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The PEG-Fluorochrome Shielding Approach for Targeted Probe Design

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Supporting Information

ABSTRACT: We provide a new approach for fluorescent probe design termed "PEG-fluorochrome shielding", where PEGylation enhances quantum yields while blocking troublesome interactions between fluorochromes and biomolecules. To demonstrate PEG-fluorochrome shielding, fluorochrome-bearing peptide probes were synthesized, three without PEG and three with a 5 kDa PEG functional group. *In vitro*, PEG blocked the interactions of fluorochrome-labeled peptide probes with each other (absorption spectra, self-quenching) and reduced nonspecific interactions with cells (by FACS). *In vivo*, PEG blocked interactions with biomolecules that lead to probe retention (by surface fluorescence). Integrin targeting *in vivo* was obtained as the differential uptake of an ^{111}In-labeled, fluorochrome-shielded, integrin-binding RGD probe and a control RAD. Using PEG to block fluorochrome-mediated interactions, rather than synthesizing *de novo* fluorochromes, can yield new approaches for the design of actively or passively targeted near-infrared fluorescent probes.

Near-infrared (NIR) fluorescent, receptor-targeted peptides have been used for pre-operative single photon emission computerized tomography (SPECT) (or positron emission tomography, PET) imaging and/or for intra-operative tumor detection in both pre-clinical[1] and clinical settings.[2] Though NIR fluorochromes are desirable because of the tissue-penetrating properties of their light and low background, they typically involve multiple unsaturated double bonds linking multiple unsaturated rings. These features lead to self-quenching fluorochrome/fluorochrome interactions, high nonspecific binding to many cells, unwanted interactions with proteins and lipids *in vivo*, and enterohepatic circulation rather than renal elimination. Efforts to obtain improved NIR fluorochromes have often followed a "medicinal chemistry" approach of synthesizing low-molecular-weight fluorochromes and screening them for desirable properties.[3]

Here we provide an example where a poly(ethylene glycol) (PEG) placed on a peptide along with a NIR fluorochrome blocks troublesome interactions between fluorochromes and biological molecules, increasing quantum yield and permitting peptide/target binding. The notion that fluorochromes can be improved not through fluorochrome redesign but rather by association with PEG can yield new avenues for probe design through PEG-fluorochrome improvement. PEG has been used to extend protein circulation and alter surface chemistry,[4] but its ability to improve fluorochrome behavior has not been recognized. To demonstrate that "PEG-fluorochrome shielding" is compatible with molecular targeting *in vivo*, we show that a PEGylated, fluorochrome-bearing RGD probe lacks unwanted fluorochrome-mediated interactions yet maintains desirable RGD/integrin interactions.

We synthesized PEGylated and un-PEGylated RGD and RAD probes bearing the CyAL5.5 fluorochrome,[5] using the strategy of attaching a multifunctional reagent module to integrin-targeted peptides[6] (see Figure 1). Here three functional groups (F1 = DOTA, F2 = fluorochrome, F3 = 5 kDa PEG) were attached to a Lys-Lys-βAla-Lys(N_3) peptide scaffold, and the multifunctional reagent module was then reacted with a linker-targeting module bearing a RGD- or RAD-targeting peptide. DOTA allowed radiometal chelation for biodistribution and/or imaging studies.

Although they differ in just a single methyl group (glycine versus alanine), RGD and RAD peptides have profoundly different strengths of interactions with integrins (see ref 7, with further support provided in Figure 4a). A copper-less "click" reaction joined the terminal dibenzylcyclooctane (DBCO) of the linker-targeting vehicle to the azide of the trifunctional reagent, avoiding the possibility of a copper reaction with the DOTA. DBCO and azide moieties were found to be highly stable and readily underwent efficient click reactions when combined. We term as "PEGylated probes" those bearing the F3 5 kDa PEG functional group, which consists of 115 PEG units rather than the short linker-PEG, which consists of just 4 such units. Complete structures of functional groups and targeting peptide structures are given in the Supporting Information, Figure S1. The molecular weights, along with a summary of our results, are provided in Table 1.

We examined the interactions between the 5 kDa PEG and the CyAL5.5 fluorochrome using the three PEGylated and three un-PEGylated compounds from Figure 1c, as shown in Figure 2. In PBS, our three un-PEGylated peptides (**5a**, **6a**, **6b**) exhibited "an aggregation peak" at ~630 nm, typical of stacked fluorochromes, while three PEGylated peptides (**5b**, **7a**, **7b**) had reduced aggregation peaks.[8] Remarkably, in methanol the three PEGylated and three un-PEGylated compounds had

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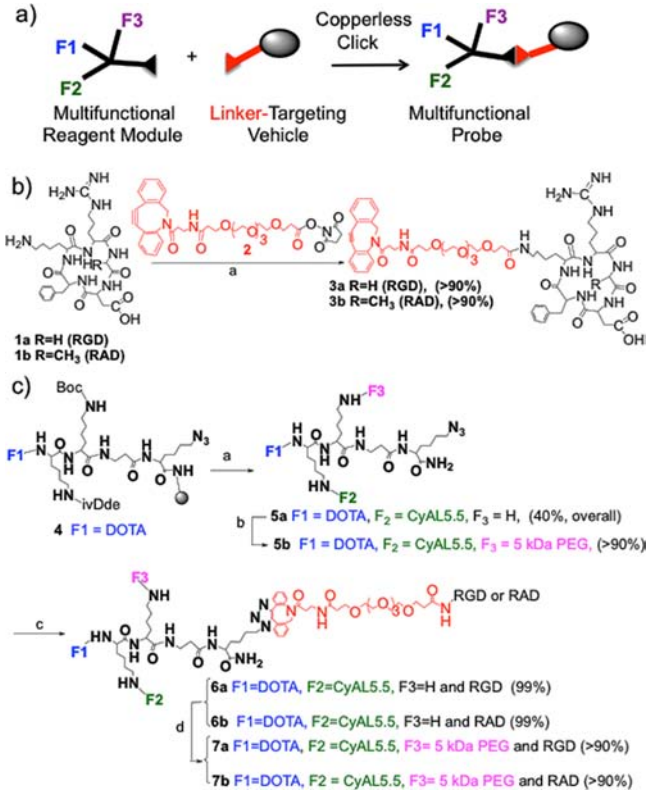

Figure 1. Synthesis of PEG-fluorochrome-shielded integrin-targeted probes. (a) A multifunctional reagent module was first synthesized and attached to a linker-targeting vehicle module via a copper-less "click" reaction, to yield a multifunctional probe. (b) Synthesis of the linker-targeting vehicles bearing RGD or RAD peptides. *a*: DMF/DiPEA (c) Synthesis of the multifunctional probes with functional groups of F1 = DOTA, F2 = CyAL5.5 fluorochrome, and F3 = 5 kDa PEG. Reaction conditions for *a*: (1) NH_2NH_2, DMF, (2) CyAL5.5 acid/PyBOP/DMF/DiPEA, (3) TFA; *b*: 5 kDa PEG-NHS, DMSO; *c*: **5a**, RGD-PEG4-DBCO (**3a**) or RAD-PEG4-DBCO (**3b**), DMSO; *d*: 5 kDa PEG-NHS. For the need of the linker arm for RGD/integrin interactions, see Figure S4. Compounds were >90% pure by RP-HPLC and FPLC (see Figure 2d).

identical absorption spectra, and those were characteristic of unaggregated fluorochromes.

We examined the effects of the F3 5 kDa PEG functional group on quantum yields (Figure 2b) and fluorescent lifetimes (Figure 2c) using the six fluorochrome-bearing compounds from Figure 1c. PEGylation produced significant ($p < 0.0001$, see Table S1) increases in quantum yield (Figure 2b, **5a** vs **5b**, **6a** vs **7a**, **6b** vs **7b**). PEGylation also produced significant increases in fluorescence lifetime (Figures 2c and S3, **5a** vs **5b**, **6a** vs **7a**, **6b** vs **7b**), with $p < 0.0001$ in all cases. PEG yielded probes with globular protein equivalent volumes of 20−25 kDa (**5b**, **7a**, **7b**, Figure 2d,e), similar to small proteins. Our three un-PEGylated probes (**5a**, **6a**, **6b**) had volumes <1 kDa. Thus, PEGylation not only blocked fluorochrome/fluorochrome interactions but also dramatically increased probe volume. To determine whether PEG-fluorochrome shielding occurred in biological systems, we examined the effects of PEGylation on the binding of probes to cells and on the elimination of probes after injection.

The ability of the F3 5 kDa PEG to reduce the nonspecific binding to cultured cells, while maintaining specific binding, is shown in Figure 3a,b, with numerical values provided in Table

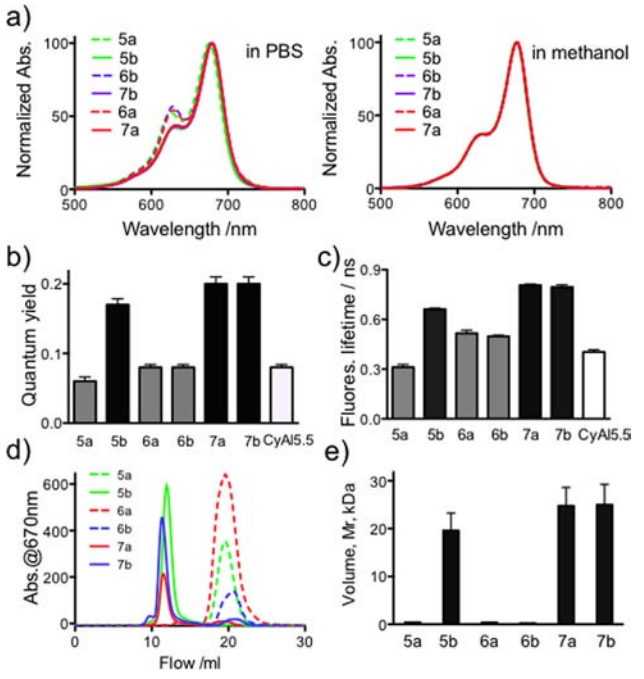

Figure 2. Physical properties of six compounds from Figure 1c. (a) Effects of 5 kDa PEG on probe absorption spectra in PBS and methanol. (b) Quantum yields. (c) Fluorescent lifetimes. (d) FPLC chromatograms. (e) Globular protein equivalent volumes of PEGylated and un-PEGylated probes. For the method of determining volume, see Figure S5.

1. Since BT-20 cells express the RGD binding integrins,[9] this cell line was used. With the PEGylation of **5a** to obtain **5b**, cell fluorescence dropped from 79.7 to 3.1 au. PEGylation also reduced the binding of RGD-bearing peptides (**6a** = 26.7 au to **7a** = 9.7 au) and RAD-bearing peptides (**6b** = 20.4 au to **7b** = 3.2 au). However, integrin-specific binding (see inset for values of **6a**, **6b**, **7a**, and **7b**) was not affected by PEGylation ($p > 0.05$). The binding of the PEGylated RAD probe **7b** to cells (3.2 ± 0.2 au) was similar to that of the PEGylated peptide lacking both RGD and RAD (**5b** , 3.1 ± 0.2 au). Therefore, our PEGylated RAD probe, **7b**, is a valid control for our PEGylated RGD probe, **7a**, because of its similar physical properties and because it does not bind integrins.

A measure of the ability of the 5 kDa PEG to block nonspecific binding *in vivo* was obtained by probe retention/elimination after an intramuscular (i.m.) injection (Figure 3c−f). Mice received dual i.m. injections of **6b** (RAD, un-PEGylated) or **7b** (RAD, PEGylated), with time dependence of surface fluorescence shown in Figure 3c−d. Two regions of interest (circles near the shoulder) were used to quantify whole-body surface fluorescence, while a single region of interest near the bladder was used to quantify bladder fluorescence and renal elimination. Results for whole-body and bladder fluorescence are quantified in Figure 3e,f. PEGylated **7b** showed pronounced renal elimination at 4 h and a lack of whole-body fluorescence. The un-PEGylated **6b** exhibited strong nonspecific interactions, evidenced by the retention of whole-body fluorescence even at 48 h post injection.

A summary of the physical properties of our compounds and their behavior in biological systems *in vitro* and *in vivo* is provided in Table 1. *In vitro* the F3 5 kDa PEG functional group reduced the interactions of fluorochromes with each

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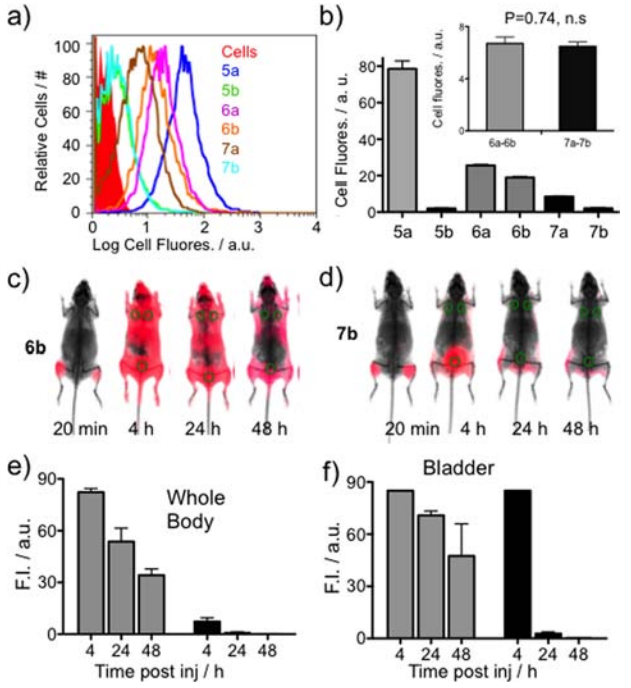

Figure 3. Effect of PEG on the specific and nonspecific binding to cells and on probe elimination. Cell fluorescence (BT-20 cells) after incubation with the six fluorochrome-bearing peptides from Figure 1c was determined by FACS as shown in (a), with peak fluorescence tabulated in (b). (c,d) Whole-body surface fluorescence of mice after **6b** (RAD control, un-PEGylated) or **7b** (RAD control, PEGylated) injection at 1 nmol/site. Circles are the regions of interest used to quantify whole-body (e) or bladder (f) surface fluorescence. (e) Quantification of whole-body surface fluorescence of seven mice; gray bars for **6b**, black bars for **7b**. (f) Quantification of bladder fluorescence for seven animals.

other (absorption spectra, quantum yield, Figure 2a,b), increased probe volume (Figure 2e), reduced the nonspecific interactions with cells (Figure 3a,b), and reduced the interactions with biomolecules that lead to the retention of fluorochrome-bearing probes after injection (Figure 3c,d).

To demonstrate that our PEG-fluorochrome shielding allowed RGD/integrin interactions *in vivo*, the DOTA functional groups of the PEGylated RGD (**7a**) and PEGylated RAD (**7b**) probes were labeled with ^{111}In and their biodistributions determined in a BT-20 xenograft system (Figure 4a, Table S2). Specific RGD binding occurred in the tumor, stomach, small intestine, lung, and heart. The differential binding of RGD and RAD probes indicates integrin-specific uptake (see above) and

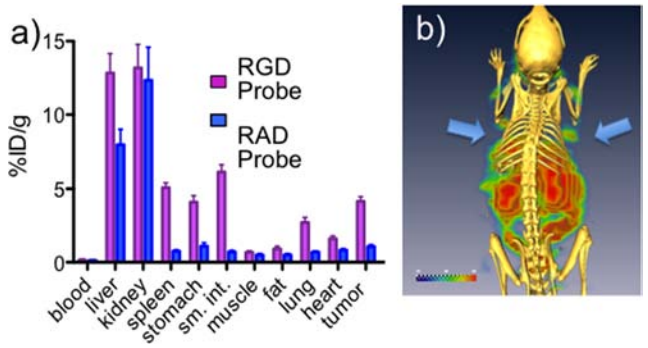

Figure 4. *In vivo* integrin targeting using the PEG-fluorochrome-shielded RGD (**7a**) and RAD (**7b**) probes. (a) Tissue concentrations of ^{111}In-labeled **7a** and **7b**. Numerical data are presented in Table S2. (b) SPECT-CT image of **7a**. Arrows indicate dual BT-20 tumors. Data are at 24 h post i.v. injection.

provides a method of determining the total RGD/integrin binding of cells (Figure 3) or tissues (Figure 4a). Integrin-specific RGD binding to the stomach, spleen, and small intestine results from a wide range of RGD integrins on diverse cells and tissues.[10] A SPECT/CT image of a **7a**-injected animal is shown in Figure 4b.

A model of the PEG-fluorochrome **7a** probe, consistent with our observations and not drawn to scale, is shown in Figure 5.

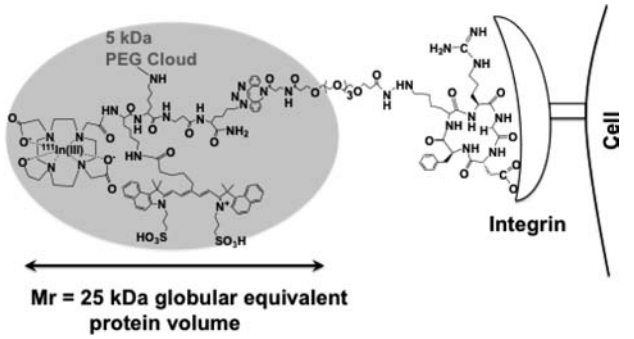

Figure 5. Model of a PEG-fluorochrome-shielded probe (**7a**) and its binding to an integrin.

The 5 kDa PEG forms a cloud around the fluorochrome, blocking nonspecific interactions (Figure 2b,c) but permitting integrin interactions (Figures 3b and 4a). PEG provides most of the probe volume (Figure 2e). However, an essential (Figure S4), short PEG linker separates the integrin-binding RGD targeting peptide from the PEG-shielded fluorochrome. With

Table 1. Physical Properties and Behavior in Biological Systems of Multifunctional Reagent Modules (5a, 5b) and Un-PEGylated (6a, 6b) and PEGylated Probes (7a, 7b)

compd	F3 5 kDa PEG	target peptide	MW (Da)	MS obsd (Da)	quantum yield[b]	fluor lifetime (ns)[c]	vol by FPLC (kDa)[d]	cell fluor (au)[e]	i.m. inject.[f]
5a	no	none	1667.0	1666.2	0.061 ± 0.006	0.31 ± 0.04	0.4	79.7 ± 4.3	
5b	**yes**[a]	**none**	**6805.1**	**6850 (peak)**	**0.17** ± 0.008	**0.66** ± 0.02	**20**	**3.1 ± 0.2**	
6a	no	RGD	2848.3	2848.3	0.082 ± 0.004	0.52 ± 0.05	0.4	26.7 ± 0.6	
6b	no	RAD	2862.4	2862.2	0.081 ± 0.004	0.50 ± 0.02	0.4	20.4 ± 0.4	retained
7a	**yes**	**RGD**	**7987.4**	**7980 (peak)**	**0.20 ± 0.01**	**0.81 ± 0.02**	**25**	**9.7 ± 0.3**	
7b	**yes**	**RAD**	**8001.4**	**8000 (peak)**	**0.20 ± 0.01**	**0.80 ± 0.03**	**25**	**3.2 ± 0.2**	**eliminated**
CyAL5.5	no	none	800.01	799.31	0.08 ± 0.004	0.40 ± 0.03	n.o.[g]	n.o	

[a]Bold = 5 kDa PEG. [b]Figure 2b. [c]Figure 2c. [d]Figure 2e. [e]Figure 3b. [f]Figure 3c,d. [g]n.o. = not observed.

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this design, PEG shielded the fluorochrome and blocked fluorochrome-mediated interactions but permitted RGD/integrin interactions.

The ability to improve fluorochromes using covalent linkages between fluorochromes and PEGs, rather than by *de novo* fluorochrome synthesis, can yield many new avenues for the design of actively or passively targeted fluorescent probes. When the goal is a receptor binding (actively targeted) probe, the strategy of attaching a PEG and a fluorochrome to a common amino acid or peptide can be employed. Our version of this general strategy employed a modular synthetic route (Figure 1a) using a trifunctional reagent module [(DOTA)-Lys(CyAL5.5)-Lys(5 kDa PEG)-βAla-Lys(N_3), or **5b**]. The amino group of an RGD-targeting peptide was reacted with a short PEG linker featuring a terminal DBCO group, and a copper-less click reaction joined the reagent module and linker RGD-targeting peptide. For the many intra-operative fluorescent imaging applications,[11] passively targeted (untargeted), PEG-shielded fluorochromes with optical properties similar to ICG, but which have lost their troublesome interactions with biological molecules, might be employed.

■ ASSOCIATED CONTENT

Supporting Information

Detailed synthesis of all compounds, methods for cell binding assay, animal model, surface fluorescence imaging, absorption spectra, quantum yield, volume determination, SPECT-CT, fluorescence lifetime, biodistribution, and additional data, including expanded structure of compounds, more absorption and emission spectra, fluorescence lifetime curve, and displacement by direct PEG probe. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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