Stable confinement of positron emission tomography and magnetic resonance agents within carbon nanotubes for bimodal imaging

Aims: Simultaneous positron emission tomography/MRI has recently been introduced to the clinic and dual positron emission tomography/MRI probes are rare and of growing interest. We have developed a strategy for producing multimodal probes based on a carbon nanotube platform without the use of chelating ligands. **Materials & methods:** Gd^{3+} and $^{64}Cu^{2+}$ ions were loaded into ultra-short single-walled carbon nanotubes by sonication. Normal, tumor-free athymic nude mice were injected intravenously with the probe and imaged over 48 h. **Results & conclusion:** The probe was stable for up to 24 h when challenged with phosphate-buffered saline and mouse serum. Positron emission tomography imaging also confirmed the stability of the probe *in vivo* for up to 48 h. The probe was quickly cleared from circulation, with enhanced accumulation in the lungs. Stable encapsulation of contrast agents within ultra-short single-walled carbon nanotubes represents a new strategy for the design of advanced imaging probes with variable multimodal imaging capabilities.

Original submitted 19 August 2013; Revised submitted 29 January 2014

KEYWORDS: carbon nanotube • contrast agent • MRI • multimodal imaging • positron emission tomography

Recent studies have demonstrated the continued progress in the development of nanoparticle-based systems for medical applications [1–3]. Among these, single-walled carbon nanotubes (SWCNTs) are of special interest due to their unique combination of notable physical and chemical properties such as high tensile strength, high aspect ratio, electrical conductivity, limited chemical reactivity and near-infrared fluorescence [4,5]. Importantly, SWCNTs have several advantages over other nanoparticle delivery systems, which make them uniquely suited for delivery of medical imaging agents. For example, unlike fullerenes, nanotubes may be filled postsynthesis, allowing for significant reduction in cost and time during preparation of the probe. Carbon nanotubes also allow intracellular delivery of imaging agents due to their ability to cross the cell membrane [6].

A key attribute of SWCNTs involves the potential to use their interior space for encapsulation of small molecules or ions [7,8]. Encapsulation offers better protection from transmetallation than metal chelation and also avoids the necessity of developing new ligands for different imaging agents and applications. For example, there are nine different commercialized gadolinium-based contrast agents (CAs) for MRI [9]. Since stable encapsulation shields the payload from biological interaction with surrounding tissue, large quantities of payload can theoretically be administered without systemic toxicities or other unwanted interactions. This structural property renders SWCNTs as a potential delivery vehicle for medical imaging agents that are otherwise toxic, such as heavy metal ions or radionuclides. Furthermore, the large, external surface area of SWCNT materials can also be used as

Brandon T Cisneros^{1,2}, Justin J Law¹, Michael L Matson¹, Ali Azhdarinia³, Eva M Seveck-Muraca³ & Lon J Wilson^{*1}

¹Department of Chemistry & Richard E Smalley Institute for Nanoscale Science & Technology, Rice University, 1900 Rice Blvd, Houston, TX 77005, USA

²Department of Surgical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

³The University of Texas Health Science Center at Houston, Center for Molecular Imaging, The Brown Foundation Institute of Molecular Medicine, Houston, TX 77030, USA

*Author for correspondence:

Tel.: +1 713 348 3268

Fax: +1 713 348 5155

durango@rice.edu

Future
Medicine

part of
fsg

a scaffold to which targeting or therapeutic moieties may be attached [10–12], allowing for targeted delivery of imaging agents and their theranostic counterparts. Compared with liposomes, which are actively used for delivery of therapeutics [13], nanotubes are better able to retain encapsulated materials, making them more effective for imaging applications in which the delivered materials should not be released.

Imaging of SWCNTs can be achieved by exploiting the intrinsic properties of the nanotube by using Raman spectroscopy, near-infrared fluorescence spectroscopy or photoacoustic tomography [14]. However, such techniques may have limited signal penetration *in vivo* and lack the widespread clinical utility observed with more conventional diagnostic imaging modalities. Thus far, there have been only a few reports of SWCNT-based probes for clinically relevant imaging modalities with full-body penetration such as computed tomography (CT), single-photon emission CT, positron emission tomography (PET) and MRI [15–17]. Both T1- and T2-weighted SWCNT-based magnetic resonance (MR) CAs have been prepared,

frequently through attachment of chelated Gd^{3+} or iron oxide on the external surface of the SWCNT material [18,19]. Radionuclides have similarly been attached to the SWCNT exterior through the use of chelating ligands [15,16]. There is also one report of molten-phase filling of SWCNTs with a radionuclide [20], but the extensive procedure required for preparation of the probe is likely prohibitive for clinical applications.

In this work, we demonstrate the synthesis of a bimodal SWCNT-based probe for PET/MRI by stable encapsulation of imaging agents (Gd^{3+} and ^{64}Cu) within ultra-short SWCNTs (US-tubes, Figure 1C). Therefore, the present study explores whether encapsulation of metal-ion-based imaging agents can offer a viable alternative to metal-ion chelation chemistry in medical imaging technology. A probe of SWCNT design (a US-tube) was chosen for these initial studies to take advantage of the fact that Gd^{3+} -ion-loaded US-tubes (gadonanotubes [GNTs]) are the highest-performing T1-weighted MRI CA material known, with a R1 relaxivity up to 40-times greater than current clinical agents such as Magnevist® (Bayer AG [Leverkusen, Germany]; gadopentetate

Figure 1. Production of the bimodal $^{64}\text{Cu}^{2+}/\text{Gd}^{3+}$ imaging probe. (A) Full-length single-walled carbon nanotubes are (B) fluorinated, pyrolyzed under argon and purified with concentrated HCl to produce (C) ultra-short single-walled carbon nanotubes with sidewall defect sites. Gd^{3+} and $^{64}\text{Cu}^{2+}$ are then internally coloaded by aqueous sonication to produce (D) $^{64}\text{Cu}@\text{GNT}$ s. (E) Excess Gd^{3+} and $^{64}\text{Cu}^{2+}$ are removed by serum challenge and washing. (F) Purified $^{64}\text{Cu}@\text{GNT}$ s are suspended in 0.1% Tween® 20 (Aurion [Wageningen, The Netherlands]) to produce surfactant-wrapped $^{64}\text{Cu}@\text{GNT}$ s for *in vivo* experiments.

$^{64}\text{Cu}@\text{GNT}$: Ultra-short single-walled carbon nanotube filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} .

dimeglumine; R1: $3.4 \text{ mM}^{-1}\text{s}^{-1}$) [21,22]. For this reason, MRI CAs and other multimodal imaging agents that incorporate a GNT structural element would be highly desirable in the clinic. Furthermore, a successful encapsulation strategy as opposed to chelation chemistry would allow for production of imaging agents with any or all whole-body imaging modalities in a single chemical entity.

To our knowledge, the present work is the first report of a SWCNT-based bimodal PET/MR probe of any nature. Since clinical instrumentation for simultaneous PET/MRI has only recently been developed [23], only a few probes utilizing both modalities are known [24,25]. As such, production of new bimodal PET/MR probes where maximized sensitivity (PET) and resolution (MRI) in medical imaging are simultaneously achieved is currently of special interest.

Materials & methods

General procedures

All reagents and solvents were analytical grade, purchased from commercial suppliers, and used without further purification unless otherwise stated. ^{64}Cu was produced by Washington University School of Medicine (MO, USA) in 0.1 M HCl and diluted with 0.1 M NaOAc (pH 6) for labeling reactions.

Preparation of US-tubes

Full-length SWCNTs (Carbon Solutions Inc. [CA, USA]) produced by the electric arc discharge method (Figure 1A) were used to prepare US-tubes by fluorination and pyrolysis (Figure 1B), as previously reported [26]. After preparation of the US-tubes, they were purified of amorphous carbon and metal impurities by sonication with concentrated HCl. The resulting purified US-tubes were then activated for internal ion loading by a brief treatment with nitric acid (6 M; 15 min reflux). The US-tubes were then reduced using Na⁰ (sodium metal) in tetrahydrofuran to further debundle them into individual US-tubes (Figure 1C) [27].

Preparation of Cu@US-tubes

US-tubes (15 mg) were bath sonicated in a solution of aqueous CuCl_2 (10 ml; 5 mM) for 60 min. The Cu@US-tube product was isolated by filtration with a coarse glass filter frit and washed with deionized water until the filtrate showed no detectable levels of Cu^{2+} , as determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; limit of detection: 0.01 ppm).

Preparation of US-tubes filled with Cu^{2+} & Gd^{3+}

US-tubes (15 mg) were sonicated in an aqueous solution of CuCl_2 and GdCl_3 (10 ml 2.5 mM CuCl_2 , 2.5 mM GdCl_3 ; or 10 ml, 1 mM CuCl_2 4 mM GdCl_3) and bath

sonicated for 60 min. NaOH (1 M; 0.6 ml) was added to adjust to neutral pH to trap the Cu^{2+} ions within the Gd^{3+} clusters within the tubes. The US-tubes filled with Cu^{2+} and Gd^{3+} (Cu@GNT) product was isolated by filtration with a coarse glass filter frit and washed with deionized water until the filtrate showed no detectable levels of Cu^{2+} or Gd^{3+} , as determined by ICP-OES.

Particle size & charge measurements

Empty US-tubes and Cu@GNTs were dispersed in 0.1% Tween[®] 20 (Aurion [Wageningen, The Netherlands]) and particle size and zeta-potential were determined using a Malvern Instruments[™] (Worcestershire, UK) Zen 3600 Zetasizer[®].

Preparation of US-tubes filled with $^{64}\text{Cu}^{2+}$ & Gd^{3+}

US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} (^{64}Cu @GNTs) production employed the same procedures as nonradioactive Cu^{2+} and was accomplished by adding GdCl_3 (0.5 ml; 1 mM) and $^{64}\text{CuCl}_2$ (25 μl ; 155.4 MBq) to a scintillation vial containing US-tubes (2.2 mg). After 30 min of bath sonication, NaOH was added to reach neutral pH. The product was isolated by ultrafiltration (Amicon[®] Ultra [Sigma-Aldrich; MO, USA]; 10 kD molecular weight cut-off) to remove nonencapsulated metal ions, then washed three times with deionized water (Figure 1D). Radiochemical purity was assessed by radio-thin-layer chromatography with Whatman[®] chromatography paper (Sigma-Aldrich) and 0.1 M ammonium acetate/0.05 M EDTA (pH 6) as the mobile phase and analyzed with an AR-2000 scanner purchased from Bioscan (Washington DC, USA).

Removal of nonspecifically bound $^{64}\text{Cu}^{2+}$ from ^{64}Cu @GNTs & aqueous suspension of ^{64}Cu @GNTs

After preparation, purified ^{64}Cu @GNT was dispersed in 0.1% Tween 20 in deionized water by brief sonication to yield surfactant-wrapped ^{64}Cu @GNT. Nonspecifically bound $^{64}\text{Cu}^{2+}$ was removed by incubation with 10 μM EDTA (40°C; 15 min) followed by incubation in 50% mouse serum (37°C; 15 min; Figure 1E). The product was isolated by ultrafiltration and washed with deionized water until the filtrate had minimal activity. The product was resuspended in 0.1% Tween 20 in phosphate-buffered saline (PBS) for the *in vivo* experiments (Figure 1F).

Stability studies

In vitro stability studies were performed on dry samples of Cu@US-tubes and Cu@GNTs to simulate *in vivo* pH, salinity and serum interaction in order to determine the retention properties of the US-tubes.

Samples were incubated in 50% (w/v) bovine serum albumin (BSA) and PBS at 37°C for 12 h, 24 h or 7 days. At each time-point, samples were purified by filtration on a coarse fritted glass filter. The recovered sample and the filtrate were analyzed for Cu^{2+} and Gd^{3+} by ICP-OES. Water, PBS and BSA used in the experiment were also analyzed for Cu^{2+} and Gd^{3+} for determination of background corrections. For the $^{64}\text{Cu@GNTs}$, 50% mouse serum was used instead of BSA with an incubation period of 24 h at 40°C in order to provide better biocompatibility for *in vivo* studies. Ultrafiltration was performed in 100 kD molecular weight cut-off centrifuge filters. All experiments were performed in triplicate.

Bimodal imaging of an aqueous suspension of $^{64}\text{Cu@GNTs}$

Images were acquired on a Siemens (TN, USA) Inveon™ MicroPET/CT scanner using the instrument parameters previously reported [28]. Phantoms were prepared at concentrations of 1.85, 3.7 and 7.4 MBq/ml with control wells containing water and Tween 20, and PET imaging was performed for 20 min (Figure 2A). The radioactivity of surfactant-wrapped $^{64}\text{Cu@GNT}$ was allowed to decay sufficiently (ten half-lives) and phantom MRIs were obtained on Philips (Amsterdam, The Netherlands) Achieva™ 1.5 T and 3 T systems. An inversion recovery sequence was used for image acquisition (repetition time: 6500 ms; echo time: 10 ms at 1.5 T; repetition time: 9000 ms, echo time: 20 ms at 3 T; Figures 2B & 2C).

In vivo PET/CT imaging

In vivo imaging studies were carried out in accordance with the standards of the University of Texas Health Science Center at Houston (USA), Center for Molecular Imaging, after review and approval of the protocol by respective Institutional Animal Care and Use or Animal Welfare Committees. Female athymic nude mice (3–4 week old; 18–22 g; Harlan Sprague–Dawley Inc. [IN, USA]) were housed five per cage in a pathogen-free mouse colony and provided with sterilized pellet chow and sterilized water. Initially, the mice were anesthetized with 1% isoflurane and a CT scan was performed. The bed position was then moved into the PET field-of-view where the mice received 7.4–11.1 MBq of surfactant-wrapped $^{64}\text{Cu@GNTs}$ or free $^{64}\text{Cu}^{2+}$ through the tail vein ($n = 3$ per group). Dynamic PET imaging was performed and region-of-interest analysis was used to generate time activity curves (TACs) for the liver, kidneys, heart, lung, muscle and bladder according to prior methods [29]. Static PET/CT images were acquired at 4, 24 and 48 h postinjection (p.i.) for each mouse and processed

with the vendor software package (Inveon™ Research Workplace [Siemens]) to quantify tracer uptake in selected organs.

Results

Preliminary studies

Studies to determine the viability of encapsulating $^{64}\text{Cu}^{2+}$ within US-tubes were successful. Nonradioactive CuCl_2 was loaded by aqueous sonication, yielding the Cu@US-tube product, which was determined to contain 2.05% (w/w) Cu^{2+} by ICP-OES. However, challenge with PBS or BSA resulted in significant Cu^{2+} leakage (>45%) within 12 h. Simultaneous loading of Cu^{2+} and Gd^{3+} was attempted using $\text{Cu}^{2+}:\text{Gd}^{3+}$ ratios of 1:1 and 1:4 and analysis of the Cu@GNT products by ICP-OES indicated Cu^{2+} contents of 0.7% (w/w) and 0.4% (w/w), respectively. Challenge with PBS or BSA over a 7-day period resulted in no detectable Cu^{2+} or Gd^{3+} leakage. T1 relaxivity values for the Cu@GNT product and GNTs produced from the same batch of US-tubes were determined to be 52.7 and 53.4 $\text{mM}^{-1}\text{s}^{-1}$, respectively. Particle size analysis by dynamic light scattering indicated Cu@GNTs are slightly larger than empty US-tubes, with an average diameter of 98 nm compared with 70 nm for empty US-tubes. Zeta-potentials for suspensions of Cu@GNTs and empty US-tubes in 0.1% Tween 20 were 23.7 and 24.6 mV, respectively, indicating stable suspensions of the probe.

Synthesis & characterization of bimodal probe

Synthesis of the bimodal probe was accomplished by replacing the nonradioactive CuCl_2 with $^{64}\text{CuCl}_2$. After washing, the product was determined to be radiochemically pure by radio-thin-layer chromatography with a specific activity of 19.69 MBq/mg. $^{64}\text{Cu}^{2+}$ labeling was found to be stable to PBS challenge over 24 h at room temperature. However, a 37% loss in activity was observed after 24 h exposure to 50% mouse serum. Further exposure of the sample to fresh serum at room temperature for an additional 24 h did not result in additional loss of $^{64}\text{Cu}^{2+}$, indicating that only a limited population of $^{64}\text{Cu}^{2+}$ can be removed by serum challenge. Phantom images of the probe indicated both PET and MRI contrast separately (Figure 2).

In vivo imaging

Dynamic imaging of mice receiving surfactant-wrapped $^{64}\text{Cu@GNTs}$ or free $^{64}\text{Cu}^{2+}$ resulted in the generation of TACs for the heart, lungs, liver, kidneys, bladder and muscle. The major site of accumulation of surfactant-wrapped $^{64}\text{Cu@GNTs}$ was in the lungs

Figure 2. Phantom images of purified US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} obtained by positron emission tomography/computed tomography and T1-weighted MRI. (A) Micro PET/CT image of purified US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} at varying concentrations. From top to bottom: Milli-Q® water (Millipore Corp. [MA, USA]), 1% Tween® 20 (Aurion [Wageningen, The Netherlands]), 7.4 MBq/ml, 3.7 MBq/ml and 1.85 MBq/ml. **(B)** MRI of purified US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} at varying concentrations for a 1.5-T field strength. From left to right: 7.4 MBq/ml, 3.7 MBq/ml, 1.85 MBq/ml and 1% Tween 20. **(C)** MRI of purified US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} at varying concentrations for a 3.0-T field strength. From left to right: 7.4 MBq/ml, 3.7 MBq/ml, 1.85 MBq/ml, and 1% Tween 20.

CT: Computed tomography; PET: Positron emission tomography.

with a peak value of 21.4 % injected dose (ID)/g at 2-min p.i. and persistent signal that was significantly higher ($p < 0.001$) than the control group throughout the scan (Figure 3A). Heart uptake was also higher in mice injected with surfactant-wrapped $^{64}\text{Cu}@GNTs$, indicating prolonged blood pool activity of the bimodal agent compared with free $^{64}\text{Cu}^{2+}$ (Figure 3B). TAC profiles for liver, kidneys and muscle revealed no significant differences between agent distribution in these organs (Figures 3C–3E). Bladder signal was approximately threefold higher in mice injected with free $^{64}\text{Cu}^{2+}$, suggesting increased renal clearance of this agent compared with surfactant-wrapped $^{64}\text{Cu}@GNTs$, although significance was not achieved (data not shown).

Delayed PET/CT imaging results correlated with the dynamic imaging data. Scans at 4 h p.i. revealed accumulation of surfactant-wrapped $^{64}\text{Cu}@GNTs$ primarily in the liver ($11.4 \pm 1.9\%$ ID/g tissue) and lungs ($11.2 \pm 2.2\%$ ID/g tissue) with minor uptake in the kidneys and GI tract, whereas free $^{64}\text{Cu}^{2+}$ showed strong liver signal and minor gastrointestinal uptake (Figure 4A). Region-of-interest values from the heart were no longer significant in mice injected with surfactant-wrapped $^{64}\text{Cu}@GNTs$ as a result of continued clearance of the agent from systemic circulation. No notable differences in kidney and muscle uptake were observed between the groups.

At 24 h, both groups continued to exhibit high liver signal, while the mice injected with surfactant-wrapped $^{64}\text{Cu}@GNTs$ also showed persistent lung uptake that was significantly higher than in control mice ($p < 0.05$; Figure 4B). Further imaging at 48 h revealed no significant changes in tissue distribution (Figure 4C). Elimination of both agents either by hepatobiliary or renal routes appeared to be slow according to the small change in % ID per gram values of the liver, kidneys and bladder from 4–48 h p.i.

Discussion

Our choice of SWCNTs as a platform for developing a bimodal PET/MR probe is based on several factors. First, we aimed to take advantage of the capability of SWCNTs to encapsulate materials for imaging or therapy [7]. Although methods exist for attaching radionuclides to the outer surface of carbon nanotubes [10], functionalization is achieved through chelators such as 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid, which may still permit loss of label through transmetallation [30]. Furthermore, many nanoparticle systems may be surface functionalized, but few afford the option of encapsulation, which offers better protection from biological interaction. Second, although there is much debate about the toxicity of carbon nanotube materials, the

Figure 3. Time-activity curves generated from dynamic positron emission tomography imaging of mice receiving surfactant-wrapped US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} and free $^{64}\text{Cu}^{2+}$. (A) Lungs, (B) heart, (C) liver, (D) kidneys and (E) muscle. The histograms show quantification of agent uptake as % injected dose/g tissue (n = 3). Error bars represent mean ± SD.

*p ≤ 0.001.

**p ≤ 0.01.

$^{64}\text{Cu}@GNT$: Ultra-short single-walled carbon nanotube filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} .

US-tubes used in this work are known to be well tolerated both *in vitro* and *in vivo* at doses up to 0.5 g/kg [31,32]. Finally, previous work has shown that US-tubes, which are produced by pyrolysis of fluorinated SWCNTs [26], can be internally loaded with aqueous solutions of Gd^{3+} . The resulting material, $\text{Gd}@US$ -tubes or GNTs, has a remarkably high T1-weighted relaxivity, which is up to 40-times greater than clinically available Gd^{3+} -based CAs under magnetic fields of 1.5 T [21,33]. Therefore, using this platform as a starting point for the development of a bimodal probe ensures a significant MR contrast component of the probe.

Preliminary studies

We initially attempted to load aqueous Cu^{2+} ions into US-tubes by aqueous sonication. Loading was successful, with 2.05% (w/w) Cu^{2+} detected. However, upon challenge with PBS or BSA, significant Cu^{2+} leakage was observed within 12 h. We then attempted to trap Cu^{2+} within the nanotube by simultaneously loading Gd^{3+} and Cu^{2+} . Although the resulting Cu^{2+} retention was reduced, no detectable Cu^{2+} or Gd^{3+} was found to leak over a 7-day period upon PBS or BSA challenge. This suggested that the pH-dependent hydrolysis chemistry of the Gd^{3+} ion is essential for retaining the Cu^{2+} . Gd^{3+} can enter the

Figure 4. Longitudinal micro-positron emission tomography/computed tomography imaging of normal mice injected intravenously with surfactant-wrapped US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} or free $^{64}\text{Cu}^{2+}$. Representative images are shown from acquisitions at (A,i) 4, (B,i) 24 and (C,i) 48 h postinjection of radiotracers. (A,ii–C,ii) Histograms show quantification of agent uptake as % injected dose/g tissue ($n = 3$). (A,ia) The arrows highlight the presence of ^{64}Cu in the liver (white arrow) and GI tract (yellow arrow) for free ^{64}Cu , (A,ib) and in the lungs (red arrow), liver and GI tract for US-tubes filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} . Error bars represent mean $\pm$ SD.

* $p \leq 0.05$.

** $p \leq 0.01$.

$^{64}\text{Cu}@GNT$: Ultra-short single-walled carbon nanotube filled with $^{64}\text{Cu}^{2+}$ and Gd^{3+} .

US-tubes through the sidewall defects under acidic conditions, but forms insoluble μ -oxo or μ -hydroxy clusters at a physiologic pH of 7.4, rendering the ions unable to exit through the same sidewall defects through which they entered [34,35]. The retained Cu^{2+} is likely either incorporated into the Gd^{3+} clusters or simply trapped within the US-tubes by the Gd^{3+} clusters at the defect sites that prevent their exit.

Next, it was demonstrated that $^{64}\text{Cu}^{2+}$ can be retained within US-tubes in quantities relevant for PET imaging. Experiments with $^{64}\text{Cu}^{2+}$ validated

the findings we observed with cold Cu^{2+} for which the loading protocol was optimized. PBS and mouse serum challenges were performed in preparation for *in vivo* experiments to examine the stability of the purified $^{64}\text{Cu}@GNT$ s (Figure 1E). Although some $^{64}\text{Cu}^{2+}$ was removed by serum challenge, the remaining population was found to be stable to further challenge. Further characterization of the bimodal probe was not attempted due to the radioactive nature of the agent and the fact that similar US-tube materials have been well studied previously [21,32,33,35].

T1-weighted relaxivity values were determined for the cold Cu@GNTs and for GNTs prepared from the same batch of US-tubes and were found to be 52.7 and 53.4 mM⁻¹s⁻¹, respectively. With so few Cu²⁺ ions present in a Cu@GNT sample, it is not surprising that the relaxivity per Gd³⁺ ion is not dramatically different from that of the parent GNTs. In both cases, the T1-weighted relaxivity values are still greatly superior to the most widely used clinical agent, Magnevist, which has an R1 of approximately 3.4 mM⁻¹s⁻¹ [22].

Phantom & *in vivo* imaging

In vivo biodistribution of a surfactant-suspended ⁶⁴Cu@GNT sample (Figure 1F) could be investigated by PET due to the extreme sensitivity of the technique; however, *in vivo* MRI was found to be impractical using the protocol of these studies due to the high dose requirements of MRI (~1 mM) and the relative low solubility of the ⁶⁴Cu@GNT/Tween 20 agent. New functionalization chemistry, such as that recently reported to yield a solubility of 50 mg/ml for SWCNT-based agents like US-tubes [36] will need to be developed for ⁶⁴Cu@GNT to achieve the solubility necessary for *in vivo* MRI. Nonetheless, the phantom images in Figure 2 clearly demonstrate the bimodal imaging capacity of the ⁶⁴Cu@GNT probe.

There have been several reports on the *in vivo* biodistribution of radiolabeled SWCNTs with surface functionalization; however, to the best of our knowledge, similar characterization of a bimodal MRI/PET SWCNT construct has yet to be performed. In our study, we developed a loading procedure with ⁶⁴Cu²⁺ and Gd³⁺ that provided sufficient stability and radioactivity, and allowed us to examine agent distribution and clearance by PET. Dynamic and static imaging studies were performed in normal mice injected with surfactant-wrapped ⁶⁴Cu@GNTs and free ⁶⁴Cu²⁺ at 4, 24 and 48 h p.i., and showed similar liver uptake for both agents at each timepoint. In the case of free ⁶⁴Cu²⁺, liver signal can be explained by chelation of the radiometal by copper-binding proteins in the liver [37], whereas the hepatic uptake of surfactant-wrapped ⁶⁴Cu@GNT occurs as a result of opsonization and is consistent with other reports showing high reticuloendothelial system uptake of radiolabeled SWCNTs [15,16]. Importantly, there was no increase in liver signal in mice injected with surfactant-wrapped ⁶⁴Cu@GNTs. It should be mentioned that since both free ⁶⁴Cu²⁺ and surfactant-wrapped ⁶⁴Cu@GNTs undergo hepatic clearance, it is difficult to distinguish if the liver signal is due to nanoparticle localization, degradation of the probe, or a combination of both.

However, given the data from the serum challenge experiments that suggest that encapsulated ⁶⁴Cu²⁺ is not expected to undergo further transchelation by serum proteins after injection, and the persistence of radioactivity in other organs such as the lungs (without notable reduction even after 48 h), we concluded that surfactant-wrapped ⁶⁴Cu@GNTs do not undergo significant degradation and its biodistribution profile reflects the *in vivo* stability of the probe.

A major difference in agent distribution was evident in the lungs where mice receiving surfactant-wrapped ⁶⁴Cu@GNTs showed prominent accumulation, which was detectable at 2 min p.i. and persisted up to 48 h p.i. In the lungs, pulmonary surfactant proteins are present in the air-liquid interface of the lungs and play an important role in defending the lungs against infection. Specifically, surfactant proteins A and D, which have been shown to selectively bind to carbon nanotubes due to the presence of surface modifications, may have played a role in the high lung accumulation observed in our study [38]. Similar lung uptake has been reported by others with radiolabeled SWCNTs and attributed to the interaction of lung-localized proteins known to interact with the *N*-acetylglucosamine molecules on the surface of the SWCNTs [20]. Although we did not perform any chemical modifications to the surface of the SWCNTs, it is conceivable that the -OH groups of Tween 20 may have reacted similarly to the -OH groups on *N*-acetylglucosamine to trigger an interaction with the pulmonary surfactant proteins. The size and shape of surfactant-wrapped ⁶⁴Cu@GNTs may also play a role in its capture and retention within the pulmonary beds, and ultimately contribute to the slow clearance of the agent from the body. From a therapeutic perspective, the robust lung uptake of surfactant-wrapped ⁶⁴Cu@GNTs is promising as it could allow for a novel approach for imaging-guided drug delivery using hybrid PET/MRI detection. For example, the loading procedure for ⁶⁴Cu²⁺ and Gd³⁺ within our probe could be modified to include a cytotoxic moiety to capitalize on lung localization and enable the use of a theranostic analog of surfactant-wrapped ⁶⁴Cu@GNTs for locoregional drug delivery for lung cancer. Noninvasive monitoring by PET/MRI could be used to examine agent biodistribution and determine the optimal dose for treatment.

Although blood was not collected from the mice, the TACs for the heart may be used as a noninvasive surrogate for blood pool retention of the probe. Images from the delayed time points revealed heart uptake values for ⁶⁴Cu@GNTs that were similar

to free $^{64}\text{Cu}^{2+}$, indicating that the agent does not localize in the myocardial tissue over time and that the early radioactive signal in the heart observed during dynamic imaging is likely due to perfusion of the organ. This observation demonstrates that the bimodal construct is effectively cleared from systemic circulation in a reasonable time frame. Conceivably, alternative coating materials could be tested with our agent to reduce lung localization, while maintaining the efficient blood clearance properties, thus creating a 'stealth' version of surfactant-wrapped $^{64}\text{Cu}@GNTs$, which could be useful not only for diagnostic purposes where it permits increased signal-to-noise ratios, but also for therapeutic use by minimizing the interaction of a cytotoxic payload (i.e., therapeutic radionuclide or chemotherapy agent) with healthy tissues.

Conclusion

The growing use of hybrid imaging instrumentation such as PET/MRI encourages development of corresponding imaging probes that are capable of maximizing the diagnostic utility of each modality. In this work, we have developed a new bimodal imaging agent for T1-weighted MRI and PET by sequestering Gd^{3+} and $^{64}\text{Cu}^{2+}$ within US-tubes to produce a SWCNT-based, biologically stable, bimodal imaging probe without the use of chelating ligands. We observed passive targeting to the lung *in vivo*, suggesting a potential use for US-tubes as a drug-delivery vehicle against various lung diseases including lung cancer [7]. Active targeting of the bimodal agent can also be achieved using proven strategies for surface modification without altering the encapsulation approach described, thus enabling increased target specificity with minimal off-target effects. The combination of these and other advances in carbon nanotube design have the potential to produce a family of imaging probes capable of targeted, multimodal imaging with any desired combination of CAs that are compatible with clinically used whole-body imaging modalities.

Future perspective

A large amount of current research is devoted to potential diagnostic and therapeutic applications of various nanoparticle platforms. Within the next decade, some of these platforms likely will gain US FDA approval for clinical use. The US-tubes described in this work are one such platform, given that they can be functionalized to render them biocompatible. Due to their ability to encapsulate small molecules and ions, US-tubes are uniquely suitable for providing contrast for virtually any medical imaging technology, as well as supporting mixed diagnostic/therapeutic applications. Further advances in high-resolution, high-sensitivity probes should lead to advanced applications such as single-cell imaging and tracking.

Acknowledgements

The authors wish to thank N Wilganowski and H Robinson for performing the *in vivo* imaging studies.

Financial & competing interests disclosure

This work was supported by the Welch Foundation (Rice University [TX, USA], C-0627; LJ Wilson), the National Science Foundation's Graduate Research Fellowship Program 0940902 (Rice University; JJ Law), and NIH U54 CA136404 (University of Texas Health Science Center [USA]; EM Sevick-Muraca). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Preliminary studies

- A bimodal positron emission tomography/MRI probe was produced by loading ultra-short single-walled carbon nanotubes with $^{64}\text{Cu}^{2+}$ and Gd^{3+} .
- The bimodal probe was produced without the use of chelating agents.
- The external surface of the probe remains unmodified, allowing for the future attachment of targeting agents.
- The probe was stable to *in vitro* challenges with EDTA, phosphate-buffered saline and serum.

Phantom & *in vivo* imaging

- Separate phantom imaging indicated the probe was both positron emission tomography and MRI active.
- *In vivo* positron emission tomography imaging indicated high lung uptake of the probe.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

- 1 Hahn MA, Singh AK, Sharma P, Brown SC, Moudgil BM. Nanoparticles as contrast agents for *in-vivo* bioimaging: current status and future perspectives. *Anal. Bioanal. Chem.* 399(1), 3–27 (2011).
- 2 Lee DE, Koo H, Sun IC, Ryu JH, Kim K, Kwon IC. Multifunctional nanoparticles for multimodal imaging and theragnosis. *Chem. Soc. Rev.* 41(7), 2656 (2012).
- 3 Sailor MJ, Park JH. Hybrid nanoparticles for detection and treatment of cancer. *Adv. Mater.* 24(28), 3779–3802 (2012).
- 4 Treacy MMJ, Ebbesen TW, Gibson JM. Exceptionally high Young's modulus observed for individual carbon nanotubes. *Nature* 381(6584), 678–680 (1996).
- 5 Ouyang M, Huang JL, Lieber CM. Fundamental electronic properties and applications of single-walled carbon nanotubes. *Acc. Chem. Res.* 35(12), 1018–1025 (2002).
- 6 Pantarotto D, Briand JP, Prato M, Bianco A. Translocation of bioactive peptides across cell membranes by carbon nanotubes. *Chem. Commun.* 1, 16–17 (2004).
- 7 Guven A, Rusakova IA, Lewis MT, Wilson LJ. Cisplatin@US-tube carbon nanocapsules for enhanced chemotherapeutic delivery. *Biomaterials* 33(5), 1455–1461 (2012).
- 8 Gautam UK, Costa PMFJ, Bando Y *et al.* Recent developments in inorganically filled carbon nanotubes: successes and challenges. *Sci. Technol. Adv. Mater.* 11, 054501 (2010).
- 9 Hao D, Ai T, Goerner F, Hu X, Runge VM, Tweedle M. MRI contrast agents: basic chemistry and safety. *J. Magn. Reson. Imaging* 36(5), 1060–1071 (2012).
- 10 Hirsch A. Functionalization of single-walled carbon nanotubes. *Angew. Chem. Int. Ed.* 41(11), 1853–1859 (2002).
- 11 Mackeyev Y, Hartman KB, Ananta JS, Lee AV, Wilson LJ. Catalytic synthesis of amino acid and peptide derivatized gadonanotubes. *J. Am. Chem. Soc.* 131(24), 8342–8343 (2009).
- 12 Bianco A, Kostarelos K, Partidos CD, Prato M. Biomedical applications of functionalised carbon nanotubes. *Chem Commun.* 5, 571–577 (2005).
- 13 Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* 65(1), 36–48 (2013).
- 14 Liu Z, Yang K, Lee ST. Single-walled carbon nanotubes in biomedical imaging. *J. Mater. Chem.* 21(3), 586 (2011).
- 15 McDevitt MR, Chattopadhyay D, Jaggi JS *et al.* PET imaging of soluble yttrium-86-labeled carbon nanotubes in mice. *PLoS ONE* 2(9), e907 (2007).
- 16 Liu Z, Cai W, He L *et al.* *In vivo* biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat. Nanotechnol.* 2(1), 47–52 (2007).
- **⁶⁴Cu-labeled single-walled carbon nanotubes target tumors.**
- 17 Luo J, Wilson JD, Zhang J *et al.* A dual PET/MR imaging nanoprobe: ¹²⁴I labeled Gd₃N@C80. *Appl. Sci.* 2(4), 465–478 (2012).
- 18 Richard C, Doan BT, Beloeil JC, Bessodes M, Toth E, Scherman D. Noncovalent functionalization of carbon nanotubes with amphiphilic Gd³⁺ chelates: toward powerful T-1 and T-2 MRI contrast agents. *Nano Lett.* 8(1), 232–236 (2008).
- 19 Choi JH, Nguyen FT, Barone PW *et al.* Multimodal biomedical imaging with asymmetric single-walled carbon nanotube/iron oxide nanoparticle complexes. *Nano Lett.* 7(4), 861–867 (2007).
- 20 Hong SY, Tobias G, Al-Jamal KT *et al.* Filled and glycosylated carbon nanotubes for *in vivo* radioemitter localization and imaging. *Nat. Mater.* 9(6), 485–490 (2010).
- **Shortened single-walled carbon nanotube accumulates in the lungs.**
- 21 Sitharaman B, Kissell KR, Hartman KB *et al.* Superparamagnetic gadonanotubes are high-performance MRI contrast agents. *Chem. Commun.* 31, 3915–3917 (2005).
- **First report of gadonanotubes.**
- 22 Laurent S, Elst LV, Muller RN. Comparative study of the physicochemical properties of six clinical low molecular weight gadolinium contrast agents. *Contrast Media Mol. Imaging* 1(3), 128–137 (2006).
- 23 Judenhofer MS, Wehrl HF, Newport DF *et al.* Simultaneous PET-MRI: a new approach for functional and morphological imaging. *Nat. Med.* 14(4), 459–465 (2008).
- **First report of simultaneous positron emission tomography/MRI.**
- 24 Jarrett BR, Gustafsson B, Kukis DL, Louie AY. Synthesis of ⁶⁴Cu-labeled magnetic nanoparticles for multimodal imaging. *Bioconjug. Chem.* 19(7), 1496–1504 (2008).
- 25 Comes Franchini M, Pucci A, Locatelli E *et al.* Biocompatible nanocomposite for PET/MRI hybrid imaging. *Int. J. Nanomedicine* 7, 6021–6033 (2012).
- 26 Gu Z, Peng H, Hauge RH, Smalley RE, Margrave JL. Cutting single-wall carbon nanotubes through fluorination. *Nano Lett.* 2(9), 1009–1013 (2002).
- 27 Ashcroft JM, Hartman KB, Mackeyev Y *et al.* Functionalization of individual ultra-short single-walled carbon nanotubes. *Nanotechnology* 17(20), 5033–5037 (2006).
- 28 Sampath L, Kwon S, Hall MA, Price RE, Sevick-Muraca EM. Detection of cancer metastases with a dual-labeled near-infrared/positron emission tomography imaging agent. *Transl. Oncol.* 3(5), 307–317 (2010).
- 29 Ghosh SC, Ghosh P, Wilganowski N *et al.* Multimodal chelation platform for near-infrared fluorescence/nuclear imaging. *J. Med. Chem.* 56(2), 406–416 (2013).
- 30 Idée J-M, Port M, Raynal I, Schaefer M, Le Gneur S, Corot C. Clinical and biological consequences of transmetallation induced by contrast agents for magnetic resonance imaging: a review. *Fundam. Clin. Pharmacol.* 20(6), 563–576 (2006).
- 31 Tran LA, Krishnamurthy R, Muthupillai R *et al.* Gadonanotubes as magnetic nanolabels for stem cell detection. *Biomaterials* 31(36), 9482–9491 (2010).
- 32 Kolosnjaj-Tabi J, Hartman KB, Boudjemaa S *et al.* *In vivo* behavior of large doses of ultrashort and full-length single-

walled carbon nanotubes after oral and intraperitoneal administration to Swiss mice. *ACS Nano*. 4(3), 1481–1492 (2012).

- **Single-walled carbon nanotubes are nontoxic *in vivo*.**

- 33 Hartman KB, Laus S, Bolskar RD *et al.* Gadonanotubes as ultrasensitive pH-smart probes for magnetic resonance imaging. *Nano Lett.* 8(2), 415–419 (2012).
- 34 Wang R, Carducci MD, Zheng Z. Direct hydrolytic route to molecular oxo-hydroxo lanthanide clusters. *Inorg. Chem.* 39(9), 1836–1837 (2000).
- 35 Sethi R, Mackeyev Y, Wilson LJ. The gadonanotubes revisited: a new frontier in MRI contrast agent design. *Inorganica Chim. Acta*. 393, 165–172 (2012).

- **Review of gadonanotubes.**

- 36 Gizzatov A, Dimiev A, Mackeyev Y, Tour JM, Wilson LJ. Highly water soluble multi-layer graphene nanoribbons and related honey-comb carbon nanostructures. *Chem. Commun.* 48(45), 5602–5604 (2012).
- 37 Rogers BE, Anderson CJ, Connett JM *et al.* Comparison of four bifunctional chelates for radiolabeling monoclonal antibodies with copper radioisotopes: biodistribution and metabolism. *Bioconjug. Chem.* 7(4), 511–522 (1996).
- 38 Salvador-Morales C, Townsend P, Flahaut E *et al.* Binding of pulmonary surfactant proteins to carbon nanotubes; potential for damage to lung immune defense mechanisms. *Carbon* 45(3), 607–617 (2007).