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The winding road for carbon nanotubes in nanomedicine

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Carbon nanotubes (CNTs) are recognized as promising nanomaterials for technological advancement. However, the stigma of structural similarity with asbestos fibers has slowed down progress of CNTs in nanomedicine. Nevertheless, it also prompted thorough studies that have revealed that functionalized CNTs (*f*CNTs) can biologically behave in a very different and safer manner. Here we review pristine and *f*CNT fate in biological settings, focusing on the importance of protein interaction, formation of the protein corona, and modulation of immune response. The emerging consensus on the desirable *f*CNT properties to achieve immunological neutrality, and even biodegradation, shows great promise for CNT adoption in medicine.

Introduction

Carbon nanotubes (CNTs) are widely known as promising nanomaterials for the advancement of technology. CNTs come in different sizes and purity grades, but they all have in common the 'one-dimensional' character and unique electronic properties that have stimulated scientists' creativity over the past twenty years. CNTs have played a key role in the nanotechnology revolution, in fields ranging from materials and electronics to nanomedicine, with CNT applications in the first progressing at a much faster rate than in the latter. On the one hand, CNTs have already reached consumers as components of a variety of marketed products ranging from batteries to sporting goods [1], and with the expectation of the 'CNT computer' [2], they are to be used as an alternative to silicon in the next generation of nanoscale processors. On the other hand, although CNTs represent an important niche in the field of innovative nanomaterials for next-generation theranostic nanomedicines (i.e. having therapeutic and diagnostic functions combined) [3], no CNT pharmaceutical product for internal use is anywhere near the market. The only two clinical trials on CNTs started in 2011 and feature them solely as components of external medical devices for cancer diagnostics (i.e. scanners for tumor imaging [4] and breath nanosensors for gastric cancer [5]). The disparity between CNT advances in the pharmaceutical industry *versus* any other field thus raises the question: what is holding back the development of CNT-based nanomedicines?

Since similarities have been drawn with asbestos [6], CNTs bear a stigma that is difficult to eradicate. In 2008, it was found that as-produced (i.e. 'pristine') CNTs of lengths in the order of 20 μm and beyond elicited asbestos-like pulmonary pathogenicity when introduced in the abdominal cavity in mice [7,8]. Those findings elicited a fear of CNTs that may have acted as an impediment for rapid biomedical applications to date. On the positive side, this has prompted intense efforts to determine the correct classification and assessment of CNT materials, their safety and their biocompatibility [9–13]. Indeed, it became soon clear that, for CNTs to successfully allow pharmaceutical development, their production had to be refined to highly homogeneous and pure samples, and *in vivo* behavior had to be established using relevant models, by avoiding the temptation to generalize results that apply to specific formulations, doses, and routes of administration [14].

Scientists who want to approach the field, firstly need to become familiar with the fact that CNTs comprise a heterogeneous population of nanomaterials that can be thought of as tubes obtained by rolling up sheets of graphene. A variety of CNT types exist and a few key aspects are to be understood as they will determine CNT

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electronic and biological behavior. It is widely known that CNTs can be metallic or semiconducting, and, dependent on the number of graphene layers that are rolled up in the form of a tube, they can be divided into single-walled (SW), double-walled (DW), or multi-walled (MW) nanotubes. Their sizes will vary accordingly, with diameters ranging from less than 1 nm up to 100 nm, and lengths that typically range from a few-hundred nanometers to several microns. An important parameter is the method used for their production and purification, which will determine the heterogeneity and purity of samples. For instance, metal residues may be present in amounts ranging from negligible traces to significant proportions (even up to 30% in weight). Readers who are unfamiliar with CNT production, properties, and classification will find detailed reviews on this topic elsewhere [15–17].

CNT functionalization

The as-produced CNTs (i.e. 'pristine') can be further purified in several ways, and with the advancement of CNT production technologies, today it is possible to achieve high-purity CNTs with less than 5% weight of residual metals and other forms of carbon, the two main kinds of contaminants. Good quality CNTs can be highly homogeneous in diameter, while their length is usually less controlled, unless further processes are applied. Amongst post-production processes, surface chemical functionalization is by far the one that offers the greatest potential to fine–tune CNT properties (e.g. length) for applications [18], including *in vivo* behavior. While CNT chemical reactivity has been well-established over the years to produce a variety of functionalization protocols over a range of conditions [19–21], new routes and mechanisms continue to emerge [22–26]. Briefly, there are two main methods: non-covalent and covalent functionalization, both aimed at reducing the tendency of hydrophobic, pristine CNTs to aggregate together into bundles that are difficult to disperse and handle. Non-covalent methods typically rely on the use of surfactants or amphiphilic polymers and macromolecules that 'wrap up' around the CNTs usually *via* hydrophobic or aromatic interactions, and that expose hydrophilic groups on the outer surface for favorable interactions with water or polar solvents. However, a disadvantage can be the potential dissociation of the non-covalently bound moieties from CNTs, with consequent release of carbon nanostructures exposing their hydrophobic surface prone to aggregation. Instead, covalent methods are more robust and exempt from this drawback. They generally exploit the presence of structural defects on the CNT surface, and/or generate new ones often *via* radical mechanisms, to create covalent bonds with several small molecules or dendrimers. In particular, chemical oxidation is a popular approach, often used alone or in addition to other methods, to generate short, oxidized CNTs that display hydrophilic functional groups (i.e. COOH, OH, C=O, among others) while removing undesired impurities (e.g. residual metal catalysts) [27].

Chemically functionalized CNTs for biological applications

Importantly, chemical functionalization is a necessary step to achieve homogeneous dispersions in aqueous media that allow CNTs to be suitable for biological applications [28]. This process dramatically changes CNT properties, pharmacokinetic profile [29,30] and, remarkably, even biodegradability. For instance, early toxicity reports indicate that pristine CNTs cannot be metabolized and their persistence *in vivo* leads to chronic inflammation. By contrast, recent findings show the remarkable biodegradation of short, oxidized SWCNTs in neutrophils and macrophages, and the biodegraded nanotubes do not generate an inflammatory response when aspirated into the lungs of mice [31]. The biodegradation of chemically functionalized CNTs (*f*CNTs) appears to be mediated by the oxidative environment in phagocytic cells, and it has been shown to occur *in vivo* [32] and *ex vivo* [33]. New reports continue to emerge on this process [32] and show it can be extended to various organs (e.g. the lung [34] and the brain [35]) and also to other *f*CNTs (e.g. amino-functionalized MW CNTs [35]). Importantly, controlled biodegradation of *f*CNTs in inflammatory cells (e.g. eosinophils) is a gateway to avoid chronic inflammation response (arising from non-biodegradable CNT accumulation), that will further allow the development of CNT-based nanomedicines [36]. Therefore, *f*CNTs should be considered as separate chemical entities relative to pristine CNTs, and, alarming conclusions on pristine CNT toxicity are not to be extended to *f*CNTs without further study.

Recently, more and more studies consistently show that pristine CNTs tend to agglomerate and accumulate in RES organs (especially liver and lungs, and, to a less extent, spleen), while *f*CNTs are better tolerated, to an extent that depends on the level and type of functionalization [29,37,38]. For instance, after half an hour of intravenous injection, MWCNT formulations accumulate in RES organs (especially liver and lungs) irrespective of their surface properties (i.e. pristine or functionalized) [37]. However, MWCNTs that tend to agglomerate more are retained for months in lungs and liver, while those that are well-dispersed and functionalized are more easily cleared from the body *via* excretion. As an example, oxidized (>3 mmol/g of carboxyl groups density), short (<500 nm) MWCNTs are not at all retained by the RES organs and are easily excreted in the urine [37]. Similar conclusions were reached also for other types of *f*CNTs with a high level of functionalization, confirming the trend [39,40].

In terms of parameters that are implicated in possible adverse effects, important factors to consider for *f*CNTs are: (1) type, (2) level, and (3) surface density of the introduced functional groups, as well as (4) purity. First, different types of chemical groups (e.g. hydrophilic or hydrophobic, charged or neutral, among others) will have an impact on the dispersibility of *f*CNTs. A key finding is that *f*CNTs that are adequately debundled (e.g. displaying ammonium-triethylene glycol moieties) do not lead to signs of inflammatory responses seen in the case of both bundled *f*CNTs (e.g. displaying hydrophobic octyl chains) and bundled pristine CNTs, both of which form large aggregates that behave more like asbestos (Fig. 1) [38]. Second, the level of functionalization has an impact on CNT cytotoxicity, presumably for similar reasons. For instance, single-dose injection of pristine MWCNTs or MWCNTs with a low-level of functionalization induce hepatic damage visible in mice at 7 days post-treatment, however, such damage was almost completely recovered after 28 days [37,41]. By contrast, *f*CNTs with a higher density of functional groups did not lead to any significant sign of toxicity in treated mice after a single administration [37]. Furthermore, metal impurities associated with CNTs play a crucial role in toxicity, which is mediated by the generation of radical oxygen species (ROS) [42] and mitigated when the CNTs are acid-oxidized and the metals thus removed [37]. This is one of the main reasons why a popular approach to functionalize CNTs is their oxidation and subsequent

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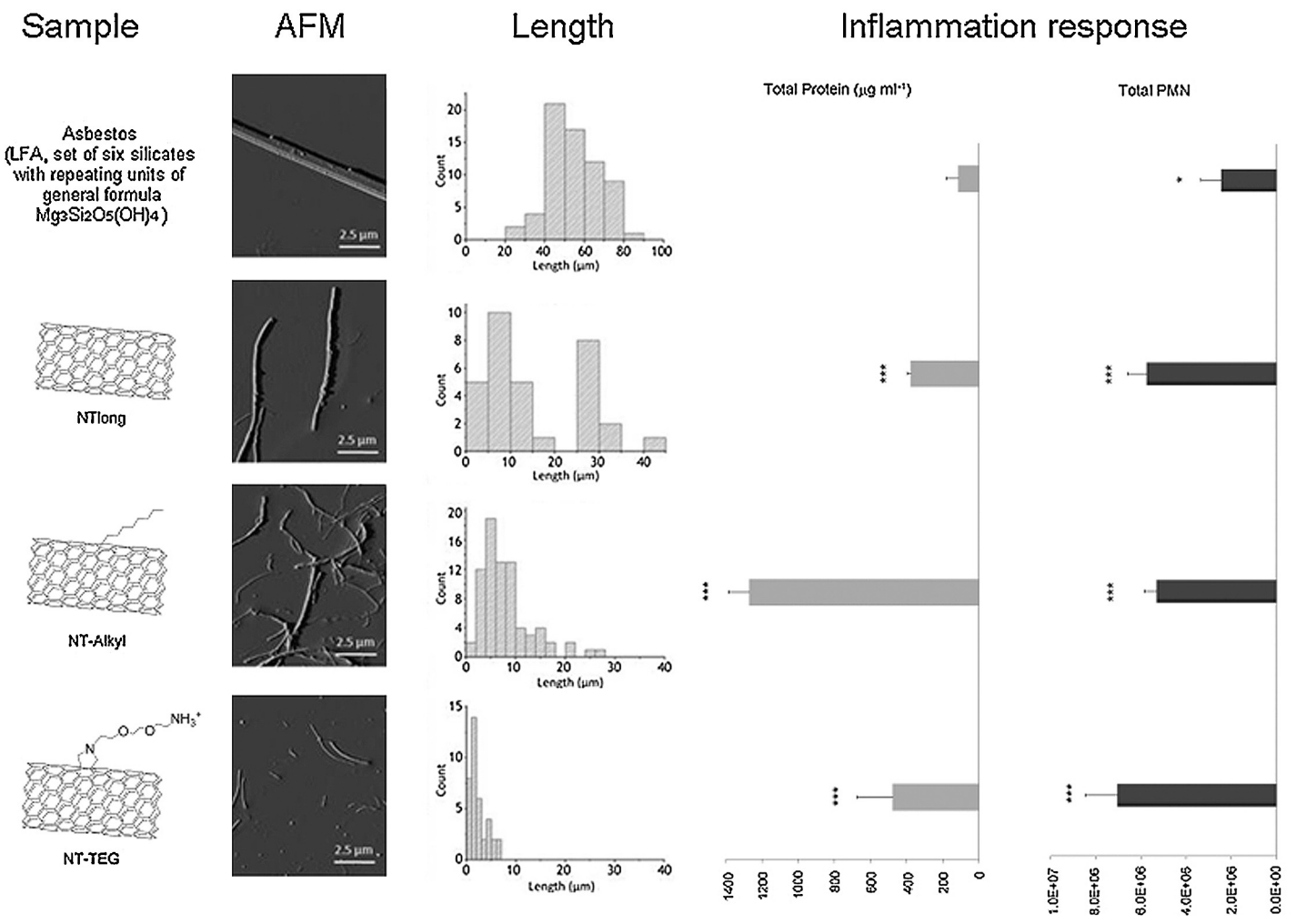

FIGURE 1

Comparison between CNT and asbestos tendency to aggregation and to trigger inflammation. From top to bottom: asbestos, long pristine CNTs, alkyl-*f*CNTs, TEG-*f*CNTs. These samples showed different propensity to form bundles, thus different structure length as seen by AFM, and elicited different levels of inflammatory response when injected into mice. Adapted with permission from Ref. [38], Copyright © 2013 Wiley-VCH.

amidation to append a variety of chemical groups or bioactive compounds. However, this approach may be suboptimal for biomedical applications, because of the non-homogeneity of the introduced functional groups on the *f*CNT surface, that tend to occur at their tips and existing defect sites [43,44]. For instance, a recent study compared the effects on the immune system exerted by either *f*CNTs displaying ammonium-terminated triethylene glycol groups (NH_3^+–TEG–CNTs) introduced *via* 1,3-dipolar cycloaddition, or *f*CNTs displaying ammonium-terminated poly(ethylene glycol) (NH_3^+–PEG–CNTs) introduced *via* oxidation and subsequent amidation. Interestingly, only the former led to homogeneous dispersions with no visible aggregates and a lack of proinflammatory response by macrophages, confirming the importance of having a homogeneous distribution of functionalization sites along the CNT surface [45]. Lack of cytotoxicity for NH_3^+–TEG–CNTs, previously shortened to about 400 nm *via* oxidation, has been confirmed also in separate studies [46,47].

PEG is a popular addition to CNTs since its biocompatibility has been well-established, and PEGylation is an effective approach to extend CNT blood circulation time and achieve stable and well-dispersed formulations [44]. Although its exact mechanism of action is still a subject of debate, it is known that PEGylation leads to reduced opsonin binding to CNTs in the blood serum, thus reduced macrophage recruitment and reduced early CNT removal [48]. A generally accepted theory is that PEG adopts a random conformation and masks the nanoparticle surface underneath from the binding of proteins that leads to the formation of the so-called protein corona and, ultimately, the activation of phagocytic cells of the RES. However, according to a recent study on PEG-SWCNT fate *in vivo* or exposed to human plasma, PEGylation does not eliminate protein binding leading to the corona, and PEG conformation appears to be a key parameter for the determination of the adsorbed protein pattern [49]. It is known that grafted polymers like PEG tend to adapt different conformations. At low grafting density, each polymer chain is isolated and 'nailed' down as a random coil to the surface; if the interaction between PEG and surface underneath (i.e. CNT) is weak, the collapsed polymer is connected to the surface through a 'stem', assuming the shape of a 'mushroom'. At high grafting densities, polymers stretch away from the interface to avoid overlapping, and adopt an extended conformation forming a so-called 'polymer brush'. When PEG is bound to SWCNTs, a brush-like conformation leads to shorter blood circulation time, faster renal excretion, and higher relative spleen *versus* liver uptake, compared to PEG that

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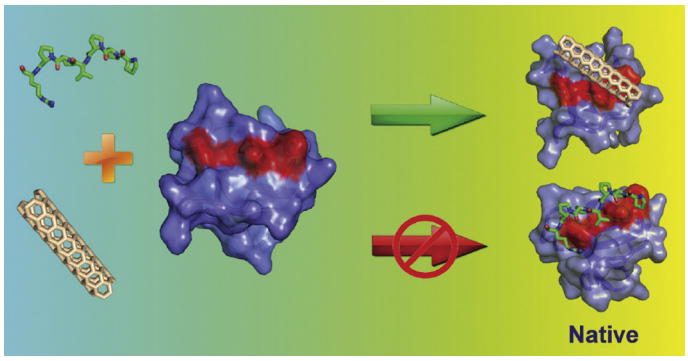

FIGURE 2

SWCNT and protein hydrophobic interaction. SWCNTs may fit into hydrophobic pockets onto proteins and interfere with natural ligand–protein interaction. Reprinted with permission from Ref. [58], Copyright © 2013 Wiley-VCH.

shows mushroom-to-brush conformation transitions. Surprisingly, the effect of PEG conformation appears more important than surface charge, and it is more pronounced when 2 kDa PEG is used, relative to 5 kDa PEG [49]. Although nanotube PEGylation appears as a promising approach to achieve successful application of CNTs in medicine, the resulting product properties will heavily depend on: (1) the CNT material used (e.g. purity, diameter and length, number of walls); (2) the PEG used (e.g. molecular weight, brush or mushroom conformation, branched or linear); and (3) the functionalization process (e.g. level, surface density, homogeneity) [44]. It is therefore not surprising that the realistic potential of PEG-CNTs in nanomedicine is still under discussion [50].

Protein corona on CNT

An interesting proposition in recent years is the importance of the protein 'corona', that is the layer(s) of biomolecules physisorbed onto nanoparticle surfaces, in the determination of their fate *in vivo*. Following initial concerns related to the high surface area presented by nanoparticles and its potential reactivity, came the realization that this surface was inevitably to allow interaction with, and eventually binding of circulating plasma proteins [51]. The process is dynamic, and the macromolecules that initially bind nanoparticles at higher rates (leading to the 'soft corona') are eventually exchanged with others that may bind more slowly, but more tightly (leading to the 'hard corona') [51]. This protein layer can significantly alter the surface properties of nanoparticles, yielding a 'new nanoscale entity' that may determine their fate *in vivo* (i.e. distribution, reactivity and degradation) [52]. CNTs are no exception and we can envisage that identification of their protein corona will assist in the design of CNTs for use in nanomedicine [53–55].

Generally, there is still controversy on the relationship between protein corona and pharmacokinetic profile for any nanoparticle [52,54,56]. The interaction between proteins and CNTs is complex, and there is an open quest toward innovative approaches and appropriate descriptors for its prediction [57]. A combination of interactions appears plausible, including π–π stacking, hydrophobic, electrostatic, π-cation. Particularly relevant for SWCNTs, which have a much smaller diameter compared to MWCNTs, is the scenario that SWCNTs fit into the hydrophobic pockets onto the protein surface, potentially able to lead to interference with protein–ligand natural interactions as well as to alterations in protein conformation (Fig. 2) [58].

An important aspect to consider is the fact that the protein corona is a dynamic entity. For instance, a combination of experimental and theoretical studies on SWCNTs binding of four different plasma proteins showed that the initial binding of transferrin and albumin is eventually replaced by the binding of fibrinogen and immunoglobulin (Fig. 3a–e). Importantly, fibrinogen binding led to reduced

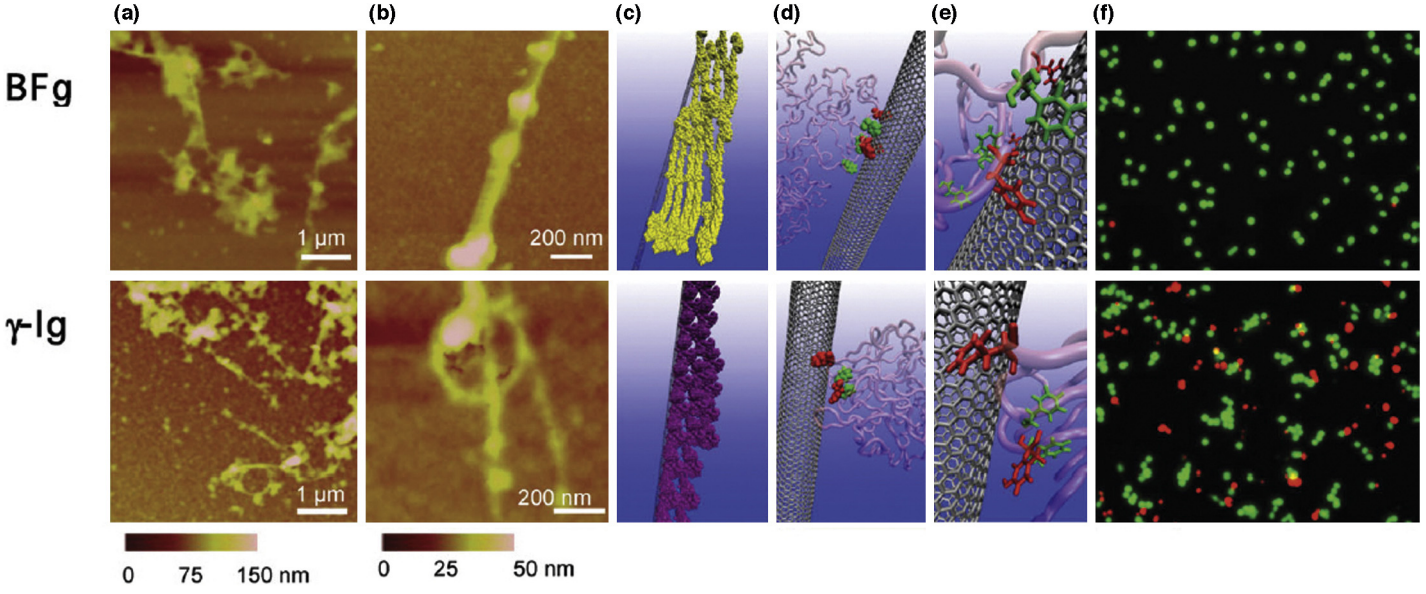

FIGURE 3

Interactions between two blood proteins (i.e. fibrinogen, BFg, *top*, and gamma-immunoglobulin, γ-Ig, *bottom*) and SWCNTs. AFM images of proteins after incubation with SWCNTs for 10 min (a) and 5 h (b). Molecular modeling illustrations for proteins (in beads representation) binding to SWCNTs after incubation for 10 min (c) and 5 h (d). (e) Locations of the most preferred binding sites for SWCNTs on proteins (pink cartoon, with highlighted tyrosine residues in red and phenylalanine residues in green). (f) The live (green) and dead (red) stains for THP-1 cells after treatment for 12 h shows reduced cytotoxicity for fibrinogen-bound CNTs (top). Reprinted with permission from Ref. [58], Copyright © 2013 Wiley-VCH.

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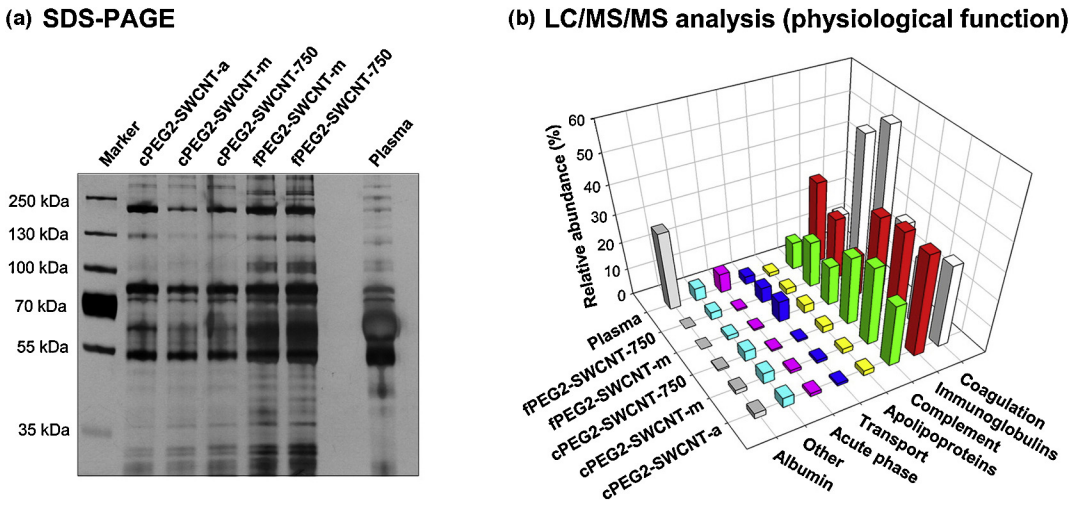

FIGURE 4

Relative abundance of human plasma proteins adsorbed onto non-covalently coated cPEG-SWCNTs and covalently functionalized fPEG-SWCNTs. Samples of PEG2-SWCNTs incubated with human plasma proteins at 37 °C and free plasma proteins were separated on 1D SDS-PAGE (A). Tryptic digested proteins were analyzed by LC/MS/MS, leading to the identification of >500 proteins, of which 240 were selected and grouped according to their physiological functions. Their relative abundance for these groups were calculated for PEG2-SWCNTs and free plasma (B). Reprinted with permission from Ref. [49]. Copyright © 2013 American Chemical Society.

cytotoxicity *in vitro* (Fig. 3f), highlighting the key role of adsorbed proteins in possibly mediating CNT biological effects [59].

Another important aspect to consider is the challenge associated with the generalization of *in vitro* results toward credible conclusions that are relevant to *in vivo* scenarios. Recent studies on the interaction between CNTs (pristine or functionalized) and plasma proteins showed that, contrary to what was suggested by earlier reports, the effect of isoelectric point, molecular weight, hydrophobicity, or number of polyaromatic residues of plasma proteins appear negligible in the determination of the adsorbed protein pattern from protein-rich samples, such as human plasma or serum-containing cell culture medium [49,60]. In addition, the relative plasma protein abundance was not reflected in the corona composition, suggesting selective adsorption was taking place (Fig. 4) [49,60]. Nevertheless, coagulation proteins (especially fibrinogen) and immunoglobulins (especially IgM) were the major plasma proteins bound to PEG-SWCNTs [49], in agreement with earlier results [59]. In addition, the different adsorption patterns of plasma proteins between differently functionalized CNTs may have been related also to the tendency toward aggregation [49,60]. For instance, it is plausible that more proteins will bind to the grooves present on 'rough' aggregates, such as those of CNTs with a non-homogeneously coated surface [60]. Indeed, the homogeneous presence of functional groups as achieved by covalent functionalization methods, and especially the 1,3-dipolar cycloaddition, is highly desirable as it allows for stable and individualized *f*-CNT dispersions [44,45].

Adsorption of proteins onto CNTs depends also on protein concentration, thus, reports that use biological samples (e.g. undiluted plasma or cell lysates) will be more relevant to the study of the adsorbed proteins, rather than those employing purified protein solutions [61]. If we also consider SWCNT interaction with protein hydrophobic pockets, this aspect may be very relevant for specific applications such as protein sensing [62], although it will not correspond to a complete picture of the *in vivo* situation. When exposed to human plasma or cell lysate, CNTs bound only a small fraction of proteins relative to other nanomaterials, such as titanium dioxide nanoparticles. Strikingly, SWCNTs (diameter <2 nm) bound almost no proteins, while MWCNTs (10–30 nm in diameter) bound mainly fibrinogen from human plasma, and filamentous or filament-forming cytoskeletal proteins from cell lysates (i.e. myosin, vimentin, actin) [61]. This was confirmed by a study on human cell lysates that showed virtually no protein binding for CNTs (both SW and MW) with diameter <10 nm [63].

The case is different for CNTs with larger (≥10 nm) diameters (Fig. 5). When exposed to cell lysates, MWCNTs bind consistent amounts of intracellular proteins. Many of these are either filamentous (i.e. vimentin) or filament-forming (i.e. actin, tubulin, keratin) cytoskeletal proteins [61,63]. Preferential binding to CNTs from proteins that bind filamentous proteins was noted in a separate study on serum-containing cell culture medium, which is also very protein-rich [60]. These results suggest shape as a relevant parameter for protein–CNT interactions *in vivo*, a concept already introduced a few years ago when CNTs were proposed as microtubule biomimetics because of the striking similarities in size and morphology between the two structures [64]. In light of these facts, it is thus perhaps not too surprising that CNTs are able to interfere with cytoskeletal organization [65] also inside cells [66–70]. Importantly, such an effect was noted only at relatively high doses (i.e. 0.3 mg/ml) and longer exposure times (≥48 h), with lack of cytotoxicity observed for lower exposure conditions, suggesting a scope for CNT use in cancer therapy [71].

Immune system activation by CNT

Among the various proteins that can bind to CNTs, an important class are the components of the complement system, which may trigger immune responses. This topic has been studied by others,

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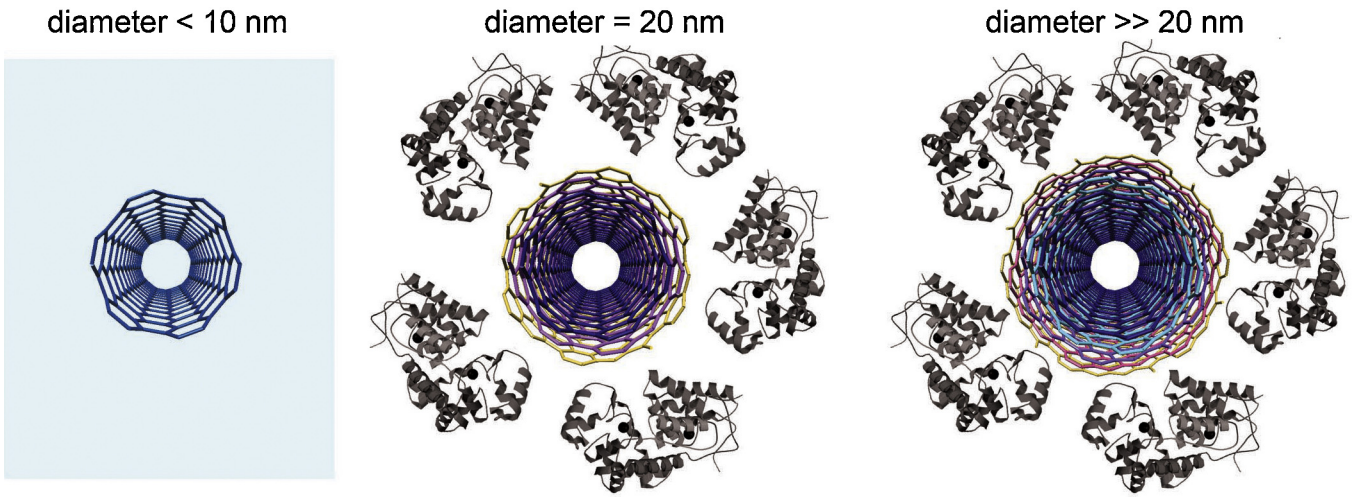

FIGURE 5

Formation of a protein corona around CNTs depends on the diameter of the tube. Nanotubes narrower than 10 nm (left) virtually bind no proteins on their surface, while for tubes with a diameter equal to or larger than 20 nm (center and right, respectively) formation of a protein corona is independent from the tubes width.

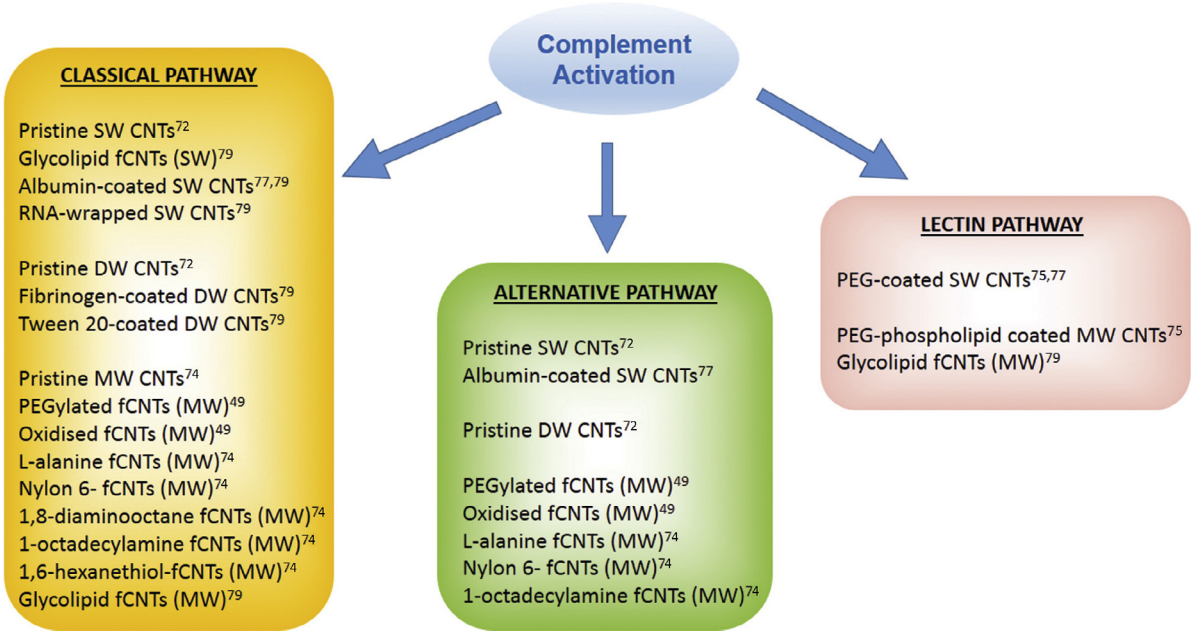

FIGURE 6

Functionalization affects complement activation pathway by CNTs. Several studies on *f*CNTs (single-walled, SW, double-walled, DW, or multi-walled, MW) reported on complement activation by CNT *via* classical (left), alternative (center), or lectin (right) pathways.

often with contradictory results based on experimental conditions and samples tested [72,73]. Recent understanding of CNT–protein interactions is suggesting convergence on the fact that CNT functionalization plays a key role in fine-tuning (and potentially reducing) protein binding [50,74–76], albeit no protocol has succeeded in completely eliminating complement activation [77]. For instance, non-covalent binding of proteins such as albumin, fibrinogen, and C1q to DWCNTs is not competitive as it occurs at different sites, therefore, complement activation is prevented by neither albumin- nor fibrinogen-coating of CNTs [78]. In fact, CNT functionalization affects not only the extent, but also the pathway of immune response activation (i.e. classical, alternative or lectin-mediated, Fig. 6), depending on the type and surface density of functional groups [48], and on the proteins that thus bind CNTs (e.g. immunoglobulins, complement proteins, or collectins) [77,79].

In general, CNTs are predominantly recognized by the immune system *via* the classical pathway, and this event can be followed by CNT phagocytosis, but not necessarily with a pro-inflammatory response [80,81]. Instead, pristine DWCNTs are the only sample shown so far to significantly activate complement *via* the alternative pathway [72,77]. In all cases, complement activation follows

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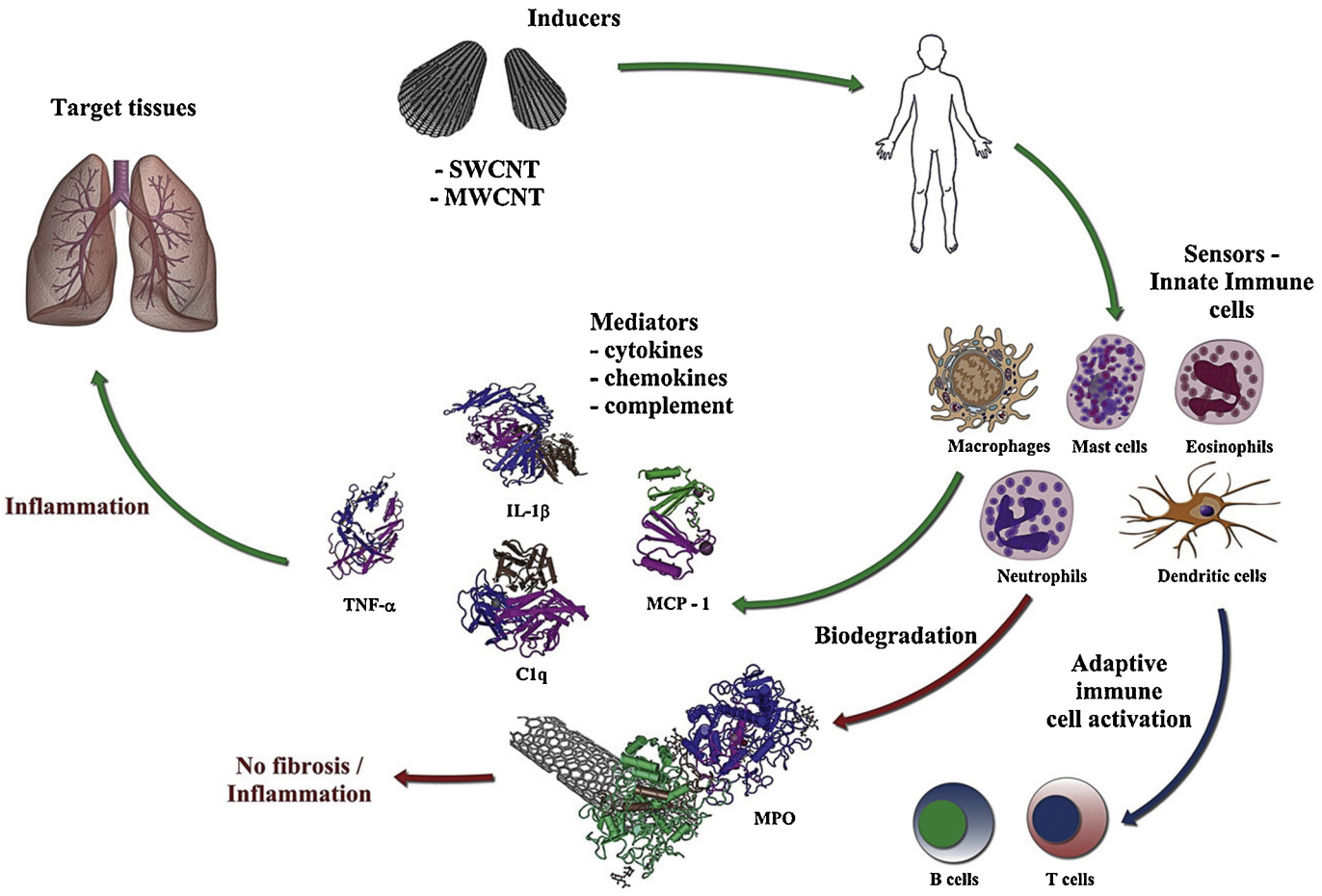

FIGURE 7

Immune response to CNTs. Three scenarios are possible: uncontrolled adverse inflammation (green pathway); biodegradation (red pathway); modulated immune response (blue pathway, e.g. for vaccine delivery). Reprinted from Ref. [36], Copyright (2013), with permission from Elsevier.

protein binding onto the CNT surface, however, it can also be reduced by the same mechanism when the bound proteins have an inhibitory effect, such as Factor H [82,83]. Alternative mechanisms that modulate immunoreactivity have also been proposed (Fig. 7). For instance, direct CNT–dendritic cell interaction [84] and use of defined MWCNT surface topography to affect dendritic cell activation [70]. Dendritic cells are the main bridge between innate and adaptive immune pathways and their activation leads to B and T cell stimulation. Therefore, appropriate choice of CNT properties (e.g. topography, chemical functionalization and diameter) can also be exploited to selectively activate either innate or adaptive immune response, depending on the intended application (e.g. CNT vaccine design) [36,46,85].

Analogous to our knowledge about the potential effects of protein corona adsorption, in the case of immune responses against CNT there is still a need for *in vivo* studies to provide relevant data for clinical applications. Although most studies mentioned above point to a certain extent of CNT adverse immunoreactivity, it was shown that complement activation can be modulated in a desirable manner, as oxidized MWCNTs injected subcutaneously into mice led to only a transient immune response with reversible effects [86]. This report adds to the emerging consensus on the desirable CNT properties to achieve immunological neutrality: short, functionalized CNTs, with smoother surface topography and even density distribution of functional groups that could also allow for their rapid biodegradation [87].

Conclusions

The winding road for carbon nanotubes in nanomedicine has provided us with useful lessons to be learnt. First and foremost that CNT functionalization, type and purity are all key parameters that will affect their fate *in vivo*. The stigma of structural similarity with asbestos fibers has been a key determinant in the relatively slow progress and adoption of CNT in nanomedicine relative to other application fields. However, it also prompted thorough studies that have revealed greater understanding on how functionalized CNTs can biologically behave in a very different and safer manner. We now know that rational chemical derivatization of CNTs can bypass adverse immune responses and accelerate biodegradation. Following the start of clinical trials of CNT-based devices, we foresee that CNT-based nanomedicines for internal use will be the next step.

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