) mice developed lung inflammation when exposed to SWCNTs. Studies carried out by Elgrabli et al. [73] showed that MWCNTs can be present in rat lungs even 6 months after intratracheal instillation and therefore may cause pathological changes in lungs. Studies on female C57BI mice revealed that exposure to

MWCNTs can be more toxic than to ozone, which is known to cause pulmonary toxicity [64]. Pathological changes in the lungs of mice exposed to aerosolized MWCNTs were reported by Li et al. [74]. Exposure to SWCNTs caused lung inflammation and granuloma formation in rats that was lethal for several animals, as shown by Warheit et al. [75]. SWCNTs caused interstitial inflammation in mice and granuloma in contrast to the lungs of mice treated with carbon black as reported by Lam et al. [76]. Suppressed immune function was observed for mice inhaling low concentrations of MWCNTs [77]. Carbon nanomaterials, among them MWCNTs suspended in saline containing SDS, can induce pathological changes in guinea pig lung tissues on intratracheal exposure, as reported by Huczko et al. [78].

As discussed above, many studies performed on rodent models revealed CNT lung immunotoxicity, but some researchers report CNTs to be non-toxic. As reported by Qu et al. [79], carboxylated MWCNTs, well-suspended in PBS containing Tween-80, did not cause pathological changes in mouse lungs and liver and are more easily eliminated from the body as they do not form as many aggregates as less dispersed MWCNTs. However, the same MWCNTs caused mouse heart injuries, but no histopathological changes were observed in the brain [79].

No acute toxicity was observed by Pulskamp et al. [80] on macrophages (NR8383) and human A549 lung cells after SWCNT and MWCNT exposure. Purified CNTs had no effect on the formation of intracellular reactive oxygen species or the MMP potential of A549 cells, but a loss of mitochondrial functionality was reported. CNTs also affected the MMP potential in mitochondria in NR8383 cells [80].

As noted above, NF-κB and AP-1 are activated by the uptake of SWCNTs by macrophages [69]. This can cause oxidative stress and release of proinflammatory cytokines. NADPH oxidase activation in macrophages can cause oxidative stress as a result of superoxide anion radical $O_2^{\cdot-}$ and hydrogen peroxide H_2O_2 generation. In the presence of transition metals the most reactive hydroxyl radical $HO^{\cdot}$ can be produced from $O_2^{\cdot-}$ [54].

Generation of $HO^{\cdot}$ from H_2O_2 is common in physiological conditions, for instance during phagocytosis of particles by alveolar macrophages. It was reported by Fenoglio et al. [81] that purified MWCNTs in aqueous suspensions did not generate $HO^{\cdot}$ and carbon-centred radicals, like $CO_2^{\cdot-}$. It was found by Shvedova et al. [82] that SWCNTs did not generate $O_2^{\cdot-}$ or the nitric oxide radical $NO^{\cdot}$ in RAW 264.7 macrophages. No $O_2^{\cdot-}$ generation was reported when high purity SWCNTs (significantly reduced metal content) were exposed to human alveolar epithelial A549 cells, but unpurified SWCNT caused peroxynitrate $ONOO^-$ and $O_2^{\cdot-}$ production [83]. No significant changes in the level of $O_2^{\cdot-}$ generation were noted after treatment of peripheral blood mononuclear cells with CNTs [84]. ROS can be generated during phagocytosis of foreign particles by the activation of the NADPH oxidase system, which catalyses the conversion of O_2 to $O_2^{\cdot-}$. CNTs can stimulate the release of TNF-α and ROS in in vitro conditions. After treatment of monocytic cells by CNTs 'frustrated phagocytosis' as a negative response to CNT was observed [84].

NADPH oxidase-derived ROS play a role in determining the route of the pulmonary response to SWCNTs [85]. Exposure of SWCNTs to NADPH oxidase-deficient C57BL/6 mice resulted in significantly higher levels of pro-inflammatory cytokines: TNF-α, IL-6 and MCP-1 compared to non-deficient control animals. The level the anti-inflammatory cytokine, TGF-β, was also shown to be significantly higher [85].

It appears that CNTs may not generate ROS if there are no catalyst impurities present. Purified MWCNTs have the ability to effectively scavenge HO·, generated by both Fenton reaction and photolysis of hydrogen peroxide H_2O_2. Superoxide anion $O_2^{\cdot-}$ can be generated by either physiological or pathophysiological processes. In the presence of MWCNTs the formation of $O_2^{\cdot-}$ was shown to decrease when detected spectrophotometrically by reduction of cytochrome *c* generated in reaction catalyzed by xanthine oxidase. Although the scavenging properties of CNTs have been observed, this is still poorly understood [81].

Regarding the toxicity of CNTs, currently relatively little is known about other organs, as most researchers carry out studies on lungs. Murray et al. used engineered skin murine epidermal cells (JB6 P+) exposed to unpurified SWCNTs containing 30% iron and this caused HO· formation and activation of AP-1, in contrast to SWCNTs containing much less iron (0.23%). Unpurified SWCNTs caused oxidative stress to immune-competent hairless SKH-1 mice [86]. Inflammatory cytokines IL-6, IL-10, TNF-α or IL-12p70, etc., production and collagen accumulation were also observed for the dermally exposed mice [86].

Observed discrepancies in reports of toxicity caused by CNTs can be a function of many factors, such as their physicochemical properties, including size, length, presence of metallic impurities on the surface, hydrophobicity or hydrophilicity of the surface, its smoothness and the degree of oxidation. Other factors along with the ability of CNTs to form long fibrous particles (like asbestos) and their surface reactivity leading to the potential generation of ROS, can considerably influence CNT toxicity. Slow clearance of long and rigid CNT in the respiratory tract and their aggregation due to van der Waals forces are potentially dangerous for the body.

The presence of structural defects on CNT surfaces can have an important impact on their observed toxicity in vitro and in vivo. There are many surface defects that can be present in nanotubes, among them topological [pentagons instead of hexagons, pentagon–heptagon (5/7) pairs in the hexagonal structure of CNTs], rehybridization (between sp^2 and sp^3 hybridization) or defects caused by incomplete bonding (vacancies) [87, 88]. The defects can change CNTs' electronic properties [89, 90]. It was reported by Muller et al. [91] that the presence of structural defects of CNTs plays an important role in MWCNT toxicity in in vivo studies.

Toxic responses have been attributed to metal contamination, CNT length, oxidation or hydrophilicity. To see how some of these properties relate to toxicity, Muller et al. [91] and Fenoglio et al. [92] took a preparation of MWCNTs and modified it by (a) grinding (introducing structural defects) and subsequently heating in a vacuum at 600°C (to reduce oxygenated carbon adducts and metallic

oxides) or in an inert gas at 2,400°C (causing elimination of metals and annealing of defects) or (b) by heating at 2,400°C in an inert gas and subsequently grinding (introducing defects in a metal-depleted carbon framework). Unlike some toxic materials, CNTs may quench oxygenated free radicals, not generate them. The capacity of the MWCNTs to scavenge hydroxyl radicals was evaluated by spin trapping. The original ground material exhibited a scavenging activity toward hydroxyl radicals, which was eliminated by heating but restored by grinding. The scavenging activity appeared to correlate with both defects and genotoxic and inflammatory activity of the MWCNTs.

7 Modification of Surface Properties to Enhance Biocompatibility

The exceptional physicochemical properties of nanoparticles make them of great interest for biomedical applications. Different classes of nanoparticles including liposomes, polymers, metallic nanoparticles, quantum dots, carbon nanomaterials, their potential toxicity, and their pharmacological applications have been reviewed by Medina et al. [93].

However, the same physicochemical properties which make nanoparticles so unique, sometimes can cause their failure in biomedical applications. The interaction of various nanoparticles, liposomes, micelles and CNTs with biological systems depends on particle size, surface properties, and surface charge. Additionally, the presence of defects on their surfaces, length, hydrophobicity and surface roughness can significantly diminish their utility in biomedical applications. Non-functionalized SWCNTs (>90% purity) and MWCNTs (>95% purity) were shown by Schrand et al. [94] to be less biocompatible than nanodiamonds in studies carried out on neuroblastoma and alveolar macrophage cell lines. Different degrees of hydrophobicity of various latex nanoparticles was shown to have an impact on the amount of protein adsorbed on their surface [95]. Lück et al. showed in vitro plasma protein adsorption was dependent on the surface properties of polymeric nanoparticles with and without covalently bound charged functional groups [e.g. NR_3^+ or $C(NH_2)_2^+$]. Such modified latex had different particle sizes, surface charge densities, electrophoretic mobilities and hydrophobicities. Different amounts of proteins adsorbed to latex with various functional groups on their surface [96]. Purification of CNTs plays an essential role in CNT biocompatibility. Chłopek et al. [97] reported good cellular biocompatibility of high purity MWCNTs on the level observed for polysulfone currently in medical use. The interaction of the MWCNTs with osteoblast cell cultures did not cause release of pro-inflammatory cytokine, IL-6, and did not initiate ROS formation.

There are many limitations for nanoparticles being used as biomedical devices or drug vehicles. Functionalization of CNTs can significantly improve their utility as long as the functionalization is not harmful for tissues by itself. Modification of nanoparticle surface properties can make them more valuable in potential

biomedical applications. Proteins, including antibodies and enzymes can be attached to the surface of CNTs and carbon nanofibres (CNF). Naguib et al. [98] reported that CNFs could be coated with monoclonal anti-CD3 antibodies. Poly (L-lysine) (PLL) enhanced the binding of proteins to CNTs. The more hydrophilic the surface of CNFs, the more protein could bind to them. Biocompatibility aspects of CNT interactions with neuronal cells, osteoblasts, fibroblasts, ion channels, cellular membranes, mono- and polyclonal antibodies and the immune system were reviewed by Smart et al. [99]. The impact of various chemical functionalizations of CNTs, including covalent modifications (1, 3-dipolar cycloaddition) and PEGylation on their biocompatibility has been reviewed [100]. Covalent binding of various biologically active molecules, including streptavidin and siRNA to different types of functionalized or non-functionalized CNTs was presented. Encapsulation of small molecules and DNA, RNA and fluorescently labeled tags was also discussed [100]. Yang et al. [101] showed that positively charged SWCNTs could be utilized to carry siRNA. These siRNA-SWCNT complexes could be used to modify of functions of dendritic cells.

As discussed above, chemical modification of CNTs can considerably alter the level of complement activation. Salvador-Morales et al. [3] showed that unmodified SWCNTs and DWCNTs activated complement via both classical and alternative pathways, which may initiate inflammation or granuloma formation. The same researchers showed that covalent modification of MWCNTs can considerably change their biochemical properties and therefore increase their biocompatibility in human blood plasma. Complement activation by classical and alternative pathways was tested on four chemically modified MWCNTs [26]. ε-Caprolactam-modified MWCNTs and L-alanine-modified MWCNTs showed significant reduction in activation of complement by the classical pathway in contrast to unmodified MWCNTs.

Studies carried out by Mooney et al. on COOH-functionalized SWCNTs and OH-functionalized MWCNTs showed that the COOH-functionalized SWCNTs were less toxic to human mesenchymal stem cells (hMSC). The localization of these SWCNTs in various cell compartments was also elucidated [102]. Biocompatibility tests were performed on fibroblast L929 mouse cells by Lobo et al. [103] for MWCNTs on titanium and silicon surfaces. Carrero-Sánchez [104] showed that MWCNTs doped with nitrogen were more tolerated by mice than pure MWCNTs.

Modification of CNTs with hydrophilic agents can influence CNT compatibility in vivo and in vitro. Coating of CNTs can modify their interaction with blood proteins and cells. Hemocompatibility studies of PEGylated alcohol/polysorbate nanoparticles (PEG-E78 NPs) and non-pegylated (E78 NPs) were performed by Koziara et al. [105]. Both types of nanoparticles had potential blood compatibility, did not activate platelets, did not cause blood cell lysis and did not have an effect on whole blood clotting time in the concentration range 0–250 μg/mL [105].

Kam et al. showed that SWCNTs functionalized by folic acid were internalized by folate receptor-overexpressing (FR^+) cells. The same SWCNTs were not internalized by normal cells without folate receptors. SWCNTs functionalized

with Cy3-labeled DNA were shown to transport DNA inside of HeLa cells. Such modified SWCNTs can potentially be used in selective targeting of cancer cells [106].

Nanoparticles can be considered as effective drug vehicles if they can deliver the drug to the site of action without causing harm to human tissues and organs. Improvement of delivery of drugs to tumors by increasing drug bioavailability and abrogating drug resistance are major purposes in targeted drug delivery. Polymer micelles with cross-linked ionic cores consist of hydrophilic nanospheres and porous ionic cores. Block ionomer complexes of poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) copolymer and Ca^{2+} cations were templates for these cross-linked polymeric micelles. The core of these cross-linked polymeric micelles is hydrophobic and consist of many functional groups which can be covalently attached to hydrophobic drugs, like cisplatin [107, 108]. Poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) [109], Pluronic P85-*b*-poly(acrylic acid) [110], F87/poly(acrylic acid) [111] and poly(ethylene oxide)-*block*-poly(*β*-benzyl-L-asparate) [112] block copolymers were studied as potential delivery systems for cationic drugs, like doxorubicin. Effective encapsulation of potential drugs in cores of micelles can protect them from premature release in the human body. Additionally, hydrophilic PEO chains can provide increased solubility and prevent interactions of plasma components with the drug encapsulated into the core of the micelles. Paclitaxel, camptothecin and other anticancer agents can be carried by micelles, polymer conjugates and liposomes and a few of them are currently in medical use. Different clinical stages of anticancer agent-incorporating micelles in oncology were reviewed by Matsumura et al. [113, 114].

The main limitation of nanoparticles in drug delivery is their rapid uptake by the mononuclear phagocyte system (MPS). The presence of PEG chains on the surface of nanoparticles can significantly change their interactions with blood proteins. Extensive studies on these aspects of biocompatibility have been carried out on nanoparticles other than CNTs, but the findings from this work are likely to be applicable to CNTs. Studies on various nanoparticles consisiting of poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) and poly(*ε*-caprolactone) (PCL) core and PEG coronas were carried out by Gref et al. [115] and showed the differences in uptake of these modified nanoparticles by polymorphonuclear cells (PMN), changes in plasma protein adsorption and zeta potentials.

The biological activity of pluronic block copolymers consisting of PEO and poly(propylene oxide) (PPO) blocks and pluronic micelles used in drug delivery was broadly reviewed by Batrakova et al. [116]. Vittaz et al. [117] showed very low complement consumption caused by poly(ethylene oxide)-poly(lactic acid) diblock copolymer (PLA-PEO) compared to poly(lactic acid) stabilized by pluronic® F68 (PLA-F68). PLA-PEO phagocytosis was significantly reduced compared to PLA-F68.

Physicochemical properties of liposomes, especially their charge, can significantly influence their interactions with blood proteins and protein adsorption patterns. Gel filtration was used by Diederichs et al. [118] for the separation of liposomes from plasma to examine bound proteins. Chonn et al. [119] showed that

liposome surface charge can determine the pathway by which complement is activated. Positively and negatively charged liposomes activated the alternative and classical pathways, respectively. Liposomes with no charge on their surface were shown not to activate the complement system in vitro.

A review by LaVan et al. [120] presents different types of nanoparticles among them liposomes, as having potential in vivo drug delivery. The authors present techniques that are useful to improve biocompatibility of potential nanoscale drug vehicles.

Prolonged circulation of nanoparticles in the bloodstream is crucial for drug delivery purposes (i.e. particles should not be cleared by the liver and spleen before reaching their target). Moghimi et al. [121] reviewed protein-binding processes to stealth nanoparticles, including the influence of surface PEGylation on complement activation and the fate of these particles.

Salvador-Morales et al. showed differences in complement activation by various surface modified lipid–polymer hybrid nanoparticles (NPs). The NPs with methoxyl surface groups and amine surface groups induced the lowest and highest, respectively, complement activation via the alternative pathway. The modification of surface properties of NPs results in different protein binding patterns. NPs consisting of a lipid monolayer between a hydrophilic polymeric shell and a hydrophobic polymeric core can be utilized as drug delivery vehicles or novel adjuvants for vaccination [122].

The modification of surface of a variety of types of nanoparticles can change their reactivity and therefore make them more biologically active. This can considerably enhance their chances to be used in pharmacology and biomedical fields.

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