

## Biodegradable pH-Sensing Dendritic Nanoprobes for Near-Infrared Fluorescence Lifetime and Intensity Imaging

Adah Almutairi,<sup>†</sup> Steven J. Guillaudeau,<sup>†</sup> Mikhail Y. Berezin,<sup>‡</sup> Samuel Achilefu,<sup>‡</sup> and Jean M. J. Fréchet<sup>\*,†</sup>

College of Chemistry, University of California, Berkeley, California 94720-1460, and Department of Radiology, School of Medicine, Washington University, St. Louis, Missouri 63110

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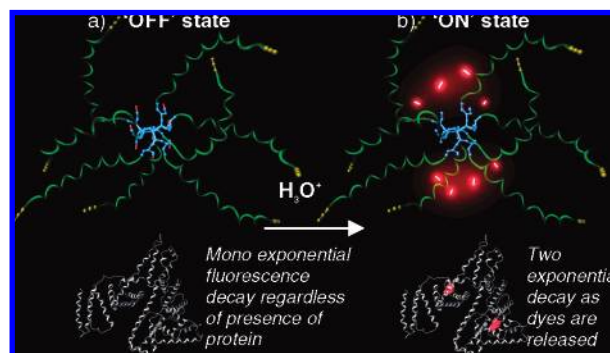
The overproduction of acidic byproducts is implicated in the development of numerous diseases, and there is currently great interest in developing contrast agents that can image acidic tissue *in vivo*.<sup>1</sup> Here we report a pH-sensing biodegradable near-infrared (NIR) nanoprobes capable of providing complementary information through both fluorescence lifetime measurements and signal amplification in acidic environments *in vivo* (Figure 1).

In the “off” state, the NIR fluorescence of this sensing nanoprobes is deactivated (Figure 2) by exploiting the polyvalent periphery of a compact biodegradable aliphatic polyester dendrimer (in blue Figure 1 and Scheme 1).<sup>2</sup> NIR dyes (in red) are attached via acid-sensitive linkages to the periphery of the dendrimer. In such close proximity, the dyes form H-type homoaggregates via face-to-face stacking (Figure 2a). This agglomeration suppresses both their fluorescence lifetime and intensity through nonradiative decay<sup>3</sup> and thereby minimizes background noise.<sup>4</sup> In acidic environments, this latent NIR nanoprobes is activated (Figure 2b) by the release of multiple copies of NIR fluorophores. As the fluorophores drift apart, their fluorescence lifetime begins to reflect the new environment which they are in, with simultaneous amplification of the fluorescence intensity signal (Figure 1). The dendrimer is PEGylated (in green Figure 1 and Scheme 1) for improved biocompatibility and to impart biological stealth.<sup>5</sup>

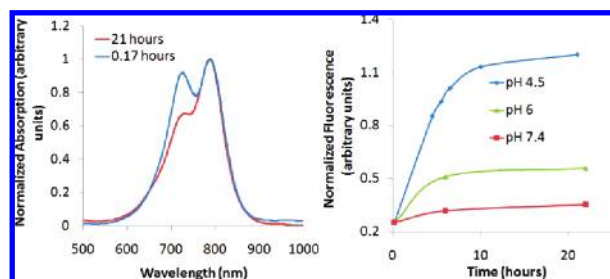
A number of small-molecule NIR dyes that operate in the tissue transparency window (750–900 nm)<sup>6</sup> respond to changes in proton concentrations at physiologically relevant pH, with a change in fluorescence intensity.<sup>7,8</sup> However, upon intravenous injection, these small-molecule NIR imaging probes immediately bind to serum proteins.<sup>9</sup> This opsonization process may lead to poor bioavailability and more importantly dominates the excited-state fluorescent properties of the fluorophores.<sup>10</sup> As a result, limited functional information on the *in vivo* environment can be extracted through lifetime imaging.<sup>10</sup> NIR probes that can be shielded from immediate opsonization and activated in desired target tissue are a promising avenue to functional fluorescence lifetime and intensity imaging.

Nanoscale probes based on polymer–dendrimer hybrids offer an ideal and modular platform for biomedical applications.<sup>11</sup> The level of branching and hydrodynamic volume of the polymeric carrier can be tuned<sup>12</sup> to suit the bioavailability needs of the application.<sup>5</sup> Furthermore, deactivation/activation of fluorescence intensity can be achieved by using a variety of environmentally sensitive linkages.<sup>8,13</sup>

In heterogeneous media, such as tissue and cells, quantitative fluorescence intensity measurements require the modeling of photon transport in the media which depends on fluorophore concentration, path length, photobleaching, and scattering.<sup>14</sup> In contrast, the excited states of NIR fluorophores are less dependent on these parameters



**Figure 1.** (a) “OFF” state: in neutral pH, found in healthy tissue, the NIR fluorescence intensity of the nanoprobes is silenced and its fluorescence lifetime properties are insensitive to the presence of *in vivo* proteins. (b) “ON” state: in acidic pH found in diseased tissue, the NIR fluorescence intensity of the nanoprobes increases as the dyes (red) are released and their fluorescence lifetime is sensitive to the presence of *in vivo* proteins.



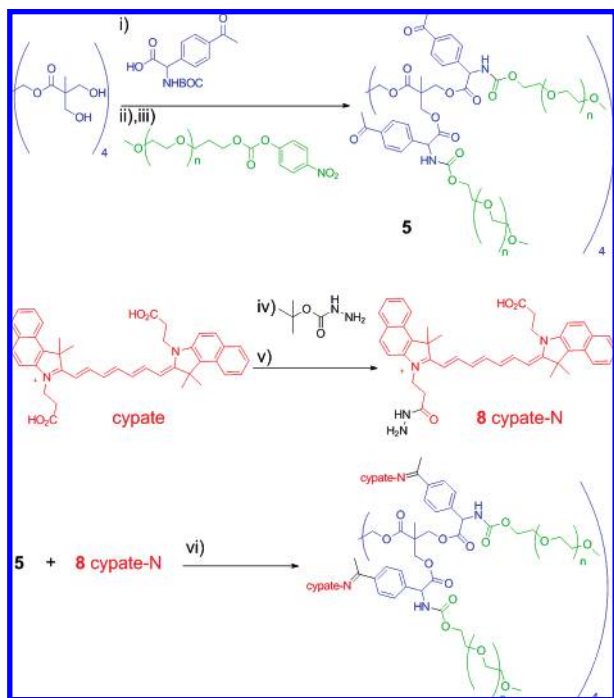
**Figure 2.** Steady-state absorption and emission spectra as a function of time and pH. (a) Absorption in pH 4.5 buffer (10% MeOH). After incubating for 21 h, the H-dimer peak is diminished, indicating hydrolysis of the acid-sensitive linkages. (b) Emission intensity as a function of time in pH 4.5 buffer (10% MeOH) mimicking lysosomal compartments, pH 6 buffer (10% MeOH) mimicking diseased tissue, and pH 7.4 buffer (10% MeOH) mimicking blood and healthy tissue.

and respond to their local environment with a sensitivity comparable to radioactive probes without the inherent danger of radioactivity.<sup>14</sup> We show here that shielding the dyes within the PEGylated carrier can control the influence of serum proteins on the fluorescence lifetime properties of the fluorophores.

Pentaerythritol was chosen as the dendrimer core for its compactness that is thought to promote aggregation. It can be divergently dendronized in one step to afford eight branching points.<sup>15</sup> Aliphatic polyester dendrimers based on the monomer 2,2-bis(hydroxymethyl) propanoic acid are especially promising for imaging applications because of their low toxicity, low immunogenicity, and biodegradable structure.<sup>2,16</sup> We introduced heterobifunctionality by coupling Boc-4-acetylphenylalanine (Scheme 1) to the pentaerythritol polyester dendrimer.<sup>17</sup> The amino group was deprotected, and eight PEG chains (5000 Da) were attached through

<sup>†</sup> University of California, Berkeley.

<sup>‡</sup> Washington University, St. Louis.

**Scheme 1.** Preparation of Acid-Activated NIR Nanoparticles<sup>a</sup>

<sup>a</sup> Conditions: (i) EDC, DMAP, DMF, 4 h, 100%; (ii) TFA, CH<sub>2</sub>Cl<sub>2</sub>, 30 min, 100%; (iii) DMAP, DIPEA, benzene, 16 h, MW = 40000, PDI = 1.06; (iv) EDC, HOBT, DMF, 0 °C, 2 h, 78%; (v) TFA, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C, 30 min, 100%; (vi) MeOH, 1:1 Pyr/AcOH, 16 h.

a carbamate bond, affording 40 000 Da polymer 5. Dynamic light scattering measurements gave a hydrodynamic volume of 8–10 nm. Nanostructures of this chemical composition and size have excellent bioavailability due to their biological stealth, as well as a diameter too large to be rapidly cleared through the renal pores.<sup>5</sup> The ketone group was subsequently reacted with NIR dyes that were modified as hydrazides (8) to form the acid-sensitive hydrazone linkage to the dendritic scaffold.

The steady-state absorption profile of the nanoprobe in pH 4.5 (Figure 2a) revealed the presence of a H-type aggregate peak that overlaps with the 712 nm shoulder from a secondary transition. After 21 h, the aggregate peak at 712 nm diminished, indicating hydrolysis of the hydrazone bonds. Fluorescence intensity measurements of our nanoprobe incubated in pH 7.4 for 24 h showed minimal NIR fluorescence signal and no significant change, while incubation in pH 4.5 overnight gave a 6-fold increase in the NIR fluorescence intensity signal (Figure 2b).

Fluorescence decay of free cypate (Scheme 1) in homogeneous solvent is mostly monoexponential, suggesting the presence of only one form of the molecule, with a fluorescence lifetime corresponding to the orientation polarizability of the solvent. In the presence of BSA, cypate reveals two fluorescence lifetimes, reflecting binding of the dye to two different pockets of this ubiquitous serum protein. In a proof of concept experiment, we measured the fluorescence lifetimes of solutions of NIR nanoprobe incubated for 4 h at pH 7 and 4 in the presence of albumin. Both pH 7 solutions, with and without albumin, had a lifetime of 0.36 ns, with 93% fractional contribution. Nanoprobes left to hydrolyze at pH 4 in albumin had two lifetimes: 0.4 and 0.98 ns with fractional contributions of 74 and 24%, respectively. These values are in close agreement with

those reported for the parent carboxylate dye interacting with albumin.<sup>18</sup> Thus, only after the NIR dyes were released from the dendrimer scaffold did they begin to interact with their environment.

In conclusion, we have developed, in six high yielding and scalable steps, a biocompatible nanoparticle capable of activating fluorescence lifetime and intensity of NIR fluorophores in acidic conditions. Both fluorescence intensity and lifetime provided complementary physiological (low pH) and molecular (released fluorophore binding to albumin) information, respectively. Near-infrared optical reporters that can noninvasively detect and analyze acidic diseased tissue as well as subcellular milieus offer a sensitive, inexpensive, safe, and decisive tool to interrogate biological processes in vivo.<sup>19</sup> We envision this system may provide information in an in vivo setting via functional fluorescence lifetime and intensity imaging. The pH-sensitive linkages are used extensively in drug delivery systems, and this noninvasive approach may help shed light on the kinetics of such drug delivery strategies in vivo in a cost-effective and more accurate manner.

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**Supporting Information Available:** Synthetic protocols and experimental procedures, including lifetime data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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