Towards nanomedicines: design protocols to assemble, visualize and test carbon nanotube probes for multi-modality biomedical imaging

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Nanomedicine is an interdisciplinary field, still in its infancy, where an accurate scientific assessment of potential risks and benefits is urgently needed, as is the engagement of end users and the public in this facet of the nanotechnology debate. There is increasing interest in improving our understanding of the interactions between nanomaterials and living systems, with regard to both the underlying chemistry and the physics of effects on the nanoscale. Ultimately, such knowledge promises new vistas for designing the ‘smart’ medicines of the future, of which targeted personalized drugs are the holy grail. Imaging and therapeutic components, including metallic radioisotopes, semiconductor quantum dots and magnetic materials, may be used to construct ‘nanocarriers’ (by encapsulation or conjugation) by rapid and simple (covalent and supramolecular) chemistry. The biomedical functions of the resulting materials are as yet largely unexplored. Encapsulation in nanocarriers could achieve delivery of the reagents (imaging and therapeutic drugs) to the sites of action in the body, while minimizing systemic toxicity and enzymatic degradation. These functional systems have the potential to become a general solution in drug delivery. Here we review recent developments concerning the applications of nanoparticles, including carbon nanotubes, as synthetic scaffolds for designing nanomedicines. This article will also focus on how understanding and design at the molecular level could help interdisciplinary teams develop research towards new diagnostics and therapeutics both in the short and the long term.

Keywords: optical imaging; positron emission tomography; single photon emission computed tomography; multi-modal imaging; nanoparticles; carbon nanotubes

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1. Introduction

Molecular imaging and applications of nanotechnology in healthcare are interlinked research areas, currently of high strategic importance to the UK and worldwide (Royal Society of Chemistry 2003). In particular, the areas of diagnostic imaging such as radiopharmaceutical imaging are crucial to healthcare and the healthcare industry.

This new discipline uses probes known as biomarkers to characterize and measure biological processes at the molecular level, also aiding the visualization of cellular function in humans and other living systems without perturbing/damaging them (figure 1). This emerging technique is of particular interest in the diagnosis of conditions such as cancer and neurological and cardiovascular diseases. The development and improvement of the ideas and methodologies underpinning molecular imaging has also enhanced the possibility of earlier and more precise diagnosis of various degenerative diseases. The most common molecular imaging modalities include molecular magnetic resonance imaging (MRI), optical bioluminescence/fluorescence, targeted ultrasound, single photon emission computed tomography (SPECT) and positron emission tomography (PET; Massoud & Gambhir 2003). Methods include chemically synthesized probes to target molecular markers at different stages of diseases. These techniques can give whole-body readouts of molecular changes with the onset and progression of different diseases that are much more reliable than those obtained from in vitro/ex vivo bioassays. Non-invasive detection of various molecular makers can allow for precise and pre-symptomatic diagnosis and early treatment of many diseases. At present, only MRI has the resolution required to accurately locate transplanted cells in adult mammals (Dunning et al. 2004). PET is also emerging as one of the primary tools for cancer diagnosis and monitoring the effects of therapy (National Cancer Research Institute 2007).

Optical fluorescence imaging does not have sufficient penetration in tissue more than a few millimetres thick, and is difficult to quantify (Jennings & Long 2009).

To date, no single molecular imaging modality is sufficient to gain all the necessary information both in vitro and in vivo. PET has very high sensitivity but poor resolution, although this continues to improve as image processing methods advance (Jennings & Long 2009). Both PET and MRI require the imaging agents to accumulate in tumours without loss of the active species en route to target. The combination of multiple molecular imaging techniques can offer synergistic advantages over single modality imaging techniques (optical or PET alone).

Cancer treatment currently uses intravenous immuno-targeted therapeutics that have a very low rate (one in 10,000) of reaching their therapeutic targets in vivo. Several drug delivery and targeting strategies have been developed based on supramolecular capsules, polymers or lipid coat carriers (Caulder & Raymond 1999; Swiegers & Malefetse 2001; Davis et al. 2002; Hof & Rebek 2002; Hof et al. 2002; Zielhuis et al. 2006; Jennings & Long 2009). Nanosystems in clinical use are few, such as liposomes, for breast and ovarian cancer, and Kaposi’s sarcoma. Gadolinium or iron oxide nanoparticles are entering clinical trials as MRI contrast agents (CAs) or lipid nanoparticles for ultrasound imaging, while paclitaxel-loaded albumin nanoparticles have been approved by the US Food and Drug Administration. For high-resolution brain imaging, the state of the art is largely limited to 18F-labelled glucose (18-FDG) for PET scans. The usefulness...
of these therapeutic or imaging agents is hampered by their lack of specificity in delivering the active molecules to the target (Peer et al. 2007; Bianco et al. 2008). Moreover, methods that would administer imaging reagents very close to target, avoiding cellular surveillance systems are lacking, particularly where delivery involves crossing the brain–blood barrier (Aulton 2007). For example, technology for the simultaneous imaging and therapy of brain diseases is just emerging, with only a handful of studies having attempted it (Liu et al. 2008a; Regina et al. 2008; van Kasterena et al. 2009).

We have been interested in developing multi-modal imaging agents (‘all-in-one’ probes) in which PET and optical probes are combined in a single system, such that they have identical pharmacodynamics (Pascu et al. 2009). This research has the potential for rapid impact: for example, PET-imaging tracers are administered in minute amounts (nM–pM), so the toxicological testing stages can take place rapidly, allowing these diagnostics to progress quickly from the chemical bench to the clinic (Bergstrom et al. 2003).

Interdisciplinary research ranging from designing new imaging probes (with complete functionality and full physico-chemical characterization) to their delivery, uptake and biodistribution testing in cells, radiolabelling (with long-lived radioisotopes, such as, for example, $^{64}$Cu for PET) and formulation of the optimized probe for in vivo evaluation (e.g. by micro-PET) are urgently needed. There is particular interest in the intracellular behaviour of target-specific imaging agents, as this might be used to inform their future design. Our understanding of the interaction between nanoscale materials and cells is limited, and hence using in vitro methods to assess their potential in diagnostic medicine is empirical at best.

The objective of newly designed drug delivery systems using nanodimensional constructs as scaffolds is to transport chemically intact medication to specific diseased cells (Allen et al. 1998). The (imaging) drug is trapped in a stable container (encapsulated) to protect it from degradation en route to the target, and delivered to the disease site (its final target), where it is released via chemical or external triggering (Pernant & Reynolds 2000). The significance of nanoscale therapeutic efficiency and advanced selectivity has been highlighted by its use in submicrometre (1–100 nm) colloidal drug delivery vehicles (Jain 2005; Lamprecht 2007). The key advantages of such systems are high stability, sizeable cavity volume, capability of encapsulating hydrophilic and hydrophobic molecules and ability to be dosed orally (Gelperina et al. 2005; Muchow et al. 2008). The size of these systems leads to enhanced bioavailability of the entrapped drug molecule, reduced systemic toxicity (Kabanov & Alakhov 1997) and selective drug targeting of particular tissues and organs. This is a consequence of intricately programmed ligand density on the nanoparticle surfaces that actively binds to receptors overexpressed on the surface of diseased cells, or within tumour microenvironment, resulting in deferred biodegradation, intracellular drug delivery (Couvreur & Vauthie 2006) and eventually tailored cancer treatments (Ferrari 2005; Ljubimova et al. 2008). The economic appeal of these systems lies with the principle of reduced dosage frequency as a consequence of enhanced targeting precision linking into personalized medicine (Allen et al. 1998; Lamprecht 2007). The development of multi-functional nanoprobe through iterative rounds of structure-led design and synthesis, and in vitro biological screens, leading to in vivo screens is the focus of attention of a number of research
Figure 1. Schematic representation of an imaging drug for targeted delivery.

groups worldwide. Currently, the only nanoparticle drug carriers approved for clinical use are synthetic polymers (Duncan 2006) and liposomes (Vasir et al. 2005; Couvreur & Vauthie 2006). However, there are other members of the nanocarrier family that have been explored for application as drug carriers, including carbon nanotubes (CNTs), dendrimers, micelles, polymers, nanoshells, liquid crystals and nanocapsules (as host–guest complexes). Among the main types of nanoparticles, those illustrated in figure 2 (e.g. polymers, micelles, quantum dots, iron oxide and gold nanomaterials) are of particular interest.

Polymeric nanoparticles show promise owing to selective uptake in tumour cells, a property that results from the enhanced permeability and retention effect, in addition to their stability, dissociation and clearance rates (Grindy & Prud’homme 2009; Park et al. 2009).

Micelles typically consist of a hydrophilic head with a lipophilic tail (figure 2c), and have traditionally been employed to deliver pharmaceutical agents of poor solubility. They have recently been used to incorporate magnetically active cores for dual MRI and fluorescence imaging. Their functionalization enabled selective targeting paving the way for personalized medicine (Mulder et al. 2009).

Drug delivery devices based on metallic nanoparticles are being developed to be stable, highly specific and with optimum clearance and drug release in vivo (Grindy & Prud’homme 2009). These have potential for delivery of low-solubility agents as well as for providing pharmaceuticals with protection from the immune system (Park et al. 2009). A tuneable selectivity can be achieved via supramolecular or covalent functionalization. Metal nanoparticles, such as Ag and Au, have been demonstrated to act as highly effective vehicles for delivery of macromolecules (Thomas & Klibanov 2003; De et al. 2008). Both iron oxide (Babincova et al. 2000) and gold nanoparticles (Ghosh et al. 2008) have been shown to provide non-toxic carriers for drug and gene delivery applications with essentially no measurable toxicity index LD₅₀. Gold nanoparticles of small diameters (less than 50nm) can form stable complexes with a variety of biomacromolecules that can efficiently penetrate cell membranes and release biomacromolecules within the cell with extremely low toxicity.

Silica-coated nanoparticles represent a well-studied area, largely owing to their ease of chemical incorporation and biocompatibility, which can be enhanced by coating with lipids, polyethylene glycol chains or polymers (van Schooneveld et al. 2008; Mulder et al. 2009). Silica mesostructures have been demonstrated to be biocompatible and suitable for delivery of protein and DNA molecules.
Figure 2. Selection of nanoparticle scaffolds used as design elements towards nanomedicines for detection and treatment of cancer: (a) single-walled carbon nanotube, (b) dendrimer, (c) micelle, (d) polymer, (e) liposome, (f) fullerene/nanoshell, (g) liquid crystals, (h) nanocapsule. Adapted from Peer et al. (2007) and Letchford & Burt (2007).

Figure 3. Quadruple-modal imaging: (a) bioluminescence, (b) micro-PET, (c) MRI and (d) fluorescence imaging. Adapted from Hwang et al. (2009).

A relatively low clearance rate, which may cause high toxicity, has led to recent developments, including smaller silica nanoparticles (diameters of 3.3 and 6 nm) that showed reduced interaction with serum proteins (Burns et al. 2009). Silica-coated nanoparticles for dual-modality imaging...
fluorescence/MRI applications have been reported, and further advances in this field have made them excellent candidates for future tri-modal imaging when PET agents have been incorporated simultaneously (Mulder et al. 2009). Quantum dots are of increasing importance in nanomedicine, thanks to their broad excitation with narrow emission spectra and have been engineered to signal drug delivery (Shashkov et al. 2008; Park et al. 2009). pH-responsive and sensing fluorescent nanoparticles have recently been reported and are particularly interesting for cancer imaging owing to the acidity of cancer cells (Khashab et al. 2009; Schulz et al. 2009).

Current trends are moving towards the design of multi-modal imaging probes, with the most frequent combination being fluorescence and MRI, owing their high sensitivity and anatomical resolution, respectively. PET and SPECT imaging methods are also of interest given their high in vivo sensitivity, which is not limited by tissue depth and could reduce dosage by two to four orders of magnitude with respect to small-molecule-based alternatives (Chen et al. 2008; Devaraj et al. 2009). Combination of ‘smart’ fluorescent probes with other modalities such as MRI/PET/SPECT is an exiting prospect for future imaging applications.

To overcome the largely qualitative historical use of quantum dots in vivo, Chen et al. designed a dual-function near-infrared fluorescent (NIRF) PET molecular probe. Quantum dots were radiolabelled with $^{64}$Cu, functionalized and found to successfully target the U87MG tumour in addition to a good correlation between NIRF and PET (Chen et al. 2008).

There has been significant research on core–shell nanoparticles, based on iron oxides, which, owing to their superparamagnetic nature, have been used as scaffolds for a number of recently developed multi-modal imaging probes. Magnetic nanoparticles have been used for targeted delivery to the brain for MRI (van Kasterena et al. 2009). Dual-modal MRI/PET nanoparticles have been reported by Lee et al. involving polyaspartic acid iron oxide nanoparticles conjugated to arginine–glycine–aspartic acid (RGD) peptide for integrin targeting and 1,4,7,10-tetraazacyclododecan-1,4,7,10-tetraacetic acid (DOTA) chelation of $^{64}$Cu for PET (Lee et al. 2008). MRI and PET both indicated selective uptake and offer the potential for higher accuracy and earlier detection of cancer. Fluorine-18 cross-linked dextran iron oxide is a modified trimodal nanoparticle with high sensitivity for PET imaging. This has been reported by Devaraj et al. to allow ‘all-in-one’ MRI, PET and fluorescence molecular tomography (Devaraj et al. 2009). Functionalization with $^{18}$F, facilitated by ‘click’ chemistry, was reported to be facile and significantly reduced the detection threshold. Chen et al. have also reported an iron oxide triple-modality PET, MRI and fluorescence nanoparticle, demonstrating tumour targeting in glioblastoma cells (Xie et al. 2009). Additionally, a quadruple-modality imaging probe, which can be detected by fluorescence, bioluminescence resonance energy transfer (BRET), PET and MRI, was reported (figure 3; Hwang et al. 2009). The probe consisted of a cobalt–ferrite nanoparticle (for MRI), incorporating rhodamine (for fluorescence) and conjugated to luciferase (for bioluminescence) and a chelating group that was tagged with $^{68}$Ga (a generator-produced PET radionuclide). Each imaging modality displayed dose dependence in vivo. Further functionalization with targeting groups could enhance the potential for effective delivery to tumours.
The emerging field of nanomedicine thus has the potential to impact greatly on the design and administration of new-generation imaging reagents and could provide a general means to drug delivery for imaging and treatment of tumours.

2. General design and synthesis considerations for biocompatible CNT-based nanomedicines

Biocompatible single-walled carbon nanotubes (SWCNTs) are likely to be particularly suited for use as multi-functional nanocarriers owing to their physico-chemical characteristics once challenges regarding advanced purification, functionalization and size consistency are overcome. CNTs have been shown to act as nanodimensional ‘scaffolds’ onto which can be attached a large number of molecules (e.g. antibodies, drugs and/or optical imaging reporter molecules).

To date, little is understood about how to most effectively assemble and deliver in a targeted manner CNT-based multi-modal CAs. This would require a precise understanding of their tissue biodistribution, their mechanisms of uptake in cells and the biological effect that these agents may have on the targeted cells. At present, the more basic aspects of carbon nanomaterial imaging probe design, such as synthesis and physical characterization, remain a matter of intense investigation (Kostarelos 2008).

A number of research groups have explored elements of synthetic design to provide the nanocarriers with groups modulating both their hydrophilicity and lipophilicity to ensure their biocompatibility and integrity, as well as their facile recovery from the body post-treatment. Water-soluble medicines tend to be excreted through the kidneys, whereas fat-soluble compounds tend to be excreted through the liver and gall bladder into the intestine (Ferrari 2008). To date only a small number of systematic in vitro studies have been carried out to determine the influence on cell toxicity mechanisms of the functionalities incorporated in these imaging probes, and a toolkit aiding their rational design has not yet been fully devised.

As such, the changes induced within cells treated with carbon nanomaterials remain a matter of much debate (Kostarelos et al. 2007, 2008). Systematic studies could imply devising hierarchical protocols that range from design and synthesis to observing molecular imaging probes in conditions mimicking physiological environment (pH and temperature). Successful surface analysis determinations (atomic force microscopy (AFM)/fluorescence imaging in cells/scanning electron microscopy) could narrow down the search for the most stable imaging probe in vitro and help rationalize the uptake, compartmentalization and mechanisms of interaction with cells and organelles, thus providing feedback to inform probe design.

Detailed attention is currently paid to purification of the CNT scaffolds to eliminate toxic catalytic particles and other carbonaceous nanostructures (Tobias et al. 2006). Established procedures involving treatment with steam yield extremely clean SWCNT outer surfaces, as demonstrated by Raman and high-resolution transmission electron microscopy (HRTEM) physical characterizations (Tobias et al. 2006). The ultimate aim is to fulfil consistently the requirement of homogeneous dimensions: recent attempts include standard cutting procedures.
and chromatographic separation (Sano et al. 2001) and/or microfluidic chips (Shin et al. 2008) aiming towards consistent lengths and shapes. SWCNTs possessing narrow, precisely engineered, diameters (e.g. ranging 1.3–1.5nm) are of ideal length (lower than 100nm; Peer et al. 2007; Bianco et al. 2008) and may be isolated by readily available techniques. The ultimate aim is that such methods will guarantee a high dimensional reproducibility with no defects. CNTs in general are well characterized both experimentally and theoretically, and a variety of methods allow their advanced purification to levels suitable for biomedical imaging, although the batch-to-batch reproducibility is still a matter of concern (Kostarelos et al. 2009).

A number of attempts were employed to externally functionalize CNTs for various purposes (Balasubramanian & Burghard 2004; Coleman 2007, 2008; Salzmann et al. 2007, 2008; Yang et al. 2007). There is increasing evidence that appropriately functionalized SWCNTs have low toxicity while intracellular: in recent works, functionalized CNTs, as opposed to unfunctionalized ones or to the long, rigid multi-walled CNTs (MWCNTs; Porter et al. 2007; Poland et al. 2008), were shown to be preferentially taken up into tumours with minimal toxic effects (Kam et al. 2004; Cherukuri et al. 2006; Ali-Boucetta et al. 2008; Kostarelos 2008; Prato et al. 2008; Salvador-Morales et al. 2008; Zavaleta et al. 2008). A number of approaches were adopted to create the biocompatible surface coatings for nanoparticles including the supramolecular wrapping of the nanotube surface and the grafting of biocompatible polymer chains by the tailor-made incorporation of biocompatible polymers (Salzmann et al. 2007, 2008; Lahiri et al. 2009). Specially designed synthetic protocols need to ensure the highly optimized biocompatible coating of the nanoprobe. Complete surface functionalization is believed to reduce the risk of partly losing the biocompatible coating in the biological environment, and consequently exposing the body to the biologically incompatible surface of the uncoated CNTs (Porter et al. 2007; Poland et al. 2008). In both approaches (covalent or supramolecular derivatization), the CNT-based probes are normally physically characterized after each derivatization step to understand their physico-chemical characteristics, and assess loading and strength of binding of multiple components.

Chemically modified CNTs have already been explored for their potential in a number of molecular imaging modalities, including MR, optical, SPECT and PET imaging. The use of CNTs as intrinsic part of the nanotheranostic design has a major advantage over other drug delivery strategies (based on polymers or lipid coat carriers or supramolecular capsules) owing to the high stability with respect to loss of the imaging reagent: this may be prevented by its encapsulation inside the nanotube. Using nanotube carriers would enable many imaging agents to reach the target efficiently and simultaneously, while keeping the doses necessary to obtain an image extremely low. Radiolabelling (for PET or SPECT) has only been reported a few times (Liu et al. 2007; Lacerda et al. 2008a) and almost exclusively on the outer SWCNT surface, where the isotopes can be lost from the carriers, while their attachment procedure cannot be easily generalized. Studies to date achieved only limited functions and the processes triggered by these in living systems are not fully understood. The isotope radiolabelling (‘hot’ chemistry) of SWCNTs by endohedral filling methods needs to be demonstrated and optimized before the most promising integral probes may be imaged in vivo by micro-PET.
Figure 4. Example of design elements incorporated into a traceable nanomedicine scaffold: HRTEM of (a) metal halide inside SWCNTs (with arrows) and (b) supramolecularly wrapped SWCNTs; (c) porphyrin tripod, tailor-made fluorescent agent and (d) AFM imaging of a porphyrin-coated SWCNT. Adapted from Pascu et al. (2008).

Design principles for the assembly of the CNT based imaging probes may involve:
— encapsulation of imaging agent (metallic ions for MRI/PET) within the CNT cavity or its appendage on the CNT surface;
— rendering the nanotubes biocompatible via supramolecular/covalent wrapping and their subsequent derivatization with chemotherapeutic payloads and/or targeting units; and
— attaching/incorporating fluorescent tags for tracking in cells, as an additional assembly step or intrinsic to the supramolecular/covalent wrapping.
(a) Imaging agent encapsulation

High-yield filling of SWCNTs with inorganic compounds (i.e. leading to continuous crystalline filling as in figure 4a) or organic molecules has been demonstrated (Thamavaranukup et al. 2004; Bendall et al. 2006; Huh et al. 2006; Ilie et al. 2006; Tobias et al. 2006; Shao et al. 2008). In proof-of-principle experiments on endohedral filling methods from molten salt routes, the ends of the nanotubes are expected to close under the reaction conditions used, which involve thermal annealing of the CNTs. For in vivo nanoSPECT applications, filling SWCNTs with the radioemitter Na\textsuperscript{125}I form the molten phase has just been demonstrated (Hong et al. 2010). Opened SWCNTs (Tobias et al. 2006; Shao et al. 2008) could be exposed to aqueous solutions of simple metal salts, such as CuX\textsubscript{2}, GdX\textsubscript{3} (X = halides such as Br\textsuperscript{−} or Cl\textsuperscript{−} or hydroxyl groups), of relevance for imaging methods. Although the filling of SWCNTs with metal species from saturated solutions is well established (and this method may be extrapolated to MRI-active species), to date it is not known if filling can be also achieved at the low concentrations in which metallic radionuclides suitable for PET are generated (N.B. \textsuperscript{64}Cu and other cyclotron-generated ions for PET imaging are typically in 10\textsuperscript{−6} to 10\textsuperscript{−8} M concentrations). It would be of interest to investigate the filling efficiency from a solution as the concentration of the metal salt is reduced (by using both ‘cold’ and hot chemistry). Any potential leaking of the encapsulated species from open SWCNTs could be prevented by the implementation of a recent encapsulation method where C\textsubscript{60} molecules are used for ‘corking’ (Shao et al. 2008).

(b) External wrapping to secure biocompatibility

CNTs have a high surface energy and a high affinity towards intermolecular aggregation; they can form large bundles easily in solution. The non-covalent wrapping of the tubular structure using various species of polymers, polynuclear aromatic compounds, surfactants and biomolecules has become an important method to prepare separated CNTs. The non-covalent interaction between CNTs and wrapping materials is based on van der Waals forces or aromatic stacking. Thus, supramolecular wrapping methods modify (or ‘decorate’) the surface of CNTs without affecting the electronic network of the tubes (Chen et al. 2003; Fernando et al. 2004).

Several approaches for the synthesis of SWCNT composites have thus been developed, including physical mixing in solution, in situ polymerization of monomers in the presence of nanotubes, surfactant-assisted processing of composites and chemical functionalization of the incorporated tubes. CNTs have been functionalized with polyethylene oxide chains for use as biosensors by immobilizing a wide range of proteins (Chen et al. 2003). To reduce or completely eliminate the toxicity associated with the nanotube sidewalls, colloidal Au nanoparticles may be used to cover the surface of CNTs with a uniform, thin layer of gold (Fullam et al. 2000). Shells of silica (SiO\textsubscript{2}) can be built around nanotubes (Guo et al. 2008) followed by further covalent functionalization of the resulting outer layer.
The dispersibility of SWCNTs in polar solvents, including water, has been achieved using supramolecular wrapping with natural-occurring carbohydrates (developed by Shinkai and co-workers (Hasegawa et al. 2004) and using schizodextrin, a naturally occurring β-1,3-glucan, with known medical applications and capable of self-targeting (Luzio et al. 1980; Morikawa et al. 1985)), oligo- and polypeptides or tailor-made, tweezers-like porphyrin tripods (figure 4c) able to fit around CNTs of appropriate diameters (Tamesue et al. 2008; figure 4d). Tripodal porphyrin hosts can ‘solubilize’ SWCNTs in dimethylformamide (DMF)–toluene mixtures. Also, this supramolecular coating material retained fluorescence and biocompatibility while helping to disperse CNTs in polar solvents (Pascu et al. 2008; figure 4).

Zinc protoporphyrin IX binds non-covalently to SWCNTs and renders them soluble in DMF or dimethylsulphoxide solution (Murakami et al. 2003; Kubat et al. 2009). Chen & Collier (2005) used water-soluble porphyrin molecules (meso-(tetrakis-4-sulphonatophenyl)porphine dihydrochloride) to wrap SWCNTs, resulting in soluble SWCNTs in porphyrin solutions that were stable for several weeks. Normally, the Soret and Q-bands owing to the porphyrin coatings in the fluorescence spectroscopy of such composites are significantly broadened and their emissions quenched in the proximity of CNT surfaces, suggesting aromatic stacking between the host and the guest.

(c) Probe tagging

Fluorescent tags (e.g. fluorescein, rhodamine, dansyl chloride, nitrobenzoxadiazoles, porphyrins) and/or targeting agents (e.g. small targeting peptides such as RGD, ocreatide or a small biological molecule such as folic acid, incorporating recognition motifs) may be attached to the supramolecular/covalent coating using mild coupling chemistry methods (i.e. mild peptide coupling, Suzuki and Sonogashira Pd-catalysed coupling, ‘click’ chemistry) to appropriate organic functional groups introduced to the coating material.

Recently, small molecules have been attached to β-D-glucan-coated SWCNTs using boronic acids having the capability to recognize and bind strongly to glucose (Tamesue et al. 2008). Die’s group reported the use of fluorescein-polyethylene glycol (Fluor-PEG) to non-covalently functionalize SWCNTs. This enhances the water-dispersibility of Fluor-PEG/SWCNT and simultaneously affords the fluorescence labels to nanotubes (Nakayama-Ratchford et al. 2007). Of course, the functionalization methodologies discussed for carbon nanomaterials are directly translatable to other nanoparticles (e.g. superparamagnetic iron nanoparticles and nanorods with comparable dimensions).

3. Assessing the toxicity of diagnostic carbon-based nanomedicines

Interesting physico-chemical properties make CNTs good candidates for drug delivery applications. These properties include: ordered structure with high aspect ratio, ultralight weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semimetallic behaviour and high surface area. There is a perceived ambiguity about the safety of CNTs owing to their...
graphite-like surfaces (Zhao et al. 2008). There are two factors that have been initially identified as acting together to induce toxicity:

— surface area of CNT, directly linked with size and shape; and
— CNT reactivity or intrinsic toxicity of the surface, directly linked with the level of functionalization.

Bare-surface (unfunctionalized) MWCNTs, of larger diameters than SWCNTs, were recently found (Chou et al. 2008) to trigger innate and adaptive immune responses in mice that led to chronic pulmonary inflammation and granuloma formation. Appropriately functionalized SWCNTs showed no acute toxicity (Schipper et al. 2008) and no toxic effects in mice over four-month observation. These quasi-monodimensional structures are ideal for modifications with biomolecules and have already been used successfully in tumour targeting (Kostarelos et al. 2008). Their relatively long circulation time and rapid renal clearance make SWCNTs suitable therapeutic and diagnostic scaffolds for nanomedicines (Singh et al. 2006). It is now believed that many toxic effects of metallic nanoparticles come from their large surface area; they are more reactive than CNTs’ sp2-bonded surface (i.e. a rather chemically inert species in vivo) and are thus capable of triggering oxidative stresses in cells. To assess CNT toxicity correctly, they have to be compared with nanomaterials such as nanographite and C60 buckyballs. Graphite is not acutely toxic but causes pneumoconiosis (Ranasinh & Uragoda 1972) characterized by granulomas, emphysema, tissue death and hardening of the blood vessels, among other symptoms. Preclinical studies on the related molecule C60 found no toxic effects in rats and no apparent genetic damage in bacteria (Mori et al. 2006). Recent reports on toxicity of ‘bare’ nanotubes suggest that this might not be entirely due to their size/aspect ratio. Assessing the effect of CNTs on living organisms is complicated by the fact that many nanotube samples still contain residues of catalytic metals (Co, Ni, Fe, Y, Mo, etc.) used for their production (Pumera & Miyahara 2009). Such metal nanoparticles and other impurities (polyaromatic hydrocarbons, graphitic nanoparticles) may have a greater effect on organisms than CNTs themselves.

Preliminary indications are that it is difficult to test the toxicity of nanoparticles in general because they agglomerate so readily. The total surface area depends on their degree of bundling and aggregation in solution. Toxicity studies showed lower cytotoxic effects for the aqueous dispersions of pristine SWCNTs stabilized with a surfactant (1% of Pluronic F108) than for the covalently functionalized SWCNTs using phenyl-SO3H or phenyl-(COOH)2 groups (Sayes et al. 2006). Synthetic chemistry-based research programmes of complex surface functionalization will eventually achieve extremely pure scaffolds and stable dispersions in biological media.

To date, limited information exists regarding the mechanisms of interactions between functional carbon nanomaterials and cells, although some details of the cytotoxicity of the raw materials have emerged and it has thus far been attributed to oxidative stress. SWCNTs penetrate the cellular and nuclear membranes by mechanisms thus far not fully understood. Research aimed at enhancing our understanding of the behaviour of simultaneously filled and functionalized CNT carriers in standard (healthy as well as cancerous) cell lines remains much needed. Step-by-step cytotoxicity tests need to be performed to help clarify under what conditions these CNTs are toxic.
The development of functionalized SWCNTs to obtain optimal biological uptake with minimum toxicity will require robust and rapid test systems to allow selection of the most promising permutations. At all stages of development, the effect of nanoprobe modifications on cell proliferation, metabolism, integrity and viability may be assessed using a number of cell lines and a variety of assays (e.g. XTT, pico green) and fluorescent probes (e.g. LIVE/DEAD) combined with microscopy and flow cytometry. Subsequent uptake and compartmentalization of the nanoprobe may be examined, for example, by live-cell confocal microscopy at different time points post-treatment. Functionalized carbon nanoprobes with low toxicity at each modification stage described above could then be carried forward to testing (e.g. in a stage-gate process). The development of a test-informed assessment protocol before designing and constructing a complete bioimaging probe and the evaluation of its functionality in cells as an intact object remain the focus of much research (Kostarelos et al. 2009).

Current research focuses on the imaging of healthy and ‘immortal’ (cancerous) cells; however, a variety of cells responsible for degenerative diseases (including neurodegenerative diseases) could be used ultimately for a general approach to studying processes in cells treated with (covalently and supramolecularly derivatized) functionalized CNTs. The understanding of processes occurring in cells and tissues treated with nanodimensional multi-functional imaging agents (monitored by PET/MRI/optical imaging and cytotoxicity assays performed in parallel for unfunctionalized radiolabelled versus functionalized radiolabelled probes) will result in a more general approach in elucidating the mechanisms of the interactions between nanomedicines and cells.

Key stages for prototype nanoprobe development are likely to involve the following.

(i) Assembly of the all-in-one biocompatible and targeted molecular imaging probe incorporating multi-modal optical/MRI and PET functionalities via simple covalent and supramolecular chemistries. These composites need to act as an intact nanoscale object capable of probing, visualizing and generalizing the processes occurring at the interactions between SWCNTs and cells.

(ii) Characterizing the nature and strength of interaction between encapsulated/attached functionalities and the nanotube scaffold within the probe after each derivatization step both in representative physiological conditions and within cells using surface analysis techniques and fluorescence imaging. Feedback to inform probe assembly.

(iii) Discovering the mechanisms of uptake and distribution in cells of the complete nanoprobes by confocal fluorescence imaging.

(iv) Characterizing nanoprobe–cell interactions, including probing the oxidative stress mechanism induced by functionalized CNT-based multi-modal imaging probes in standard cell lines (transformed and non-transformed cell lines).

(v) Comparison of functionalized versus unfunctionalized nanoparticles (by supramolecular versus covalent chemistry), tagged and targeted versus raw (including detailed size/shape considerations).
(vi) Assessing the toxicity of the probe in bulk through cytotoxicity assays (MTT, LDH, flow cytometry, etc.).

(vii) Radiolabelling the most promising SWCNT probes emerging from (i) to (v) using long-lived PET or SPECT isotopes (e.g. $^{64}$Cu, $^{111}$In) that are likely to be compatible with the in vivo circulation of large nanoparticulate probes.

(viii) In vitro toxicity tests for the radiolabelled nanoprobes and selection for the most promising probe for in vivo testing and preclinical investigations.

The toxicity risk of nanotubes in living systems is widely cited as a matter of concern, particularly in the media. It is likely that the toxicity of SWCNTs is somewhat overstated at present, and more detailed studies are needed to clarify this: for example, murine models demonstrated minimal brain inflammatory responses to SWCNTs (VanHandel et al. 2009). Bearing in mind the extremely low dose of the overall imaging probe to be administrated for the multi-modality optical/PET/MRI probe, its potential use for the early diagnosis of a number of conditions (such as, for example, aggressive tumours with survival typically weeks to months where current therapies are unlikely to effect cure) should be extremely valuable. In such situations, theoretical risks of long-term toxicity are of less significance and are unlikely to prevent in vivo work from proceeding.

For metallic species (radionuclides or magnetic materials), details of cellular uptake and distribution remain poorly understood, while quantifying the uptake in the cell nucleus can greatly impact on calculations of the safe dose that can be tolerated. A clear advantage of the encapsulation method proposed here is that multiple metal ions are guided to the target, minimizing the dose to be delivered, and hence the side effects of the drug. This would also ensure the high resolution required for imaging (with resolution ranging from nanometre to millimetre scales) while working with extremely low concentrations.

In vitro evaluation tests to date are centred on the methods (i)–(iii) outlined below.

(i) Cell penetration and localization studies. Fluorescence microscopy studies in cancer cells need to be carried out for each of the completed probes to assess their potential for cell penetration. The evaluation of probe interactions with healthy cells, as well as with a variety of immortal cells (i.e. adherent cancer cells), has been central to many recent studies: for proof of principle, probes derivatized with known targeting units such as folic acid (for uptake into HeLa cells, an immortal cell line derived from cervical cancer; Sun et al. 2006; Dhar et al. 2008) may be used. Findings from epi- and laser scanning confocal fluorescence microscopies can be reinforced by dual AFM/epi-fluorescence microscopies that offer increased resolution. Following dynamic events in cells, such as real-time monitoring, the molecular interactions with cell surfaces may lead to increased understanding of the uptake and biolocalization of nanoparticles in vitro. This can be achieved through time-resolved fluorescence imaging techniques and single-molecule fluorescence microscopies (e.g. FLIM), but thus far remains an undeveloped area of research.
(ii) Cytotoxicity tests. This is currently assessed by flow cytometry (FACS) as well as via standard MTT and LDH assays for all types of nanoparticles (including CNTs) after each of the functionalization steps in both healthy and cancer cells, to differentiate between live, apoptotic and necrotic cells. There are mechanistic implications for the interaction between cells and the nanoprobe, and the effect of the filling material on the probe cytotoxicity is not understood thus far. Flow cytometry is a routine method of investigation, via ‘live and dead’ cell reagents and annexin V testing, which helps distinguish between necrotic and apoptotic cells upon incubation with the nanomaterial (Al-Jamal & Kostarelos 2010). Standard LDH and MTT assays are also employed. These may reflect membrane damage since membranes are the most sensitive target of oxidative damage (Kang 2008; Herzog et al. 2009; Shvedova et al. 2009; Yang et al. 2009). In addition to the standard adherent, immortal cell types (e.g. HeLa, MCF-7, IGROV, etc.), primary bronchial epithelial cells and A549 alveolar epithelial carcinoma cells need to be used in standard toxicity evaluation protocols since the lung has been highlighted as a major target of potential toxicity of much larger airborne nanoparticles, such as MWCNTs.

(iii) Probe the mechanisms of interactions: bioimaging probes and cells. Several tests on stress protein induction in certain raw SWCNTs were recently reported, and used microarrays to look at global gene expression (Sarkar et al. 2007) and HO-1 (Li et al. 2007), essentially using general markers of oxidative stress for nanotoxicity screening. The precise relationship between the functionality type and degree of surface modification and oxidative stress response needs to be fully addressed. The link between the level of oxidative stress and functional outcome should generate a better understanding of the cell membrane damage by nanoparticles and apoptosis-altered gene expression profiles both in cancerous and non-cancerous cell lines. For example, it would be interesting to understand how simultaneous filling and external functionalization of CNTs may affect the mechanistic aspects of cytotoxicity.

If such integrated protocols prove successful, a new class of entirely safe nanocarriers for biomedical imaging applications could emerge.

4. Traceable carbon nanomaterials in living systems and selected cancer imaging applications

Accurate diagnostic imaging is crucial for the effective management of cancer; in all cancer treatments, it is vital to be able assess treatment response. This is usually done using anatomical imaging, such as computed tomography or MRI, to determine whether there has been a physical reduction in the size of the tumour. This method of assessment is not always ideal as the physical size of a tumour does not always equate to biological activity of tissue. Functional imaging offers the opportunity to select non-responders from responders earlier in their treatment course and alter their therapeutic regime to increase the chance of treatment success.

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The growth of new blood vessels, angiogenesis, is one of the fundamental processes required for tumour growth and metastases and is largely mediated by hypoxia-induced vascular endothelial growth factor (VEGF) signalling. Non-invasive imaging strategies play a critical role in assessing the efficacy of angiogenesis therapies. Angiogenic gene expression is regulated by proteins such as hypoxia-inducible factor-1, which are produced in response to low tissue oxygen concentrations. The integrin (i.e. integral protein attached to the cellular membrane) is activated in angiogenic vessels and represents a potential target for angiogenesis imaging. By using radiolabelled biomarkers against components of the VEGF signalling cascade, valuable information about the nature of the disease can be obtained. Furthermore, labelled biomarkers can be used to monitor the circulation, delivery and uptake of anti-angiogenic drugs and have the potential to unravel unanswered questions regarding VEGF biology. Many clinically approved drug treatments are expensive in clinical use; employing radiolabelled versions of these drugs will provide a means of assessing likely therapeutic response prior to embarking on the course of treatment (Hurwitz et al. 2004; Duda et al. 2007; Escudier 2007; Motzer et al. 2007; Beer & Schwaiger 2008).

In the last 5 years, a number of anti-angiogenic chemotherapeutic agents, such as antibodies against VEGF (e.g. bevacizumab) or low-molecular-weight drugs (e.g. SU5416, Sutent, etc.), that inhibit VEGF signalling have been studied in clinical trials and found to be of benefit (Hurwitz et al. 2004; Demetri et al. 2006; Sandler et al. 2006; Duda et al. 2007; Escudier 2007; Motzer et al. 2007). 99mTc-labelled peptides selectively localize to endothelial cells in regions of increased angiogenesis and could be used for non-invasive imaging of angiogenesis. This targeted radiotracer imaging approach is a major advance in tracking therapeutic myocardial angiogenesis and has important clinical potential (Hua et al. 2005).

A potential approach involving nanomedicines could be the use of biocompatible, functionalized nanocarriers (ranging from metallic nanoparticles to CNTs) as scaffolds for targeting molecules and reporter molecules in order to image tumour angiogenesis (figure 1). A number of researchers currently examine the hypothesis that multi-functional nanoparticles (including CNTs) can be used for the functional imaging of cancer by targeting tumour angiogenesis, as detailed below (Duda et al. 2007; Beer & Schwaiger 2008; Ou et al. 2009).

One such study evaluated the potential application of SWCNT scaffolds for targeted radioisotope drug delivery where the SWCNTs were conjugated to both $^{64}$Cu and c(RGDyK), a potent integrin $\alpha_v\beta_3$ antagonist, aimed at \textit{in vivo} targeting of integrin $\alpha_v\beta_3$-positive tumours in mice via specific RGD–integrin $\alpha_v\beta_3$ binding. Covalently linked $^{86}$Y-derivatized DOTA chelates have been used to label SWCNTs. This construct was obtained from amine-functionalized, water-dispersed CNTs (McDevitt et al. 2007). The distribution and clearance of $^{86}$Y (a PET isotope with half-life of 14.7h) \textit{in vivo} was studied at 3 and 24h post-injection in murine models using micro-PET. It has been shown that $^{86}$Y disappeared from the blood within 3 hours and accumulated predominantly in the kidneys, liver, spleen and, to a lesser extent, the bones. Wang et al. (2004) studied the distribution of $^{125}$I-radiolabelled water-dispersed hydroxylated SWCNTs in mice ($^{125}$I is a gamma emitter with a half-life of 60.28 days). This study demonstrated, for the first time, that a quantitative analysis of CNTs accumulated in animal tissues may be performed. $^{111}$In-labelled water-dispersed SWCNTs have also been used: SPECT imaging revealed that SWCNTs were

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not retained in any of the reticuloendothelial system (RES) organs (liver or spleen) and were rapidly cleared from systemic blood circulation through the renal excretion route (Singh et al. 2006). In vivo biodistribution of $^{64}$Cu-appended SWCNTs has also been studied using micro-PET. The SWCNTs used in this research were non-covalently functionalized with phospholipid–PEG (PL–PEG) and these composites were surprisingly stable in vivo. Effectively PEGylated SWCNTs thus exhibited relatively long blood circulation times and low uptake by the RES. DOTA was attached to the termini of the PEG chains and used to conjugate the positron-emitting radionuclide $^{64}$Cu. Dai et al. reported that SWCNTs conjugated with various targeting ligands, including the breast cancer therapeutic Herceptin (anti-Her2), Erbitux (anti-Her1) and RGD peptide (for targeting integrin, an essential angiogenesis marker), exhibited distinct Raman G-band peaks and can be used for Raman imaging of biological systems (Liu et al. 2008b). The targeting effects and localization of RGD-tagged SWCNTs have been evaluated and their effect compared with that of untagged, yet biocompatible, SWCNTs. The imaging results showed increased accumulation of RGD-SWCNTs in tumours compared with plain SWCNTs over 72 h.

Other in vivo applications involved addressing brain imaging with nanoparticles. There are concerns that large nanoconstructs would not diffuse through the extracellular matrix (ECM) of the brain passively, when the drugs are administered intravenously. However, a recent study has described methods to increase the effective pore size of the ECM to allow convection of nanoparticles smaller than 50 nm (Neeves et al. 2007). A widely used cancer chemotherapy drug (paclitaxel) attached via PEG to water-soluble CNTs increased the drug delivery in a murine cancer model compared with conventional methods. This also demonstrated delivery of paclitaxel to brain tissue, thus crossing the brain–blood barrier (Neeves et al. 2007; Liu et al. 2008a; Regina et al. 2008).

Radiolabelling performed strictly as the last functionalization step is mainly designed to account for short-lived isotopes ($^{11}$C and $^{18}$F). Liu et al. (2009) described the attachment of $^{64}$Cu to SWCNTs as the final step, by using a DOTA ligand on the outside of the nanotube. Alternative approaches, involving encapsulating the radionuclide within the tube, have not been investigated thus far. In principle, this approach would offer the advantage of protecting the radionuclide en route to target and keeping the overall probe intact as ligands such as DOTA are not sufficiently kinetically stable to avoid loss of the radionuclide ion from the probe in vivo. Ideally, having only targeting groups and fluorescent tags on the outside of the CNTs, unperturbed by radionuclides and their ligands, would mean that binding to targets should be greater and more specific.

In addition to radiolabelling biomarkers, the availability of a second optical imaging mode for the same probe (in an all-in-one approach) could provide a valuable method of confirming biodistribution via fluorescence imaging of tissue samples from biopsy and for investigating details of cellular uptake. SWCNTs labelled with fluorescent markers have been exploited for optical imaging applications. Dai et al. used PL-PEG-NH$_2$-functionalized semiconducting SWCNTs as NIRF tags for probing of cell-surface receptors and cell imaging (Welsher et al. 2008). PL-PEG-NH$_2$-functionalized semiconducting SWCNTs were attached to antibody Rituxan to recognize CD20 cell-surface receptors on B-cells and Herceptin to recognize HER2/neu-positive breast cancer cells. Bottini et al. (2006) reported the application of streptavidin-conjugated
quantum-dot-decorated SWCNTs in both optical and confocal fluorescence microscopies. The supramolecular luminescent nanocomplex can stably disperse under physiological conditions, and these complexes could be internalized by Jurkat T leukaemia cells by multi-valent CD3 receptor-mediated endocytosis.

Recent studies showed the potential use of SWCNTs in MRI. The loading of ultrashort SWCNTs with aquated Gd$^{3+}$ clusters is believed to occur through the side-wall defects or end-of-tube openings in the tubes, and give linear superparamagnetic molecular magnets with efficacies 40 to 90 times larger than any small-molecule Gd$^{3+}$-based CAs in clinical use (Sitharaman et al. 2005). Such Gd$^{3+}$-cluster-filled tubes were confirmed as ultrasensitive pH-smart probes from pH 7.0 to 7.4, and thus good candidates for detection of early-stage onset of cancer where the extracellular pH value can drop below pH = 7 (Hartman et al. 2008). Strano and co-workers (Choi et al. 2007) reported that complexes formed by magnetic iron oxide nanoparticles and NIRF SWCNTs can be used as dual MRI and fluorescent imaging agents. Iron oxide particle-enriched (Fe-enriched) DNA-SWCNT solutions have been analysed. These have magnetization saturation, magnetic relaxation time-scale ratio and spin-spin relaxation time significantly more favourable for MRI than typical ferromagnetic particles alone.

5. Ethical considerations on nanomedicines for diagnostics

There is a perceived ambiguity about the safety of CNTs and their possible harmful effects on living cells and multicellular organisms (Chou et al. 2008; Schipper et al. 2008). Currently, there is no common understanding of, and no comprehensive approach in, assessing the risk profile of nanotechnology in general and carbon nanomaterials in particular, although there have been reports aiming to address the toxicology of nanomaterials. The need for systematic risk assessment and categorization of nanomaterials has been acknowledged (Maynard et al. 2006) as there is no commonly accepted standard guidance on physico-chemical characterization of engineered nanoscale materials for the toxicological assessment and the safe handling and disposal of manufactured nanomaterials. Self-regulation, a long-standing tradition in the professions, is widespread when tackling the safety issues relating to nanotechnology, and at each of the product development stages (figure 5). Toxicity tests need to be increasingly used for the evaluation of hazards and risks in production, functionalization and manufacture of nanomedicines as well as the hazards and risks during the use of products by the end user. The impact on the environment needs to be assessed during the life cycle of the nanomaterials synthesized. In the long term, robust and standard systems suitable to evaluate health and environmental impact of therapeutic nanomaterials will eventually emerge as a result.

There is an increasing awareness of the ongoing controversy affecting the public perception of nanotechnology (Burri 2008). Much of the fear over new technologies concerns the risk of the effects that new, less well-known and less understood products may have on human tissue and on the environment. The appreciation of these risks requires a detailed understanding of the properties of nanostructures and of their interaction with living cells. The priorities of research in nanomedicine design and evaluation need to align with public views on nanotechnology and
efforts need to be made at all stages of the development of new systems for the dissemination and communication of the results. Research in this field will need to follow the standard approval procedure regarding ethics and research governance, which differs from country to country. In vivo experiments need to be kept to a minimum, and the development of high-throughput in vitro tests will be necessary.

Addressing nanosafety in terms of risks (real and perceived) and benefits needs to become a key activity within the overall research programmes aimed at nanomedicines design. As a result of rigorous cytotoxicity tests after each derivatization step (as described above), the behaviour in cells will be better understood for the nanomedicines designed and an evaluation of the real risks can be made during fabrication/synthesis in the laboratory and during the lifetime of the product development and accidental human exposure/environmental exposure.

Mechanisms for the dissemination and communication of the results with the involvement of all stakeholders (researchers, collaborating institutions and end users) need to be employed throughout such programmes. Issues of ethics, safety, environmental impact and exposure need to be considered using multi-criteria decision analysis versus constructive technology assessment tools (Porter et al. 2007; Lacerda et al. 2008a), taking into account considerations of clinical (safety, efficacy and effectiveness), economic (cost-effectiveness), patient related (quality of life, ethical/juridical and psychosocial) and organizational aspects (diffusion and adoption) and scenario drafting.

The delivery of the new generation of therapeutic agents through a fully integrated research approach (e.g. nanomedicines design) would be ideally performed by multi-disciplinary teams (spanning chemistry, materials, physics, chemical engineering, cell and molecular biology involving imaging specialists and clinicians) with expertise in interfacing biomedical research, physical sciences and medicine, whereby multi-functional nanoprobes are developed through iterative rounds of structure-led design and synthesis, and in vitro biological screens, leading to in vivo screens.
6. Conclusions and outlook

While some initial progress has been made, there remains a clear need for the detailed mechanistic understanding of the delivery of diagnostic and/or therapeutic agents encapsulated inside nanodimensional carriers, ranging from metallic nanoparticles to polymer micelles to biocompatible SWCNTs. These scaffolds may be equipped with targeting biomolecules and multiple reporting units and have the potential to change the current approaches to the design of imaging and therapeutic agents. This will require long-term large-scale integrated projects but could have the potential for rapid, high-impact knowledge transfer, particularly in the biomedical imaging field, as (radio) imaging agents can be administered in minute amounts such that the toxicological testing may be rapid and nanocarriers could progress quickly from the chemical bench to the clinic.

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