Published in final edited form as:

Macromol Biosci. 2011 July 7; 11(7): 927–937. doi:10.1002/mabi.201100043.

Selective Imaging and Killing of Cancer Cells with Protein-Activated Near-Infrared Fluorescing Nanoparticles

Parul Rungta¹, Yuriy P. Bandera¹, Ryan D. Roeder¹, Yangchun Li², William S. Baldwin², Deepti Sharma³, Michael G. Sehorn³, Igor Luzinov¹, and Stephen H. Foulger¹

Stephen H. Foulger: foulger@clemson.edu

¹Center for Optical Materials Science and Engineering, Technologies, School of Materials Science and Engineering, Clemson University, Clemson, SC 29634-0971, USA

²Department of Biological Sciences, Environmental Toxicology Program, Clemson University, Clemson, SC 29634-0971, USA

³Department of Genetics and Biochemistry, Clemson University, Clemson, SC 29634-0971, USA

Abstract

We present a general approach for the selective imaging and killing of cancer cells using protein-activated near-infrared emitting and cytotoxic oxygen generating nanoparticles. Poly(propargyl acrylate) (PA) particles were surface modified through the copper-catalyzed azide/alkyne cycloaddition of azide-terminated indocyanine green (azICG), a near-infrared emitter, and poly(ethylene glycol) (azPEG) chains of various molecular weights. The placement of azICG onto the surface of the particles allowed for the chromophores to complex with bovine serum albumin when dispersed in PBS that resulted in an enhancement of the dye emission. In addition, the inclusion of azPEG with the chromophores onto the particle surface resulted in a synergistic ninefold enhancement of the fluorescence intensity, with azPEGs of increasing molecular weight amplifying the response. Human liver carcinoma cells (HepG2) overexpress albumin proteins and could be employed to activate the fluorescence of the nanoparticles. Preliminary PDT studies with HepG2 cells combined with the modified particles indicated that a minor exposure of 780 nm radiation resulted in a statistically significant reduction in cell growth.

Keywords

fluorescence; imaging; nanoparticles; photophysics; protein; sensors

Introduction

Sub-100 nm sized colloidal particles that are functionalized with multiple moieties have the potential to combine imaging, early detection, prevention, and the treatment of cancer with a single type of colloidal “nanodevice.”^[1–5] Specifically, the design of a nanodevice that exhibits a cancer cell activated near-infrared fluorescence, which is coupled with a photodynamic response, is of particular interest. Photodynamic therapy (PDT) is a relatively new medical technology for the treatment of cancer that requires a photosensitizer and oxygen to be in the proximity of the afflicted tissue.^[6–7] The photo-excitation of the photosensitizer results in the promotion from the ground singlet state (S_0) to an excited singlet state (S_1) with some excited singlets undergoing inter-system crossing to a longer-lived excited triplet state (T_1). Oxygen resident in tissue has a ground triplet state and energy transferred from the photosensitizer to the oxygen can result in the formation of the highly cytotoxic singlet oxygen (1O_2), resulting in destruction of the afflicted tissue. Tissue damage is localized due to the short lifetime of singlet oxygen in biological systems ($<0.04 \mu s$) and its corresponding short radius of action ($<0.02 \mu m$).^[8]

A number of photosensitizers have been developed for commercial use in PDT, though there is still a need for systems that exhibit absorption of light in the infrared spectrum (NIR), which provides a maximal penetration of light into tissue. A NIR fluoroprobe that is the only NIR organic dye approved by the US Food and Drug Administration (FDA) for human use is indocyanine green (ICG).^[9] ICG has been widely investigated for use in deep-tissue imaging and is an amphiphilic carbocyanine dye that exhibits absorption at ca. 780 nm and an emission maxima at ca. 820 nm. ICG exhibits very low toxicity effects to humans^[10,11] and has resulted in its clinical use as a contrast agent in angiography,^[12] facilitating directed biopsies,^[13] and for assessing blood flow and hepatic function.^[14–16] The yield of triplet formation of free ICG through S_1-T_1 intersystem-crossing is 14% in water and 11% in an aqueous albumin solution.^[17] This triplet yield, coupled with the ubiquitous nature of ICG in medical diagnostic technologies, may allow ICG to operate as a photosensitizer in PDT.^[18] Nonetheless, ICG has many of the shortcomings associated with organic dye molecules. One of the major challenges in its *in vivo* application is its low fluorescence quantum yield^[19] and non-specific quenching.^[20–23] In addition, free ICG will bind to proteins^[24] which leads to aggregation and subsequent elimination from the body. One approach to remedy this shortcoming is the inclusion of the chromophores inside colloidal particles^[25–27] and results in an extension of circulation half-life and enhanced *in vivo* stability relative to the free fluoroprobe molecules.^[26] Specifically with regard to ICG, a number of researchers have employed a doped particulate approach to address the intrinsic issues of ICG degradation and rapid blood clearance.^[27–31] Although all of these approaches have utilized an encapsulation approach that affords a static environment to the ICG and reduces environmental degradation, encapsulation prevents both (i) the chromophore from being spatially proximal to resident oxygen in afflicted tissue and (ii) the dye from participating in advantageous host/guest assemblies. Photoluminescent dyes can be utilized as biologically based sensors or switches due to the noticeable variations in their emission spectra when they form supramolecular host/guest assemblies or complex with biomacromolecules such as proteins.^[32,33] The environmental sensitivity in the emission properties of the chromophores can also be exploited to assist in refining the proposed parameters that govern molecular recognition.^[34–36] For example, the fluorescence quantum yield of ICG is limited to a few percent by internal conversion under optimal environmental conditions, while in water, the hydrophobic dye exhibits dimerization at low concentrations which even further reduces the fluorescence quantum yield. Nonetheless, the strong affinity of ICG to serum albumin in water shifts the onset of dimerization to higher concentrations,^[12,20] effectively increasing the quantum yield. Recently, it has been reported

that molecules possessing higher affinity for serum albumin are found to exhibit efficient PDT applications.

In the current effort, ICG and poly(ethylene glycol) (PEG) of various molecular weights were modified with attachment of a terminal azide and then attached to poly(propargyl acrylate) (PA) colloids through a copper-catalyzed azide/alkyne cycloaddition (CuAAC) performed in water. The placement of ICG onto the surface of the particles allows for the chromophore to complex with proteins that resulted in the alteration and enhancement of the emission of the dye. In addition, the inclusion of PEG with ICG onto the particle surface resulted in a synergistic enhancement of the fluorescence intensity, with PEGs of increasing molecular weight amplifying the response. The surface attachment of ICG and its availability to be spatially adjacent to molecular oxygen when the particles are dispersed in tissue, coupled with protein enhanced fluorescence, may make these particles a valuable resource in PDT.^[37]

Results and Discussion

A schematic of the three particle-based system studied in this effort is presented in Figure 1. The PA colloids were prepared using a standard aqueous emulsion polymerization technique with sodium dodecyl sulfate as the surfactant, potassium persulfate as the initiator, and divinyl benzene as a crosslinker, resulting in spheres of diameter of 73 ± 7 nm (mean and standard deviation). To functionalize the surface of the particles, a multiple step “click” reaction was performed to produce PA particles that had both ICG and PEG on their surface. An azide-modified indocyanine green (azICG) was attached to the PA particles through a CuAAC (“click” transformation) performed in water. To attach the chromophores to the particles, the azICG was initially clicked onto the particles for 10 min and then the reaction was stopped by the removal of unreacted azICG, sodium ascorbate, and Cu(II)SO₄ through a repeated particle washing procedure consisting of centrifugation and redispersion in methanol. The cleaned PA/azICG particles were subsequently utilized in a secondary click transformation with azide-modified PEG chains with molecular weights of 1000 (azPEG_{1K}) or 5000 (azPEG_{5K}) that was allowed to run for 24 h and then washed to remove unreacted species; these particles are referred to as PA/azICG/azPEG_{1K} and PA/azICG/azPEG_{5K}, respectively.

Emission of azICG

The emission characteristics of the azide-functionalized ICG are similar to ICG (not presented) when dispersed in methanol. Figure 2a presents the molar extinction coefficient and photoluminescence of the free azide-functionalized indocyanine green (azICG) dispersed in methanol. In this solvent, azICG has a peak absorption maximum at 785 nm, while the corresponding emission peak is at 830 nm, for a relatively small Stokes shift of 45 nm. The symmetry for the absorption and emission spectra is evident, though the absorption exhibits a small peak at 915 nm. This lower energy absorption is often seen in concentrated aqueous solutions of ICG after the formation of J-aggregates;^[20,38–41] the appearance of this peak in the freshly prepared dilute methanol solution of azICG is surprising and may suggest a higher self-affinity with the substitution of the sodium salt for the azide. The relative fluorescence quantum yield in methanol for azICG was $\phi = 0.044 \pm 0.004$, while the unfunctionalized ICG had a quantum yield $\phi = 0.036 \pm 0.002$, which suggests there is little effect in the emission characteristics of ICG with the addition of the azide moiety.

In comparison, Figure 2b presents the absorption and photoluminescence spectra of PA particles after they have been modified with the attachment of azICG to their surface (PA/azICG particles) and dispersed in methanol. The absorption spectra of the PA/azICG particles indicate an absorption maximum that is at a wavelength of ca. 800 nm, a 15 nm

bathochromic shift from the free dye. It is well known that ICG exhibits a molar extinction coefficient that is both concentration and solvent dependent.^[38] Previous studies on free ICG have indicated that there is a strong affinity of the dye to methanol, which results in a reduction of the probability of dimer formation, and only at high dye concentrations does the inter-dye separation become small and closely spaced pairs and larger aggregates are formed.^[20] Establishing the molar extinction coefficient in the dilute regime for the free azICG in methanol (cf. Figure 2) can allow for the estimation of the number of chromophores attached to the particles. Following this approach, the PA/azICG particles had a grafting density of $1.84 \pm 0.50 \text{ ICG} \cdot \text{nm}^{-2}$ when analyzed over the wavelength range of 700–800 nm. This grafting density would result in each azICG being statistically ca. 8 Å away from its nearest azICG neighbor. Similarly, elemental (combustion) analysis of the PA/azICG particles resulted in a grafting density of $1.5 \text{ ICG} \cdot \text{nm}^{-2}$ with an inter-azICG spacing of ca. 9 Å. Even though the azICG is attached to the particle surface through a short aliphatic spacer and triazole ring, the large dimensions of the planar azICG, which are approximately 25 Å by 12 Å, would suggest that the molecules are packed relatively densely on the surface of the particles.

Figure 2b presents the photoluminescence of the PA/azICG particles and indicates that the emission peak is at a wavelength of 820 nm, a 10 nm hypsochromic shift from the free azICG, with a resulting Stokes shift for the surface attached dyes of 20 nm. The relative fluorescence quantum yield for the PA/azICG particles was $\phi = 0.017$. As indicated earlier, the free azICG exhibited a quantum yield of $\phi = 0.044$ and the observed quantum yield for the surface-attached azICG moieties indicate that attaching the chromophores to the particles does reduce the quantum efficiency when the modified particles are dispersed in methanol. Particles may act as quenching centers of fluorescence for chromophores that are adsorbed onto their surface since (i) the planar surface offers a constrained two-dimensional region on which the chromophores can dimerize^[42] and (ii) a non-radiative energy transfer can occur from the excited molecules to the particle,^[20] though recent studies have indicated that dye-doped particles result in an optical system that underestimates the quantum efficiency of the dye using established procedures.^[43] Nonetheless, dispersing the PEGylated PA/azICG particles (both with azPEG_{1K} or azPEG_{5K}) in methanol resulted in the modified particles exhibiting a similar photoluminescence spectrum (not presented) relative to the PA/azICG particles. In addition, the quantum efficiency of the PEGylated PA/azICG particles was similar to the neat PA/azICG particles in methanol (cf. Table 1), suggesting that the attachment of the PEG chains did not influence the emission characteristics of the attached ICG.

Emission Enhancement with BSA Concentration

The utilization of an azICG-modified particle for any in vivo or in vitro imaging application will require the particles to be dispersed in an aqueous environment. The replacement of methanol for a PBS solution in the PA/azICG particles resulted in a total quenching of fluorescence. Due to the hydrophobic nature of the dye, the employment of PBS is speculated to have forced the bound azICG to sequester to the particle surface and dimerize.^[20] Previous studies of free ICG in water indicated that physically bound ground state dimer formation occurred at low dye concentrations resulting in low fluorescence quantum yield of ca. 4×10^{-5} at an ICG concentration of ca. $2.7 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$; this concentration of free dye would result in a theoretical inter-ICG distance of 80–90 Å.^[20] In the current system, the attachment of the azICG to the surface of the particles results in the “local” concentration of chromophores to be significantly higher with an inter-azICG distance of 8–9 Å, promoting the dimerization in a poor solvent (cf. Figure 1). The hydrophobic nature of the neat PA and PA/azICG particles was confirmed by contact angle measurements of films composed of the particles. The neat PA particles are relatively

hydrophobic by the appearance of a contact angle of 73° , though with the grafting of azICG to their surface, the hydrophobicity of the particles is enhanced as indicated by the increase in contact angle to 87° . The surface of the particles was PEGylated in an attempt to enhance the hydrophilicity of the particles and offer a way to separate the attached azICG. Though the attachment of the azPEG_{1K} and azPEG_{5K} chains to the particles did significantly reduce the contact angle of the particles to water to ca. 24° (cf. Table 1), there was no discernible photoluminescence of the particles in water or PBS. Figure 3a presents the fluorescence of the PA/azICG/azPEG_{5K} particles when dispersed in PBS, indicating an almost total quenching of fluorescence.

It has been established that the fluorescence quantum yield of ICG changes when the dye is adsorbed to macromolecules such as proteins and enzymes,^[20, 44–46] and this response can be employed as a fluorescent diagnostic probe in medical and biological applications. Previous studies have established that ICG adsorption occurs on the ca. 10 nm sized human albumin protein and that dye adsorption will dominate over dye dimerization when the dye number density is less than a few times the protein number density.^[20] In the current system, the PA/azICG/azPEG_{5K} particles in PBS were mixed with bovine serum albumin (BSA) at various concentrations. With the addition of BSA to the solution, the observed fluorescence intensity of the modified particles increased dramatically. Serum albumin is a major protein constituent of blood plasma and this protein facilitates the disposition and transport of a variety of exogenous and endogenous ligands to specific regions. The delivery of ligands originates from two structurally selective binding sites where the binding affinity originates from a combination of hydrophobic, hydrogen bonding, and electrostatic interactions. The long term increase in emission output is presented in Figure 3a for the addition of 0.025 and 0.25 mM BSA to the particles; these concentrations represent a ratio of 0.39 and 3.93 BSA molecules to every azICG in the system, respectively. The emission intensity in Figure 3a exhibited an immediate increase within the first 30 min that accounted for ca. 25% of the total increase in intensity followed by a long term gradual increase. Figure 3b presents the intensity ratio (I/I_0) for the modified particles for various BSA concentrations after the systems have equilibrated for 4 days. The PA/azICG particles exhibit the least improvement in fluorescence emission with the addition of BSA, though with almost all BSA concentrations resulting in at least a 50% increase. The PA/azICG/azPEG_{1K} particles exhibit a long term increase in intensity ratio of ca. 5 for the highest BSA concentration, although all concentrations of BSA resulted in significant emission enhancements, while the PA/azICG/azPEG_{5K} particles, with the longer surface-attached PEG chains, offer the greatest improvement in emission intensity with the 4 day emission ratio being 8.5 for the majority of all BSA concentrations above $0.05 \times 10^{-3}\text{M}$. At a BSA concentration of ca. $0.07 \times 10^{-3}\text{M}$, the number of BSA molecules is approximately equal to the total number of azICG in the system. The observed increase in fluorescence intensity with BSA binding likely results from the ability of the protein to “dissolve” hydrophobically aggregated azICG on the surface of the particles. The superior enhancement in emission intensity with longer PEG chains is speculated to be due to an entrapment process. As presented in the schematic of Figure 1, it is assumed that the BSA proteins are continually absorbing and de-absorbing onto the particles at equilibrium, but as the PEG chains become longer, their extended length acts to retard the desorption through entanglement effects, thus forcing the proteins to spend more time near the surface of the particle and enhancing the time in which a protein is complexed with a surface tethered azICG.

This was confirmed by measuring the concentration of BSA entangled with the particles through the Bradford protein assay. For the particles employed in Figure 3 with $0.1 \times 10^{-3}\text{M}$ BSA, the assay indicated that ≈ 233 BSA molecules were associated with every PA/azICG particle, while the PA/azICG/azPEG_{1K} particles had 380 BSA molecules and the PA/azICG/azPEG_{5K} particles had 411 BSA molecules. On average, for every particle, there is a single

BSA molecule for 130 azICG chromophores on the PA/azICG particles. This ratio is reduced for the PA/azICG/azPEG_{1K} particles to 80 azICG/BSA, while for the PA/azICG/azPEG_{5K} particles this ratio is 74.

Emission Enhancement with Time

Figure 4a presents the time evolution of the emission intensity ratio (I/I_0 measured at 819 nm) for the first 1 200 min of PA/azICG/azPEG_{1K} particles when dispersed in PBS and mixed with 0.014×10^{-3} M BSA. In this system, the particles were mixed with BSA at a particle/protein 1:7 000 ratio (azICG/protein 1:0.22) and the photoluminescence spectrum (cf. Figure 4a inset) of the particles was measured every 30 min. There is an immediate increase in the emission intensity followed by a slower growth; the increase can be roughly modeled by an exponential rise to a maximum curve, which results in a time constant of ca. 15 min. For potential PDT applications, the PA/azICG/azPEG_{1K} particles achieve 63% of their total 1 day emission increase within the first 15 min. Figure 4b presents an optical micrograph of the PA/azICG/azPEG_{1K} particles in water, PBS, and PBS after a 2 h exposure to 0.014×10^{-3} M BSA. These images demonstrate that (i) the quenched fluorescence of the modified particles in PBS can be “turned on” by the addition of BSA and (ii) even though the PA/azICG/azPEG_{1K} particles do not exhibit as pronounced an emission increase relative to the PA/azICG/azPEG_{5K} particles (cf. Figure 3b), the increase in azICG emission is still clearly discernible. This latter feature facilitates the potential use of these particles in a combined in vivo luminescent imaging and PDT study of tumors.

In vitro Studies with Cancer Cells

To assess the potential application of the PA/azICG/azPEG particles as photosensitizers for PDT and to determine whether the particles pose a toxic concern, cytotoxicity tests were carried out in HepG2 cancer cells; cell viability was determined via the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium, inner salt (MTS) assay. Figure 5 presents the relative growth of the cells with both neat PA (blank) and PA/azICG/azPEG_{1K} particles. At the lower particle concentrations, the blank nanoparticles (*P*) exhibited a statistically significant reduction in growth for the HepG2 cells, while there was statistically significant growth for the blank particles at the highest concentrations. The surface modified particles (SMP) reduced the growth of the HepG2 cells for all PA/azICG/azPEG_{1K} particle concentrations, though the reduction was not significant. The all *trans*-conformer of the azPEG_{1K} chain measures ca. 10 nm and its attachment to the particles should increase their diameter from approximately 70 to 90 nm. Due to the hydrophilic nature of PEG, the DLS determined diameter of the modified particles in water was >100 nm, larger than the predicted value. The large hydrodynamic size of the PA/azICG/azPEG_{1K} particles relative to the neat PA particles may result in a less efficient cellular uptake at these concentrations.

In addition, preliminary cell viability was studied with NIR light exposure following published procedures^[47] to assess potential PDT performance. HepG2 cells secrete a variety of major plasma proteins; e.g., albumin,^[48, 49] which could enable the fluorescence of the PA/azICG/azPEG particles. The fluorescence is indicative of the electronic promotion from the ground singlet state to an excited singlet state with some excited singlets undergoing inter-system crossing to a longer-lived excited triplet state. Oxygen which is adjacent to the cells and modified particles may accept energy from the excited azICG chromophores and form the cytotoxic singlet oxygen, resulting in destruction of the cell. The preliminary PDT study performed with the PA/azICG/azPEG_{1K} particles is presented in Figure 6 and indicates a decrease in HepG2 cell growth after a 24 h incubation time post 15 min exposure to 780 nm light at a $0.04 \text{ mW} \cdot \text{cm}^{-2}$ flux. Even for this relatively minor exposure, the cells exhibited a reduced growth with both particle densities. There appears to be an enhanced sensitivity to the higher concentration of particles, with the highest dose of particles

resulting in a statistically significant reduction in growth ($p < 0.01$); this would correlate to the higher number of azICG chromophores. To insure that exposure to the light alone did not result in the observed reduced growth, the inset in Figure 6 presents the relative growth of the neat cells with and without light exposure, indicating no sensitivity to the radiation. Although this PDT study is promising, more thorough studies with the modified particles are currently being pursued to verify their PDT performance.

Conclusion

A general strategy for the preparation of particles with surface attached ICG, a near-infrared emitter, and PEG chains is described. PEGs of various molecular weights and ICG were modified with the addition of a terminal azide (azPEG and azICG) and then attached to PA colloids through a CuAAC performed in water. The placement of azICG onto the surface of the particles allowed for the chromophores to complex with proteins that resulted in the enhancement of the dye emission. In addition, the inclusion of azPEG with azICG onto the particle surface resulted in a synergistic enhancement of the fluorescence intensity, with azPEGs of increasing molecular weight amplifying the response. Preliminary PDT studies with HepG2 cells combined with particles surface-decorated with both azICG and azPEG indicated that a minor exposure to 780 nm radiation resulted in a statistically significant reduction in cell growth. These results suggest that the surface attachment of azide-modified ICG to particles and its availability to spatially adjacent molecular oxygen, coupled with protein enhanced fluorescence, may make these particles a valuable resource in the treatment of cancer.

Reagents and Solvents

All the commercial reagents were purchased from Sigma–Aldrich and used without further purification. All the solvents were dried according to standard methods. Deionized water was obtained from a Thermo Scientific Barnstead NANOpure Water Purification System and exhibited a resistivity of ca. $10^{18} \cdot \Omega^{-1} \cdot \text{cm}^{-1}$.

Chemical Characterization Methods

^1H and ^{13}C NMR spectra were recorded on a JEOL ECX300 spectrometer (300 MHz for proton and 76 MHz for carbon). Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent (CDCl_3 : δ 7.26 ppm, $\text{DMSO}-d_6$: δ 2.50 ppm). Chemical shifts for carbon is reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonances of the solvent (CDCl_3 : δ 77.16). Electrospray ionization (ESI) mass spectra were obtained using the Finnigan LCQ classic spectrometer + HP 1 100 (HPLC). The IR spectra were recorded at room temperature in the wavenumber range of 400–4 000 cm^{-1} and referenced against air with a Nicolet 6700 FTIR instrument. A total of 32 scans were averaged for each sample at 2 cm^{-1} resolution.

Preparation of Azide-Modified Indocyanine Green (azICG)

3-(3-Azidopropyl)-1,1,2-Trimethyl-1H-Benzo[e]Indolium Iodide (1)—The solution of 2,3,3-trimethyl-4,5-benzo-3H-indole (1 g, 4.78 mmol) and 1-azido-3-iodopropane (2 g, 9.56 mmol) in acetonitrile (50 mL) was refluxed for 72 h. The solvent was evaporated under vacuum and the residue was dissolved in dichloromethane (10 mL). This solution was added dropwise to diethyl ether (80 mL) to precipitate a product (Figure 7). This purification was done three times and the solid obtained was filtered and dried under vacuum (hygroscopic) to give a dark-brown product. Yield: 1.61 g (80%). ^1H NMR (CDCl_3) 1.87 (s, 6H), 2.37 (m, 2H, $^3J_{\text{HH}} = 5.9$ Hz, $^3J_{\text{HH}} = 6.9$ Hz), 3.25 (s, 3H), 3.76 (t, 2H, $^3J_{\text{HH}} = 5.9$ Hz), 5.00 (t,

2H, $^3J_{\text{HH}} = 6.9$ Hz), 7.70 (m, 3H, $^3J_{\text{HH}} = 8.6$ Hz, $^4J_{\text{HH}} = 1.4$ Hz, $^4J_{\text{HH}} = 1.7$ Hz), 7.96 (d, 1H, $^3J_{\text{HH}} = 8.9$ Hz), 8.10 (m, 2H, $^3J_{\text{HH}} = 8.6$ Hz, $^3J_{\text{HH}} = 8.9$ Hz). ^{13}C NMR (CDCl_3) δ 16.9, 22.5, 27.4, 47.6, 48.8, 55.8, 112.7, 122.8, 127.5, 127.6, 128.5, 129.9, 131.4, 133.5, 136.8, 138.1, 196.0.

4-(1,1,2-Trimethyl-1H-Benzo[e]Indolium-3-yl)Butane-1-Sulfonate (2)—The solution of 2,3,3-trimethyl-4,5-benzo-3H-indole (0.6 g, 2.87 mmol) in 1,4-butane sultone (1.17 g, 8.59 mmol) was heated at 120 °C for 2 h. After cooling, the crystallized product was washed with acetone, filtered, and dried to give the compound (2) as a white solid. Yield: 0.92 g (93%). ^1H NMR ($(\text{CD}_3)_2\text{SO}$) 1.75 (s, 6H), 1.78 (m, 2H, $^3J_{\text{HH}} = 7.2$ Hz), 2.03 (m, 2H, $^3J_{\text{HH}} = 7.6$ Hz), 2.52 (t, 2H, $^3J_{\text{HH}} = 7.2$ Hz), 2.95 (s, 3H), 4.61 (t, 2H, $^3J_{\text{HH}} = 7.6$ Hz), 7.69–7.80 (m, 2H), 8.20 (d, 2H, $^3J_{\text{HH}} = 8.9$ Hz), 8.27 (d, 1H, $^3J_{\text{HH}} = 8.9$ Hz), 8.36 (d, 1H, $^3J_{\text{HH}} = 7.9$ Hz).

4-2-[(1E,3E,5E)-6-(Acetylanilino)-1,3,5-Hexatrienyl]-1,1-Dimethyl-1H-Benzo[e]Indolium-3-yl-1-Butanesulfonate (3)—The mixture of (2) (0.3 g, 0.88 mmol) and glutaconaldehyde dianil hydrochloride (0.27 g, 0.96 mmol) in acetic anhydride (4 mL) and acetic acid (1 mL) was heated at 110 °C for 2 h. After cooling, the solution was added dropwise to diethyl ether to precipitate a product. The solvent was decanted and the residue was dissolved in dichloromethane (3 mL) and precipitated from diethyl ether again. The solid was filtered, washed with water and dried under vacuum to give the product as a dark-purple solid. Yield: 0.36 g (76%). Melting point: 170 °C with a destruction. ^1H NMR ($(\text{CD}_3)_2\text{SO}$) 1.75 (m, 2H), 1.90 (m, 11H), 2.50 (t, 2H), 4.49 (t, 2H, $^3J_{\text{HH}} = 7.9$ Hz), 5.23 (d, 1H, $^3J_{\text{HH}} = 11.7$ Hz), 6.59 (d, 1H, $^3J_{\text{HH}} = 11.0$ Hz), 7.06 (d, 1H, $^3J_{\text{HH}} = 15.1$ Hz), 7.41 (d, 2H, 6.9 Hz), 7.60 (m, 4H), 7.76 (m, 2H), 8.15 (m, 5H), 8.38 (d, 1H, $^3J_{\text{HH}} = 8.3$ Hz).

4-(2-(1E,3E,5E,7E)-7-[3-(3-Azidopropyl)-1,1-Dimethyl-1,3-Dihydro-2H-Benzo[e]Indol-2-Ylidene]-1,3,5-Heptatrienyl)-1,1-Dimethyl-1H-Benzo[e]Indolium-3-yl)-1-Butanesulfonate (azICG) (4)—The solution of (3) (1.7 g, 3.13 mmol) and (1) (1.44 g, 3.44 mmol) in the mixture of pyridine (20 mL), acetic acid (2 mL) and acetic anhydride (2 mL) was heated at 50 °C for 4 h. After cooling, this solution was added dropwise to diethyl ether to precipitate a crude product. This product was filtered and dried, dissolved in pyridine (4 mL) and quenched with diethyl ether once again. The solid obtained was filtered, washed with water and dried to give a dark-green product. Yield: 2.1 g (96%, purity: 90%). ^1H NMR ($(\text{CD}_3)_2\text{SO}$) 1.91 (m, 18H), 2.5 (t, 2H), 3.54 (t, 2H, $^3J_{\text{HH}} = 6.5$ Hz), 4.22 (m, 4H), 6.34 (d, 1H, $^3J_{\text{HH}} = 13.4$ Hz), 6.59 (m, 3H), 7.50 (m, 2H), 7.66 (m, 3H), 7.80 (m, 2H), 7.90–8.10 (m, 6H), 8.25 (m, 2H). ESI-Mass (m/z ; rel. intensity %): 700 ($M +$; 90), 564 (50), 408 (35), 346(100). FTIR (cm^{-1}): 993, 1 054 ($\text{s}, =\text{C-H}$); 1 350, 1 399 (s, CH_2); 2 089 (N_3).

Preparation of Azide-Modified Polyethylene Glycol (azPEG)

Mono-Methoxy-PEG1000-Methansulfonate (6)—Triethylamine (0.39 g, 3.9 mmol) was added dropwise at room temperature to a stirred solution of mono-methoxy-PEG1000 (3 g, 3 mmol) and methylsulfonyl chloride (0.41 g, 3.6 mmol) in dichloromethane (30 mL). The solution was stirred at 20 °C for 4 h, then washed with water and the organic layer was dried with Na_2SO_4 with further filtration (Figure 8). The solvent was evaporated under vacuum to give the product (6) as a white solid. Yield: 3 g (93%). ^1H NMR (CDCl_3) 3.07 (s, 3H), 3.36 (s, 3H), 3.53 (m, 2H), 3.62 (m, 78H), 3.74 (m, 2H), 4.36 (m, 2H).

Mono-Methoxy-PEG1000-Azide (7)—The mixture of (6) (3 g, 2.78 mmol) and sodium azide (0.47 g, 7.23 mmol) in acetonitrile (30 mL) was refluxed and stirred for 6 h. After cooling, the mixture was filtered and the solvent was evaporated. The residue was dissolved

in dichloromethane and washed with water, organic layer was separated, dried with Na₂SO₄ and filtered. The solvent was evaporated, the crystalline residue was washed with hexane, filtered, and dried in air to give the product as a white solid. Yield: 2.8 g (98%). ¹H NMR (CDCl₃) 3.37 (s, 3H), 3.39 (m, 2H), 3.55 (m, 2H), 3.64 (m, 80H). FTIR (cm⁻¹): 1 095 (s, C-O-C); 1 340, 1 465 (CH₂); 2 100 (N₃); 2 880 (s, CH₂).

A similar procedure was employed for the synthesis of mono-methoxy-PEG5000-azide.

Preparation of the Particles

Monodisperse cross-linked PA particles were prepared using an emulsion polymerization procedure. A standard emulsion apparatus was utilized where 0.05 g of sodium dodecyl sulfate (SDS, 99% Aldrich) was added to 75 mL of 18.2 MΩ water; this solution was allowed to stir at 250 rpm at 83 °C under a nitrogen purge. After a 60 min purge, 9 mL of the propargyl acrylate (PA) (98% Aldrich) and 1.8 mL of the divinyl benzene (DVB, 80% Aldrich) was added dropwise to the solution. The PA and the DVB were passed over alumina basic to remove inhibitors prior to being added to the solution. Once the addition of the PA/DVB mixture was completed, 0.393 mL of 3-allyloxy-2-hydroxy-1 propanesulfonic acid sodium salt (COPS-1, 40 wt.-% soln. Aldrich) and 5 mL deionized water was added dropwise to the solution. After the COPS-1 was completely added, the solution was allowed to stir for an additional 5 min before 0.1 g potassium persulfate (KPS, 99 + % Aldrich), which was mixed with 5 mL deionized water, was added to the solution. The emulsion polymerization was carried out under a nitrogen atmosphere for 40 min. The resulting particles were dialyzed against deionized water for 2 weeks at 60 °C using a dialysis bag with a 50 000 MWCO and then shaken with an excess of mixed bed ion-exchange resin (Bio-Rad AG-501-X8(D)). After the cleaning procedure, the particle diameter was measured to be 73 ± 7 nm (average and standard deviation), as indicated by a Coulter N4Plus dynamic light scattering (DLS) analyzer.

For a typical surface modification of the particles, for example, the grafting of azICG and azide-modified PEG chains with molecular weight of 1 000 (azPEG_{1K}) onto the particles, 1 mL PA particles and 10.9 mg azICG were added to 2 mL of deionized water. Solutions of 0.07624 g copper(II) sulfate (99.999% Aldrich) in 10 mL deionized water and 0.3024 g sodium ascorbate (99% Aldrich) in 10 mL deionized water were made. Initially, 0.5 mL of the Cu(II)SO₄ solution was added to the PA/azICG solution, followed by 0.5 mL of the sodium ascorbate solution. The resulting mixture was maintained at a temperature of ca. 28 °C for 10 min and then the reaction was stopped by the removal of unreacted azICG, sodium ascorbate, and Cu(II)SO₄ through a repeated particle washing procedure consisting of centrifugation and redispersion in methanol. The cleaned PA/azICG particles in water were subsequently utilized in a secondary click transformation with 39.12 mg azPEG_{1K}, and previously presented Cu(II)SO₄ and sodium ascorbate solutions. The reaction was allowed to run for 24 h and then washed to remove unreacted species as determined by photoluminescence measurements; these particles are referred to as PA/azICG/azPEG_{1K} particles.

Bradford Protein Assay

Protein determination per functionalized particle was determined using established procedures.^[50] PA/azICG, PA/azICG/azPEG_{1K}, and PA/azICG/azPEG_{5K} particles incubated in the presence or absence of BSA (final 0.15 × 10⁻³M) were collected by centrifugation at 15 000 g. After the supernatant was removed, the particles were washed with water. The supernatant and respective particles were incubated separately with the Bradford's reagent (Bio-Rad) for 10 min at 37 °C and the absorbance was measured at 595 nm using a spectrophotometer (Amersham Biosciences). The amount of BSA interacting

with the particles was calculated using a BSA standard curve prepared with known BSA concentration standards.

Cell Analysis

(Cell culture) HepG2 cells are a human hepatoma cell line and were obtained from ATCC (Rockville, MD). All cells were cultured in phenol red-free Dulbeccos modified Eagles media (DMEM) containing 5% fetal bovine serum (FBS), 1% Penicillin–Streptomycin, and supplemented with glutamine (Invitrogen, Carlsbad, CA). Cells were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO₂. (Non-radioactive cell proliferation (MTS) assay) HepG2 cells were plated in 96 well plates at 20 000 cells per well, and exposed to 9.45×10^8 , 9.45×10^{10} , and 9.45×10^{12} particles · mL⁻¹ 24 h after initial plating. Proliferation was assayed colorimetrically 96 h after particle exposure with the Cell-Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, medium was decanted and a solution containing 100 µL 10% FBS with DMEM media and 20 µL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulfate (PMS) was added to each well. After 60 min, the wells were scanned colorimetrically at 490 nm on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA). The conversion of MTS into an aqueous soluble formazan product is achieved only by dehydrogenase enzymes which are present in metabolically active cells; the absorbance at 490 nm from the formazan product is directly proportional to the number of living cells in culture.

Optical Characterization Methods

Absorption spectra were taken using a Perkin-Elmer Lambda 900 UV/VIS/NIR spectrophotometer. Photoluminescence (PL) spectra were collected using a Thermo Oriel xenon arc lamp (Thermo Oriel 66 902) mated with a Thermo Oriel Cornerstone 7 400 1/8 m monochromator (Thermo Oriel 7400) and a Horiba Jobin-Yvon MicroHR spectrometer coupled to a Synapse CCD detector. Quantum yields (ϕ) of the dyes and modified particles were determined relative to the reference dye 1,1',3,3',3'-hexamethylindotricarbocyanine iodide (HITCI) in methanol, which has a fluorescence quantum yield of $\phi_{\text{ref}} = 0.12$,^[51, 20] employing established procedures.^[52]

Acknowledgments

The authors thank DARPA (grant number: N66001-04-1-8933), NIH (grant number: R15-ES017321), the State of South Carolina, and the Gregg-Graniteville Foundation for financial support.

References

1. Gupta AK, Gupta M. Biomaterials. 2005; 26:3995. [PubMed: 15626447]
2. Lu AH, Salabas EL, Schuth F. Angew Chem, Int Ed. 2007; 46:1222.
3. Medina C, Santos-Martinez MJ, Radomski A, Corrigan OI, Radomski MW. Br J Pharmacol. 2007; 150:552. [PubMed: 17245366]
4. De Jong WH, Borm PJA. Int J Nanomed. 2008; 3:133.
5. Torchilin VP. Adv Drug Delivery Rev. 2006; 58:1532.
6. Brown SB, Brown EA, Walker I. Lancet Oncol. 2004; 5:497. [PubMed: 15288239]
7. Huang Z. Technol Cancer Res Treatment. 2005; 4:283.
8. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbek M, Moan J, Peng Q. J Natl Cancer Inst. 1998; 90:889. [PubMed: 9637138]
9. Malicka J, Gryczynski I, Geddes CD, Lakowicz JR. J Biomed Opt. 2003; 8:472. [PubMed: 12880353]
10. Frangioni JV. Curr Opin Chem Biol. 2003; 7:626. [PubMed: 14580568]

11. Olsen TW, Lim JI, Capone A, Myles RA, Gilman JP. Arch Ophthalmol. 1996; 114:97. [PubMed: 8540861]
12. Benson RC, Kues HA. Phys Med Biol. 1978; 23:159. [PubMed: 635011]
13. Motomura K, Inaji H, Komoike Y, Kasugai T, Noguchi S, Koyama H. Jpn J Clin Oncol. 1999; 29:604. [PubMed: 10721942]
14. Ishihara H, Okawa H, Iwakawa T, Umegaki N, Tsubo T, Matsuki A. Anesth Analg. 2002; 94:781. [PubMed: 11916773]
15. Ott P. Pharmacol Toxicol. 1998; 83:7.
16. Paumgart G, Probst P, Kraines R, Leevy CM. Ann NY Acad Sci. 1970; 170:134.
17. Reindl S, Penzkofer A, Gong SH, Landthaler M, Szeimies RM, Abels C, Baumler W. J Photochem Photobiol AChem. 1997; 105:65.
18. Baumler W, Abels C, Karrer S, Weiss T, Messmann H, Landthaler M, Szeimies RM. Br J Cancer. 1999; 80:360. [PubMed: 10408838]
19. Desmettre T, Devoisselle JM, Mordon S. Surv Ophthalmol. 2000; 45:15. [PubMed: 10946079]
20. Philip R, Penzkofer A, Baumler W, Szeimies RM, Abels C. J Photochem Photobiol A-Chem. 1996; 96:137.
21. Soper SA, Mattingly QL. J Am Chem Soc. 1994; 116:3744.
22. Simmons R, Shephard RJ. J Appl Physiol. 1971; 30:502. [PubMed: 4929468]
23. Gathje J, Steuer RR, Nicholes KR. J Appl Physiol. 1970; 29:181. [PubMed: 4913806]
24. Muckle TJ. Biochem Med. 1976; 15(17):936. Macromol Biosci. 2011; 11:927–937. [PubMed: 21480531]
25. Sharma P, Brown S, Walter G, Santra S, Moudgil B. Adv Colloid Interface Sci. 2006; 123:471. [PubMed: 16890182]
26. Jin S, Ye KM. Biotechnol Prog. 2007; 23:32. [PubMed: 17269667]
27. Yu J, Yaseen MA, Anvari B, Wong MS. Chem Mater. 2007; 19:1277.
28. Altinoglu EI, Russin TJ, Kaiser JM, Barth BM, Eklund PC, Kester M, Adair JH. ACS Nano. 2008; 2:2075. [PubMed: 19206454]
29. Saxena V, Sadoqi M, Shao J. J Photochem Photobiol B-Biol. 2004; 74:29.
30. Rodriguez VB, Henry SM, Hoffman AS, Stayton PS, Li XD, Pun SH. J Biomed Opt. 2008; 13:014025. [PubMed: 18315383]
31. Kim G, Huang SW, Day KC, O'Donnell M, Agayan RR, Day MA, Kopelman R, Ashkenazi S. J Biomed Opt. 2007; 12:044020. [PubMed: 17867824]
32. Bhasikuttan AC, Mohanty J, Nau WM, Pal H. Angew Chem, Int Ed. 2007; 46:4120.
33. Zadnard R, Schrader T. J Am Chem Soc. 2005; 127:904. [PubMed: 15656628]
34. Babendure JR, Adams SR, Tsien RY. J Am Chem Soc. 2003; 125:14716. [PubMed: 14640641]
35. Jisha VS, Arun KT, Hariharan M, Ramaiah D. J Phys Chem B. 2010; 114:5912. [PubMed: 20380473]
36. Jisha VS, Arun KT, Hariharan M, Ramaiah D. J Am Chem Soc. 2006; 128:6024. [PubMed: 16669657]
37. Fickweiler S, Szeimies RM, Baumler W, Steinbach P, Karrer S, Goetz AE, Abels C, Hofstadter F, Landthaler M. J Photochem Photobiol B-Biol. 1997; 38:178.
38. Landsman MLJ, Kwant G, Mook GA, Zijlstra WG. J Appl Physiol. 1976; 40:575. [PubMed: 776922]
39. Rotermund F, Weigand R, Penzkofer A. Chem Phys. 1997; 220:385.
40. Weigand R, Rotermund F, Penzkofer A. Chem Phys. 1997; 220:373.
41. Kirchherr AK, Briel A, Mader K. Mol Pharm. 2009; 6:480. [PubMed: 19228053]
42. Mubarekyan E, Santore M. Langmuir. 1998; 14:1597.
43. Russin TJ, Altinoglu EI, Adair JH, Eklund PC. J Phys: Condens Matter. 2010; 22:334217. [PubMed: 21386507]
44. Ketley JN, Habig WH, Jakoby WB. J Biol Chem. 1975; 250:8670. [PubMed: 1184584]
45. Sauda K, Imasaka T, Ishibashi N. Anal Chem. 1986; 58:2649. [PubMed: 3813009]

46. Imasaka T, Okazaki T, Ishibashi N. *Anal Chim Acta*. 1988; 208:325.
47. Bozkulak O, Yamaci RF, Tabakoglu O, Gulsoy M. *Photodiag Photodyn Ther*. 2009; 6:117.
48. Knowles BB, Howe CC, Aden DP. *Science*. 1980; 209:497. [PubMed: 6248960]
49. Zhu XH, Wang CH, Tong YW. *J Biomed Mater Res Part BAppl Biomater*. 2007; 82B:7.
50. Bradford MM. *Anal Biochem*. 1976; 72:248. [PubMed: 942051]
51. Sperber P, Spangler W, Meier B, Penzkofer A. *Opt Quantum Electron*. 1988; 20:395.
52. Williams ATR, Winfield SA, Miller JN. *Analyst*. 1983; 108:1067.

Figure 1.

Schematic of various PA particles surface modified with (i) an azide-terminated indocyanine green (azICG), (ii) azICG and an azide-terminated PEG with molecular weight of 1K (azPEG_{1K}), (iii) azICG and azPEG with molecular weight of 5K (azPEG_{5K}). Particles modified through a CuAAC in water.

Figure 2. (a) Molar extinction coefficient ($5 \mu\text{g} \cdot \text{mL}^{-1}$) (○) and photoluminescence (●) of azide-functionalized indocyanine green (azICG) in methanol. (b) Absorbance (○) and photoluminescence (●) spectra of PA/azICG particles in methanol. Excitation energy at a wavelength of 710 nm.

Figure 3.

(a) Photoluminescence of PA/azICG/azPEG_{5K} particles dispersed in a PBS solution without BSA (●) and after 0.025 mM BSA (○) and 0.25 mM BSA (▽) had been added; time duration of ca. 4 days. Inset presents difference between initial and final emission spectra of particles with 0.25 mM BSA. (b) Increase in maximum photoluminescence intensity of particles composed of PA/azICG (●) and PA/azICG/azPEG with PEG of molecular weight of 1 000 (○) and 5 000 (▽) with varying amounts of added BSA; particles dispersed in a PBS solution with particle density of $1.259 \times 10^{12} \text{ cm}^{-3}$. Excitation energy at a wavelength of 710 nm and emission intensity measured at 825 nm.

Figure 4.

(a) Increase in photoluminescence intensity ratio of PA/azICG/azPEG_{1K} particles dispersed in a PBS solution with the addition of 0.014 mM BSA; time evolution of the intensity at 819 nm relative to the initial intensity. Inset presents photoluminescence of particles after 2 min (●), 37 min (○), and 1174 min (▽). Excitation energy at a wavelength of 710 nm; particle density of $1.259 \times 10^{12} \text{ cm}^{-3}$. (b) Optical image of fluorescence intensity of PA/azICG/azPEG_{1K} particles in deionized water (far left), PBS (center), and 2 h after the addition of 0.014 mM BSA to the PBS solution (far right); images taken with a Caliper Xenogen IVIS Lumina II XR Instrument with 745 nm excitation filter and ICG emission filter; particle density of $1.259 \times 10^{12} \text{ cm}^{-3}$.

Figure 5. Proliferation of HepG2 cells after 4 days of incubation with neat PA (P) and PA/azICG/azPEG_{1K} (SMP) particles at concentrations of 9.45×10^8 (8.97), 9.45×10^{10} (10.97), and 9.45×10^{12} (12.97) particles $\cdot$ mL⁻¹. Each condition was tested in three replicates with the high/low values being presented as error bars. An asterisk indicates statistical significance from the control by ANOVA followed by Tukeys multiple comparisons test ($p < 0.01$).

Figure 6.

Proliferation of HepG2 cells with 24 h of incubation time with PA/azICG/azPEG_{1K} (SMP) particles at concentrations of 9.45×10^{10} (10.97) and 9.45×10^{12} (12.97) particles $\cdot$ mL⁻¹ after exposure for 15 min to 780 nm light at a 0.04 mW $\cdot$ cm⁻² flux. Each condition was tested in three replicates with the high/low values being presented as error bars. Inset presents proliferation of neat cells with and without light exposure. An asterisk indicates statistical significance from the control by ANOVA followed by Tukeys multiple comparisons test ($p < 0.01$).

Figure 7. Synthetic route for 2-[(1E,3E,5E)-7-[(2E)-3-(3-azidopropyl)-1,1-dimethyl-1H,2H,3H-naphtho[2,1-b]pyrrol-2-ylidene]hepta-1,3,5-trien-1-yl]-1,1-dimethyl-3-(4-sulfonatobutyl)-1H-naphtho[2,1-b]pyrrol-3-ium (azICG).

Figure 8.
Synthetic route for azide-modified polyethylene glycol (azPEG).

Table 1

Contact angle (θ) and quantum efficiency (ϕ) of surface functionalized PA particles.

Solvent	θ	ϕ
	Water	Methanol
PA	73 ± 9	–
PA/azICG	87 ± 2	0.017 ± 0.004
PA/azICG/azPEG _{1K}	23 ± 3	0.015 ± 0.004
PA/azICG/azPEG _{5K}	25 ± 1	0.017 ± 0.004