Encapsulation of α-Particle–Emitting $^{225}Ac^{3+}$ Ions Within Carbon Nanotubes

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$^{225}Ac^{3+}$ is a generator of α-particle–emitting radionuclides with 4 net α-particle decays that can be used therapeutically. Targeting $^{225}Ac^{3+}$ by use of ligands conjugated to traditional bifunctional chelates limits the amount of $^{225}Ac^{3+}$ that can be delivered. Ultrashort, single-walled carbon nanotubes (US-tubes), previously demonstrated as sequestering agents of trivalent lanthanide ions and small molecules, also successfully incorporate $^{225}Ac^{3+}$. **Methods:** Aqueous loading of both $^{225}Ac^{3+}$ ions and Gd^{3+} ions via bath sonication was used to construct ^{225}Ac@gadonanotubes (^{225}Ac@GNTs). The ^{225}Ac@GNTs were subsequently challenged with heat, time, and human serum. **Results:** US-tubes internally loaded with both $^{225}Ac^{3+}$ ions and Gd^{3+} ions show 2 distinct populations of $^{225}Ac^{3+}$ ions: one rapidly lost in human serum and one that remains bound to the US-tubes despite additional challenge with heat, time, and serum. The presence of the latter population depended on cosequestration of Gd^{3+} and $^{225}Ac^{3+}$ ions. **Conclusion:** US-tubes successfully sequester $^{225}Ac^{3+}$ ions in the presence of Gd^{3+} ions and retain them after a human serum challenge, rendering ^{225}Ac@GNTs candidates for radioimmunotherapy for delivery of $^{225}Ac^{3+}$ ions at higher concentrations than is currently possible for traditional ligand carriers.

Key Words: ^{225}Ac; carbon nanotube; alpha therapy; nanotechnology

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Single-walled carbon nanotubes (SWNTs, Fig. 1A) are cylinder-shaped, "rolled-up" graphene sheets that possess a graphitic carbon exterior allowing for covalent functionalization with disease-targeting agents (*1–5*). Moreover, when SWNTs are properly prepared (*6*), they possess small defects along their sidewalls that allow for the encapsulation of small molecules and ions within their hollow interior (*7–9*). These features render SWNT-based materials potentially useful as delivery platforms for targeted α-particle–emitting radionuclides, for radioimmunotherapy of cancer (*10–12*). Here, we examine the ability of modified SWNTs (under 100 nm in length, known as ultrashort tubes [US-tubes], Fig. 1C) to encapsulate the potent α-particle ($^4He^{2+}$) generator $^{225}Ac^{3+}$.

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α-particle emitters, such as ^{213}Bi, ^{211}At, and ^{225}Ac, possess a far greater linear energy transfer (5,000–8,000 keV) and shorter range (50–80 μm, or a few cell diameters) than the β-particle (e^-) emitters currently used for radioimmunotherapy, such as the Food and Drug Administration–approved ^{90}Y and ^{131}I (*8,11,13,14*). These properties of α-particle emitters make them preferred for the specific killing of small-volume cancers such as single cells or micrometastatic lesions. Moreover, Monte Carlo simulations suggest that a solitary α particle can have a cytotoxic effect equivalent to that of over a thousand β particles (*15*). $^{225}Ac^{3+}$ is particularly potent because of the yield of 4 α particles in the decay pathway to ^{209}Bi. Traditional radiometal-labeling requires the use of bifunctional chelates, such as DOTA or diethylenetriaminepentaacetic acid, to bind the free metal radionuclides to the targeting ligand. Recently, chelates have been used to attach $^{225}Ac^{3+}$ to ammonium functionalized carbon nanotubes for therapy of model human lymphoma (*16*). Although these macrocyclic chelates have provided several clinical successes, typically less than 1 chelated α-emitting atom is attached for every 100–1,000 targeting ligands, suggesting an ongoing need to identify alternative chelating agents. The use of selective targeting agents, such as monoclonal antibodies, for the delivery of therapeutics to specific sites in vivo may decrease radioimmunotherapy toxicity (*13*). There is an inherent preference for intracellular uptake and retention of radiometals over radiohalides (*17*), and US-tubes are also inherently bioinert, intracellular agents (*18,19*). Another potential radioimmunotherapy agent, ^{211}At, has been previously reported to be encapsulated within US-tubes by oxidation to form the mixed halide $^{211}AtCl_2$ (*8*). However, ^{211}At possesses a short half-life (7.21 h) compared with $^{225}Ac^{3+}$ (10 d), making the $^{225}Ac^{3+}$ approach more practical. The successful targeting of a $^{225}Ac^{3+}$/US-tube construct could lead to rapid uptake of $^{225}Ac^{3+}$ within targeted diseased cells. In this article, we report a new synthetic approach to the internalization and stable retention of $^{225}Ac^{3+}$ ions within US-tubes (accomplished through the addition of Gd^{3+} ions) as displayed in Figure 2.

MATERIALS AND METHODS

$^{225}Ac^{3+}$

A dried $^{225}AcNO_3$ residue obtained from the Department of Energy (Oak Ridge National Laboratory) was dissolved in 0.1 mL of 0.2 M HCl (Optima grade; Fisher Scientific). ^{225}Ac activity was measured using a drop well dose calibrator (CRC-17 radioisotope calibrator; E.R. Squibb and Sons, Inc.) set at 775, with displayed activity value multiplied by 5.

US-Tube Synthesis

US-tubes were prepared following a previously established synthetic method (Fig. 1) (*6*). Briefly, 1.0 g of full-length SWNTs produced

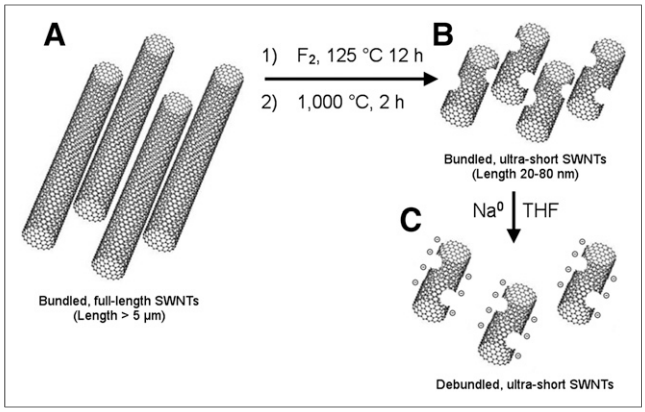

FIGURE 1. Synthetic scheme for production of US-tubes.

in an electric arc discharge with nickel–yttrium catalyst (AP grade; CarboLex, Inc.) (Fig. 1A) was exposed to a fluorinating gas mixture (5.2% helium–diluted F_2) at 125°C for 12 h. The resulting fluorinated SWNTs were pyrolyzed at 1,000°C for 2 h under argon gas, rendering ultrashort nanotubes (US-tubes), 20–80 nm in length, with sidewall defects that facilitate the aqueous internal loading of ions (Fig. 1B) (*7–9*). The US-tubes were then purified of nickel–yttrium catalyst impurities via bath sonication in concentrated HCl and chemically reduced in an amended Birch reduction with potassium in anhydrous tetrahydrofuran (*3*). The reduction yields individualized US-tubes as opposed to small bundles of US-tubes (Fig. 1C).

$^{225}Ac^{3+}$ Labeling

US-tubes were dispersed via bath sonication to a concentration of 5 g/L in metal-free water obtained from a Purelab Plus system (United States Filter Corp.). Three separate aqueous loading techniques were examined: the first was addition of $^{225}Ac^{3+}$ ions alone, the second was addition of a mixture of Gd^{3+} ions and $^{225}Ac^{3+}$ ions, and the third was sequential addition of Gd^{3+} ions followed by $^{225}Ac^{3+}$ ions. For $^{225}Ac^{3+}$ ions alone, 3.0 μL of $^{225}AcCl_3$ (7.0 MBq) were diluted in 200 μL of metal-free water and mixed with 50 μL of US-tube suspension. For the mixture, 3 μL of $^{225}AcCl_3$ (7.0 MBq) were diluted in 150 μL of metal-free water, mixed with 50 μL of 19 mM $GdCl_3$ (Aldrich Chemical) and 50 μL of the US-tube suspension. Finally, for the sequential addition, 150 μL of metal-free water were mixed with 50 μL of 19 mM $GdCl_3$ and 50 μL of the US-tube suspension (the previously established method for creation of the gadonanotubes); after 24 h, 7.0 MBq of $^{225}AcCl_3$ were added to the $GdCl_3$/US-tube solution. Activity was determined using a Cobra γ counter (Packard Instrument Co., Inc.) with a 340- to 540-keV window.

Previous work detailing the loading of Gd^{3+} into US-tubes produced the desired product by 1 h of bath sonication followed by overnight equilibration (*9*). To help ensure that ^{225}Ac could be encapsulated, samples were bath-sonicated for 2 h and allowed to equilibrate overnight. The next day, all samples were washed with 250 μL of metal-free water and filtered using a Handee Micro-Spin Column (Thermo Scientific Pierce) with a paper membrane (pore size, ~10 μm) until no activity was detected in the filtrate via drop well dose calibrator. After filtration, all samples were resuspended in 200 μL of distilled water by pipette aspiration and the activity measured. All loading procedures were performed in triplicate.

Human Serum Challenge Experiments

To simulate in vivo conditions, challenge experiments were conducted monitoring the effect of heat, time, or human serum on ^{225}Ac@US-tube suspensions. For each serum challenge, 20 μL of the resulting ^{225}Ac@US-tube (12.5 μg) suspension from the above 3 loading techniques were added to 180 μL of normal human AB serum (Sigma Chemical Co.). The mixture was then stirred and incubated at 37°C for 2 h. After incubation, the mixture was filtered using a centrifugal device (100*g*, 5 min.), and the serum filtrate was counted in scintillation fluid using a β counter (samples were allowed to reach equilibrium overnight). Standards of ^{225}Ac@gadonanotubes (^{225}Ac@GNTs) (prepared by the second technique above) were counted as a reference. For rechallenging the samples, 200 μL of serum were again added to the filter device, and the mixture was resuspended and reincubated at 37°C for 2 h. The filtration process was repeated. As a control, $^{225}AcCl_3$ alone in serum was spun in the filter device. Samples also were challenged for longer times (4 and 12 h) and at both room temperature and 37°C to compare the effects of temperature and time in serum.

RESULTS

$^{225}Ac^{3+}$ Labeling

Serial washings via centrifuge filtration were used to determine the extent of $^{225}Ac^{3+}$ labeling. For the loading protocol involving only $^{225}Ac^{3+}$, over 95% (6.7 MBq) of the initial activity remained with the US-tubes after washing. For both loading techniques involving Gd^{3+} ions with $^{225}Ac^{3+}$ ions (simultaneous and sequential), only 50% (3.5 MBq) of the initial activity remained associated with the US-tubes. This reduction in yield is likely due to the large excess of Gd^{3+} ions saturating potential binding sites for ^{225}Ac at the US-tube sidewall defects. All 3 loading techniques ($^{225}Ac^{3+}$ only, $^{225}Ac^{3+}$ and Gd^{3+} simultaneously, and $^{225}Ac^{3+}$ and Gd^{3+} sequentially) showed no radioactivity in the centrifugal filtrate after the third washing of 250 μL, implying that only 750 μL of metal-free water was required to separate the $^{225}Ac^{3+}$ ions not bound by the US-tubes in suspension.

Human Serum Challenge Experiments

The ^{225}Ac@US-tubes and ^{225}Ac@GNTs were challenged with human serum (Fig. 3). For the ^{225}Ac@US-tubes labeled with only $^{225}Ac^{3+}$ ions, only 40.0% (2.7 MBq) of the loaded $^{225}Ac^{3+}$

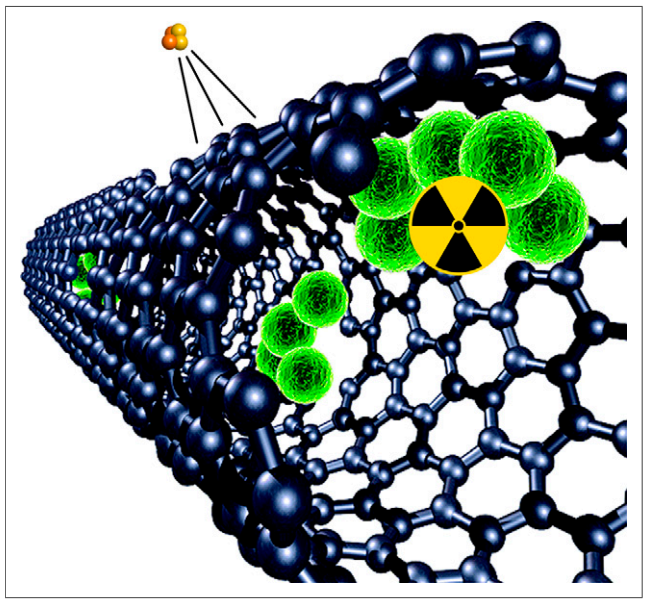

FIGURE 2. Representation of ^{225}Ac@GNT construct. Green represents Gd^{3+} ions, yellow with radioactive symbol represents $^{225}Ac^{3+}$ ion, and orange and yellow cluster represents emitted α particle.

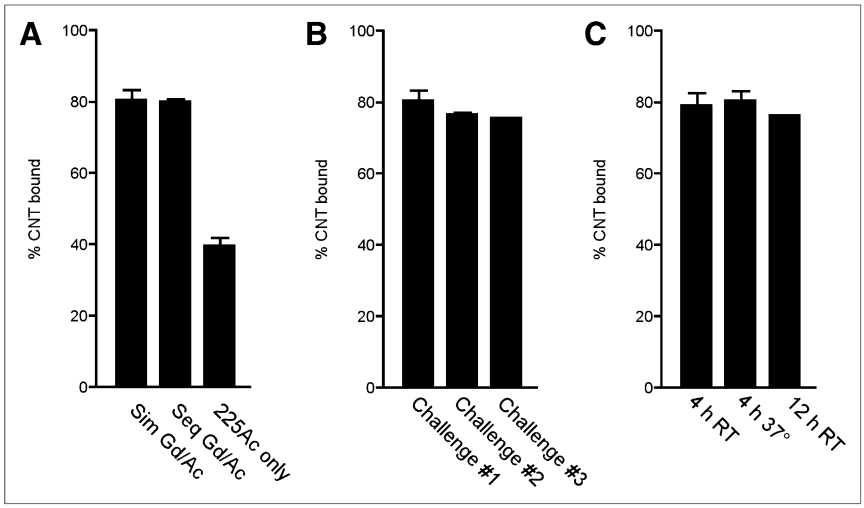

FIGURE 3. Results of human serum challenge experiments. (A) Time and temperature challenge experiments performed on 3 prepared ^{225}Ac materials: simultaneous $Gd^{3+}/^{225}Ac^{3+}$ loading, sequential $Gd^{3+}/^{225}Ac^{3+}$ loading, and $^{225}Ac^{3+}$-only loading. (B) Effect of repeated serum challenges on activity of simultaneously loaded ^{225}Ac@GNTs. (C) Effect of various serum challenge conditions on activity of simultaneously loaded ^{225}Ac@GNTs. RT = room temperature.

remained after the initial human AB serum washing. There was little difference in $^{225}Ac^{3+}$ retention after serum challenge of the US-tubes loaded via sequential and simultaneous techniques with $^{225}Ac^{3+}$ and Gd^{3+} ions within the US-tubes. The simultaneous loading technique retained 77% (2.7 MBq), and the sequential technique retained 80% (2.8 MBq) (Fig. 3A). Subsequent serum challenges of the ^{225}Ac@US-tubes loaded with Gd^{3+} ions had no measurable effect on removing ^{225}Ac; however, subsequent challenges of the ^{225}Ac@US-tubes with no Gd^{3+} ions present continued to further remove ^{225}Ac, until less than 5.0% (0.34 MBq) of the original ^{225}Ac activity remained bound by the US-tubes. Conversely, subsequent challenges of the ^{225}Ac@GNTs (after the initial ~20% activity loss) through both simultaneous and sequential loading revealed no measurable quantity of $^{225}Ac^{3+}$ in the filtrate and approximately 100% retention of the remaining $^{225}Ac^{3+}$ ions with the ^{225}Ac@GNTs in the filter (Fig. 3B). Moreover, the additional challenges of time (both 4 and 12 h) and elevated temperature (37°C) of the ^{225}Ac@GNTs using the simultaneous loading technique did not produce additional losses of $^{225}Ac^{3+}$ (Fig. 3C). Control experiments (free ^{225}AcCl in serum) showed no significant nonspecific retention of $^{225}Ac^{3+}$ on the filter devices. These results indicate that a serum challenge may be used as a stripping procedure to purify the material by removing loosely bound $^{225}Ac^{3+}$ ions that are not embedded within Gd^{3+} ion clusters and encapsulated within the nanotube, as reported previously for US-tubes filled with ^{64}Cu (*20*).

DISCUSSION

Previous work has shown that ^{225}Ac may be attached to carbon nanotubes by chelation with the macrocyclic chelator DOTA, which may be covalently linked to the external sidewall of the nanotube (*16*). In the same manner, antibodies may be attached for in vivo targeting of the construct (*12,21*). In demonstrating that ^{225}Ac may be encapsulated within carbon nanotubes, we have simplified the production of these targeted nanoconstructs by eliminating the need for attachment of the macrocyclic chelator. Retention of the radioisotope within the nanotube reduces exposure to serum proteins and increases the area on the external sidewall that may be used for attaching targeting peptides or antibodies while retaining the therapeutic efficacy. Furthermore, the encapsulation of gadolinium within the carbon nanotubes has previously been shown to enhance MR imaging contrast (*22*), and a bimodal PET/MR contrast agent has been produced by trapping $^{64}Cu^{2+}$ within Gd^{3+} clusters (*20*).

The above results are consistent with the hypothesis that the $^{225}Ac^{3+}$ ions should behave similarly to the Gd^{3+} ions within US-tubes because of the similar solution chemistries of trivalent actinide ions and trivalent lanthanide ions (*19*). One exception was the continual leakage of $^{225}Ac^{3+}$ ions from the US-tubes for the ^{225}Ac@US-tubes on repetitive serum challenges, whereas Gd^{3+} ions show no leakage (*23*). Therefore, it is unclear whether the internalization of the $^{225}Ac^{3+}$ ions directly mimics the Gd^{3+} ion internalization of the gadonanotubes. Previous high-resolution transmission electron microscopy images revealed that the Gd^{3+} ions in the gadonanotubes exist in small (1 nm × 2–5 nm) clusters that correspond to roughly 5–10 Gd^{3+} ions per cluster (*9*); additionally, the gadonanotubes average 2%–5% Gd^{3+} by mass as determined by inductively coupled plasma optical emission spectrometry. Assuming a mean length of 50 nm and approximately 120 carbon atoms per nanometer, each US-tube therefore contains about 10–20 Gd^{3+} ions. Although the synthetic method herein uses the minimum concentration of US-tubes that can be reliably manipulated for filtration experiments, practical considerations (i.e., amount of radionuclide readily available) limited the concentration of $^{225}Ac^{3+}$ ions such that the conditions provided for one $^{225}Ac^{3+}$ ion for roughly every 250 US-tubes. At this low concentration, there are not enough $^{225}Ac^{3+}$ ions present to form $^{225}Ac^{3+}$ clusters that would directly mimic the stable Gd^{3+} ion clusters inside the gadonanotubes. However, at this concentration, the $^{225}Ac^{3+}$ ions behave similarly to the Gd^{3+} ions when admixed into the US-tubes with Gd^{3+} carrier ions, allowing for use of the nonradioactive ions for most of the cluster formation. Interestingly, the methodology of loading (sequential vs. simultaneous) appears to have little or no effect on the final product. This result should prove beneficial to future synthetic routes with regard to the time of production of $^{225}Ac^{3+}$/gadonanotube conjugates, and the solution chemistry of the $^{225}Ac^{3+}$ ions within the gadonanotubes further supports the idea that production of an ion cluster is critical for their stable embedment within the US-tubes and their resistance to serum challenge.

CONCLUSION

^{225}Ac@GNTs provide a novel alternative to chelation for radiometal ions useful for radioimmunotherapy. In addition to sequestering the therapeutic radioisotope, ultrashort carbon nanotubes may also be used to encapsulate elements for imaging by MR or PET and may be functionalized with antibodies, creating a single construct capable of targeted imaging and therapy.

DISCLOSURE

The costs of publication of this article were defrayed in part by the payment of page charges. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734. The Welch Foundation (grant C-0627) and the NIH (grant R01CA055349) provided partial support of this work. David A. Scheinberg is a consultant to Ensyce Biosciences, Inc. No other potential conflict of interest relevant to this article was reported.

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