Published in final edited form as:

Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2009 ; 1(2): 156–167. doi:10.1002/wnan.7.

***In vivo* fluorescence imaging: a personal perspective**

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Abstract

In vivo fluorescence imaging with near-infrared (NIR) light holds enormous potential for a wide variety of molecular diagnostic and therapeutic applications. Because of its quantitative sensitivity, inherent biological safety, and relative ease of use (i.e., with respect to cost, time, mobility, and its familiarity to a diverse population of investigators), fluorescence-based imaging techniques are being increasingly utilized in small-animal research. Moreover, there is substantial interest in the translation of novel optical techniques into the clinic, where they will prospectively aid in noninvasive and quantitative screening, disease diagnosis, and post-treatment monitoring of patients. Effective deep-tissue fluorescence imaging requires the application of exogenous NIR-emissive contrast agents. Currently, available probes fall into two major categories: organic and inorganic NIR fluorophores (NIRFs). In the studies reviewed herein, we utilized polymersomes (50 nm to 50 μ m diameter polymer vesicles) for the incorporation and delivery of large numbers of highly emissive oligo (porphyrin)-based, organic NIRFs.

Organic fluorophores are either free emissive molecules [e.g., indocyanine green (ICG)] or conjugates of single fluorescent dyes to biological targeting moieties such as antibodies, antibody fragments, proteins, peptides, nucleic acids, polysaccharides, or small molecules. Owing to the small numbers of their biologically expressed targets, as well as their short circulation half-lives, low photo-bleaching thresholds, and lack of strong near-infrared (NIR) absorption and emission, organic fluorophores are inherently limited in their ability to generate fluorescent signals at significant tissue depths (of greater than 1–2 cm). Semiconductor nanoparticles, also called quantum dots, classify as an important family of inorganic fluorophores. Quantum dots possess vastly superior optical characteristics when compared to traditional organic fluorophores. Moreover, these nanoparticles can be decorated with polymers and biological conjugates that increase their blood circulation times and augment the affinity of these probes for specific *in vivo* molecular targets. As a result, quantum dots offer more sensitive NIR fluorescence detection with respect to their molecular organic fluorophore benchmarks, but at the cost of decreased safety, as their composite materials are toxic in their elemental forms. As a result, there is no currently available NIR-emissive agent that possesses the ideal properties for human application.

For several years, we have focused our efforts on the development of the first generation of organic-based fluorescent nanoparticles capable of deep-tissue optical imaging. Such agents have been designed to possess good water solubility, large NIR absorption extinction

coefficients, high NIR fluorescence quantum yields, high photobleaching thresholds, no phototoxicity, as well as safe and complete *in vivo* biodegradation. Highly sensitive and specific deep-tissue fluorescence-based imaging has required the co-development of improved organic NIR fluorophores (NIRFs) and novel nanoparticle delivery vehicles capable of multi-avidity targeting. In the studies reviewed herein, we utilized polymersomes (50 nm–50µm diameter polymer vesicles) for the incorporation and delivery of large numbers of highly emissive, oligo(porphyrin)-based, supermolecular NIRFs. The methodology necessary to engineer the physical, optical, and biomaterial properties of these NIR-emissive polymersomal assemblies was systematically developed in order to optimize their *in vivo* efficacy.

Over the past several decades, fluorescent agents have been extensively utilized for *in vitro* biological research. Similar to their use in cellular microscopy and flow cytometry applications, exogenous fluorescent agents can be employed, after successful conjugation to bioactive molecules (such as antibodies), to image molecular targets in live animals. Further, novel activatable organic probes have been generated that produce highly sensitive signals only upon specific interactions with their molecular targets. In order to image structures deeper than several millimeters, exogenous NIR-emissive agents must be utilized. Currently, there are two classes of NIR-emissive contrast agents: organic and inorganic NIRFs. Figure 1 depicts a schematic comparison of unconjugated, targeted, and activatable exogenous fluorescent agents for *in vivo* optical imaging. General principles underlying their use in fluorescence-based imaging applications are further elaborated upon below.

CONVENTIONAL AGENTS FOR *IN VIVO* FLUORESCENCE IMAGING

Throughout the visible spectrum, intracellular structures such as mitochondria contribute considerably to optical scatter. With longer wavelength (λ), light scattering decreases appreciably (as $1/\lambda^4$) and photon absorption by endogenous oxygenated and deoxy-generated hemoglobin lessens, approaching a nadir over the NIR spectrum (650–1000 nm)¹ (see Figure 2). Fluorescence-based imaging in this NIR spectral window is entirely biologically safe and enjoys an outstanding signal-to-noise ratio (SNR) owing to minimum interference from tissue auto-fluorescence. As a result, NIR optical imaging can prospectively be used to resolve molecular events at tissue depths of up to 12 cm.² Realization of the full potential for fluorescence-based *in vivo* imaging has been critically dependent upon the design of exogenous contrast agents that both absorb and emit NIR light.^{1,3–7}

Unconjugated Organic Near-Infrared Fluorophores

The earliest NIR *in vivo* imaging applications utilized the unconjugated fluorophore ICG for the noninvasive detection of tumors in both animals and patients.⁶ ICG is a diagnostic agent that was first approved by the FDA in the 1970s in order to test hepatic function.⁸ Since then, it has also been used in investigations of cardiac physiology and in ophthalmologic procedures for fluorescence angiography.^{9,10} ICG is a small molecule (MW = 775 g/mol) that emits NIR light at 800 nm upon optical excitation at 780 nm.⁹ It exhibits strong albumin binding and shows preferential uptake and retention in tumors as a result of increased vascular permeability through surrounding leaky blood vessels.^{11,12} ICG possesses a short circulation half-life, as it is highly susceptible to the body's first-pass metabolism, which allows its rapid clearance from the blood stream. As a result, good tumor accumulation (~2:1 signal-to-background ratio) can be obtained within approximately 30 min of intravenous injection.¹³

When bound to albumin, ICG's spectra are altered¹⁴ and its quantum yield significantly decreases (from approximately 15% in DI water to 5% in blood).¹⁵ In order to improve these optical characteristics, and to augment ICG's tumor uptake and contrast capabilities, cyanine

derivatives that possess better water solubility, decreased protein binding, and more favorable pharmacokinetics have been synthesized.^{15,16} In general, ICG and its derivatives offer basic physiological information such as tissue perfusion and accumulation effects with no additional information about dynamic molecular interactions or cellular signaling pathways. Hydrophilic organic fluorophores, however, can be further conjugated to biological moieties to bind to specific targets and enable *in vivo* molecular imaging.

Targeted Organic Near-Infrared Fluorophores

A number of water-soluble cyanine and rhodamine/fluorescein dyes have been conjugated to targeting moieties such as antibodies, antibody fragments, proteins, peptides, and small molecules to facilitate specific molecular binding and detection.^{7,17–19} In general, the use of antibody fragments, peptides, or other small molecules is preferable to that of whole antibodies, as these agents typically possess better contrast kinetics dependent upon target binding and clearance of unbound fractions.⁶ Further, antibodies are more difficult to produce in large quantities and are often immunogenic. Organic NIR dyes have successfully been bound to many biological ligands, including octreotate (a somatostatin analog),²⁰ bombesin,²¹ vasoactive intestinal peptide,²² transferrin,^{23,24} Annexin V,^{25,26} and low-density lipoprotein (LDL),²⁷ among other biological ligands. Another innovative strategy has involved the conjugation of fluorophores to naturally occurring small molecules and metabolites (e.g., a bisphosphonate-derivative-conjugated cyanine dye bound to hydroxyapatite affords visualization of osteoblastic activity in mice²⁸). In general, bioconjugates of organic fluorophores possess high target specificity; but, their *in vivo* imaging is often limited by a relatively low signal-to-background ratio, since nonbound probes are also fluorescent and contribute to the background noise.^{6,29,30}

Activatable Organic Near-Infrared Fluorophores

‘Activatable’ contrast agents take advantage of the fact that a molecule’s fluorescence is intimately related to its physical and chemical environment. When two fluorochromes are in close proximity (e.g., at molecular separations shorter than typical Foster energy transfer distances of 5–6 nm), fluorescence is significantly quenched.³¹ Two different types of activatable organic fluorophores have been designed in order to study dynamic *in vivo* molecular events. Both of these agents (i.e., molecular beacons and enzyme-activatable probes) provide outstanding imaging signal-to-background ratios upon binding to their molecular targets. Molecular beacons are single-stranded DNA fragments that have a fluorophore attached to one end and a quencher to the other.^{32,33} The unhybridized beacon forms a hairpin structure so that the quencher and fluorophore are in close proximity, resulting in quiescent emission. When the probe binds to a complementary strand of endogenous DNA or RNA within the cell, it unwinds and the fluorescence signal is generated. The fluorescence signal is typically weak because of the small number of nucleic acid target molecules. Further, presently there is no effective method for *in vivo* intracellular delivery of the beacons so as to enable systemic molecular imaging in live animals.

The second class of activatable probes consists of multiple organic fluorophores bound closely to one another on a poly(L-lysine)/poly(ethylene glycol) backbone (MW = 1000 kDa).^{34–36} The emissive agents are either conjugated directly or via a peptide spacer. Within the body, protease enzymes cleave either the construct backbone (e.g., lysosomal cystine/serine proteases such as cathepsin-B that recognize lys–lys amide bonds) or the peptide spacer (e.g., matrix-metallo-proteinase 2, cathepsin D, or thrombin) resulting in a strong (up to several hundredfold) increase in fluorescence signal-to-background (i.e., ‘dequenching’).^{34–40} These enzyme-sensing probes are useful for assessing activity in a number of diverse diseases, including mouse models of dysplastic intestinal adenomas, rheumatoid arthritis, and atherosclerosis.³⁹

Inorganic Near-Infrared Fluorophores (Quantum Dots)

The energy gap law states that as the energy difference between a fluorophore's ground and excited state decreases, nonradiative decay pathways increasingly dominate its excited state relaxation.³¹ As a result, there is a relative paucity of organic-based NIRFs. Those that do exist (e.g., ICG and its water-soluble cyanine derivatives¹⁵) often lack the ideal properties for generating strong fluorescent signals through deep tissue sections. The design of high emission dipole strength fluorophores is necessary for both large-animal and clinical imaging applications. The ideal NIR agent should possess:

1. large NIR absorption extinction coefficients
2. high NIR fluorescence quantum yields
3. high photobleaching thresholds
4. no photo-based cellular toxicity
5. good water solubility
6. safe and complete *in vivo* clearance

One alternative strategy to circumvent the imaging limitations associated with conventional organic NIRFs is to use inorganic semiconductor nanoparticles (i.e., quantum dots).^{41–45} Quantum dots are nanocrystals with a core/shell structure composed of transition metals (normally from groups II–V or II–VI of the periodic table—e.g., Cd, Se, Te, S, and/or Zn) surrounded by a water-soluble organic coating. Controlling the precise size of the inorganic core/shell sets the dot's band gap and enables tuning of its narrow fluorescence emission band (FWHM ~25–35 nm) with considerable accuracy. In addition to absorption extinction coefficients and fluorescence quantum yields (~15–30%) that are high with respect to most organic NIRFs, emissive quantum dots also possess high photobleaching thresholds.⁴⁶ Further, the commercial development of these agents offers exciting opportunities for bioconjugation and detection of molecular targets in both *in vitro* and *in vivo* small-animal investigations.^{45,47–49} The major limitations of quantum dots include their tendency to aggregate in aqueous solution (thereby losing their fluorescence—albeit this can be largely mitigated by the selection of an appropriate organic coating), their construction from toxic elemental materials, and the relatively large sizes of their NIR-emissive compositions (hydrodynamic radii = ~20 nm—too large to be cleared via renal filtration resulting in high fluorescence background noise and increased potential for *in vivo* toxicity).⁵⁰

NEAR-INFRARED EMISSIVE POLYMERSOMES

Several years ago, we set out to develop the first generation of organic-based fluorescent nanoparticles capable of deep-tissue optical imaging. For our emissive components, we chose a series of oligo(porphyrin)-based NIRFs that possess large NIR absorption extinction coefficients,^{51–54} high NIR fluorescence quantum yields,^{55,56} high photobleaching thresholds,^{56,57} no chemical or photo-based *in vitro* toxicity,⁵⁸ and whose design was based on the structures of natural biodegradable pigments (i.e., porphyrins). Highly sensitive and specific deep-tissue fluorescence imaging further required the development of nanoparticle delivery vehicles that were capable of multi-avidity molecular targeting and that were fully biodegradable and safe with complete *in vivo* clearance. In the studies described herein, polymersomes (50 nm – 50 μm diameter polymer vesicles)^{59–63} were used for the incorporation and delivery of large numbers of highly emissive, oligo(porphyrin)-based NIRFs. When compared to natural nanoparticles composed of phospholipids (e.g., LDL⁵⁸ or liposomes), polymersomes are uniquely capable of incorporating and uniformly distributing numerous large, hydrophobic NIRFs exclusively in their lamellar membranes.⁶⁴ Within these sequestered compartments, long polymer chains regulate the mean fluorophore–

fluorophore interspatial separation as well as the fluorophore-localized electronic environment.

Porphyrin-based NIRFs manifest photophysical properties within the polymersomal matrix akin to those established for high emission dipole strength fluorophores in organic solvents, thereby yielding uniquely emissive vesicles. Further, the total fluorescence emanating from the assemblies gives rise to a localized optical signal of sufficient intensity to penetrate through significant tissue depths.⁶⁴ Polymersomes thus define a *soft matter* platform with exceptional potential to facilitate *in vivo* fluorescence imaging for clinical diagnostic and drug-delivery applications. Methodology necessary to engineer the physical, optical, and biomaterial properties of these polymersomal assemblies has been systematically developed in order to optimize their *in vivo* activity.

Physical Properties of Near-Infrared-Emissive Polymersomes

NIR-emissive polymersomes are generated through cooperative self-assembly of amphiphilic diblock copolymers and conjugated multi(porphyrin)-based NIRFs⁶⁴ (see Figure 3). When confined within organic nanoparticles, NIRF emission is highly dependent on the precise regulation of the average fluorophore–fluorophore spatial separation as well as the mean fluorophore electronic environment. Such control is necessary in order to minimize pathways for singlet excited-state deactivation and the subsequent loss of fluorescence quantum yield, which is anathema to most NIRF-containing materials. Our initial studies focused on defining the relationship between the thickness of polymersome membrane and its capacity to stably incorporate hydrophobic oligo-porphyrin (PZn)-based fluorophores (PBFs) of various sizes.⁶⁵ The effects of vesicle loading on the absorptive and emissive properties of individual membrane-embedded PBFs, as well as whole polymersomal ensembles, were examined. In addition, changes in the mechanical stabilities of the overall assemblies dependent upon the degree of PBF-membrane incorporation were also determined.⁶⁵ The studies showed that polymersomes can accommodate a variety of hydrophobic, oligo(porphyrin)-based NIRFs at >10 mol/wt% loadings without significantly compromising the robust mechanical stabilities of these synthetic organic nanoparticles.⁶⁵ Although the fluorescence signal intensity per emitter decreases with larger fluorophore loadings, the total integrated emission on per nanoparticle basis was maximized at 5 mol% NIRF/polymer (within the poly(ethylene oxide)-block-poly(1,2 butadiene)-based polymersome compositions).^{64,65}

Optical Properties of Near-Infrared-Emissive Polymersomes

The bulk optical properties of emissive polymersomes were determined through steady-state spectroscopic experiments. A large body of literature focused upon the utilization of porphyrin monomers as photosensitizers has previously determined that noncovalent confinement in nano-assemblies greatly influences the sensitizer's photophysical properties. Specifically, the type and number of substituents, the central metal, axial ligands, charge, degree of aggregation, and packing effects predetermine the type of interactions with the surrounding microenvironment and are manifested by changes in absorption, fluorescence, and the kinetics of excited-state deactivation. As such, we examined the essential structural requirements that enable control over transition dipole moment magnitude and direction, the anisotropy of the singlet excited state, the absorption profile, emission wavelength, as well as the extent of excited-state interchromophore electronic coupling in emissive polymersomal assemblies.⁶⁶

Ultra-fast excited state dynamics of nanoscale NIR-emissive polymersomes incorporating a series of related ethynyl-bridged oligo(porphyrinato)zinc(II) (PZn)-based supermolecular fluorophores were further studied.⁶⁷ Within polymersome membrane environments, long

polymer chains uniformly disperse and effectively constrain fluorophore conformeric populations, giving rise to excitation dynamics within the nanoparticles that bear many features in common with those that are manifest by these species in dilute organic solvent.^{64,66} NIR-emissive polymersomes formed from various molar ratios of NIRF-to-polymer, varying membrane loading per nanoparticle between 0.1–10 mol/wt%, further enabled exploration of the concentration-dependent mechanisms for nonradiative decay and excitation energy transfer.^{65,67}

These studies successfully helped to clarify essential fluorophore and vesicle structural requirements that enable control over relative fluorophore transition dipole moment alignment and the extent to which radiative processes dominate NIR-emissive polymersome excited-state deactivation. Specifically, we determined that the NIRFs' emission wavelengths,⁶⁶ as well as their concentration-dependent fluorescence quantum yields,^{65,67} are intimately related to specific intramembranous polymer–fluorophore interactions within polymersome membranes. The nature and extent of these interactions are precisely controlled by varying the position and chemical composition of the NIRF's ancillary substituent groups, and by engineering the relative membrane thickness and dielectric environment of the polymersomal carrier.^{66,67} To date, a large number of optically unique NIR-emissive polymersomes have been developed; together, they effectively define a family of fluorescent organic nanoparticles that exhibits precise emission energy modulation over a broad domain of the visible and NIR spectrum (600–850 nm) (see Figure 3).

Biomaterial Properties of Near-Infrared-Emissive Polymersomes

The future clinical utility of polymer vesicles in drug delivery and/or NIR imaging applications is incumbent upon the generation of polymersome formulations that are not only biocompatible but also fully biodegradable. As such, we focused on developing the first self-assembled polymersomes composed entirely of biodegradable diblock copolymers whose composite materials had previously been approved for human use by the United States Food and Drug Administration (FDA). Fully bioresorbable diblock copolymers of poly(ethylene oxide)-block-poly(ϵ -caprolactone) (PEO-b-PCL)⁶⁸ and the related diblock copolymer poly(ethylene oxide)-block-poly(γ -methyl ϵ -caprolactone) (PEO-b-PMCL)⁶⁹ were synthesized and extensively characterized (see Figure 3b). As the generation of bilayered vesicles via self-assembly is dependent upon prescribed molecular geometric constraints on the composite amphiphile, the aqueous phase behaviors of the resultant PEO-b-PCL and PEO-b-PMCL copolymers were determined.

Steady-state absorption and relative concentration-dependent emission of various porphyrin-based NIRFs were studied when loaded within these two biodegradable polymersomes systems (PEO-b-PCL- and PEO-b-PMCL-based vesicles).⁷⁰ Appropriate selection of fluorophore ancillary aryl group substituents and choice of polymer chain chemistries were found to dictate fluorophore solvation, mean electronic environment, and concentration-dependent fluorescence⁷⁰ (Figure 3c and d). The selection of precise ancillary aryl group substituents drives fluorophore solvation into membranous regions with matching dielectric properties.^{66,67,70} The more hydrophobic NIRFs exhibit greater fluorescence within the relatively apolar poly(γ -methyl ϵ -caprolactone)-based membranes, while those with increased amphiphilicity are better dissolved within more polar poly(ϵ -caprolactone)-based bilayers.⁷⁰ Detailed examinations of these biodegradable emissive assemblies are necessary in order to assess their structural stabilities, blood circulation times, degradation kinetics, and clearance of *in vivo* by-products.

CONCLUSION

Current State of the Art in Near-Infrared-Emissive Polymersomes

For any future *in vivo* application, it is crucial to determine how the optical signal strength varies as a function of depth of tissue penetration and NIRF concentration. Such information can be ascertained by utilizing tissue phantoms that reconstitute biological scattering/absorption media *ex vivo*. Appropriate comparisons have been made between NIR-emissive polymersomes and the only FDA-approved exogenous NIRF, ICG. The premise behind utilizing polymersomes as optical contrast agents is that they can uniformly disperse thousands of highly emissive NIRFs within a single nanoparticle; long polymer chains effectively segregate the fluorophores into isolated domains that help to minimize intermolecular energy transfer within the polymersomes' membranes. As a result, a single polymersome incorporating numerous highly fluorescent NIRFs can generate an enormous emissive output signal per nanoparticle. At equivalent solution concentrations to albumin-bound ICG, NIR-emissive polymersomes greatly increase the sensitivity of the fluorescence signal that can be detected through deep tissue sections.

Liquid tissue phantoms are an established method to reconstitute, *ex vivo*, the absorption and scattering properties of human breast^{71–78} (Figure 4). Tris(porphyrinato)zinc(II) (PZn₃)-based NIRFs (incorporated within aqueous suspensions of polymersomes) and equivalent emitter numbers of ICG (dissolved in the same total aqueous volumes) were utilized in order to quantify the tissue depths at which optical signals could be accurately detected for a given number of fluorophores. Figure 5 compares the depth of tissue penetration as a function of total emitter numbers for these two different NIRFs. When compared on an emitter-to-emitter basis with albumin-bound ICG, polymersome-incorporated PZn₃ exhibits similar fluorescence intensities at analogous emitter numbers and generates optical signals that can be effectively detected at equivalent tissue depths (Figure 5c).

It is important to note that although ICG and PZn₃ fluorophores (loaded at a 1 : 100 molar ratio of NIRF-to-polymer within PEO₈₀-b-PBD₁₂₅ (OB18)-based polymersomes) exhibit comparable optical properties (on a per molecule basis), a single 150-nm-diameter polymersome incorporates 1280 copies of emissive PZn₃-based NIRFs within its membrane. As a result, compared to an ICG-bound albumin molecule, a 150-nm diameter NIR-emissive polymersome possesses an absorption extinction coefficient that is 3 orders of magnitude greater and is able to generate an emissive signal that is correspondingly more intense. PZn₃ loaded at a 1 : 100 molar ratio of NIRF-to-polymer in OB18-based polymersomes is utilized as a standard, as this NIRF exhibited the largest per molecule fluorescence at this loading condition when compared to the various other polymersome formulations examined to date.⁷⁰ Equivalent depth-dependent optical signal strengths can be obtained from other NIR-emissive polymersome systems varying in emitter numbers and polymer compositions; several notable formulations (e.g., ones useful for bioactive coupling as well as those capable of full biodegradation) are compared in Table 1.

Ongoing Studies with Near-Infrared-Emissive Polymersomes

Extensive *in vivo* imaging experiments on small animals (e.g., tumor-bearing mice) are necessary in order to determine precise pharmacokinetics and quantitative biodistribution data for nontargeted NIR-emissive polymersomes. Nanometer-sized polymersomes are small enough to traverse compromised endothelial cell barriers and passively accumulate in tumor tissues by the enhanced permeability and retention (EPR) effect (associated with leaky tumor microvasculature) (Figure 6). An 'active targeting' mode can be further engineered by chemical functionalization of vesicle surfaces with biological moieties (e.g., antibodies, peptides, DNA/RNA, small molecules). These agents have enabled the

generation of polymersomes that are capable of specific binding to molecular targets.^{79,80} *In vivo* delivery experiments with targeted NIR-emissive polymersomes are further necessary in order to determine their efficacy as highly sensitive and specific contrast agents for deep-tissue molecular imaging. Together with the methodology developed to date, these investigations will help to establish NIR-emissive polymersomes as the first-generation of organic fluorescent contrast agents capable of *in vivo* clinical diagnosis.

Acknowledgments

The authors are grateful to Mr Samuel Bernard, Mr Ali Dhanaliwalia, and Ms Dalia Levine for assistance with the tissue phantom experiments as well as Drs Ramachandran Murali, Geng (Georgia) Zhang, and Hongtao Zhang for helping to conduct the animal work. Daniel A. Hammer and Michael J. Therien acknowledge funding support from the National Institutes of Health (MJT - R01CA115229). Peter Ghoroghchian is grateful for fellowship support from the Whitaker Foundation and the NIH Physician Scientist Training Program.

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Organic NIR Fluorophores

Inorganic NIR Fluorophores (Quantum Dots)

FIGURE 1. Schematic of organic versus quantum dot-based contrast agents used for *in vivo* fluorescence imaging.

FIGURE 2.

Absorption spectra of oxyhemoglobin (HbO₂) and deoxyhemoglobin (Hb) in whole blood. Spectra are displayed in units of absorption extinction coefficient ($M^{-1} cm^{-1}$) versus wavelength (nm) and are plotted on a log scale. The NIR window corresponds to a spectral region from 650 to 1000 nm where there is a nadir in the physiological absorption of light by endogenous chromophores. Utilizing exogenous NIR fluorescent contrast agents enables effective deep-tissue *in vivo* imaging.

FIGURE 3.

Schematic depiction of the incorporation of various oligo(porphyrin)-based NIRFs within polymersomes. (a) The NIRFs vary with respect to the number of porphyrin subunits (N), the linkage topology between porphyrin monomers, and the nature and position of ancillary aryl-group substituents (R). (b) Various diblock copolymer compositions have been utilized to form NIR-emissive polymersomes. (c) Membranous interactions between polymers and specific ancillary aryl group substituents vary the conformational populations assumed by the NIRF and can be used to tune its emission wavelengths. (d) Engineering the chemical composition and thickness of the polymersome membrane (d) helps to drive individual

NIRFs into dielectric environments of matching polarity. (e) a family of nanoscale NIR-emissive polymersomes.

Human Breast Phantom
 $(\mu_a = 0.04 \text{ cm}^{-1}, \mu_s' = 10.0 \text{ cm}^{-1} \text{ at } \lambda = 785 \text{ nm})$

PZn₃ in OB18 Polymersomes
 150 nm; $\lambda_{\text{em}} \text{ max} = 818 \text{ nm}$

Albumin-bound ICG
 3x3x8 nm; $\lambda_{\text{em}} \text{ max} = 810 \text{ nm}$

FIGURE 4.

Schematic of the liquid tissue phantom arrangement. Imaging experiments on human breast phantoms were done with a GE eXplore Optix imaging instrument.

Tris(porphyrinato)zinc(II) (PZn₃)-based NIRFs (incorporated within aqueous suspensions of OB-18 polymersomes composed of a 1 :100 molar ratio of NIRF-to-polymer) and equivalent numbers of ICG (dissolved in the same total volume of dilute bovine albumin solution) were utilized in order to quantify the tissue depths at which optical signals could be accurately detected for a given number of fluorophores. Samples were excited and emission was detected in the reflectance geometry; the liquid absorption/scattering media consisted of India ink in an intralipid solution ($\mu_a = 0.04 \text{ cm}^{-1}$, $\mu_s' = 10.0 \text{ cm}^{-1}$ at $\lambda = 785 \text{ nm}$).

FIGURE 5.

Determination of fluorophore optical signal strength versus depth of tissue penetration. (a) Attenuation of optical signal sensitivity (signal-to-noise ratio; SNR) versus depth of immersion in the liquid phantom for various amounts of albumin-bound ICG emitters. (b) Attenuation of SNR versus depth of immersion in the liquid phantom for various amounts of PZn₃-based NIRFs (incorporated within aqueous suspensions of OB-18 polymersomes composed of a 1 : 100 molar ratio of NIRF-to-polymer). (c) Comparison of the maximum tissue depth of fluorescence signal penetration versus number of albumin-bound ICG and polymersome-incorporated PZn₃ emitters in solution. NIRF solutions were embedded within liquid tissue phantoms and compared on an emitter-to-emitter basis. Experimental

conditions: emissive signals emanating from the liquid tissue phantoms were detected by NIR fluorescence imaging utilizing a GE eXplore Optix instrument (λ_{ex} = 785 nm, λ_{em} = 830–900 nm).

FIGURE 6.

In vivo fluorescence imaging experiments with nontargeted NIR-emissive polymersomes. (a) Image of a tumor-bearing mouse (in the supine position) taken 6 h after tail-vein injection of nontargeted, PZn₃-based, NIR-emissive polymersomes. The 150-nm diameter polymersomes do not accumulate in the lungs. As such, they neither aggregate *in vivo*, as is often witnessed with quantum dots, nor do they cause any microvascular injury during their circulation, *vide infra*. (b) Images of a tumor-bearing mouse (in the prone position) taken 6 h after tail-vein injection of nontargeted, PZn₃-based, NIR-emissive polymersomes. The 150-nm diameter polymersomes are able to preferentially accumulate and remain (for greater than 24 h) in tumor tissues as a result of the enhanced permeability and retention (EPR)

effect. Experimental conditions: 1.5 nmol equivalent of the 3',5'-alk-PZn₃ NIRF loaded in 150-nm diameter nondegradable polymersomes (composed of a 1 : 1 molar mixture of PEO₃₀-b-PBD₄₆ and PEO₈₀-b-PBD₁₂₅; 1 : 40 NIRF-to-total-polymer molar ratio) were imaged after tail-vein injection of tumor-bearing mice utilizing a GE eXplore Optix instrument ($\lambda_{\text{ex}} = 785 \text{ nm}$, $\lambda_{\text{em}} = 830\text{--}900 \text{ nm}$).

Comparison of the Optical Properties of Albumin-bound ICG and Various PZn₃-based Polymersome Formulations

TABLE 1

	Albumin-bound ICG	3',5'-alk-PZn ₃ in PEO ₈₀ -b-PBD ₁₂₅	3',5'-alk-PZn ₃ in PEO ₃₀ -b-PBD ₄₆	3',5'-alk-PZn ₃ in PEO ₄₃ -b-PMCL ₆₆	3',5'-peg-PZn ₃ in PEO ₄₅ -b-PCL ₁₀₅
Molar ratio NIRF-to-polymer		1 : 100	1 : 20 : 20	1 : 40	1 : 40
Relative emission per molecule ^a	1	1	0.46	0.13	0.12
Absorption $\epsilon(\text{M}^{-1} \text{cm}^{-1})$ per molecule	1.2×10^5	1.3×10^5	1.3×10^5	3.5×10^4	6.9×10^4
No. of NIRFs per nanoparticle ^b	1	1280	3260	3160	3040
Nanoparticle characteristics	FDA-approved, nontargeted	Biocompatible, nontargeted	Biocompatible, targeted	Biodegradable	Biodegradable
Absorption $\epsilon(\text{M}^{-1} \text{cm}^{-1})$ per nanoparticle ^b	1.2×10^5	1.7×10^8	4.2×10^8	1.1×10^8	2.1×10^8
Relative emission per nanoparticle ^{a,b}	1	1280	1500	410	360

^a Experimental conditions: emissive signals emanating from liquid tissue phantoms as detected by NIR reflectance imaging utilizing a GE eXplore Optix instrument ($\lambda_{\text{ex}} = 785 \text{ nm}$, $\lambda_{\text{em}} = 830\text{--}900 \text{ nm}$).

^b Calculations based on 150-nm-diameter polymersome; 1 nm^2 interfacial surface area per polymer chain comprising the vesicle membrane; and, polymersome membrane core thicknesses of 14.8 nm (PEO₈₀-b-PBD₁₂₅), 12.1 nm (1 : 1 PEO₃₀-b-PBD₄₆; PEO₈₀-b-PBD₁₂₅), 16.6 nm (PEO₄₃-b-PMCL₆₆), and 22.5 nm (PEO₄₅-b-PCL₁₀₅), respectively.

3',5'-alk-PZn₃; 5,15-Bis[(5'-10,20-bis[3',5'-di(3,3-dimethyl-1-butyloxy)phenyl]porphinato)zinc(II)ethynyl]-10,20-bis[2',6'-di(3,3-dimethyl-1-butyloxy)phenyl]porphinato]zinc(II).

3',5'-peg-PZn₃; 5,15-Bis[(5',10',20'-bis[3,5-di(3,3-dimethyl-1-butyloxy)phenyl]porphinato)zinc(II)ethynyl]-10,20-bis[3,5-di(9-methoxy-1,4,7-trioxanonyl)phenyl]porphinato]zinc(II).