The winding road for carbon nanotubes in nanomedicine

Silvia Marchesan[1,*], Kostas Kostarelos[2], Alberto Bianco[3] and Maurizio Prato[1]

[1] Dipartimento di Scienze Chimiche e Farmaceutiche, University of Trieste, Piazzale Europa 1, 34127 Trieste, Italy
[2] Nanomedicine Lab, Faculty of Medical & Human Sciences, University of Manchester, AV Hill Building, Manchester M13 9PT, United Kingdom
[3] CNRS, Institut de Biologie Moléculaire et Cellulaire, Laboratoire d'Immunopathologie et Chimie Thérapeutique, 67000 Strasbourg, France

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

*Corresponding author:. Marchesan, S. (smarchesan@units.it)

1369-7021/
http://dx.doi.org/10.1016/j.mattod.2014.07.009

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