Ultrahigh Sensitivity Carbon Nanotube Agents for Photoacoustic Molecular Imaging in Living Mice

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ABSTRACT Photoacoustic imaging is an emerging modality that overcomes to a great extent the resolution and depth limitations of optical imaging while maintaining relatively high-contrast. However, since many diseases will not manifest an endogenous photoacoustic contrast, it is essential to develop exogenous photoacoustic contrast agents that can target diseased tissue(s). Here we present a novel photoacoustic contrast agent, Indocyanine Green dye-enhanced single walled carbon nanotube (SWNT-ICG). We conjugated this contrast agent with cyclic Arg-Gly-Asp (RGD) peptides to molecularly target the αvβ3 integrins, which are associated with tumor angiogenesis. Intravenous administration of this tumor-targeted contrast agent to tumor-bearing mice showed significantly higher photoacoustic signal in the tumor than in mice injected with the untargeted contrast agent. The new contrast agent gave a markedly 500 times higher photoacoustic contrast in living tissues than previously reported SWNTs, leading to suprananomolar sensitivities. Finally, we show that the new contrast agent can detect ~20 times fewer cancer cells than previously reported SWNTs.

KEYWORDS Photoacoustic molecular imaging, carbon nanotube

Photoacoustic imaging is an emerging modality based on the photoacoustic effect where light is converted into ultrasound waves that are detected outside the subject of interest.¹ Photoacoustic imaging has been used in numerous applications where intrinsic contrast is available such as visualizing blood vessels structure,² thermal burns,³ and melanoma.⁴ However, most diseases will not show photoacoustic contrast, thereby requiring the use of an exogenous contrast agent that will target the diseased tissue. The main challenge in designing such contrast agent remains creating an agent that produces sufficient photoacoustic signal in order to be detected in low concentration, while being able to target the diseased tissue(s). In this work, we developed a new contrast agent that targets cancer-specific receptor in tumor-bearing mice while producing unprecedented sensitivity.

We have recently reported on the conjugation of cyclic Arg-Gly-Asp (RGD) peptides to pegylated single-walled carbon nanotubes⁵ (SWNT-RGD) and their use as photoacoustic imaging agents⁶ to image αvβ3 integrins, which are overexpressed in tumor vasculature. The minimal detectable concentration of SWNT-RGD in living mice was previously calculated to be ~50 nM. In this work, we enhanced the photoacoustic signal of the SWNT-RGD, by attaching Indocyanine Green (ICG) dye to the surface of the nanotubes through π–π stacking interactions⁷ (see Supporting Information for more details). The ultrahigh surface area of the nanotubes allows for highly efficient loading of aromatic molecules such as ICG on the nanotube surface creating a new kind of photoacoustic agent, SWNT-ICG-RGD (Figure 1a). Control untargeted particles were conjugated to a mutated nontargeted peptide, RAD that does not bind to αvβ3 integrins.

The optical absorbance spectrum of the new SWNT-ICG nanoparticle reveals that at its peak absorbance at 780 nm the SWNT-ICG particles exhibited a 20-fold higher absorbance as compared with plain SWNTs (Figure 1b). Importantly, SWNT-ICG-RAD had very similar optical spectrum as SWNT-ICG-RAD. We constructed a nonabsorbing and non-scattering agarose phantom with inclusions of SWNT-ICG-RAD at increasing concentrations from 0.5 nM to 121.5 nM in multiples of 3 (n = 3 inclusions of each concentration). The photoacoustic signal produced by the SWNT-ICG-RGD particles correlated highly with the nanoparticle concentration (R² = 0.983) (Figure 1c).

We further validated that the new particles are stable in serum (see Supporting Information and Figure S1). The particle’s photobleaching (i.e., loss of optical absorption due to continuous light exposure of the dye component of the
nanoparticle was characterized and found to be relatively small, only 30% reduction in optical absorption after 60 min of laser irradiation at normal power density of 8 mJ/cm² (see Supporting Information and Figure S2). Finally, cell uptake studies showed specific binding of SWNT-ICG-RGD to U87MG cells compared with the control particles SWNT-ICG-RAD (see Supporting Information and Figure S3).

We then tested the particle’s sensitivity in living subjects by subcutaneously injecting the lower back of mice with 30 µL of SWNT-ICG-RAD mixed with matrigel at increasing concentrations of 820 pM to 200 nM in multiples of 3. Matrigel alone produced no significant photoacoustic signal (data not shown). All animal experiments were performed in compliance with the Guidelines for the Care and Use of Research Animals established by the Stanford University Animal Studies Committee. Upon injection, the matrigel solidified, fixing the SWNT-ICG-RAD in place and three-dimensional (3D) ultrasound and photoacoustic images of the inclusions were acquired (Figure 2a). While the ultrasound image visualized the mouse anatomy (e.g., skin and inclusion edges), the photoacoustic image revealed the SWNT-ICG-RAD contrast in the

FIGURE 1. Characterization of the ICG dye-enhanced SWNT. (A) Illustration of a SWNT-ICG particle. ICG molecules (red) are attached to the SWNT surface through noncovalent π-π stacking bonds. Polyethylene glycol-5000 (blue) is conjugated to a targeting peptide in one end and to the SWNT surface on the other end through phospholipids. (B) Optical spectra of plain SWNT (black), SWNT-ICG-RGD (blue), and SWNT-ICG-RAD (red). ICG dye-enhanced SWNTs particles showed 20 times higher optical absorption than plain SWNT at the peak absorption wavelength, 780 nm. The similarity of SWNT-ICG-RAD and SWNT-ICG-RGD spectra suggests that the peptide conjugation does not notably perturb the photoacoustic signal. (C) The photoacoustic signal produced by SWNT-ICG was observed to be linearly dependent on the concentration ($R^2 = 0.9833$).
The photoacoustic signal from each inclusion was quantified using a three-dimensional region of interest (ROI) drawn over the inclusion volume. We observed a linear correlation ($R^2 = 0.97$) between the SWNT-ICG-RAD concentration and the corresponding photoacoustic signal (Figure 2b). Tissue background signal was calculated as the average photoacoustic signal in areas where no contrast agent was injected. Extrapolation of the signal-concentration graph reveals that 170 pM of SWNT-ICG-RAD gives the equivalent photoacoustic signal as the tissue background (i.e., signal to background ratio = 1). This value represents over 300 times improvement in sensitivity compared to plain SWNTs.

Finally, we tested the nanoparticles targeting ability in living mice. Mice bearing U87MG tumor xenografts (150 mm$^3$ in size) were injected through the tail vein (IV) with 200 µL of either SWNT-ICG-RGD (targeted) or SWNT-ICG-RAD (untargeted control) particles ($n = 4$ mice per group) at a concentration of 1.2 µM. We acquired 3D photoacoustic and ultrasound images of the entire tumor area before and up to 4 h after the injection. Mice injected with SWNT-ICG-RGD showed significantly higher photoacoustic signal in the tumor compared with the control group injected with SWNT-ICG-RAD (Figure 3a). The ultrasound images were used for visualizing the boundaries of the tumor as well as to validate that no significant movement (beyond 100 µm) had occurred throughout the experiment. While the tumor’s photoacoustic signal before the injection is primarily due to the tumor’s blood content, the photoacoustic signal postinjection is due to both the blood and the SWNT-ICG particles. To subtract out the background blood signal, a subtraction image calculated as the 2 h postinjection minus the preinjection image was calculated (Figure 3a). Measurement of the photoacoustic signal from a 3D ROI around the tumor (Figure 3b) showed that the photoacoustic signal in the tumor was significantly higher in mice injected with SWNT-ICG-RGD as compared with the control particles SWNT-ICG-RAD ($p < 0.001$). For example, at 2 h postinjection mice injected with SWNT-ICG-RGD showed over 100% higher photoacoustic signal in the tumor than mice injected with the control SWNT-ICG-RAD.

To compare the performance of plain SWNT-RGD to the dye-enhanced SWNT-ICG-RGD, we incubated U87MG cells, which express the target αvβ3 on their surface, with either particle solution for 2 h. After incubation, the cells were washed three times with cold saline to remove unbound particles and placed in a clear agarose phantom at increasing concentrations from $25 \times 10^3$ to $6 \times 10^6$ cells per well ($n = 3$ samples per group) and imaged with the photoacoustic system (Figure 4a). Quantitative analysis of the photoacoustic signal from the phantom revealed that cells exposed to SWNT-ICG-RGD were detected at 20 times lower concentration than cells exposed to plain SWNT-RGD ($p < 0.0001$) (Figure 4a,b). These observations are consistent with the optical absorbance of SWNT-ICG-RGD being ~20 times higher than plain SWNT-RGD.

We have synthesized, characterized, and demonstrated the application of dye-enhanced SWNTs as ultrahigh sensitivity photoacoustic imaging agents. A concentration of 170 pM was estimated to produce an equivalent photoacoustic signal as tissue background signal, representing 300 times improvement in sensitivity as compared with plain SWNTs in living mice. This improvement is likely due to both the higher optical absorption of the particles as well as the fact that the new particle’s absorption peak is at 780 nm where the background tissue photoacoustic signal is greatly reduced. Intravenous injection of RGD-targeted SWNT-ICG particles to tumor-bearing mice led to significantly greater accumulation of the particles in the tumor compared to nontargeted control particles. This in vivo targeting study results are likely negatively influenced by the effect of photobleaching, where continued laser light exposure of the
tumor caused reduction in the optical absorption (and photoacoustic signal) of the particles that were bound to the tumor. This primarily affects the targeted group, SWNT-ICG-RGD, and to a much lesser extent the untargeted group, SWNT-ICG-RAD, which continued to circulate through the animal’s bloodstream unexposed to laser irradiation. Therefore, it is likely that the difference between these two groups is even greater in reality than reflected in the results. Finally, we demonstrated the ability to detect 20 times fewer cancer cells when using SWNT-ICG-RGD as the imaging agent, as compared with plain SWNT-RGD. These results agree with the fact that SWNT-ICG has ~20 times greater optical absorbance compared to plain SWNT. Applications of the enhanced particles may therefore be exploited to lead to the earlier detection of cancer by providing the ability to detect smaller tumors.

Most of the work done on photoacoustic contrast agents has been focused on gold nanoparticles8–10 as well as other kinds of nanoparticles.11,12 However, the main challenge that has yet been solved is the delivery of such agents to the tumor in sufficient amounts to create detectable and specific signal. This is likely due to the particles’ large size that leads to rapid clearance by the reticuloendothelial system (RES) upon intravenous injection, preventing the particles from accumulating at the tumor site. In contrast, the SWNTs used here are 1–2 nm in diameter and 50–300 nm in length. Since the dye we used was attached to the surface of the SWNTs under the PEG, it is expected that the total particle size was not significantly changed, thereby allowing the particles to keep a favorable biodistribution as previously reported.5 Hence, the dye-enhanced SWNTs presented in this work offer unprecedented photoacoustic signal strengths while maintaining relatively small size allowing them to target tumors upon intravenous injection. We have also previously published pilot toxicology studies of the SWNTs with encouraging results in mouse models13 as well as observed they are able to be excreted via the biliary pathway.14

The reason for loading a SWNT with many small ICG dye molecules is the high efficiency of optical absorption of ICG dye compared to its weight. According to the parameter of optical absorption divided by weight, ICG is 7 times more efficient than SWNTs and ~8500 times more efficient than commercial gold nanorods with a peak absorption at 780 nm.

The dye-enhanced SWNT photoacoustic contrast agents reported here have the capability to bind to molecular targets in living animals while maintaining a very high photoacoustic signal. No other imaging modality or imaging agent can achieve sub-nM sensitivity at large depths of penetration and

FIGURE 4. Comparison of plain SWNT-RGD to SWNT-ICG-RGD. (A) Photoacoustic vertical slice image through an agarose phantom containing decreasing number of U87 cancer cells exposed to SWNT-ICG-RGD and plain SWNT-RGD particles. While 1.7 x 10^6 cells exposed to SWNT-RGD are barely seen on the image, a clear photoacoustic signal was observed from 1.4 x 10^5 cells exposed to SWNT-ICG-RGD. The signal inside the ROI (dotted white boxes) is not homogeneous due to possible aggregates of cells. (B) Quantitative analysis of the photoacoustic signals from the phantom (n = 3) showed that SWNT-ICG-RGD can visualize ~20 times less cancer cells than SWNT-RGD can (p < 0.0001). The background line represents the average background signal in the phantom. Linear regression was calculated on the linear regime of both curves.
submillimeter spatial resolution as can be achieved with photoacoustic imaging of dye-enhanced SWNTs.

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Supporting Information Available. Description of particles synthesis, photoacoustic imaging instrument, experimental procedures, statistical methods, and in vitro characterization of the particles including serum-stability, photobleaching, and cell-uptake study. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES AND NOTES