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Layer-by-layer assembled fluorescent probes in the second near-infrared window for systemic delivery and detection of ovarian cancer

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Fluorescence imaging in the second near-infrared window (NIR-II, 1,000–1,700 nm) features deep tissue penetration, reduced tissue scattering, and diminishing tissue autofluorescence. Here, NIR-II fluorescent probes, including down-conversion nanoparticles, quantum dots, single-walled carbon nanotubes, and organic dyes, are constructed into biocompatible nanoparticles using the layer-by-layer (LbL) platform due to its modular and versatile nature. The LbL platform has previously been demonstrated to enable incorporation of diagnostic agents, drugs, and nucleic acids such as siRNA while providing enhanced blood plasma half-life and tumor targeting. This work carries out head-to-head comparisons of currently available NIR-II probes with identical LbL coatings with regard to their biodistribution, pharmacokinetics, and toxicities. Overall, rare-earth-based down-conversion nanoparticles demonstrate optimal biological and optical performance and are evaluated as a diagnostic probe for high-grade serous ovarian cancer, typically diagnosed at late stage. Successful detection of orthotopic ovarian tumors is achieved by in vivo NIR-II imaging and confirmed by ex vivo microscopic imaging. Collectively, these results indicate that LbL-based NIR-II probes can serve as a promising theranostic platform to effectively and noninvasively monitor the progression and treatment of serious ovarian cancer.

Significance

Survival of cancer patients can be greatly improved by an ideal theranostic platform capable of early detection and effective treatment of tumors. Layer-by-layer (LbL) assembly is a well-established technology that matches the requirements of modularity and versatility to develop theranostic platforms for different NIR-II probes. LbL assembly allows for the construction of hierarchical and multifunctional polyelectrolyte multilayers on a charged colloidal core substrate (13–15), and it is possible to incorporate therapeutics such as siRNA, inhibitors, or proteins in the multilayers around the core nanoparticle (NP) (16). The LbL platform provides improved biocompatibility that reduces off-target toxicity of the delivered payloads (17), and the LbL stealth coatings provide extended blood plasma half-life when applied to liposomes, QDs, gold, and other NP systems (15, 18). Recent work using the LbL platform has led to a demonstration of staged siRNA/chemotherapy using a single lipid, polymer, protein, or bacteriophage (6–12). However, these delivery carriers lack the modularity and versatility to include drugs effectively for theranostic platforms and do not as readily enable the incorporation of complex or multiple drug payloads. Consequently, a modular delivery system of the NIR-II probes is more attractive because it allows incorporation of imaging agents and multiple drugs independently and ability of staged release of therapeutics.

Layer-by-layer (LbL) assembly is a well-established technology that matches the requirements for modularity and versatility to develop theranostic platforms for different NIR-II probes. LbL assembly allows for the construction of hierarchical and multifunctional polyelectrolyte multilayers on a charged colloidal core substrate (13–15), and it is possible to incorporate therapeutics such as siRNA, inhibitors, or proteins in the multilayers around the core nanoparticle (NP) (16). The LbL platform provides improved biocompatibility that reduces off-target toxicity of the delivered payloads (17), and the LbL stealth coatings provide extended blood plasma half-life when applied to liposomes, QDs, gold, and other NP systems (15, 18). Recent work using the LbL platform has led to a demonstration of staged siRNA/chemotherapy using a single lipid, polymer, protein, or bacteriophage (6–12). However, these delivery carriers lack the modularity and versatility to include drugs effectively for theranostic platforms and do not as readily enable the incorporation of complex or multiple drug payloads. Consequently, a modular delivery system of the NIR-II probes is more attractive because it allows incorporation of imaging agents and multiple drugs independently and ability of staged release of therapeutics.
combination release in a triple-negative breast cancer model (13). Furthermore, the LbL system can be used to generate highly effective, dual targeting of outer layers that enables accumulation both through stimuli-responsive behavior triggered by the hypoxic tumor microenvironment and through the binding of ligands overexpressed on tumor cell membranes (14). The considerable control and flexibility of the LbL platform makes it ideal for preparing theranostic nanomedicines, because it can load both therapeutics and diagnostics with high capacity (19, 20) and can coat a broad range of nanomaterials down to 10 nm in size while maintaining uniformity, shape, and structure (18).

Despite current efforts of applying NIR-II probes for bioimaging, some of the essential properties relevant for the clinical translation of these probes are either missing or insufficiently characterized for in vivo biomedical applications. Particularly, SWNTs suffer from poor circulation in either lipid-coated or bacteriophage-bound form (11, 21), the pharmacokinetics of NIR-II emissive QDs and organic dyes are rarely reported (8, 10), and real-time whole-body imaging and pharmacokinetics for DCNPs are not reported (9). Furthermore, each material was studied with different delivery systems and instrumentation, contributing to the observed variations in performance across reports. Therefore, we leverage the LbL platform to generate NIR-II probes with a reproducible and biocompatible targeting stealth coating to facilitate the head-to-head comparison of these materials in vivo.

In this paper, we generate LbL NIR-II NPs with identical polymer multilayer modifications to provide a comprehensive side-by-side evaluation of the available NIR-II materials for in vivo real-time whole-body circulation, pharmacokinetics, biodistribution, toxicities, and applications in disease detection. Different LbL NIR-II NPs broadly exhibit prolonged blood circulation, a critical factor that allows NPs to accumulate in diseased sites. Comparison of these NPs affords clinically relevant information on currently available NIR-II probes and reveals their benefits and drawbacks, providing guidance for potential clinical translation. Overall, LbL-modified DCNPs exhibit excellent signal-to-noise ratio, low toxicity, and long circulation and are selected to demonstrate diagnostic capabilities within ovarian tumor, typically diagnosed at advanced stage. Both in vivo imaging and histology of the diseased tissues suggest preferential accumulation of LbL DCNPs in the tumors, indicating that LbL NIR-II NPs may act as an effective diagnostic platform. Moreover, the modular nature of the LbL platform allows us to further functionalize these materials, particularly through the incorporation of therapeutics to transform the formulations discussed in this work into theranostic NPs.

Results and Discussion
Preparation and Characterization of LbL NIR-II NPs. LbL NPs were constructed for NIR-II probes, including an organic dye (IR1061), SWNT, QD (PbS), and DCNP (NaY) (Fig. 1A and B). Each of the hydrophobic nanoscale NIR-II probes (dye, QD, and DCNP) was first encapsulated in the amphiphilic partially alkyl amide functionalized poly(acrylic acid) (PAA) to yield a net negatively charged core (~100 nm in diameter) for LbL assembly. In the case of the SWNT system, a negatively charged core was first created using sodium cholate stabilized SWNTs to undergo ligand exchange with the modified PAA. Biocompatible poly(−arginine) (PLA, 10 kDa) and dextran sulfate (DxS, 10 kDa) were the barrier layers. Hyaluronic acid (HA, 40 kDa) for the outermost layer is a natural polysaccharide that extends blood circulation, targets CD44 (a largely expressed receptor in many cancer cell lines), and provides tunable surface chemistry for further modifications. Successful LbL assembly was confirmed by dynamic light scattering (DLS) size measurements that indicated a 10-nm growth following the deposition of each barrier layer and a 40-nm growth following the deposition of terminal HA layer (Fig. 1C). Further validation of layer deposition was provided by electrophoretic measurements that indicated a complete reversal of surface charge following each layer deposition (Fig. 1C). The completed LbL NPs, with a layered structure consisting of NIR-II emissive core/PLA/DxS/PLA/HA, possessed zeta potentials of approximately −30 mV and hydrodynamic diameters within the optimal range (10−200 nm) for systemic delivery (22, 23), except the SWNT (280 nm) because its unique elongated shape was not recognized correctly by DLS measurement. Whereas the polydispersity index (PDI) of dye, QD, and DCNP systems falls between 0.1 and 0.2, indicating monodisperse LbL NPs, the higher PDI of 0.3 for LbL SWNTs was owing to the variation in length of the starting SWNTs (Fig. 1C).

To evaluate the LbL films consist of highly interpenetrated carbon-based polyion blends, and thus individual polyelectrolyte layers were indistinguishable in TEM. SWNTs of 3 nm in diameter were observed as singular nanotubes on a holey grid after LbL assembly, indicating that nanotubes presented primarily as individual rather than aggregate structures. It was suggested that the charged SWNTs remained sterically stabilized during LbL assembly, thus retaining efficient fluorescence. Before studying the LbL NIR-II NPs for in vivo imaging, optical absorption and emission spectra were measured (Fig. 1D and E and Fig. S1). NIR-II probes were excited with either an 808-nm or a 980-nm laser depending on the unique excitation properties of each probe, while maintaining a large spectral separation between excitation wavelengths and main emission peaks (SI Results and Discussion and Fig. S2). The excitation wavelengths and emission peaks (shown as 2λex/2λem in nanometers) of LbL NPs were 808/1,100 for dye complex, 808/1,225 for SWNT, 980/1,350 for QD, and 980/1,575 for DCNP system (Fig. 1E). Under these excitation and emission conditions, the dye complex (3,285 cm−1) and OD (2,797 cm−1) possessed smaller Stokes shift than the SWNT (4,213 cm−1) and DCNP (3,855 cm−1) systems.

Biodistribution and Pharmacokinetics of LbL NIR-II NPs. To evaluate and compare the different LbL-coated NIR-II probes for
biomedical applications, biodistribution and pharmacokinetics studies of each LbL NP type were performed in BALB/c female mice. Whole-body real-time fluorescence imaging was carried out using a custom-built imager, consisting of 808-nm and 980-nm lasers, a silicon camera for bright-field images, and an InGaAs camera taking NIR-II fluorescence images. During whole-body imaging, mice were placed under anesthesia and arranged in either the dorsal or lateral position and injected with LbL NPs via a catheterized tail vein. Immediately following the bolus injections of the NPs, fluorescence images were acquired continuously for 5 min; during this immediate time period, because the NPs were introduced rapidly throughout the bloodstream most organs were clearly recognized (Movie S1, with play speed 10× faster). To study the long-term distributions of the NPs, the whole-body bright-field and fluorescence images were collected at multiple time points ranging from 5 min to 72 h postinjection (Fig. 2). At the same time points, blood samples were drawn and analyzed to assess NP pharmacokinetics (Fig. 2D). Additionally, time-dependent biodistribution of LbL NPs was quantified from injection to 72 h postinjection based on the in vivo fluorescence images (Fig. 2E), and end-point ex vivo biodistribution was quantified based on the fluorescence images of the harvested organs at 72 h postinjection (Fig. 2F).

Several similar features of the biodistribution and pharmacokinetics were observed among the LbL NIR-II NPs. For the biodistribution study, the NPs localized to the heart within 10 s and began to accumulate in the lungs, liver, spleen, and circulatory system at ∼30 s (Movie S1 and Fig. 2E). The fluorescence intensities of various major organs remained relatively stable during the remaining part of the video and for up to 1 h. At later time points, fluorescent signals decayed in the major organs for most of the LbL NPs except dye, indicating the clearance of the

![Fig. 2.](image-url)
NPs prevailed over their accumulation. As expected, the liver and spleen were the main sources of fluorescent signals over the 72-h treatment period (Fig. 2F), consistent with the role of these organs in the reticuloendothelial system. Interestingly, NP accumulation was observed in osseous tissues from 30 s to 48 h postinjection, including the spine, femur, and tibia (Fig. 2A). Tracking of NPs to osseous tissues demonstrated the benefit of deep penetration gained with NIR-II imaging, because it was difficult to observe with visible and NIR-I imaging.

The pharmacokinetic analysis of the LbL NPs indicated that the probe concentrations in blood experienced a two-phase decay, including the processes of initial rapid distribution and following elimination from tissues. Notably, all LbL NPs, with the exception of the QD system, possessed extended half-lives as long as 24 h (Fig. 2D), likely owing to the role of the highly hydrophilic terminal HA layer preventing protein adsorption and opsonization, and the formation of a particularly dense layer in LbL systems achieved upon adsorption to the underlying PLA layer. We have previously examined these PLA/HA systems and found that these extended half-lives were characteristic of weak LbL systems with HA (13, 14, 17). The similarities observed for different NIR-II NPs were likely attributable to the identical LbL surface modifications and demonstrated the efficacy of the LbL platform for facilitating the systemic delivery of diverse material systems. It is noted that the delivery of the LbL coating on the surfaces of these different NIR-II probes is key to achieving the long-term blood circulation required for systemic applications, particularly when examining accumulations that can take place over a period of several days (Fig. S3). The QD system, however, had a half-life of 9 h, much shorter than that of the other systems and different from our observations of LbL QD systems examined in earlier work (15). We believe this difference may be related to the nature of the modified PAA coating on the QD surface, which may not have formed as cohesive an interface with the LbL layers as that achieved with direct layering of a QD synthesized with negatively charged ligands.

Despite the common characteristics among the LbL NIR-II NPs, unique features were observed for each probe. First, for LbL DCNPs at 72 h postinjection, fluorescence intensities were predominantly detected in liver and spleen both in vivo and ex vivo, and the relative intensities from other organs were much lower than those observed for the other probes due to the long emission wavelength of DCNP, further explained in the next section (Fig. 2F). Second, LbL SWNTs exhibited the quickest first-phase decay from blood circulation (0.1 h), followed by a slow second-phase decay (24.1 h) (Fig. 2D), in accordance with their rapid initial accumulation and sluggish clearance from the major organs (Fig. 2E). For instance, osseous organs including sternum, femur, and spine were identified as at late as 48 h postinjection (Fig. 2A), and the relative fluorescence intensities of SWNTs in excised organs were higher than those of DCNPs (Fig. 2F). This was attributed to the elongated shape of SWNTs, which may promote tissue penetration and therefore rapidly reduce the concentration of SWNTs in the circulatory system immediately after injection, as well as promote entrapment by the organs to slow excretion from tissues. Third, in contrast to SWNTs, LbL QDs exhibited the slowest first-phase decay (0.63 h) and fastest second-phase decay (9.77 h) (Fig. 2D), in agreement with the extended ascending and sudden descending fluorescence profiles of the major organs (Fig. 2E). In addition, QDs exhibited the lowest fluorescence intensities in the harvested organs (Fig. 2F). Based on previous reports (24, 25), it is thought that QDs (~6 nm), first encapsulated in amphiphilic PAA, can diffuse out of the LbL film and eventually be cleared via the renal system (26, 27). Finally, the LbL dye complex presented an unusual fluorescence profile of the organs, in which the fluorescence intensity peaked at ~24 h postinjection (Fig. 2E), suggesting that the dye complex maintained a high concentration in the blood (Fig. 2D) and continuously accumulated in the organs for a long time before tissue clearance dominated. It was concluded that, among these LbL NIR-II NPs, DCNPs exhibited the most favorable biodistribution and pharmacokinetics profile, because they offered prolonged first- and second-phase decays, as well as a regular pattern of tissue clearance.

The distinguishing optical properties of various NIR-II probes likely contributed to the observed discrepancies for in vivo and ex vivo fluorescence imaging. In general, probes with longer emission wavelengths can be imaged with higher quality owing to reduced light scattering and tissue autofluorescence. In particular, light scattering decreases monotonically as emission wavelength increases (9, 28, 29). According to their fluorescence emission spectra, we chose optical filter sets of two 1,400-nm long pass + two 1,575-nm band pass, two 1,300-nm long pass + two 1,375-nm band pass, two 1,300-nm long pass, and two 1,100-nm long pass + two 1,125-nm band pass for DCNPs, QDs, SWNTs, and dye complex, respectively, to maximize each probe’s signal-to-noise ratio (Table S1). As a result, LbL DCNPs offered NIR-II images with the least scattering and correspondingly defined the vascular and skeletal structures with the highest resolution out of all of the probes (Movie S1 and Fig. 2A).

The different scattering of fluorescent signals emitted by NIR-II probes was further investigated using breast-mimic phantoms of various thicknesses. It was observed that the degree of scattering decreased as the emission spectra moved toward longer wavelengths (Fig. 2B). The other major source of background noise is autofluorescence, which is known to decrease with larger spectral separation between excitation and emission wavelengths. As mentioned previously, DCNPs and SWNTs process Stokes shift close to or larger than 4,000 cm⁻¹, which is out of the range of the Raman shift of most organic molecules, providing the low level of autofluorescence observed with these probes (30, 31). Instead, QDs and dye complex possess Stokes shift around 3,000 cm⁻¹, strongly overlapping with the Raman shift of organic molecules, resulting in greater autofluorescence, especially in the regions of abdominal cavity and skin (Fig. 2A and C). In addition, the excitation wavelength of 808 nm (for dye or SWNTs) resulted in more autofluorescence than the excitation wavelength of 980 nm (for QDs or DCNPs), albeit with similar Raman shift (dye and QDs, or SWNTs and DCNPs), because Raman intensity is inversely proportional to the fourth power of the excitation wavelength (32). The high level of autofluorescence observed for NIR-II imaging with the dye complex likely contributed to the irregular fluorescent signal profiles and low quality of images (Fig. 2A and C and Movie S1), as well as the highest fluorescence intensities from excised organs (Fig. 2F). Overall, LbL DCNPs offered the optimal optical properties for NIR-II imaging, with the least interference from scattering and autofluorescence, and seemed to be a promising tool for biomedical imaging.

To the best of our knowledge, this is the first report to provide a comprehensive investigation as well as comparison of the biodistribution and pharmacokinetics of the currently available NIR-II fluorescent probes. Further, it is a first look, to our knowledge, at the potential for LbL coatings to address the enhancement of biodistribution for each of four very different NIR-II emissive material systems. All NIR-II probes possessed identical LbL modifications and allowed us to attribute the similarities and differences across these probes to their intrinsic characteristics, morphological or optical. In contrast, the observed variations of performance from different probes among previous reports could be partly owing to extrinsic properties, such as surface charge, surface chemistry, or targeting ligands.

To assess the capabilities of NIR-II imaging to obtain anatomical information, principle component analysis (PCA) was performed to group image pixels with similar time-dependent fluorescence intensities (33). PCA of the video frames of first 5 min postinjection generated composite images that distinguished
various organs with different assigned colors (Fig. 3). For all LbL NIR-II NPs, the main clearance organs (lungs, liver, and spleen) were the major ones identified. Although PCA of SWNTs, QDs, and dye systems showed organ resolution comparable to previous reports (8, 10, 33), video-rate whole-body imaging immediately following i.v. injection of LbL DCNPs and associated PCA is, to our knowledge, first reported in this study. Further, PCA of DCNPs system produced clearer anatomical features, as well as more identified organs, such as pancreas, skin vascular network, and spine, due to the advantages of longer emission wavelength such as deeper penetration, less light scattering, and reduced autofluorescence.

**Toxicities of LbL NIR-II NPs.** To evaluate the toxicities of the LbL NPs in vivo, the vital organs, including liver, spleen, heart, lungs, and kidneys, were excised and fixed with formalin at 72 h post-injection. Standard H&E staining of the major organs’ cross-sections was performed. As examples show in Fig. S4, for tissues from mice treated with all of the probes (i) cardiac fibers in the hearts maintained integrity; (ii) white pulps, red pulps, and trabecular arteries in the spleens were observed without major damage; and (iii) irregular macrophage accumulation in the lungs was not observed in the alveolar space, indicating that LbL NPs did not trigger a severe immune response. In addition, no major damage in the liver or kidneys was observed in mice treated with the dye, SWNTs, and DCNPs systems. However, severe toxicities in liver and kidneys were detected in QDs-treated mice. For instance, focal necrosis and dilated hepatocytes were identified in the liver (circled in Fig. S4C), and swollen tubule, a sign of kidney atrophy, was also noted (Fig. S4C). Hepato-renal toxicity is a well-known issue preventing the approval of QDs for biomedical applications, and the observed damage is likely attributed to the leakage of the QDs out of the LbL shell (34, 35). To our knowledge, this is the first report of the head-to-head comparison of toxicities of NIR-II probes in an identical LbL platform. In summary, we observed that the LbL-modified dye complex, SWNTs, and DCNPs were functionally nontoxic for biomedical applications. In contrast, these LbL QDs presented severe tissue toxicities; however, past work with LbL QDs suggests that this toxicity is related to leakage of individual QDs from the inner modified PAA encapsulation and LbL films (24, 25, 36).

**Detection of Orthotopic Ovarian Tumors Using LbL DCNPs.** Owing to their superior biodistribution, pharmacokinetics, and optical properties, LbL DCNPs were used to detect the presence of ovarian tumors in an orthotopic murine model. In this study, COV362 cells were selected based on their genetic similarity to high-grade serous ovarian cancer (HGSOC), the most aggressive ovarian carcinoma subtype (37). The orthotopic tumors were typically formed and disseminated in the cavity after 2 wk following the i.p. implantation of cancer cells into the nude mice. To detect the tumors, the mice received LbL DCNPs as a single i.p. dose. NIR-II fluorescence images of the whole body and excised organs were captured at 72 h postinjection, showing individual disseminated tumor nodules and tumor nodes on normal tissues (Fig. 4A and Fig. S5; the bright spots indicate the location of the tumors). Tumors and organs of interest were extracted, fixed with formalin, and stained with H&E. The formation of orthotopic ovarian tumors was confirmed by histopathological features such as irregular cellular shape and crowding, high nuclear-to-cytoplasmic ratio, and a distinct necrotic core (Fig. 4B) (7). Furthermore, up-converted visible light by DCNPs (38) allowed us to determine the colocalization of DCNPs and tumorous tissue on the cellular level using two-photon confocal microscopy. The DCNPs were found predominantly in the tumor nodes (Fig. 4B), which preferentially uptook LbL DCNPs relative to normal tissues such as the pancreas and intestine (Fig. 4C and D). The selectivity is likely due to the HA terminal layer, which binds to the CD44 receptor overexpressed by the COV362 cell line. Notably, in certain instances tumor cell crowding was observed inside the liver, indicative of tumor invasion, where relatively fewer DCNPs were detected in the tumor area (Fig. 4E). To our knowledge, this approach to determine colocalization of NPs and tumorous tissue on the cellular level using any other NIR-II probes has not been reported, because most intravitral confocal microscopes are not equipped with necessary NIR-II detectors. Instead, DCNPs offer up-converted visible emission for cellular-level detection with low autofluorescence and down-converted NIR-II emission for in vivo imaging with deep penetration. Herein, we provide the first

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**Fig. 3.** (A–D) PCA of the videos acquired from injection time to 5 min postinjection. For each type of NPs, the lateral (left) and dorsal (right) positions are shown. For each composite image, the red, green, and blue channels represent the combined positive and negative areas of the second, third, and fourth principle components, respectively, from PCA.

**Fig. 4.** Targeted detection of orthotopic ovarian tumors. (A) NIR-II images of the whole mouse (left) and the excised organs (from left to right and top to bottom are, spleen, ovaries, kidneys, pancreas, liver, stomach, and intestines). The bright spots indicate tumor nodules. (B–E) Slices of tumor (B), tumor nodules on pancreas (C), intestine (D), and the tumor invaded the liver (E). Each pair show an H&E-stained slice of tissue (Left) and the registered multiphoton confocal microscopy (Right) where signals from DCNPs (green), hematoxylin (blue), and eosin (red) were composited. (Scale bars: 100 μm.)
proof-of-concept study, to our knowledge, using LbL DCNPs for cancer detection in an HGSOC model and demonstrate the effectiveness and versatility of these modular systems for promising translational applications, from bioimaging to theranostics.

Conclusion. In summary, we constructed LbL-modified NIR-II NPs from currently available NIR-II fluorescent materials to perform a side-by-side investigation and comparison to the biodistribution, pharmacokinetics, and toxicities of these probes. Despite prior research efforts, many benefits and drawbacks among current NIR-II probes remained unexplored. For the first time to our knowledge, these NIR-II probes were directly compared to determine clinically relevant information using the same delivery platform and imaging instrumentation, eliminating previously observed discrepancies generated by such external factors. As a consequence, the findings and achievements in this study are of great interest for research endeavors in NIR-II imaging and provide guidance when applying NIR-II fluorescent probes for biomedical applications.

After weighing both the optical and the pharmacokinetic characteristics of these NIR-II probes, LbL-modified DCNPs provided superior imaging performance and were evaluated as a dual-modality system with an orthotropic model of HGSOC. The ovarian tumors, either distributed within the abdominal cavity or associated with vital organs such as liver, pancreas, intestine, and so on, were successfully detected in a noninvasive manner. This study concludes that LbL NIR-II NPs can serve as an imaging tool to monitor tumor dissemination, invasion, metastasis, and treatment response, as well as real-time imaging-guided surgery.

Materials and Methods
All materials are provided in Supporting Information. Experimental procedures were provided in Supporting Methods, including synthesis of DCNPs and SWNTs, fabrication and characterization of LbL NIR-II NPs, mouse handling and injection, whole-body imaging, pharmacokinetics, toxicity, cell culture, and tumor induction procedures, calculation of scattering width and signal-to-fluorescence ratio, PCA, and multiphoton confocal microscopy.

All in vivo experiments were performed under the supervision of the Division of Comparative Medicine, Massachusetts Institute of Technology, and in compliance with the principles of laboratory animal care of the National Institutes of Health.

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