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Toward the suppression of cellular toxicity from single-walled carbon nanotubes

Zhenghong Gao,^{a,b} Juan A. Varela,^{c,d} Laurent Groc,^{c,d} Brahim Lounis^{a,b} and Laurent Cognet^{*a,b}

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In the multidisciplinary fields of nanobiology and nanomedicine, single-walled carbon nanotubes (SWCNTs) have shown great promise due to their unique morphological, physical and chemical properties. However, understanding and suppressing their cellular toxicity is a mandatory step before promoting their biomedical applications. In light of the flourishing recent literature, we provide here an extensive review on SWCNT cellular toxicity and an attempt to identify the key parameters to be considered in order to obtain SWCNT samples with minimal or no cellular toxicity.

1. Introduction

Over the past two decades, nanoscience and nanotechnology have been largely impacted by the development of carbon-

based nanomaterials, such as fullerenes,¹ nanodiamonds,^{2,3} graphene,⁴ and carbon nanotubes.^{5–7} Carbon nanotubes are attractive due to their outstanding electrical,⁸ optical,⁹ mechanical,¹⁰ and thermal properties.¹¹ Apart from applications in materials science, electronics or photonics,¹² carbon nanotubes also have multiple promising applications in biomedicine, serving as biosensors,^{13,14} bioprobes,^{15,16} drug carriers,¹⁷ photothermal therapy enhancers¹⁸ and molecular imaging contrast agents.¹⁹ SWCNTs distinguish themselves from double-walled carbon nanotubes²⁰ and multi-walled carbon nanotubes⁵ by their single-layer cylindrical sidewall structure, which provides them more finely tuned physical and chemical

^aUniv. Bordeaux, Laboratoire Photonique Numérique et Nanosciences, UMR 5298, F-33400 Talence, France

^bInstitut d'Optique & CNRS, LP2N UMR 5298, F-33400 Talence, France.

E-mail: laurent.cognet@u-bordeaux.fr

^cUniv. Bordeaux, Interdisciplinary Institute for Neuroscience, UMR 5297, F-33000 Bordeaux, France

^dCNRS, IINS UMR 5297, F-33000 Bordeaux, France



Zhenghong Gao

Zhenghong Gao received his Ph.D. in Advanced Interdisciplinary Studies in 2012 from the University of Tokyo, Japan, under the co-supervision of Prof. Takeshi Serizawa and Prof. Mokoto Komiyama. He then worked as a postdoctoral researcher with Dr Laurent Cognet and Prof. Brahim Lounis at Institut d'Optique, CNRS and University of Bordeaux, France for developing ultra-small nanoprobe and exploring their applications in

neuroscience. Currently, he is a research fellow at the University of Michigan-Ann Arbor, USA. His current research focuses on the development of novel theranostic nanosystems and small molecule probes for applications in early-stage diagnosis and therapy of cancer.



Juan A. Varela

Juan A. Varela studied physics at Univ. de la República in Uruguay and did his Ph.D. in Ireland at the Centre for Bio-Nano Interactions, University College Dublin. He subsequently spent four years as a postdoc at the Interdisciplinary Institute for Neurosciences in Bordeaux (France). Juan is currently working in the Department of Chemistry at the University of Cambridge (UK). His main research interests are the use of

nanotechnology and high resolution microscopy to understand and cure neurodegenerative diseases.

properties for applications as compared to other carbon structures. Due to their ultra-small diameter, high curvature and large surface area ($1315 \text{ m}^2 \text{ g}^{-1}$),²¹ they possess highly reactive surfaces²² and they can interact with biomolecules present in biological systems, like proteins,²³ DNA²⁴ and lipids²⁵ by weak interactions (e.g. van der Waals, π -stacking, hydrophobic interactions, and hydrogen bonds). Reactive surfaces of SWCNTs may offer vast opportunities for surface modification and have potential for a variety of applications.^{26–28} Conversely, direct interactions of SWCNTs with biomolecules might make them deleterious to the integrity of cells and organs. In this regard, SWCNTs' impact on human health is a rising concern within the scientific, industrial, and public communities.^{29,30}

Cell-based experiments are considered the preliminary test for assessing the biological safety of nanoparticles before practical applications in biology and medicine. Toxicity of nanoparticles to cells can be evaluated at different levels, ranging from evaluation of the plasma membrane integrity to the activation of late intracellular proteolytic cascades and DNA fragmentation. Typical tests include the examination of cell growth, cell viability, membrane permeability, mitochondrial activity, metabolic activity, oxidative stress, immune response, DNA fragmentation, DNA repairing enzymes cleavage, *etc.* Over the past few years, a high number of studies have been performed to understand SWCNTs' cellular toxicity. Many published results are however contradictory and a full knowledge concerning nanotube cellular toxicity remains to be established.

The purpose of this article is to review the current knowledge of SWCNTs' cellular toxicity, which differs from that of their multiwalled counterparts. Previous review articles³¹ generally addressed the toxicity of carbon nanotubes regardless of their type (single *versus* multiwalled). Our aim is to attempt to identify the critical parameters to be taken into account to

understand and further minimize SWCNTs' cellular toxicity. We will consider several aspects of SWCNT sample properties (see Fig. 1); among them, nanotube synthesis and purification processes will first be discussed. Generally, the as-produced SWCNT samples are a heterogeneous mixture of nanotubes with impurities (mainly metal catalysts and carbon by-products)³² and multiple step post-synthesis purification procedures are commonly employed to remove such impurities.³³ In addition, SWCNTs do not consist of single molecular species, but instead different chiral angles and diameters

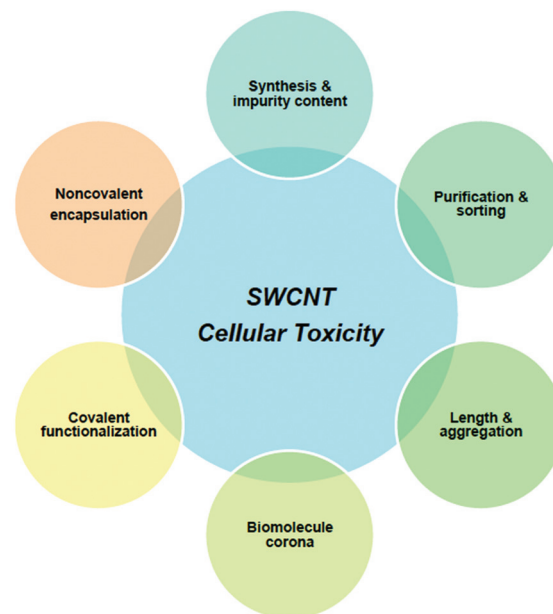


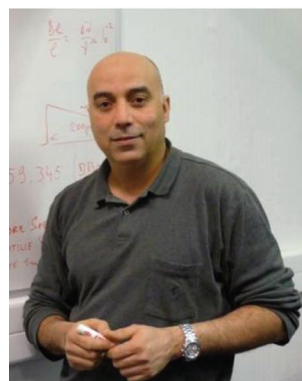
Fig. 1 Key parameters associated with SWCNT cellular toxicity.



Laurent Groc

Laurent Groc is a CNRS Senior Investigator and director of the team "Development and Adaptation of Neuronal Circuits" at IINS – University of Bordeaux. After his Ph.D. at the Wayne State University (Michigan, USA) and Université Claude Bernard (Lyon, France), LG investigated the maturation of glutamate synapse at the Department of Physiology (Goteborg, Sweden). He then combined electrophysiological and single molecule

imaging expertise in Bordeaux to investigate the fast dynamics of glutamate receptor, unraveling the key role of this process in neuronal communication. His team investigates the molecular and cellular basis of brain cell development in physiology and in models of neuropsychiatric disorders.



Brahim Lounis

Brahim Lounis obtained his Ph.D. in laser cooling and trapping of atoms at the Ecole Normale Supérieure. He was appointed as an Assistant Professor at Bordeaux University in 1993, where he performed pioneering studies on quantum optics with single molecules and developed the first single photon sources. After a sabbatical at Stanford University, he becomes a Professor at Bordeaux University, where he leads the Nanophotonics group.

His research interests concern the interaction of light and matter and nano-optics. He develops optical methods for detection and spectroscopy of individual nano-objects and explores their applications in quantum optics, condensed matter physics and bioimaging.

provide them distinct molecular structures. This can induce specific species-related physical properties and (bio)chemical molecular affinities^{34–36} which could further result in distinct cellular toxicity.^{37,38} Vast improvements of sorting techniques have arisen over recent years,^{39–41} which could greatly help in the understanding of SWCNT cellular toxicity. Furthermore, the strong inter-tube van der Waals interactions among pristine SWCNTs can be up to $500 \text{ eV } \mu\text{m}^{-1}$,^{42,43} which renders pristine nanotubes insoluble in common physiological media.⁴⁴ In order to overcome this issue, nanotube encapsulation using amphiphilic molecular moieties (commonly called surfactants) is generally used to individualize and solubilize SWCNT in aqueous media.⁴⁴ Cellular toxicity arising from surfactants rather than nanotubes themselves must also be considered. An alternative route for solubilizing SWCNTs consists of functionalizing the nanotube surface, thus creating defects on the pristine SWCNT backbone structures.^{45,46} The contribution of these functional groups on SWCNT cellular toxicity will also be discussed in this review.

2. Effect of synthesis and impurities

Several synthesis methods are commonly used for producing SWCNTs, such as chemical vapor deposition (CVD),⁴⁷ laser-ablation⁴⁸ and arc-discharge.⁴⁹ Different types of metal particles are used as catalysts in the synthesis formulations in order to reduce activation energy barriers of the chemical reaction and/or to control nanotube growth orientation. The most commonly used metals are Fe, Ni, Co, Mo and Y,⁵⁰ which can therefore be present in as-produced nanotube samples. In addition, carbonaceous by-products may also be produced, such as nanocrystalline graphite, amorphous carbon, and fullerenes.⁵¹ As a result, as-produced SWCNT samples not only contain nanotubes but also many impurities which depend on the synthesis formulations. Impurities can be embedded

within the inner channels of nanotubes making them difficult to be removed completely (see Fig. 2a and b).⁵² They might therefore interfere with the pristine properties of SWCNTs and affect the interplay between nanotubes and cells.⁵³ Indeed, metal elements commonly co-exist with many proteins/enzymes and also participate in various biological pathways.⁵⁴ Loading metal particles in cells may cause multiple types of toxic effects,^{55,56} such as gene silencing and hypoxia signal induction,⁵⁷ ion channel inhibition,⁵⁸ production of reactive oxygen species (ROS),⁵⁹ lipid peroxidation⁶⁰ and formation of massive mitochondrial and nuclear DNA adducts.⁶¹ The impact of carbonaceous particles on cell integrity might also be significant.^{62,63} In order to reduce the influence of impurities, the as-produced SWCNT samples might require thorough purification through various chemical and physical treatments,⁶⁴ such as harsh acid washing,⁶⁵ low temperature oxidation,⁶⁶ polymer wrapping extraction,⁶⁷ in combination with ultracentrifugation or other sorting techniques. Applications of such strategies depend on the SWCNT synthesis methods as will be discussed below.

2.1. CVD SWCNTs

CVD methods allow large-yield production of SWCNTs. CVD commonly produces nanotubes through carbon monoxide (CO) disproportionation either under high-pressure (HiPco sample)⁶⁸ or with Co–Mo as supported catalysts (CoMoCAT sample).⁶⁹ The former technique produces nanotubes with interesting pristine optical properties for biological imaging.⁷⁰ The latter allows narrow nanotube chirality distributions. We discuss below the impact of the impurities contained in HiPco and CoMoCAT nanotube samples on cellular toxicity.

2.1.1. HiPco SWCNTs. HiPco samples contain a mixture of nanotubes with different chiralities.⁷¹ They can also contain various metal catalysts used during the synthesis process. A number of studies that focused on the interplay between SWCNTs and cells employed unpurified nanotube samples, and such impurities played a critical role in cellular toxicity.^{72–74} Davoren *et al.* studied the impact of HiPco SWCNTs containing 10 wt% Fe on primary human lung epithelial cells (A549),⁷⁵ as lung exposure is a primary pathway for human contact with nanoparticles. After 24 hours exposure at a dosage of $800 \mu\text{g mL}^{-1}$, low acute toxicity was reported but apparent changes in cell morphology were visualized with a transmission electron microscope (TEM). No individual SWCNTs were observed inside cells, while an increased number of multilamellar and vesicular bodies were observed, which was hypothesized to arise from a defensive response of lung cells. A549 and human bronchial epithelial cells (NHBE) further showed suppressed inflammatory and increased oxidative stress responses after exposure to the same nanotubes coated with dipalmitoylphosphatidylcholine (DPPC).^{76,77} DPPC coating improved the individualization degree of SWCNTs in cell culture medium and led to increased toxicity in A549 cells after 48 hours exposure but had no effect on NHBE cells. This induced toxicity can be attributed to impurities (10 wt% in this study) released from DPPC-coated nanotubes when incu-



Laurent Cognet

Laurent Cognet is CNRS Research Director at Institut d'Optique – University of Bordeaux. After his Ph.D. in atom optics at Institut d'Optique (Paris XI University) he performed one of the first experiments about the detection of single fluorescent molecules in living cells at Leiden University (NL). In 2000, he was hired by CNRS at the University of Bordeaux and in 2006 he spent a sabbatical year at Rice University

(Texas) as a Fulbright scholar to develop single molecule imaging and spectroscopy of single-walled carbon nanotubes. His research interests concern the development of high-resolution imaging strategies of individual nanoobjects and their application in biology.

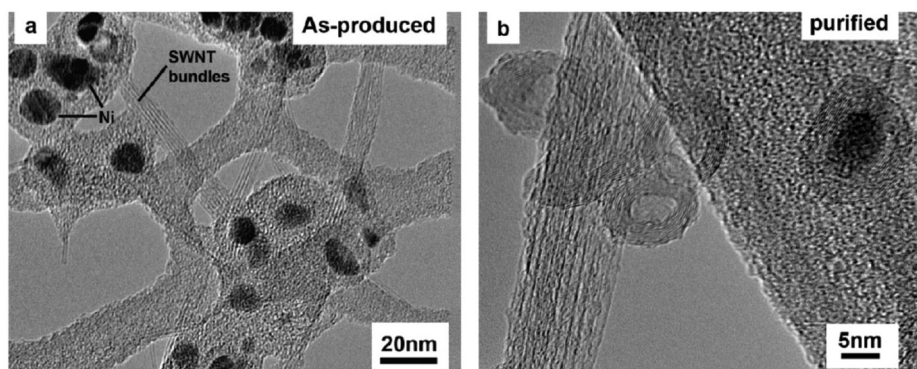


Fig. 2 Typical electron microscopy micrographs of Ni-containing SWCNT samples before (a), and after purification (b). Reproduced from ref. 55 with permission from John Wiley & Sons.

bated with A549 cells.⁷⁸ This observation also indicates that SWCNT toxicity depends on the cell type. To also address another important pathway of nanoparticle body penetration, Murray *et al.*⁷⁹ studied the effect of unpurified nanotubes on skin cells. Cell oxidative and inflammatory effects were evaluated on the EpiDerm-FT engineered skin of murine epidermal cells (JB6 P+) through administration of unpurified HiPco SWCNTs containing 30 wt% Fe and their purified counterparts containing only 0.23 wt% Fe.⁷⁹ A significant induction of activator protein 1 (a transcription factor regulating gene expression in response to various stimuli)⁸⁰ was observed upon exposing unpurified HiPco SWCNTs to JB6 P+ cells, but no activation was observed using purified HiPco nanotubes. Further topical exposure of unpurified HiPco nanotubes to immune-competent hairless SKH-1 mice after 5 days at a daily dosage of 40 μg per mouse led to increased oxidative stress, depletion of glutathione, oxidation of protein thiols and carbonyls, and elevated myeloperoxidase activity, thereby resulting in an increase of dermal cell numbers and thickening of the animal skin. In an attempt to obtain bio-compatible nanotube samples by coating them with biomolecules, Boyer *et al.*⁸¹ used bovine serum albumin (BSA), a bioactive blood protein widely used as blocking reagents for reducing non-specific bindings, as an encapsulating macromolecule. The authors reported the cellular uptake of HiPco SWCNTs (containing 5 wt% carbonaceous and 0.3 wt% metallic impurities) by murine macrophage-like cells (J774A.1) and NIH-3T3 cells.⁸¹ BSA-coated SWCNTs reduced cell proliferation in a dose-dependent manner and increased cell sizes at a dosage level of 30 $\mu\text{g mL}^{-1}$, most likely due to increased amounts of impurities. More recently, Holt and coworkers reported that BSA-coated SWCNTs were taken up by human mesenchymal stem cells and HeLa cells without apparent acute effects.⁸²

Altogether the investigations mentioned above suggest that the impurity content in HiPco nanotube samples and the role of encapsulating agents are important parameters in cellular toxicity. Sample purification might be employed to reduce impurities and therefore toxicity. The impact of encapsulating compounds will be detailed in section 4.

2.1.2. CoMoCAT SWCNTs. CoMoCAT nanotube samples have a narrow chirality distribution, generally enriched in (6,5) nanotubes.⁶⁹ CoMoCAT formulation involves the use of Co–Mo bi-metallic catalysts supported on a SiO_2 substrate.⁸³ Purification processes are usually achieved by low temperature oxidation to remove amorphous carbon, hydrofluoric acid washing to remove the SiO_2 substrate, and hydrochloric acid (HCl) treatment to remove Co–Mo bi-metallic catalysts that mostly attach to nanotube ends. Yehia *et al.* investigated the cellular toxicity of CoMoCAT SWCNTs (thoroughly purified sample containing 6.64 ppm Co and 1.55 ppm Mo; dosage of 50 $\mu\text{g mL}^{-1}$; 100–400 nm in length) in HeLa cells.⁸⁴ Nanotubes were suspended in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS) 5 v/v% (DM-SWCNTs). The MitoRox™ Red assay suggested that superoxide levels in mitochondria were similar for both incubations, with and without nanotubes. The HeLa cell morphology and proliferation showed no apparent change after exposure to DM-SWCNTs compared with unexposed cells over 4 days. This investigation indicated that the DM-SWCNTs were not inherently toxic because the impurities were efficiently removed. The CoMoCAT samples with low amounts of impurities (Co–Mo 1.8 wt%, dosage of 10 $\mu\text{g mL}^{-1}$) similarly showed low cellular toxicity to *E. coli* K12 cells after 1 hour exposure.⁸⁵

The cellular toxicity of CoMoCAT coated with biomolecules was investigated by Bertulli *et al.*, assessing the long-term effects of BSA-coated CoMoCAT SWCNTs, purified by low temperature oxidation and acid treatments 200–300 nm in length, dosage of 8 $\mu\text{g mL}^{-1}$ on macrophages.⁸⁶ No significant difference in cell proliferation and viability was observed between cells exposed to reference and BSA-coated SWCNTs for 65 hours (corresponding to three cell division cycles). This result supports the observations made on HiPco nanotubes suspended in BSA where toxicity was primarily attributed to sample impurities.⁸¹ Ge and coworkers further examined the cellular toxicity of SWCNTs (Co as catalysts) coated by different blood proteins, including bovine fibrinogen (BFG), gamma globulin, transferrin, and BSA in human acute monocytic leukemia (THP-1) and human umbilical vein endothelial cells

(HUVECs).⁸⁷ Interestingly, BFG-coated SWCNTs showed the lowest toxicity, which might be due to the tighter binding of BFG proteins to nanotubes preventing direct contact of the nanotube backbone with cellular components. These results suggest that blood proteins can be promising candidates for coating SWCNTs by reducing interactions between nanotubes and cellular components. They might also suggest that bare nanotubes can adsorb proteins in the bloodstream, although the fate of nanotubes in the bloodstream is not yet understood.

2.1.3. Arc-discharge SWCNTs. The synthesis of SWCNTs by arc-discharge utilizes a composite anode, usually placed under a hydrogen or an argon atmosphere.⁸⁸ The anode is made of graphite and a metal, such as Ni, Fe, Co, Pd, Ag, Pt, *etc.*; or the mixture of Co, Fe, and Ni with other elements. Arc-discharge SWCNTs have diameters of 1.4–2 nm and lengths of several μm . The reaction products include many metal catalyst residues and unexpected products such as MWCNTs and fullerenes.⁴⁹ Nimmagadda *et al.* reported the influence of arc-discharge SWCNTs on 3T3 mouse fibroblasts.⁸⁹ As-produced (AP-SWCNT, Ni 0.73 wt%, Y 0.38 wt%), purified (Pur-SWCNT, Ni 0.07 wt%, Ni 0.04 wt%), and glucosamine-modified (GA-SWCNT, Ni 0.09 wt%, Y 0.03 wt%) arc-discharged nanotubes were incubated with 3T3 mouse fibroblasts for 3 days at concentrations up to 0.1 wt%. It was found that 3T3 cell viability and metabolic activity strongly depend on nanotube preparation, purification, and concentration. The AP-SWCNTs showed the largest cellular toxicity, the Pur-SWCNTs showed a mild toxicity while the GA-SWCNTs showed the lowest cellular toxicity. These results demonstrated again that the impurity content of carbon nanotube samples significantly affects cellular toxicity for the HiPco and CoMoCAT nanotubes.

2.1.4. Laser-ablation SWCNTs. Laser-ablation formulation produces SWCNT samples containing a high content of carbon arc-materials and metal catalysts that require to be removed by extensive purification.⁹⁰ Warheit *et al.* investigated the cellular toxicity of laser-ablation SWCNTs to pulmonary cells. SWCNT samples containing 30–40 wt% amorphous carbon and 5 wt% Ni and Co⁹¹ were directly dispersed in phosphate-buffered saline (PBS) containing 1 wt% Tween80 and used in cell experiments without further purification. No significant change in cell proliferation was found compared to control samples following 24 hours cell exposure at a dosage of $5 \mu\text{g mL}^{-1}$. The SWCNT samples used in this study were however rarely found as individualized nanotubes, but rather in agglomerated ropes. The effects of nanotube aggregation on cellular toxicity will be discussed in Section 3.2.

Pulskamp *et al.* studied the responses of lung macrophages (NR8383) and A549 cells to the exposure of laser-ablation SWCNTs purified by acid treatment containing traces of Ni/Co catalysts.⁹² After 24 hours nanotube exposure to cells at a dosage of $100 \mu\text{g mL}^{-1}$, no acute toxicity was reported on cell viability. In contrast, the use of unpurified commercial SWCNTs (CVD, Nanostructured & Amorphous Materials Inc., Los Alamos, USA) indicated a dose- and time-dependent increase of intracellular reactive oxygen species and a decrease

of mitochondrial membrane potential in both NR8383 and A549 cell lines, revealing the impact of impurities in the laser-ablation nanotube sample on cellular toxicity.

2.1.5. Comparison between SWCNT synthesis methods and toxicity mechanism. In order to gain insight into the cellular toxicity of different SWCNT formulations, Chowdhury *et al.* tested on bacteria the toxicity of SWCNTs produced by HiPco, CoMoCAT, and arc-discharge methods.⁸⁵ SWCNTs were dispersed in water containing 2 wt% F108 copolymer, and their toxicity was determined by Live/Dead BacLight bacterial viability tests. This study suggested that the HiPco (Fe 6.52 wt%) nanotubes have a greater impact on cell viability as compared to the CoMoCAT (Co–Mo 1.80 wt%) and arc-discharge nanotubes (Y–Ni 0.21 wt%) at the same dosage of $10 \mu\text{g mL}^{-1}$ after 1 hour exposure.

The observations mentioned above suggest that regardless of the synthesis method, metal catalysts/impurities embedded in SWCNT samples have a deep impact on cellular toxicity. A possible molecular mechanism of cytotoxicity induced by cell exposure to carbon nanotubes and leached metallic particles has recently been proposed.⁷² The cellular toxicity of SWCNTs was suggested to be mediated by ROS and the related disorder of intracellular metabolic pathways.^{93,94}

As a first conclusion, the use of SWCNT samples containing low metal impurities is important to obtain reduced cellular toxicity. Control of SWCNTs' cellular toxicity primarily requires control on nanotube sample impurity contents through synthesis and purification processes.

3. Effect of nanotube length and aggregation

The impact of nanoparticles on living cells depends on several parameters such as their physical size, hydrodynamic volume, aggregation states, colloidal stability in physiological environments, *etc.*⁹⁵ It is well known that SWCNTs in colloidal suspensions are relatively heterogeneous in length, and contain both individualized nanotubes and bundles. In this section, we summarize the reported effects of nanotube lengths and aggregation states on cellular toxicity.

3.1. Length

SWCNTs have very large aspect-ratios (length-to-diameter), which complicates the understanding of size effects involved in cellular toxicity. The lengths of SWCNTs are believed to play essential roles in nanotube internalization pathways,^{96,97} cellular responses⁹⁸ and subcellular distribution.⁹⁹ Kang *et al.* reported the effect of SWCNT lengths (chitosan-coated nanotubes, dosage of $50 \mu\text{g mL}^{-1}$) on cell internalization pathways⁹⁹ and suggested that 100–200 nm long nanotubes are internalized in cells through clathrin-coated vesicles and the caveolin-dependent pathways. In contrast, 50 nm short nanotubes could directly enter cells through an energy-independent pathway involving insertion and diffusion across the cell membrane. After internalization, 100–200 nm long nanotubes were

found to localize mainly in the cytoplasm, while 50–100 nm short nanotubes were found to distribute closer to the cell nucleus. Sato and coworkers' investigations indicated that cell toxicity caused by exposure to 220 nm long nanotubes was weaker than the toxicity induced by 825 nm long nanotubes.¹⁰⁰ Donkor *et al.* reported that short SWCNTs (coated with 6-arm branched PEG) with a length below 35 nm could efficiently deliver 4700 bp plasmid DNA molecules into HeLa cells in 24 hours to obtain transfected cells.¹⁰¹ Moreover, short nanotubes were reported to be less hazardous to DNA than their longer counterparts.¹⁰² A very recent study suggested that short SWCNTs (1.5 nm in diameter, 10 nm in length, coated by 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) at a concentration of 10 mg mL⁻¹) were able to spontaneously insert into the lipid bilayer of cell membranes. This leads to the formation of artificial 'molecule channels' capable of translocating water, ions, protons and DNA molecules.¹⁰³ From a bio-safety point of view, these results imply that extremely short DOPC-SWCNT complexes may interfere with the structure and function of cell membranes.

3.2. Aggregation

The aggregation state of SWCNTs is also an important parameter for minimizing their cellular toxicity.¹⁰⁴ SWCNTs can form large aggregates (micrometers in diameter) in cell culture medium or inside the cell body.¹⁰⁵ Umemoto and coworkers¹⁰⁶ reported that nanotube aggregates induced relocation of cell clathrin complexes in mast cells (RBL2H3) just after 10 min of exposure, and reduced the total clathrin level after 1 hour (NanoLabs, Detroit, USA. CVD, 5 wt% Fe impurities, 10–100 µg mL⁻¹). Further studies revealed striking membrane perturbations and rearrangements around nanotube aggregation zones in mast cells as a consequence of a strong disruption of the cortical actin cytoskeleton. Characterization performed at the molecular level indicated that nanotube aggregations induced biphasic calcium response and phosphorylation of post-receptor kinases related to FCER1 receptors (a high affinity receptor for the Fc region of immunoglobulin E).

Altogether, these observations suggest that nanotube aggregates activate pro-inflammatory responses of mast cells. Belyanskaya *et al.*¹⁰⁷ compared the cellular toxicity of well-dispersed SWCNTs (arc-discharged nanotubes with Ni and Y catalyst, polyoxyethylene sorbitan monooleate (PS80)-coated nanotubes, dosage of 50 µg mL⁻¹) as well as purified rope-like aggregated nanotubes (HCl treatment, 15 minute) and soot-like nanotubes-pellet fractions (centrifugation pellet) with commercial asbestos as a reference in human MSTO-211H cells. Cell morphology analyses suggested that well-suspended SWCNTs were less toxic than asbestos and that rope-like nanotube aggregates induced more pronounced toxicity than asbestos fibers at identical concentrations. Raja *et al.*¹⁰⁵ examined the impact of SWCNTs (0.1 mg mL⁻¹ HiPco, sonicated in a 3 : 1 (v/v) mixture of H₂SO₄ and HNO₃) on rat aortic smooth muscle cells after 3.5 days of incubation. Unfiltered samples containing nanotube aggregates significantly decreased cell-growth rates compared to filtered samples.

From these studies, it clearly appears that aggregation of SWCNTs should be avoided and that nanotube individualization is a key parameter to minimize cellular toxicity.

4. Surface modification of SWCNTs

As already suggested above, cellular toxicity of SWCNTs can also be influenced by nanotube surface exposure to the cellular environment.^{96,108,109} In this section, we now discuss the impact of diverse surface modification strategies (see Fig. 3) used in biological studies to introduce nanotubes into biological samples.

Pristine SWCNTs are insoluble in water due to their hydrophobic surfaces and direct exposure of pristine nanotubes to biological systems might lead to interactions with various biomolecules. For example, in the bloodstream, many proteins and biochemical species can adsorb onto nanotubes in an unspecific way.¹¹⁰ In this sense, it is important to shield or modify the surface of pristine SWCNTs to make them inert to

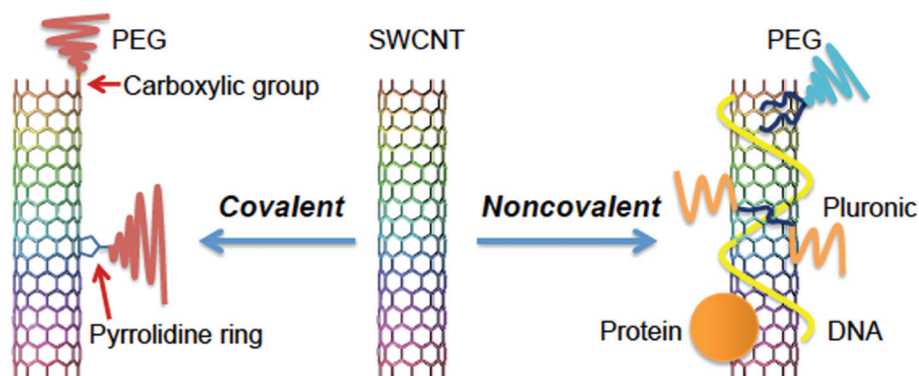


Fig. 3 Surface modification of SWCNTs for bio-applications. Covalent approaches frequently consist of grafting moieties from pyrrolidine rings or carboxylic groups while noncovalent approaches are based on SWCNT encapsulation using amphiphilic molecules. Examples of PEG (e.g. PL-PEG), pluronic (e.g. F108), proteins (e.g. BSA) and single-strand DNA are pictured.

chemical and biological components of the cells. The surface of SWCNTs can also contain various defects and chemical groups¹¹¹ that might play a role in nanotube interactions with biological molecules. Conversely, certain covalently doped SWCNTs have been shown to provide new optical or chemical properties promising for bioimaging or sensing applications,^{112–114} such that controlling nanotube surface exposure to their bio-environments might also be promising to retain their properties.

Encapsulation of nanotubes by hydrophilic moieties and chemical grafting of solubilizing agents on nanotube surfaces are the two commonly used approaches for preparing nanotube aqueous solutions with solubility up to concentrations in the order of g mL^{-1} .¹¹⁵ For bio-applications, many small biomolecules, polymers and surfactants have been used. Among them, poly(ethylene glycol) (PEG) has arisen as the most widely used biocompatible moiety for modifying nanoparticle surfaces (termed as PEGylation) due to its proven biological inertness and hydrophilic properties.¹¹⁶ PEG chains are relatively flexible in physiological environments and are able to undergo long-time circulation in blood due to the strong resistance against non-specific protein absorption. It was also suggested that PEG modified nanoparticles are promising for penetrating various biophysical barriers, such as the reticulo-endothelial system and blood–brain barriers.¹¹⁷ This is essential for increasing the accessibility of nanoparticles and the specificity for *in vivo* targeting applications.

We summarize below the cellular toxicity of SWCNTs coated by either covalent or non-covalent approaches. We outline several widely employed surface coatings and put a particular focus on PEG-based strategies due to their wide applicability in biomedical usages.

4.1. Covalent modification

Chemical modification of SWCNT sidewalls is a widely used approach for solubilizing nanotubes in aqueous media. It is usually achieved by grafting functional moieties onto initially oxidized SWCNT sidewalls (see Fig. 3).^{118,119} Oxidation of nanotubes is performed using H_2SO_4 and/or HNO_3 to create carboxylic groups. Importantly, oxidized SWCNTs used without further coating were reported to induce multiple toxic effects to cells. For example, Singh *et al.* reported cell chirality loss, centrosome disintegration, tubulin network disorganization, adhesion complex maturation and decrease of migration ability during multicellular alignment or migration after cellular exposure to oxidized SWCNTs.¹²⁰ Such oxidized SWCNTs were also reported to induce multipolar spindles and abnormal mitosis.¹²¹ Luanpitpong *et al.* reported that direct chronic exposure of oxidized SWCNTs to lung epithelial cells would induce the production of cancer stem cells with malignant properties¹²² and that these cells could become aggressive and develop tumors. These nanotubes might thus be more toxic than pristine unpurified nanotubes, which reflects the impact of surface defects and chemical groups introduced by oxidation.¹²³ Therefore, oxidized SWCNTs without further

surface modification should be used with caution for both *in vitro* and *in vivo* applications.

In this context, it was shown that grafting anti-fouling polymers onto nanotube backbones provides an efficient approach for minimizing SWCNTs' direct interactions with cellular components. For instance, PEG-modified SWCNTs are commonly used and are usually produced by an amidation reaction of $-\text{COOH}$ groups of oxidized nanotubes with $-\text{NH}_2$ groups of PEG. PEG-modified SWCNTs produced with this approach stabilize nanotubes as individual and/or small bundles in colloidal suspensions and are stable in a high salt and serum containing environment.¹²⁴ Zhang *et al.* investigated the toxic effects of linear PEG-modified SWCNTs and compared it to non-modified SWCNTs on PC12 cells.¹²⁵ Using a high concentration of $100 \mu\text{g mL}^{-1}$ for 24 hours exposure, linear PEG-modified SWCNTs were found to be much less toxic than the non-modified counterparts (as indicated by the water soluble tetrazolium reduction assay). Similarly, based on lactate dehydrogenase release assays, it was found that linear PEG-modified SWCNTs caused less cell membrane damage than the non-modified nanotubes. The morphology of these cells was also differentially affected, as cells treated with oxidized SWCNTs had an elongated shape while linear PEG-modified nanotubes did not induce such morphological changes. Dose-dependent ROS and significant glutathione depletion were found after 24 hours administration of both materials. However, linear PEG-modified SWCNTs showed a considerably less effect to oxidative stress related genes in PC12 cells compared to non-modified nanotubes. Once again, these findings indicate that the cellular toxicity mechanism of nanotubes is associated with oxidative stress. Importantly, these results suggest that PEG modification reduces oxygen species generation induced by nanotube administration.

In order to study the effect of PEG structures on nanotube cellular toxicity, Heister *et al.* compared HeLa cell viability after exposure to branched PEG-modified and non-modified SWCNTs.¹²⁶ Unlike non-modified SWCNTs which formed clusters/precipitates in cell culture media, branched PEG-modified nanotubes (10 kDa) were more stable and dispersed. In addition cell viability stayed at 100% over the whole dosage range from 0.01 to $100 \mu\text{g mL}^{-1}$ after 4 days of incubation. This observation suggests that branched PEG-modified SWCNTs are able to isolate nanotube surfaces more efficiently from exposure to cellular components compared to linear PEG chains.

Covalent PEG modifications can also be achieved through grafting PEG molecules onto $-\text{NH}_2$ groups. These groups are introduced onto the SWCNT surfaces by adding pyrrolidine rings to nanotubes *via* 1,3 dipolar cycloaddition reaction in azomethine ylides with subsequent thermal condensation in α -amino acids and aldehydes.¹¹⁵ These PEG modified nanotubes are stable in various colloidal suspensions. They do not induce any complement reaction due to high structural stability.¹²⁷ Indeed, it was shown that the PEG chains on SWCNTs are unable to interact with the natural anti-PEG factor that is involved in the complement reaction induced by PEG molecules in biological systems.

Altogether, these studies indicate that PEG covalent grafting strategies dramatically reduce the cellular toxicity of oxidized SWCNTs. The branched PEG-modified SWCNTs appear to be more biocompatible than the linear PEG-modified counterparts due to compact binding and large coverage of nanotubes, which limits the nanotube backbone exposure to cellular components. Yet, the effects of the PEG density, length and branch degree are yet to be systematically investigated to fully understand their impact on cellular responses and toxicity.

4.2. Non-covalent encapsulation

The covalent modification of nanotube surfaces discussed above induces sp^3 hybridization bonds through the introduction of chemical groups. These modifications can have important detrimental implications on the mechanical, physical or chemical properties of nanotubes. For instance, heavy covalent nanotube functionalization generally suppresses the intrinsic near-infrared (NIR) photoluminescence properties of nanotubes¹²⁸ whereas many bio-applications of SWCNTs are based on these properties.¹²⁹ For such applications, non-covalent-based nanotube solubilization using biologically compatible amphiphilic materials (soft polymers and biomolecules) is a widely used strategy (see Fig. 3).⁷⁰

In general, cationic, anionic or nonionic charged surface coating can be used to encapsulate SWCNTs and to control the outer charge of the coated nanotubes. As the plasma membrane is negatively charged, the surface charge of encapsulated SWCNTs is a key parameter for controlling nanotube–cell membrane interactions, nanotube internalization pathways and intracellular fate. Usually, cationic nanoparticles interact more strongly with the cell membrane and therefore show higher uptake efficiency compared to anionic and neutral nanoparticles.¹³⁰ Negatively charged nanoparticles are also known to be taken up efficiently by pinocytosis or following membrane diffusion.^{131–133} Keeping nanotube surfaces neutrally charged thus appears to be the key for reducing nanotubes non-specific binding to cell membranes and serum proteins. In addition, nanoparticle hydrophobicity should also be controlled as it plays an important role in cellular uptake processes and subcellular fate.^{134,135}

More specifically, ionic detergents (such as sodium dodecyl benzene sulphonate (SDBS), sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide, sodium cholate or sodium deoxycholate) are frequently used as excellent suspension agents of SWCNTs.^{136,137} However, because of the well-known cytotoxicity of these ionic surfactants, they are not ideal for SWCNT biological applications. On the other hand, nonionic surfactants (*e.g.* pluronic) can also disperse SWCNTs and be used in cellular applications.^{138,139} Low cellular toxicity of pluronic F108-coated nanotubes was previously reported,¹⁴⁰ however, recent developments illustrate that serum proteins in the bloodstream can replace F108 molecules and stick to SWCNT surfaces in physiological environments, which could restrict the performance of this surfactant.¹⁴¹ Interestingly, collagen has also been used to suspend SWCNTs with no apparent

effects on viability of bovine chondrocytes at a high dosage ($15 \mu\text{g mL}^{-1}$) after 15 days exposure.¹⁴²

Phospholipid–polyethylene glycol (PL–PEG) deserves a particular focus since it has become a widely used non-covalent suspension agent for bio-applications of SWCNTs both *in vitro* and *in vivo*.^{70,143–146} The hydrophobic PL chains attach to the surface of nanotubes, with the hydrophilic PEG chains increasing the solubility and stability of nanotubes in a high salt and serum containing environment. It is worth noting that 5 kDa linear PL–PEG-coated SWCNTs have been reported to trigger complement system reactions *in vitro*, but neither acute nor chronic toxicity was observed for these materials both for *in vitro* and *in vivo* tests as a consequence of complement activation.¹⁴⁷ It was similarly found that PL–PEG incorporated into liposomes activates the complement system in human serum through an alternative pathway *via* interaction with naturally existent anti-PEG antibodies.¹⁴⁸ We note that BSA-, RNA-, glycolipid-, and non-coated SWCNTs were also reported to trigger complement activation.¹²⁷ However, recent studies suggested that complement activation reaction could be avoided by slightly modifying the chemical structure of the surfactants. For example, complement activation by PL–PEG could be avoided by mutating an anionic phosphate site into a methylated site¹⁴⁷ since its activation arises from the anionic phosphate site in the PL–PEG structure.

DNA molecules represent another class of biomolecules commonly used to solubilize SWCNTs in the context of potential biomedical applications.²⁴ DNA is a flexible biopolymer capable of adjusting its molecular geometry to wrap around the SWCNT outside walls by forming a helical structure.^{24,36} This is achieved by non-covalent interactions (mainly π -stacking interactions) between SWCNT backbones and the aromatic nucleotides along the DNA perpendicular axis. This compact DNA wrapping can be SWCNT chirality selective depending on DNA sequences.¹⁴⁹ Importantly, DNA being naturally biocompatible, DNA-coated SWCNTs can be expected to show little cytotoxicity. Dong *et al.* demonstrated that low dose (GT)₁₅-coated SWCNTs ($0.8 \mu\text{g mL}^{-1}$) have no detectable impact on human astrocytoma cell morphology, proliferation, or viability after 24 hours exposure, as opposed to nanotubes coated by SDBS or SDS.¹⁵⁰ Accordingly, Jin *et al.* reported no apparent cytotoxicity in live NIH 3T3 cells exposed for several hours to (GT)₁₅-coated SWCNTs at the same dose. Such low SWCNT doses are typically used in live cell experiments aiming at studying nanotube endocytosis and intracellular trafficking at the single nanotube level.^{96,151} Indeed, DNA-coated nanotubes are usually found to strongly interact with cells and thus get internalized. The absence of cytotoxicity at higher doses of DNA-coated SWCNTs is yet to be determined. In addition, several studies have suggested that the adsorption of biomolecules present in cellular serums on DNA-coated SWCNTs and the nanotube cellular uptake might depend on the DNA sequence, nanotube chirality¹⁵² and length.¹⁰⁸ Additional studies are required to fully understand the nature and stability of DNA–nanotube interactions and the interplay of DNA-coated SWCNTs with biological systems.

Interestingly, several studies have also explored peptide-PEG¹⁵³ and DNA-PEG¹⁴ conjugations as new SWCNT coatings. The impact of these surfactant-nanotube complexes on cellular toxicity remains to be investigated.

4.3. Surface coverage density

The density of chemical functionalities on the sidewall of SWCNTs significantly affects the surface properties of the nanotubes and their fate in biological systems. For instance, Sayes *et al.* examined the cellular toxicity of HiPco SWCNTs in human dermal fibroblasts (HDF) by varying the density of phenyl-SO₃X functional groups covalently grafted on the nanotubes (purified by acid treatment, containing 1 wt% impurities).¹⁵⁴ The average density of phenyl-SO₃X groups was controlled by the carbon/phenyl-SO₃X ratio (18, 41, and 80). This study indicated that the SWCNTs were less cytotoxic as the degree of sidewall functional density increased.

Similar to covalently modified SWCNTs, non-covalently suspended carbon nanotubes showed improved solubility⁷⁰ and reduced toxicity with an increasing coverage of coating molecules. For example, Liu *et al.* studied the effect of the PEG length and nanotube surface coverage density on the nanotube circulating lifetime and accumulation in live mice skin.¹⁵⁵ It was found that poly(maleic anhydride-*alt*-1-octadecene)-poly(ethylene glycol) (C18PMH-PEG) coated SWCNTs showed the longest circulating lifetime (up to 20 hours) due to their compactness upon nanotube binding and large surface coverage. The same group reported that branched PEG chains could also

be used. The extension of hydrophilic star-like PEG chains in biological surroundings allowed imaging the nanotubes for up to 10 hours in mice.

Another study addressed the impact of the PEG length and nanotube surface coverage density by monitoring protein adsorption on SWCNTs using either covalently grafted or non-covalently attached PEG moieties.¹⁵⁶ It was shown that PEG conformational transition from the mushroom to brush state was the key for reducing non-specific protein absorption,¹⁵⁶ which may help to reduce nanotube toxicity and prolong circulation lifetime in the bloodstream.¹⁵⁷

For both covalent and non-covalent surface coverage, increasing the PEG density and branched degree not only improved the solubility of SWCNTs in biological fluids, but also made nanotubes less toxic by reducing unspecific interactions with biological components, especially proteins.¹²⁶

5. Effect of SWCNT-protein corona

In biological fluids, binding of biomolecules onto nanoparticle surfaces might lead to the formation of supramolecular complexes, usually called the nanoparticle-biomolecule corona.¹⁵⁸ Knowledge of the dynamic rates, affinities, and stoichiometries of nanoparticle-protein association/dissociation is fundamental for understanding the properties of these complexes.¹⁵⁹ In particular, these coronae directly impact the fate of nanoparticles in biological systems, in terms of pathophysiology,¹⁶⁰

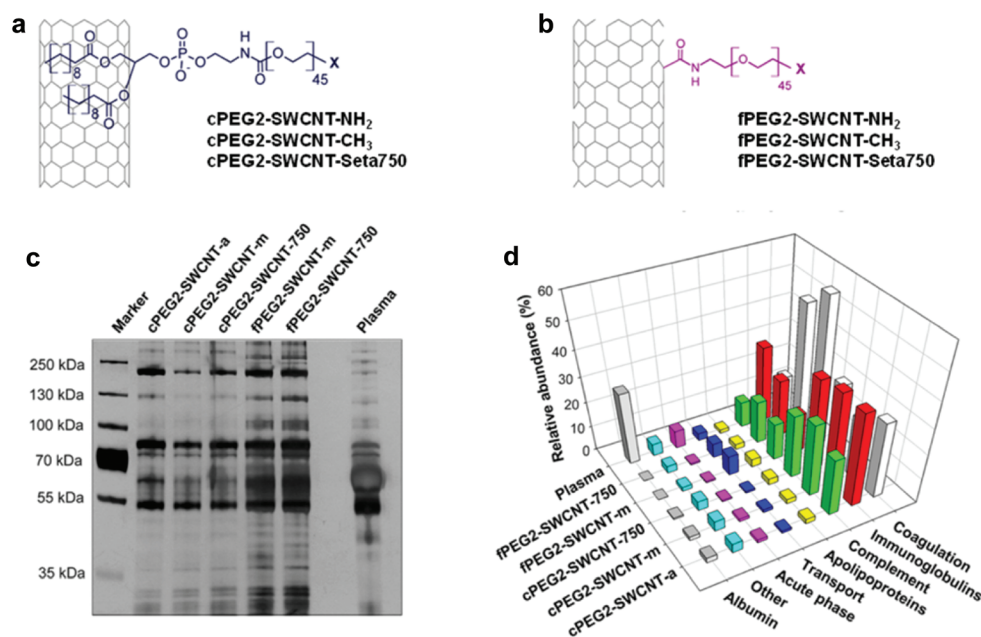


Fig. 4 PEG2k-SWCNT interactions with human plasma proteins. (a) PEG(2k)-coated SWCNTs (cPEG2-SWCNTs). (b) and PEG(2k)-functionalized SWCNTs (fPEG2-SWCNTs). Different linear 2 kDa molecular weight PEG chains having amino groups, methyl groups, or NIR-emitting dyes (Seta750) at their distal ends were used to investigate the protein corona. (c, d) Relative abundance of human plasma proteins adsorbed onto PEG2-SWCNTs. Samples of PEG2-SWCNTs were incubated with human plasma proteins at 37 °C and free plasma proteins were separated on 1D SDS-PAGE (c); the donor-averaged relative abundances for these groups were calculated for PEG2k-SWCNTs and free plasma (d). Reproduced from ref. 156 with permission from the American Chemical Society.

biological performance,¹⁶¹ and toxicity.^{159,161} In this section, we focus on the impact of PEG-modified SWCNT–protein coronae on cellular toxicity. Indeed, PEG chains are used to minimize the formation of the protein corona, thus allowing better control on the nanoparticle fate in many applications.^{162,163} However, PEG molecules do not completely prevent dynamic protein binding, especially when the PEG coverage of nanoparticle surface is not total.¹⁶⁴ In addition, and as mentioned above, covalent and non-covalent approaches can be used to coat SWCNT surfaces. Sacchetti *et al.* systematically investigated the protein corona properties of arc discharge SWCNTs surface coated with 2 kDa PEG chains through either covalent or non-covalent approaches (see Fig. 4a and b).¹⁵⁷ In this study, a non-uniform coating was observed on the sidewall of non-covalent PEG-coated SWCNTs (terms as cPEG-SWCNTs) leaving open areas that extend up to tens of nanometers that could directly expose the nanotube surface to biological environments. In contrast, covalent PEG-functionalized SWCNTs (fPEG-SWCNTs) displayed a denser and more uniform PEG layer on nanotube surfaces. After removing unbound PEG, cPEG2-SWCNTs and fPEG2-SWCNTs showed respectively a PEG density of 0.1 mmol (PEG average height 1 nm, mushroom conformation) and 0.4 mmol (PEG average height 7 nm, mushroom–brush transition conformation) per gram of nanotubes. Analysis of human plasma proteins adsorbed on nanotubes was studied by mapping with 1D SDS-polyacrylamide gel electrophoresis combined with mass spectrometry after incubating plasma with nanotubes. The results revealed clear differences in the composition of PEG2k-SWCNT coronae as a function of nanotube surface properties and protein properties: fPEG2-SWCNTs mainly adsorbed coagulation proteins whereas cPEG2-SWCNTs mainly adsorbed immunoglobulin proteins (see Fig. 4c and d). In addition, some plasma proteins were selectively enriched or

depleted depending on the type of PEG coating used. The influence of PEG conformation was subsequently tested by *in vivo* distribution studies using PEG2k-SWCNTs¹⁵⁷ confirming that surface modification (covalent *vs.* non-covalent), size, and PEG conformation together influence the composition and dynamics of nanotube–protein corona.¹⁶⁵

6. Conclusions and challenges

In this review, we present recent advances in the understanding of SWCNTs' cellular toxicity. We showed that SWCNTs' cellular toxicity is related to many factors, such as nanotube synthesis, impurity content, surface modification, nanotube length, nanotube aggregation state and protein corona formation. In addition, the reliability and accuracy of the methods used for assessing the toxicity of the nanotubes may need to be considered.^{166,167} Interdisciplinary knowledge on nano–bio interfaces in materials science, physics, chemistry, biology, and medical engineering is thus required to understand SWCNT toxicity and move towards better control of the nanotube impact on biological specimens.

We can attempt to draw a general strategy (although not a complete one) for minimizing SWCNT cellular toxicity (Fig. 5). First, regardless of the nanotube synthesis method, metallic impurities and carbon byproducts present in SWCNT samples need to be entirely removed by using dedicated purification processes. The cellular toxicity is also strongly associated with SWCNT properties (*e.g.* length, aggregation, and stability) in various suspensions. These need to be rationally controlled during preparation. Second, in order to reduce non-specific absorption of biomolecules, it is necessary to incorporate robust anti-fouling coatings on nanotube surfaces in order to isolate the nanotube backbone from biological environments

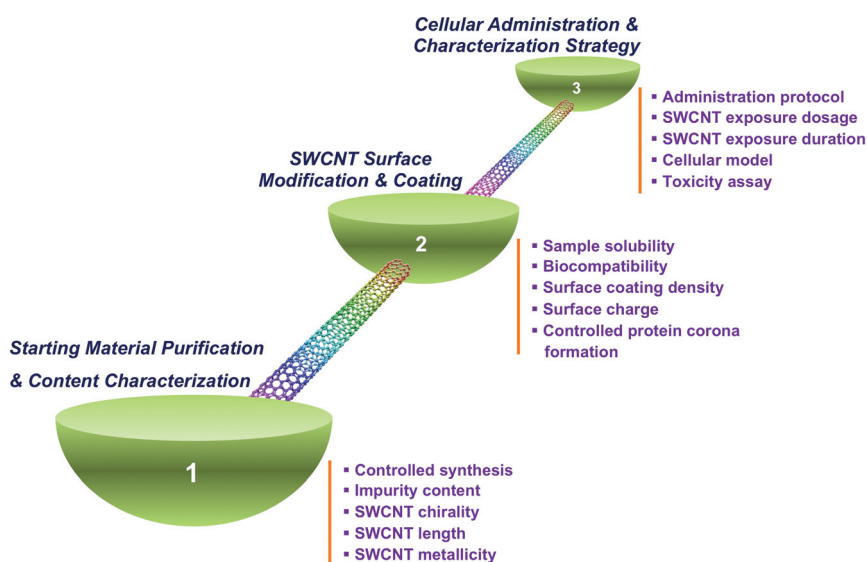


Fig. 5 General strategy for minimizing SWCNT cellular toxicity.

and further minimize the formation of nanotube-protein corona. It is the key to achieve a full coverage of nanotube surfaces by incorporating a dense, compact, and biocompatible coating. Many natural biomolecules and synthetic polymers can be good candidates for nanotube coating but PEG surface modification (PEGylation) currently seems to provide the best option. PEG indeed gathers a combination of excellent properties: water solubility, low absorption to proteins, low toxicity, and long lifetime circulation. Third, the administration conditions (incubation approach, time, concentration, etc.) need to be rationally chosen.

In this context, several challenges still remain to fully understand and minimize SWCNT cellular toxicity. A major challenge is certainly to produce high-purity and chirality-controlled nanotube samples.^{168–170} Although PEG–nanotube complexes are widely used, new biocompatible surface coatings with ultra-low nonspecific protein absorption must be prepared when the use of PEG should be avoided.^{171,172}

More generally, one should emphasize that further studies on SWCNT clearance, removal, and degradation in living systems will be necessary in order to fully understand their fate.^{173,174} The dosage and hydrodynamic size of nanotubes after binding plasma proteins need to be correctly monitored.^{175–177} In addition there is no standard tool for assessing SWCNT cellular toxicity.¹⁷⁸ In many experiments, the chemical reagents used for assessing toxicity directly interact with nanotubes in the absence of cells and can lead to false results and misunderstanding¹⁷⁹ which hinder fair comparison between reports. Finally, investigations at the molecular and genetic levels will certainly be needed. This would allow the molecular mechanisms of nanotube induced genetic toxicity, such as DNA damage,^{180,181} multiple poles in cell mitosis,¹²¹ and interruption to chromosome¹⁸² to be uncovered.

In spite of clear challenges, SWCNT-based biomedical materials and devices have shown spectacular progress in recent years and as such they hold great promise for innovations in biology and medicine.¹⁸³

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