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Non-Covalently Pre-Assembled High-Performance Near-Infrared Fluorescent Molecular Probes for Cancer Imaging

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Abstract

New fluorescent molecular probes, which can selectively target specific cell surface receptors, are needed for microscopy, in vivo imaging, and image guided surgery. The preparation of multivalent probes using standard synthetic chemistry can be a laborious process due to low reaction yields caused by steric effects. In this study, fluorescent molecular probes were prepared by a programmed non-covalent pre-assembly process that used a nearinfrared fluorescent squaraine dye to thread a macrocycle bearing a cyclic arginine-glycine-aspartate peptide antagonist (cRGDfK) as a cancer targeting unit. Cell microscopy studies using OVCAR-4 (ovarian cancer) and A549 (lung cancer) cells that express high levels of the integrin $\alpha v \beta 3$ or $\alpha v \beta 5$ receptors, respectively, revealed a multivalent cell targeting effect. That is, there was comparatively more cell uptake of a pre-assembled probe equipped with two copies of the cRGDfK antagonist than a pre-assembled probe with only one appended cRGDfK antagonist. The remarkably high photostability and low phototoxicity of these near-infrared probes allowed acquisition of long-term fluorescence movies showing endosome trafficking in living cells. In vivo near-infrared fluorescence imaging experiments compared the biodistribution of a targeted and untargeted probe in a xenograft mouse tumor model. The average tumor-to-muscle ratio for the pre-assembled targeted probe was 3.6 which matches the tumor targeting performance reported for analogous cRGDfK-based probes that were prepared entirely by covalent synthesis. The capability to excite these pre-assembled near-infrared fluorescent probes with blue or deep-red excitation light makes it possible to determine if a target site is located superficially or buried in tissue, a probe performance feature that is likely to be very helpful for eventual applications such as fluorescence guided surgery.

Keywords

Supramolecular chemistry; Host-Guest systems; Self-assembly; Fluorescent probes; Cancer

Introduction

There is increasing demand for molecular imaging agents which can selectively target specific cell surface receptors as biomarkers of cancer.^[1–5] In particular, fluorescent molecular probes have broad utility in biochemical assays, cell microscopy, flow cytometry, preclinical in vivo imaging, clinical fluorescence guided surgery, and histopathology.^[6–9] Fluorescent molecular probes with near-infrared excitation/emission wavelengths have

inherent advantages for in vivo imaging due to deeper tissue penetration of the light and reduced background signal caused by autofluorescence.^[10] Almost all in vivo fluorescent probe designs can be divided into two distinct strategies: targeted probes that accumulate over time at a region of interest in the living subject, or activatable probes that “switch on” fluorescence emission at the target site.^[10] Our focus is on the former, and we are especially interested in developing multivalent fluorescent probes that selectively target the exterior surface of cancer cells. Multivalent targeting of cell surface biomarkers has the potential of achieving high affinity and high selectivity for cancer cells which overexpress the biomarker.^[11,12] But preparing multivalent probes using standard covalent chemistry can be a laborious process, especially when there is a large number of appended targeting units because steric effects can lower the reaction yields.^[13] An alternative approach to probe fabrication is to employ the principles of supramolecular chemistry and non-covalently self-assemble a collection of molecular components that are strategically equipped with targeting and reporting units.^[14] There is a growing number of self-assembly methods that generate nanoscale objects such as vesicles, micelles, and related nanoparticles, but the relatively large size and statistical structure of these objects is a potential drawback in some imaging applications.^[15,16] A refined version of this approach is to employ highly complementary host/guest partners that associate with strong affinity in water under very mild conditions and create discrete molecular objects with precise structures and well-defined stoichiometries. At present there are very few synthetic host/guest pairs that have the requisite supramolecular and optical properties to enable self-assembly of multivalent fluorescent probes for effective in vivo imaging. Literature studies have examined cyclodextrins, calixarenes, pillararenes, or cucurbiturils as the host partner.^[14] But most of these host systems are limited by either insufficient host/guest affinity, or the need to append near-infrared fluorophores by additional covalent synthesis steps.

We are developing a multivalent near-infrared probe pre-assembly method using Synthavidin (synthetic avidin) technology.^[17–19] To briefly summarize, we have discovered that suitably modified tetralactam macrocycles can be threaded in near-quantitative yield by fluorescent squaraine dyes with flanking polyethylene glycol (PEG) chains to produce noncovalent complexes with sufficient stability in water to enable in vivo imaging of living subjects.^[20–22] A major attraction with this approach is the possibility of mixing different squaraine and macrocycle components in combinatorial fashion and producing libraries of pre-assembled probes for subsequent testing. Our previous studies threaded a bis(thiophene)squaraine dye platform with two different types of functionalized macrocycles. In one case, the macrocycle was equipped with multiple iminodiacetate groups that permitted targeting of the threaded complex to the surface of bone,^[20] and in a second case, the macrocycle contained zinc dipicolylamine groups that targeted the anionic surfaces of bacteria and necrotic tissue.^[21,22] In both of these examples, probe targeting was driven by strong electrostatic interactions with the surface of the in vivo target, and the probes exhibited several necessary properties for successful in vivo operation such as high mechanical and chemical stability. Furthermore, the probe pharmacokinetics could be controlled in a predictable manner by modifying the length of the appended PEG chains. The next step in the research is to substantially broaden the technological scope by creating pre-assembled probes with low molecular weight antagonists as cell receptor targeting units.

From a supramolecular perspective, the utilization of receptor antagonists for probe targeting is a more demanding challenge, compared to our previous systems, for three reasons: (a) the increased number and variety of functional groups within the antagonist structure might inhibit the Synthavidin preassembly process, (b) the PEG chains appended to the preassembled probes might lower antagonist affinity for the receptor target,^[23] and (c) the appended antagonist(s) might alter probe biodistribution in undesirable ways.^[24]

Herein, we report two important technical and conceptual advances that help produce high performance pre-assembled fluorescent probes for preclinical cancer imaging and eventually clinical fluorescence guided surgery. The first advance is a demonstration that the Synthavidin pre-assembly process can be used to efficiently fabricate threaded probes equipped with one or more copies of a small peptide antagonist that has selective affinity for a cell surface receptor. The arginine-glycine-aspartate peptide motif was chosen as the cancer targeting unit. More specifically we used the cyclic peptide cRGDfK, a validated highaffinity antagonist for several types of integrin receptors which are overexpressed in many forms of cancer.^[6] The second advance is a major improvement in the photostability of the pre-assembled probes, which was achieved by replacing the bis(thiophene)squaraine dye platform with a more photostable bis(hydroxy)phenyl squaraine platform.^[25–27] At the project's outset there was some literature precedence supporting the feasibility of each structural change as a separate modification to the Synthavidin pre-assembly process, but it was not certain if both modifications could be incorporated simultaneously. Schematically, the project goal was to prepare the two targeted pre-assembled probes shown in Scheme 1. One probe has a single peptide antagonist threaded onto a single bis(hydroxy)phenyl squaraine dye, and the other has two copies of the antagonist threaded on to a linked bis(hydroxy)phenyl squaraine dimer. We also prepared control versions of these preassembled probes that lacked targeting units. The first step in the project was to synthesize the requisite squaraine and macrocycle building blocks and then demonstrate that they can be preassembled under mild conditions to make stable fluorescent probes in near-quantitative yield. The next step was to assess the performance of these new integrin-targeted near-infrared fluorescent probes for microscopic imaging of cancer cells and in vivo imaging of tumors in a mouse model of cancer.

Results and Discussion

Shown in Scheme 2a are the individual squaraine and macrocycle components that were used to pre-assemble the five near-infrared fluorescent probes for this study. The synthesis and spectral characterization of each compound is provided in the Supporting Information (SI). In Scheme 2b is a schematic summary of the pre-assembled probes that were prepared by simply mixing the corresponding components in water. The three squaraine derivatives, bis(thiophene)squaraine **T**, bis(hydroxyphenyl)squaraine **H**, and dimeric bis(hydroxyphenyl)squaraine **H3H**, were threaded by either the symmetric macrocycle **C** with six appended carboxylate groups, or the unsymmetric macrocycle **R**. At one end of **R** is a copy of the cRGDfK antagonist, and at the other end is an appended tricarboxylate unit which ensured good water solubility.^[28] In Scheme 2c is a molecular model showing how the tetralactam macrocycle encapsulates a bis(hydroxyphenyl)squaraine dye. The association constant for threading is close to 10^8 M^{-1} in water and the complex is stabilized by a

combination of four hydrogen bonds between the squaraine oxygens and the macrocycle NH residues, and hydrophobic stacking of the dye's aromatic surfaces against the macrocycle's anthracene side walls.^[26,27] A key structural feature within **H** and **H3H** is the terminal *N*-propyl group at each end of the bis(hydroxyphenyl)squaraine chromophores which we knew would act as a "steric speed bump" and slow the kinetics for threading/dethreading but not alter the association constant.^[29] Thus, we expected that probe preassembly could be achieved in very high yield and short timeframe if the squaraine and macrocycle components were mixed in water at micromolar concentration. Indeed, preliminary threading studies showed that near-quantitative threading occurred when **H** was mixed with one molar equivalent of **R** or **C**, or **H3H** was mixed with two equivalents of **R** or **C**. In Figure 1a-b are representative absorption spectra of the squaraine dyes before and after probe pre-assembly. The absorption profile for free dimeric **H3H** is extremely broad due to folding of the structure and stacking of its two squaraine chromophores.^[20,22] But after addition of macrocycle, near-quantitative threading and probe pre-assembly was indicated by the appearance of diagnostic narrow and red-shifted squaraine absorbance maxima. In Figure 1c-d are two representative sets of absorption spectra showing the spectral changes that occur during the probe pre-assembly process. The threaded probes exhibit sharp squaraine absorption maxima bands indicating little propensity for intermolecular aggregation, a conclusion that was supported by Dynamic Light Scattering analysis.

As a standard procedure, a 15 hour incubation period was employed to pre-assemble a 50 μ M stock solution of each probe in water. In Table 1 is a summary of the photophysical properties of the free squaraine dyes and the pre-assembled probes in water. In agreement with expectations, the quantum yield for free squaraine dye **H** in water was relatively high but it was extremely low for the free dimeric squaraine **H3H** due to its folded and selfquenched structure.^[20,22] Likewise, the relatively low quantum yields for the dimeric threaded probes **2(C) $\supset$ H3H** and **2(R) $\supset$ H3H** are also attributed to their partially folded and quenched structures in water.

Additional spectral proof that macrocycle threading had occurred was gained by observing efficient internal energy transfer after excitation of the anthracene sidewalls in the macrocycle with 350 nm light (Figure S1-S2). In the cases of **C $\supset$ H** and **R $\supset$ H**, excitation of the anthracene sidewalls produced a quenched anthracene emission band (relative to the emission spectrum of free macrocycle) and strong squaraine emission at 710 nm. In the cases of **H3H** and **2(R) $\supset$ H3H**, the emission band for each threaded probe was partially quenched but the squaraine emission was also weak due to the probe's partially folded and self-quenched structure.

Further independent proof of the structures for **R $\supset$ H** and **2(R) $\supset$ H3H** was provided by high resolution mass spectroscopy with electrospray ionization, which identified molecular ion peaks that were emblematic of the threaded complexes (Figures S3-S4). ¹H NMR spectra of the threaded complexes were quite broad at the relatively high millimolar concentrations needed for spectral acquisition and thus not very informative,^[20] but purity analysis by agarose gel electrophoresis proved to be very insightful (Figure 2). Individual samples of **H**, **H3H**, **R $\supset$ H**, and **2(R) $\supset$ H3H** were loaded onto an agarose gel and separated according to their different charge-to-mass ratios at pH 8.3. For uncharged **H** and

H3H there was little or no electrophoretic migration, while the double threaded probe **2(R) ⊃ H3H** with much higher charge-to-mass ratio migrated much further than the singly threaded, **R ⊃ H** (Figure 2a). A fifth well was loaded with an equal volume mixture of the four pre-assembled probes. The well-resolved bands observed in this “mixture” lane matched those seen in the individual lanes, and demonstrated that **H**, **H3H**, **R ⊃ H**, and **2(R) ⊃ H3H** were all stable and that the threaded probes were formed in near-quantitative yield. The fluorescent properties of these compounds allowed the gels to be imaged using a deep-red excitation/near-infrared emission filter set (Figure 2b) and also a blue excitation/near-infrared emission filter set (Figure 2c). In the case of deep-red excitation, both the free squaraine dyes and threaded complexes were observed with brightness commensurate to the quantum yields listed in Table 1 (for **H3H**, this means almost no emission was observed). In the case of blue excitation, only the threaded complexes were observed to give near-infrared emission due to their unique capacity for internal energy transfer.

Taken together, the independent spectral, mass spectrometry and electrophoresis data prove that probe pre-assembly at 50 μM in water occurs in near-quantitative yield. Furthermore, probe stability experiments indicated that negligible dethreading of the pre-assembled probes occurred over 17 hours after an aliquot of probe sample was diluted to 2 μM in fetal bovine serum (Figure S5). This finding is consistent with our previous work showing that Synthavidin pre-assembled probes have sufficiently high kinetic and thermodynamic stability to be effective biological imaging agents.^[20–22]

The cancer targeting capabilities of **R ⊃ H** and **2(R) ⊃ H3H** were assessed using the integrin expressing cell lines OVCAR-4 and A549. OVCAR-4 is an excellent cell model of high-grade serous ovarian carcinoma because it has a very similar genomic sequence and response to chemotherapeutic drugs.^[32] We have recently demonstrated that the $\alpha\text{v}\beta 3$ integrin receptor is expressed at high levels ($[9.8 \pm 2.5] \times 10^4/\text{cell}$) on the surface of OVCAR-4 cells, and that the cells are amenable to molecular targeting and imaging using covalently-conjugated fluorophores bearing the cRGDfK antagonist.^[33] In contrast A549 adenocarcinoma cells do not express $\alpha\text{v}\beta 3$, but they do express the integrin $\alpha\text{v}\beta 5$ receptor, which also has good affinity for the cRGDfK motif.^[34] Expression of $\alpha\text{v}\beta 5$ in A549 cells was corroborated using immunocytochemistry staining with an anti- $\beta 5$ FITC-conjugated antibody (Figure S6). A quantitative flow cytometry study, using this antibody in conjunction with a fluorescence calibration kit, found that there was an average of $[10.2 \pm 3.7] \times 10^4$ $\beta 5$ proteins on the surface of each A549 cell (Figure S7). Furthermore, microscopy studies using both cell lines showed binding and uptake of a commercially available orange fluorescent cRGDfK-Cy3 probe, along with blocking by free cRGDfK (structures and micrographs in Figure S8). Subsequent fluorescence microscopy studies quantified the binding and uptake of the pre-assembled near-infrared targeted and untargeted probes. The microscopy experiments incubated separate samples of the cells with one of the probes for 30 min, then fixed the cells and washed them several times to remove unbound probe from the cell surface. Additionally, deconvolution microscopy techniques were employed to remove erroneous signal emanating from the cell exterior and thus allowed the amount of internalized probe to be quantified. Both OVCAR-4 and A549 cells exhibited high uptake of the cRGDfK-targeted probes **R ⊃ H** and **2(R) ⊃ H3H** with the fluorescent

signal localized to punctate compartments inside the cell (Figure 3a). In contrast, uptake of the analogous untargeted probes **C** $\supset$ **H** and **2(C)** $\supset$ **H3H** was significantly lower. Receptor blocking experiments also showed that internalization of the cRGDfK-targeted probes could be inhibited by an excess of free cRGDfK. The amount of cell internalization for monovalent **R** $\supset$ **H** and divalent **2(R)** $\supset$ **H3H** was compared under cell incubation conditions that used the same concentration (2 μ M) of cRGDfK targeting unit, and in both cell lines there was greater uptake of the divalent targeted **2(R)** $\supset$ **H3H** (Figure 3b,c), indicating an enhanced multivalent targeting effect.

One of the major aims of the project was to improve probe photostability by changing the structure of the encapsulated squaraine dyes from bis(thiophene)squaraine (**T**) to bis(hydroxy)phenyl squaraine (**H**). Enhanced photostability was first demonstrated by conducting simple cuvette experiments that illuminated two separate solutions containing the threaded probes, **C** $\supset$ **T** or **C** $\supset$ **H** (Figure 4a-b). The decrease in squaraine absorption for **C** $\supset$ **T** indicated moderate photobleaching compared to no change in absorption for **C** $\supset$ **T**. Confirmation that **C** $\supset$ **T** was a stronger oxygen photosensitizer than **C** $\supset$ **H** was gained by conducting an experiment that used the commercial indicator dye Singlet Oxygen Sensor Green (SOSG) to trap and quantify a higher amount of photogenerated singlet oxygen (Figure S9). This improvement in probe photostability was maintained during live cell microscopy experiments. For example, separate samples of cells were incubated with **C** $\supset$ **T** or **C** $\supset$ **H** for several hours to permit non-specific uptake, and an epifluorescence microscope was used to collect movies of probe-labelled endosome trafficking in each sample (see SI for movies and Figure S10 for movie montage). The movies show significant photobleaching for the sample containing **C** $\supset$ **T** and much greater stability for the sample containing **C** $\supset$ **H**. Additional live cell fluorescence microscopy studies found that the cRGDfK-targeted probes **R** $\supset$ **H** and especially **2(R)** $\supset$ **H3H** probes were also remarkably photostable. In the Supporting Information is a movie showing trafficking of endosomes containing either near-infrared **2(R)** $\supset$ **H3H** or the orange emitting cRGDfK-Cy3, and analyses of these movies are provided in Figure 4c-d. The Cy3 fluorophore is well-known for its very high photostability,^[35] but the fluorescence micrographs of A549 cells containing cRGDfK-Cy3 were almost completely bleached after 15 min of imaging. Remarkably, under the same conditions, there was only a 30% drop in fluorescence intensity for cell micrographs containing **2(R)** $\supset$ **H3H**, making it a very rare example of a near-infrared fluorescent probe that is more photostable than Cy3.^[36]

The last stage of the project was to determine the in vivo cancer targeting capability of divalent cRGDfK-targeted **2(R)** $\supset$ **H3H** in a xenograft mouse tumor model. Our goal was to make a preliminary assessment of probe potential for fluorescence-guided surgery of cancer where the desired imaging outcome is accurate identification of a tumor within a couple of hours after dosing the subject with a fluorescent probe.^[37] Sixteen athymic nude mice were injected subcutaneously with A549 cells in their rear flanks and tumors were allowed to develop over one month. The anesthetized mice were randomly split into two cohorts and given tail vein injections of either cRGDfK-targeted **2(R)** $\supset$ **H3H** or the untargeted control **2(C)** $\supset$ **H3H**. A series of live animal fluorescence imaging experiments was conducted using an In Vivo Imaging Station (IVIS). Whole mouse images collected at 90 and 180

minutes showed significantly higher tumor accumulation of targeted **2(R) ⊃ H3H** than untargeted **2(C) ⊃ H3H** (Figure 5a). Quantitative analysis of the live mouse images (Figure S11) provided two conclusions. At the 90 minute time point, the tumor could be unambiguously identified in all mice treated with the targeted probe. In addition, there was high accumulation of the probe in the kidneys indicating reasonably rapid renal clearance of the probe from the blood stream, which is a desirable attribute for high contrast imaging. The live animal images at the 180 minute time point showed that probe accumulation in tumor or kidney had hardly changed so the mice were sacrificed and the excised organs imaged using the IVIS (Figures 5b). The ratio of mean pixel intensity (MPI) for near-infrared probe fluorescence in each organ relative to that of excised thigh muscle and the data is plotted in Figure 5c.^[38] The average tumor-to-muscle ratio of 3.6 for mice that were treated with targeted **2(R) ⊃ H3H** is similar to the tumor selectivity ratios reported for analogous but completely covalent RGD-targeted molecular probes in similar mouse tumor models.^[33,39–41] Moreover, a tumor selectivity ratio of 3 or more is a desired benchmark for effective fluorescence guided surgery.^[42,43]

A final demonstration of the potential of these pre-assembled fluorescent probes for fluorescence guided surgery was gained by conducting a phantom imaging experiment that showcased a unique performance feature, namely, the capability to determine if a target site is exposed to the camera or screened by a layer of tissue. Two wells within a multiwell plate were filled with solutions of pre-assembled **C ⊃ H** and simultaneously imaged in the IVIS using either blue excitation (ex: 410 nm) with near-infrared emission (enabled by energy transfer from the excited anthracene sidewalls of the surrounding macrocycle to the encapsulated squaraine dye within **C ⊃ H**) or direct deep-red excitation of the encapsulated squaraine (ex: 670 nm). As illustrated in Figure 6, the light path of one of the wells was screened with a 0.5 cm thick slice of chicken breast. Inspection of the near-infrared fluorescence images shows that screening of a cuvette with tissue barely decreased emission intensity when the samples were illuminated with deep-red (670 nm) light, but there was 70% decrease in emission intensity for a screened cuvette when it was illuminated with blue (410 nm) light whose intensity was strongly attenuated by the tissue. Thus, by simply switching between blue and deep-red illumination light, an observer can rapidly and conveniently determine if a fluorescence signal is radiating from an exposed target site or from a site that is buried underneath tissue. This is a key question that has to be answered often during fluorescence guided surgery of cancer.^[37]

Conclusions

A new class of non-covalently pre-assembled near-infrared molecular probes was prepared with appended cRGDfK antagonist(s) that have selective affinity for integrin receptors that are often overexpressed in many forms of cancer. The probes were pre-assembled by threading a functionalized tetralactam macrocycle with a squaraine dye that had flanking PEG chains. Quantitative probe pre-assembly in water was confirmed by a collection of independent fluorescence, mass spectral, and electrophoresis data. The new pre-assembled probes are based on bis(hydroxy)phenyl squaraine dyes which are much more photostable and generate less singlet oxygen than analogous probes based on bis(thiophene)squaraine dyes. This significant improvement made it possible to acquire long-term microcopy movies

of probe-labeled living cells. Fluorescence microscopy studies using OVCAR-4 (ovarian cancer) and A549 (lung cancer) cells that expressed high levels of the integrin $\alpha v\beta 3$ or $\alpha v\beta 5$ receptors, respectively, found a multivalent cell targeting effect. That is, there was comparatively more cell uptake of a preassembled probe equipped with two copies of the cRGDfK antagonist than a pre-assembled probe with only one appended cRGDfK antagonist. In vivo near-infrared fluorescence imaging experiments compared the biodistribution of targeted **2(R) $\supset$ H3H** and untargeted **2(C) $\supset$ H3H** in a xenograft mouse tumor model. The average tumor-to-muscle ratio for the targeted probe was 3.6 which reaches the tumor selectivity benchmark that is needed for effective fluorescence guided surgery. A unique performance feature of these pre-assembled probes is the capability to excite the near-infrared fluorescence with blue or deep-red excitation light. It should be possible to exploit this feature, as a convenient means to determine if a fluorescent target site is located superficially or buried in tissue.

Experimental Section

Materials

Antibodies were purchased from ThermoFischer: Anti-Human CD51/CD61 (Integrin $\alpha v\beta 3$) FITC, catalog number 11-0519-42; Integrin $\beta 5$ Monoclonal Antibody (KN52) FITC, 11-0497-4; Mouse IgG1 kappa Isotype Control FITC, catalog number 11-4714-42. cRGDfK-Cy3 was purchased from Molecular Targeting Technologies, catalog number RG-1002. Agarose gel and Tris/Borate/EDTA (TBE) Buffer materials were purchased from Sigma Aldrich. Singlet Oxygen Sensor Green (SOSG) was purchased from Thermo Fischer.

Synthesis

The synthetic methods used to prepare the squaraine dyes and macrocycles as well as the compound characterization are described in the Supporting Information.

Stock Preparation

Stock solutions (1 mM) of squaraine and macrocycle components **C**, **R**, **T**, or **H** were prepared in water. A stock solution (1 mM) of **H3H** was prepared in DMSO. Stock solutions (50 μ M) of pre-assembled probes, **C $\supset$ T**, **C $\supset$ H**, **R $\supset$ H**, **2(R) $\supset$ H3H**, or **2(R) $\supset$ H3H** were prepared by mixing the macrocycle and squaraine components in water at appropriate stoichiometries (1:1 or 2:1) and allowing the sample to shake gently for 15 hours at room temperature. After spectral confirmation that each preassembled structure was formed in near-quantitative yield, the stock solutions of pre-assembled probe were used for bioimaging experiments.

Photophysical studies

Fluorescence spectra were taken and analyzed on a Horiba Fluoromax-4 Fluorometer with FluorEssence software. Absorption spectra were collected on an Evolution 201 UV/Vis Spectrometer with Thermo Insight software. All spectra were obtained using spectrophotometric grade solvent at 20 °C with either a glass cuvette or quartz cuvette (1 mL, 10 mm path length). Quantum yield measurements used methylene blue ($\Phi_f = 0.02$ in H₂O) as a standard. The sample concentrations were adjusted to have an absorption of 0.08

at 600 nm. The fluorescence spectrum of each solution was obtained by excitation at 600 nm and the integrated area was used to calculate the quantum yield. The estimated error of this method is $\pm 10\%$.^[30]

Photobleaching and singlet oxygen photogeneration studies examined separate 2 μM solutions of **C** $\supset$ **T** or **C** $\supset$ **H** in water, with or without SOSG (30 $\mu\text{g/mL}$). The samples were exposed to air and irradiated with a Xenon lamp (150 W, 620 nm long-pass filter) for 20 min. The photobleaching experiment monitored loss of the squaraine absorbance maxima band. The singlet oxygen photogeneration experiment monitored fluorescence emission between 500–650 nm (ex: 490 nm) with fluorescence increase indicating singlet oxygen photogeneration.

Gel Electrophoresis

Separate solutions of pre-assembled probes **H**, **R** $\supset$ **H**, **H3H**, and **2(R)** $\supset$ **H3H** (193 μM) were added (20 μL) to separate wells in a 2% agarose gel. A mixture of all four probe solutions (5 μL each) was added into a fifth well. Cold TBE buffer (pH 8.3, 8.9 mM Tris, 8.9 mM borate, 200 μM) was filled to the top of the gel (but not covering the wells, to prevent the compounds from leaking into the buffer). The gel was run for at 100 V for 30 min to load the compounds into the gel. The entire gel was subsequently covered with TBE buffer and run at 125 V for 2 hr. The gel was photographed in ambient light, and a fluorescent image was acquired using an In Vivo Imaging Station with the following parameters: Cy5.5 filter set (ex: 640/25 nm, em: 732/38 nm, exposure: 7 s, F-stop: 2, binning: small), or GFP/Cy5.5 (ex: 468/22 nm, em: 732/38 nm, exposure: 7 s, F-stop: 2 binning: small). Overlaid images were created using ImageJ software.

Cell Culture

OVCAR-4 human ovarian carcinoma cells (DCTD Tumor Repository, National Cancer Institute at Frederick, Maryland) and A549 human lung carcinoma cells (ATCC CCL-185) were cultured in RPMI-1640 or F-12K media (Thermo Fischer) supplemented with 10% (w/w) fetal bovine serum, 1% streptomycin-penicillin, 5% L-Glutamine (only in RPMI media), and 0.1 mM NEAA in a humidified incubator at 37 °C, 5% CO₂ over air.

Flow Cytometry

A549 cells were dissociated from a confluent plate with non-enzymatic dissociation solution (Gibco). The cells were then incubated for 30 min at 4 °C with either 0.375 $\mu\text{g/mL}$ of FITC conjugated $\beta 5$ antibody or 7.5 $\mu\text{g/mL}$ of FITC conjugated IgG1 kappa isotype control. After incubation, the cells were washed and fixed with IC fixation buffer (ThermoFisher Scientific). Using a BD LSRFortessa X-20 flow cytometer, the cells were excited at 488 nm and emission was collected with a FITC 520/530 nm filter (max: 20,000 events). The data was then analyzed using FlowJo software, where the geometric mean fluorescence of each sample was calculated by subtracting the isotype control fluorescence from the sample fluorescence. The geometric mean fluorescence was then plotted to a calibration plot created using a Quantum™ FITC-5 MESF kit. This identified the number of FITC fluorophores per cell which was subsequently divided by the number of FITC fluorophores per antibody ($\beta 5$: 5.5 ± 1.0). A resulting value of $([10.2 \pm 3.7] \times 10^4 \beta 5 \text{ proteins/cell})$ was determined,

assuming only one antibody could bind at a time. Experiments were repeated independently three times in duplicate

Microscopic Imaging of Cancer Cells

Sample preparation—Cells were seeded onto an 8-well chambered coverglass (Lab-Tek, Nunc, USA) and grown to 80% confluence (48 h). Prior to treatment with probes, the media was replaced with a supplemented HEPES buffer (25 mM HEPES, 130 mM NaCl, 3.8 mM KCl, 0.35 g/L Na_2HCO_3 , 7.0 mM glucose, 2 mM sodium pyruvate, 1x MEM NEAA, pH 7.4) for 20 min. The cells were then incubated with 200 μL **R** $\supset$ **H** (2 μM) or **2(R)** $\supset$ **H3H** (1 μM) in buffer at 37 °C for 30 min. For live cell experiments, cells were washed and costained with 3 μM Hoechst for 10 min, then washed twice and imaged under supplemented HEPES buffer. For fixed cell experiments, endocytosis was stopped by the addition of 100 μL ice cold 4% paraformaldehyde for 5 min. Blocking experiments used free cRGDfk (100 μM) applied both during the 20 min FBS starvation treatment and during the cell staining treatment. Control experiments were conducted in an analogous manner with orange emitting cRGDfk-Cy3. Immunocytochemistry experiments for $\alpha\text{v}\beta 5$ expression on A549 cells used FITC conjugated $\beta 5$ monoclonal antibody (KN52) (0.125 $\mu\text{g}/100 \mu\text{L}$, eBioscience 11–0497-41) where the antibody was administered for 20 min in supplemented HEPES buffer followed by two wash steps, fixation as before, and imaging under PBS.

Fluorescence microscopy—Fluorescence microscopy was conducted on a GE Healthcare DeltaVision Deconvolution epifluorescence microscope equipped with a X-cite 120 fluorescence illumination system and the following filter sets: near-infrared (ex: 660/20 nm, em: 700/30 nm), orange (ex: 555/28, em: 617/73), green (ex: 490/20 nm, em: 528/38 nm), ultraviolet (ex: 360/40 nm, em: 457/50 nm). Images were collected in softWoRx using a Photometrics Cascade II:512 EMCCD camera operating in CCD mode with 1 s acquisition times, 1 MHz readout speed. Images were deconvolved in softWoRx, and a 50 pnt. rolling background was subtracted in ImageJ. The mean fluorescence intensity (MFI) for each micrograph was calculated from the average of 20 randomly generated 25 $\times$ 25 pixel extra-nuclear ROIs. Averages and SEM were calculated and plotted in GraphPad Prism using 3 images each from 3 biological replicate experiments. Timelapse image sets of live cells were acquired with the same settings, every 10 s, for 15 min. For plots of photobleaching: (1) a plot of the micrograph MFI vs time was generated, (2) the plot was fit with a one phase decay and the baseline of the fit was used to approximate the MFI of the fully photobleached sample, (3) this baseline MFI was subtracted from the MFI of each micrograph, (4) the baseline adjusted MFI was normalized to the baseline adjusted MFI at time = 0 min and plotted.

In vivo Imaging

All mouse experiments were approved by the University of Notre Dame Institutional Animal Care and Use Committee. A549 cells were grown to confluency in complete F12K growth medium supplemented with 10% fetal bovine serum. Female Foxn1 nude mice (Charles River Laboratories) were injected subcutaneously on the right rear flank with A549 cells (1×10^6 cells/mouse, 100 μL) in 1:1 Matrigel(Corning):media. Approximately, one month later, the mice were randomly divided into two cohorts of eight, and each mouse received an

intravenous injection of either **2(C) ⊃ H3H** or **2(R) ⊃ H3H** (170 μL, 5 nmol/mouse). The mice were then anesthetized with 23% isoflurane (oxygen rate of 2 L/min) for imaging. A preliminary fluorescence image was acquired in an In vivo Imaging System (IVIS) using a Cy5.5 filter set (ex: 640/25 nm, em: 732/38 nm, exposure: 3 s, Fstop: 2, binning: small). The mice were imaged again at 90 min and 180 min and the images analyzed using ImageJ software to calculate tumorto-background ratio (TBR) and kidney-to-background ratio (KBR). After 180 min, the mice were anesthetized, sacrificed via cervical dislocation, and the major organs were removed (liver, heart, lungs, spleen, and kidneys, along with thigh muscle, blood, and tumor). The organs were imaged following the above protocol. Images were processed and analyzed using ImageJ software. For whole mouse images and tumor images, a 1000 pnt. rolling ball radius was applied for background subtraction and all images were scaled against the highest intensity in the set. The biodistribution analysis involved: (1) subtracting a 1000 point rolling background, (2) creating a region of interest (ROI) around each organ using an intensity threshold, (3) measuring the mean pixel intensity (MPI), (4) normalizing the MPI to that of the thigh muscle, (5) averaging and plotting the normalized MPI with its SEM. It is important to realize that the threaded probe has to be the source of the near-infrared fluorescence signal in the mouse images because the IVIS does not detect the blueshifted and very weakly fluorescent squaraine dye **H3H** that is produced by probe unthreading (Figure S12).

Light Penetration through Tissue

Separate solutions of **C ⊃ H** (2.5 mL, 5 μM) were added to two wells in a multi-well plate. A 0.5 cm thick slice of chicken breast was placed under one of the wells to screen the excitation/emission light path. The wells were imaged with a Bruker Xtreme with ex: 410/10 nm or 670/10 nm and em: 700/17.5 nm. The fluorescence images were processed in ImageJ by creating an ROI around the perimeter of each well and measuring the MPI. The MPI for the image of the screened well was normalized to that of the exposed well for both the 410 nm excitation and 670 nm excitation image sets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- [38]. It is worth noting two peripheral points concerning the probe biodistribution data in Figure 5c. First, the tumor-to-muscle ratio for the mice treated with untargeted probe **2(C)** $\supset$ **H3H** is

1.8±0.2. This small but statistically significant amount of tumor accumulation is likely a manifestation of the enhanced permeation and retention (EPR) effect, which is further increased in the case of targeted probe **2(R) ⊃ H3H**. Second, there is comparatively slightly higher accumulation of the targeted probe in the liver and lungs, which is not surprising given the known propensity of multiple-peptide targeted probes to accumulate in these organs. For example, see: Cao Q, Li ZB, Chen K, Wu Z, He L, Neamati N, Chen X, Eur. J. Nucl. Med. Mol. Imaging 2008, 35, 1489–1498. [PubMed: 18373091] Jin Z-H, Furukawa T, Degardin M, Sugyo A, Tsuji AB, Yamasaki T, Kawamura K, Fujibayashi Y, Zhang M-R, Boturn D, et al., Mol. Cancer Ther 2016, 15, 2076–2085. [PubMed: 27422811]

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Figure 1.

Absorption spectra in water for: (a) free **H** and threaded probes **C ⊃ H** and **R ⊃ H**, (b) free **H3H** and threaded probes **2(C) ⊃ H3H** and **2(R) ⊃ H3H**. For all samples, the molecular concentration was 5 μM . Representative absorption spectra showing spectral changes that occur over time due to macrocycle threading and probe pre-assembly in water and 25 $^{\circ}\text{C}$; (c) mixing **H** and **R** (1:1 molar ratio) to make **R ⊃ H**, monitored over 140 minutes, (d) mixing **H3H** and **R** (1:2 molar ratio) to make **R ⊃ H**, monitored over 200 minutes.

Figure 2.

Gel electrophoresis using 2% agarose gel loaded with free squaraine dyes or threaded probes (193 μM /well). The three images show the same gel after electrophoresis (100 V for 30 min, then 125 V for 2 h, pH = 8.3). (a) White light image, dashed circles denote location of sample bands. (b) Fluorescence image with ex: 640/25 nm, em: 732/38 nm. (c) Fluorescence image with ex: 468/22, em: 732/38 nm.

Figure 3.

(a) Representative fluorescence micrographs showing relative amounts of near-infrared probes taken up by OVCAR-4 or A549 cells after 30 min: 2.0 μ M **C** $\supset$ **H**, 2.0 μ M **R** $\supset$ **H**, 2.0 μ M **R** $\supset$ **H** + 100 μ M cRGDfK, 1.0 μ M **2(C)** $\supset$ **H3H**, 1.0 μ M **2(R)** $\supset$ **H3H**, or 1.0 μ M **2(R)** $\supset$ **H3H** + 100 μ M cRGDfK. Blue fluorescence shows Hoechst nuclear stain. Scale bar = 30 μ m. (b,c) Quantification of mean near-infrared intracellular fluorescence intensities as a measure of probe internalization.

Figure 4.

Absorption spectra of: (a) $\text{C} \equiv \text{H}$ and (b) $\text{C} \equiv \text{T}$ ($2 \mu\text{M}$ in H_2O) before (black line) and after (red line) irradiation with red light for 20 min. (c) Plots showing relative photobleaching over 15 min for fluorescent micrographs of separate samples of live A549 cells containing near-infrared $2(\text{R}) \equiv \text{H3H}$ or orange emitting cRGDfk-Cy3. The micrographs were acquired every 10 s using 2 s exposure times. (d) Representative fluorescence micrographs at time = 0 min and 15 min. The gray/white color indicates $2(\text{R}) \equiv \text{H3H}$ or cRGDfk-Cy3 fluorescence, and blue shows Hoechst nuclear stain

Figure 5.

(a) Representative near-infrared fluorescence images of tumor-bearing mice at -15 min, 90 min, and 180 min after intravenous injection of 2(C) or 2(R) (5 nmol/mouse). (b) Near-infrared fluorescence images of tumors removed from the mice at 3 hours after probe injection. (c) Biodistribution of fluorescent probes in organs removed from the mice (N = 8 for each cohort). The mean pixel intensity (MPI) for near-infrared probe fluorescence in each organ is relative to the MPI for thigh muscle from the same animal; error bars indicate $\pm$ SEM. Insert shows scatter plot for tumor MPI data.

Figure 6.

- (a) Illustration of phantom imaging experiment to assess fluorescence screening by tissue.
- (b) Fluorescence images of two wells, each containing a solution of $C \supset H$ (5 μM) with ex: 410/10 nm or 670/10 nm and em: 700/17.5 nm. A 0.5 cm thick slice of chicken breast screened the light path of the upper well, while the lower well was exposed to the camera.
- (c) Quantification of the two sets of images (ex: 410 nm or 670 nm) as a ratio of fluorescence mean pixel intensity (MPI) for the screened well divided by the MPI for the exposed well.

Scheme 1.

Illustration of Synthavidin pre-assembly, using a macrocycle (red) with a conjugated antagonist as targeting unit (green) and a monomeric or linked dimeric near-infrared fluorescent squaraine dye (blue).

Scheme 2.

(a) Compounds used to pre-assemble the different fluorescent probes. (b) Molecular model of tetralactam macrocycle threaded by bis(hydroxyphenyl)squaraine dye illustrating the important hydrogen bonding and hydrophobic stacking interactions. (c) Descriptors of the pre-assembled fluorescent probes used in this study.

Table 1.

Photophysical properties of free dyes and preassembled probes in water.

Compound	Abs (nm)	Em (nm)	Φ ^[a]
T	666	678	0.04 ^[b]
C $\supset$ T	677	710	0.18 ^[b]
H	652	675	0.24
C $\supset$ H	671	711	0.14
R $\supset$ H	669	707	0.18
H3H	644	668	0.005
2(C) $\supset$ H3H	670	712	0.027
2(R) $\supset$ H3H	671	710	0.024

^[a]Relative to a standard solution of methylene blue in water ($\Phi_f = 0.02$)[30,31]^[b]From reference[29]