Toward carbon nanotube-based imaging agents for the clinic

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Abstract

Among the many applications for carbon nanotubes (CNTs), their use in medicine has drawn special attention due to their potential for a variety of therapeutic and diagnostic applications. As progress toward clinical applications continues, monitoring CNTs *in vivo* will be essential to evaluate their biodistribution, potential toxicity, therapeutic activity, and any physiological modifications that the material may induce in specific tissues. There are many different imaging modalities to visualize and track CNTs *in vivo*, yet only a few are full-body penetrating, a central characteristic that widens their clinical utility. In order to visualize CNTs, chemical modification is often required for the material to be used as a platform to carry imaging agents compatible with one or more of the clinical imaging techniques. Here, we focus on the most recent work involving the use of CNTs as imaging agents for the non-invasive, full-body penetrating clinical modalities of MRI, PET, SPECT, and X-ray CT. The synthesis and modification of the CNT materials are discussed, as well as relevant preclinical studies.

Keywords: carbon nanotubes; clinical imaging; MRI; PET; SPECT; X-ray CT

Abbreviations: acetylacetone (acac); carbon nanotubes (CNTs); computed tomography (CT); contrast agent (CA); diethylenetriaminepentaacetic dianhydride (DTPA); doxorubicin (DOX); extended X-ray absorption fine structure (EXAFS); Gadonanotube (GNT); hexafluoroacetylacetone (hfac); high pressure carbon monoxide (HiPCO); high resolution transmission electron microscopy (HR-TEM); inductively-coupled plasma mass spectrometry (ICP-MS); intravenous (i.v.); inversion time (Ti); magnetic resonance imaging (MRI); multi-walled carbon nanotubes (MWCNTs); p-carboxyphenyldiazonium (PCP); percent injected dose per gram of tissue (%ID/g); phosphate buffered saline (PBS); poly(allylamine hydrochloride) (PAH); poly(diallyldimethylammonium chloride) (PDDA); polyethylene glycol (PEG); positron emission tomography (PET); scanning transmission electron microscopy (STEM); single-photon emission computed tomography (SPECT); single-walled carbon nanotubes (SWCNTs); superparamagnetic iron oxide nanoparticles (SPIONs); 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA); 2,2,6,6,-tetramethyl-3,5-heptanedione (thd); Tesla (T); transmission electron microscopy (TEM); ultra-short SWCNTs (US-SWCNTs)

1. Introduction

Among the many nanoparticles currently under investigation, carbon nanotubes have been and continue to be a key material for their unique properties, cost effectiveness, and extreme diversity of applications. Carbon nanotubes (CNTs) can be described as graphene sheets that are rolled in a cylindrical shape and have different electrical and optical properties depending on the axis about which they are rolled, which is called chirality or the “twist” of the nanotube. The different CNT chiralities include the armchair structure, in which the C-C bonds are perpendicular to the tube axis; the zig-zag structure, in which the C-C bonds are parallel to the tube axis; and the chiral structure, in which the C-C bonds lie at an angle with respect to the tube axis [1,2]. CNTs are either single-walled (SWCNTs) with a diameter around 1-2 nm, or multi-walled (MWCNTs), which are composed of 2-30 concentric SWCNTs with an outer diameter ranging from 10-100 nm. Some of the methods that are generally used to produce CNTs are arc-discharge, laser-ablation, and chemical vapor deposition, including the high-pressure carbon monoxide (HiPCO) synthesis. These processes usually occur in the presence of transition metal (Co, Fe, Ni) or rare earth (Gd, Y) catalysts, which remain in the pristine CNT product [3–9]; significant progress has been accomplished in the removal of trace amounts of these catalysts [9–13], although more work is currently underway. Since their discovery [14], CNTs have been of great interest because of their unique structural and chemical properties, such as their high tensile strength, high aspect ratio, and the capability to be chemically functionalized, while remaining relatively inert [15–18]. This interest has led to CNTs being used in a variety of applications in electronics [19,20], material composites [21], energy [22], catalyst supports [23,24], and particularly those in the medical field [25–27]. Within the emerging field of nanomedicine, CNTs have been investigated as drug delivery vectors [28,29], therapeutic agents exploiting microwave-, photo-, or radiofrequency-induced thermal effects [30–33], scaffolds for tissue engineering [34], and diagnostic imaging agents [35,36].

For diagnostic and therapeutic applications, it is essential to determine the CNT biodistribution and pharmacokinetic profile to study the biological effects of the material on specific tissues. To this end, researchers have used a wide range of imaging modalities that rely on the intrinsic properties of CNTs. These techniques include Raman scattering [37], high optical and near infrared (NIR) absorbance, and photoluminescence [38–40], photoacoustic [41–44], thermoacoustic [45], and echogenic properties [46]. However, some of these imaging techniques suffer from penetration depth limitations, poor spatial resolution, or poor soft-tissue contrast [47,48]. To combat these limitations, CNTs may be employed as a scaffold or capsule for

radionuclides or ions that are used for the noninvasive, full-body penetrating clinical modalities, such as magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), positron emission tomography (PET), and X-ray computed tomography (X-ray CT) (see Figure 1).

Figure 1: Schematic of the versatile CNT platform. Various ways to modify CNTs: attaching chelated ions or nanoparticles, such as superparamagnetic iron oxide nanoparticles (SPIONs), to the outer surface of the material or loading other agents or ions within the hollow interior of the material.

While common imaging contrast agents (CAs) generally are not able to cross the cell membrane, the use of CNTs allows for these agents to be delivered intracellularly for cell tracking and sometimes selectively with the use of biological targeting moieties. Packaging the imaging agents onto or inside the CNTs enables them to be internalized by cells [49] without the need of cytotoxic transfection agents [50,51]. However, the highly hydrophobic nature of CNTs also prohibits their suspension in aqueous media, requiring covalent or non-covalent functionalization techniques to produce biocompatibility and high aqueous suspendability (see Figure 2). Several reviews discussing extensively the different methods for CNT functionalization have been previously published [52–54], as well as reviews on the use of CNTs for imaging [35,36,55–57]; however, the rapid growth in the field constantly prompts an updated discussion of current findings. For this reason, this review focuses primarily on recent clinically relevant work involving exclusively non-invasive full-body penetrating techniques reported within the past five years.

Figure 2: Common CNT functionalization techniques. Common strategies to render CNTs water-suspendable including (A) non-covalent functionalization with surfactants that wrap the CNT according to van der Waals forces, (B) oxidation of the CNT surface using strong acids, (C) covalent functionalization of the surface, where R is usually a peptide chain or another hydrophilic moiety (Note: 1,3-dipolar cycloaddition of azomethine ylides and cyclopropanation are typically performed separately on different materials), and (D) non-covalent functionalization with amphiphilic peptides (blue) where a hydrophobic chain (red) interacts with the material by van der Waals forces.

2. CNT-based Agents for MRI

Among the imaging techniques used in the clinic, MRI offers the advantage of high-quality anatomical images with high spatial resolution without the use of ionizing radiation. Instead, MRI utilizes a high-intensity magnetic field that aligns the nuclear magnetization of hydrogen atoms in water molecules within the body, taking advantage of the difference in water concentration among tissues to produce images. Common MRI CAs are chemical compounds containing a chelated paramagnetic metal ion (such as Gd^{3+} , Mn^{2+} , etc.) with larger effects on T_1 shortening (spin-lattice relaxation agents), or superparamagnetic materials such as iron oxide

nanoparticles with larger effects on T_2 shortening (spin-spin relaxation agents), where T_1 and T_2 are the proton relaxation times. The theoretical basis for the relaxivity of paramagnetic and superparamagnetic agents has been extensively described in the literature [58–62]. In order to produce an MRI-active CNT CA, a paramagnetic or superparamagnetic agent must be conjugated to or encapsulated within the CNT platform.

2.1 Gadolinium-based Contrast Agents

The first CNT-based MRI CA containing Gd^{3+} was reported in 2005 by Sitharaman *et al.*, and was termed the “Gadonanotube” or GNT [63,64]. The CA consisted of ultra-short (20-80 nm) single-walled carbon nanotubes (US-SWCNTs) loaded with Gd^{3+} ion clusters (3-4% Gd by weight, see Figure 3A and 3B). GNTs displayed 40 to 90 times superior relaxivity values compared to the clinically used MRI CA Magnevist[®]. Furthermore, the Gd^{3+} ion cluster are so tightly contained by the CNT platform that the ions do not leak out of the CNTs under biological conditions [65], which is critical for clinical applications given the high toxicity of free Gd^{3+} [66]. Usually, a 0.17% Pluronic[®] F-108 solution is used to suspend the GNTs in an aqueous medium for *in vitro* studies. In subsequent reports, GNTs were used to label macrophage cells [67], mesenchymal stem cells (see Figure 3C and 3D) [68,69], and HeLa cells [70]. In all cases, GNTs localized in the cytoplasm of the cell, delivering about 10^9 Gd^{3+} ions per cell. In another study, GNTs were covalently derivatized with D,L-serine amino acid substituents to produce water-suspendable (2 mg/mL) “ser-GNTs,” which were then used to label MCF-7 human breast cancer cells with no observable cytotoxic effects or morphologic changes [71].

Figure 3: Gadonanotubes (GNTs) as an intracellular MRI CA. (A) Illustration of a single US-SWCNT loaded with Gd^{3+} ions (GNT). (B) High resolution transmission electron microscopy (HR-TEM) image of the bundled GNTs showing the Gd^{3+} clusters (arrows) formed within the US-SWCNTs. (C) TEM image of

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4 a GNT-labeled MSC. Red arrows point to GNT aggregates in the cytoplasm of the cell. **(D)** T_1 -weighted
5 MR images at 1.5 T and 25 °C of (left to right) unlabeled MSCs, Gd-DTPA (diethylenetriaminepentaacetic
6 dianhydride)-labeled MSCs (for comparison), and GNT-labeled MSCs at $T_i = 500$ ms (T_i = inversion
7 time). **(A)** and **(B)** adapted from ref. [63], Copyright (2005), with permission from Royal Society of
8 Chemistry. **(C)** and **(D)** adapted from ref. [68], Copyright (2010), with permission from Elsevier.

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11 In a follow-up to the original GNT report, three new Gd-based CAs, derived from
12 confinement of three different gadolinium chelates within the cavities of the same US-SWCNTs
13 were recently reported. The chelates $\text{Gd}(\text{acac})_3 \cdot 2\text{H}_2\text{O}$, $\text{Gd}(\text{hfac})_3 \cdot 2\text{H}_2\text{O}$, and $\text{Gd}(\text{thd})_3$ (acac =
14 acetylacetone; hfac = hexafluoroacetylacetone; thd = 2,2,6,6-tetramethyl-3,5-heptanedione),
15 were used to evaluate the effect of the number of water molecules coordinated to the Gd^{3+} ion (q),
16 which is one of the factors that influences the inner-sphere relaxivity of the CA [72]. In doing so,
17 it was proven that the encapsulation of the free Gd^{3+} ions (non-chelated) provided a better MR
18 outcome than the chelated ions, because the Gd^{3+} ions within the US-SWCNTs have a q of
19 approximately 3, while the chelated ions have a q value of 0 or 2, depending on the chelate. The
20 amount of Gd within the US-SWCNTs for each sample was reported to be 7.91, 4.45, and 7.73
21 wt. % for the acac, hfac, and thd chelates, respectively. To further decipher the structural origin of
22 the exceedingly high-performance MRI contrast of the GNTs, a more detailed extended X-ray
23 absorption fine structure (EXAFS) study was performed [73], revealing essential information
24 about the dynamic system around the clustered Gd^{3+} ions within the US-SWCNTs.

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26 In another attempt to produce highly water-suspendable GNTs, following the
27 encapsulation of the Gd^{3+} ions, the surface of the US-SWCNTs was functionalized with *p*-
28 carboxyphenyldiazonium (PCP) tetrafluoroborate salt, which covalently attached benzoic acid
29 moieties, making the material highly water-suspendable (35 mg/mL) [74]. However, 90% of the
30 Gd^{3+} ions leaked from the SWCNTs due to the acidic conditions of the functionalization process.
31 To address this issue, US-SWCNTs, encapsulating the $\text{Gd}(\text{acac})_3 \cdot 2\text{H}_2\text{O}$ and $\text{Gd}(\text{hfac})_3 \cdot 2\text{H}_2\text{O}$
32 chelates were synthesized and the surface of the resulting material was functionalized with PCP,
33 producing stable, highly water-suspendable US-SWCNTs containing 2.5 – 3.8% Gd by weight.
34 These water-suspendable US-SWCNTs, encapsulating $\text{Gd}(\text{acac})_3 \cdot 2\text{H}_2\text{O}$, were then used to label
35 porcine mesenchymal stem cells, making the cells MRI active without affecting cell viability
36 [74].

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38 Recently, the magnetic properties of metal impurities present in raw, commercially
39 available CNTs (mainly from iron and nickel catalysts) have been studied for T_2 -based contrast
40 signal enhancement. A similar approach was employed to synthesize a Gd-based CA using Gd_2O_3
41 nanoparticles as catalysts to synthesize SWCNTs (Gd-SWCNTs) via chemical vapor deposition
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for T_1 -weighted MRI [75–77]. The Gd^{3+} content of the final product was reported to be 6.2% and aqueous suspendability was achieved by non-covalent functionalization with a phospholipid polymer, PEG₅₄₀₀-DSPE (PEG = polyethylene glycol, DSPE = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine) [77]. The cell-labeling efficacy was tested for NIH/3T3 fibroblast cells, which showed no change in viability, proliferation, or changes in their division cycle compared to untreated controls. Gd-SWCNTs localized to the cytoplasm and delivered 10^8 - 10^9 Gd^{3+} ions per cell. Phantom studies of the labeled cells using a T_1 -weighted MRI at 3 T showed a four-fold increase in MR signal intensities compared to unlabeled cells. A continuation study [78] by the same group evaluated the *in vivo* pharmacodynamics of Gd-SWCNTs after intravenous (i.v.) injection (0.5 mg/kg) in male Wistar rats, which was assessed by bright-field optical microscopy. The macroscale distribution of the material showed uptake in the lungs, liver, kidneys, brain, and spleen. The histological distribution was also assessed and the molecular and genetic responses were evaluated for up to 30 days. After 24 h, the Gd-SWCNT material accumulated in different organs leaving the circulatory system, with total elimination occurring in 10 days. However, *in vivo* MRI data for the Gd-SWCNTs were not obtained in any of the studies discussed above.

An alternative approach to synthesize CNT-based CAs containing Gd^{3+} is to use the CNT as a scaffold to covalently attach Gd-containing moieties to the surface of the material. A study performed by Marangon *et al.* reported the use of oxidized multi-walled carbon nanotubes (MWCNTs, 20-30 nm in diameter and ~400 nm in length) functionalized with the chelating ligand diethylenetriaminepentaacetic dianhydride (DTPA), followed by metallation with Gd^{3+} (see Figure 4) [79]. RAW 264.7 mouse macrophage cells easily internalized the MWCNTs functionalized with Gd-DTPA, but showed little uptake of the free Gd-DTPA chelate itself. Cells labeled with increasing concentrations of the CNT-based material were dispersed in an agarose gel mimicking the cell density of tissue and MR images were acquired (Figure 5). For T_1 -weighted images, the signal increased nearly two fold for the highest concentration of the CA with respect to unlabeled cells. *In vivo* MRI studies were performed on female C57/Bl6 mice after i.v. administration of the material at two different doses (2.5 mg/kg and 12.5 mg/kg). At five hours following injection, MRI studies showed very large uptake of the Gd-based CA in the bladder, in addition to the liver and spleen. MRI, electron microscopy, and histological data suggested that a significant amount of the MWCNTs functionalized with Gd-DTPA is eliminated by renal clearance.

Figure 4: Synthesis of CNTs functionalized with Gd-DTPA. Schematic of the synthesis of a MWCNT functionalized with Gd-DTPA, showing the oxidation of the CNT with a mixture of $\text{H}_2\text{SO}_4/\text{HNO}_3$ in the first step to produce structure **1**. This was followed by the functionalization with the chelating ligand and later hydrolysis to produce MWCNT-DTPA **4**. Chelation of the metal ion to produce the Gd^{3+} -containing MWCNT **5** is shown in the final step. Adapted with permission from ref. [79], Copyright (2014) John Wiley and Sons.

Figure 5: MR images of cells labeled with MWCNT functionalized with Gd-DTPA.

T_1 -weighted (A) and T_2 -weighted (B) fast spin echo MR images of compacted cells in agarose gel mimicking tissue (70 μL) labeled with different concentrations (10, 20, 50 and 100 $\mu\text{g/mL}$) of MWCNTs functionalized with Gd-DTPA or oxidized MWCNTs (ox-CNT). Images of agarose gel alone (agar), water, or unlabeled cells (cells) are also displayed. (C) Representative bright field and corresponding dark field images of cells for different concentrations of MWCNTs functionalized with Gd-DTPA. Black spots on bright field images and bright spots on dark field images are indicative of the material uptake. Adapted with permission from ref. [79], Copyright (2014) John Wiley and Sons.

2.2 Iron-based Contrast Agents

As mentioned above, superparamagnetic iron oxide nanoparticles (SPIONs) have also been used as MRI CAs. The synthesis and general applications of CNTs filled with ferromagnetic materials have been under investigation for several years and previously reviewed [80]. More recently, Wu *et al.* reported the *in situ* loading of Fe₃O₄ nanoparticles on the surface of MWCNTs (10-30 nm in diameter, 0.5-2 μ m in length) [81]. The MWCNT/Fe₃O₄ hybrid material was synthesized by a solvothermal method using diethylene glycol and diethanolamine as solvents, producing a water-suspendable CA with superparamagnetic properties at room temperature and a T_2 -relaxivity of 175.5 Fe mM⁻¹s⁻¹. The amount of Fe₃O₄ in the MWCNT/Fe₃O₄ hybrids was estimated to be 41.3%. For *in vitro* studies, human breast adenocarcinoma cells (MCF-7) and mouse C3H/An connective tissue cells (L929) were incubated with MWCNT/Fe₃O₄, maintaining cell viability greater than 80% after 24 h of treatment. *In vivo* MR images were acquired after i.v. administration of the MWCNT/Fe₃O₄ into mice, showing uptake in the liver, lungs, and spleen.

A similar approach was performed by Al Faraj and coworkers [82,83]. A mixture of H₂SO₄ and HNO₃ was used to oxidize SWCNTs, generating oxidized nanotubes, 200-300 nm in length, which were mixed with polyvinylpyrrolidone (PVP) polymer to obtain a stable suspension of the material in water. Iron chloride salts and ammonium hydroxide were employed to grow ferrous nanoparticle crystals on the SWCNTs. In these studies, the Fe-tagged SWCNT material was used as an MRI CA, as well as a magnetic agent to deliver drugs to tumors guided by an external magnetic field. Inductively-coupled plasma mass spectrometry (ICP-MS) revealed that the CNTs contained 40% (w/w) iron oxide nanoparticles. This theranostic (*therapeutic* and *diagnostic*) agent was also conjugated with mouse Endoglin/CD105 monoclonal antibodies for specific active targeting of breast cancer tumors and with the anti-cancer drug, doxorubicin (DOX). A small magnet was placed over the tumor site to enable magnetic targeting. Tumor growth and progression were monitored using MRI, showing that DOX delivery through antibody-conjugated magnetic SWCNTs improved the efficacy (decreased tumor size) of DOX over control groups by improving targeting to the tumor site [82].

Another similar CNT-based MRI CA was reported by Liu *et al.*, incorporating a lactose-glycine adduct around the SPION as a liver targeting functionality, while also preventing its oxidation [84]. MWCNTs were oxidized with a mixture of H₂SO₄ and HNO₃, and further treated with citric acid to produce water-suspendable MWCNTs. Poly(diallyldimethylammonium chloride) (PDDA) was coated on the surface of the acid-treated MWCNTs by electrostatic interactions and SPIONs, previously modified with the targeting agent by a co-precipitation

method, were subsequently immobilized on the surface of the PDDA-MWCNTs. The hybrid material exhibited a T_2 relaxivity of $186 \text{ mM}^{-1}\text{s}^{-1}$ and was tracked *in vivo* by T_2 -weighted MRI and found to accumulate in a murine orthotopic liver tumor model following i.v. injection (10 mg/kg) with low observed toxicity. Subsequently, the same group reported SPIONs within the inner cavity of MWCNTs, ensuring high mechanical stability of the nanoparticles [85], although with lower relaxivity values than the previously reported material ($R_2 = 85 \text{ mM}^{-1}\text{s}^{-1}$). The synthesis was achieved by mixing the same acid-treated MWCNTs (20-50 nm inner diameter) with $\text{Fe}(\text{acac})_3$, followed by its reduction to Fe_3O_4 with N_2H_4 using a microwave reactor. Finally, the nanostructure was coated with diblock copolymers PMETAC-*b*-PEGMA, (PMETAC: 2-(Methacryloxy)ethyl trimethylammonium chloride, PEGMA: Poly(ethylene glycol) methacrylate) to achieve high suspendability in water. Diblock copolymers were prepared with different PEGMA block lengths (degree of polymerization = 20, 60, 89, and 118, named P₁-P₄, respectively) and the same PMETAC block length (degree of polymerization = 10). *In vitro* studies performed with two cancer cell lines demonstrated low cytotoxicity using up to 200 $\mu\text{g/mL}$, and T_2 -weighted MR images of a cancerous mouse liver showed an increased tumor/liver contrast ratio after administration of the material (Figure 6).

Figure 6: Properties and imaging of CNT- Fe_3O_4 . (A) Last step of the CNT- Fe_3O_4 polymer preparation. (B) TEM images of CNT- Fe_3O_4 -P₄. Cytotoxicity results of CHO-GFP (C) and Huh7 (D) cell lines labeled with the CNT- Fe_3O_4 derivatives (CNT- Fe_3O_4 , CNT- Fe_3O_4 -P₁, and CNT- Fe_3O_4 -P₄). (E)(F) *In vivo* MR

images of mouse liver pre- and post-injection of CNT-Fe₃O₄-P₄ at a dose of 100 mg/kg, with white arrows indicating tumors. Adapted with permission from ref. [85], Copyright (2015) American Chemical Society.

Another theranostic agent consisting of a ferromagnetic material encapsulated inside MWCNTs has been reported by Peci *et al.*, however in this case, the magnetic material was in the form of a wire [86]. The iron nanowire, termed iron-filled MWCNTs (Fe-MWCNTs), displayed high coercivity due to shape anisotropy, promoting its use in cancer therapy as a magnetic hyperthermia agent. In this case, the authors added Gd³⁺ ions to the surface of the Fe-MWCNTs by sonication with GdCl₃, which resulted in attachment of Gd³⁺ ions on the MWCNT sidewalls. This resulted in a dual-function agent owing to the integration of both the heating element (the Fe nanowire) and the paramagnetic Gd³⁺ ions that generated MRI contrast. Although the structures were well characterized, no MR images were reported and the biological stability of the Gd³⁺ ions was not evaluated.

A *T*₂-weighted MRI signal is also observed from CNTs containing metal impurities such as iron and nickel. Such a phenomenon was exploited to study the chronic toxic effect of raw SWCNTs to the lungs through a long-term (3 months) follow-up study that was achieved by hyperpolarized helium-3 (HP-³He) MRI [87], a technique that is used for clinical pulmonary research and has been described previously [88]. Intratracheal instillation of SWCNTs was performed in rats and the amount of metal impurities in the CNTs were large enough to produce a significant drop in magnetic field homogeneity as detected in the ³He MR images. In another study, MRI in conjunction with Raman spectroscopy and iron bio-assays, was used to assess the biodistribution of intravenously injected pristine (raw and purified) and functionalized SWCNTs in a two-week study [89]. The intrinsic iron impurities (11% by weight) in the raw sample allowed the *in vivo* detection of SWCNTs by MRI. In a similar report, commercial HiPCO SWCNTs were non-covalently coated with amine-terminated PEG and relaxivity values, as well as two-dimensional images of solid phantoms were acquired [90]. The trace amount of iron in the sample was sufficient to distinguish a difference from the background in the MRI scan at nanomolar concentrations.

The potential of MWCNTs with low metal impurities (2.57% Fe by weight) as intracellular MRI CAs was exploited by Vittorio and coworkers [91]. A solution of 0.1% Pluronic[®] F127 in phosphate-buffered saline (PBS) was used to make an aqueous suspension of the MWCNTs and mesenchymal stem cells from the bone marrow of rats were used for *in vitro* studies. MR images of the CNT suspension and of labeled cells at different CNT concentrations showed that the low Fe content in the CNTs was sufficient to visualize the cells by *T*₂-weighted MRI. As shown above, MRI provides a very efficient method for tracking CNTs as well as

tracking cells using CNT-based intracellular probes since MR images elucidate anatomical structures with very high resolution. However, MRI is limited by its low sensitivity, which requires large concentrations (e.g. 1 mM) in order to produce high quality images when using a CA.

3. CNT-based Agents for Nuclear Imaging

Among the nuclear imaging techniques, SPECT and PET have become important tools in both clinical and research settings. The way a SPECT image is generated is by having one or two detectors rotate to identify gamma (γ) rays emitted from a decaying radionuclide inside the patient and convert the detected energy into an electrical signal, which is then processed into an image. In PET imaging, radionuclei release positrons (β^+), which interact with electrons in their vicinity and annihilate one another, emitting two γ rays 180° with respect to each other with identical energies of 511 keV. The γ rays are detected by a ring of detectors that register coincident rays coming from a single event along a given line of response. In both types of imaging, two-dimensional cross-sections of the subject from multiple angles can be reconstructed and stacked to create a three-dimensional image [92]. When compared to MR and X-ray imaging techniques, which offer structural information with very high resolution, nuclear imaging techniques offer functional information and the ability to quantitatively track material *in vivo* with very high sensitivity. PET is a far more sensitive technique than SPECT, requiring material concentrations of only 10^{-10} to 10^{-12} M, whereas SPECT requires concentrations of about 10^{-6} M. Furthermore, PET often has higher resolution (2-3 mm) than SPECT (6-8 mm) [93,94].

3.1 CNT-based Agents for SPECT

Hong *et al.* filled short SWCNTs (approximately 300 nm in length) with Na^{125}I ($t_{1/2} = 59$ d) and determined tissue localization using SPECT [95]. In this report, the authors first employed a steam purification technique to shorten the SWCNTs and remove any graphitic and metal impurities. SWCNTs were subsequently loaded with Na^{125}I by molten-phase capillary wetting and covalently surface-functionalized with *N*-acetylglucosamine sugar molecules. The material was then characterized using TEM and scanning transmission electron microscopy (STEM). The radiolabeled, functionalized SWCNTs were injected i.v. in mice and imaged using SPECT, showing localization predominately in the lungs with about 80% injected dose per gram of tissue (%ID/g) up to 24 h, and a negligible amount was found in the blood 3 min after injection. Histological analysis of lungs, liver, spleen, and kidneys corroborated the SPECT imaging data and showed no sign of tissue necrosis or other acute (24 h) or long-term (30 d) abnormalities.

Another study by Al-Jamal *et al.* used SPECT imaging of MWCNTs radiolabeled with ^{111}In ($t_{1/2} = 67.5$ h) to determine effects of specific chemical functionalization on the distribution and excretion of the MWCNTs [96]. The authors investigated three different samples: MWCNTs **6**, **7**, and **8**. The first sample, MWCNT **6**, was functionalized solely by 1,3-dipolar cycloaddition [97] and then conjugated to DTPA. The other two samples, MWCNTs **7** and **8**, were first oxidized, shortening and introducing carboxylate groups to the surface of the nanotubes, followed by amidation with diaminotriethyleneglycol, introducing amino groups to the surface of the nanotubes, and then finally conjugating DTPA to the material using amide coupling. MWCNT **8** was functionalized for a second time after learning that the MWCNT **7** sample had extra free amino groups on the surface after coupling to DTPA and therefore had approximately twice the amount of functionalization as MWCNTs **6** and **7**. TEM indicated that the mean diameter of the MWCNTs was 34.1 ± 10.2 nm with lengths of 773 ± 425 nm for the precursors of MWCNT **6** and 354 ± 170 nm for the precursors of MWCNTs **7** and **8**. The study reported that the sample with the highest degree of functionalization (MWCNT **8**) accumulated in the spleen and lungs. Meanwhile, MWCNT **8** showed reduced liver accumulation and greater renal excretion in contrast with the other samples, which aggregated and remained in the liver, spleen, and lungs (see Figure 7). The authors of this study and others have also previously reported [98,99] that only individualized (non-bundled) CNTs with average diameters of 20-30 nm can be excreted through the kidneys.

Figure 7: SPECT/CT fused images of the whole mouse post-administration of ^{111}In -DTPA-MWCNTs. Images were taken at 0.5, 3.5 and 24 h post-injection for MWCNTs 6, 7, and 8 after injection of 50 μg of ^{111}In -DTPA-MWCNTs with a scanning time up to 0.5 h. Reproduced with permission from ref. [96], Copyright (2012) John Wiley and Sons.

3.2 CNT-based Agents for PET

McDevitt and coworkers developed an amine-functionalized SWCNT material that was conjugated to both the tumor neovascular-targeting antibody E4G10 and to one of two different metal chelates that contained either $^{225}\text{Ac}^{3+}$, an alpha emitter for radiotherapy ($t_{1/2} = 10$ d) chelated by 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), or $^{89}\text{Zr}^{4+}$, a β^+ emitter for PET imaging ($t_{1/2} = 78$ h) chelated by desferrioxamine B [100]. Using the targeted, radiolabeled SWCNT material resulted in an approximately five-fold increase in specific activity, which improved the signal-to-noise ratio of the image. Another study by Huang *et al.* examined the effects on fetotoxicity of water-suspendable SWCNTs and MWCNTs and used PET to study material localization in pregnant mice and their fetuses [101]. The CNTs were non-covalently functionalized using an amino-phospholipid-poly(ethylene glycol) (PL-PEG-NH₂) derivative as previously reported [102] with DOTA then conjugated to the CNTs in order to radiolabel the material with $^{64}\text{Cu}^{2+}$ ($t_{1/2} = 12.7$ h). The samples tested were short SWCNTs and MWCNTs of similar lengths (0.5-2 μm), with various outer diameters of MWCNTs: < 8 nm (MWCNT-8), 20-30 nm (MWCNT-20), and ~ 50 nm (MWCNT-50). Toxicity results indicated that MWCNT-50 had a more toxic effect on fetuses than the SWCNT, MWCNT-8, and MWCNT-20 samples at the same dose. MWCNT-50 also was shown to decrease the long-term survival of the offspring, with a > 20% lethality rate. PET imaging results showed that the material accumulated in the liver with high uptake values (10-12 %ID/g), as well as in the uterus of pregnant mice at 48 h post-injection. The fetuses were then harvested and imaged at 48 h post-injection and showed CNT uptake in the fetal liver and placenta. The authors concluded that functionalized MWCNTs (particularly MWCNT-50) more readily induced toxicity in mouse fetuses than other derivatives at similar doses. Since the biodistribution and the uptake values of the CNTs were similar, the study concluded that any toxicity that was observed must not be from the amount of uptake, but from other properties of the CNTs, such as the diameter, agglomeration state, etc. These and other studies have concluded that among all the nuclear medicine techniques, SPECT and PET can be used to effectively track minute amounts of material *in vivo* due to their high sensitivity.

4. CNT-based Agents for X-ray CT

Despite the introduction and growth of MR, ultrasound, and nuclear imaging systems, X-ray remains one of the main imaging modalities today due to its cost-effectiveness and diverse applicability. X-ray CT combines multiple X-ray projections taken from different angles to produce detailed cross-sectional images of the body. Contrast enhancement comes largely from the photoelectron effect due to high atomic numbers, thus hard tissue such as bone and cartilage have high attenuation compared to soft tissues due to the high concentration of calcium. Currently, there are two types of approved X-ray CAs for human use: barium sulfate suspensions (strictly used for imaging the gastrointestinal tract) and iodinated molecules. There are many elements with larger atomic numbers than iodine, thus the current use of iodinated CAs is mostly based on their superior safety and cost rather than their optimal efficacy as X-ray attenuators [103]. Some metals, metal salts, and metal particulates have been shown to be excellent X-ray CAs, such as bismuth sulfide [104,105] and gold nanoparticles [106,107].

Little research involving CNT-based CAs for X-ray has been developed, mostly due to the high CA concentration needed to produce good contrast and the poor water solubility and dispersion achieved with CNTs in aqueous media. Previously, functionalized US-SWCNTs (20-80 nm) filled with I₂ were synthesized and the X-ray attenuation was tested, although no *in vitro* or *in vivo* studies were performed [108]. More recently, Bi³⁺ ions and Bi₂O₃ were encapsulated in US-SWCNTs using BiCl₃ as the bismuth source, followed by suspension of the material in a 0.17% Pluronic[®] solution. Bismuth was chosen as a substitute for iodide due to its higher atomic number and lower toxicity [109,110]. *In vitro* studies were performed in porcine mesenchymal stem cells, causing no observed cytotoxicity to the cells while increasing the X-ray attenuation when compared with control cells [111]. With today's technology, single-cell tracking using CT is not promising [112], however, 3D structures of cells such as stem cell-engineered cell sheets, cells encapsulated in hydrogels or capsules, among others, can be monitored with CT [113–115]. In general, CT still is one of the most used clinical modalities and more efforts regarding the development of new CAs should be considered in order to limit the radiation dose to the patient.

5. CNT-based Agents for Multimodal Imaging

Currently, nuclear images are often overlaid with a CT image to generate a composite image, which provides anatomical information for image analysis. However, due to the much higher resolution of MRI over CT, there is an increasing interest in combining the ultra-high resolution of MRI with the high sensitivity of SPECT and PET in a single image. Recently, there have been many reports of creating multimodal imaging agents for new multimodal

instrumentation, in order to prevent image distortion that may result if the two imaging agents are injected separately [116,117]. For example, Cisneros and coworkers developed GNTs loaded with both Gd^{3+} and $^{64}Cu^{2+}$ ions for combined PET/MR imaging (referred to as $^{64}Cu@GNT$) [118]. PET imaging results at 4 h post-injection showed the material predominantly localized to the lungs (~ 11.4 %ID/g) and liver (~ 11.2 %ID/g), with minor uptake in the kidneys and gastrointestinal tract (see Figure 8A-8B). Although $^{64}Cu^{2+}$ -labeled SWCNTs and free $^{64}Cu^{2+}$ ions both localized to the liver, since the $^{64}Cu^{2+}$ -labeled SWCNTs also concentrated in the lungs up to 48 h post-injection (Figure 8E-8F), the authors concluded that there was no transmetallation or degradation of the material. This particular study included both PET and MR phantom images, showing the efficacy of the bimodal agent, however, no *in vivo* MR or PET/MR images were obtained, due to the high dose required for MRI (~ 1 mM) and the comparatively low aqueous suspendability of the agent.

Figure 8: Bimodal imaging of MRI/PET CNT agents. (A, C, E) Micro-PET/CT images of normal mice injected with surfactant-wrapped US-SWCNTs filled with $^{64}Cu^{2+}$ and Gd^{3+} (referred to as $^{64}Cu@GNT$) or free $^{64}Cu^{2+}$. (B, D, F) Histograms show quantification of the material at 4, 24, and 48 h postinjection. (A.a.) The arrows highlight the presence of $^{64}Cu^{2+}$ in the liver (white arrow) and gastrointestinal tract (yellow arrow) for free $^{64}Cu^{2+}$, (A.b.) and in the lungs (red arrow). Error bars represent mean $\pm$ SD. * $p \leq 0.05$; ** $p \leq 0.01$. Adapted with permission from ref. [118], Copyright (2014) Future Medicine Ltd.

Wang *et al.* also developed a bimodal imaging agent based on MWCNTs that were conjugated to SPIONs for MRI contrast and then radiolabeled with ^{99m}Tc ($t_{1/2} = 6$ h) for SPECT imaging [119]. Functionalized MWCNTs were mixed with iron acetate, which served as the precursor to the SPIONs that grew along the backbone of the MWCNTs with different amounts of SPIONs loaded on the MWCNTs, henceforth referred to as S-MWCNTs (superparamagnetic MWCNTs). The two main samples that were studied were S-MWCNT-2 (28.0% SPION by weight) and S-MWCNT-3 (41.5% SPION by weight). Relaxation measurements that were performed on the S-MWCNTs 2 and 3 showed r_2^* relaxivities of $343 \text{ mM}^{-1}\text{s}^{-1}$ and $425 \text{ mM}^{-1}\text{s}^{-1}$, respectively. The SPION-MWCNT conjugates were then radiolabeled with ^{99m}Tc with good efficiency by using dipicolylamine-alendronate as a linker between the radionuclide and the SPIONs, although S-MWCNT-3 showed the best radiolabeling yields and biological stability and was exclusively used in subsequent imaging studies. Tissue distribution from whole-body SPECT/CT imaging showed prevalent accumulation in the lungs, liver, and spleen within 30 min of i.v. injection. After 4 h post-injection, while the liver signals remained constant, there was a clear decrease in lung uptake and SPECT results were verified with post-mortem gamma-scintigraphy. *In vivo* MR images were also obtained of the liver using S-MWCNT-3, which showed good relaxation rates that increased linearly with increasing dose of material. *In vitro* studies of S-MWCNT-3 using a mouse macrophage cell line with concentrations up to $100 \mu\text{g/mL}$ after 24 h incubation found no significant cytotoxicity in addition to any histological abnormalities in mouse tissues up to 30 days.

Similarly, Al Faraj and coworkers recently developed a multifunctional SWCNT agent conjugating SPIONs and gallium-67 ($t_{1/2} = 3.26$ days) for MRI and SPECT, respectively, in order to target and image breast cancer stem cells [120]. SWCNTs were oxidized and shortened to about 200 nm in length. The oxidized CNTs were then PEGylated and conjugated to SPIONs and DOTA to chelate gallium-67 for imaging, in addition to CD44 monoclonal antibodies for cancer stem cell targeting. SPECT imaging studies in mice showed significant material accumulation in reticuloendothelial organs (liver and spleen) as well as the kidneys and heart, but to a lesser extent. Tumor homing was enhanced after injection of the antibody-conjugated samples compared to untargeted controls. SPECT data were confirmed using MRI and post-mortem gallium-67 quantification using a gamma counter. Since the amount of material accumulation at 2, 24, and 48 h time points was statistically similar, the authors suggested that the material was cleared slowly from the mouse. The authors also substituted the gallium-67 for a near-infrared fluorescence probe and conducted imaging studies, which yielded very similar results as the SPECT and MRI studies. With the development of multimodal imaging instrumentation, it is

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4 expected that this field will grow and more multipurpose agents, both for imaging and therapeutic
5 applications will begin to appear in the literature.
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8 9 **6. Conclusion**

10 This review documents many examples of CNT materials, which have been labeled with
11 one or more imaging agents, and functionalized for water suspendability to image non-invasively.
12 While other CNT-based CAs have been synthesized for other imaging techniques, we have
13 chosen to focus on those full-body imaging modalities most used in the clinic today (MRI, X-ray
14 CT, SPECT, and PET), and therefore exhibit the most translational potential. The examples
15 presented here have shown that appropriately functionalized CNT materials are a versatile
16 platform with potential for a wide-range of imaging and therapeutic applications in medicine. In
17 general, the studies reviewed have also shown that properly purified and derivatized CNT
18 materials are non-toxic at reasonable clinical concentrations. While this is true for the nuclear
19 imaging techniques of SPECT and PET, this is less certain for MR and X-ray CT imaging where
20 concentrations in the millimolar range are required. In fact, new functionalization procedures
21 aimed at increasing CNT agent suspendability into the millimolar range or above are still needed
22 for toxicity and performance testing at these levels. For a complete discussion on the toxicity of
23 CNTs, the authors direct the readers to the following review articles [121–123].
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26 Some of the currently accepted criteria for clinical imaging agents are that the agent
27 should (1) be safe (non-toxic), (2) have sufficiently high performance for the imaging techniques
28 needed and (3) be stable *in vivo* long enough to perform the imaging function until eventual
29 elimination or biodegradation. In fact, recent research has focused on peroxidase-driven
30 biodegradation of CNTs, and other natural enzymatic catalysis [124–126]. Finally and most
31 importantly, the agent must be reproducibly produced using good manufacturing practices as
32 required by the U.S. Food and Drug Administration. This requirement implies that SWCNTs
33 should be superior to MWCNTs for CNT-based agent design simply because MWCNTs are a far
34 less uniform material.
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37 While several CNT-based materials in the literature have already satisfied most of the
38 above criteria, two major problems linger. Namely, more work is needed to create a more uniform
39 particle distribution in both size (length) and degree of functionalization. Secondly, more
40 preclinical data is needed which quantifies and potentially proves total elimination of the CNT
41 agents from the body over time. Finally, CNT-based materials have been shown to surpass the
42 performance of all other nanoparticle and small molecule CAs, while maintaining high
43 biocompatibility. For example, the “Gadonanotubes” provide unrivaled performance as MRI CAs
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with relaxivities up to 40 times greater (on a per Gd^{3+} -ion basis) than current Gd^{3+} -based CAs in the clinic [63]. Studies have shown that this phenomenon is due to the encapsulation of Gd^{3+} -ion clusters within the cavities of the US-SWCNTs and the special Gd^{3+} ion-water interaction for that specific environment. Such findings provide us with valuable information about the physics behind the performance of the agents and about the contrast enhancement phenomenon, which allow us to possibly design more powerful CAs. This is just one of the many surprises that other new CNT-based CA might also provide. Therefore, the search for new CNT-based CAs should go on to explore the possibilities, while parallel efforts are also needed to seek pathways toward clinical approval for key CNT-based CAs that emerge from such studies.

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