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Promises, facts and challenges for carbon nanotubes in imaging and therapeutics

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The use of carbon nanotubes in medicine is now at the crossroads between a proof-of-principle concept and an established preclinical candidate for a variety of therapeutic and diagnostic applications. Progress towards clinical trials will depend on the outcomes of efficacy and toxicology studies, which will provide the necessary risk-to-benefit assessments for carbon-nanotube-based materials. Here we focus on carbon nanotubes that have been studied in preclinical animal models, and draw attention to the promises, facts and challenges of these materials as they transition from research to the clinical phase. We address common questions regarding the use of carbon nanotubes in disease imaging and therapy, and highlight the opportunities and challenges ahead.

One of the key advantages of carbon nanotubes in biomedical applications is that they can be easily internalized by cells, and therefore can act as delivery vehicles for a variety of molecules relevant to therapy and diagnosis. Moreover, their unique electrical, thermal and spectroscopic properties in a biological context offer further advances in the detection, monitoring and therapy of diseases. The therapeutic and diagnostic applications of nanotubes are intensively explored by many academic and industrial laboratories around the world, in parallel with their toxicological profile and any potential adverse pathogenic reactions from exposure. The risk–benefit balance for these materials — to be reached by combining both areas of investigation — will ultimately determine their clinical fate.

As-produced carbon nanotubes are insoluble in most organic or aqueous solvents, therefore for any type of biological application the nanotube surface needs to be modified. For example, chemically functionalized carbon nanotubes have been shown to act as unique non-viral delivery systems for the transfer of nucleic acids. They have been explored for targeted delivery of small organic molecules (for example, antibiotics and anticancer agents), the development of peptide-based synthetic vaccines, as a platform for antibody targeting, and for the transportation of proteins and sugar mimetics[1]. However, carbon nanotubes are still at the very early stages of their clinical development, and their efficacy and limitations must be carefully determined and addressed. Concerns about the toxic effects of these materials are under intensive debate[2]. It is imperative to determine the impact of nanotube exposure on biological components at the cellular, tissue and overall physiological level.

A further challenge is related to the lack of an accepted protocol to determine the degree of purity of the carbon nanotube material used[3]. Standard chromatographic techniques — such as thin-layer chromatography, high-pressure liquid chromatography and gel permeation chromatography — have achieved limited success, with no general or reproducible outcomes. Such technical limitations will need to be addressed, and standard procedures for the production and purification of nanotubes or functionalized nanotube materials should be developed to enable a move into large-scale multi-centre clinical trials.

As these challenges are confronted, it is necessary to show unequivocally the therapeutic efficacy of these nanotubes over established alternatives if industrial biotechnology and pharmaceutical investments are to continue and grow. Demonstrating the benefits of nanotubes in medicine is also needed to avoid common unrealistic expectations that may prove to be counter-productive to the development of the field overall. In this article, we examine the state-of-the-art in the use of carbon nanotubes for imaging and therapeutics, focusing only on studies that use *in vivo* models, and highlight the opportunities and challenges as these materials move closer to the clinical setting.

Carbon nanotubes used in imaging and therapy

Carbon nanotubes can be single-walled[4] or multiwalled[5] and are now produced in substantial quantities for a variety of commercial applications[6]. They have diameters in the nanometre range and their length can reach several microns. In terms of their use in biomedical applications, the initial hurdle has been the insolubility of carbon nanotubes in most solvents, and particularly in biologically compatible buffers and conditions. Different strategies have been developed to make carbon nanotubes compatible with the biological milieu[7]. The two main methodologies are based on the non-covalent coating of nanotubes with amphiphilic molecules (for example, lipids and polymers), and the covalent functionalization of the nanotube surface by grafting various chemical groups directly onto the backbone.

Figure 1 illustrates the types of carbon nanotubes that have been explored in biomedical studies using *in vivo* preclinical models. The three categories shown have different structural and surface characteristics that critically influence their biological performance. Pristine nanotubes (Fig. 1a) are the prototype materials produced and are the most difficult to handle biologically because they are hardly dispersed in aqueous solutions and have a strong tendency to interact hydrophobically and aggregate. Interestingly, pristine nanotubes — mostly poor-quality aqueous dispersions — have been the predominant type used in most toxicology studies[2]. Their dispersion is dramatically improved by coating the nanotube surface with different amphiphilic macromolecules such as lipid–polyethylene glycol (PEG) conjugates (Fig. 1b)[8], copolymers and

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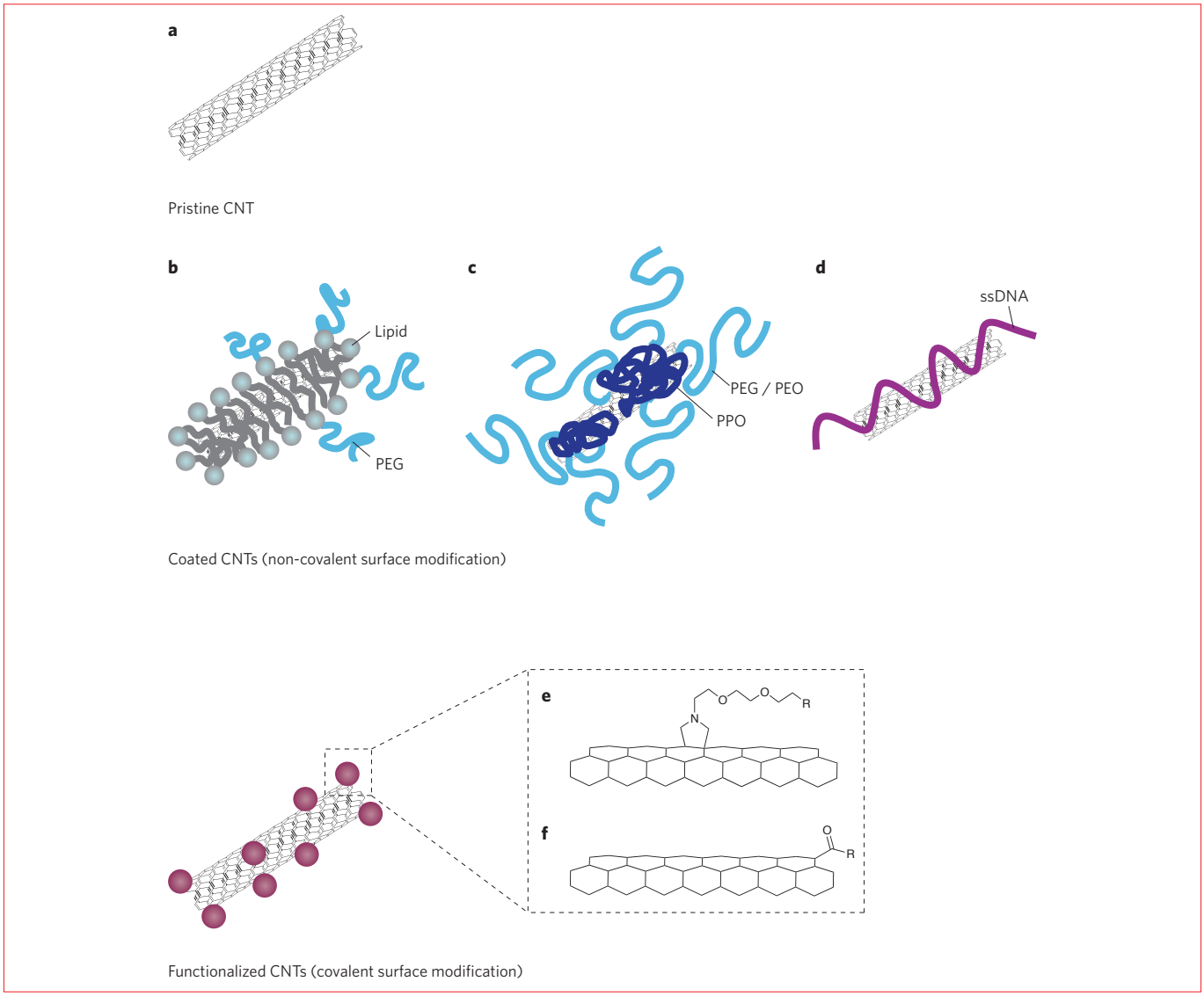


Figure 1 | Types of carbon nanotube studied *in vivo* for imaging and therapy. a, Pristine carbon nanotubes (CNTs) are those without any surface modification. **b**, Lipid-coated nanotubes (primarily single-walled nanotubes) with or without PEGylated lipids and other versions of further modified lipid molecules. **c**, Copolymer or surfactant-coated nanotubes (primarily single-walled nanotubes). PEO is polyethylene oxide; PPO is polypropylene oxide. **d**, Single-stranded DNA (ssDNA)-coated nanotubes (primarily single-walled nanotubes). **e**,**f**, Chemically functionalized nanotubes (both single-walled and multiwalled nanotubes) by 1,3 dipolar cycloaddition (**e**) and by acid oxidation (**f**).

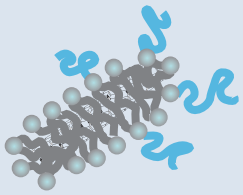
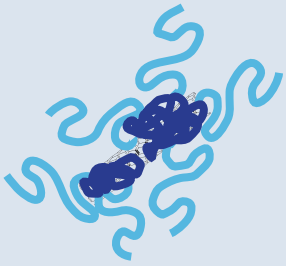
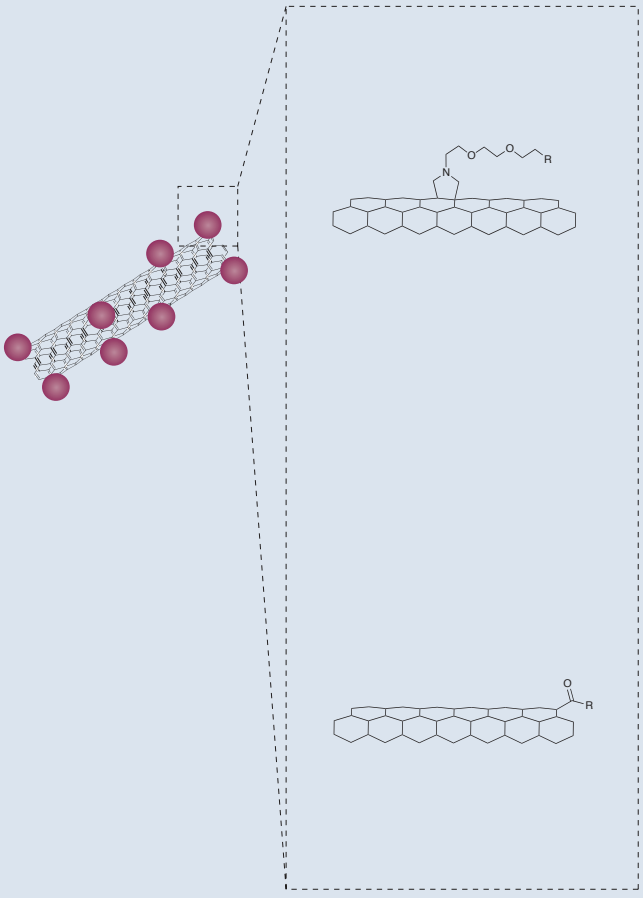
surfactants (for example, Kentera and Pluronic F127) (Fig. 1c)[9,10] and single-stranded DNA (Fig. 1d)[11]. Covalently functionalized nanotubes that have been used in biomedical applications are made of pristine materials that have undergone surface modification by using either cycloaddition reactions to attach ammonium groups (Fig. 1e) or strong acid treatment to generate carboxylic acid groups (Fig. 1f)[7]. Both types of chemical functionalization strategies remarkably improve the water dispersibility of the nanotubes, and at the same time offer a flexible platform for further derivatization.

Of the many strategies developed to modify the surface of nanotubes used in medicine, it is clear that the degree of aggregation and the individualization of nanotube materials in the biological milieu (blood, intraperitoneal, interstitial fluids, and so on) have an important role in their pharmacological performance.

Lessons learnt from preclinical *in vivo* studies

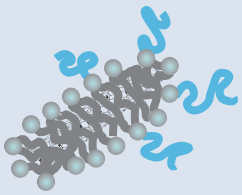
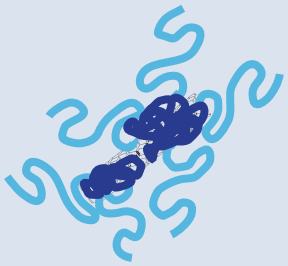
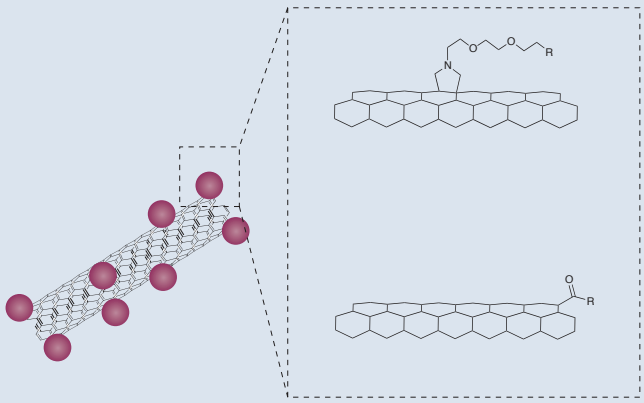
All *in vivo* studies using carbon nanotubes reported so far have used one of the types described above (Fig. 1), and the majority of preclinical models have focused on oncology — making cancer the primary disease candidate for future clinical trials. Carbon nanotubes offer many potential advantages over other types of nanoparticles used or developed for cancer therapy. For example, covalently functionalized carbon nanotubes are able to evade the endosomal compartment and translocate directly into the cytoplasm of different types of cells[12]. Furthermore, their unique physical properties permit efficient electromagnetic stimulation and highly sensitive detection using various imaging modalities. Their large surface area and internal volume also allows drugs and a variety of small molecules, such as contrast agents, to be loaded onto the nanotube. Carbon nanotubes have been used to halt tumour growth in the context of various therapeutic modalities including chemotherapy, hyperthermia and gene silencing. Also, *in vivo* tumour targeting by systemic administration has been described using both coated and covalently functionalized nanotubes. Despite the advances made, comparative *in vivo* studies against other 'benchmark' nanoparticles with a proven clinical record

Table 1 | Preclinical *in vivo* studies using carbon nanotubes.

Type of carbon nanotube*	Biomedical application	Therapeutic/ imaging agent	Preclinical model	Refs
	Cancer imaging (systemic)	Radionuclide (^{64}Cu)	Solid-tumour model	8
	Cancer treatment (systemic)	Paclitaxel	Solid-tumour model	16
	Cancer imaging (systemic)	Intrinsic Raman and ultrasound signal	Solid-tumour model	15
	Cancer imaging (localized)	Hyperthermia by radiofrequency activation	Solid-tumour model	10
	Vaccination	FMDV-derived peptides	Normal mice	21
	Vaccination	(AMA-1) peptide	Rodent malaria	20
	Imaging (systemic)	Radionuclide (^{111}In and ^{86}Y)	Normal mice	17, 18,19
	Cancer imaging (systemic)	Monoclonal antibody (Rituximab)	Bone-marrow-tumour model	22
	Cancer treatment (localized)	Gene silencing (siRNA)	Solid-tumour model	13
	Cancer imaging (systemic)	Radionuclide (^{125}I and ^{14}C)	Normal mice	23, 24
	Cancer treatment (localized)	Gene silencing (siRNA)	Solid-tumour model	26
	Tumour vaccination	Tumour-lysate proteins	Solid-tumour model	25
	Cancer treatment (systemic)	Cisplatin	Solid-tumour model	27

*See Fig. 1 for explanation of carbon nanotube types.

Table 2 | Toxicity studies of carbon nanotubes developed for medical imaging and therapy.

Type of carbon nanotube*	Route and method of detection	Organ accumulation and excretion route	Model and adverse effects	Refs
	Intravenous • Raman spectroscopy • Optical microscopy (Hematoxylin–eosin histology)	Liver accumulation Slow faecal excretion	Normal animals (mouse) No adverse effects	36,37
	Intravenous • Near-infrared fluorescence • Mass spectrometry (^{13}C)	Liver accumulation No excretion studied	Normal animals (rabbit and mouse) No adverse effects	34,35,38
	Intravenous • Optical microscopy (Hematoxylin–eosin histology) • Electron miscrosopy (urine, kidney sections)	Limited organ (liver and lung) accumulation Urinary excretion	Normal animals (mouse and rat) No adverse effects	19,40,41
	Intravenous • Electron microscopy (liver sections)	Liver and lung accumulation Slow faecal and urinary excretion	Normal animals (mouse) No adverse effects	23

*See Fig. 1 for explanation of carbon nanotube types.

(such as liposomes) are lacking, with only a single such study recently reported[13]. Table 1 describes all the *in vivo* studies published so far according to the type of carbon nanotube used.

The strategy of coating nanotubes with lipopolymer molecules has been pioneered by the Dai group[14], and more recently explored as a delivery system for cancer therapy. According to this strategy, nanotubes are surface-coated with the lipopolymers to achieve aqueous dispersibility. The distal end of the PEG chain is generally used to link all other molecules, such as targeting moieties (for example, the RGD sequence)[8,15], radionuclides (^{64}Cu)[8] and drugs (Paclitaxel)[16]. Tumour accumulation has been reported for nanotubes coated with a phospholipid–PEG–RGD peptide conjugate, and imaged by using the photoacoustic and Raman signatures of the carbon nanotubes. In the only therapeutic study using lipopolymer-coated nanotubes, the water-insoluble drug Paclitaxel was conjugated at the tip of the PEG chain and reportedly led to relative tumour volume suppression in a murine cancer model[16], even in the absence of a targeting ligand. However, direct comparisons with approved therapeutics based on alternative drug delivery systems (for example, Abraxane and Doxil) are needed to establish improved efficacy and to illustrate the advantages offered by carbon nanotubes.

Another strategy using coated carbon nanotubes to treat cancer has been based on the capacity of nanotubes to convert electromagnetic radiation into heat. Carbon-nanotube-mediated hyperthermic treatment of tumour tissue by radiofrequency activation after intratumoural administration of pristine tubes coated with Kentera (a polymer based on polyphenylene ethynylene) has been described[10]. Even though different radiofrequency pulse sequences seemed to ablate cancer cells when the nanotubes were injected directly into the tumour, no quantitative efficacy data has yet been reported with this methodology. More *in vivo* studies using nanotubes to achieve established solid tumour ablation by hyperthermia are expected in the near future by different groups.

Chemical functionalization of carbon nanotubes offers the advantage that the functional groups, further modified with a therapeutic, targeting or imaging molecule, are stably attached on the nanotube backbone, and therefore avoid the risk of macromolecule desorption or exchange with serum proteins and other blood components following administration. As can be seen from Table 1, a wider range of therapeutic models have been explored using functionalized nanotubes, compared with coated ones.

One type of functionalized carbon nanotube explored in biomedical applications is based on the covalent surface modification methodologies developed by our laboratories. These nanotubes have now been studied for various applications, including imaging using various radionuclides (^{111}In, ^{86}Y)[17–19], vaccination using immune-activating peptides (FMDV-derived peptide, AMA-1 peptide)[20,21], and cancer therapy using monoclonal antibody (Rituximab)[22] and small interfering RNA (siRNA)[13] therapeutics (using two different human-tumour models of lymphoma and lung cancer, respectively). The lung cancer study by Podesta and colleagues[13] indicated that carbon nanotubes may be more effective in prolonging the survival of tumour-bearing animals, compared with cationic liposomes, when administered locally (intratumorally) — presumably owing to their more facile translocation into the tumour cell cytoplasm. Another type of functionalized nanotube that has been explored *in vivo* is based on the chemical modification of carboxylic acid groups at the nanotube tips and sidewalls introduced after strong acid treatment. These nanotubes have also been studied using tracing radionuclides (^{125}I, ^{14}C)[23,24], as well as for cancer treatment using vaccination strategies (tumour-lysate proteins)[25], siRNA gene-silencing (TERT gene)[26] and chemotherapy (cisplatin)[27]. All of these studies have reported tumour growth delay with no adverse reactions.

Overall, the *in vivo* studies performed so far indicate that carbon nanotubes could potentially have a significant contribution to efficacious treatment of diseases. However, even in the case of cancer therapy where most *in vivo* studies have been attempted, it is extremely difficult to correlate and compare the data generated. Different tumour models, administration routes, nanotube doses, ways to describe tumour-volume data and therapeutic modalities (chemotherapy, hyperthermia, gene silencing, immunotherapy) have been used. Moreover, the lack of comparisons with well-established alternatives (for example, liposomes, polymers) in the design of most studies prevents a clear determination of the advantages and limitations of carbon nanotubes compared with existing technologies.

Current status of carbon nanotube toxicity in biomedicine

Generally, very limited toxicity data exist on the types of carbon nanotubes described above that have been studied for medical applications. The majority of toxicological studies have focused on pristine non-functionalized nanotubes that are dispersed in different buffers with a huge variation in the quality of the dispersions. These studies mostly consider nanotube exposure in the context of occupational and environmental health, and are mainly designed by administration of nanotubes through the pulmonary route (for example, intratracheal, intranasal)[28–31]. A few of these studies have indicated serious risks associated with carbon nanotube exposure, however, the material, dosing and administration routes used are not directly relevant to those explored for medical applications. There is evidence based on some toxicological studies that prolonged accumulation of long (>10 μm), rigid, pristine nanotubes in tissues may be associated with health risks such as carcinogenesis and should be avoided[32,33]. These studies assessed the risk for mesothelioma formation following exposure (through intraperitoneal injection) and, even though they have their limitations, they have generated serious discussion regarding the overall toxicological profile of uncoated and coated nanotubes when these consist of long pristine materials.

In Table 2, the *in vivo* studies that address some of the toxicity considerations of the nanotubes explored for therapy and diagnostics are described. These reports aim mainly at elucidating the tissue distribution, organ accumulation, excretion route and any physiological abnormalities caused from intravenous administration of carbon nanotubes, using a variety of detection methodologies. An overall conclusion from these studies is the absence of acute or other adverse reactions between one week and three months following nanotube administration. However, none of these studies were designed with a toxicology model or specific mechanism under consideration. This is needed to determine the overall toxicity profile of carbon nanotubes — particularly in comparison with known toxins and other nanoparticle types — and to elucidate structure–function relationships that will help engineer effective and safe nanotube materials.

Among the studies examining the lipopolymer- or surfactant-coated nanotubes, there is evidence that: 1) some degree of desorption of the molecules coating the nanotube backbone occurs *in vivo* on administration[34]; 2) the liver is the predominant organ where coated nanotubes tended to accumulate[34–37]; and 3) new methodologies based on the Raman signature of carbon nanotubes can help determine the tissue accumulation of coated nanotubes[37,38]. Prolonged (months-long) accumulation of coated nanotubes in the liver has been described[36,38], with slow excretion mainly through the bile and the faecal pathway[37]. Although these studies confirm that carbon nanotubes are not biodegradable and are not metabolized by the liver, as is the case with small molecules or other delivery systems such as liposomes, the overall toxicological implications from such *in vivo* behaviour remains unresolved.

Shortening of nanotubes (to <1 μm) from chemical treatment may be responsible for the improved toxicological profile of functionalized nanotubes that have been used in *in vivo* studies[39]. The excretion of a considerable fraction of intravenously administered functionalized nanotubes has been shown independently by two groups[18,19] to be taking place by means of rapid translocation through the glomerular filter[40]. This effect is thought to depend mainly on how well the nanotubes are individually separated in the administered functionalized nanotube dispersion; the higher the number of individually separated nanotubes, the higher their urinary excretion and the lower their accumulation in tissues. However, in view of medical applications based on systemic administration, the rapid excretion of functionalized nanotubes poses the challenge of maintaining the injected material within the blood circulation for longer periods of time to allow targeting and interaction with diseased tissue. The type and degree of chemical functionalization on the nanotube surface is considered critically important in determining tissue accumulation and excretion[41], particularly for functionalization chemistries that may not lead to shortened length and dispersions of individual nanotubes. Overall, there is good agreement between studies that urinary excretion of functionalized nanotubes and low organ accumulation is thought to be due to shortened and better dispersed nanotubes[18,19,24].

Adverse reactions following intravenous administration of the types of nanotubes studied *in vivo* for medical applications have not been reported so far. There is agreement among studies using coated nanotubes that indicate mainly liver accumulation whereas, studies using functionalized nanotubes show significant urinary excretion that is strongly dependent on the fraction of individualized nanotubes in the injected dispersion. The route of administration is also a determining factor for tissue accumulation and possible toxic responses. More studies exploring different routes are therefore needed. Moreover, toxicological studies using the types of nanotubes explored for therapy and diagnostics should be done in collaboration with toxicologists, using toxicology models to determine whether the mechanisms of activation that are described for pristine nanotubes can be alleviated by coating or covalent functionalization.

Opportunities and challenges

As clearly illustrated in the sections above, intense efforts and interest have been invested by a growing number of laboratories to explore carbon nanotubes in medicine. This is based on the range of advantages that carbon nanotubes may offer over alternative systems in therapeutic or diagnostic applications. However, it is

Box 1 | Carbon nanotubes in medicine: questions and answers.

Q: Are carbon nanotubes really useful in medicine?
A: Proof-of-principle studies using *in vitro* and *in vivo* models indicate that carbon nanotubes may help treat various diseases (cancer, AIDS, malaria, metabolic diseases), but only one study[13] so far has reported a therapeutic outcome (prolonged survival) in a preclinical human-tumour model.

Challenges:
- Nanotubes may not treat disease more effectively than established technologies.
- The risk-to-benefit ratio offered by nanotube-based therapeutics and diagnostics may weigh towards the risk.

Opportunities:
- The possible contributions of nanotubes in medicine are almost unlimited and wide-ranging, from advanced delivery systems, electrodes and biosensors to probes for diagnostics and treatment-monitoring devices.

Q: Can carbon nanotubes help cure cancer?
A: It is too early to determine because only early-stage preclinical studies are available and at present there are no clinical studies underway.

Challenges:
- Unrealistic claims and expectations of tumour-targeted carbon nanotubes could cause a backlash.
- Carbon nanotubes may offer a poor risk-to-benefit ratio in oncology settings.

Opportunities:
- Cancer is a challenging disease. Patients and clinicians will be keen to explore new therapeutic concepts such as carbon-nanotube-based treatments, as long as these offer benefits.

Q: Can carbon nanotubes act as 'nanorobots' in the blood stream?
A: Injectable nanorobots have not yet been developed, and active navigation of nanoparticles in the blood stream has not been achieved. Therefore, nanotubes can neither act as nanorobots nor be navigated in the blood stream.

Challenges:
- The association between the fear of 'self-replicating nanorobots' and carbon nanotubes.
- Nanotubes as components of nanorobots and other nanomachines that may accumulate and intoxicate the body.

Opportunities:
- Carbon nanotubes can act as components of nanofabricated machinery and offer tremendous capabilities — for example in wireless communication and monitoring between the patient and the clinician.

Q: Are carbon nanotubes biocompatible and what does that mean?
A: The term 'biocompatibility' is commonly ill-defined. In most cases, it implies the ability to interact with the biological milieu without adverse reactions. Chemically functionalized nanotubes have been shown by many groups to be more biocompatible (no immune or acute inflammatory responses)[21] than pristine nanotubes.

Challenges:
- Some types of carbon nanotubes or their impurities may accumulate in the body, leading to deposits that may cause unwanted side effects in the long-term.

Opportunities:
- New carbon nanotube materials and strategies to make them biocompatible are actively pursued.

Q: Are carbon nanotubes toxic?
A: Toxicity depends strongly on the type of nanotube, the dose, the route of administration and the tissue that is most affected. Pristine nanotubes have been shown to activate various mechanisms associated with toxicity, however these effects are shown to be remarkably reduced when properly functionalized with chemical groups. So far, no *in vivo* study using the types of nanotubes developed for medical purposes has reported adverse effects, however, studies with established toxicology models are much needed.

Challenges:
- The structural similarity and association between carbon nanotubes and the carcinogenic asbestos fibres.
- Absolute statements such as 'carbon nanotubes are toxic' can be very damaging.

Opportunities:
- Systematic toxicological studies of carbon nanotubes to make them the 'standard' fibrilar nanomaterial.
- Need to determine the extent of toxicological risks from using nanotubes, their doses, types and route of administration.

Q: Should regulatory authorities restrict the use of carbon nanotubes?
A: No regulatory authority around the world has recommended or enforced a ruling on restricted use of carbon nanotubes. For medical applications, the very strict regulatory and authorization framework for new drugs is considered adequate.

Challenges:
- Restrictions posed on the use of carbon nanotubes based on premature evidence of adverse effects and unsubstantiated 'nanofear'.

Opportunities:
- Clinical development of a carbon-nanotube-based therapeutic or diagnostic will act as a proof-of-principle nanomedical product.

important to recognize that translating carbon nanotubes from an interesting nanomaterial to an effective pharmaceutical product is still at the nascent stages of development. At the same time, blinded 'fear' of the risks associated with any new nanomaterial should not impede investment and investigation.

Common questions regarding the use of carbon nanotubes in medicine are addressed in Box 1. The answers to these questions are based on peer-reviewed published data. Interestingly, each of these questions raise a set of challenges and opportunities associated with the use of nanotubes in the diagnosis and treatment of diseases.

The use of carbon nanotubes in medicine, as first suggested a few years ago, has already resulted in a number of studies using various *in vivo* models. Many more studies from a wider spectrum of laboratories and applications are expected in the immediate future as more disease-oriented groups will be motivated to study the utility of carbon nanotubes in their models. Moreover, there are other biomedical applications using carbon nanotube materials that are also being explored — mainly in the engineering of devices such as electrodes (for example, neurological tissue stimulation)[42], scaffolds for tissue regeneration (for example, orthopaedic and dental implants)[43,44] and *ex vivo* biosensors[45] — which were deemed

outside the scope of this article. Such applications, particularly those that will not require interaction with living tissue (such as biosensors) may indeed be developed sooner than others. However, irrespective of application and type of carbon nanotubes used, their clinical development will be determined by solid evidence on the advantages offered compared with established technologies.

In this article we have attempted to highlight the progress made so far, focusing only on nanotubes that have been explored for medical imaging and therapeutics using preclinical models. It is now apparent that the use of carbon nanotubes in therapy and diagnosis of diseases will be determined by the systematic determination of the benefit they offer against the risk they pose. The biomedical and toxicological investigations of these materials will have to take place simultaneously to transform carbon nanotubes into a clinical reality.

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