Deep, noninvasive imaging and surgical guidance of submillimeter tumors using targeted M13-stabilized single-walled carbon nanotubes

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Highly sensitive detection of small, deep tumors for early diagnosis and surgical interventions remains a challenge for conventional imaging modalities. Second-window near-infrared light (NIR2, 950–1,400 nm) is promising for in vivo fluorescence imaging due to deep tissue penetration and low tissue autofluorescence. With their intrinsic fluorescence in the NIR2 regime and lack of photobleaching, single-walled carbon nanotubes (SWNTs) are potentially attractive contrast agents to detect tumors. Here, targeted M13 virus-stabilized SWNTs are used to visualize deep, disseminated tumors in vivo. This targeted nanoprobe, which uses M13 to stably display both tumor-targeting peptides and an SWNT imaging probe, demonstrates excellent tumor-to-background uptake and exhibits higher signal-to-noise performance compared with visible and near-infrared (NIR1) dyes for delineating tumor nodules. Detection and excision of tumors by a gynecological surgeon improved with SWNT image guidance and led to the identification of submillimeter tumors. Collectively, these findings demonstrate the promise of targeted SWNT nanoprobes for noninvasive disease monitoring and guided surgery.

cancer imaging | fluorescence-guided surgery | M13 bacteriophage

In clinical oncology, in vivo fluorescence imaging has emerged as a valuable tool for improving diagnosis, staging tumors, monitoring response to therapy, and detecting recurrent or residual disease. Compared with existing imaging modalities, fluorescence imaging offers a low-cost, portable, and safe alternative (i.e., nonionizing radiation), with key advantages including real-time imaging, superior resolution, and high specificity for small tumor nodules during diagnostic and intraoperative surgical procedures (1, 2). Although efforts have focused on using visible and short near-infrared (NIR1, 650–900 nm) wavelength fluorescent dyes as contrast agents for delineating tumor margins in both preclinical cancer models (2, 3) and human patients (4), these agents are suboptimal for noninvasive, reflectance-based imaging due to limited penetration depth (3–5 mm) and high tissue autofluorescence. During intraoperative surgery, these dyes may additionally undergo photobleaching, thereby reducing the ability of the surgeon to readily locate and resect tumors. Alternative approaches to specifically permit noninvasive imaging and limited photobleaching would be highly desirable for diagnostic and surgical applications.

Single-walled carbon nanotubes (SWNTs) hold great promise as fluorescence imaging agents due to the large interband difference between their excitation and emission wavelengths, resulting in minimal spectral overlap and tissue autofluorescence. In particular, the low tissue autofluorescence observed with SWNTs greatly enhances target-to-background ratios (TBRs) necessary for improved detection of small tumor nodules in confined anatomic regions. SWNT emission at longer wavelengths in the

near-infrared second window (NIR2, 950–1,400 nm) results in less optical scattering and deeper tissue penetration compared with shorter wavelength visible and NIR1 imaging agents. Simulations (5) and experimental results (6) suggest the greatest tissue penetration depth is achieved in the NIR2 regime, which further supports the potential value for use of SWNTs for biological imaging applications. Additionally, unlike visible and near-infrared dyes, well-functionalized SWNTs are less susceptible to photobleaching or quenching effects (7), which make them attractive for continuous, long-term imaging required during many surgical procedures. Previously, we have demonstrated that M13 bacteriophage-stabilized SWNTs can target s.c. prostate tumors in preclinical models for fluorescence imaging in the second optical window (8). SWNTs have also been used for vascular and deep tissue fluorescence imaging (9). Importantly, M13 serves as a scaffold to couple both targeting and imaging moieties while allowing them to retain their functionalities (10); in comparison,

Significance

Early detection of cancer positively impacts diagnosis and treatment, ultimately improving patient survival. Using fluorescence imaging offers the promise of safe, noninvasive detection with excellent resolution and guides surgical removal of tumors to improve patient outcomes. However, the success of current optical probes is limited due to high background from tissue autofluorescence, poor penetration depth, and inherently low signal stability. Here, we engineered M13 bacteriophage to stabilize single-walled carbon nanotubes for selective, targeted imaging of ovarian tumors. These nanoprobes fluoresce at longer near-infrared wavelengths than current probes, thereby improving noninvasive detection of small, deep tumors and guidance for surgical removal of submillimeter tumors. This material-based approach may be attractive to guide surgical interventions where deep tissue molecular imaging is informative.

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Here we report an M13-stabilized SWNT probe that selectively targets Secreted Protein, Acidic and Rich in Cysteines (SPARC)-expressing tumor nodules in an orthotopic mouse model of human ovarian cancer. Ovarian cancer remains a major health care problem for women. Annually, 225,000 women worldwide are diagnosed with epithelial ovarian cancer (EOC) and ~140,000 women die as a result (15). Although women with early-stage ovarian cancer [International Federation of Gynecology and Obstetrics (FIGO) stage I/II] can be cured, advanced-stage ovarian cancer (FIGO III/IV) remains considerably more difficult to treat. Unfortunately, 80% of women with EOC have metastatic disease at the time of diagnosis, and many undergo a treatment regimen of surgery and chemotherapy. Our study focused on ovarian cancer because clinical evidence indicates that optimal surgery can significantly prolong the median overall survival of patients as well as reduce disease morbidity (16). Using the NIR2 emission of these fluorescence probes, we determine the detection limit of labeled tumors and their TBRs. We demonstrate that this SWNT-based probe detects tumors that were missed when either visible or NIR1 dyes were used, thereby aiding the discovery of smaller tumors during surgery. Collectively, our results highlight the potential for affinity-targeted NIR2 fluorescence probes to monitor disease processes such as cancer with enhanced sensitivity compared with current state-of-the-art optical probes.

probes will be stable in the vascular and lymphatic systems and the peritoneal cavity and for cellular uptake. We also confirmed the targeted probes are not acutely cytotoxic to primary human endothelial cells and ovarian carcinoma cell line 8 (OVCAR8) (*SI Appendix*, Figs. S5 and S6, respectively), which builds upon previous studies on cell-type dependence of nanomaterial toxicity (24) and underscores their potential for in vivo imaging applications.

We next examined the sensitivity of the probe in terms of its capacity to target OVCAR8 ovarian cancer cells in vitro. Serial 10-fold dilutions of OVCAR8 cells were incubated with SBP-M13-SWNT for 24 h, and cell lysates were collected. Measuring the fluorescence intensity of the SBP-M13-SWNT incubated cells, we observed that as few as ~10,000 cells incubated with SBP-M13-SWNT exceeded the minimum level of detection (Fig. 1C).

To test SBP-M13-SWNTs for risk of photobleaching, we exposed them to an 808-nm laser for a continuous, 30-min period and measured fluorescence intensity in 5-min intervals up to 30 min postirradiation. No appreciable loss of fluorescence of SBP-M13-SWNTs was observed during this period. However, the intensity of fluorescein isothiocyanate (FITC), a fluorescein derivative that has been used to molecularly image and guide intraoperative resection of ovarian tumors in humans (4), exponentially decreased in response to the same light exposure kinetics (Fig. 1D). The observations that SWNTs do not photobleach and maintain their optical properties illustrate their potential to assist surgeons in visualizing tumors during resection.

Another potential advantage of SWNT-based imaging compared with FITC-based imaging is the potential to detect tumors located at greater depths in the body. To investigate the depth of detection that can be achieved with our probe, we harvested ovarian tumors that had been treated with SBP-M13-SWNTs and imaged the small tumor fragments (~1-mm diameter) at various depths within a tissue “phantom” construct, which mimics the optical properties of human tissue. Using our NIR2 fluorescence reflectance imaging system (8), we could detect SWNT-containing tumors to depths as great as 9.7–18.2 mm (*SI Appendix*, Fig. S7). To our knowledge, this is the best reported quantifiable tumor depth using reflectance imaging, relative to previously reported values (3). By permitting deeper imaging, SBP-M13-SWNT therefore offers the potential for noninvasive detection before surgery and improved resection of tumors during surgery.

In Vivo Characterization of the SBP-M13-SWNT Probe. Having demonstrated in vitro stability and fluorescence of SBP-M13-SWNTs, we next characterized their in vivo properties in an orthotopic model of ovarian cancer. The OVCAR8 human cell line was used to create the orthotopic model, as it overexpresses SPARC, as confirmed by ONCOMINE (25) analysis and immunohistochemistry (*SI Appendix*, Fig. S8). To compare routes of administration, tumor-bearing animals were injected intraperitoneally or i.v., and the circulating probe concentration was monitored via SWNT fluorescence in the blood. The i.v.-administered SBP-M13-SWNTs reached a peak concentration in the circulation ~10 min after injection, and circulating levels became negligible after 150 min (Fig. 1E). Notably, SBP-M13-SWNTs injected intraperitoneally led to negligible elevations in blood-borne SWNT fluorescence for at least 24 h, suggesting that the majority of SBP-M13-SWNTs remain in the peritoneum postinjection (Fig. 1E). This finding was verified by the observation that, following a transient increase in NIR2 fluorescence in the peritoneum, the overall intensity in this location stabilizes for periods up to 24 h following injection (Fig. 2A and B). Lastly, we studied the biodistribution of SBP-M13-SWNTs administered into the peritoneal cavity of tumored mice (*SI Appendix*, Fig. S9). The accumulation of SBP-M13-SWNTs was greatest in tumors at days 1 and 7 postinjection, with additional signal observed in

Fig. 2. Noninvasive tumor imaging with SBP-M13-SWNTs. (A) Representative whole-abdomen NIR2 imaging series following i.p. administration of SBP-M13-SWNTs. (Inset) Surgically excised tumor nodule (denoted by red arrow) observed 24 h postinjection of SBP-M13-SWNTs. (B) NIR2 fluorescence intensity in the abdomen of tumor-bearing animals following IP administration of SBP-M13-SWNTs up to 24 h postinjection ($n = 6$; error bars represent SE). (C) Noninvasive imaging of ovarian tumors using SBP-M13 conjugated to SWNTs (NIR2), AlexaFluor750 (NIR1), and FITC (Visible) (top to bottom). Arrows in the SWNT panel denote nodules visible only by SWNTs ($n = 3$ animals). (D and E) Tumor-to-muscle ratio and TBR from noninvasive images obtained with SWNTs, AF750, and FITC ($n = 3$ per group; *** $P < 0.001$; * $P < 0.05$; one-way ANOVA and Tukey posttests). Error bars, s.d. [Scale bar, 1 cm (A)].

liver, spleen, kidney, and peritoneum in the vicinity of the injection site. At day 7 postinjection, the lowest signals observed were in heart and lung tissue, supporting limited systemic exposure of SBP-M13-SWNT after i.p. administration. Importantly, analysis of serum from tumor-bearing animals following the administration of SBP-M13-SWNTs into the peritoneal cavity revealed no evidence of systemic hepatic, renal, metabolic, or hematologic acute toxicities within the first week following administration (*SI Appendix*, Fig. S10).

Improved Signal-to-Noise Performance in Vivo Using SBP-M13-SWNT in the NIR2 Wavelength Regime. Signal-to-noise behavior is a critical parameter for sensitive detection of tumors. To directly compare the signal-to-noise ratios between SWNTs and dyes in the NIR1 or visible regimes, SBP-M13 viruses were either complexed to SWNTs or conjugated with AlexaFluor750 dye (SBP-M13-AF750) or FITC (SBP-M13-FITC). Probes were then combined and added in fivefold excess of their minimum detection limits to mice bearing disseminated tumors. Noninvasive images were acquired through the fully intact skin of tumor-bearing animals (Fig. 2C), and fluorescence intensities from tumors in the peritoneal cavity, muscle, and background were determined. The tumor-to-muscle ratio of SBP-M13-SWNTs was 5.5 ± 1.2 (mean $\pm$ SD), which was significantly higher than ratios calculated for SBP-M13-AF750 (3.1 ± 0.42) and SBP-M13-FITC (0.96 ± 0.10) (Fig. 2D). The TBR (i.e., intensity of tumor to image

Image Guidance Using SBP-M13-SWNT Improves Surgical Resection of Tumors. To assess the potential clinical utility of SBP-M13-SWNTs for reduction of tumor burden, a gynecological surgeon performed surgeries on orthotopic models of ovarian cancer that were imaged at various points during the surgical procedures. Approximately 15–25 min were spent on procedures in each experiment, and tumor implants were predominantly distributed in the bowel mesentery, peritoneal wall, subdiaphragmatic surfaces, and surfaces of organs including the liver, spleen, pancreas, and within the pelvic cavity. H&E-stained tissue sections were prepared from all excised nodules and assessed by a pathologist. With the exception of two non-tumor-containing mesenteric lymph nodes, all tissues examined ($n = 197$) were positive for ovarian tumor tissue, indicating an accuracy of 98.9% of our probe for ovarian tumors. Surgery was first performed with preoperative image guidance to assess whether this addition to the process would be beneficial to the surgical procedure based on the distribution of excised tumor nodule sizes. A comparative analysis of excised tumors revealed that a significantly higher number of submillimeter tumor nodules were discovered in the image-guided cohorts versus the non-image-guided cohorts (12 and 0 nodules, respectively; *SI Appendix, Fig. S11*). Using image guidance, there were also greater numbers of excised tumors from 1.3 to 3 mm; however, there was no appreciable difference for larger tumors (>3 mm) between image-guided and non-image-guided cohorts. We additionally assessed the impact of performing surgery in a serial manner, with an initial round of non-image-guided surgery, followed by image acquisition and a second round of image-guided surgery (Fig. 4A). We observed reduction of tumor burden from non-image-guided surgery to image-guided surgery

Fig. 4. Surgical removal of tumors with SBP-M13-SWNT guidance. (A) Schematic of serial surgical procedure. (B) Representative whole-abdomen NIR2 images before injection of SBP-M13-SWNT, before surgery, after an initial unguided surgery, and after subsequent SBP-M13-SWNT-guided surgery. White arrow indicates a SWNT-positive nodule detected only during image-guided surgery. (C) TBRs during surgery. Muscle from the hind limb was used for background ($**P < 0.01$; $****P < 0.0001$; two-tailed *t* tests; error bars represent SE). (D) Photographs and NIR2 images of excised tumor nodules following unguided and SWNT-guided surgery. (E) Histogram of tumor diameters removed with and without guidance. (F) Dot plot of individual tumor nodule diameters excised with and without SWNT-guidance (guided, $n = 74$; unguided, $n = 146$; $**P < 0.01$; two-tailed *t* test). [Scale bars, 1 cm (B), 1 cm (C, photograph), and 1 cm (C, NIR2).]

(Fig. 4B). We determined the TBR of overall tumor burden to background muscle by region of interest analysis and confirmed reduction of overall tumor burden due to image-guided reduction (Fig. 4C). Using both SWNT imaging (Fig. 4B) and quantification of excised tumor nodule diameters, we observed a greater number of submillimeter excised tumors in the groups assisted by SWNT image guidance (30 versus 4 nodules; Fig. 4D and E). Overall, significantly more, smaller diameter tumors were excised using SWNT-based image guidance as opposed to unguided surgeries (Fig. 4F).

Discussion

This study describes the development and use of a single fluorescence imaging agent for high-contrast, detection and guidance for surgical removal of disseminated ovarian tumors. NIR2-emitting SWNT probes offer significantly improved signal-to-noise performance compared with visible and near-infrared dyes and detect tumors not visualized using the optical dyes. These targeted, M13-stabilized SWNT probes assist surgical removal of ovarian tumors with excellent sensitivity, as confirmed by subsequent pathological examination. The probe is sensitive for identifying tumor nodules located on several abdominal viscera, the peritoneal wall, and the bowel mesentery. Importantly, compared with fluorescent probes in the visible or NIR1 regimes, the fluorescence of SWNTs is not limited by quenching, allowing for long-term, continuous imaging. With the development of advanced imaging platforms, surgeons may be able to visualize tumors both before and throughout surgical procedures, thereby significantly improving fluorescence-guided tumor resection. This study demonstrates that surgery accompanied by image guidance leads to identification and removal of smaller tumor nodules. Although NIR2 images could not provide 3D localization of the tumor implants, they provided information about the

sites of disease burden requiring closer surgical examination. Imaging of regions in which the surgeon was initially reluctant to explore in an effort to minimize morbidity by risking excessive blood loss, but were shown to harbor a positive NIR2 signal, often led to the identification and excision of additional tumor nodules missed on non-image-guided approaches. Although we did not investigate longitudinal survival rates after image-guided surgery due to surgical constraints in our small-animal models, the majority of clinical evidence suggests that optimal surgery, currently defined as the removal of tumors with diameters of 1 cm and larger, is correlated with improved overall survival rates (16). SWNT-based affinity probes may aid in surgical planning and resection to help achieve a reduction in mortality rates in the future.

We achieve detection of submillimeter tumors with excellent TBRs using M13-stabilized SWNTs, in part due to properties of the particles that lead to low tissue scattering and minimal tissue autofluorescence in the NIR2 optical window. In comparing excised tumors with unaffected intestinal tissues as a background measurement, we observed high TBRs of ~ 112 using our SPARC-targeted M13-SWNT probes. Following i.p. administration, some uptake is observed using nontargeted SWNT probes, which is most likely due to nonspecific binding interactions or convective flow patterns present within the peritoneal cavity. Whereas many nanoparticles include targeting peptides conjugated directly to the nanoparticle, our nanoprobe takes advantage of the genetically encoded M13 scaffold to spatially uncouple the targeting peptide (SBP) from the imaging probe (SWNT). This separation of targeting and imaging moieties circumvents direct and excessive conjugation, which may abolish the functionality of each component (11, 26); however, how this spatial uncoupling affects both targeting and nanomaterial functionality is an area requiring further study.

Fluorescence imaging in the second optical window offers the promise of imaging at greater penetration depths (>3 – 5 mm) with reduced optical scattering within the tissue. In comparing the performance of visible, NIR1, and NIR2 probes, the M13-SWNT probe achieved the highest tumor-to-muscle ratios and TBRs, most likely due to less tissue absorbance and autofluorescence in the NIR2 regime. In addition, we were able to see tumors not observed in the visible and NIR channels, highlighting the improved tissue penetration and detection in the NIR2 regime. Using our reflectance imaging system, we can detect 1-mm-diameter tumors up to a maximal depth between 9.7 and 18.2 mm. To our knowledge, this represents the highest reported depth of detection using fluorescence reflectance and is also higher than previous reports that detected mammary tumors labeled with activatable Cy5 probes (3). Future work to enhance the fluorescence of M13-SWNTs using plasmonic nanomaterials (27) and affinity targeting using other ligand–receptor interactions including the folate receptor (4) may offer further improvements on current limits of detection and resolution. These longer wavelength-emitting probes will greatly aid in locating ovarian tumors confined to deep anatomical regions.

SBP-M13-SWNTs injected intraperitoneally colocalized with stromal SPARC expression on the periphery of the ovarian tumor nodules. Tumor nodules labeled with the probes exhibited high signal with low background in the surrounding healthy tissues, including liver, spleen, and intestine. These high organ-specific TBRs contributed to more accurate surgical resection of tumor nodules localized to the organ surfaces. Because our probes can visualize the tumor margins, they have potential to assist the surgeon in delineating tumors from healthy tissue for improved resection of other solid tumors, as also demonstrated by approaches using activatable peptides (3, 28) and dyes (2), fluorescein conjugates (4), and multimodal nanoparticles (29).

The use of nanomaterials as clinical imaging probes is a rapidly evolving area. As new materials emerge from the community, their attractiveness for clinical applications is typically

informed first by their *in vitro* properties, then by their behavior in animal models, and ultimately by their performance in humans. In this context, M13–SWNT probes exhibit many desirable *in vitro* properties such as solution stability, retention of optical properties under various pH and physiological environments and under constant excitation, and the dual capability of genetically encoding affinity ligands and solubilizing SWNTs. The next step in assessing their attractiveness for clinical translation is to assess their performance in animal models. Thus, this study explores the roles of targeting, acute biocompatibility via *i.p.* delivery, and optical emission in NIR2 in a preclinical mouse model of ovarian cancer. Our acute toxicity studies are consistent with several other preliminary studies exploring the safety of M13 and SWNTs, in that a panel of serum biomarkers were not elevated (30–33). Collectively, we believe these findings suggest that these materials warrant further consideration for clinical translation, which would include the development of instruments for real-time imaging, outcomes research such as survival studies in preclinical models, and rigorous safety evaluation through the National Characterization Laboratory (National Cancer Institute) and others to assess long-term trafficking and clearance of these materials as well as chronic toxicity studies.

Although in this study we use SWNTs for fluorescence imaging, others have demonstrated their utility as carriers for therapeutic cargoes (reviewed in ref. 34) as well as photothermal ablative therapy (35). We have begun to explore the utility of SBP–M13–SWNTs for *in vivo* heating of the tumor microenvironment to assess their potential as sensitizing agents to multimodal imaging and therapeutic agents.

To advance our findings closer to clinical translation, new instrumentation will be required to allow for real-time intraoperative guidance and 3D tomography for quantitative analysis and more accurate localization of tumors. Simulations suggest that at near-infrared wavelengths that define the NIR2 window,

SWNT-based probes may be detectable at depths up to 10 cm on improved imaging platforms, highlighting the potential utility of these particles in human subjects (36). This new platform would allow for real-time, noninvasive imaging and processing for visualization of tumors during tumor staging, presurgical planning, and procedures. Coupling improved instrumentation with probe development will greatly improve the ability to detect tumors at earlier stages. The instrumentation should also be compatible with other NIR2 window optical probes as they are developed. Early detection of smaller tumors may also provide fundamental insights into tumorigenesis and disease progression, as well as allow clinicians to better monitor therapeutic responses and recurrence of disease.

Materials and Methods

All materials are provided in *SI Appendix, SI Materials and Methods*. *In vitro* and *in vivo* characterization of the SBP–M13–SWNT probe, including optical properties, stability, detection sensitivity, biodistribution, and serum chemistry assays, are described in detail in *SI Appendix, SI Materials and Methods*. Tumor induction procedure, fluorescence imaging, and analysis of SBP–M13–SWNT targeting *in vivo* and *ex vivo* are detailed in *SI Appendix, SI Materials and Methods*. Comparison of noninvasive imaging between SBP–M13–SWNT with visible and NIR1 imaging probes and image-guided surgeries is described in *SI Appendix, SI Materials and Methods*.

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