Family of Enhanced Photoacoustic Imaging Agents for High Sensitivity and Multiplexing Studies in Living Mice

Adam de la Zerda[1,2], Sunil Bodapati[1], Robert Teed[1], Salomon Y. May[1], Scott M. Tabakman[3], Zhuang Liu[3,4], Butrus T. Khuri-Yakub[2], Xiaoyuan Chen[1,5], Hongjie Dai[3], Sanjiv Sam Gambhir[1,6]

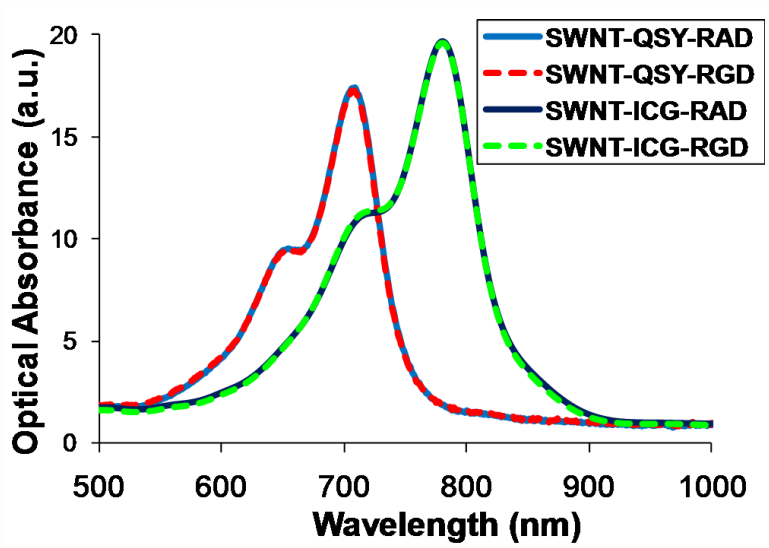
[1]Molecular Imaging Program at Stanford, Department of Radiology and Bio-X Program, the [2]Department of Electrical Engineering and the [3]Department of Chemistry, Stanford University, Palo Alto, CA 94305, USA, [4]Institute of Functional Nano & Soft Materials (FUNSOM), Soochow University, Suzhou, Jiangsu, 215123, China, [5]Laboratory of Molecular Imaging and Nanomedicine, National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, MD 20892, USA, The [6]Department of Bioengineering and the Department of Materials Science & Engineering, Stanford University, Palo Alto, CA 94305, USA.

* e-mail: sgambhir@stanford.edu

Supplementary Notes

In vitro Characterization of Dye-enhanced SWNTs

We measured the absorbance spectra of SWNT-QSY-RGD, SWNT-QSY-RAD, SWNT-ICG-RGD and SWNT-ICG-RAD to verify that the particle absorbance spectrum is independent of the peptide conjugation. As shown in Supplementary Figure 1, particles conjugated with RGD showed nearly identical absorption spectrum and intensity as particles conjugated with RAD.

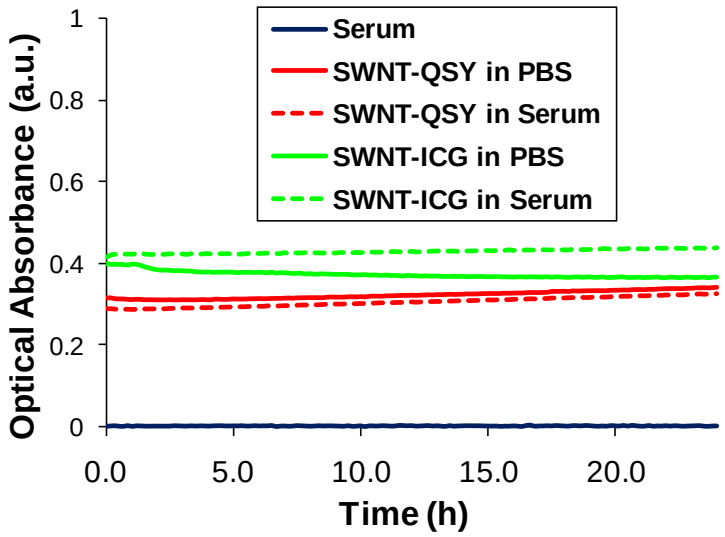


Supplementary Figure 1. Absorbance spectra of RGD- vs. RAD-conjugated SWNT. SWNT-QSY-RAD (light blue) exhibited the same optical absorbance spectrum as its corresponding RGD-conjugated particle (dashed red). SWNT-ICG-RAD (dark blue) also showed an identical optical absorbance spectrum as its corresponding RGD-conjugated particle (dashed green). This indicated that the optical absorbance of the SWNT-dye particles is independent of the peptide that is conjugated to the SWNT.

Serum Stability

The stability of the SWNT-dye particles in serum was verified. We incubated 10 nM of SWNT-QSY-RGD or SWNT-ICG-RGD with 10% fetal bovine serum (FBS) and 90% PBS 1X and monitored the optical absorbance of the solution at 710nm and 780nm respectively every 10 min for a period of 24 h. Control solutions included 10% serum in PBS only or SWNT-QSY-

RGD in PBS or SWNT-ICG-RGD in PBS (no serum). The optical absorbance remained steady during the 24 h of measurement (standard deviation of the absorbance was 4% and 2% of the average absorbance and the maximum deviation from average was below 10% and 7% for SWNT-QSY-RGD and SWNT-ICG-RGD, respectively) (**Supplementary Figure 2**).

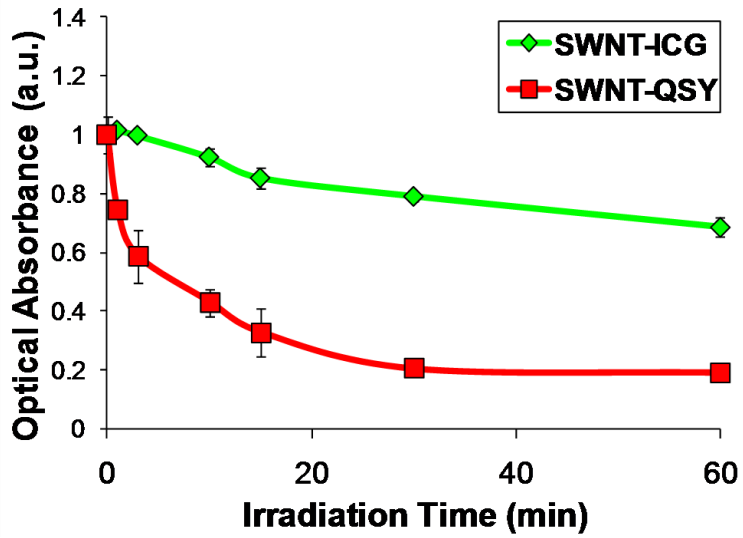


Supplementary Figure 2. Serum stability of SWNT-dye particles. SWNT-QSY-RGD and SWNT-ICG-RGD particles were exposed to 10% serum and their optical absorbance was measured over a period of 24 h (red and green dashed lines, respectively). Control solutions included SWNT-QSY-RGD in PBS and SWNT-ICG-RGD in PBS only (solid red and green lines, respectively) and serum only (blue). All solutions showed steady optical absorption within ±5% over the 24 h period.

Photobleaching

We tested the optical stability of SWNT-QSY-RGD and SWNT-ICG-RGD particles under increasing durations of light exposure (photobleaching). SWNT-ICG-RGD were found to be more photo-stable, losing only 30% of their optical absorbance after 60 min of continuous laser irradiation at 780 nm and 8 mJ/cm^2 (the maximal skin exposure used in the experiments described here). However, SWNT-QSY-RGD exhibited higher photobleaching with 50% decrease in optical absorbance after 7 min of irradiation at 710 nm wavelength and laser intensity of 8 mJ/cm^2 (**Supplementary Figure 3**). Importantly, in living animals, an additional layer of

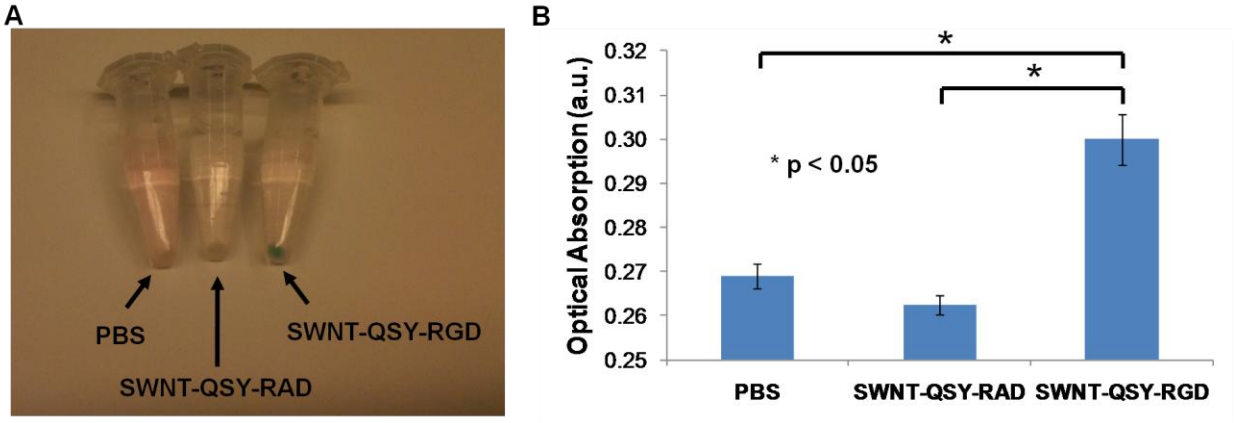
skin is protecting the animal and hence, the effective laser intensity that reached the tumor and the particles was likely lower than 8 mJ/cm^2. Moreover, since the average mouse imaging session requires ~10 min, during which the laser light illuminates different areas over the tumor, it is expected that photobleaching is not affecting the imaging results significantly.



Supplementary Figure 3. Photobleaching of SWNT-dye particles. SWNT-ICG-RGD and SWNT-QSY-RGD samples (n = 3 for each) were exposed to increasing durations of 780 nm and 710 nm laser light, respectively, at power density of 8 mJ/cm^2 (the maximal skin exposure used in the animal experiments). After 60 min of laser exposure, the optical absorption of the SWNT-ICG-RGD particles was reduced by ~30%, whereas the SWNT-QSY-RGD absorbance was reduced by ~80%.

Cell Uptake Study

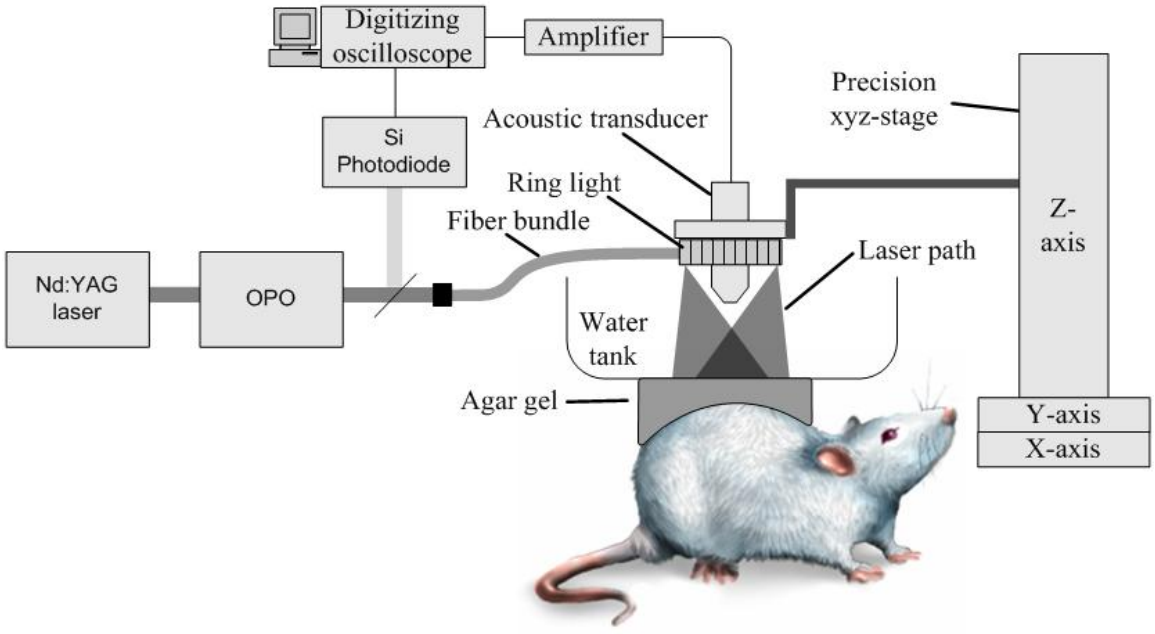
We tested the particle's in-vitro targeting capability by incubating 10^6 U87MG cells in 800 µl of cell media with 200 µl of SWNT-QSY-RGD or SWNT-QSY-RAD at 100 nM concentration for 2 h at 4^oC (n = 3 samples per particle type). Control cells were incubated the same way, only with 200 µl of PBS only (n = 3). During the incubation period, the vials containing the cells were placed on a rocker to allow homogeneous exposure of the cells to the particles. After incubation, the cells were washed with cell medium twice. After the wash, cells exposed to SWNT-QSY-RGD were still colored in blue, while cells exposed to PBS or to SWNT-QSY-RAD, had no color (**Supplementary Figure 4A**). We then vortexed the cells for 10 s to reconstitute the cells in 1 ml volume and measured the solution's absorbance at 710 nm (**Supplementary Figure 4B**). Control cells incubated with either saline or SWNT-QSY-RAD showed a baseline absorbance, primarily due to the cells optical scattering properties. However, cells incubated with SWNT-QSY-RGD showed significantly higher absorbance ($p < 0.05$ compared to either cells group). In a previous paper,[1] we have already shown the specific cell uptake of SWNT-ICG-RGD versus SWNT-ICG-RAD and saline only.



Supplementary Figure 4. Cell uptake study of SWNT-ICG. U87MG cells were exposed to RGD-targeted SWNT-QSY, untargeted SWNT-QSY, or saline (control) for 2 h and then washed twice to remove any unbound particles. **(A)** Cells exposed to PBS or to SWNT-QSY-RAD showed no color, while cells incubated with SWNT-QSY-RGD showed a strong blue color, characteristic of the SWNT-QSY particles. **(B)** The binding level was measured by the total optical absorbance of the cell solution. Cells exposed to SWNT-QSY-RGD showed significantly higher optical absorbance than cells exposed to SWNT-QSY-RAD or to PBS only ($p < 0.05$).

The background absorbance of ~0.26 is due to the endogenous optical scattering of the cells. Error bars in the figure represent standard error (n = 3)

Supplementary Figures



Supplementary Figure 5. Photoacoustic imaging instrument. A tunable pulsed laser (Nd:YAG laser and OPO) illuminated the subject through a fiber optic ring light. The photoacoustic signals produced by the sample were acquired using a 5 MHz focused transducer. A precision xyz-stage was used to move the transducer and the fiber ring along a planar 2D trajectory. The time of arrival and the intensity of the laser pulses were recorded using a silicon photodiode. This information was used to synchronize the acquisition and compensate for pulse-to-pulse variations in laser intensity. The analog photoacoustic signals were amplified using a variable-gain preamplifier and digitized using an oscilloscope.

Supplementary Information References

1. de la Zerda, A.; Liu, Z.; Bodapati, S.; Teed, R.; Vaithilingam, S.; Khuri-Yakub, B. T.; Chen, X.; Dai, H.; Gambhir, S. S. Ultrahigh sensitivity carbon nanotube agents for photoacoustic molecular imaging in living mice. *Nano Letters* **2010,** *10*, 2168-2172.