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Synthesis and Biodistribution of Oligonucleotide-Functionalized, Tumor-Targetable Carbon Nanotubes

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Abstract

Single-wall carbon nanotubes (SWNT) show promise as nanoscale vehicles for targeted therapies. We have functionalized SWNT using regioselective chemistries to confer capabilities of selective targeting using RGD ligands, radiotracing using radiometal chelates, and self-assembly using oligonucleotides. The constructs contained approximately 2–7 phosphorothioate oligonucleotide chains and 50–75 amines per 100 nm length of SWNT, based on a loading of 0.01–0.05 mmol/g and 0.3–0.6 mmol/g, respectively. Dynamic light scattering suggested the functionalized SWNT were well dispersed, without formation of large aggregates in physiologic solutions. The SWNT–oligonucleotide conjugate annealed with a complementary oligonucleotide sequence had a melting temperature of 54 °C. Biodistribution in mice was quantified using radiolabeled SWNT–oligonucleotide conjugates. Appended RGD ligands allowed for specific binding to tumor cells in a flow cytometric assay. The techniques employed should enable the synthesis of multifunctional SWNT capable of self-assembly in biological settings.

Nanoscale particles have significant potential as drug delivery and molecular imaging platforms.¹ The optimal delivery platform can be chosen from a wide array of available nanomaterials based on their unique properties. Carbon nanotubes in particular have generated significant interest in medical applications.^{2–7} The small diameter of carbon nanotubes results in high aspect ratios (>200:1 length:width for HiPCO-produced single-wall tubes) that afford them unique biological properties, particularly with respect to their pharmacokinetics^{3,5–9} and cellular interactions.^{10–12} The ability to radiolabel carbon nanotubes to high specific activities also offers potential in amplification of radioisotope delivery.^{4,6,7} Although pristine, as-produced carbon nanotubes are completely insoluble in

aqueous media, a variety of chemistries has been applied to nanotubes in order to successfully functionalize them and render them highly biocompatible^{6,10,12} and dispersable^{13,14} in aqueous media. In contrast to studies using long (microscale lengths), unmodified, and insoluble carbon nanotubes,^{15,16} several recent studies have used carbon nanotubes in animal models with little to no sign of toxicity for shorter, well-functionalized, and well-dispersed materials.^{17–19}

Processing of pristine carbon nanotubes by oxidation in strong acids serves to remove residual metallic catalyst impurities and, along with sonication, shortens SWNTs. This process also introduces carboxylic acid moieties, which form preferentially at the tube ends and defect sites.²⁰ The resulting acid modified SWNT (SWNT-COOH) are more readily dispersed, but still highly insoluble. Further functionalization through the covalent addition of azomethine ylides^{21–23} onto the π -bonding system on the nanotube sidewalls can serve to solubilize the SWNT when the appended moiety is hydrophilic. This approach has led to soluble, highly biocompatible nanotubes bearing primary amines that are readily functionalized with commercially available cross-linking reagents. In addition to sidewall amines, the carboxylic acid groups are available for diimide activation and reaction with primary amine containing compounds,^{24,25} permitting regioselective syntheses.²⁶ By using these orthogonal chemistries, one can create complex multifunctional SWNT platforms.

In previous studies, we demonstrated that single-wall carbon nanotubes can be oxidized, shortened, and function-alized with primary amines that allow for subsequent attachment of isotope chelators (for imaging or therapy)⁹ and monoclonal antibodies (for tumor targeting).⁶ In addition, a large number of copies of these moieties can be appended, which can be used to achieve either multivalency of the biologic targeting agent or amplification of a therapeutic payload. Such nanoscale constructs are exciting candidates for multifunctional therapeutic and diagnostic agents.

To further develop the SWNT as a platform for drug delivery, we appended oligonucleotide ligands that allow for addressable cross-linking, resulting in a biocompatible SWNT device capable of self-assembly. DNA oligonucleotide analogs have been successfully applied as complementary pairs in antibody-based pretargeting therapy²⁷ where hybridization also occurs in vivo. DNA modified or wrapped nanotubes have also been investigated as building blocks for self-assembled nanodevices.^{28–30} Although such studies are mostly focused on nanotubes' use in nanoelectronics and biosensors, we propose that these approaches should also enable the creation of SWNT drug-delivery constructs capable of in situ self-assembly. In this study, we synthesized covalently modified SWNT bearing single stranded oligonucleotide analogues, radiotracing moieties, and targeting peptides (Figure 1). These constructs serve as a prototype for targetable nanotube platforms capable of hybridizing cDNA addresses. Further, we provide pharmacokinetic data of these novel materials in mice, and investigated the impact of DNA modification on pharmacokinetics.

In a typical preparation, 450 mg of pristine, as-produced HiPCO single wall carbon nanotubes (SWNT, Carbon Nanotechnologies Inc., 1–1.5 nm diameter) were first dispersed in 500 mL of 7 M nitric acid (HNO₃) using bath sonication (VWR 550HT) for 30 min. This step serves to both disperse and cut the nanotubes to shorter lengths. After sonication, the

SWNT formed a black slurry of insoluble material. This mixture was then heated in the acid at reflux for a period of 5 h. The reaction conditions were chosen to minimize loss of SWNT material while ensuring adequate removal of metallic impurities.²⁰ The oxidation step serves to introduce carboxylic acid groups primarily onto nanotube ends and defect sites. Oxidized metal catalyst impurities were subsequently washed away through filtration over a fine glass frit filter. The SWNT-COOH slurry was then extensively washed with deionized water until the pH of the washes reached 4.5. We confirmed that the SWNT were effectively chemically modified, purified, and shortened by both TEM and Raman analysis (see the Supporting Information). The Raman spectra demonstrated a rise in the disorder band (D-band), suggesting successful chemical modification.³¹ The SWNT-COOH material was then lyophilized resulting in a black powder with a typical recovery of 60–80% by weight.

Amine functionalization was accomplished by reaction of azomethine ylides onto the nanotube sidewalls (Figure 1). In a typical reaction, 134 mg of SWNT-COOH powder was dispersed in 300 mL of *N,N*-dimethylformamide (DMF, 99.9%+, Sigma) using bath sonication for a period of up to 3 h. Effective dispersion was vital to ensure adequate reaction yields. A 6-fold excess by weight (804 mg) of paraformaldehyde (>95%, Fluka) that had been previously suspended in 100 mL of DMF was added to the SWNT-COOH dispersion. The SWNT-COOH/paraformaldehyde suspension was heated to 130 °C and 25 mL of 8 mg/mL HOOC-CH₂NH-(C₂H₄O)₂-C₂H₄NHBoc linker⁶ was added. The reaction mixture was continually heated and stirred with monitoring of temperature for a period of 5 days. On days 2, 3, and 4, additional linker (200 mg) was added (800 mg total). After 5 days, the reaction was allowed to cool and centrifuged at 1000× g for 5 min to remove unreacted particulate aggregates. The supernatant was filtered and then placed on a rotary evaporator to remove the DMF. The resulting residue was dissolved in 100 mL of chloroform and this organic phase was washed three times with equal volumes of distilled water. The solvent was once again evaporated and the residue (the crude SWNT-NHBoc-COOH) dissolved in minimal methanol. The pure SWNT-NHBoc-COOH was then precipitated by addition of excess diethyl ether and collected via filtration. The SWNT-NHBoc-COOH product (Figure 1, [1]) was dried under a vacuum, resulting in 35.9 mg of dry product (26.8% by weight of starting SWNT-COOH product). This product was highly dispersible in DMF. On the basis of previous studies with similarly oxidized and amine-functionalized SWNT,⁹ we expected the functionalized SWNT-NHBoc-COOH to be effectively shortened to less than 100 nm, with the presence of both small bundles and individualized tubes.

Phosphorothioate backbone modified DNA oligonucleotides (ODNFAM, 5' to 3' sequence: GTC-CCT-TCG-TCA-ACA-CTA) with a 3' amino group and 5' fluorescein were custom synthesized by Tri-Link Biotechnologies (San Diego, CA). The sequence was based on previously successful antibody-based pretargeting using oligonucleotides³² and the modified backbone was chosen to minimize sensitivity to endogenous exo- and endonucleases and promote stability in vivo.³³ The primary amine on the 3' end of the ODN was coupled to SWNT-COOH via carbodiimide activation of the carboxylic acids generated in the oxidation of the SWNT.³⁴ Twenty-five mg of ODN-FAM (3.9 μmol) was dissolved in 20 mL of 0.1 M sodium phosphate buffer, pH 6.5. The 35.9 mg of SWNT-NHBoc-COOH were then

dispersed in 5 mL of 99.9%+ DMF. The SWNT-NHBoc-COOH solution in DMF was added to the aqueous ODN-FAM solution and 150 mg (final concentration) 31 mM = of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Pierce) was added. This mixture was reacted overnight with stirring and protected from light. The next day the reaction mixture was placed under argon, frozen to -80°C , protected from ambient light, and lyophilized to yield a crude product residue of SWNT-NHBoc-ODNFAM (Figure 1, [2]).

The crude SWNT-NHBoc-ODNFAM product was then treated with 6 mL of neat trifluoroacetic acid (TFA, Sigma) at room temperature and protected from light for up to 2 h in order to deprotect the primary amines on the nanotube sidewalls. The stability of the ODN-FAM in TFA was confirmed via gel electrophoresis in a 15% TBE-UREA gel (Biorad) demonstrating minimal breakdown of the oligonucleotide alone after TFA treatment (data not shown). The final crude product, SWNT-NH₂-ODNFAM (Figure 1, [3]), was then placed on a rotary evaporator to remove the TFA and the residue was reconstituted in a 0.1 M sodium phosphate buffer (pH 6.5) with addition of NaOH in order to neutralize the product and allow for dissolution in the aqueous phase. The resulting aqueous solution of SWNT-NH₂-ODNFAM (20 mL) was then purified using an Amicon Ultra centrifugal filter device (30K MWCO, Millipore). The device was prerinsed with metal-free water and the sample spun at 2,000xg for 20 min, resuspended in 0.1 M sodium phosphate, and then respun. This process was repeated three times. Each centrifugation step resulted in some aggregation and settling of SWNT material that was resuspended by washing the membrane with a bicarbonate buffer (pH 10). To further purify the product, we then extensively dialyzed the mixture into distilled H₂O using a 20 000 MWCO dialysis cassette (Slide-a-lyzer, Pierce).

The final purified SWNT-NH₂-ODNFAM product was then characterized by Kaiser assay (see the Supporting Information) for amine content,³⁵ UV/vis spectroscopy to verify oligonucleotide, fluorescein, and SWNT content, and both gel-permeation (panels a and b in Figure 2) and reverse phase HPLC analysis. Transmission electron micrographs were also obtained (Figure 2c). When observed by TEM, small bundles of SWNT-ODNFAM-NH₂ constructs were visible, with approximate lengths of 50–100 nm. A typical SWNT-NH₂-ODNFAM product was found to contain roughly 0.33–0.56 mmol/g of amine and 0.01–0.05 mmol/g of oligonucleotide. This represents roughly 50–75 amines and 2–7 oligonucleotides per 100 nm of SWNT length. This loading is consistent with the expected yields for the chemistries utilized. The mole ratios of ODN and FAM were determined to be equal on the basis of the spectral contribution of DNA absorbance at 260 nm or the fluorescein absorbance at 495 nm, suggesting that the DNA–fluorophore linkages remained intact.

The aggregation states and length distributions of the SWNT-oligonucleotide constructs when in physiologic solutions (such as aqueous buffers, serum, etc.) are unclear. Although the constructs were stably dispersed, it is possible that the functionalized nanotubes exist as larger nanotube aggregate “ropes”, small bundles, or individual tubes, resulting in a heterogeneous mixture of nanotube lengths. To better define the dispersion of SWNT-ODNFAM-NH₂ constructs, samples reconstituted in phosphate buffered saline (pH 7.2) were analyzed by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern)

equipped with a narrow bandpass filter (see the Supporting Information). These studies found that the both the nanotube-oligonucleotide conjugates and nanotubes alone had an apparent hydrodynamic radius of $230 \text{ nm} \pm 60 \text{ nm}$, suggesting they are not forming large, aggregated “ropes” both before and after derivitization with the oligonucleotides. A small peak at a smaller radius of 4–9 nm was observed in the size distribution, which is believed to be a rotational mode of nanotube motion and is inline with the anisotropic nature of the rodlike nanotubes. Note that the exact size values are unlikely to be a quantitatively precise reflection of the true tube lengths due to the application of sphere-based (Stokes-Einstein) models to the SWNT as well as the polydispersity of lengths expected in the synthetic techniques used.

The SWNT-NH₂-ODNFAM (Figure 1, [3]) was tested for its ability to specifically hybridize a complementary ODN sequence by tracking absorbance changes with increase in temperature at 260 nm. Typical DNA hybridization behavior should result in an inflection point at the melting temperature (T_m) of the oligonucleotide complex. To accomplish this, a dilute solution of SWNT-NH₂-ODNFAM in hybridization buffer (10 mM HEPES, 0.14 M NaCl, 1 mM EDTA, pH 7.6) was prepared in a quartz cuvette, and an equimolar (DNA content measured by A_{260}) amount of complementary phosphorothioate oligonucleotide (cODN, 5' to 3' sequence = TAG-TGT-TGA-CGA-AGG-GAC, Tri-Link Biotechnologies) added. This cuvette was referenced against an identical solution containing SWNT-NH₂-ODNFAM alone. The cuvette was placed in a Cary 200 UV/vis spectrophotometer (Varian) equipped with a multichamber, temperature-controlled Peltier heating block. After a minute of stirring, the absorbance was then read as the temperature was increased at a rate of 5 °C/min. The absorbance rose in the expected sigmoidal pattern where the inflection point represents the melting temperature of the oligonucleotide strands. The SWNT-NH₂-ODNFAM/cODN complex was found to have a melting temperature of 54 °C, very close to that of the DNA oligonucleotides alone (Figure 3). This suggests that the oligonucleotides that were appended on the nanotubes can still hybridize their complementary pairs with normal behavior.

To determine whether the SWNT-NH₂-ODNFAM can serve as a building block for oligonucleotide-directed self-assembly in vivo in diagnostic or therapeutic applications, the pharmacokinetics and biodistribution of the material were characterized. The biodistribution studies of SWNT-NH-ODNFAM used traditional radiotracer techniques with a bifunctional radiometal chelator. An amine-reactive benzyl isothiocyanate derivative of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA-SCN, Macrocylics) was first reconstituted in metal free dH₂O to a concentration of 10 g/L. One-hundred microliters of this solution was then added to 70 μL of 3 M ammonium acetate and 15 μL (4.86 mCi) of ¹¹¹InCl₃ (Perkin-Elmer). This labeling reaction was adjusted to pH 5.0 and heated at 58 °C for 30 min. 100 μL of the chelated ¹¹¹In-DOTA-SCN reaction mixture (3.09 mCi) was then added to 139 μL of SWNT-NH₂-ODNFAM (~250 μg) and 250 μL of 1 M sodium carbonate to raise the pH to 9–9.5. This reaction was carried out at room temperature for 35 min at which point 10 μL of 50 mM DTPA was added to chelate any free radiometal. The reaction mixture was then purified using a PD-10 gel filtration column (Biorad), with elution of the product into bacteriostatic 0.9% sodium chloride (Hospira Inc.) containing 1% human

serum albumin (Swiss Red Cross, Bern, Switzerland). This formulation was used as the injection medium without further treatment or modifications. As a control, a similar procedure was used to label ODN-FAM-NH₂ alone to yield ODNFAM-¹¹¹In(DOTA). The SWNT-¹¹¹In(DOTA)-ODNFAM (Figure 1, [4]) product was found to label to a specific activity of 0.7 $\mu\text{Ci}/\mu\text{g}$, whereas the ODNFAM-¹¹¹In(DOTA) contained 0.1 $\mu\text{Ci}/\mu\text{g}$. Radiochemical purities were confirmed by ITLC and RP-HPLC methods (see the Supporting Information), confirming that the radiolabel was associated with the SWNT construct and the oligonucleotide alone, respectively.

One group of 15 mice (NCI nu/nu, National Cancer Institute) received 70 μL of SWNT-¹¹¹In(DOTA)-ODNFAM ($\sim 7 \mu\text{g}$, 5 μCi) each, and another group received 70 μL of In¹¹¹(DOTA)-ODNFAM (2.3 μg , 0.25 μCi) each. The constructs were injected intravenously via the retroorbital sinus. All mice were female, 4–6 weeks old, and all animal studies were conducted under the approval of the Institutional Animal Care and Use Committee. At each time point (1, 24, and 96 h), 5 mice from each group were sacrificed, the organs harvested and weighed, and the radioactivity measured using a Packard Cobra II gamma counting instrument. Blood was collected immediately after sacrifice via direct cardiac puncture.

The SWNT-¹¹¹In(DOTA)-ODNFAM cleared the blood compartment quickly (Figure 4), with 0.4% ID/g in the blood at 24 h post injection. The blood clearance was similar to previous SWNT constructs.⁶ In addition, the %ID/g remaining in the kidney for the oligonucleotide functionalized SWNT was lower at all time points compared to nonoligonucleotide functionalized SWNT. The ¹¹¹In(DOTA)-ODN-FAM alone accumulated in the liver and spleen due to serum protein binding.³² In future work, other modified backbone DNA analogs such as morpholinos are likely to offer an advantage over the phosphorothioate derivatives as they accumulate significantly less in these organs.³⁶ The SWNT-NH₂-ODNFAM appeared to clear the kidney more rapidly than liver and spleen. The liver (at all time points) and spleen (at 1 and 24 h) accumulation are significantly lower ($p < 0.05$) than that seen for the oligonucleotide alone. The observed pharmacokinetic profile is encouraging as rapid clearance and minimization of kidney uptake may be favorable properties for both targeted and pretargeted self-assembling drug delivery vehicles. Other groups have found that similar covalently functionalized nanotube materials are also cleared rapidly through the kidneys,^{3,17} although there are differences in accumulation in organs such as spleen and liver. The injection formulation was chosen based on our experience with radiotherapeutics in animal models³⁷ and the effect of the injection formulation on nanoparticle dispersions such as those used in these studies remains to be determined.

Kidney retention was significantly reduced versus previous studies with nonoligonucleotide-modified SWNTs.⁶ Previous studies found $\sim 40\%$ ID/g was retained in the kidney at 24 h, while the oligonucleotide functionalized SWNT materials in these studies were approximately 2-fold less at $\sim 18\%$ ID/g. We speculated that this may be a charge effect where negative surface charge imparted by the backbone of the appended oligonucleotides impacts both aggregation state and cellular interactions, particularly in the renal cortex. To confirm the importance of charge, biodistribution of SWNT-DOTA conjugates was conducted on materials with a variable number of negatively charged DOTA groups

appended to the SWNT sidewalls (see the Supporting Information). A similar charge effect was seen in that the SWNT-DOTA conjugates with greater loading of negatively charged DOTA groups had significantly reduced nonspecific organ retention. Therefore, it appears that increasing the number of negatively charged groups appended to the SWNT minimizes renal uptake. This is consistent with studies that show radiolabeled cationic proteins are retained in the proximal tubules of the renal cortex.³⁸ SWNT constructs also appear to be retained in the renal cortex.⁹ These findings suggest that the construct design and charge can also be optimized to enhance renal clearance. More extensive studies, such as correlation of zeta potential with biodistribution and cellular and tissue interactions should better determine the effect of surface charge. Although renal excretion is supported by the presence of the SWNT constructs in the urine of injected animals, the exact clearance mechanism is under investigation.

After demonstrating the hybridizing ability and biodistribution of SWNT-NH₂-ODNFAM constructs, we wished to determine whether targeting moieties could be appended to the SWNT-NH₂-ODNFAM sidewalls to form an anchor construct that would specifically target tumors. $\alpha_V\beta_3$ integrin has been extensively explored as a target in tumor neovasculature and has the benefit of the well-established cyclic RGD peptide as a targeting moiety.³⁹ Cyclic RGD binds to the integrin when it is expressed in the disrupted endothelium of neovasculature, and has specificity in vivo for tumors.⁴⁰ Vascular targets are particularly advantageous as the relatively large and complex constructs proposed may have limited penetration into the tumor.

To couple cyclic RGD peptides to the nanotube sidewall amines, we used an approach based on common protein cross-linking techniques. The heterobifunctional cross-linker succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate] (LC-SMCC, Pierce) contains an amine reactive NHS ester on one end, and a sulfhydryl reactive maleimide at the other end. The LC-SMCC was first reacted onto SWNT-NH₂-ODNFAM by reacting the material in 0.1 M sodium phosphate (pH 7.6) buffer, with a 10:1 excess of LC-SMCC to amine content as determined by the Kaiser assay. This reaction introduces thiol reactive maleimides to the SWNT sidewalls (Figure 1, [5]). This crude mixture was then treated with an excess of 10:1 cyclic RGD peptide (Ansynth, Netherlands) to maleimide. The RGD peptide contains an acetyl protected cysteine residue which was deprotected in situ by addition of a 30-fold excess of hydroxylamine hydrochloride (neutralized to pH 7 with NaOH). After allowing the reaction to occur for 1 h, the crude SWNT-RGD-ODNFAM (Figure 1, [6]) product was prepurified using a Centricon centrifugal filter device (30K MWCO, Millipore). The device was spun twice at 2000 × g for 30 min, with resuspension into 0.1 M sodium phosphate (pH 7.6). This prepurified product was then further purified using dialysis into dH₂O using a 20K MWCO dialysis cassette (Slide-a-lyzer, Pierce). A nontargeting cyclic RAD peptide sequence was employed in a parallel synthesis and purification to yield a nontargeting control construct. The final purified products were lyophilized until reconstitution in dH₂O.

To verify specific antigen targeting of the nanotube-oligonucleotide conjugates, an $\alpha_V\beta_3$ positive human coronary artery endothelial cell line (HCEC, Lonza, Switzerland) was used as a model in a flow cytometric assay for binding specificity (Figure 5). The expression of

the $\alpha_V\beta_3$ integrin on the cells was confirmed by flow cytometric assay using an $\alpha_V\beta_3$ specific monoclonal antibody labeled directly with fluorescein dye (anti-CD51/CD61, Becton Dickinson) in comparison to an anti-CD3 negative control. The analysis demonstrated a significant increase in median fluorescence intensity of cells treated with the SWNT-RGD-ODNFAM over the isotype control SWNT-RAD-ODNFAM. This result demonstrated that addition of the RGD targeting moiety to the SWNT-oligonucleotide construct allowed for specific binding, and should allow the SWNT-RGD-ODNFAM to serve as an anchor construct in a neovasculature targeted self-assembly approach.

By taking advantage of orthogonal chemistries, we synthesized a SWNT construct with potential for self-assembly (through oligonucleotide analogs), radiotracing, and targeting (through appended peptides) that has considerable promise as a unique nanotechnology-based drug delivery platform. HPLC analysis demonstrated that the SWNT-NH₂ODNFAM construct could be produced in high purity and could be chromatographed through both gel permeation and reverse phase techniques. This material was easily and stably dispersed in aqueous solutions at normal physiologic pH ranges. Using quantitative tests of amine content (Kaiser assay) and spectroscopic measurement of DNA content, we determined that for a shortened SWNT of ~100 nm length we can expect roughly 50–75 amines and 2–7 oligonucleotides per construct. The SWNT–oligonucleotide construct was able to hybridize its complementary sequence with melting temperatures close to that of the oligonucleotide pair alone, suggesting normal base-pairing behavior. Although dynamic light scattering suggested that the nanotubes were well-dispersed without formation of large aggregates, a quantitatively precise determination of nanotube length and bundling remains to be determined. Much attention has been recently placed on production of carbon nanotubes that are monodisperse with regard to their conductivity, diameter, and lengths.⁴¹ Such advances will be critical for the production of homogeneous materials required for the ultimate aim of using these materials in biomedical applications.

We conducted biodistribution studies of the radiolabeled SWNT-oligonucleotide construct and found rapid clearance from the blood compartment with significant retention seen only in the kidney, liver, and spleen. In previous work, we found that despite the large molecular weights for individual constructs, the SWNT constructs were excreted predominantly through the kidney, resulting in clearance from the animal, with significant retention seen only in spleen, liver, and kidney.⁶ On the basis of the similar pharmacokinetic pattern observed in these studies, the SWNT-oligonucleotide conjugates appear to have the same clearance pathways. For a therapeutic or imaging agent, rapid clearance may offer an advantage in building constructs through pretargeted self-assembly.⁴² Furthermore, it appears the appended moieties significantly alter the pharmacokinetic profile with surface charge appearing to have an important role. Although phosphorothioate oligonucleotides alone are known to accumulate in liver and spleen because of binding to serum proteins,³² the SWNT–oligonucleotide constructs resulted in significantly less accumulation in the liver and spleen versus oligonucleotide alone. Other DNA analogs, such as morpholino oligonucleotides, are likely to be preferable for future in vivo studies and may further reduce nonspecific liver and spleen retention.

Finally, we demonstrated that targeting moieties appended to the nanotube sidewall amines resulted in specific targeting and binding to tumor cells. While we chose to demonstrate targeting of oligonucleotide-functionalized SWNT through bound RGD peptide ligands, other ligands or monoclonal antibodies⁶ are likely to work as well. Although multivalency is likely to be advantageous, the effect of the targeting moiety copy number on ligand affinity with modified SWNTs also remains to be determined. SWNT–oligonucleotide conjugates are nanoscale platforms capable of specifically recognizing complementary sequences and may be useful for targeted self-assembly in vivo of complex therapeutic nano-structures. In the current study we synthesized and characterized a prototype anchor construct. By validating this construct in vitro and characterizing the pharmacokinetics we can now implement these devices in xenograft tumor models and demonstrate self-assembly in vivo. One can envision new therapeutic approaches such as the targeted self-assembly in vivo of radioisotope nanogenerators³⁷ or even site-specific cross-linking to infarct tumor neovasculature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Synthetic scheme for the production of targetable, oligonucleotide-functionalized single-wall carbon nanotubes. **[4]** was used for biodistribution studies, whereas **[6]** was used to study tumor-cell specific binding.

Figure 2.

(a) Gel permeation chromatograph of SWNT-NH₂-ODNFAM in isocratic aqueous mobile phase using photodiode array detector at 260 nm. (b) Spectrum of chromatograph peak ($t = 26.8$ min) using in-line detector capable of real-time spectral scanning. The spectrum shows the characteristic features of the oligonucleotide and fluorescein (at 260 and 495 nm, respectively) convoluted with the SWNT spectrum (broad absorbance which decreases with wavelength). (c) Transmission electron micrograph of SWNT-NH₂-ODNFAM in small bundles adsorbed onto a copper grid. The oligonucleotide functionalized nanotubes appear to be 50–100 nm in length.

Figure 3.

(a) DNA hybridization and (b) corresponding derivative curves for (solid) SWNT-NH₂-ODNFAM + cODN, $T_m = 54.8$ and (dashed) ODNFAM + cODN, $T_m = 52.4$. The similar melting temperatures indicate that the SWNT-oligonucleotide conjugate is able to engage in normal DNA base-pairing and that hybridization can occur at normal physiologic temperatures ($T_m > 37$ °C).

Figure 4. Biodistribution of ¹¹¹In(DOTA)-ODNFAM and SWNT-¹¹¹In(DOTA)-ODNFAM. For administration of each compound ($n = 5$ for each group), the mice were anesthetized with isoflurane, and injection was via the retroorbital sinus. For each measurement, the radioactivity remaining in each organ was measured versus a standard of the injected formulation. The data were then expressed as percentage of injected dose per gram of tissue.

Figure 5.

Increased binding of SWNT-RGD-ODNFAM (solid) versus isotype nontargeting control SWNT-RAD-ODNFAM (dashed) to $\alpha_V\beta_3$ integrin-expressing HCEC cells. Median fluorescence intensities were 9982 and 4340, respectively. Cells were cultured in EBM media supplemented with growth factors and 10% fetal bovine serum and kept at 37 °C in a 5% CO₂ atmosphere. Cells were washed with PBS and resuspended in PBS with 0.5% BSA as a blocking agent. To the cell suspension was added 10 ug/mL SWNT-RGD-ODNFAM or SWNT-RAD-ODNFAM with the concentration determined through spectrophotometric quantitation of SWNT absorbance at 600 nm. The cell suspensions were placed on ice for 90 min, after which the cells were washed and resuspended in PBS. To measure cell binding, an Accuri C6 flow cytometer (Accuri Cytometers, Inc.) was set to detect the fluorescein moiety of the oligonucleotide in the SWNT-RGD/RAD-ODNFAM constructs. ~20 000 events per run were collected, and the data were analyzed using FlowJo cytometry software (TreeStar, Inc.).