VIP Imaging Probes

DOI: 10.1002/ange.200701491

Squaraine Rotaxanes: Superior Substitutes for Cy-5 in Molecular Probes for Near-Infrared Fluorescence Cell Imaging**

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Recent breakthroughs in fluorescence microscopy are producing notable improvements in imaging resolution.[1] These technical advances should have a major positive impact on the emerging field of cell imaging with single-molecule methods and help merge the subdisciplines of cell and molecular biology.[2] However, the full potential of many of these new high-resolution imaging methods will only be achieved if they employ extremely bright and highly stable luminescent probes that do not undergo photobleaching or photoblinking.

Luminescent probes can be grouped into two major classes: synthetic systems, such as organic dyes, inorganic nanoparticles, and lanthanide coordination complexes,[3] and biological systems, such as fluorescent and bioluminescent proteins.[4] It is unlikely that any one luminescent system will emerge as the universal solution for all optical applications, and each class warrants further development. Herein we focus on fluorescent organic dyes that emit in the near-infrared (NIR) region (650–900 nm). NIR dyes are particularly attractive for fluorescence imaging of biomedical samples because there is very little undesired absorption and autofluorescence by common biomolecules, and diminished Rayleigh–Tyndall scattering of light.[5] Indeed, wavelengths above 650 nm can penetrate through skin and tissue,[6] and fluorescent NIR imaging probes are increasingly used for in vivo optical imaging of live animals.[7] At present, the most commonly used fluorescent NIR dyes are sulfonated carbocyanine dyes (Cy-5, Cy-5.5, Cy-7) and their derivatives.[8] Although they are quite popular, the performance of Cy dyes is limited by molecular properties such as moderate to poor photostability, undesired reactivity with nucleophiles, and a propensity to self-quench upon dye aggregation.[9] Furthermore, cyanine dyes are known to undergo photoblinking[10] and light-driven fluorescence switching,[11] which makes them inherently unsuitable as FRET acceptors in single-molecule studies (FRET = fluorescence resonance energy transfer).[12] New classes of high-performance NIR fluorophores are under development by various commercial and academic research groups around the world.[13,14] In particular, significant progress has been made with Bodipy,[15] and Rylene[16] dyes, which exhibit excellent photostability; however, most of these fluorophores have not been incorporated into molecular probes for cell imaging.

Our interest is in squaraine dyes, which have very attractive photophysical properties for biological imaging.[17] However, they suffer from two important limitations: chemical instability as a result of attack by strong biological nucleophiles and a tendency to form nonfluorescent self-aggregates. We have discovered that both problems can be greatly attenuated by encapsulating the dye as the thread component inside an interlocked rotaxane structure.[18] The synthetic strategy to produce squaraine rotaxanes has already been described;[19] the key reaction is a templated Leigh-type macrocyclization that connects five building blocks in a single step. X-ray crystal structures show that the surrounding macrocycle sits perfectly over both faces of the electrophilic cyclobutene core of the squaraine thread and blocks nucleophilic attack.[20] The steric protection provided by the surrounding macrocycle also explains why there is no aggregation-induced broadening of absorption or self-quenching of fluorescence. Even when aggregated, the inner squaraine chromophores are unable to get close enough to interact. Furthermore, non-halogenated squaraine rotaxanes are poor photosensitizers of singlet oxygen and do not react readily with it;[21] thus, squaraine rotaxanes are likely to be non-phototoxic and highly resistant to photobleaching. Herein, we demonstrate that squaraine rotaxanes are versatile, high-performance molecular probes for in vitro and in vivo fluorescence imaging of cells. Three different types of probe structures are shown to target different cellular locations, namely, the organelle membrane, the internal aqueous phase, and the exterior cell surface.

Squaraine rotaxanes **1**–**4** have absorption and emission profiles that closely match the Cy-5 chromophore in control compound **5** (Table 1). Thus, all probes can be conveniently imaged by using an epifluorescence microscope and a standard Cy-5 filter set (exciter: HQ620/60X, dichroic: 660LP, emitter: HQ700/75m). We find that the relatively nonpolar squaraine rotaxane **1** interacts with cells in a very similar way to the well-known lipophilic stain Nile red.[22] As shown in Figure 1, probe **1** rapidly accumulates at lipophilic sites inside a living cell, such as the endoplasmic reticulum

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[**] We warmly thank the NIH (GM059078 and P50 CA94056) and Notre Dame CEST for funding support.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

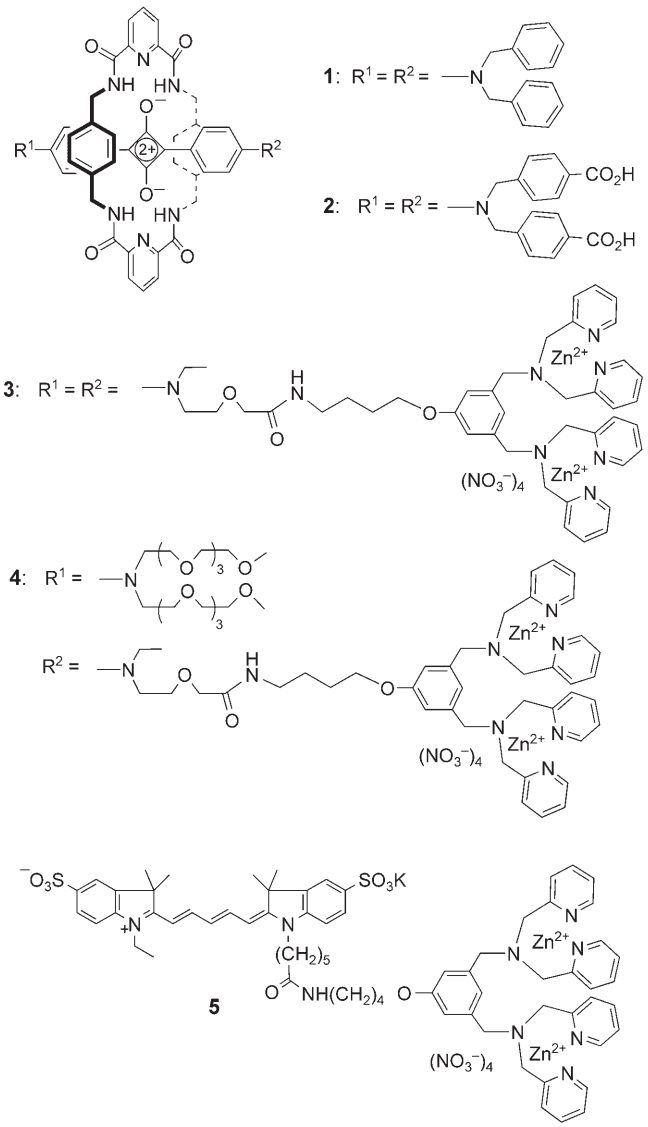

Table 1: Photophysical properties in water.

Compound	λ_{abs} [nm]	λ_{em} [nm]	Φ_f[d]
1[a]	639	659	0.70
2[b]	653	675	0.25
3[c]	653	675	0.20
4	650	669	0.08
5	648, 603	671	0.25

[a] Measurements were carried out in THF. [b] Excited-state lifetime in water was found to be 1.5 ns. [c] Molar absorptivity (ε) in water was found to be 121.052 M^{-1} L ($\log\varepsilon = 5.1$). [d] Solutions were excited at optically matching wavelengths and emission monitored in the region 600–900 nm. Fluorescence quantum yields were determined using 4,4-[bis(*N*,*N*-dimethylamino)phenyl]squaraine dye as the standard ($\Phi_f = 0.70$ in $CHCl_3$); error limit ±5%.

(ER) and intracellular lipid droplets. For example, probe localization with Chinese hamster ovary (CHO) cells is primarily in the ER (Figure 1A), whereas, with human lung carcinoma (A549) cells, uptake is mainly in lipid droplets (Figure 1B). Confirmation of these localization sites was

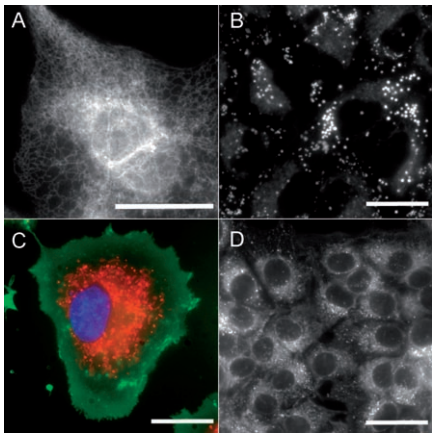

Figure 1. Fluorescence-microscopy images of live mammalian cells treated with **1**: A) Endoplasmic-reticulum staining of a single CHO cell. B) Lipid-droplet staining in A549 cells. C) Multicolor image of a single COS-7 cell treated with H33342 (blue), FM1-43 (green), and **1** (red). D) MCF7 cells 144 h after a single treatment with **1**. Single-color images were left in grayscale to preserve image detail and contrast. Scale bars corresponds to 20 μm.

obtained by co-staining the lipid droplets with Nile red, and the ER with blue-emitting ER-Tracker Blue-White DPX (Molecular Probes)[23] (see the Supporting Information). The red emission band for probe **1** is quite narrow and permits the acquisition of multicolor images. For example, shown in Figure 1C is a monkey kidney (COS-7) cell that has been treated with the DNA stain H33342 (blue), a lipophilic stain administered to label the plasma membrane (FM1-43, green), and probe **1** (red). Figure 1D illustrates the high chemical stability and low toxicity of **1**. Even after eight days, the staining pattern in live human breast cancer (MCF7) cells remains the same as obtained on day one, and the fluorescence intensity remains strong in all cells; thus, it appears that the dye is transferred efficiently to subsequent daughter cells. Another notable feature with probe **1** is that its staining is unaffected by cell fixation with paraformaldehyde (see the Supporting Information).

The tetracarboxylic acid squaraine rotaxane **2** is soluble in solutions at physiological pH and acts as an excellent fluorescent marker of encapsulated aqueous phases inside living cells. For example, treatment of COS-7 cells with **2** leads to accumulation of the dye in small punctate compartments within the cell that are trafficked quite rapidly. The high photostability of **2** allows this fascinating trafficking process to be monitored as a real-time movie, with constant sample irradiation, over many minutes (see the Supporting Information). This type of movie cannot be acquired with currently available NIR fluorescent probes, such as the amphiphilic styryl dye FM4-64 and water-soluble dextran Alexa-647 conjugate, because they are rapidly photobleached.[16]

Fluorescent probes **3**–**5** have appended zinc(II)–dipicolylamine (Zn–DPA) coordination centers as cell-targeting ligands. We have recently demonstrated that fluorescent conjugates with attached Zn–DPA groups will selectively associate with the anionic surfaces of bacterial cells in preference to the zwitterionic membrane surfaces of healthy

animal cells.[24] To compare the targeting ability and photostability of a squaraine rotaxane directly with a related Cy-5 probe, we prepared three fluorophore Zn–DPA conjugates; squaraine rotaxane **3** with two Zn–DPA targeting ligands, squaraine rotaxane **4** with one Zn–DPA targeting ligand, and Cy-5 probe **5** with one Zn–DPA targeting ligand. As expected, all three probes strongly associate with the periphery of bacterial cells, which allowed photobleaching experiments to be conducted. Three separate samples of bacterial cells (*E. coli*), each stained with equal amounts of probes **3**, **4**, or **5**, were irradiated continuously with light ($\lambda = (620 \pm 30)$ nm) from an X-cite 120 fluorescence illumination system through a Nikon TE2000-U epifluorescence microscope. The observed photobleaching half-lives were: 1080 s for **3**, 197 s for **4**, and 11 s for Cy-5 conjugate **5**. Thus, direct comparison between squaraine rotaxane **4** and Cy-5 probe **5** suggests that the squaraine rotaxane is almost 20 times more photostable. The additional fivefold increase in half-life with doubly valent squaraine rotaxane **3** is attributed to its stronger cell-surface affinity, leading to a slower off rate for the probe. In other words, there is diminished signal loss with **3** as a result of slower probe diffusion away from the cell surface.

The remarkable stability of probe **3** permits fluorescence-imaging experiments that are impossible with probes based on NIR cyanine dyes. For example, we have acquired real-time fluorescence-microscopy movies of bacteria undergoing cell division. Shown in Figure 2 is a montage of images at

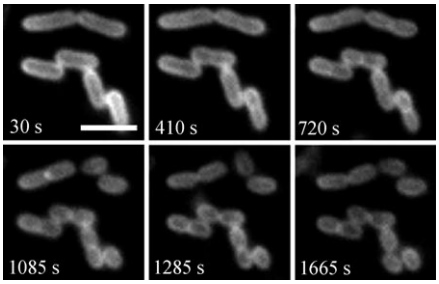

Figure 2. Binary fission of *E. coli* cells stained with **3**. The cells were imaged every 5 s for 30 min by using fluorescence microscopy. The times stated in each panel correspond to the movie time point and the scale bar represents 2 μm.

various time points from a 30-minute movie that monitors binary fission of *E. coli* cells stained with **3**. The morphological changes associated with binary fission, such as formation of the septum between the daughter cells, are easily resolved. These microscopy images of cell division highlight the extreme photostability of squaraine-rotaxane probe **3** and also its low phototoxicity. Since the NIR fluorescence from probe **3** can penetrate through the skin and tissue of a living animal, it can be used to image bacteria in vivo. To demonstrate this imaging property, separate populations of *E. coli* and *S. aureus* bacteria were incubated with **3**, washed, and then injected subcutaneously near the posterior thigh muscles of a living nude mouse. A fluorescence image of the entire animal was subsequently acquired by using a Kodak 4000MM fluorescence-imaging station. As illustrated in Figure 3, both sites of bacterial inoculation are very apparent, because the fluorescence emission intensities are about 100 times greater than the background signal from other anatomical parts of the mouse.

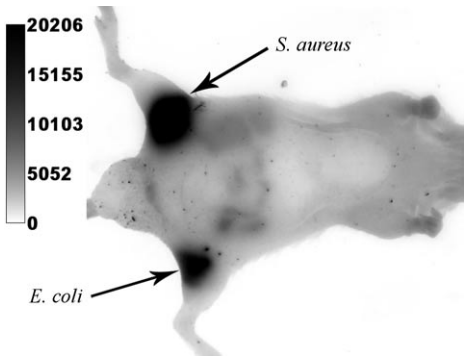

Figure 3. Optical image of a live mouse with subcutaneous injections of *S. aureus* and *E. coli* that were prelabeled with **3**. The entire animal was irradiated with filtered light at $\lambda = (625 \pm 40)$ nm and an image of emission intensity at $\lambda = (670 \pm 20)$ nm was collected by a CCD camera during a 5-s acquisition period.

In summary, squaraine-rotaxane dyes can be readily converted into extremely bright and highly stable NIR fluorescent probes for in vitro and in vivo optical imaging of live and fixed cells. The probes can be structurally modified for targeting of quite different cellular locations. Squaraine rotaxanes are likely to be superior substitutes for Cy-5 in many biotechnology and imaging applications that require NIR fluorescent dyes.

Received: April 5, 2007
Published online: June 22, 2007

Keywords: cell recognition · dyes/pigments · fluorescence · imaging agents · rotaxanes

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