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Fluorescent Probes

Deoxyribonucleoside-Modified Squaraines as Near-IR Viscosity Sensors

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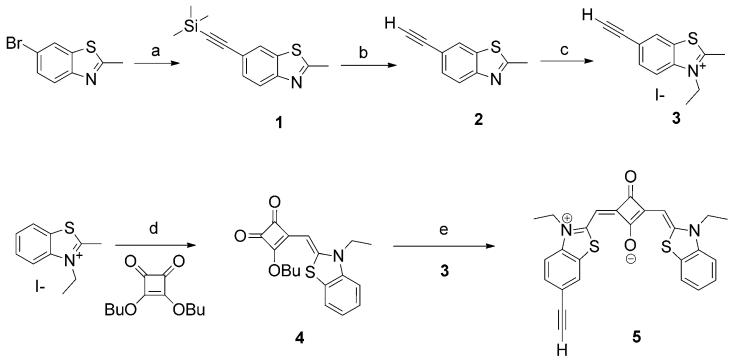
Abstract: Deoxyribonucleoside-modified squaraines were synthesized by Sonogashira coupling reactions using an unsymmetrical, terminal alkynylated benzothiazolium squaraine dye. These non-natural nucleosides exhibited fluorescent 'turn-on' properties in viscous conditions with an enhancement of $>$300-fold. The viscosity-dependent fluorescence enhancement was attributed to a combination of hampering both molecular aggregation and intramolecular bond rotation of the squaraine probe. Fluorescence microscopy allowed visualization of highly viscous regions during various stages of cellular mitosis.

Visualizing viscous regions at the cellular and organism levels are of substantial interest, in part due to potential strategies for disease detection.[1] Fluorescence sensors with enhancements on the order of 30-fold were achieved in viscous environments. This fluorescence 'turn-on' behavior was explained by a so-called "molecular rotor" mechanism,[2] in which the nonradiative rotational and/or vibrational processes in the excited state can be confined in a viscous medium, and consequently increase the fluorescence quantum yield. Based on this theory, a number of fluorescent 'turn-on' viscosity probes were developed with various chromophores for intracellular viscosity studies,[3] including amino cinnamonitrile, porphyrin, cyanine, and BODIPY.

Our interests include squaraine chromophores as near-IR emitting dyes for bioimaging,[4] photovoltaics,[5] and supramolecular assembly through liquid-crystal-mediated and polyelectrolyte-templated self-assembly.[6] Particularly interesting properties of squaraine dyes are their absorption and emission in the far-red to near-IR,[7] a range that is beyond the self-absorption and scattering of biomolecules and an attractive window for use as sensors and probes in biological media.[8] In addition, due to the generally hydrophobic nature of squaraine dyes, they exhibit a strong tendency to form nonfluorescent aggregates.[9] This characteristic was utilized to enhance the fluorescence response to analytes, since a dark background can be achieved by starting with nonfluorescent aggregates.[10] Recently, by studying the photophysical properties of an indolium squaraine dye in different solvent systems, solvent polarity dependent emission was found and the lowest rate constant of nonradiative deactivation (k_{nr}) was obtained in a viscous alcohol solvent.[11] However, further biological applications may be hindered by poor water solubility.

Modifying hydrophobic chromophores using biomolecules is of particular interest,[12] particularly the use of nucleosides as building blocks.[13] In addition to enhanced water solubility and reduced toxicity, a modified compound may have a number of intriguing applications in chemistry and biology.[14] Herein, we report deoxyuridine and deoxyadenosine-modified squaraine analogues synthesized by Sonogashira coupling reactions. The hydrophilic and biocompatible properties of deoxyribonucleosides were incorporated into the new molecules. Both squaraine aggregation and molecular rotor mechanisms facilitated achieving over 300-fold fluorescence enhancement in viscous media. Preliminary demonstration of the new compounds to probe viscosity at cellular levels was conducted by in vitro fluorescence microscopy studies.



Scheme 1. Synthesis of alkyne-functionalized benzothiazolium squaraine dye **5**. a) [Pd-(PPh_3)$_2$$Cl_2$], CuI, CH_3CN, triethylamine (TEA), pyridine, trimethylsilylacetylene, 50 °C; b) NaOH, CH_2Cl_2, MeOH; c) microwave reaction, CH_3CH_2I, CH_3CN, 110 °C, 120 min; d) squaric acid dibutyl ester, EtOH, TEA, reflux; e) i) EtOH, NaOH, ii) HCl, iii) *n*BuOH, pyridine, **3**.

The synthesis of the alkyne-containing benzothiazolium squaraine dye **5** was achieved as shown in Scheme 1. 5-Ethynyl-3-ethyl-2-methylbenzothiazolium iodide (**3**) was synthesized

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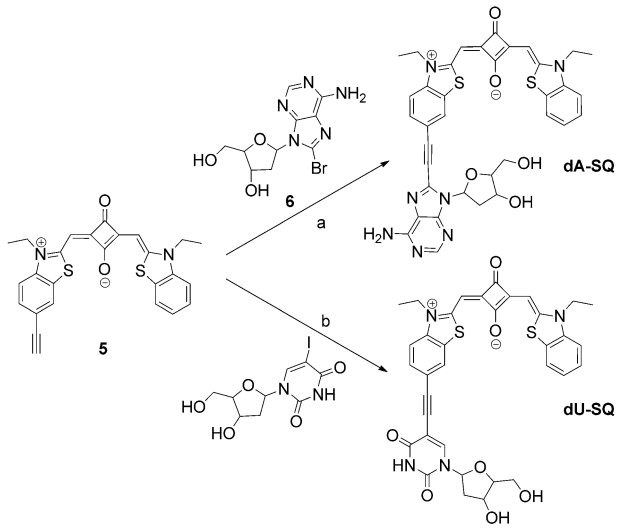
from 5-bromo-2-methylbenzothiazole in three steps. Substitution of bromine by the ethynyltrimethylsilyl group afforded compound **1** in excellent yield (91%). After removal of the $SiMe_3$ group under basic conditions, **2** was alkylated with iodoethane in a microwave reactor at 110 °C for 2 h, providing **3** in 38% yield. Accordingly, hemisquaraine **4** was prepared following reported procedures,[15] that is, by refluxing a mixture of dibutyl squaraine ester with 3-ethyl-2-methylbenzothiazolium iodide in ethanol. Reaction of hemisquaraine **4** with the alkynylated benzothiazolium **3** afforded the alkyne-containing squaraine dye **5** in 35% yield. HRMS spectra indicated a strong tendency of **5** to form aggregates,[16] with $[M+H]^+ = 457.1039$, $[2M+Na]^+ = 935.1825$, and $[3M+Na]^+ = 1391.2744$.

Nucleoside conjugates with fluorescent compounds through chemical linkage at the C-position of bases are receiving particular interest nowadays for a number of applications.[17] Among the collection of methods that can achieve nucleoside C-modification, Pd-catalyzed coupling reactions have proven to be practical.[18] The deoxyribonucleoside precursors can exist with or without protected hydroxyl groups. Even though deoxyribonucleosides with protected hydroxyl groups exhibit better solubility in normal organic solvents, deprotection is required after the coupling reactions, and, consequently, lead to more transformations and, all too often, low overall yield. An alternative strategy is to conduct the coupling reaction with unprotected nucleosides in very polar solvents, a more efficient strategy.[19]

In our case, deoxyadenosine monohydrate was first converted to 8-bromo-deoxyadenosine (**6**) by using bromine in NaOAc/AcOH buffer.[20] The squaraine and deoxyadenosine moieties were linked together through a triple bond construct by Sonogashira coupling (Scheme 2). Both 5′-OH and NH_2 groups of 8-bromo-deoxyadenosine were unprotected, and reaction conditions were optimized (see Supporting Information). In general, the best conditions for this coupling reaction



Scheme 2. Synthesis of squaraine-modified deoxyribonucloside analogues by Sonogashira coupling reaction: a) $[Pd(PPh_3)_4]$, CuI, *N*,*N*-diisopropylethylamine (DIPEA), DMF; b) $[Pd(PPh_3)_4]$, CuI, Amberlite IRA-67, DMF.

was using DIPEA as base and 20 mol% CuI in DMF. After purification by column chromatography, deoxyadenosine-modified squaraine **dA-SQ** was obtained in 11% yield. Similarly, **dU-SQ** was obtained by Sonogashira coupling between (+)-5-iodo-2′-deoxyuridine and alkynylated squaraine **5**, using Amberlite IRA-67 as base and 20 mol% CuI, in 14% yield after purification by column chromatography.

In DMSO solution, **dA-SQ** and **dU-SQ** exhibited identical maximum absorption at 681 nm and an emission maximum at 695 nm. However, once water was introduced to the system, H-aggregation occurred along with fluorescence quenching. (Supporting Information, Figures S1 and S2). Photophysical properties of **dU-SQ** in variable DMSO/water concentrations were then studied. Consistent with previous photophysical studies of indolium squaraine dyes,[11,21] for **dU-SQ**, a linear correlation was observed between the wavelength of absorption maxima and the Bayliss function and Lorentz–Lorenz function, in the nonaggregate domain window, exhibiting solvent-polarity-dependent properties (Supporting Information, Figures S3 and S4). Such solvent-polarity influence, plus the contribution of water-induced H-aggregation, contributed to a 30-fold fluorescence enhancement in DMSO/water mixtures with high DMSO proportions. In addition, the impact of aggregation on emission intensity was studied for **dU-SQ** in a DMSO/water (1:3, v/v) mixture. Dissociation of H-aggregation as a function of temperature led to an approximately 10-fold fluorescence-intensity enhancement (Supporting Information, Figure S5).

When **dU-SQ** was dissolved in glycerol/methanol mixed solvents with variable proportions of the alcohols, no significant polarity-induced shift in either absorption or emission maximum wavelength was observed (Figure 1). However, a prominent increase in emission intensity with increasing viscosity (by increasing the volume ratio of glycerol) was observed. This phenomenon is likely related to the "molecular-rotor" mechanism,[2] as both aggregation and solvent-polarity effects do not occur under these conditions.

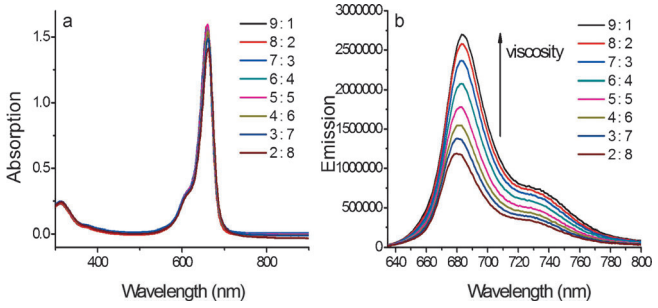


Figure 1. a) Absorption and b) emission spectra of **dU-SQ** ([dU-SQ] = 1.0×10^{-5} M) in methanol with varying glycerol content. Volume ratios of glycerol/methanol varied from 2:8 to 9:1. Excitation wavelength was 625 nm.

The photophysical properties of the squaraine compound before conjugation with the deoxyribonucleosides were also investigated. Relative to **dU-SQ**, identical behavior was observed for alkynylated squaraine **5** in glycerol/methanol mixed

solvents (Supporting Information, Figure S6), whereas similar properties in DMSO/water solutions were also observed (Supporting Information, Figures S7 and S8). However, it is worth noting that the aggregation tendency of **dU-SQ** was much stronger than **5** in DMSO/water mixtures, as evidenced by their absorption spectra; for example, 50% water can lead to obvious H-aggregation of **dU-SQ** but 80% water was needed to trigger the same behavior in **5**. This is likely due to the more planar structures and extended π-systems after conjugating with the deoxyribonucleosides.

In addition, because of the highly hydrophobic zwitterion structure, **5** was insoluble in water and precipitation occurred in glycerol/water mixtures, which hampered further biological applications. However, after chemically linking the squaraine to hydrophilic deoxyribonucleosides, the squaraine analogs **dA-SQ** and **dU-SQ** were more soluble in polar protic solvents. Hydrogen bonding between deoxyribonucleoside and solvent molecules likely facilitates this aqueous solubility.

In glycerol/water mixed solvents, negligible impact of solvent polarity was evident. However, viscosity-dependent optical behaviors were observed for both deoxyribonucleoside-modified squaraines. When the viscosity of the solution increased from 1.7 cP (20% glycerol, v/v) to 209 cP (90% glycerol, v/v) at 25 °C,[22] the absorption spectra of **dU-SQ** changed as viscosity increased, with the monomer band increasing with a concomitant decrease of the H-aggregate band. Meanwhile, when excited at 625 nm, the fluorescence intensity of **dU-SQ** was greatly enhanced (310-fold) in highly viscous solvents and the quantum yield reached 0.16 in 90% glycerol solution (Figure 2), a relatively high value for a squaraine dye in a polar

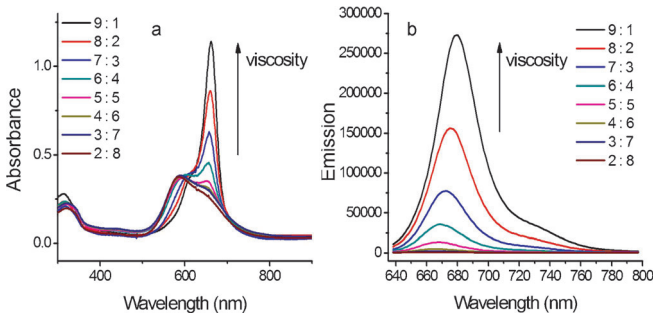


Figure 2. a) Absorption and b) emission spectra of **dU-SQ** ([dU-SQ] = 1.0×10^{-5} M) in glycerol with varying water content. Volume ratio of glycerol/water varied from 9:1 to 2:8. Excitation wavelength was 625 nm.

or aqueous solvent system. **dA-SQ** exhibited the same behavior, with a nearly 300-fold increase in fluorescence intensity in 90% glycerol solution and fluorescence quantum yield of 0.12 (Supporting Information, Figure S9).

Furthermore, at low viscosities (from 2:8 to 4:6 glycerol/water), at which there was little change in the H-aggregates according to the absorption spectra, the increase in fluorescence intensity was still very clear (Figure 2 and Supporting Information, Figure S9). Thus, this 'turn-on' fluorescence in glycerol/water mixtures can be attributed to impeding the aggregation of the squaraine moiety while slowing down molecular rotation.[3] For normal "molecular-rotor-mechanism"-based viscosity sensors, their viscosity-dependent fluorescence intensity can be described by the Förster–Hoffmann equation.[23] Even though aggregation effects are added to the "molecular-rotor mechanism" for deoxyribonucleoside-modified squaraines in glycerol/water solvents, log–log analysis of the emission intensity data as a function of solvent viscosity generated acceptable linear relationships for **dU-SQ** with $R^2 = 0.93$ and **dA-SQ** with $R^2 = 0.94$ (Supporting Information, Figures S10 and S11).

dU-SQ was later selected to perform viscosity-sensing experiments at the cellular level because of its higher fluorescence compared with **dA-SQ**. Since the biological environment is complex within cells, and some squaraine structures have shown enhanced emission upon addition of serum albumin[24] and lipid membranes,[4b,25] studies of the interaction of **dU-SQ** with biomolecules were performed in the presence of calf thymus DNA, bovine serum albumin, and biomimetic conditions (e.g., liposomes and pluronic micelles), and none of them resulted in a significant change in the optical properties of the dye (Supporting Information, Figure S12).

In some intra- and intercellular regions, high viscosity values have been reported, which were estimated to be 100–400 cP.[3c] We hypothesized that if the dye is in high viscosity regions, fluorescence enhancement of **dU-SQ** will result with bright emission signals collected from a dark background. After incubation with HTC 116 (human colorectal cancer cells) for 1 h, **dU-SQ** appeared to readily enter the cells, yielding clear fluorescence images (Figure 3 and Supporting Information, Figure S13). Hoechst was used as a nuclear stain to visualize the cell nucleus for reference.

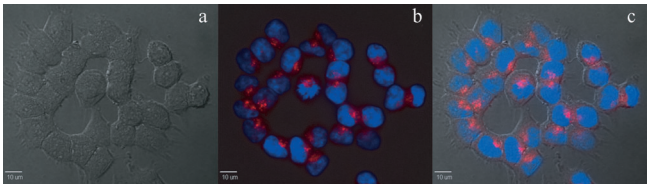


Figure 3. Images of HCT 116 cells incubated with **dU-SQ** (10 μM) and Hoechst (5 μg mL^{-1}): a) DIC, b) overlay of fluorescence microscopy images of **dU-SQ** (red, Ex 562/40, DM 593, Em 654/40) and Hoechst (blue, Ex 377/50, DM 409, Em 460/50), c) overlay of (a) and (b). Scale bars = 10 μm.

High viscosities may be generated during mitosis and in the vicinity of microtubules (MTs).[26] Recent studies showed that the viscosity was dependent on MT crosslinking and density.[27] In order to confirm this, COS 7 (monkey kidney tissue cells) were incubated with **dU-SQ** and Hoechst. MTs were stained (by incubation with primary antibody anti-Vinculin mouse mAb and subsequent staining with FITC-labeled anti-mouse goat IgG) and different stages of mitosis were captured. During metaphase and anaphase stages the viscosity appears higher than telophase, as determined by the intensity (brightness) of the fluorescence (viscosity-dependent fluorescence enhancement), as shown in Figure 4 and Figure S14 in the Supporting Information. Figure 4 presents micrographs at different stages of mitosis, showing the DIC imaging, nuclear and MT staining,

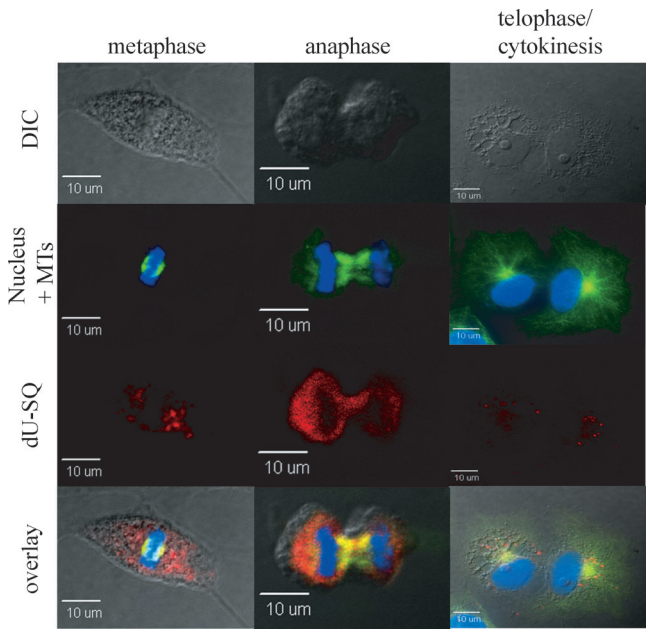


Figure 4. Fluorescence microscopy images of COS 7 cells incubated with **dU-SQ** (10 μM) and Hoechst (5 μg mL^{-1}) followed by microtubule staining. Nucleus stained with Hoechst (blue, Ex 377/50, DM 409, Em 460/50), microtubules stained with FTIC-secondary antibody - anti-mouse goat IgG (green, Ex 477/50, DM 507, Em 536/40) and **dU-SQ** (red, Ex 562/40, DM 593, Em 654/40). Scale bars = 10 μm.

the **dU-SQ** viscosity probe, and finally, an overlay of all three sets of images for each stage. The location of the bright-red fluorescence from **dU-SQ** was primarily in the vicinity of spindle poles and/or locations where the MTs were mostly condensed and crosslinked, indicative of regions of higher viscosity.

In conclusion, deoxyribonucleoside-modified squaraine derivatives were prepared and characterized. Through the squaraine chromophore, these non-natural deoxyribonucleosides exhibited absorption and emission in the far-red to near-IR region, a region very attractive for in vitro and in vivo imaging. Both **dA-SQ** and **dU-SQ** probes exhibited viscosity-dependent properties in glycerol/water and glycerol/methanol mixed solvents. With the combined effect of reducing aggregation and intramolecular rotation in glycerol/water systems, >300-fold fluorescence enhancement in high viscosity environments was achieved, the highest viscosity-dependent enhancement reported thus far for chromophore-based (vs. nanoparticle-based) fluorescent viscosity sensors. Significantly, in vitro investigations provided near-IR fluorescence viscosity sensing at the cellular level, allowing visualization of different stages of mitosis and highly viscous regions of microtubules. Hence, these deoxyribonucleoside-modified squaraine analogues are a promising class of near-IR probes and viscosity sensors for bioimaging.

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Keywords: aggregation · deoxyribonucleosides · near-IR imaging · squaraine dyes · viscosity sensors

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