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Development of NIR Fluorescent Dyes Based on Si−rhodamine for in Vivo Imaging

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Supporting Information

ABSTRACT: We have developed a series of novel near-infrared (NIR) wavelength-excitable fluorescent dyes, SiR-NIRs, by modifying the Si−rhodamine scaffold to obtain emission in the range suitable for in vivo imaging. Among them, SiR680 and SiR700 showed sufficiently high quantum efficiency in aqueous media. Both antibody-bound and free dye exhibited high tolerance to photobleaching in aqueous solution. Subcutaneous xenograft tumors were successfully visualized in a mouse tumor model using SiR700-labeled anti-tenascin-C (TN-C) antibody, SiR700-RCB1. SiR-NIRs are expected to be useful as labeling agents for in vivo imaging studies including multicolor imaging, and also as scaffolds for NIR fluorescence probes.

Near-infrared (NIR) (650−900 nm) wavelength-excitable fluorescent dyes are attractive for biological applications because of minimal tissue absorption and low background autofluorescence from serum, proteins, and other biomolecules in the NIR range, so that high contrast can be obtained between target and background tissue.[1,2] Consequently, NIR fluorescent dyes have been applied for in vivo imaging to diagnose or to elucidate the mechanisms of various diseases, including cancer,[3,4] atherosclerosis,[5] rheumatoid arthritis,[6] and inflammation.[7] Although many NIR light-excitable fluorescent scaffolds have been reported, most of them do not emit adequate fluorescence in aqueous media due to molecular stacking or poor water-solubility, and the only widely used fluorophore excitable in the NIR region is cyanine dye, as exemplified by Cy5.5 and Cy7.[3−7] However, even cyanine dyes have some deficiencies for bioimaging; for example, in many cases they suffer photobleaching.[8] Accordingly, the development of a novel fluorescent scaffold that is excitable in the NIR region, photostable, and usable in aqueous media is still an important challenge. Further, novel NIR fluorescent dyes with favorable photophysical characteristics would be alternatives to cyanine dyes as fluorescent labeling agents. Herein, we report the development of a series of novel NIR wavelength-excitable fluorescent dyes based on the rhodamine scaffold, and we demonstrate their superior characteristics for in vivo fluorescence imaging.

Group 14 rhodamines, containing silicon, germanium, or tin at the 10 position of the xanthene chromophore, show large bathochromic shifts compared to the original rhodamines, but still retain the advantages of the original rhodamines, including high quantum efficiency in aqueous media (Φ_{fl} = 0.3−0.45), tolerance to photobleaching, and sufficient water solubility for biological applications.[9−11] Although the emission maximum of conventional Si−rhodamine (called SiR650 here; Figure 1), one of the group 14 rhodamines, is as long as around 660 nm, this is not favorable for in vivo imaging. In view of the wavelength dependence of the tissue transparency and the requirement for sufficiently low background fluorescence, two wavelength windows at 700 and 800 nm are often used as emission channels for practical in vivo imaging.[12−15] So, we set out to develop novel NIR fluorescent dyes by modifying the Si−rhodamine scaffold to obtain emission in the >700 nm region required for practical in vivo imaging. Since it has been reported that the emission wavelength of rhodamine can be elongated by expansion of the xanthene ring, as seen in the Alexa Fluor series,[16] we applied this strategy to the Si−rhodamine scaffold and obtained a series of NIR fluorescent dyes, named SiR-NIRs. The structures of SiR680, SiR700, and SiR720 are shown in Figure 1.

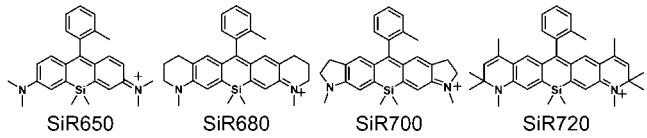

Figure 1. Chemical structures of SiR650 and newly synthesized NIR wavelength-excitable fluorescence dyes, SiR-NIRs.

First, we examined the photophysical properties of SiR-NIRs in aqueous media. As we had expected, all three dyes exhibited absorption and emission maxima in the NIR region (670−740 nm) (Figure 2), and the fluorescence quantum efficiency decreased as the wavelength became longer. It is noteworthy that SiR680 and SiR700 exhibit sufficiently high fluorescence quantum efficiency for application as NIR fluorescence dyes (Φ_{fl} = 0.35 and 0.12) based on the comparison with Cy5.5 (Φ_{fl}

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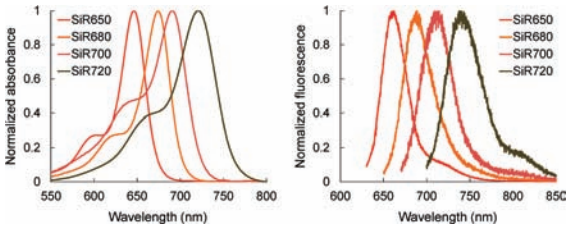

Figure 2. Normalized absorption (left) and emission (right) spectra of 1 μM SiR-NIRs, measured in PBS at pH 7.4.

= 0.23), which is widely used as a NIR fluorescence labeling agent (Table 1). We next examined their photostability. Cy5.5 showed relatively rapid photobleaching, but little decrease of absorption and emission was observed in the cases of SiR680 and SiR700 (Figure 3A and Figure S10) and these SiR-NIRs showed excellent tolerance to photobleaching. This result suggests that SiR-NIRs may be suitable for various applications requiring high photostability, including fluorescence imaging with a high-power laser or repeated irradiation.

Table 1. Photophysical Properties of SiR-NIRs, Measured in PBS at pH 7.4

dye	λ_{abs} (nm)	λ_{fl} (nm)	ε ($M^{-1}cm^{-1}$)	Φ_{fl}[a]
SiR650	646	660	1.1×10^5	0.31[b]
SiR680	674	689	1.3×10^5	0.35
SiR700	691	712	1.0×10^5	0.12
SiR720	721	740	1.6×10^5	0.05

[a]For determination of the fluorescence quantum efficiencies (Φ_{fl}), Cy5.5 in PBS at pH 7.4 (Φ_{fl} = 0.23) was used as a fluorescence standard.[17] [b]This value was taken from ref 9.

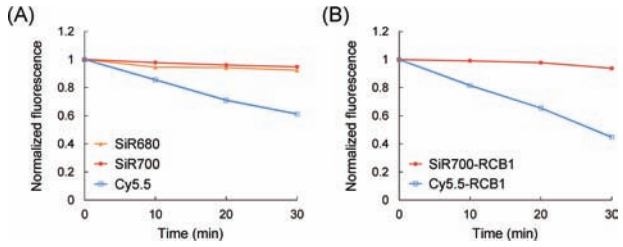

Figure 3. Photobleaching experiments. Normalized fluorescence changes of 1 μM SiR680, SiR700 and Cy5.5 (A), and 0.3 μM SiR700-RCB1 and Cy5.5-RCB1 (dye per antibody ratio (D/A) = 2.0) (B) in PBS at pH 7.4 were measured during light irradiation (40 mW/cm^2, Cy5 filter) for 0, 10, 20, and 30 min.

Next, we prepared amine-reactive succinimidyl ester-bearing SiR700, 2-Me-4-COOSu SiR700 (Figure 4A), for labeling of biomolecules such as antibodies and proteins, and applied it for in vivo tumor imaging targeted to the extracellular matrix glycoprotein tanascin-C (TN-C), a glycoprotein known to be ubiquitously expressed by malignant gliomas.[18] For this purpose, we prepared SiR700-labeled anti-TN-C-antibody (SiR700-RCB1). Initially, we examined the tolerance of SiR700-RCB1 to photobleaching. The photostability of antibody-bound SiR700 was extremely high (Figure 3B), being similar to that of free SiR700 in aqueous solution. We then intravenously injected SiR700-RCB1 into a mouse xenograft tumor model prepared with human malignant meningioma HKBMM cells. The fluorescence signal of SiR700 in the tumor was clearly observed at 24 h after the injection, and remained

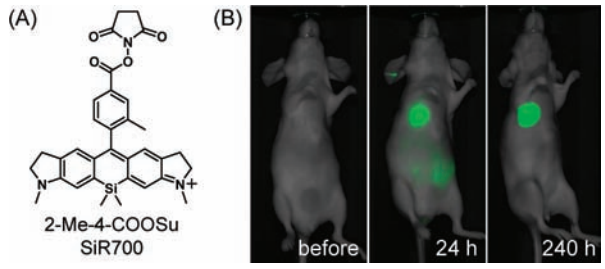

Figure 4. In vivo tumor imaging with SiR700-labeled anti-tenascin-C antibody (SiR700-RCB1). Xenograft tumor model mice prepared with human malignant meningioma HKBMM cells were intravenously injected with 300 μL of 130 μg/mL SiR700-RCB1 (D/A = 1.6). The tumor fluorescence images were obtained before and at 24 h after the injection.

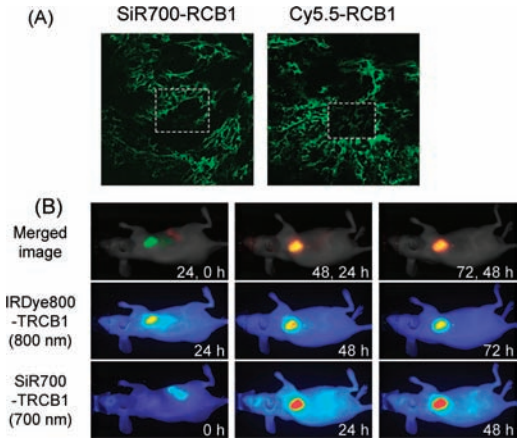

Figure 5. (A) Fluorescence images of frozen sections of tumors harvested 3 days after intravenous injection of SiR700- or Cy5.5-labeled anti-tenascin-C antibody (SiR700- or Cy5.5-RCB1). Dotted squares were irradiated at 670 nm for 5 min. Fluoromount/Plus (COSMO BIO) was used as a mounting medium for preparing the slide samples. (B) In vivo imaging using a Pearl Impulse Imager with a 700 nm channel and an 800 nm channel. SiR700-RCB1 was iv injected into a mouse xenograft tumor model prepared with human malignant meningioma HKBMM cells at 24 h after the iv injection of IRDye800-RCB1. The tumor was at the base of the right forefoot. SiR700-RCB1 (bottom row) and IRDye800-RCB1 (middle row) were separately monitored in two wavelength windows, and merged images (800 nm: green, 700 nm: red) are also shown (top row). Wavelength maxima for excitation/emission filters were 685/720 nm (700 nm channel) for SiR700, and 785/820 nm (800 nm channel) for IRDye800. Fluorescence images were acquired at 24 h (left column), 48 h (middle column) and 72 h (right column) after injection of IRDye800-RCB1 and at 0 h (left column), 24 h (middle column) and 48 h (right column) after injection of SiR700-RCB1 into the mouse xenograft tumor model.

observable for more than 10 days (Figure 4B). Observation of the frozen tumor sections confirmed that the fluorescence was localized in the extracellular matrix (Supporting Figure S1), in accordance with fluorescence microscopic images presented previously.[19,20] We also examined the photostability of SiR700 in the frozen sections. Four commercially available mounting media containing an antifading agent were used to prepare slide samples. In two cases, the fluorescence of Cy5.5 faded significantly during continuous scanning for 5 min by a confocal microscope operating at 670 nm, whereas no fading of SiR700 was observed in any of the media (Figure 5A and Supporting Figures S2−S5). This result indicates that SiR700 is

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more suitable than Cy5.5 for longer-term observation under high-power laser irradiation. To examine further the practical usefulness of SiR700, we conducted in vivo experiments using two kinds of dye-labeled RCB1, SiR700-RCB1 and IRDye800-RCB1. The *N*-hydroxysuccinimide (NHS) ester of IRDye 800CW was purchased from LI-COR Bioscience (Lincoln, NE). One conjugate was injected at 24 h after injection of the other conjugate, followed by observation of the fluorescence in the tumor region using two wavelength windows (700 nm for SiR700, and 800 nm for IRDye800). The tumor region was well imaged with a high signal-to-noise ratio by using either SiR700-RCB1 or IRDye800-RCB1. Further, the fluorescence of SiR700-RCB1 and IRDye800-RCB1 in the tumor could be separately monitored (Figure 5B), so that multicolor imaging with these dyes is feasible.

In summary, we have developed a series of novel NIR wavelength-excitable fluorescent dyes, SiR-NIRs, by modifying the Si−rhodamine scaffold to obtain longer-wavelength emission in the range suitable for in vivo imaging. Among them, SiR680 and SiR700 showed sufficiently high quantum efficiency in aqueous media for fluorescence imaging, and exhibited high tolerance to photobleaching in the free or protein-bound state in aqueous solution. This characteristic is favorable for prolonged observation, and we also confirmed that the fluorescence remained observable after prolonged storage of frozen sections (for at least 3 months; Supporting Figures S6−S9). Further, subcutaneous xenograft tumors in a mouse tumor model were visualized with SiR700-labeled anti-TN-C antibody. SiR-NIRs are expected to be useful as labeling tools for a wide range of in vivo imaging studies including multicolor imaging, and also as a novel scaffold for NIR fluorescence probes. Development of NIR fluorescence probes based on these dyes is in progress.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis, experimental details and characterization of SiR-NIRs, and results of imaging experiments in a mouse tumor model. 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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