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SQUARAIN DYES, DESIGN AND SYNTHESIS FOR VARIOUS FUNCTIONAL MATERIALS APPLICATIONS

by

YUANWEI ZHANG
M.S. Nankai University, 2008

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Science at the University of Central Florida Orlando, Florida

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Major Professor: Kevin D. Belfield
ABSTRACT

This dissertation contains the synthesis and characterization of squaraine based new functional materials. In the first part of this thesis work, a water soluble benzothiazolium squaraine dye was synthesized with pyridium pendants, and controlled aggregation properties were achieved. After formation of partially reversible J-aggregation on a polyelectrolyte (poly(acryl acid) sodium salt) template, the nonlinear, two-photon absorption cross section per repeat unit was found to be above 30-fold enhanced compared with nonaggregate and/or low aggregates. Using a similar strategy, sulfonate anions were introduced into the squaraine structure, and the resulting compounds exhibited good water solubilities. A ‘turn on’ fluorescence was discovered when these squaraine dyes interacted with bovine serum albumin (BSA), titration studies by BSA site selective reagents show these squaraine dyes can bind to both site I and II of BSA, with a preference of site II. Introduction of these squaraine dyes to BSA nanoparticles generated near-IR protein nano fabricates, and cell images were collected. Metal sensing properties were also studied using the sulfonates containing a benzoindolium squaraine dye, and the linear response of the absorption of the squaraine dye to the concentration of Hg$^{2+}$ makes it a good heavy metal-selective sensing material that can be carried out in aqueous solution. Later, a squaraine scaffold was attached to deoxyribonucleosides by Sonogashira coupling reactions, in which the reaction conditions were modified. Iodo-deoxyuridine and bromo-deoxyadenosine were used as the deoxyribonucleosides building blocks, and the resulting squaraine dye-modified deoxyribonucleosides exhibited near-IR absorption and emission properties due to the squaraine chromophore. Interestingly, these non-natural deoxyribonucleosides showed viscosity dependent photophysical properties, which make them nice candidates for fluorescence viscosity sensors at the cellular level. After incubation with cells, these
viscosity sensors were readily uptaken by cell, and images were obtained showing regions of high viscosity in cells.
To my parents and family
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CHAPTER 1. BACKGROUND OF SQUARAINE DYSES

1.1 General Introduction

Squaraines are a family of chromophores containing structures such as cyanine dyes, two donor groups conjugated to an electron deficient oxocyclobutenolate core, leading to a highly electron delocalized structure that can be exemplified as zwitterions. Generally, squaraine dyes with donor-acceptor-donor (D-A-D) structures are synthesized by the condensation reaction of 3,4-dihydroxy-3-cyclobutene-1,2-dione (squaric acid) with activated aromatic or heterocyclic components.\(^1\) Figure 1-1 shows the synthesis of aniline based symmetrical squaraine dyes with the corresponding zwitterionic structures. In 1965, Treibs and Jacob obtained a red-violet solid when they reacted squaric acid with pyrrole, which is the first report related to squaraine dye synthesis.\(^2\) Later, this class of dyes was named “squaraine” by Schmidt.\(^3\) Since then, numerous studies have been reported in the 1990s on squaraine dyes, covering various areas from synthetic methods and reaction mechanisms to physical properties.\(^4\)-\(^6\)

\[\begin{array}{c}
\text{R} \quad \text{N} \quad \text{aryl} \\
\text{R} \quad \text{O} \quad \text{O} \\
\text{OH} \quad \text{OH} \\
\end{array} \quad \text{R} \quad \text{N} \quad \text{aryl} \\
\text{R} \quad \text{O} \quad \text{O} \\
\text{OH} \quad \text{OH} \]

Figure 1-1: Synthesis of symmetrical aniline-based squaraine dyes and zwitterions resonance forms.

Due to their planar structures and zwitterionic properties, squaraine dyes exhibit strong absorption (\(\varepsilon > 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}\)) and emission in the near-IR region. By modifying the aromatic or heterocyclic donor moiety (e.g., N,N-dialkylanilines, phenols, pyrroles, indoles, benzothiazoles and quinolones) it is easy to modify the chromophore structure to tune the optical properties.\(^7\) A large number of squaraine dyes have been synthesized with different donor groups producing absorption and emission in the range of 550 to 850
Due to the advantage of intensive absorption with tunable wavelength, squaraine dyes are very good candidates for diverse applications, such as colorimetric sensors,\textsuperscript{9-10} nonlinear optical devices,\textsuperscript{11,12} and organic solar cells.\textsuperscript{13,14} In addition, the rigid and planar conjugation structure makes squaraine dyes suitable for applications of aggregation related physical properties.\textsuperscript{15-20} More important, the near-IR absorption and emission, which are outside the self-absorption and self-fluorescence region of biological media, makes squaraine dyes nice chromophores for studies associated with photodynamic therapy,\textsuperscript{21,22} bio-imaging, and biochemical labeling.\textsuperscript{23-27}

1.2 Synthesis of Squaraine Dyes

1.2.1 Symmetrical Squaraine Dyes

The starting material for squaraine synthesis, squaric acid (diketocyclobutenediol), is a colorless solid at room temperature, which was first synthesized by Cohen and coworkers, and a symmetrical resonance stabilized anion form ($\text{C}_4\text{O}_2^-$) was proposed.\textsuperscript{28} Using Raman and infrared spectroscopy, Ito and West confirmed the flexible bonded dianion structures of squaric acid in which the C-C bonds may be treated as bent bonds.\textsuperscript{29} Due to the resonance stabilization of the anionic structure, squaric acid exhibits a highly acidity behavior, with pKa = 2.2.

![ Structures of squaraine dyes 1-3 synthesized from pyrrole, azulene and dimethylaniline, respectively, along with their maximum absorption wavelengths in CHCl$_3$.](image)

Figure 1-2: Structures of squaraine dyes 1-3 synthesized from pyrrole, azulene and dimethylaniline, respectively, along with their maximum absorption wavelengths in CHCl$_3$. 
Shortly after the first synthesis of red-violet squaraine dye 1 from the condensation of squaric acid and pyrroles, Ziegenbein and Sprenger added more structures to the squaraine family by replacing pyrrole with azulene and dialkylaniline, which generated compounds 2 and 3 (Figure 1-2). These condensation reactions were carried out in n-butanol/benzene mixed solvent, meanwhile the water generated was removed from the refluxing system by azeotropic distillation; this protocol later became a classical way for synthesizing symmetrical squaraine dyes. Under these reaction conditions, the condensation mechanism was suggested by Sprenger and Ziegenbein, Scheme 1-1. Briefly, squaric acid is first activated by n-butanol and formed monobutyl squarate upon losing one molecule of water, followed by nucleophilic attack of an activated aromatic compound (Ar-H). The arylated squaric acid intermediate loses one n-butanol, and later a second aromatic molecule attacks the electron deficient carbonyl carbon. The target squaraine dye product was obtained after the second dehydration procedure. Using 2-propanol as the main solvent instead of n-butanol, with an additional 3 equivalents of tributylorthoformate, Law et al. reported a higher yield synthetic method. Other reactions (to produce, e.g., N,N-dimethylaminophenyl and 4-methoxyphenyl squaraines) have been studied and the results shown for this method were better than the one reported by Sprenger.

Scheme 1-1: Suggested mechanism for squaraine formation in n-BuOH/benzene mixed solvent.
1.2.2 Unsymmetrical Squaraine Dyes

According to structures of squaraine dyes, they can be classified into two groups, symmetrical and unsymmetrical squaraines. Symmetrical squaraines have the same electron-donating groups on each side of the oxocyclobutenolate core, so the synthesis involves 2 equivalents of electron-rich aromatic or heterocyclic compounds and 1 equivalent of squaric acid. Upon refluxing in n-butanol/benzene mixture, symmetrical squaraine can be obtained with high yields. Unsymmetrical squaraines, however, have different electron-donating groups on each side, thus the preparation is more complicated. In general, the synthesis and separation of the semi-squaraine intermediate is required and crucial in order to form unsymmetric squaraine dyes. There are two synthetic routes to afford unsymmetrical squaraine dyes, and both start from derivatives of squaric acid. The first strategy is using thionyl chloride\textsuperscript{34} or oxalyl chloride\textsuperscript{35} to prepare 3,4-dichloro-cyclobut-3-ene-1,2-dione,\textsuperscript{36} then reacting this with one equivalent of an activated aromatic compound, followed by immediate hydrolysis to obtain the intermediate semi-squaraine, as shown in Scheme 1-2. Then the semi-squaraine was allowed to react with a different aromatic compound and afford the final unsymmetric structure.

In another strategy, unsymmetric squaraine dyes containing more reactive N-alkylated heterocyclic structures, like alkyl-indolium, alkyl-benzothiazolium and alkyl-quinaldium, can be synthesized through the intermediate of 3,4-dialkoxy-cyclobut-3-ene-1,2-dione (Scheme 1-2).\textsuperscript{37,38} Activated heterocyclic compounds react with 3,4-dialkoxy-cyclobut-3-ene-1,2-dione in a 1:1 ratio in ethanol with triethylamine (TEA), resulting in the semi-squaraine intermediate. The semi-squaraine is then brought to reaction with another half heterocyclic moiety, generating the anticipated unsymmetric structure. However, the regioselectivity should not be ignored when condensing of semi-squaraine with the second equivalent of
donor, during which procedure minus of 1,2-condensation byproduct can be formed along with the desired 1,3-condensation product.

Scheme 1-2: Synthetic strategies to achieve unsymmetrical squaraine dyes.

Moreover, Jyothish et al. showed recently that one can achieve semi-squaraine and symmetrical squaraine depending on the electronic properties of the donors. For example, the quinaldium scaffold with electron-withdrawing groups (Br, I, CN and NO₂) make the heterocyclic ring electron deficient and the symmetrical product 4c was produced via the intermediate compound 4b. Meanwhile, electron-donating groups (OH and OEt) containing a quinaldium scaffold can only generate semi-squaraine intermediate compound 4a (Scheme 1-3). The different behavior on the same platform was explained by influencing the activity of the 2-methyl protons, which were involved in the nucleophilic attack steps. While electron-donating groups were attached, the acidity of the 2-methyl protons was reduced, which consequently lowered the proficiency in forming the final symmetrical squaraines. Meanwhile, when linked with electron-withdrawing groups, an opposite influence takes place. This detailed study of
squaraine dye synthesis controlled by electronic properties provided an alternative strategy of incorporating heterocyclic structures into the squaraine chromophore.\textsuperscript{40}

\textbf{Scheme 1-3:} The synthesis of semi-squaraines and symmetric squaraines dependent on the electronic properties of the quinaldium donors.

Starting from the semi-squaraines, condensation reaction conditions with a second moiety could be different, depending on the reactivity of the second donor. When an activated arene is used as the second donor, the best yield could be achieved by refluxing the reaction mixture in 2-propanol with the assistance of tributyl orthoformate as dehydration reagent. For example, semi-squaraine 3-hydroxy-4-(4-methoxyphenyl)cyclobut-3-ene-1,2-dione was used to condense with 3-fluoro-N,N-dimethylaniline under this condition and generated unsymmetric squaraine 5 in a yield of 60\%.\textsuperscript{41} In the case of anhydrobase (activated heterocyclic structures), which can be obtained both \textit{in situ} or \textit{ex situ}, the process of condensation was carried out in a mixed solvent system of either n-BuOH/toluene or i-PrOH/benzene and addition of organic base. For example, unsymmetric squaraine 6 was achieved in good yield (68\%) by using this method (\textbf{Scheme 1-4}).\textsuperscript{42}
Scheme 1-4: Example of synthesis unsymmetric squaraine from semi-squaraine.

**1.3 Physical Properties of Squaraine Dyes**

**1.3.1 Optical Properties**

Squaraine dyes exhibit a sharp and intense absorption band with high molar extinction coefficients ($\varepsilon > 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$).\(^{43,44}\) By varying donor moieties and increasing the $\pi$-conjugated systems, the absorption wavelength can cover the long wavelength visible to near-IR region from 550 to 850 nm.\(^{11,45}\) Characteristic narrow and strong absorption makes squaraine dyes ideal candidates for the application of optical materials that need very specific narrow wavelength. For example, in sensor design the sensitivity is the main concern, squaraine dyes can carry out this duty with changes of color or absorption intensities even at very low concentrations. Furthermore, squaraine dyes are fluorescent in organic solvents with moderate fluorescent quantum yields ($\Phi_f$) of ca. 0.30 in the wavelength range of 650 – 850 nm\(^{40}\) and a Stokes shift of 10–30 nm.

The research on squaraines as potential materials for two-photon absorption (2PA) applications was pioneered by Scherer et al. After systematic study of dimethylindolium squaraines, they reported two-photon absorption cross-section ($\delta_{2PA}$) as high as 5000 GM (Goppert-Mayer units, $1 \times 10^{-50} \text{ cm}^4 \text{s photon}^{-1}$).
molecules\textsuperscript{1}).\textsuperscript{46} Later, two-photon absorption properties of indolium squaraines were reported by Pagani et al. The structures of the squaraine compounds 7 (black), 8 (red), and their linear (lines) and two-photon absorption properties (data points and lines) were measured in DCM and the spectrum is shown in Figure 1-3. These chromophores exhibited efficient quadrupolar two-photon absorption in the range of 700–900 nm with δ\textsubscript{2PA} as high as 450 GM, which is impressive considering the short conjugated systems.\textsuperscript{47}

Figure 1-3: structures of indolium compounds 7 and 8. Their linear and nonlinear absorption spectrum. Ref. 47, copyright 2008 American Chemical Society.

Marder and coworkers later discovered that the δ\textsubscript{2PA} could be further amplified by extending the π-conjugated system. Pyrrolium squaraines with extended conjugation were achieved by adding more pyrrole and benzene to end capping the donor groups. The δ\textsubscript{2PA} value in THF reached 30,000 GM.\textsuperscript{48} In another study of porphyrin-squaraine-porphyrin triad, Marder et al. reported very high δ\textsubscript{2PA} data over a broad range of wavelengths.\textsuperscript{12} Our research group is interested in squaraine dyes for their applications in advanced photonic materials, and the two-photon absorption properties were measured for various derivatives. The structure of a π conjugation extended pyrrolium squaraine dye 9 is shown in Figure 1-4 along with it is linear and nonlinear absorption spectrum.\textsuperscript{27}
Figure 1-4: Structure of compound 9 and it is linear and nonlinear absorption spectrum. Ref 27, copyright 2011 American Chemical Society.

When squaraine dyes are dissolved in organic solvents, the chromophore interacts with the solvent molecules producing solute – solvent complexes. The interaction force is dependent on the D–A–D charge transfer character of the chromophore conjugate. In the solid state, however, the squaraine dyes like to assembled as aggregates and exhibit strong intermolecular charge transfer interactions, which led to the broad range of the absorption spectrum. This absorption spectrum broadening properties of solid state squaraine dyes have been utilized in dye-sensitized solar cells (DSSC).

1.3.2 Thermal Stability

In general, squaraine dyes are thermally stable, exhibiting melting and/or decomposition temperatures above 100 °C. Although, thermal properties are very important for applications such as photosensitizers for DSSC and photodynamic therapy, very limited studies has been reported about their thermotropic behaviors. Qaddoura and Belfield recently studied the aggregation and thermotropic behavior of a series of 2,4-bis[4-(N,N-dialkylamino)-2-hydroxyphenyl]squaraines. In this work they showed the length of the alkyl chain can greatly affect the thermal stability in the series. Higher decomposition temperatures were
associated with the squaraine structures possessing shorter alkyl chains while longer alkyl chains can weaken the intermolecular van der Waals’ and hydrogen bonding, forces, resulting in lower melting temperatures. For example, the compound with ethyl chains showed no detectable melting point before decomposition at 240 °C, while the derivative with propyl chains had a melting point at 221 °C. For longer alkyl chain compounds (butyl and heptyl) the melting temperature was 196 and 185 °C, respectively. The lowest melting point was observed for the hexyl squaraine derivative with a melting point of 135 °C.

1.4 Applications of Squaraine Dye Materials

1.4.1 Supramolecular Assembly of Squaraine Aggregates

Organic dyes are capable of forming supramolecular assemblies composed of multiple ordered subunits linked through non-covalent bonds, such as van der Waals forces, π–π stacking, hydrophobic and/or hydrophilic interactions, H–bonds, and electrostatic forces.\(^{49,50}\) This phenomenon is also common when biomolecules are concerned, in which H–bond and π–π stacking are very important interactions from DNA to proteins. Squaraine dyes are very likely to form H- or J-aggregates by strong π–π stacking interactions due to their rigid and planar zwitterions structures. However, it is still challenging to control and harness ideal self-assembled functional materials based on squaraine chromophores.\(^{7}\)

In order to use the properties of H- and J-aggregates in advanced material devices, one first needs to identify them. An easy method uses absorption spectroscopy, H-aggregates are characterized by shorter absorption wavelength absorption compared to the monomer band.\(^{51}\) While, aggregates that exhibit a narrow red-shifted band in contrast with the monomer band are generally termed J-aggregates.\(^{52,53}\) Dye molecules that aggregate in parallel with a plane-to-plane stacking are called H-aggregates while a head-to-tail stacking can generate J-aggregates. The different modes of assembling are illustrated in **Figure 1-5**
with a schematic explanation of spectral shifts based on molecular exciton theory. Upon excitation, a transition from ground state to upper state in H-aggregates and to lower state in J-aggregates leads to the shifts in absorption wavelength to blue and red, respectively.

Figure 1-5: Illustration of H- and J-aggregation and spectral shift based on molecular exciton theory.

The significance of J-aggregates was recognized shortly after its application in spectral sensitization of photographic processes with silver halides. After that, tremendous work was carried out in order to provoke and control J-aggregates. During the last three decades, chromophores like cyanines, merocyanines, squaraines, and other natural light harvesting pigments have been used as the repeat unit to generate J-aggregates, which have been summarized by Würthner et al., recently. Compared to other chromophores that can form J-aggregates under certain conditions, the reports about exclusive and stable J-aggregates of squaraine dyes are sparse, and most investigations were focused on solid thin films. Among the earliest work is that reported by Law and Whitten; the study of aggregation formed from aniline based hydrophobic unsymmetrical squaraine dyes in Langmuir–Blodgett (LB) films showed very interesting temperature-dependent assembling pattern. 4-(Distearylamino)phenyl-4′-(dimethylamino)phenylsquinacridone was one of the squaraine compounds studied, which showed a typical sharp absorption at 633 nm in chloroform solution. However, when it was incorporated into LB films on
glass, blue-shifted aggregates were generated, and the absorption maximum wavelength moved to 530 nm. After the LB films were heat at 65 °C for 1–2 h, the initial blue-shifted aggregate species converted to red-shifted aggregates compared with the absorption wavelength of the monomer, with a broadened absorption peak centered at 660 nm. More interesting, with even stronger heating (105 °C), both the blue-shifted and red-shifted aggregates reorganized to a new and sharp aggregate with absorption centered at 690 nm, which was established to J-aggregation.\(^{15}\)

After the study of hydrophobic squaraines, Law and Whitten continued to investigate the properties of LB films composed of surfactant squaraines. By employing the squaraines containing a hydrophobic alkyl chain and a hydrophilic carboxylic acid, the formation of aggregates was detected even in relatively dilute concentration at the air–water interface, and the aggregation pattern was found to be responsive to surface pressure. In LB films, a mixture of red- and blue-shifted aggregation was generated initially. However, when heat was applied to the solid support, pure red-shifted aggregates were obtained. This was explained by the facilitation of squaraine–squaraine interaction in LB films after water was evaporated upon heating, and consequently enabled the formation of slipped stack arrangement.\(^{16}\)

![Figure 1-6: Structures of hydroxyaniline squaraine dye compound 10 (A), 11 (B) and their absorption spectra (1) after thermal annealing, (2) after spin coating and (3) in CHCl₃ solution. Ref 59, copyright 2011 Taylor & Francis Group.](image-url)
Aggregated squaraines in the microcrystalline state have been studied and reported for xerographic applications. Recently, our research group has been interested in squaraine dyes for their nonlinear optical and photovoltage applications. After synthesizing a series of 2,4-bis[4-(N, N-di-n-alkylamino)-2-hydroxyphenyl] squaraines, UV-vis spectroscopy was used to study their behavior in thin films formed by spin coating before and after thermal annealing. H- and/or J-aggregates were observed in the thin films made from several of the squaraine derivatives. Figure 1-6 shows two of the hydroxyaniline squaraine derivatives 10 and 11 synthesized by Qaddoura, followed by their absorption studies under different solid and solution conditions. After 10 was spin coated on glass slides, multiple absorption peaks from were detected, including H-aggregates (503 to 533 nm) and J-aggregates (732 to 787 nm). Further annealing enhanced the H-bands at the expense of J-bands, according to the absorption spectra. Similarly, squaraine dye 11 formed H-aggregates on spin coated glass slides, and the H-band shifted from 537 to 570 nm. Controlling the aggregation states of squaraine compound 11 in the active layer of photovoltaic cells resulted in an enhancement in short-circuit current. This might open a new strategy to enhance photon-voltage conversion efficiency using organic dye aggregate assemblies.

Even though J-aggregate assembly could alter the original optical behavior of the material and exhibit valuable photophysical properties, reports of squaraine dye J-aggregate in solution are very limited. In 1993, Das and George et al. reported the formation of J-aggregates in acetonitrile based on simple squaraine structures, bis(2,4-dihydroxyphenyl)squaraine and bis(2,4,6-trihydroxyphenyl)squaraine. Intermolecular hydrogen bonding between monomer molecules was explained to be the main factor for aggregate formation.

Aggregation behavior of symmetrical aniline based squaraines with N-alkyl chain length from n-butyl to n-octyl to n-dodecyl was studied by Wojtyk et al. in DMSO/water mixture. Driven by hydrophobic forces,
J-aggregates are triggered and stabilized in a high percentage of water, whereas H-aggregates were dominant with a low percentage of water. For this squaraine series, the author classified the driving forces for J-aggregate formation into two main interactions. The first is intermolecular charge transfer between the donor and acceptor part in the squaraine structure. Secondly, the N-alkyl chain length controlled hydrophobic interactions. It is a combination of these two types of interactions that stabilized the J-aggregation in high percentage of water and the dynamic conversion to H-aggregation was impeded under these conditions.  

\[ \text{Scheme 1-5: Synthesis of chiral squaraine 12 through a two-step, one-pot reaction. Ref 18, copyright 2006 Wiley-VCH Verlag & Co. kGaA.} \]

A novel chiral squaraine dye 12 was reported by Rabe and Hecht et al., synthesized from L-proline. Multiple methods were used to study aggregation behavior, including spectroscopic measurements and STM (scanning tunneling microscopy) and STS (scanning tunneling spectroscopy). Scheme 1-5 shows the structure and synthetic route of a chiral squaraine dye 12 through a two-step, one-pot reaction. In acetonitrile solution, with increasing portion of water, J-aggregation of 12 could be triggered and increased with increased water percentages, shown in Figure 1-7. Corresponding circular dichroism (CD) spectra were recorded, the strong negative Cotton effect at the red shifted absorption band position.
verified the formation of J-aggregates. Similar to the report by Wojtyk et al., the generation of aggregates was attributed to the forces driven by alkyl chain substrates as well as squaraine–squaraine interactions.

**Figure 1-7**: UV-vis absorption (a) and circular dichroism spectra (b) of 12 in acetonitrile with increasing water portion at 25 °C. Ref 18, copyright 2006 Wiley-VCH Verlag & Co. kGaA.

Most recently, Pang et al. found that a series of squaraine derivatives have a high tendency to form nonfluorescent H- and/or J-aggregates in aqueous solutions with the addition of 0.05% sodium dodecyl sulfate (SDS). The authors pointed out that the bulkiness of the alkyl chain in the squaraine molecule structure could play an important role in H- or J-aggregate formation. However, the reasons for selectively triggering of H- and J-aggregate formation are not fully understood.

### 1.4.2 Biomolecule Sensors and Bioimaging Materials

The strong absorption and emission signal of squaraine chromophores are derived from charge transfer transitions, thus sensitive to the neighboring environment, e.g., solvent polarity, temperature, and other provocations. By evaluating the change of optical properties, the changes in environment can be monitored, which makes squaraine dyes good candidates as chemosensors. Using an indoleninium
squaraine, Ioffe et al. successfully detected alterations in lipid bilayer membranes. Switching from water solution to a lipidic environment, the emission intensity of the squaraine probe increased significantly along with a red shift. Moreover, the absorption and emission responses of the squaraine sensor in different liposome media were related to the bilayer polarity varied by addition of positively charged electrolyte cetyltrimethylammonium bromide (CTAB), anionic phospholipid cardiolipin (CL) and neutral sterol (Chol). This study showed the applications of squaraine chromophores as a fluorescent probe for membrane related processes.  

More interesting behavior was found based on bis(2,4,6-trihydroxyphenyl)squaraine, which can form a 2:1 inclusion complex with β-cyclodextrin (β-CD) in aqueous solution. In the absence of β-CD, the fluorescence intensity from squaraine dyes was quenched, due to the formation of hydrogen bonding between hydroxyl groups in squaraines and solvent water, which disturbed the intramolecular hydrogen bond interactions. β-CD encapsulation generated a hydrophobic environment in addition with reduced rotation of the squaraine chromophore, resulting in a great enhancement of fluorescence intensity with an enhancement of 90-fold.  

Squaraine dyes applied for protein sensing and labeling are basically studied by model proteins such as bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin, and avidin. Suzuki and Yokoyama used a very simple squaraine 13 as a BSA protein sensor in 2007. Upon the addition of BSA, squaraine 13 noncovalently interacts with proteins, resulting in an observable color change from orange to deep purple (Figure 1-8). Squaraine 13 showed consistent concentration response to various proteins, which is promising compared with other commercially available protein detection materials. The author also found that after the formation of 13-BSA complex, the stability to other nonprotein substances was also very
good. Finally, studies of protein staining on 1D SDS-PAGE minigels using protein sensor 13 was performed; showing an easy procedure with high sensitivity.66

Figure 1-8: Structure of squaraine dye 13 and (a) absorption spectra of 13 before and after the addition of BSA, (b) visual color change upon BSA addition. Ref 66, copyright 2007 Wiley-VCH Verlag & Co. kGaA.

Bis(2,4,6-trihydroxyphenyl)squaraine 14, which has four more hydroxyl groups compared with compound 13 was used to act as protein sensor with site selective binding with BSA. Similar to 13, squaraine 14 also can noncovalently interact with BSA and show a red-shifted absorption with a huge enhancement of the fluorescent intensity (Figure 1-9). The optical property change of 14 upon interaction with BSA makes it a good candidate for a dual mode recognition reagent for serum albumin. Through a site selectivity binding experiment, Ramaiah et al. pointed out the huge change in optical spectra were derived from the environmental change of 14 after it binds at site II of BSA, in which the binding position has a larger cavity size compared with site I with possible noncovalent binding forces include a combination of electrostatic interactions, hydrogen bonding, and hydrophobic forces.67

In a work by Ramaiah and coworkers, bis(3,5-dibromo-2,4,6-trihydroxyphenyl)squaraine and bis(3,5-diiodo-2,4,6-trihydroxyphenyl)squaraine were synthesized and their binding properties with BSA and HSA were studied and compared with compound 14. Even though, these compounds are slightly different in structure, all of them exhibited size-dependent selectivity binding at site II of serum albumins with “turn-
on” fluorescence intensity along with increased triplet excited state lifetimes and quantum yields. These results indicated the potential applications of squaraine dyes as near-IR protein labeling and photodynamic therapeutic reagents.⁶⁸

Figure 1-9: Structure of squaraine dye 14 and (A) change in fluorescence spectrum with addition of BSA in phosphate buffer, (B) detection of BSA through fluorescence turn on. Ref 67, copyright 2006 American Chemical Society.

Pang et al. carried out detailed studies of the interactions between squaraine dyes and BSA. Different strategies were used to trigger the nonfluorescent aggregates of squaraine dyes before interaction with BSA and consequently result in a tremendous fluorescence enhancement upon BSA addition. 200-fold fluorescence “turn on” was reported related with the conversion from aggregated states to monomer species, using SDS as the aggregation introduction reagent.⁶² With the same squaraine dye structures, chemically converted graphene (CCG) was employed to modulate the aggregation of hydrophobic squaraine dyes, thus quench the initial fluorescent. Later BSA addition resulted in an 80-fold fluorescence “turn on” behavior. A site-selectivity experiment was carried out, and the fluorescence response with the concentration of replacing ligands indicate these squaraine dyes were binding to both site I and site II of BSA with preference to site I.⁶⁹
Red-shifted absorption, enhanced fluorescence intensity, and enlarged fluorescence lifetime are typical optical responses of squaraine dyes with BSA. Volkova et al. reported a 190-time fluorescence increase after the formation of dye-BSA complex, and the analogous dye-HSA and dye-ovalbumin complexes exhibit less enhancement of fluorescence intensity, of up to 24-fold compared with the absence of protein. Taraerts and Patsenker et al. reported the optical properties of a series of water soluble indolenium squaraine dyes (Figure 1-10) before and after complexion with BSA. When these dyes were dissolved in water, all exhibited a low quantum yield of 0.02 to 0.10, along with short fluorescence lifetime. However, upon interaction with BSA through noncovalent bonding, the fluorescence intensity increased and achieved a quantum yield of 0.13 to 0.45 and fluorescence lifetime up to 1.30 to 3.32 ns.

![Figure 1-10: Structures of indolenium squaraine dyes used for BSA sensitivity studies.](image)

Squaraine dyes have been widely applied as protein labels due to their intense near-IR fluorescence. Besides noncovalent interactions with serum albumin, studies have also been reported using covalent bonding to link modified squaraine dyes with BSA and/or HSA. Oswald et al. synthesized squaraine dyes with succinimide groups, followed by covalent bonding to serum albumin through succinidyl esters bonds. After chemically binding, the fluorescence quantum yield of the final complexes increased from 0.15 to 0.6 – 0.7, which facilitated the fluorescence measurement in whole blood with a detection minimum twice as low as assays using cyanine chromophores. These promising results led to further development of a
class of commercially available squaraine dyes, in which the commercial name is Seta dyes. Studies using Seta dyes to conjugate with lysozyme for protein–lipid interaction detection\textsuperscript{73} and with biotin to probe biotin–antibiotin interactions have been carried out by Terpetschnig and Patsenker \textit{et al.}\textsuperscript{74}

As discussed previously, squaraine scaffolds can sufficiently absorb and emit in the near-IR region, which is a suitable window for the studies under biological conditions. A lot of efforts have been reported to enhance squaraine dye’s water solubility in order to perform their duties in biological environments. In general, the water solubility of squaraine dyes can be improved by introduction of polar functional groups to the squaraine scaffolds, such as sulfonate groups,\textsuperscript{70,71} carbohydrates,\textsuperscript{71} polyethylene glycol residues,\textsuperscript{75} hydroxy groups,\textsuperscript{67} phosphonic acid,\textsuperscript{76} and imidazolium pendants.\textsuperscript{25} On the other hand, different strategies have been used to overcome the drawbacks of squaraine dyes including chemical fragility due to the nucleophilic attack (by cyanide anion, amine- and thiol-containing biomolecules) and strong tendency to form nonfluorescent aggregates in biological media, which can further hamper the applications of squaraine dyes for bioimaging.

One strategy to sterically protect the squaraine core was introduced by Smith and coworkers. In general, the idea is to encapsulate the squaraine dye inside a molecular rotaxane. The four-membered ring core of squaraine dyes was enclosed by the rotaxane through the interaction of hydrogen bonding that protects the core from further nucleophilic attack, also the bulky rotaxane effectively reduced non-fluorescent aggregation in biological media. The first example of a squaraine-derived rotaxane \textbf{16} was synthesized using normal squaraine structures as a pre-organized template for Leigh type amide rotaxane reaction (\textbf{Figure 1-11}).\textsuperscript{77,78} The chemical and photophysical stabilities of the resulting squaraine-derived rotaxanes were greatly improved compared with the non-encapsulated one, and the aggregation tendency was minimized after encapsulation.\textsuperscript{79} This pioneering study opened the possibility to prepare a
wide range of squaraine-derived rotaxanes to match the requirement of photochemical and photophysical properties needed for using the squaraine scaffold as bioimaging materials. Squaraine-derived rotaxane 17 was later synthesized by Smith et al. containing terminal acetylene residues, which can be further modified by a copper catalyzed Huisgen cycloaddition reaction.24

![Structure for squaraine-derived rotaxane compounds 16 and 17.](image)

**Figure 1-11:** Structure for squaraine-derived rotaxane compounds 16 and 17.

With the enhanced stability in bio-environment, squaraine-derived rotaxanes are nice candidates for near-IR imaging probes, since their emission above 650 nm can easily penetrate skin and tissue.80,81 Smith et al. reported the synthesis and application of squaraine-derived rotaxane 18 and it is derivatives for the studies in live cell imaging and live mouse imaging. The relatively nonpolar compound 18 transports within cells in manner similar to lipophilic stain Nile red.82 **Figure 1-12a** shows the multicolor image of a single COS-7 cell (monkey kidney cell line) costained with DNA labeling reagent H33342 (blue color), plasma membrane labeling compound FM1-43 (green color) and the squaraine-derived rotaxane probe 18 (red color). In addition, the stability and low toxicity of 18 in biological environment was shown in **Figure 1-12b**, which is an image of MCF7 cells (human breast cancer cell line) incubated with compound 18 for 144 h. The staining pattern and fluorescence intensity remain after eight days incubation, and it appears that the squaraine complex also transferred to mitotic daughter cells.23
Another strategy was encapsulating the squaraine dye into a nano-sized enclosure. Most recently, Zhao et al. introduced graphene oxide enwrapped mesoporous silica nanoparticles as protective vessels for squaraine chromophores. Squaraine 14 was used in this study. The procedure included loading of 14 into the mesoporous silica nanoparticles followed by wrapping graphene oxide around the surfaces of the porous through electrostatic forces (Scheme 1-6). The resulting squaraine dye-contained hybrid exhibited a remarkable stability and inertness to nucleophilicities of thiol groups in aqueous solutions. After the mesoporous pores were covered by graphene oxide, the leakage of the squaraine dyes could also be reduced. 83

This stabilized squaraine containing mesoporous silica nanoparticles was later used for bio-imaging studies with HeLa cancer cells. Figure 1-13 show the microscopy images of HeLa cells treated with squaraine containing mesoporous silica nanoparticles: (a) shows the location of nuclei stained with 4',6-diamidino-2-phenylindole (DAPI), (b) is the bright red fluorescence coming from enclosed squaraine dyes, (c) is overlay of (a) and (b), and (d) is overlay of (c) with the phase contrast image. The author claimed that the nano hybrid accumulated in the cytoplasm of the cells. 83

Figure 1-12: Structures of squaraine-derived rotaxane compound 18, and it is application in cell imaging studies. Ref 23, copyright 2007 Wiley-VCH Verlag & Co. KGaA.
Scheme 1-6: Preparation of graphene oxide enwrapped squaraine dye loaded mesoporous silica nanoparticles. Ref 83, copyright 2012 American Chemical Society.

Figure 1-13: Fluorescence microscopy images of squaraine-containing mesoporous silica nanoparticles incubated with HeLa cells. Ref 83, copyright 2012 American Chemical Society.

Our research group also reported using near-IR emitting squaraine dyes for multiphoton fluorescence imaging studies. Squaraine dyes were introduced into micelles of Pluronic F127, and the resulting squaraine dye-doped micelle exhibited high photostability and thermostability compared with
commercially available cyanine dye Cy5. Subsequent microscopic images were taken under one-photon and two-photon excitation, providing high contrast images. These results suggest the utilities of squaraine dyes for two-photon or even multiphoton fluorescence based ex vivo and in vivo bioimaging.27

1.4.3 Colorimetric Sensors for Metal Ion Detection

Selective sensing and probing metallic ions is important in biological conditions, such as sensing Na\(^+\), K\(^+\), Ca\(^{2+}\), and Zn\(^{2+}\). And detection of transition heavy metal ions like Cd\(^{2+}\), Cu\(^{2+}\), Pd\(^{2+}\), Hg\(^{2+}\) and Ag\(^+\) is also crucial for industrial and environmental applications. Organic chromophores have been used as indicating units for a long time due to their intense and sharp absorption and emission. Signals can be generated from the chemosensors when interacting with metal ions, and the changes in optical properties can be achieved by electron transfer, energy transfer, and formation of excimers. In 2002, Yagi and coworkers reported a bisquarylium dye and metal coordination studies that showed an exclusive interaction with transition metal ions with a hypsochromic shift in the absorption spectrum.84

Similar to methane bridged bisquarylium structures, squaraine derivatives were also been employed as probes for the detection of various metal ions. Ajayaghosh et al. developed a series of squaraine based sensors can specific detection for Ca\(^{2+}\) and Mg\(^{2+}\). One of the sensor structures is shown in Figure 1-14, in which ether bridges link two squaraine chromophores. Once the oxygen in ether and nitrogen in aniline interact with metal ions, two chromophores forced closer enough to induce electronic transitions and signal occurs through the formation of 1 : 1 or 2 : 1 squaraine–metal complexes. Figure 1-14 shows the schematic representation of a chemosensor based on cation driven exciton interaction. According to Job plots, there were two different coordination ratios, chromophores formed a 2:1 sandwich dimer when interacting with calcium ions, while the squaraine podant enfolding around the magnesium ions generated a 1:1 folded dimer.85 By extending the linker from an ether to a polyether backbone in between
two squaraine dyes, Ajayaghosh et al. reported the reversibility of wrapping around calcium ions upon addition of EDTA.\textsuperscript{86}

![Figure 1-14: Structure of squaraine dye 19 and schematic representation of chemosensor based on cation driven exciton interaction. Ref 1, copyright 2005 American Chemical Society.](image)

In order to have strong bonding abilities of squaraine dyes to selected metal ions, a lariat-crown ether cation receptor moiety was linked to the aniline based squaraine chromophore by Oguz et al. The crown ether moiety was designed to interact and grab Na\textsuperscript{+}, along with the conjugated squaraine chromophore, which has very intensive absorption in the near-IR. The combination resulted in this metal sensor with good water solubility and selective sensitivity of Na\textsuperscript{+}.\textsuperscript{87} In another example, a crown ether ring designed for affinity to Hg\textsuperscript{2+} was attached to a squaraine chromophore, and the chemosensor exhibited good sensitivity in near-IR region.\textsuperscript{88} Additionally, by employing sulfur hybrid crown ether moieties, Martínez-Mañez et al. reported the synthesis and application of an Hg\textsuperscript{2+} sensor (Figure 1-15). When compound 20 was dissolved in acetonitrile in the presence of water, there shows a typical H-aggregation band in the absorption spectrum. With the addition of Hg\textsuperscript{2+}, the squaraine can bind Hg\textsuperscript{2+} through the dithia-dioxa-aza crown, consequently changing the optical properties of the squaraine chromophore. The fully bleached monomer and H-aggregate bands upon addition of Hg\textsuperscript{2+} were reversible if stronger chelating ligands are
involved. The influence of Ag⁺ is different somehow, which can induce disaggregation and regenerate the monomer species.⁸⁹ These works reinforce the potential application of squaraine dyes as chemosensors in the near-IR region of the spectrum, where background absorption in the visible wavelength can be eliminated.

![Image](image.png)

**Figure 1-15**: Structure of compound 20 and absorbance in the presence of 5 equivalents of Hg²⁺ and Ag⁺ in acetonitrile/water (80 : 20, v/v) mixed solvent. Ref. 89, copyright 2004 American Chemical Society.

Anslyn and coworkers developed a Pd²⁺ sensor based on squaraine structures; the mechanism is shown in **Scheme 1-7**. The cyclobutene core of the squaraine dyes undergo a nucleophilic attacked upon ethanethiol addition, resulting in a colorless intermediate SQ:Set. Once this intermediate was mixed with Pd²⁺, a turn on absorption and emission was detected based on the thiol scavenging ability of Pd²⁺ ion. By using this method, the concentration limit for detection of Pd²⁺ by eye was as low as 0.5 ppm, and the minimum detection by instrumental means was as low as 100 ppb.⁹⁰ Similarly, detection of very dilute Hg²⁺ was achieved because Hg²⁺ also can act as a thiol scavenging reagent.⁹¹
This year, Das and coworkers reported another squaraine dye-based chemosensors for the detection of Hg$^{2+}$. One example of an unsymmetrical squaraine dye used as chemosensors are shown in **Figure 1-16**, with the changes in absorption and emission spectra of **21** in methanol upon addition of Hg$^{2+}$. Squaraine **21** can form unusual complexes with Hg$^{2+}$ through the phenyl-squaraine carbon – carbon bond, thus disturbing the conjugated system and consequently changing its original absorption and emission properties. With the concentration of Hg$^{2+}$ increasing, the absorption and emission intensity decreases; when the concentration of Hg$^{2+}$ reached 45 μM the absorption intensity at 590 nm decreased 87% accompanied with 88% reduction of fluorescence intensity. Addition of Fe$^{3+}$ can lead to color fading of squaraine dyes along with a formation of a new peak at 340 nm. The author explained that the instant decrease in absorption upon adding Fe$^{3+}$ was attributed to oxidation of the squaraine, because squaraines are well known to undergo many redox reactions.
Sensing and detection of iron is also important both in biological and environmental applications.\textsuperscript{96,97} Anslyn and coworkers reported a method to detect iron using UV-vis spectroscopy based on a squaraine dye structure. The squaraine had four hydroxyl groups on the benzene ring oriented to the four-membered core, potential hydrogen bonding stabilizes the squaraine core and reduces the possibility of nucleophilic attack (Figure 1-17). Compound 22 acted like a bidentate ligand when interacting with Fe\textsuperscript{3+} between the deprotonated phenol hydroxyl group and the carbonyl group of the cyclobutadiene ring. Upon addition of Fe\textsuperscript{3+}, the absorption spectrum of the squaraine dye shifted to longer wavelength.\textsuperscript{8}
After iron and zinc, copper is the third most abundant essential trace element in the human body. Lan and Chen et al. developed a copper sensor based on squaraine scaffolds by employing a 2-piccolyl anchor to give efficient copper chelating positions. The colorimetric Cu$^{2+}$ sensor 23 is shown in Figure 1-18. When combined with Cu$^{2+}$, the original absorption band at 514 nm shifted largely to longer wavelength at 675 nm, but no significant changes in absorption spectrum were found when other metal ions were added. Job plot analysis shows 1:1 complexes of the 23-Cu$^{2+}$ interchelates. Studies were carried out to outline the possibility of using 23 as a fluorescent probe for Cu$^{2+}$ in living cells. The authors showed that such Cu$^{2+}$ sensor would be potentially used in biological systems for revealing the roles of copper either in vitro or in vivo.$^{10}$

![Figure 1-18](image)

**Figure 1-18**: Colorimetric sensor for Cu$^{2+}$ and the color changes upon addition of various metal ions in HEPES/THF (60 : 40, v/v). Ref 10, copyright 2010 Elsevier Ltd.

1.4.4 Photoconduction Materials Based on Squaraine Dyes

Due to the famine of fossil energies, searching for substitute energy resources has become one of the most important contemporary research topics. Among them harvesting energy by solar cells is rapidly developing with a significant number of organic dye-based devices having been reported in recent years. Concerning the possibility of large scale factory productions, two strategies are widely accepted: the bulk heterojunction organic photovoltaic (BHJ-OPV) solar cells$^{98,99}$ and dye sensitized solar cells (DSSC).$^{100}$
Structurally modified squaraine dyes have been used in both technologies, as squaraine chromophores exhibit several important features related to photovoltaic applications.\textsuperscript{101}

Over the past decades, single layered organic photovoltaics (OPVs) composed of squaraine derivatives were made and their power conversion efficiencies (PCEs) were studied, generating 0.02\% maximum efficiency.\textsuperscript{102-104} The organic dyes are generally acting as a donor in a mixture with a fullerene-based electron acceptor (6,6)-phenyl-C\textsubscript{61}-butyric acid methyl ester (PCBM), which has been widely used in organic solar cells studies. The organization and aggregation patterns of squaraine dyes in the solid state are also very important to develop high performance BHJ-OPV materials, which can be modified by using linear or branched alkyl chain substituted squaraines.

Squaraine derivatives 24\textsuperscript{a} and 24\textsuperscript{b} with extended conjugation systems were synthesized based on pyrridinium structures (Figure 1-19). The strong absorption over a wide wavelength range is a key factor for the applications in OPVs. As can be seen from the absorption spectrum of 24 in thin films, the absorption band is significantly broadened compared with the sharp band in organic solvents. In addition using cyclic voltammetry (CV), the HOMO and LUMO levels of 24\textsuperscript{a} and 24\textsuperscript{b} are estimated to be -3.3 eV and -5.0 eV, which fall in the range to perform sufficient charge and/or energy transfer when PCBM was used as the acceptor. Compared with polymer-based devices, the BHJ-OPV device fabricated with squaraine dyes can be prepared easily simply by spin coating under ambient atmosphere. The maximum efficiency recorded using squaraine 24 reached 1.26\% with a short circuit current as high as 5.70 mA/cm\textsuperscript{2}.\textsuperscript{105-107}
**Figure 1-19:** (A) Normalized absorption spectra of 24a and 24b in thin film and in CHCl₃ solutions. (B) HOMO/LUMO levels for 24a and 24b vs. PCBM. Ref 104, copyright 2008 American Chemical Society.

**Scheme 1-8:** Synthesis route for squaraine ensemble 25. Ref 109, copyright 2008 The Royal Society of Chemistry.

A supramolecular strategy was introduced by Torres and Pagani et al. for the design and synthesis of a squaraine-phthalocyanine ensemble with the aim of broadening the absorption spectrum of the final compound. The ensemble 25 was prepared by joining Ru-phthalocyanine with a bidentate squaraine ligand together at a 2:1 ratio (Scheme 1-8). The absorption window of the resulting ensemble 25 processes the original absorption properties of the squaraine dye and Ru-phthalocyanine, covering wavelengths...
from 550 to 850 nm. Due to the interaction between the squaraine moiety and the coordinated ruthenium metal, the absorption band at 706 nm from the squaraine part is about 25 nm red-shifted compared with the free base. The authors reported a conversion efficiency of 0.3%,109 which related closely to low short circuit current.110

The device structure and design of a DSSC is different from BHJ-OPV based solar cells. In general, organic dyes absorb the light and reach an electronically excited state, then an electron of the excited state molecule is transferred to an inorganic titania semiconductor, the separated charge was consequently collected by a metal electrode. During this process, the full cycle photovoltaic conversion is completed by counter transportation of a hole. Normally, organometallic-based solar cell sensitizers have good absorbing properties in the visible region; however, at longer wavelength in the near-IR region they perform less efficient. Among the class of chromophores designed for red or near-IR photosensitizers, squaraine dyes are highly regarded for their strong absorption located in the 600–800 nm window and they exhibit good thermal stability.

Das et al. first studied the possibility of using squaraine chromophores for DSSC applications. A series of squaraine dyes were designed and synthesized with a carboxylic acid group, which acted as a grafting group to link the organic dye to the titania surface. By comparing the conversion efficiencies between

![Figure 1-20: Squaraine dyes for applications in DSSCs.](image-url)
symmetric and nonsymmetric squaraines, the nonsymmetric ones are far more efficient, since directional HOMO to LUMO photon excitation could be achieved.\textsuperscript{14} Further studies by Burke and Grätzel showed that symmetric squaraine dyes can also be modified and performed appropriate photovoltage conversions. The resolution is to use the ammonium salt form of squaraine dyes and constrain the squaraine dye’s aggregation by mixing with chenodeoxycholic acid. When compound 26 was used in the fabrication of solar cells, a high efficiency of 3.7% was obtained.\textsuperscript{111} Structurally similar compounds 27 and 28 have been synthesized containing indolium and/or benzoindolium moieties with a carboxylic acid group to react with the titania layer. With direct linkage of carboxylic acid to the squaraine main conjugate, a much more efficient coupling between surface conductor and sensitizers was achieved. The best efficiencies for solar cells based on squaraine dyes 27 and 28 are 4.5% and 5.4%, respectively.\textsuperscript{112}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme.png}
\caption{Synthetic route for $\pi$-conjugation extended squaraine dyes.}
\end{figure}

Extending the $\pi$-conjugation systems can also achieve the goal of a broad absorption spectrum.\textsuperscript{113} Maeda and coworkers recently studied a series of linearly extended $\pi$-conjugated squaraine dyes 29abc, which were synthesized by cross coupling reactions using stannylcyclobutenediones as the starting material (Scheme 1-9). Not only in the visible region, these squaraine compounds also exhibited strong
photo-electron conversion responses in the near-IR range. The DSSCs composed of the squaraine series 29, afforded a converting efficiency of 2%. This strategy makes broad wavelength light harvesting possible, through simple π-system extension. The authors also pointed out the modest value of the overall conversion efficiencies may be due to back electron transfer and self quenching effects in aggregated squaraine dyes.114

1.4.5 Photosensitizers for Photodynamic Therapy

Squaraine dyes have also been considered and used as second generation photosensitizers for photodynamic therapy (PDT). First in vivo injection of photosensitizer materials was conducted, followed by irradiation of certain areas using a particular wavelength, singlet oxygen is then produced indirectly and consequently causes damage to cells. Squaraine dyes are attractive for application as photosensitizers because of their intensive absorption and emission located in the near-IR window, which is relatively transparent for biological tissues. After a complex synthesis, a few squaraine dyes have been studied on tumor cells and/or on small animals. Ramaiah and coworkers found that the halogenated product from compound 14 can generate efficient singlet oxygen for PDT treatment. A comparison experiment was carried out to study the photocytotoxicity and mutagenicity induced by the halogenated squaraine and nonhalogenated squaraine. The nonhalogenated squaraine dye exhibited completely negligible triplet single oxygen generation, while halogenated ones showed toxicity to cells upon suitable wavelength excitation.115 Among the structures studied, the tetraiodo derivative bis(3,5-diiodo-2,4,6-trihydroxyphenyl)squaraine was further used in PDT trials in male Swiss albino mice. The potential clinical applications were demonstrated since the tetraiodo derivative can reduce the tumor size and exhibit strong therapeutic effects without affecting the neighboring normal cells.116
More detailed research was carried out by Pagani and coworkers, the photosensitizing properties were tested for both mono- and bis-squaraines based on benzothiazolium structures. According to cell toxicity studies, the squaraine dyes are generally nontoxic in dark, however, they exhibit strong photodynamic effects on tumor cells when excited with white light.117 Importantly, the authors also demonstrated that the squaraine dyes can efficiently localize in the cytoplasm and not in the nucleus of the cells. Upon irradiation, the squaraine dyes undergo a type I radical chain process, generating peroxide and hydroperoxide radicals. For application of photosensitizers, water solubility and cell membrane permeability are very critical to its performance. Like use for biological fluorescent labels, squaraine dyes for PDT sensitizers also need good aqueous solubility and aggregation prevention.

1.5 Summary and Research Goals

In summary, the squaraine family is rich in promising candidates for many applications due to their unique optical and electronic properties. The goal of this research is to develop new squaraine dyes for potential applications as mentioned above. A major aim is to understand their structure property relationships to better design particular derivatives for specific applications.

For aggregation related applications, such as 2PA cross section enhancement and optical switching materials, controlling the aggregation state is the most difficult aspect in order to develop advanced devices. We are particularly interested in supramolecular J-aggregation, which can give a red shift in the absorption spectrum, generally with an enhancement of emission and 2PA cross section. In the first part this dissertation, squaraine dyes with pyridinium pendants were synthesized to both improve the water solubility and promote interaction with a polyelectrolyte template. By introducing and removing the polyelectrolyte template, controllable J-aggregation was achieved, and the resulting J-aggregate exhibited
enhanced 2PA. Thus, the optical properties were modulated through reversible and controlled supramolecular assembly.

For biomolecular sensing and bioimaging applications, water solubility is crucial. The interaction forces between squaraine dyes and biomolecules need to be considered. Sulfonate groups were introduced to the squaraine structures to improve water solubility, while the negative charges also play an important role in complexation with bovine serum albumin. Different from the aforementioned methods to protect the squaraine core from nucleophilic attack and prevent self-aggregation fluorescence quenching, the squaraine chromophores were encapsulated within BSA nanoparticles with enhanced water solubility and cell membrane permeability. Bright near-IR fluorescence cell imaging was achieved to afford a protein fluorescence tracking agent in live cells.

To explore further applications of squaraine dyes in biological environments, deoxynucleosides were linked to an unsymmetrical squaraine structure through a triple bond. Basically, the deoxynucleosides were first modified by halides and then conjugated to squaraine dye containing a terminal alkyne group by Sonagashira coupling. The coupling reaction conditions were modified by testing different organic bases and varying the amount of catalyst. The aggregation properties were studied in DMSO/water mixed solvent, and the squaraine modified deoxynucleosides exhibit temperature dependent aggregation behavior and viscosity-dependent emission, which may be further applied as a biologically friendly detectors and fluorescence probes.

For metal sensing and detection, a benzoindolium squaraine dye with sulfonate groups was employed. The sulfonated squaraine dye, with its excellent water solubility, facilitated ion detection in aqueous solution. Metal selectivity and sensitivity was studied using a broad range of metal ions, including alkali
metals, alkaline earth metals, and some transition metals. From absorption titration, emission titration, and $^1$H NMR titration, the squaraine dye’s metal complexation abilities were examined.

1.6 References


CHAPTER 2. J-AGGREGATION OF SQUARAINE DYES IN AQUEOUS SOLUTION*

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2.1 Introduction

In recent decades, J-aggregation formed by organic dyes, such as cyanines, merocyanines, chlorophyll dyes, and porphyrin and phthalocyanine dyes have been extensively studied.\textsuperscript{1,2} It is critical to control the aggregation behavior to understand and construct new devices utilizing the unique photophysical and optical properties of dye aggregates.\textsuperscript{3,4,5} J-aggregates are formed via noncovalent forces, and the characteristic long wavelength absorption of a J-aggregate is oriented from the exciton delocalization within the assembly.\textsuperscript{6,7} However, squaraine dyes do not readily form well-organized J-aggregates that are stable over a wide concentration range, especially in water.\textsuperscript{8-17}

In general, there are four main strategies to introduce organic dyes J-aggregation in solution, including: (1) inorganic salt triggered J-aggregation,\textsuperscript{18,19} (2) polyelectrolyte induced organic dye J-aggregation,\textsuperscript{2,20-22} (3) J-aggregation formed on bio-supramolecular (like DNA and poly-l-lysine) templates,\textsuperscript{23-25} and (4) J-aggregation of organic dyes modified with chiral structures.\textsuperscript{16} The resulting J-aggregates showed their potential application as a sensing unit for biomolecules and a photosensitizer for photosynthetic devices.

For the J-aggregation formation under conditions of (1) and (2), net electronic charges are required in the structure of organic dyes, which can interact with the aggregation introduction reagent through electrostatic interactions. In 2006, Belfield et al. first studied the nonlinear optical properties of a J-
aggregate assembly made of pseudoisocyanine dye under inorganic salt condition. The result J-band exhibit 10 times of enhancement for the value of 2PA cross section compared with non-aggregate monomer.\textsuperscript{19} In the same year, Whitten \textit{et al.} reported a J-aggregation example of positively charged cyanine dye in the presence of negatively charged polyelectrolyte carboxymethyl amylase (CMA).\textsuperscript{20} The changes in absorption and emission spectrum of CMA titration in pseudoisocyanine methanol/water mixed solution showed the formation of a sharp and red-shifted J-band (Figure 2-1). The helix transformation of CMA compared with random coil morphology was confirmed by the circular dichroism (CD) signal from achiral cyanine dye J-aggregation.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2-1.png}
\caption{Pseudoisocyanine dye and UV-vis absorption (a) and fluorescence (b) ([pseudoisocyanine] = 1 × 10\textsuperscript{-5} M, excited at 460 nm) titration of CMA to pseudoisocyanine dye in 1 : 4 methanol/water mixture. ([CMA] = 0 to 30 × 10\textsuperscript{-5} M). Ref. 20, copyright 2006 American Chemistry Society.}
\end{figure}

Most recently, Biswas and Belfield reported a porphyrin J-aggregation which J-band enhanced by norbornene based polymer. Both porphyrin structures selected containing sulfonate groups which can aid the water solubility and interact with positively charged norbornene polymer. The structures of the resulting complexes are shown in Figure 2-2, and formation of J-band was confirmed by Uv-vis absorption and time resolved fluorescence spectroscopy. By studying the 2PA cross section using Z-scan method, the
enlarged J-band on norborene polymer template exhibit strong 2PA cross section value as high as 10000 GM, which is over 30 times enhancement in contrast with non-aggregate monomer. Leading by this concern, we designed and synthesized a squaraine dye SQ-Py contains two positively charged pyridium pendants to act as the handle involved in the interactions with aggregation induced reagents. And the aggregation properties of SQ-Py were studied under both salt and polyelectrolyte conditions.

![Figure 2-2: Structures of porphyrin J-aggregation on norborene polymer template. Ref 2, copyright 2012 American Chemistry Society.](image)

When strategy (3) was employed as the aggregation method, the organic dyes can be non-covalently bond to biomolecules (such as intercalating at DNA major or minor grooves) or more commonly through a chemical bond. The advantage of J-aggregation formation through chemical bonding is the easier control of aggregation sequence and pattern for molecular building blocks. One example of chemical bond conjugated system is studied by Whitten and coworkers, in this work cyanine dye was used as the aggregation chromophore and poly-l-lysine was employed as the template (Figure 2-3). Whitten et al.
found that by extending the cyanine J aggregate length on poly-l-lysine scaffold J-aggregation band becomes more sharp and red-shifted. In our work an unsymmetrical squaraine dye SQ-COOH containing carboxylic acid group was designed, synthesized and be ready to condensation with biomolecular template through ester bond. The aggregation property was studied using chiral poly-l-lysine as the template.

![Diagram of monomeric cyanine dye and cyanine-pendant poly-l-lysine assembly]

**Figure 2-3**: The monomeric cyanine dye and cyanine-pendant poly-l-lysine assembly.

### 2.2 Structure Design and Synthesis Route

The synthesis of squaraine dye SQ-Py is shown in **Scheme 2-1**. Intermediate compound 3-(4-iodobutyl)-2-methylbenzothiazol-3-ium iodide was synthesized by a literature method, and the resulting $^1$H NMR matched the reported data. The pyridium pendant was introduced by replacing the iodo atom in 3-(4-iodobutyl)-2-methylbenzothiazol-3-ium iodide in a one-pot reaction along with the condensation reaction with squaric acid. Hence, **SQ-Py** was synthesized by condensation of squaric acid with benzothiazolium intermediate in a 4:1 mixture of n-butanol and pyridine. The pyridine employed in the reaction acted as both base and as reaction reagent to replace the iodo atom and form the pyridium pendant. **SQ-Py** with the pyridium pendant exhibited enhanced water solubility, and the introduction of cations allowed us to control the J-aggregation by electrostatic interactions with aggregation induce reagents.
Unsymmetric squaraine dye with a carboxylic acid group was synthesized (Scheme 2-2). First, 2-methylbenzothiazole was converted to 3-(2-carboxyethyl)-2-methylbenzo[d]thiazol-3-ium iodide by reacting with 3-iodopropanoic acid. And the result benzothiazolium salt was carried on to react with half squaraine dye in a solvent mixture composed of 4 : 1 n-butanol and pyridine. After reflux under a Dean-Stark trap, luster green solid were precipitate and collected. After washing and recrystalization in methanol, the final compound SQ-COOH was obtained with a yield of 44%.

Synthesis of poly-l-lysine template SQ-COOH was illustrated in Scheme 2-3. First 10 mg of poly-l-lysine hydrobromide (M.W. = 4000-15000, MP Biomedicals, LLC) was dissolved in dry DMF solution, followed by adjusting the pH value to 7 ~ 8 using TEA as neutralizing base. After that 44 mg of SQ-COOH DCM solution was added, the final SQ-COOH/poly-l-lysine repeat unit was 1.2 : 1, which make the SQ-COOH to be the
excessive species. Dehydration reagent DCC was added and the result mixture was carried out under stirring overnight at room temperature. The final mixture was acidified using 2 N HCl solution, and the pH value was brought to 3 ~ 4, in which range poly-l-lysine was be protonated (enhance water solubility) and unreacted SQ-COOH would have no solubility in water. The aqueous phase was washed with DCM twice to extract unreacted SQ-COOH and also extra DCC. Final, the water solution was neutralized again and dialysised using Slide-A-Lyzer Dialysis Cassette (2000 MWCO, 0.5 – 3.0 ml capacity, Thermo Scientific) for 48 h.

Scheme 2-3: Illustration of the structure J-aggregates of poly-l-lysine template SQ-COOH. (SQ-COOH repeat unit may be random on the poly-l-lysine scaffold).

2.3 Materials and General Techniques

Synthetic reagents were generally used as received unless specifically stated. 1,4-Diiodobutane and n-butanol were purchased from Aldrich. 2-methylbenzothiazole and pyridine were purchased from Alfa-Aesar. Squaric acid was purchased from MP Biomedicals, LLC. Poly(acrylic acid), Avg. Mw = 2000, was purchased from Aldrich. Deuterated DMSO was purchased from Cambridge Isotope Laboratories. Suitable
water was obtained using an ELGA/purelab ultra water purification system. $^1$H NMR and $^{13}$C NMR spectra were recorded at 300 MHz and 125 MHz, respectively. High-resolution mass spectrometry analysis was performed at the Department of Chemistry, University of Florida. The melting points are uncorrected.

Squaraine dye solutions were prepared in ultrapure water with or without the addition of NaCl and PAA-Na polyelectrolyte. The concentrations of SQ-Py in solution were kept nearly constant during absorption measurements ([$C$] ~ $10^{-5}$ M). PAA-Na of different concentrations was prepared by mixing poly(acrylic acid) (PAA, avg. Mw = 2000) with NaOH, at a 1:1 ratio of [COOH]/[OH$^-$]. Time-dependent UV-vis absorption spectroscopy was used to study the aggregation properties of SQ-Py after the introduction of salt and polyelectrolyte. The absorption spectra were measured using an Agilent 8453 UV-vis spectrophotometer with 10 mm quartz cuvettes. Fluorescence emission spectra were measured using a PTI Quantamaster spectrofluorimeter with a Hamamatsu R928 photomultiplier tube detector. Quantum yield measurements were conducted using cresyl violet as a reference. Spectral sensitivity of the PTI emission monochromator and photomultiplier tube detector were corrected for fluorescence spectra. All absorption and emission measurements were made at 15 °C unless specifically stated. Measurements at different temperatures were performed using a thermostated assembly.

A 2PA cross-section ($\delta_{2PA}$) was measured by the open aperture Z-scan method, using an amplified Ti:sapphire laser system (Legend Elite with Verdi-pumped Mira 900) with an optical parametric amplifier (Coherent OPerA Solo) in a 1 mm quartz cuvette. The laser pulses were ~100 fs full width at half-maximum duration at a 1 kHz repetition rate.
2.4 Results and Discussion

2.4.1 Absorption and Emission Properties of SQ-Py

**SQ-Py** was readily soluble in DMSO, yielding a blue solution with sharp absorption maxima located in the near-IR region as well as intense fluorescence. In DMSO, the absorption maximum was independent of concentration, appearing at 677 nm (**Figure 2-4**), with an extinction coefficient (molar absorptivity) of $2.1 \times 10^5$ M$^{-1}$ cm$^{-1}$. The fluorescence maximum in DMSO was observed at 687 nm with a small (10 nm) Stokes shift. And the fluorescence quantum yield was 0.36 when excited at 660 nm, using cresyl violate in methanol as reference. These values are typical for squaraine dyes in monomeric form.$^{29}$

![Normalized UV-vis absorption (solid line) and emission (dashed line) of SQ-Py in water (black) and DMSO (gray).](image)

**Figure 2-4**: Normalized UV-vis absorption (solid line) and emission (dashed line) of **SQ-Py** in water (black) and DMSO (gray).

In aqueous solution, however, the absorption spectra of **SQ-Py** exhibit concentration dependence behavior (**Figure 2-5**). At a low concentration (below $7.5 \times 10^{-7}$ mol/L), there was only one peak (absorption maximum of 643 nm), which represented the monomer band. When the concentration increased above $1.5 \times 10^{-6}$ mol/L, another absorption maximum began to appear at shorter wavelength. The absorption
band observed at higher dye concentrations, centered at 592 nm, was attributed to the formation of SQ-Py dimer. Organic dye molecules usually undergo self-aggregation, driven by a hydrophobic effect, removing water molecules from the surrounding dye aromatic surfaces. This arrangement of the dye molecules results in aggregate formation, which is balanced and stabilized by the combined effects of electrostatic repulsion, π-electron interactions, and hydrophobic surface stabilization. The squaraine dye SQ-Py contained two positively charged pyridium pendants and a relatively large conjugate surface. When SQ-Py was dissolved in water, significant electrostatic repulsion was at work due to the positively charged pendants. This electrostatic repulsion can be overcome somewhat by decreasing the overall hydrophobic moieties that are exposed to water molecules, which results in the formation of a dimer. The proportion of the dimer increased as the total concentration of SQ-Py increased. Compared with DMSO solution, both absorption and emission peaks shifted to shorter wavelength and the relative quantum yield from the SQ-Py monomer in water dropped to 0.02 when excited at 630 nm (Figure 2-4). In this spectrum, Absorption of SQ-Py in water was measured at $1.2 \times 10^{-5}$ mol/L to show the absorption from the monomer and the dimer, while the emission was measured at $7.5 \times 10^{-7}$ mol/L.

![Concentration-dependent absorption spectrum of SQ-Py in water solution. The concentration was decreased from $2.4 \times 10^{-5}$ to $7.5 \times 10^{-7}$ mol/L.](image)

**Figure 2-5:** Concentration-dependent absorption spectrum of SQ-Py in water solution. The concentration was decreased from $2.4 \times 10^{-5}$ to $7.5 \times 10^{-7}$ mol/L.
In the absorption spectrum of SQ-Py in water, the absorption wavelength of the monomer and the dimer were separated quite well, and the extinction coefficient of monomer at 643 nm can be calculated from dilute solutions where the Beer-Lambert law is operative. It is assumed that, because the overlap between the monomer and the dimer in the absorption spectrum is negligible, the concentration of the dimer can be calculated and its extinction coefficient at the maximum wavelength can be estimated. As with other squaraine dyes, dimers have a higher extinction coefficient. In pure water solution, the calculated extinction coefficient of SQ-Py for the dimer was $1.9 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, while the extinction coefficient for the monomer was $1.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

2.4.2 NaCl Induced J-Aggregation of SQ-Py

The absorption spectra of SQ-Py (1.4 × 10^{-5} \text{ mol/L}) dissolved in different concentrations of aqueous NaCl solution were measured. In aqueous solution, SQ-Py remains monomers and dimers, in part due to the electrostatic cationic repulsion of the pyridinium groups. When the concentration of NaCl increased from 0.001 to 0.1 mol/L, the formation of dimers was enhanced, accompanied by a decrease of the monomer band, as shown in Figure 2-6. At the initial state, when no NaCl was involved, the SQ-Py shows a dimer band and a monomer band located at 592 and 643 nm, respectively. Corresponding absorption intensity ratio of $A_d/A_m$ is 0.60, which the monomer species exhibit stronger absorption intensity than dimers. Using the afore mentioned extinction coefficient to calculate the concentration ratio of [dimer]/[monomer] = 0.5. However, at the NaCl concentration of 0.1 mol/L, the absorption intensity ratio of $A_d/A_m$ increased to 1.25, and corresponding concentration ratio reached [dimer]/[monomer] = 1.1. In this range of NaCl concentration, the inorganic salt plays an important role to control the ratio of SQ-Py dimer and monomer, which higher concentration of salt induced and stabilized the formation of dimers.
Figure 2-6: Absorption spectra for SQ-Py (1.5 × 10^{-5} mol/L) in water with different NaCl concentrations increased from 0 to 0.1 mol/L.

However, when the concentration of salt increased above 0.2 mol/L, the absorption spectrum of the solution changed very significantly. A new absorption band at 779 nm began to appear with a full width at the half maximum (FWHM) of 62 nm, when the concentration of NaCl was higher than 0.5 mol/L, attributed to the J-aggregate (Figure 2-7). The intensity of this J-band increased along with the concentration of NaCl, with a clear isosbestic point at 660 nm, and the reducing of associating dimer and monomer bands are very clear. When the concentration of NaCl increased from 0.5 to 1.0 mol/L, in the absorption spectrum there is huge decrease of the dimer and monomer bands along with a surging enhancement of J-band. Even the concentration of NaCl reached in that high rang, the solutions did not show noticeable increases in viscosity. And also no noticeable precipitate was observed during the whole procedure, which means the introduction of NaCl induced J-aggregation of SQ-Py. Meanwhile, the maximum absorption wavelength belongs to dimer, monomer, and J-aggregate did not change when the concentration of NaCl was varied. This indicate in higher concentration of NaCl, the J-aggregate of SQ-Py
are generated and stabilized through a combination of electrostatic forces and hydrophobic interactions of squaraine conjugates.

![Absorption spectra for SQ-Py (1.5 × 10⁻⁵ mol/L) in water with different NaCl concentrations increased from 0.2 to 2.0 mol/L.](image)

**Figure 2-7**: Absorption spectra for **SQ-Py** (1.5 × 10⁻⁵ mol/L) in water with different NaCl concentrations increased from 0.2 to 2.0 mol/L.

In order to better understand the procedure of NaCl induced **SQ-Py** aggregation, absorption kinetics of **SQ-Py** (1.2 × 10⁻⁵ mol/L) in high concentrations of NaCl were studied. In 1.0 mol/L aqueous NaCl solution, the J-band absorption at 779 nm monotonically increased with time, accompanied by a decrease of both the dimer (D) and monomer (M) bands at 592 nm and 643 nm, respectively (**Figure 2-8**). The rate of J-band incensement slowed down after 510 s, and the J-band absorption intensity remained constant after 540 s. The time for the formation of 50% of the absorbance ($t_{1/2}$) of the J-band ($A_{779}$) was 270 s. At equilibrium, a majority of the squaraine dye still dispersed in the solution in the form of dimer and monomer. According to the absorption intensity before and after reaching equilibrium, the dimer band decreased 50% and monomer band reduced 20%. The result J-band exhibit a broad full width at a half maximum (FWHM) of 80 nm.
In 2.0 mol/L aqueous NaCl solution, the aggregation behavior of SQ-Py is very similar compared with in 1.0 mol/L NaCl solution. The J-band absorption at 779 nm increased gradually, accompanied by a decrease of both the dimer (D) and monomer (M) bands at 592 nm and 643 nm, respectively (Figure 2-9). However, when the concentration of NaCl was increased to 2.0 mol/L, a faster conversion rate to J-aggregate was found. The resulting t₁/₂ was 120 s, which was twice as fast as when 1.0 mol/L NaCl was used. Furthermore, the value of the optical density (OD) of the J-band at equilibrium in 2.0 mol/L NaCl was almost twice as high as that in 1.0 mol/L NaCl. The values of the remaining absorbance of the D- and M-band, as well as the J-band, were dependent on the NaCl concentration, decreasing with increasing salt concentration for both the dimer and the monomer and increasing for the J-aggregate. Interestingly, the final J-band induced in 2.0 mol/L NaCl exhibit stronger absorption intensity than dimer and monomer bands. And again, the J-band generated in NaCl solution shows a broad FWHM of 75 nm.
Thus far, the results were obtained at a temperature of 15 °C. When the temperature was increased, the rate of J-aggregate formation decreased. This is shown in Figure 2-10; the change in temperature from 15 to 35 °C resulted in a change in the rate by a factor of 2 - 5. The same effect of temperature was found in the salt-induced J-aggregation of cyanine dyes and bacteriochlorophyll. A possible explanation is a decrease in J-aggregation because they tend to dissociate into free monomers when the temperature is increased. This effect is quite reasonable for SQ-Py in aqueous NaCl solution at higher temperature, as a majority of the SQ-Py exists as monomer and dimers. At higher temperatures, the equilibrium of forming J-aggregate from monomers and dimers shifts toward less aggregated forms, resulting in a negative temperature coefficient.

Figure 2-9: Absorption spectra of SQ-Py (1.2 × 10⁻⁵ mol/L) in 2.0 mol/L NaCl as a function of time. Insert shows kinetic curves at 592, 643, and 779 nm.
**Figure 2-10:** Kinetic curves of the J-aggregation of SQ-Py (1.2 × 10^{-5} mol/L) in the presence of 2.0 mol/L NaCl at different temperatures. X-axis is time in second, y-axis is absorbance of J-band at wavelength of 779 nm.

2.4.3 **SQ-Py J-Aggregation on PAA-Na Template**

Driven by their hydrophobic nature, organic dye molecules are well known to form H- or J-aggregates with the addition of salt and/or polyelectrolytes. We also investigated the aggregation properties of the cationic squaraine dye SQ-Py in the presence of polyelectrolyte PAA-Na. Even though SQ-Py can aggregate and form a J-band in aqueous NaCl solution, a rather high concentration of NaCl was needed, and the resulting J-band was much broader than when PAA-Na was used as a template. The addition of PAA-Na to an SQ-Py solution resulted in the formation of a new, low-energy absorption band at 765 nm; the bathochromic shift of about 122 nm compared with the monomer band indicates the formation of J-aggregates (**Figure 2-11**). In contract with the J-band formed in high NaCl solution with maximum absorption wavelength of 779 nm, the J-band induced by PAA-Na was located in a shorter wavelength at 765 nm.
Figure 2-11: Absorption spectra of SQ-Py (1.5 × 10^{-5} mol/L) in water with variable PAA-Na concentrations. The concentration of PAA-Na solutions was increased from 0.01 mol/L to 0.25 mol/L. Spectrums were recorded after 10 min of equilibrium.

In addition, the formation of this nonfluorescent J-band was accompanied by reduction of absorption bands at shorter wavelengths belonging to the dimer and the monomer. A control experiment has been carried out, and the aggregation behavior of SQ-Py in NaOH solution alone was studied. From low concentration to high concentration, the aggregation behavior of SQ-Py was very similar to NaCl solutions, which only the ratio of dimer/monomer changed but no obvious J-band formed (Figure 2-12). Since NaOH base conditions alone could not trigger the formation of a J-band, indicating that it is the polyelectrolyte PAA-Na served as template in the process of SQ-Py J-aggregate formation. The critical concentration of PAA-Na to trigger the formation of J-aggregates was 0.01 mol/L, and the resulting J-band had a very narrow full width at a half maximum (FWHM) of 15 nm. The development of the J-band was at a maximum at 0.01 mol/L PAA-Na; if this optimum condition was not met; J-aggregate formation could be hampered. Interestingly, a decrease in the J-band absorption was found upon the further addition of PAA-Na, and the
J-band completely disappeared after the concentration of PAA-Na reached 0.25 mol/L. At this point, the J-aggregates were completely eliminated, and both the monomer and the dimer absorption band were completely recovered (Figure 2-11).

![Absorption spectra of SQ-Py (1.1 × 10⁻⁵ mol/L) in different NaOH concentrations.](image)

**Figure 2-12:** Absorption spectra of SQ-Py (1.1 × 10⁻⁵ mol/L) in different NaOH concentrations.

During SQ-Py aggregation, the chains of polyelectrolytes PAA-Na likely provide an electrostatic scaffold that triggered and stabilized dye aggregation by partly neutralizing the ionic repulsions among squaraine molecules. One can think of this as the cationic squaraine molecules being brought together to the surrounding negatively charged polyelectrolyte via electrostatic forces, leading to aggregate formation driven by hydrophobic forces. During this process, an excess of negatively charged carboxylate groups are required to trigger the assembly of the cationic squaraine dyes gathered around the polyelectrolyte backbone. However, as the concentration of PAA-Na increases, the excessive negative ions begin to provide a great deal more anionic positions where the positively charged squaraine dyes can interact; consequently, the physical distances between neighboring dye molecules increase, disrupting the
aggregation. In our case, the absorption of the J-band decreased with the increasing addition of excess PAA-Na polyelectrolyte beyond the optimal concentration, accompanied by the regeneration of the original monomer and dimer absorbance bands.

The absorption spectra of SQ in the presence of 0.01 mol/L PAA-Na were studied as a function of time (Figure 2-13). Fifteen seconds after the squaraine dye solution was mixed with polyelectrolyte, a new absorbance with a maximum at 560 nm, assigned to the H-aggregate, was observed, presumably due to the dye molecules accumulating around the polyelectrolyte. Before the introduction of PAA-Na template, in the water solution there were already large amount of dimmers (H-dimer) which process the head-to-head structure. Once interacted with polyelectrolyte, it is more likely, the H-dimmer was moved as whole parts, which consequently generate the H-aggregates. Later, there was a gradual decrease of the H-band accompanied by an increase in J-aggregates (viz-a-viz absorbance at 765 nm). This might be attributing to the flexibility of polyelectrolyte backbone, which can slides and/or rotate, leading the transformation of head-to-head (H-aggregates) to head-to-tail (J-aggregates). The time dependence of the absorbances at 560 and 765 nm was characterized by the presence of an induction period and an inflection point. The half-life, t_{1/2}, of the J-aggregate formation was 105 s, and more than 70% of the H-aggregates were converted to J-aggregates after 210 s. After reaching equilibrium, a majority of the dye remained as J-aggregates, with a small amount of dimer and monomer being observed at 592 and 643 nm, respectively. A similar time-dependent transformation of H-aggregates to J-aggregates was found recently when 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TSPP) was aggregated on a cationic polymer template.²
Figure 2-13: Absorption spectra of SQ-Py ($1.2 \times 10^{-5}$ mol/L) in 0.01 mol/L PAA-Na water solution, as a function of time. Insert shows kinetic curves at 560 and 765 nm.

2.4.4 Control of J-Aggregation of SQ-Py on a PAA-Na Template Using Ca$^{2+}$

In aqueous solution, the polyelectrolyte PAA-Na served as a template for SQ-Py J-aggregate formation, i.e., the morphology of PAA-Na in water was critical for the aggregation of SQ-Py. Since PAA-Na can strongly interact with Ca$^{2+}$ ions and water, the complex of PAA-Na with Ca$^{2+}$ ions in aqueous solution has been investigated.$^{31-34}$ Two COO$^-$ groups are likely to chelate with one Ca$^{2+}$; for this to occur, torsion of the PAA-Na chain is required. One interesting feature of the interaction between the PAA-Na chains and Ca$^{2+}$ ions was that the dissolved polyacrylate chains undergo conformational changes, in which a coil shrinking process was induced after interacting with Ca$^{2+}$ ions.$^{33,34}$ At the molecular scale, the carboxylate group chelates with Ca$^{2+}$ ions in three ways: unidentate, bidentate and bridging.$^{35}$
Figure 2-14: a) Visible absorption spectra of a solution initially containing $1.0 \times 10^{-5}$ mol/L SQ-Py and 0.01 mol/L PAA-Na (black line) after increasing addition of CaCl$_2$. [Ca$^{2+}$]/[COONa] increased from 0 to 0.6. b) Evolution of the absorbance at 765 nm as a function of added CaCl$_2$ equivalents (vs. COONa).

We conducted studies out to understand how the Ca$^{2+}$ influences the PAA-Na attraction that resulted in the change of the aggregate form of SQ-Py. Solutions containing SQ-Py and PAA-Na were prepared at the optimized concentration ratio to maximize the formation of J-aggregates. Upon the introduction of CaCl$_2$, there was a noticeable effect on the absorption spectrum; the J-band absorption decreased gradually with increasing amounts of CaCl$_2$, indicating that the Ca$^{2+}$ ions disrupted the formation of J-aggregates. The decrease in J-band upon CaCl$_2$ addition correlated with an increase of the monomer and dimer absorption bands. As shown in Figure 2-14a, the decrease of the J-band reached equilibrium when [Ca$^{2+}$]/[COONa]=0.5; at this point, 80% of J-aggregates were converted back to dimer and monomer. Further addition of CaCl$_2$ had no significant effect on the absorption spectrum. A naked-eye-detectable color change of the solution was observed when the main stable component transformed from the dimer and monomer (deep blue) to the PAA-Na templated J-aggregate (light blue) and then back to dimer and monomer upon the addition of CaCl$_2$. Evolution of the absorption at 765 nm, correlating to the ratio of
[Ca^{2+}]/[COONa], was observed (Figure 2-14b). The decrease of the J-band slowed down after [Ca^{2+}]/[COONa] was higher than 0.5, indicating that the majority of COO\(^{-}\) had coordinated with Ca\(^{2+}\) ions at that level when two COO\(^{-}\) groups chelated with one Ca\(^{2+}\). During the CaCl\(_2\) addition, no observable precipitate formed.

Scheme 2-4: An illustration of SQ-Py J-aggregate formation in the presence of a PAA-Na template; the calcium ions disrupted SQ-Py aggregation by chelating with the PAA-Na template. The representation of the aggregate is not intended as a rigorous structural proposal.

To complete the cycle of switching from monomer and dimer to J-aggregates, an in situ experiment was carried out as shown in Scheme 2-4. Starting with SQ-Py (1.2 \(\times\) 10\(^{-5}\) mol/L) in pure water, when there were only monomer and dimer in solution, J-aggregation was triggered by adding the PAA-Na polyelectrolyte. Calculated from the absorption intensity at equilibrium, 70% of dimers and 80% of monomers were converted to J-aggregates. After CaCl\(_2\) was added to the this solution, the PAA-Na template chelated with calcium ions, liberating the squaraine dye molecules from the template, driving them back to solution where they formed dimers and monomers again. Even though there was still a J-band absorption tail
apparent in the absorption spectrum more than 80% of the J-aggregates were dissociated. The J-band reformed after the PAA-Na polyelectrolyte was added again. The regenerated J-band reached the same absorption maximum wavelength compared to the previous sharp J-band, with 60% recovery of intensity (Figure 2-15), demonstrated modulation of supramolecular assembly and disassembly.

**Figure 2-15: In situ supramolecular assembly modulation.** SQ-Py (1.2 × 10^{-5} mol/L) in water (blue solid line), the introduction of H-aggregate (magenta solid line) and later J-aggregate after mixing with PAA-Na (red solid line), followed by the addition of CaCl₂ (blue dashed line) to free the squaraine molecules from the template, forming H-aggregate (magenta dashed line) and then J-aggregates (red dashed line) again with PAA-Na. Spectra were recorded after 20 min of equilibration for J-aggregation.

### 2.4.5 Nonlinear Absorption Studies of SQ-Py Before and After J-Aggregation

Two-photon absorption (2PA) cross-sections (δ_{2PA}) were measured by the open aperture Z-scan method in a 1 mm quartz cuvette to help ascertain the potential applications of SQ-Py J-aggregates. A Ti:sapphire amplified system with an optical parametric amplifier, providing laser pulses of ~100 fs (fwhm) duration with a 1 kHz repetition rate. The concentration of SQ-Py monomers and dimers for Z-scan measurements was 10^{-3} M, at with most of the SQ-Py existing as dimers. A PAA-Na-templated SQ-Py J-aggregate solution
was used at $10^{-4}$ M due to broadening of the J-band at higher concentrations. As shown in Figure 2-16, the 2PA cross section of SQ-Py dimers and monomers was below 30 GM (Goppert-Mayer units, $1 \times 10^{-50}$ cm$^4$ s photon$^{-1}$ molecule$^{-1}$) per unit from 920 nm to 1000 nm. However, after aggregation induced by the PAA-Na template, the 2PA cross-section per repeat unit of the J-aggregates increased significantly. At 920 nm, the 2PA cross-section value increased from 20 to 460 GM, an impressive 23-fold enhancement upon formation of J-aggregates. The 2PA cross-section of J-aggregates was 460 GM at an excitation wavelength of 920 nm and dropped to 55 GM at 1000 nm. Even at 1000 nm, the 2PA cross-section values still increased by a factor of 5 per repeat unit relative to that of the dimer and monomer. The controllable polyelectrolyte template formation of SQ-Py J-aggregates provides a way to modulate both linear and nonlinear absorption, an aspect that may be interesting for a number of emerging applications, such as reversible photo switches.

Figure 2-16: Linear absorption spectra (lower x-axis and left y-axis) of SQ-Py, $10^{-3}$ M, in water (blue solid line), polyelectrolyte-templated SQ-Py J-aggregate, $10^{-4}$ M, (red solid line), and corresponding 2PA cross section (upper x-axis and right y-axis, each half-filled symbol).
2.4.6 SQ-COOH J-Aggregation on Poly-L-Lysine Template

![Absorption spectrum of SQ-COOH](image)

**Figure 2-17:** Absorption spectrum of SQ-COOH in dichloromethane (DCM) (black line) and poly-l-lysine template SQ-COOH assembly in water solution (red line).

When SQ-COOH was dissolved in DCM, it showed a maximum absorption peak at 647 nm. However, once SQ-COOH was covalently linked to a poly-l-lysine template through an amide bond, the complex exhibited an absorption peak at 753 nm in water solution (**Figure 2-17**). This 105 nm red shift in the absorption spectrum indicated formation of J-aggregates on the poly-l-lysine scaffold. However, the resulting J-band was very broad, and the extended absorption in the longer wavelength region interfered with the measurement of the 2PA cross section in that wavelength range. Further dilution of the J-aggregate solutions showed no change of the J-band absorbance structure and maximum absorption wavelength (**Figure 2-18**). This confirmed that once the squaraine dye was linked to the poly-l-lysine back bone and J-aggregation was triggered, the resulting complex exhibited an absorption spectrum in which the shape and absorption maximum were not dependent on concentration. Since the morphology of poly-l-lysine is well known, and in water solution it processes a helical structure, the poly amino acid-templated
aggregation would be very interesting in advanced materials development for organized assemblies of organic chromophores.

![Graph](image)

**Figure 2-18:** Decreasing the concentration of poly-l-lysine template SQ-COOH in water solution.

### 2.5 Summary

We investigated the conditions for J-aggregation formation of a new benzothiazolium squaraine dye SQ-Py, which contain pyridium pendants to enhance water solubility. The nonfluorescent J-band triggered by 0.01 mol/L PAA-Na has a more intense and sharp absorption compared with the J-band formed in high concentration of NaCl, which indicate that the polyelectrolyte PAA-Na served as a good template in the procedures of J-aggregates formation. Furthermore, changing the morphology of PAA-Na template by introducing more strong calcium ion chelates could result a dissociation of J-aggregates, and at equilibrium most of the SQ-Py molecular restored as monomer and dimer. These results show the potential usage of templates to trigger and control J-aggregation in aqueous solutions through interactions between anionic polyelectrolyte and cationic hydrophobic squaraine dye. In order to complete the cycle, switching from monomer, dimer to J-aggregate, in situ experiment was carried out.
At the second cycle, the regenerated J-band kept the same absorption maximum wavelength compared to the previous sharp J-band, with 60% recovery of intensity. Significantly, two-photon absorption property of each repeat unit of the **SQ-Py** J-aggregates was higher than the unaggregated or lower aggregated dimers, with a 23 times enhancement of 2PA cross section at wavelength of 920 nm. The controllable J-aggregate formation provides a way to modulate the linear and nonlinear absorptivity of SQ and achieve relatively high 2PA cross section, which is interesting for a number of potential applications, for example, optical data storage, optical switch and optical power limiting. In addition, the poly-l-lysine-templated J-aggregation was also studied, and the result showed this optional strategy could be potentially interesting.

### 2.6 Experimental Section

**Synthesis of 3-(4-iodobutyl)-2-methylbenzothiazol-3-iium iodide.** 3-(4-Iodobutyl)-2-methylbenzothiazol-3-iium iodide was prepared as previously reported.\(^{26}\) A mixture of 2-methylbenzothiazole (2.54 mL, 0.02 mol) and 1,4-diiodobutane (6.6 mL, 0.05 mol) in 10 mL dioxane was refluxed for 8 h. A pale yellow solid was obtained after precipitation with diethyl ether, filtered off, and washed with isopropyl alcohol and diethyl ether. The yield was 6.5 g (71%): m.p. 196-198 °C (dec.); \(^1\)H NMR (DMSO-\(d_6\), 300 MHz) \(\delta\) 8.45 (d, \(J=8.0\) Hz, 1H), 8.36 (d, \(J=8.2\) Hz, 1H), 7.89 (td, 1H), 7.80 (td, 1H), 4.77 (t, 2H), 3.30 (t, 2H), 3.20 (s, 3H), 1.94 (m, 4H).

**Synthesis of 4-(pyridinium-1-yl)butylbenzothiazolium squaraine dye (SQ-Py).** 3-(4-Iodobutyl)-2-methylbenzothiazol-3-iium iodide (460 mg, 1 mmol) and squaric acid (60 mg, 0.5 mmol) were suspended in 8 mL of n-butanol and 2 mL of pyridine. The mixture was refluxed using a Dean-Stark apparatus for 4 h. The reaction was then brought to room temperature; the crude product was collected by filtration, followed by washing with dichloromethane and diethyl ether. After recrystallization in methanol twice,
purple solid was obtained (170 mg, 38%): m.p. 247-249 °C (dec.); $^1$H NMR (DMSO-d$_6$, 300 MHz): δ 9.11 (d, J=5.9 Hz, 4H), 8.62 (t, J=7.8 Hz, 2H), 8.18 (m, 4H), 7.88 (d, J=7.6 Hz, 2H), 7.54 (d, J=7.9 Hz, 2H), 7.45 (m, 2H), 7.28 (m, 2H), 5.82 (s, 2H), 4.69 (t, J=6.9 Hz, 4H), 4.31 (t, 4H), 2.11 (m, 4H), 1.72 (m, 4H). $^{13}$C NMR (DMSO-d$_6$, 125 MHz): δ 175.72, 158.74, 146.09, 145.23, 141.37, 128.66, 127.76, 124.45, 122.98, 112.95, 109.99, 85.63, 85.61, 60.70, 45.18, 28.25, 23.96. ESI-MS: Calculated for [M-2I]$^{2+}$ = 322.1134; [M-I]$^+$ = 771.1319; found [M-2I]$^{2+}$ = 322.1141; [M-I]$^+$ = 771.1318.

Synthesis of 3-butoxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione. Compound was synthesized according to literature method.$^{36,37}$ Briefly, 3-ethyl-2-methylbenzothiazoleium iodide (300 mg, 0.98 mmol) and dibutyl squarate (220 mg, 0.97 mmol) were mixed in 10 ml of ethanol. Then 0.2 ml of triethyl amine was added, the reaction was brought to refluxing for 30 min. After the reaction was cooled to room temperature, solvent was removed under reduced pressure. Purification was carried out by flash column chromatography (silica gel) with hexane/ethyl acetate (1 : 1) as eluent, providing 230 mg of yellow crystal. Yield: 71.2%, m.p.: 139 – 140 °C. $^1$H NMR (500 MHz, CDCl$_3$) δ: 7.49 (dd, J = 7.8 Hz, 1H), 7.35 (m, 1H), 7.16 (m, 1H), 7.09 (d, J = 8.3, 1H), 5.46 (s, 1H), 4.79 (t, J = 6.6 Hz, 2H), 4.06 (q, J = 7.3 Hz, 2H), 1.85 (m, 2H), 1.52 (m, 2H), 1.41 (t, J = 7.3, 3H), 0.99 (t, J = 7.3 Hz, 3H). $^{13}$C NMR (125 Hz, CDCl$_3$) δ: 185.75, 185.40, 159.14, 140.60, 126.98, 126.84, 123.48, 121.98, 110.67, 78.73, 73.49, 40.57, 32.12, 18.72, 13.72, 11.81 ppm.

3-(2-carboxyethyl)-2-methylbenzo[d]thiazol-3-ium iodide. 2-methylbenzothiazole (2g, 13.4 mmol) was mixed with 3-iodopropanic acid (13.4 g, 67.0 mmol) in 20 ml of toluene. This mixture was heated to reflux under a water condenser for 8 hours. After cooling to room temperature, off-white precipitate formed, and collected by filtration. The solid was further washed with dichloromethane and diethyl ether. After drying, 3.3 g off-white product was obtained with a yield of 71.0%. m.p.: 224 – 226 °C (dec.). $^1$H NMR (500 MHz, DMSO-d$_6$) δ: 8.47 (dd, J = 5.1 Hz, 1H), 8.43 (dd, J = 5.0 Hz, 1H), 7.88 (m, 1H), 7.83 (m, 1H), 7.26 (m, 2H), 5.86 (s, 2H), 4.68 (t, J=6.9 Hz, 4H), 4.15 (t, 4H), 1.73 (m, 4H).
4.74 (t, 2H), 3.25 (s, 6H), 2.96 (t, 2H). $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ: 178.53, 171.90, 141.16, 129.83, 129.36, 128.49, 125.13, 117.38, 45.51, 32.27, 17.75 ppm.

(E)-2-((Z)-(3-(2-carboxyethyl)benzo[d]thiazol-2(3H)-ylidene)methyl)-4-((3-ethylbenzo[d]thiazol-3-iium-2-yl)methylene)-3-oxocyclobut-1-enolate (SQ-COOH). Compound SQ-COOH was synthesized by two continues steps. 3-butoxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione (330 mg, 1.0 mmol) was dissolved in 20 ml of ethanol. After the solution was heated to reflux, 0.3 ml of 40% NaOH water solution (w/w) was added, and the reaction was kept at refluxing for 30 min before cooled to room temperature. The pH of the solution was adjusted to 3 ~ 4 using 2N HCl. Then extracted with dichloromethane two times, organic layers were combined and the solvent was removed under reduced pressure. This resulting intermediate 3-hydroxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione was used immediately without further purifcation. This intermediate and 3-(2-carboxyethyl)-2-methylbenzo[d]thiazol-3-iium iodide (294 mg, 0.84 mmol) were dissolved in 10 ml mixed n-BuOH/pyridine solvent (4 : 1, v/v). The mixture was refluxed with a Dean-Stark apparatus for 4 h. During which time a lot of luster green solid precipitated. After cooled to room temperature, the green solid was collected by filtration. Further washed with diethyl ether and then recrystalized twice in methanol solution, providing 210 mg of green powder. Yield: 44%, m.p. : 186-188 °C (dec.). $^1$H NMR (500 MHz, DMSO-d$_6$) δ: 12.59 (s, 1H, COOH), 7.85 (m, 2H), 7.43-7.58 (m, 4H), 7.17 (m, 2H), 5.77 (m, 2H), 4.37 (m, 4H), 2.78 (m, 2H), 1.27 (t, 3H).
2.7 Spectrum Appendix

$^1$H NMR of 3-(4-iodobutyl)-2-methylbenzothiazol-3-ium iodide

$^1$H NMR of 4-(pyridinium-1-yl)butylbenzothiazolium squaraine dye SQ-Py.
$^{13}$C NMR of 4-(pyridinium-1-yl)butylbenzothiazolium squaraine dye SQ-Py.

ESI-MS of 4-(pyridinium-1-yl)butylbenzothiazolium squaraine dye SQ-Py.
\[ ^1\text{H NMR of compound 3-butoxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione}. \]

\[ ^13\text{C NMR of compound 3-butoxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione}. \]
$^1$H NMR of 3-(2-carboxyethyl)-2-methylbenzo[d]thiazol-3-ium iodide.

$^{13}$C NMR of 3-(2-carboxyethyl)-2-methylbenzo[d]thiazol-3-ium iodide.
$^1$H NMR for compound SQ-COOH.

2.8 References


(4) J-aggregates (Ed.: T. Kobayashi), World Scientific, Singapore, **1996**.


CHAPTER 3. SENSING AND LABELING BSA USING WATER SOLUBLE SQUARAIN DYES*


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3.1 Introduction

Since proteins play very important roles in biological systems, there is growing interest in developing probes that can noncovalently bind and show fluorescent response to individual proteins. Several fluorescent compounds have been synthesized and used as detection reagents of proteins in solution. These dyes exhibited “turn on” fluorescence intensity and increased lifetime, which was explained by changes in the environment due to protein micro-encapsulation. In addition, the exploration of fluorescent protein labels may contribute to the development of imaging materials, which are expected to recognize and sense cellular analytes.¹,²

For instance, the labeling molecule may contain reactive anchors for chemical conjugation to the biomolecules of interest. Alternatively, fluorescent labels may noncovalently bind with biomolecules in a process that involves a combination of hydrophobic, electrostatic, and/or hydrogen bonding interactions, resulting in the formation of a stabilized complex. Although the noncovalent interactions are normally weaker than chemical bond linkage, they generally occur at a faster rate and in a physiological pH range. When the noncovalent binding constant is large and the stoichiometry of the complex is known, purification steps are usually unnecessary. Moreover, noncovalent fluorescent labels are usually
encapsulated by the biomolecules, shielded from quenchers that are abundant in biological environments, thus adding stability advantages while reducing the tendency to form fluorescence-quenching aggregates.

Thiazole organe and malachite green derivatives have been developed by Szent-Gyorgyi and Waggoner et al. to act as fluorogenic dyes, which exhibit dark fluorescence alone but bright fluorescence when activating by specific human single-chain antibodies (scFv). The unique scFv as the fluorogen activating proteins (FAPs) have been isolated by screening method from a library of human scFvs. After these fluorogenic dyes incubated with yeast or mammalian cells, followed by interacting with the FAPs on the cell surface, the fluorescence increased thousands folds and through microscope imaging these FAPs were located. A class of dendron based fluorogenic dyes (dyedrons) was developed by Szent-Gyorgyi and Bruchez. In the fluorogenic dye structure, there includes multiple cyanine (Cy3) donors linked to a single malachite green acceptor. When the malachite green acceptor noncovalently interacted with a unique scFv the fluorescence will be enhanced (Figure 3-1). Later, Armitage and Waggoner et al. expanded the fluorogen dye derivatives which are potentially interesting for cell biological studies, and showing the possibility of performing multicolor imaging.

Figure 3-1: Binding activation of fluorescence for dyedrons developed by Szent-Gyorgyi. Ref 4, copyright 2010 American Chemistry Society.
Serum albumin is the major protein in blood plasma and is well known for its ability to bind and to transport various ligands to specific sites, both exogenous and endogenous. This unique ligand-delivery property benefits from two major binding sites of serum albumins, namely, site I and site II (Figure 3-2). Both binding sites are structurally selective; noncovalent binding at site I is primarily driven by hydrophobic forces, while a combination of hydrophobic and electrostatic interactions are involved when small molecules bind at site II. Bovine serum albumin (BSA) is one of the most widely used serum albumins and has been employed as a model structure for studies of protein morphology and drug delivery, since serum albumin conjugates can enhance the solubility of hydrophobic drugs in plasma and play an important role in modulating drug delivery in biological environments.

Figure 3-2: Structure of human serum albumin and identification of drug binding site I, II and fatty acid (FA) site 6. Ref 7, copyright 2013 American Chemistry Society.

Nanoparticles (NPs) made of albumins garnered interest recently as carriers to encapsulate hydrophobic drugs as well. After a breakthrough in the clinical application of albumin NPs for breast cancer treatment, albumin NPs have been extensively studied as nano-scale medicine delivery systems. Compared to other NPs, the biodegradable and biocompatible albumin NPs are less toxic and
easier to make, simply by water-in-oil emulsion\textsuperscript{12} or desolvation methods\textsuperscript{13,14} followed by cross-linking with glutaraldehyde.\textsuperscript{15}

Recently, Franchini and coworkers using BSA as nanocarrier encapsulating and stabilizing inorganic spinel cobalt ferrite NPs (CoFe\textsubscript{2}O\textsubscript{4}), for the application of MRI diagnosis and hyperthermic therapy.\textsuperscript{16} The CoFe\textsubscript{2}O\textsubscript{4} NPs were capped with a hydroxamic acid, ethyl-12-(hydroxyamino)-12-oxododecanoate (EHO) at the surface in order to increase the solubility in acetone. Later, the nanocarrier was prepared in acetone with poly(lactic-co-glycolic acid) (PLGA) cross linking BSA (\textbf{Scheme 3-1}). MRI experiment in vivo showed BSA-CoFe\textsubscript{2}O\textsubscript{4} has good efficiency in contrasting images of liver at short times. Moreover, BSA has been used widely to modify the surface of NPs in order to enhance water solubility, cell permeability and lower toxicity in bio-environment.

\begin{center}
\textbf{Scheme 3-1}: Capping of CoFe\textsubscript{2}O\textsubscript{4} with EHO and synthesis of the magnetic nanocarrier BSA-CoFe\textsubscript{2}O\textsubscript{4}. Ref 16, copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
\end{center}

\textbf{3.2 Structure Design and Synthesis Route}

Two squaraine dyes were designed with different heterocyclic donor, thus with different photophysical and aggregation behaviors. Both squaraine dyes have sulfonate pendants, so as exhibit nice water solubility and also plays an important role when interacting with BSA. The squaraine dyes were
synthesized by condensation reactions between squaric acid and according sulfonate inner salt, 3-(2-methylbenzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate and 3-(1',1',2'-trimethyl-1'H-3'-benz[e]indolio)propanesulfonate, as shown in Scheme 3-2.

**Scheme 3-2:** Synthesis route of SQ-SO1 and SQ-SO2.

BSA was labeled with squaraine dyes SQ-SO1 and SQ-SO2 by combining them together in water (3:1 molar ratio of BSA to SQ). BSA NPs were prepared by the desolvation technique. Briefly, BSA in DI-water (20 mg/mL) was adjusted to pH ≈ 7.4 using 0.01 M NaOH. Under constant stirring at 500 rpm, acetone was added continuously at a rate of 0.5 mL/min until the solution became turbid. Then, 20 μL of 8% glutaraldehyde solution was added, and the BSA particles, formed during desolvation, were crosslinked. After 24 h incubation at room temperature under continuous stirring, the resultant NPs were purified three times by centrifugation and re-dispersed in DI-water in an ultrasonication bath. The solution was filtered before use, and the concentration of the BSA-SQ was determined via UV-vis spectrophotometry.
using the molar absorptivity (BSA-SQ-SO1 $\varepsilon_{660} = 1.6 \times 10^5$ M$^{-1}$ cm$^{-1}$ and BSA-SQ-SO2 $\varepsilon_{675} = 2.1 \times 10^5$ M$^{-1}$ cm$^{-1}$).

### 3.3 Materials and General Techniques

Squaric acid and 2-methylbenzothiazole were purchased from Alfa Aesar, and 1,1,2-trimethylbenz[e]indole was purchased from Fluka. Propane sultone was purchased from TCI. 5-Dimethylamino-1-naphthalenesulfonamide (DNSA) and dansylproline (DP) were purchased from BroadPharm and were used without further purification. All solvents were used as received from commercial suppliers. Doubly distilled water was employed in all studies. $^1$H and $^{13}$C NMR spectra were recorded on an NMR spectrometer at 500 and 125 MHz, respectively. MS analyses were performed at the University of Florida. Melting points are uncorrected. Particle sizes of BSA-SQ NPs were analyzed by the light-scattering method using Zetasizer Nano-ZS90 (Malvern Instruments).

Linear absorption was measured using an Agilent 8453 UV-vis spectrophotometer. Fluorescence spectra were measured with a PTI Quantamaster spectrofluorimeter, which was equipped with a photomultiplier tube (PMT) detector. Emission spectra were corrected for the spectral sensitivity of the PMT. Emission spectra were used for calculation of the relative fluorescence quantum yield, using cresyl violet ($\Phi_{FL} = 0.54$) as a reference. $^{18}$ Lifetime measurements were performed using a tunable Ti:sapphire laser system with pulse duration $\sim 200$ fs/pulse, and repetition rate of 76 MHz. For the lifetime measurements, a band-pass filter was placed in front of an avalanche photodiode detector (APD), allowing the collection of suitable emission wavelengths. Data were acquired with a time-correlated single photon counting system (PicoHarp300).
For cell toxicity studies, HCT 116 cells (America Type Culture Collection, Manassas, VA) were placed in 96 well plates and incubated until there were no less than $6 \times 10^3$ cells per well for the cytotoxicity assays. After that, the cells were incubated at different concentrations of BSA-SQ NPs for an additional 20 h. Next, 10 μL of CellTiter 96 Aqueous One solution reagent was added into each well and incubated for 4 h. The relative cell viability, incubated with BSA-SQ NPs and a control for untreated cells, was determined by measuring the MTS-formazan absorbance on a microplate reader at 490 nm. All absorbance values were subtracted from the blank volume from a cell-free control. The results are given from the average of the three individual experiments.

For bioimaging studies, HTC 116 cells were placed onto poly-D-lysine-coated glass coverslips, and the cells were incubated for 48 h in order to grow a suitable amount for imaging. After that, the cells were incubated with BSA-SQ NPs at a concentration of 10 μM for 1 h; the incubation growth medium was made by diluting BSA-SQ NPs stock solution with PBS buffer. For the co-staining experiment, HTC 116 cells were incubated with a mixture of BSA-SQ NPs and Lysosensor Green (Invitrogen). After the incubation with dyes, the cells were washed three times using PBS buffer and then fixed using 3.7% formaldehyde solution for 15 min. NaBH₄ solution in PBS (1 mg/mL, pH = 8.0) was added to each well for 15 min, followed by washing with PBS and water again. Finally, glass coverslips were mounted on glass slides using Prolong Gold (Invitrogen) mounting media for microscopy imaging. Fluorescence images were obtained using a Leica TCS SP5 II laser-scanning confocal microscope. For BSA-SQ NPs, cells were excited at 622 nm, and fluorescence emission was collected at longer wavelengths. A confocal pinhole was applied for better image quality.
3.4 Results and Discussion

3.4.1 Interations of Squaraines with BSA

The absorption and emission of squaraine dyes are sensitive to the environment. Both absorption and emission spectra of squaraine dyes exhibited large changes in the presence of BSA, characterized by red shifts in wavelength. In the case of SQ-SO1, the initial absorption contains a monomer peak at 641 nm and a dimer peak located at a shorter wavelength (590 nm). The addition of BSA decreased absorption corresponding to both the monomer and dimer at first. However, continuous addition resulted in a gradual increase in absorption intensity with a new band at 660 nm (Figure 3-3a). When the concentration of BSA and SQ dyes reached the ratio of 2 : 1, the absorption of dimer completely disappeared.

Figure 3-3: Changes in the absorption (a) and emission (b) spectra of SQ-SO1 (5.0 μM) with addition of BSA. BSA concentration increased from 0 to 12 μM.

According to changes observed in absorption spectra, the noncovalent binding strength of SQ-SO1 with BSA was strong enough to cause dissociation of dimer aggregates with aggregation tendency highly reduced after the formation of the BSA-SQ-SO1 complex. Figure 3-3b shows the corresponding changes
in fluorescence spectra of **SQ-SO1** with increasing BSA concentration. Increasing the concentration of BSA resulted in a corresponding increase in fluorescence intensity, and there was a red shift of the maximum emission wavelength from 656 to 674 nm. This significant change in fluorescence with increasing intensity and bathochromic shift of approximately 20 nm facilitated naked eye detection.

Similar to the behavior of **SQ-SO1**, **SQ-SO2** exhibited the same tendency to conjugate with BSA. In the initial state, when **SQ-SO2** was dissolved in pure water, no dimer absorption was apparent in the absorption spectrum. This was probably due to the four methyl groups in the benzoindole structures, which hampered the aggregation of hydrophobic squaraine chromophore. The maximum absorption wavelength was 656 nm, which was 15 nm longer than that of the **SQ-SO1** monomer.

![Absorption and Emission Spectra](image)

**Figure 3-4**: Changes in the absorption (a) and emission (b) spectra of **SQ-SO2** (5.0 μM) with addition of BSA. BSA concentration increased from 0 to 6 μM.

When BSA was added to the solution, the absorption spectra showed an initial decrease and then increased with a new absorbance appearing at 675 nm. Relative fluorescence intensities of **SQ-SO2** increased with the addition of BSA until they reached a saturation point (Figure 3.4). Then, 5 μM of BSA
was needed in order to saturate the fluorescence intensity of 5 μM SQ-SO2, resulting a 1:1 stoichiometry for the complex between SQ-SO2 and BSA. Compared to SQ-SO2, more BSA was needed to bring the fluorescence of 5 μM SQ-SO1 to saturation; this may be because extra BSA was required to dissociate SQ-SO1 dimers. Thus, SQ-SO2 exhibited higher association constants with BSA of $7.6 \times 10^7$ M$^{-1}$ compared to $6.2 \times 10^6$ M$^{-1}$ for the BSA-SQ-SO1 complex.

In order to gain a better understanding of the interaction between these squaraine dyes and BSA, time-resolved fluorescence studies were conducted. Table 3-1 shows the fluorescence quantum yields and lifetimes of SQ-SO1 and SQ-SO2 in the presence and absence of BSA. SQ-SO1 and SQ-SO2 exhibited very weak fluorescent and low quantum yield in the absence of BSA, whereas in the presence of BSA, these two squaraine dyes showed 10-fold emission enhancement. In addition, both dyes dissolved in water without BSA and exhibited similarly short lifetimes of approximately 0.3 ns. However, after the introduction of BSA, the lifetime increased to 3.74 and 2.64 ns, respectively (Figure 3-5). The observed red shift and extended fluorescence lifetime after the introduction of BSA indicated environmental changes surrounding the squaraine chromophore, likely caused by the strong noncovalent interactions.

**Figure 3-5:** Picosecond time resolved fluorescence decay profiles of the SQ-SO1 (a) and SQ-SO2 (b) in the absence (blue line) and presence (red line) of BSA. In the presence of BSA, concentration of squaraine dye to BSA was 1:2 for SQ-SO1, and 1:1 for SQ-SO2.
Table 3-1: Photophysical properties of SQ-SO1 and SQ-SO2 in the presence and absence of BSA in aqueous solution. Absorption ($\lambda_{\text{Abs}}$), fluorescence ($\lambda_{\text{Em}}$), Stokes shift ($\Delta\lambda$), fluorescence quantum yield ($\Phi_f$), and fluorescence lifetime ($\tau_{\text{exp}}$). Fluorescence quantum yields were calculated relative to cresyl violet as the reference standard in methanol ($\Phi_f = 0.52$).

<table>
<thead>
<tr>
<th>Dyes</th>
<th>$\lambda_{\text{Abs}}$ (nm)</th>
<th>$\lambda_{\text{Em}}$ (nm)</th>
<th>$\Delta\lambda$ (nm)</th>
<th>$\Phi_f$</th>
<th>$\tau_{\text{exp}}$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ-SO1</td>
<td>641 ± 1</td>
<td>652 ± 1</td>
<td>11 ± 2</td>
<td>0.03 ± 0.006</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>SQ-SO1 + BSA</td>
<td>660 ± 1</td>
<td>669 ± 1</td>
<td>9 ± 2</td>
<td>0.27 ± 0.03</td>
<td>3.74 ± 0.2</td>
</tr>
<tr>
<td>SQ-SO2</td>
<td>655 ± 1</td>
<td>668 ± 1</td>
<td>13 ± 2</td>
<td>0.03 ± 0.005</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>SQ-SO2 + BSA</td>
<td>675 ± 1</td>
<td>684 ± 1</td>
<td>9 ± 2</td>
<td>0.24 ± 0.02</td>
<td>2.64 ± 0.1</td>
</tr>
</tbody>
</table>

3.4.2 Investigation of Site-Selective Binding of Squaraine Dyes to BSA

The complex formation and binding position between squaraine dyes and BSA was further studied by ligand displacement experiments; BSA site-specific binding reagents were used, dansylamide (DNSA) for site I selectivity and dansylproline (DP) for site II binding.\textsuperscript{20-22} When DP was added to a solution of the BSA-SQ-SO1 complex, a gradual decrease of the original absorption band at 660 nm was observed with an increase of a new band at 600 nm (Figure 3-6). Meanwhile, the emission intensity decreased gradually with an increase in DP concentration; an effective displacement of about 90% was achieved at 0.2 mM DP. One the other hand, with the addition of DNSA to the BSA-SQ-SO1 complex, there only result a slit change in both absorption and emission spectrum (Figure 3-7). Only approximately 10% effective displacement was found at a high concentration of DNSA. According to the changes of the absorption and emission spectrum upon addition of site special replacing reagent DNSA and DP, the squaraine dye SQ-SO1 could bind at both binding positions of site I and II, with preference of site II.
Figure 3-6: Absorption and emission changes of SQ-SO1 (5 μM) complexes with BSA (10 μM), followed by the addition of DP. DP concentration increased from 0 to 0.2 mM.

Figure 3-7: Absorption and emission changes of SQ-SO1 (5 μM) complexed with BSA (10 μM), followed by the addition of DNSA.

On the other hand, the BSA-SQ-SO2 complex exhibited similar properties (Figure 3-8). Interestingly, by introduction of DP to BSA-SQ-SO2 complex, the absorption spectrum exhibit the constant absorption wavelength, compared with the influence of DP to BSA-SQ-SO1 complex which caused the shift of absorption band. When the concentration of DP increased above 0.4 mM, the further addition of DP can
only cause small absorption and emission changes in contrast with the initial introduction. Meanwhile, the introduction of DNSA process little effect to both absorption and emission spectrum of BSA-SQ-SO2 complex (Figure 3-9). An effective displacement of 46% was achieved with increasing DP concentration, whereas DNSA showed only 17% displacement according to fluorescence intensity changes.

**Figure 3-8**: Absorption and emission changes of SQ-SO2 (5 μM) complexes with BSA (5 μM), followed by the addition of DP. DP concentration increased from 0 to 0.6 mM.

**Figure 3-9**: Absorption and emission changes of SQ-SO2 (5 μM) complexed with BSA (5 μM), followed by the addition of DNSA.
Control experiments were carried out in order to show the shifting and reduction of BSA-SQ absorption and emission are due to the addition of exchanging ligands. SQ-SO1 was dissolved in pure water without adding BSA, followed by titrations of DP and DNSA to the solution (Figure 3-10 and Figure 3-11). Both absorption and emission spectrum were recorded and the effects were estimated upon the intensity of emission band. In the absorption spectrum, DP can slightly reduce SQ-SO2 absorbance, while the introduction of DNSA has almost no effect on the absorption intensity. DP and DNSA can lead to 10% and 7% reduction of emission intensity of SQ-SO1, however, taking consider of the weaker fluorescent intensity of SQ-SO1 compared with BSA-SQ-SO1 complex, the effect of adding ligand replacing reagent is negligible.

Figure 3-10: Change of absorption (a) and emission (b) spectra of SQ-SO1 (5 μM) with increasing DP concentration.
Figure 3-11: Change of absorption (a) and emission (b) spectra of SQ-SO1 (5 μM) with increasing DNSA concentration.

Figure 3-12: Change of absorption (a) and emission (b) spectra of SQ-SO2 (5 μM) with increasing DNSA concentration.
**Figure 3-13**: Change of absorption (a) and emission (b) spectra of SQ-SO2 (5 μM) with increasing DP concentration.

SQ-SO2 alone also exhibit small changes in both absorption and emission spectra upon the introduction of DNSA and DP (Figure 3-12 and Figure 3-13). There are almost no effect in absorption and emission spectrum upon the addition of DNSA. And the influence with introduction of DP are also ignorable compared with the changes when BSA-SQ-SO2 is involved. These studies indicate that the photophysical changes observed for BSA-SQ complexes were mainly caused by displacement of SQ dyes by the binding ligands DNSA and DP. The absorption and emission response of BSA-SQ complexes to the added ligands revealed that SQ-SO1 and SQ-SO2 could bind to both site I and site II of BSA, with preference to site II.

### 3.4.3 Characterization of SQ-BSA NPs

The BSA particles with SQ label were prepared and cross linked via the well known procedure. Particles sizes were analyzed by dynamic light scattering (DLS) using Zetasizer Nano-ZS90 (Malvern Instruments). The samples were measured at 25°C and a scattering angle of 90°. **Figure 3-14** shows the number-averaged particle size distribution results for BSA-SQ-SO1 and BSA-SQ-SO2 NPs. BSA-SQ-SO1 and BSA-SQ-
SO2 NPs show similar size distribution results with average particle sizes of 90 and 130 nm, respectively. The absorption and emission of BSA-SQ NPs were measured, and the maximum intensity corresponded to that of the BSQ-SQ complex, with bathochromic shifts of 20 nm compared with SQ in aqueous solution without BSA (Figure 3-15).

**Figure 3-14:** BSA-SQ-SO1 NPs (up) and BSA-SQ-SO2 NPs (down) number-averaged particle size distribution measured by dynamic light scattering.

**Figure 3-15:** Absorption and normalized emission spectra of (a) BSA-SQ-SO1 NPs and (b) BSA-SQ-SO2 NPs.
3.4.4 Cytotoxicity of SQ-BSA NPs

Before using BSA-SQ NPs for potential protein tracking and bioimaging applications, biocompatibility was tested. A cytotoxicity assay was performed using the HCT 116 cell line (a common human colorectal cancer cell line). Since the squaraine dyes were noncovalently bound with BSA and were stabilized and shielded by a BSA pocket through electrostatic and hydrophobic interactions, the toxicity should be low enough even at high concentrations of NPs. The concentrations of BSA-SQ NPs tested ranged from 1.56 to 25 μM, even at the highest concentration of 25 μM. After 20 h incubation with cells the percentage of viable cells remained above 80% and 90% for BSA-SQ-SO1 NPs and BSA-SQ-SO2 NPs (Figure 3-16). For the following cell imaging studies, all BSA-SQ NPs were incubation with cells at the concentration of 10 μM, at which concentration BSA-SQ NPs exhibit almost non-toxicity.

![Graph showing cell viability of HCT 116 cell line with different concentrations of BSA-SQ-SO1 NPs (white bar) and BSA-SQ-SO2 NPs (black bar).](image)

**Figure 3-16:** Cell viability of HCT 116 cell line with different concentrations of BSA-SQ-SO1 NPs (white bar) and BSA-SQ-SO2 NPs (black bar).
3.4.5 Fluorescence Microscopy Imaging of Living Cells

Uptake of the NPs was conveniently monitored using fluorescence microscopy by determining the red fluorescence emitted from the cells that internalized the BSA-SQ NPs. HCT 116 cells were incubated with 10 μM of BSA-SQ NPs for 1 h, and, after fixing and washing, glass coverslips were mounted using Prolong Gold before imaging. For both BSA-SQ-SO1 and BSA-SQ-SO2 NP-incubated cells, the excitation wavelength was 622 nm, which largely avoids self-absorption and autofluorescence of biological specimens. Fluorescence images were collected at longer wavelengths from 640 to 750 nm. Figure 3-17 shows the differential interference contrast (DIC) and fluorescence images of HCT 116 cells after incubation with BSA-SQ NPs. From the fluorescence images, we can clearly see the uptake of the BSA-SQ NPs by the HTC 116 cells and exhibit dotted fluorescent particles which could be the organelles within cells.

Figure 3-17: Images of HCT 116 incubated with 10 μM BSA-SQ-SO1 NPs, DIC (upper left), fluorescence microscopy imaging (upper right). Images of HCT 116 incubated with 10 μM BSA-SQ-SO2 NPs, DIC (lower left), fluorescence microscopy imaging (lower right). Scale bar is 50 μm.
3.4.6 Intracellular Distribution of BSA-SQ Nanoparticles

Figure 3-18: Confocal fluorescence images of HCT 116 cells incubated with BSA-SQ-2 NPs (10 μM, 2 h) and LysoSensor Green (75 nM, 2 h). (a) DIC, (b) fluorescence image with BSA-SQ-2, (c) LysoSensor Green, & (d) colocalization imaging, overlay of (b) and (c). Scale bar is 50 μm.

It is well known that serum proteins can accelerate the cellular uptake of nanoscale particles through receptor-mediated endocytosis. At the initial stage of this process, the exogenous nanoparticles are enclosed in endosomes, which later mature into late endosomes and end by fusing with lysosomes. For internalized proteins, late endocytic transportation delivers them to lysosomes for degradation. Both BSA NPs and BSA-coated NPs have been reported to be uptaken by cells into the endosomes and/or lysosomes. To track the BSA-SQ NPs following their uptake, the lysosomal compartments of HCT 116 cells were coincubated with LysoSensor Green. LysoSensor Green is widely used to stain the lysosomes related
organelles at cellular level. **Figure 3-18** shows, that after incubation with BSA-SQ-SO2 NPs for 1 h, large amounts of red fluorescent particles overlapped with LysoSensor-stained lysosomes (green color), as seen by the yellow areas in the merged images. A similar result was observed for BSA-SQ-SO1 NPs. After uptake by cells, the BSA NPs localize in lysosomes (**Figure 3-19**). The bright red to near-IR fluorescent micrographs collected from BSA-SQ NPs provides potential for *in vivo* protein tracking and bioimaging.

**Figure 3-19**: Confocal fluorescence images of HCT 116 cells incubated with BSA-SQ-SO1 NPs (10 μM, 1 h) and LysoSensor Green (75 nM, 1 h). (a) DIC, (b) fluorescence image showing BSA-SQ-SO1, (c) and LysoSensor Green, and (d) colocalization imaging, overlay of (b) and (c). Scale bar is 25 μm.

### 3.4.7 BSA Tracking in Fixed Cells

Since **SQ-SO1** dye can exhibit fluorescence “turn on” upon interacting with BSA, a fix cell fluorescence imaging study was moved on in order to show the possibility of using **SQ-SO1** to tracking the location of
BSA at cellular level. Chinese hamster ovary (CHO) cells were placed onto poly-D-lysine coated glass coverslips in a 24-well plates. Incubation of 48 h, and the CHO cells grows to around $3 \times 10^4$ cells per well. After incubation, the cells were fixed with 3.7% formaldehyde PBS solution for 15 min at 37 °C. The fixed cells were washed with PBS buffer five times, followed by adding 0.5 mL of 1 mg/mL NaBH₄ PBS solution to each well for 15 min. The wells were then washed with PBS buffer two times and then DI water. Later on, the cells were treated with Triton-X solution for 15 min in order to permeate the cells membranes. Then again washed with PBS buffer two times and DI water, after that, the permealized cells were incubated with 50 μM of SQ-SO1 dye solution for 1 h for the sample of dye stained cells. For the controlled sample, the cells were incubated for 1 h without adding the SQ-SO1 dye solution. After washing with PBS and DI water again, the plates were then mounted on glass coverslips using Prolong Gold mounting media (Invitrogen) for later microscopy imaing. Fluorescence images were collected using Olympus IX70 microscope equipped with a cooled DCC. Band-pass filter was used to collect emission at suitable wavelength. Samples were exited at Texas Red channel and emission was recorded at longer wavelength.

The fixed cell imaging is shown in Figure 3-20, as we can see, in the control experiment, no emission observed in the wavelength collecting window. After stained with SQ-SO1, however, the squaraine dyes interacts with BSA within the cells, and exhibit a strong fluorescence and the corresponding imaging was collected. For the controlled sample there was no fluorescence detected since no squaraine chromophores were incubated. However, for the one stained using SQ-SO1 solution, there was a bright fluorescent showing in the near-IR wavelength. Since after the cells were fixed and permealized, they were treated with BSA solution, which blocked the proteins within the cells, after further incubated with squaraine dye, the complex of BSA-SQ formed and the bright near-IR fluorescence was detected by the microscope. This result shows that the water soluble squaraine dyes behaved like a potential candidate
for ‘turn-on’ fluorescence detection of proteins within cells. This study shows the BSA tracking application of water soluble SQ dyes at cellular level.

![Figure 3-20: CHO cell imagings with or without SQ-SO1 dye staining.](image)

### 3.5 Summary

In summary, two squaraine dyes, SQ-SO1 and SQ-SO2 were synthesized with sulfonate pendants with enhanced water solubility. SQ-SO2 with benzoiindolium structure process longer absorption and emission wavelength compared with benzothiazolium SQ-SO1, while SQ-SO1 showed stronger tendency to form aggregate dimer in aqueous solution. Through noncovalent interactions with biomacromolecule BSA, both
squamaine dyes exhibit large fluorescence enhancement and extended life time. This observed large fluorescence turn-on is believed due to the change of environment, after strongly complexation with protein BSA. Site selective experiment show these squaraine dyes were binding to both site I and site II of BSA with preference to site II. BSA-SQ complexes were used to make BSA NPs with average particle size around 100 nm. Utilizing the reduce tendency of aggregation and increased near IR fluorescent intensity, BSA-SQ NPs were incubated with HCT 116 cells and fluorescence microscope images were collected. To track the BSA-SQ NPs following their uptake by HCT 116 cells, the lysosomal compartment of HCT 116 cells were stained with LysoSensor Green, and there were large amounts of red fluorescent emitted from BSA-SQ particles overlapped with lysosomes. Fixed cells were also used to studied the BSA location sensing properties of the squaraine dye, and bright emissions were collected after the staining with SQ-SO2, showing the ex vivo applications. These two water soluble squaraine dyes, act as fluorogenic sensing to BSA through noncovalent interactions, showed the potential application as a near IR protein labeling reagent for protein tracking and both ex vivo and in vivo imaging.

3.6 Experimental Section

3-(2-methylbenzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate. Compound was obtained by literature method.30 In general, 2-methylbenzothiazole (2g, 13.4 mmol) was mixed with propane sultone (1.2 ml, 13.4 mmol) in 6 ml of toluene. The solution was brought to reflux with a water condenser for 8 hours. After cooling to room temperature, precipitate was filtrated and washed with dichloromethane and diethyl ether. 2.3 g off-white solid was obtained with a yield of 63.2%. m.p.: 284 – 286 °C (dec.). $^1$H NMR (500 MHz, DMSO-d$_6$) δ: 8.43 (t, J = 8.8 Hz, 2H), 7.89 (m, 1H), 7.8 (m, 1H), 4.92 (t, 2H), 3.19 (s, 3H), 2.64 (t, J = 6.4 Hz, 2H), 2.16 (m, 2H).
**N-propanesulfonate-benzothiazolium squaraine (SQ-SO1).** A mixture of 3-(2-methylbenzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate (1 g, 3.69 mmol) and squaric acid (210 mg, 1.84 mmol) in 8 ml toluene and 2ml pyridine was refluxed with a Dean-Stark apparatus for 3 h. Upon cooling, diethyl ether was added and the resulting precipitate was collected by filtration. Purified by recrystallization in methanol twice gave 800 mg of product SQ-SO1 as a blue solid. (yield 54.3%) mp 261-263 °C (dec.). $^1$H NMR (500 MHz, DMSO-d$_6$) δ: 8.93 (d, 4H), 8.58 (m, 2H), 8.06 (m, 4H), 7.91 (d, 2H), 7.74 (d, 2H), 7.47 (t, 2H), 7.30 (m, 2H), 5.92 (s, 2H), 4.43 (m, 4H), 2.61 (m, 4H), 2.02 (m, 4H). $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ: 178.45, 159.16, 142.72, 140.93, 127.36, 126.55, 124.24, 121.94, 112.73, 84.97, 48.48, 47.72, 44.73, 23.34. HRMS (ESI) theoretical [M-2C$_5$H$_6$N]$^2^-$ = 309.0135, [M-2C$_5$H$_6$N+Na]$^-$ = 641.0312, found [M-2C$_5$H$_6$N]$^2^-$ = 309.0140, [M-2C$_5$H$_6$N+Na]$^-$ = 641.0161.

**3-(1',1',2'-trimethyl-1'H-3'-benz[e]indolio)propanesulfonate.** Compound was obtained by literature method.$^{31}$ In general, 1,1,2-trimethyl-1H-benz[e]indole (2 g, 9.56 mmol) was mixed with propanesultone (1 ml, 11.17 mmol) in 10 ml of p-xylene. The solution was brought to reflux under a water condenser for 6 hours. After cooling to room temperature, precipitate was filtrated and washed with dichloromethane and diethyl ether. 2.1 g yellow solid was obtained with a yield of 66.2%. m.p. : 273 – 275 °C (dec.). $^1$H NMR (500 MHz, DMSO-d$_6$) δ: 8.35 (d, J = 8.3 Hz, 1H), 8.24 (m, 3H), 7.77 (t, J = 8 Hz, 1H), 7.71 (t, J = 8 Hz, 1H), 4.77 (t, J = Hz, 2H), 2.93 (s, 3H), 2.66 (t, J = 6.4 Hz, 2H), 2.21 (m, 2H), 1.75 (s, 6H).

**N-propanesulfonate-benzoindoilum squaraine (SQ-SO2).** A mixture of 3-(1',1',2'-trimethyl-1'H-3'-benz[e]indolio)propanesulfonate (1.5 g, 4.52 mmol) and squaric acid (250 mg, 2.19 mmol) in 10 ml n-butanol and 3ml pyridine was refluxed with a Dean-Stark apparatus for 5 h. Upon cooling, diethyl ether was added and the resulting precipitate was collected by filtration. Purified by recrystallization in methanol three times gave 1.4 g of product SQ-SO2 as a green solid. (yield 35.6%) mp 283-285 °C (dec.).
$^1$H NMR (500 MHz, DMSO-d$_6$) $\delta$: 8.89 (d, 4H), 8.50 (t, 2H), 8.22 (m, 2H), 8.00 (m, 8H), 7.83 (d, 2H), 7.62 (d, 2H), 7.45 (m, 2H), 5.90 (s, 2H), 4.37 (t, 4H), 2.61 (t, 4H), 2.08 (m, 4H), 1.97 (s, 6H). $^{13}$C NMR (125 MHz, DMSO-d$_6$) $\delta$: 196.69, 139.19, 137.38, 133.27, 130.98, 130.48, 128.93, 127.70, 123.83, 113.80, 55.57, 47.85, 24.42, 21.88, 13.92. HRMS (ESI) theoretical [M-2C$_5$H$_6$N]$^-$ = 369.1040, [M-2C$_5$H$_6$N+Na]$^-$ = 761.1973, found [M-2C$_5$H$_6$N]$^-$ = 369.1044, [M-2C$_5$H$_6$N+Na]$^-$ = 761.1965.

### 3.7 Spectrum Appendix

![Spectrum Image](image)

$^1$H NMR of 3-(2-methylbenzo[d]thiazol-3-ium-3-yl)propane-1-sulfonate.
$^1$H NMR of SQ-S01.

$^{13}$C NMR of SQ-S01.
MS spectrum of SQ-S01.

$^1$H NMR of 3-$(1',1',2'-\text{trimethyl}-1' H-3'$-benz[e]indolio)propanesulfonate.
$^1$H NMR of SQ-SO2.

$^{13}$C NMR of SQ-SO2.
MS spectrum of SQ-SO2.

3.8 References


CHAPTER 4. SQUARAINÉ MODIFIED DEOXYRIBONUCLEOSIDES AS NEAR-IR FLORESCENCE VISCOITY SENSOR

4.1 Introduction

Synthesis of modified nucleosides with functional structures has received increasing interest in recent years, due to their attractive applications in the field of both chemistry and biology. In the past decades, a number of modified or mutate nucleobase have been reported carrying functional moieties, like, aminoalkynyl and aminoalkyl, biotin, ferrocene, amino acids, fluorescein, porphyrin, cyanine dye, isoindoline-derived nitrooxide, and desmethyl thiazole orange. The examples of functional group modified deoxyuridine catalogue are show in Figure 4-1. These artificial non-nature nucleosides have been synthesized and applied to study nucleic acids structures, and their roles in biological systems. Among the non-nature nucleosides with purine or pyrimidine bases, the ones containing alkynyl group have received growing interest because of their potential usage as anticancer and antiviral drugs, and shows to be good candidates for raman imaing in living cells. In addition, the 5-alkynyl-substituted uridine derivatives does not change the conformations of oligonucleotides significantly, when the modified uridine bases were incorporated into the oligomer. These potential applications of artificial non-natural nucleosides have aroused continuous efforts to develop new functional groups for modification as well as efficient synthetic methods.
When a large scale of fluid is brought under shear forces, the internal friction leaded resistance against the shear force could be measured and the character is related to viscosity. The definition of viscosity is the ratio of shear stress vs. shear rate, and the general measuring methods including falling ball viscometer, capillary viscometer and cone-and-plate viscometer. However, all these above mentioned methods are unable to carry out the due of measuring viscosity in a small scale, for instance at cellular levels. Measuring fluid viscosity using fluorescence based method offered an opportunity to study at tiny scale owing to the sensitivity of fluorescent techniques. Fluorescent molecular for the application of viscosity sensors normally process twisted intramolecular charge transfer (TICT) states, which can lead to viscosity dependent fluorescent intensity and quantum yield ($\Phi_f$). The relationship between quantum

Figure 4-1: Example of functional group labeled deoxyuridine complexes. Ref 10, copyright 2003 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
yield and viscosity is well described by Förster-Hoffmann equation, \( \Phi_f = C \times \eta^x \), where in \( \eta \) is the viscosity, \( C \) and \( x \) are experiment condition dependent constants. The fluorescent viscosity sensors can measure a relatively large range from 1 to 1000 centipoise (cP). After the pioneering work done by Haidekker and Theodorakis, the fluorescent viscosity sensors have been developed rapidly and numbers of chromophores were used in order to achieve different detection wavelength. \(^{27,28}\)

**Figure 4-2:** Structure of RET pair based fluorescent viscosity sensor and emission spectrum upon changing viscosity in glycerol/ethylene glycol mixed solvent. Ref 29, copyright 2007 Nature Publishing Group.

Haidekker and Theodorakis developed a ratiometric fluorescent viscosity sensor based on a resonance energy transfer (RET) pair, composed of a donor fluorophore and an acceptor fluorophore (Figure 4-2). While the viscosity varied, the coumarin part (shown in blue) exhibit a viscosity independent emission while the amino cinnaminitrile moiety (shown in red) is viscosity sensitive. However, this RET pair is working under excitation at a wavelength of 365 nm, which is very close to biofluids absorbing range (280 nm for Trp) and autofluorescence of the fluid. Thus the excitation wavelength was adjusted to 390 nm to minimized the disturbing from fluid itself. \(^{29}\)
Kuimova and Ogilby introduced longer wavelength absorption and emission fluorescent viscosity sensor based on a porphyrin dimer.\textsuperscript{30} Even though the viscosity variation can not cause significant change in absorption spectrum, in the emission spectrum there are two well resolved bands located at 710 and 780 nm, the author assigned these two bands to emission from twisted and planar conformations of the dimer, respectively. This strategy offered a convenient way for monitoring dynamic processes at cellular level. In addition this compound has been used to create efficient photosensitizer for photodynamic therapy (PDT) due to the photophysical and photochemical properties of phorphyrin.

More recently, Peng \textit{et al.} reported a viscosity sensor based on cyanine dye, the authors announce with the help of the rotation of aldehyde group in the structure viscosity dependent emission was achieved.\textsuperscript{31} When dissolved in nonviscous solvent, the dye shows very weak fluorescence, however, upon increasing viscosity, the red emission is largely increased with a 12-fold of enhancement. After introducing into living cells, the intracellular viscosity imaging was collected and the inner cellular viscosity was reported to as high as 900 cP in some areas. Later Kang and Kim reported a BODIPY based fluorescent viscosity sensor aimed at the viscosity measurement for mitochondria in cells.\textsuperscript{32}
Figure 4-4: Cyanine dye based viscosity sensor with hypothetical rotation aldehyde group and viscosity dependent emission spectrum in glycerol/water mixed solvent. Ref. 31, copyright 2011 American Chemistry Society.

4.2 Structure Design and Synthesis Route

Scheme 4-1: Synthesis of alkyne functionalized benzothiazolium squaraine dye 5. a) Pd(dppf)Cl₂, Cul, CH₂CN, TEA, pyridine, trimethylsilylacetylene, 50°C, b) NaOH, DCM, MeOH, c) microwave reaction, EtI, CH₂CN, 110°C, 120 min, d) 1) EtOH, NaOH, 2) HCl 3) nBuOH, pyridine, 4.
Synthesis of alkyne containing benzothiazolium squaraine dye 5 was achieved as shown in Scheme 4-1. 5-ethynyl-3-ethyl-2-methylbenzothiazolium iodide was synthesized from 5-bromo-2-methylbenzothiazole in three steps. By replacing the 5-bromo with trimethylsilyl group, compound 1 was obtained with a yield of 91%. Followed by removing SiMe\textsubscript{3} group under NaOH base condition, the resulting deprotected compound 2 was carried on to alkylation with iodoethane. The alkylation of compound 2 was performed in a microwave reactor, at 110°C reaction temperature for 2 hr, after precipitation and washing product was obtained with a yield of 38%. Accordingly, semi-squaraine compound 4 was prepared following reported procedures,\textsuperscript{33} simply made by refluxing the mixture of dibutyl squarate with 3-ethyl-2-methylbenzothiazolium iodide in ethanol. Jointing semi-squaraine 4 with compound 3 afford the final alkyne squaraine dye 5 with overall yield of 35%.

Nucleosides modified by fluorescent compounds through chemical linkage at C-position of bases are valuable, which can be used as probes for the detection and structure studies of nucleic acid. Among the wide range of methods that can perform the nucleosides C-modification, coupling reactions catalyzed by palladium have been extensively studied and proved to be a feasible strategy.\textsuperscript{34} When nucleosides with protected hydroxyl groups are chosen as the starting material for coupling reactions, the hydrophobic properties help them more soluble in the organic reaction solvent, compared with unprotected ones. However, following this synthetic route, deprotection step is needed after the coupling reactions, consequently led to more operations and sometimes low overall yield. The alternative is to run the coupling reactions in high polar solvents starting with unprotected nucleosides, which shown to be more efficient. Both unprotected 5-halopyrimidine and 8-halopurine analogues have been reported to undergo the coupling reaction and afford high alkynylation yield.\textsuperscript{35,36}
Commercially available deoxyadenosine monohydrate was first converted to 8-bromo-deoxyadenosine compound 6, using bromine in NaOAc/AcOH buffer. The squaraine part and the deoxyadenosine moiety were linked together through a triple bond by Sonagashira coupling, scheme 4-2. Both 5’-OH and NH$_2$ groups in 8-bromo-deoxyadenosine were unproctected, and reaction conditions for Sonagashira coupling were optimized. N,N-Diisopropylethylamine (DIPEA) was proved to be a suitable base for the coupling reaction starting with BrdA, and the base of Amberlite IRA-67 beads was only a little less efficient compared with DIPEA. In general, the best conditions for this particular Sonagashira coupling reaction was using DIPEA as base and 20 mol% of CuI in DMF, after purification by column chromatography, squaraine dye modified deoxyadenosine dA-SQ was obtained at a yield of 11%.

Similar to the synthesis of dA-SQ, dU-SQ was also obtained by Sonagashira coupling between (+)-5-iodo-2′-deoxyuridine and alkyne squaraine 5, using a optimized condition using Amberlite IRA-67 as base and 20 mol% of CuI, and the yield was 14% after purification by column chromatography. Other organic bases, like TEA and DIPEA, are far less efficient compared with Amberlite IRA-67, when iodo-deoxyuridine was used as the starting mate. Meaning while, the 5’-OH of (+)-5-iodo-2′-deoxyuridine could be replaced by other functional group, like protection group dimethoxytrityl (DMT) in order to increase the solubility in general organic solvents. The resulting DMT protected deoxyuridine 7 was used to react with alkyne squaraine 5 by sonagashira coupling to afford the compound dU(DMT)-SQ. Protected product dU(DMT)-SQ was gained with a much higher yield of 23%, compared with the non-protected compound dU-SQ after purification by silica column.
Scheme 4-2: Synthesis of squaraine dye modified deoxynucleoside analogues by Sonagashira coupling reaction. a) NaOAc/AcOH buffer, Br₂, NaHSO₃, b) Pd(pph₃)₄, CuI, DIPEA, DMF. c) pyridine, DMTr-Cl, d) Pd(pph₃)₄, CuI, Amberlite IRA-67, DMF.

With the help of deoxynucleotide moiety, the resulting dA-SQ and dU-SQ are more bio-friendly and readily be soluble in glycerol/water mixture. When dissolved in glycerol/water mixed solvent, the hydrogen bond between the deoxynucleotide part and the hydroxyl groups from the solvent plays an important role both improved the solubility and control aggregation in polar solvent. In addition, the triple bond linking the deoxynucleotide and squaraine could perform a rotation in low viscous solvent, but not
in a highly viscous environment, which resulted in the viscosity dependent behavior. Viscosity dependent absorption and emission measurement were carried out.

### 4.3 Materials and General Techniques

2-methylbenzothiazole, 3,4-di-n-butoxy-3-cyclobutene-1,2-dione and (+)-5-iodo-2'-deoxyuridine were purchased from Alfa Aesar. 5-bromo-2-methylbenzothiazole and 3-ethyl-2-methylbenzothiazolium iodide were purchased from TCI. 2'-deoxyadenosine monohydrate was purchased from Acros. All other reagents and solvents were used as received from commercial suppliers. $^1$H and $^{13}$C NMR spectra were recorded on NMR spectrometer at 500 and 125 MHz, respectively. MS analyses were performed at University of Florida. Melting points were collected using Laboratory Devices Meltemp (50/60 cycles, 200 watts). Decomposing temperature of dA-SQ and dU-SQ were performed by Thermogravimetric Analysis (TGA) instrument Q5000 (V3.12, build 260).

UV-vis absorption spectra were measured using Agilent 8453 spectrophotometer. Fluorescence emission spectra were measured with PTI Quantamaster spectrofluorimeter, photomultiplier tube (PMT) was Hamamatsu R928. Cresyl violet was used as reference when fluorescence quantum yields were calculated. Lifetime experiments were carried out using a tunable Ti:sapphire laser system, Coherent Verdi-V10 and MIRA 900, with a pulse duration of ~200 fs/pulse and 76 Hz repetition rate. Band-pass filter was equipped in front of the avalanche photodiode detector in order to collect suitable emission wavelength.

Calculated fluorescence lifetime were determined by fluorescence quantum yield ($\Phi$) and natural lifetime ($\tau_N$) from the equation $\tau_{\text{cal}} = \tau_N \times \Phi^{38}$. $\tau_N$ was calculated by the following equation,
\[ 1/T_N = 2.88 \times 10^{-9} \times n^2 \times \int \frac{F(\nu)d\nu}{[F(\nu)/\nu^3]d\nu} \times \int \frac{\varepsilon(\nu)}{\nu} d\nu \]

wherein \( n \) is the refractive index of the solvent used for measurement, \( F(\nu) \) is the quantum fluorescence intensity, \( \varepsilon(\nu) \) is the absorption extinction coefficient, and wave number \( \nu \) is given by the unit of cm\(^{-1}\).

For bioimaging studies, HTC 116 cells were placed onto poly-D-lysine-coated glass coverslips, and the cells were incubated for 48 h in order to grow a suitable amount for imaging. After that, the cells were incubated with dU-SQ at a concentration of 8 μM (2% DMSO in PBS buffer) with or without 50 nM Hoechst for 1 h. After the incubation, the cells were washed three times using PBS buffer and then fixed using 3.7% formaldehyde solution for 15 min. NaBH\(_4\) solution in PBS (1 mg/mL, pH = 8.0) was added to each well for 15 min, followed by washing with PBS and water again. Finally, glass coverslips were mounted on glass slides using Prolong Gold (Invitrogen) mounting media for microscopy imaging. Fluorescence images were obtained using Olympus IX70, equipped with QImaging cooled CCD. Nucleus stained with Hoechst (blue, Ex 377/50, DM 409, Em 460/50), microtubules stained with FTIC-antibody (green, Ex 477/50, DM 507, Em 536/40) and dU-SQ (red, Ex 562/40, DM 593, Em 654/40). A confocal pinhole was applied for better image quality.

### 4.4 Results and Discussion

#### 4.4.1 Optimizing Reaction Conditions for Sonagashira Coupling

The key reaction for the linkage of squaraine part with deoxynucleotide is the final sonagashira coupling. Both organic base and amount of catalyst are carefully studied in order to optimizing the reaction condition, and the results are summarized in Table 4-1.
Table 4-1: Optimization of the conditions for the coupling of halid deoxynucleoside and alkyne squaraine dye.[a]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Deoxynucleoside</th>
<th>Solvent</th>
<th>Base</th>
<th>Cul (mol-%)</th>
<th>Product (yield)[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BrdA</td>
<td>Dioxane</td>
<td>TEA</td>
<td>10</td>
<td>No reaction</td>
</tr>
<tr>
<td>2</td>
<td>BrdA</td>
<td>Dioxane</td>
<td>DIPEA</td>
<td>10</td>
<td>No reaction</td>
</tr>
<tr>
<td>3</td>
<td>BrdA</td>
<td>DMF</td>
<td>TEA</td>
<td>10</td>
<td>Complex mixture</td>
</tr>
<tr>
<td>4</td>
<td>BrdA</td>
<td>DMF</td>
<td>DIPEA</td>
<td>10</td>
<td>dA-SQ (8%)</td>
</tr>
<tr>
<td>5</td>
<td>BrdA</td>
<td>DMF</td>
<td>DIPEA</td>
<td>20</td>
<td>dA-SQ (11%)</td>
</tr>
<tr>
<td>6</td>
<td>BrdA</td>
<td>DMF</td>
<td>Amberlite IRA-67</td>
<td>20</td>
<td>dA-SQ (9%)</td>
</tr>
<tr>
<td>7</td>
<td>IdU</td>
<td>DMF</td>
<td>TEA</td>
<td>20</td>
<td>Complex mixture</td>
</tr>
<tr>
<td>8</td>
<td>IdU</td>
<td>DMF</td>
<td>DIPEA</td>
<td>20</td>
<td>Complex mixture</td>
</tr>
<tr>
<td>9</td>
<td>IdU</td>
<td>DMF</td>
<td>Amberlite IRA-67</td>
<td>10</td>
<td>dU-SQ (6%)</td>
</tr>
<tr>
<td>10</td>
<td>IdU</td>
<td>DMF</td>
<td>Amberlite IRA-67</td>
<td>20</td>
<td>dU-SQ (14%)</td>
</tr>
<tr>
<td>11</td>
<td>IdU^{DMT}</td>
<td>DMF</td>
<td>DIPEA</td>
<td>20</td>
<td>dU(DMT)-SQ (10%)</td>
</tr>
<tr>
<td>12</td>
<td>IdU^{DMT}</td>
<td>DMF</td>
<td>Amberlite IRA-67</td>
<td>20</td>
<td>dU(DMT)-SQ (23%)</td>
</tr>
</tbody>
</table>

[a] Reactions were run under the conditions above using 10 mol-% Pd(PPh₃)₄, room temperature and continues stirring for 24 h. [b] Average isolated yield from two or more trials.
When Br-dA was used as the starting material, there were no reactions when dioxane was employed as the reaction solvent, either using TEA or DIPEA as the base. However, in more polar solvent DMF, under the same conditions, there could afford either a complex mixture or a 8% yield of dA-SQ after purification by silica columns. Keeping DIPEA as the base and increase the amount of CuI catalyst could generate a little bit higher yield of dA-SQ, and by replacing DIPEA with Amberlite IRA-67 beads, as milder base, the yield of final product could be affected, and the yield decreased a little bit to 9%. Starting with I-dU to perform the sonagashira coupling reaction, however, the option of organic base was narrowed. With 20% mole ratio of CuI, both TEA and DIPEA could generate the final product in a mess way companied by a lot of by-products. When switched to amberlite IRA-67, the reaction became much cleaner and the final dU-SQ could be isolated at a yield of 14%. Similar to dU-SQ, dU(DMT)-SQ could be afforded under the same conditions with a total yield of 23% after purified by silica column.

4.4.2 Physical Properties of dA-SQ and dU-SQ

![Normalized absorption (black line) and emission (red line) of dA-SQ (left) and dU-SQ (right) in DMSO.](image)

Figure 4-5: Normalized absorption (black line) and emission (red line) of dA-SQ (left) and dU-SQ (right) in DMSO.
In DMSO solution, \textbf{dA-SQ} and \textbf{dU-SQ} exhibit identical absorption and emission properties, with maximum absorption at 681 nm and emission maximum at 695 nm (Figure 4-5). The Stoke shifts are around 14 nm, typical for the squaraine analogue. The summary of absorption and emission data was given in Table 4-2, along with their quantum yield and fluorescent life-time. Generally, fluorescent quantum yield ($\Phi_f$) is correlated strongly of fluorescent life-time, which is consistent with our studies.\textsuperscript{39} \textbf{dA-SQ} had a lower $\Phi_f$, corresponding with a shorter life-time of 2.08 ns, while the fluorescent life-time of \textbf{dU-SQ} was 2.18 ns along with a higher $\Phi_f$ of 0.37. For the lifetime spectrum (Figure 4-6) measured in DMSO solution, both modified deoxynucleosides exhibited single exponential fluorescence decay. And the calculated fluorescent lifetime matches the experimental data in an acceptable range.

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{Abs}}$ (nm)</th>
<th>$\lambda_{\text{Em}}$ (nm)</th>
<th>$\Delta\lambda$ (nm)</th>
<th>$\Phi_f$</th>
<th>$\tau_{\text{exp}}$ (ns)</th>
<th>$\tau_{\text{cal}}$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dA-SQ</td>
<td>681 ± 1</td>
<td>695 ± 1</td>
<td>14 ± 2</td>
<td>0.30 ± 0.02</td>
<td>2.08 ± 0.10</td>
<td>1.53 ± 0.12</td>
</tr>
<tr>
<td>dU-SQ</td>
<td>682 ± 1</td>
<td>695 ± 1</td>
<td>13 ± 2</td>
<td>0.37 ± 0.04</td>
<td>2.18 ± 0.11</td>
<td>1.84 ± 0.15</td>
</tr>
</tbody>
</table>

\textbf{Table 4-2:} Photophysical properties of \textbf{dA-SQ} and \textbf{dU-SQ} in DMSO solution. Absorption ($\lambda_{\text{Abs}}$), fluorescence ($\lambda_{\text{Em}}$), stock shift ($\Delta\lambda$), fluorescence quantum yield ($\Phi_f$) and fluorescence life time from experimental ($\tau_{\text{exp}}$) and calculation ($\tau_{\text{cal}}$).

\textbf{Figure 4-6.} Fluorescent life-time measurements of \textbf{dA-SQ} (a) and \textbf{dU-SQ} (b).
When the squaraine modified deoxynucleotides were dispersed into 3 : 1 water/DMSO (v/v) mixed solvent, H-aggregation generated located at shorter wavelength in the absorption spectrum. When excited at the original monomer wavelength of 625 nm, the emission intensity decreased a lot, compared with the emission intensities in DMSO (Figure 4-7). The quantum yields under this condition were dropped to 0.0018 and 0.0021 for dA-SQ and dU-SQ, respectively. Increasing the amount of water, even lower quantum yield could be resulted until almost no detectable fluorescent signal.

![Figure 4-7](image)

**Figure 4-7**: Absorption (black line) and normalized emission (red dot) of dA-SQ (a) and dU-SQ (b) in 3 : 1 water/DMSO (v/v) mixed solvent. Excitation wavelength of 630 nm. Quantum yield under this condition were 0.0021 and 0.0016, respectively.

In addition, the thermal stability properties were measured, and the resulting dA-SQ and dU-SQ exhibit quite stable thermal behavior. Thermogravimetric analysis were carried out using TGA instrument Q5000 (V3.12, build 260) with platinum pan. Ramp method was selected with a heating rate of 10 °C/min. According to the result, dA-SQ exhibit three decomposition temperatures, at 180, 338 and 608 °C, respectively. dU-SQ however, is slightly stable than dA-SQ, process only two decomposition temperatures, at 203 and 538 °C, and the two decomposition temperatures are separated by a slow weight decreasing platform. When the temperature reached 700 °C, there was only 4% remaining mass. (Figure 4-8)
4.4.3 Viscosity Dependent Optical Properties

With the help of hydrophilic deoxynucleoside moiety, dA-SQ and dU-SQ are more biological environment friendly and readily soluble in glycerol/water mixed solvent. In glycerol/water mixed solvent, hydrogen bond could be generated between the deoxynucleotide part and hydroxyl groups from the solvent, which improved the solubility and modulate the aggregation from squaraine moiety. When dissolved in glycerol/water mixed solvent, both absorption and emission are highly dependent on the viscosity of the binarial solvent. By adding glycerol to water, the viscosity of the solution increased from 1.7 cP (20% glycerol, v/v) to 209 cP (90% glycerol, v/v) at 25 °C.\(^{40}\) Absorption spectrum changed as viscosity increased, with the monomer band decreasing companied by the formation of H-aggregation band. On the other hand, fluorescence of dU-SQ was greatly enhanced by 310-folds and the quantum yield reached 0.16 in 90% glycerol solutions (Figure 4-9). Viscosity dependent fluorescent intensity is described by Förster-Hoffmann equation,\(^{26}\) and widely used for viscosity sensors.\(^{25,27,31}\)
Figure 4-9: Absorption (a) and emission (b) spectrum of dU-SQ ([dU-SQ] = \(1.0 \times 10^{-5}\) mol/L) in glycerol upon increasing amount of water. Volumn of glycerol/water varied from 9 : 1 to 8 : 2. Insert of (b) shows the linearity of log I (emission intensity at 675 nm) versus log η (viscosity, cP) of dU-SQ in glycerol/water mixed solvent. Excitation wavelength of 625 nm.

Figure 4-10: Absorption (a) and emission (b) spectrum of dA-SQ ([dA-SQ] = \(1.0 \times 10^{-5}\) mol/L) in glycerol upon increasing amount of water. Volumn of glycerol/water varied from 9 : 1 to 8 : 2. Insert of (b) shows the linearity of log I (emission intensity at 675 nm) versus log η (viscosity, cP) of dA-SQ in glycerol/water mixed solvent. Excitation wavelength of 625 nm.

dA-SQ shows similar properties under the same condition. From 20% to 80% glycerol solution, the fluorescent intensity increased almost 320-folds, and the Förster-Hoffmann linear plot of \(R^2 = 0.94\) (Figure
4-10). In addition the “turn on” fluorescence in glycerol also showed the potential application of these squaraine modified deoxynucleotides as fluorogenic dyes.⁴¹

4.4.4 Effect of Aggregation

When the viscosity increased, the aggregation of **dU-SQ** was hampered along with the rotation of triple bond linking nucleobase and squaraine dye, all contributed to the “turn on” fluorescent with increasing viscosity. Continuous studies were performed in the purpose of identifying the effect of aggregation on fluorescence intensity. Solvent system of water/DMSO was chosen because of similar polarity and dielectric constant compared with water/glycerol binary solvents. In 3 : 1 water/DMSO, **dU-SQ** can be readily dissolved and exhibit a big hump of H-aggregation, and the shape of the absorption spectrum was closed to the frame obtained in low viscosity glycerol/water. Upon increasing the temperature, the H-aggregation dissociated and the monomer accumulated, which process identical to the results in viscosity experiments. At the final stage, at temperature of 70 °C nearly all the H-aggregation were dissembled and the monomer absorption intensity was comparable to the ones in high viscosity systems. However, according to the fluorescence intensity, the enhancement of dissociation of H-aggregation can only lead to 13-folds of fluorescence increase when the samples were exited at the wavelength of 633 nm (Figure 4-11). Similar results were obtained when using **dA-SQ**, H-aggregation dissociated companied with increasing monomer band and 8-folds of fluorescence increasement were generated with increasing temperature (Figure 4-12). Thus, the impact of H-aggregation alone can not lead to the huge 310-folds of fluorescence enhancement from low viscosity solvent to high viscosity glycerol/DMSO solvent. The influence of rotation restriction is another factor that consequently contributes to the fluorescence ‘turn on’.
Figure 4-11: Temperature dependent of absorption (a) and emission (b) spectrum of dU-SQ ([dU-SQ] = 8.0 × 10^{-6} mol/L) in 3:1 water/DMSO solvent. Temperature increased from 20 °C to 70 °C. Emission spectrums were recorded with 633 nm excitation wavelength.

Figure 4-12: Temperature dependent of absorption (a) and emission (b) spectrum of dA-SQ ([dA-SQ] = 1.0 × 10^{-5} mol/L) in 3 : 1 water/DMSO solvent. Temperature decreased from 70 °C to 20 °C. Emission spectrums were recorded with 633 nm excitation wavelength.

4.4.5 Bio-Environment Pre-Testing

Since the final purpose is to introduce the squaraine dye modified deoxyribonucleosides into cells and monitoring the viscosity at cellular level, bio-environment pre-testing was carried out in order to have an
idea of how those biomolecules will impact the duty of viscosity sensors. **dU-SQ** was selected as the model compound for the testing and further cell incubation, because of higher fluorescence intensity compared with **dA-SQ**. Conditions were chosen to mimic the bio-environments from cell membrane to nucleic, such as liposome, BSA, CT-DNA and pluronic micelles. The results are showing in Figure 4-13. By eye detection, only the sample in 90% glycerol exhibit a light green color showing the predominate of monomer species, while all the others in DMSO/water conditions all performed blue colors as the formation of H-aggregation. Under the excitation using long wavelength UV lamp, only the sample in glycerol viscous binary solution showing a bright red fluorescence, in the meantime, other samples with the addition of biomolecules have no affect on the fluorescence. The dim blue fluorescent from sample 3 may be due to the organic molecules contained in the liposome structure, like proteins and cholesterol.

![Image of fluorescence samples](image)

**Figure 4-13:** Absorption (up) and emission (bottom) of **dU-SQ** under different conditions. (1) 9 : 1 glycerol/water (v/v), (2) 9 : 1 water/DMSO (v/v), (3) liposome, (4) 10 mg/ml BSA, (5) 0.5 mg/ml CT-DNA, (6) pluronic F127 micelle (2 wt%) in 9 : 1 water/DMSO (v/v). Concentration of **dU-SQ** around 5 × 10⁻⁵ mol/L.

### 4.4.6 Cell Imaging

High viscosity values have been reported in some intra- and intercellular regions, which were estimated to be 100 – 400 cP. We hypothesized that high viscosity will lead to the fluorescence enhancement of
**dU-SQ** 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 4-14** and **4-15**). Hoechst was used as a nuclear stain to visualize the cell nucleus for reference.

**Figure 4-14:** Images of HCT 116 cells incubated with 8 μM **dU-SQ** (a) DIC, (b) fluorescence microscope overlaid with DIC. Scale bar = 10 μm.

**Figure 4-15:** Images of HCT 116 cells incubated with **dU-SQ** (10 μM) and Hoechst (5 μg/mL) (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 bar = 10 μm.
High viscosities can be generated during mitosis and in the vicinity of microtubules (MTs). Recent studies showed that the viscosity was dependent on MTs cross-linking and density. In order to confirm this, COS 7 (monkey kidney tissue cells) were incubated with dU-SQ and Hoechst. MTs were stained and different stages of mitosis were captured. During metaphase and anaphase stages the viscosity appears higher than telophase, viz-a-viz the intensity (brightness) of the fluorescence (viscosity-dependent fluorescence enhancement), as shown in Figures 4-16. Figure 4-16 presents micrographs at different stages of mitosis, visualizing the DIC imaging, nuclear and MTs staining, the dU-SQ viscosity probe, and

Figure 4-16: Fluorescence microscopy images of COS 7 cells incubated with dU-SQ (10 μM) and Hoechst (5 μg/mL) followed by microtubule staining. Nucleus stained with Hoechst (blue, Ex 377/50, DM 409, Em 460/50), microtubules stained with FTIC-antibody (green, Ex 477/50, DM 507, Em 536/40) and dU-SQ (red, Ex 562/40, DM 593, Em 654/40). Scale bar = 10 μm.
finally, 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 cross-linked.

### 4.5 Summary

In conclusion, we have demonstrated and optimized the reaction conditions of squaraine dye modified deoxyadenosine and deoxyuridine compounds. These non-native deoxynucleosides with C-modified squaraine chromophore exhibit sharp and intensive absorption in the near IR region, along with identical fluorescence emission at 695 nm in DMSO solution. Both absorption and emission spectra of these squaraine dye modified deoxynucleosides show viscosity dependent properties in glycerol/water mixed solvents. Under the combined effect of aggregation and rotation, the enhancement of fluorescence under highly viscous solvent could reach above 300-fold, which is the highest value reported so far. Hence, these non-native deoxynucleosides can have the potential applications for the studies such as near-IR nucleoside tracking and viscosity related sensors at cellular level.

### 4.6 Experimental Section

**2-methyl-5-((trimethylsilyl)ethynyl)benzothiazole (1).** Under a nitrogen atmosphere 5-bromo-2-methylbenzothiazole (1 g, 4.38 mmol), bis(triphenylphosphine)palladium(II) dichloride (300 mg, 0.43 mmol), copper iodide (100 mg, 0.53 mmol) were mixed in 20 ml degassed acetonitrile and triethyl amine solution (1 : 1, v/v). 2 ml of pyridine was added, and the resulted mixture was stirred at 50 °C for 16 h. After cooled to room temperature, solvent was removed under reduced pressure and the solid residue
was purified by flash column chromatography (silica gel, degrade elution hexane/ethyl acetate from 10 : 1 to 5 : 1). 0.98 g of white solid was obtained. Yield: 91%, m.p.: 126 – 127.5 °C. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta:\) 8.02 (d, \(J = 1\) Hz, 1H), 7.72 (d, \(J = 8.3\) Hz, 1H), 7.43 (dd, \(J = 8.3, 1.5\) Hz, 1H), 2.82 (s, 3H), 0.28 (s, 9H). \(^{13}\)C NMR (125 Hz, CDCl\(_3\)) \(\delta:\) 167.91, 153.25, 138.06, 128.31, 125.83, 121.04, 104.77, 94.38, 20.21, 0.00 ppm. HRMS (ESI) theoretical \([M+H]^+ = 246.0767\), found \([M+H]^+ = 246.0777\).

**5-ethynyl-2-methylbenzothiazole (2).** 2-methyl-6-((trimethylsilyl)ethynyl)benzo[d]thiazole (2 g, 8.15 mmol) was dissolved in 30 ml of dichloromethane, and 30 ml methanol NaOH solution (3%, w/w) was added slowly. The mixture was allowed to stir at room temperature for 2 h, followed by removing the organic solvent. The solid residue was further purified by column chromatography (silica gel, degrade elution hexane/ethyl acetate from 10 : 1 to 5 : 1). 1.1 g pale yellow crystal was obtained. Yield: 78%, m.p. : 66 – 67 °C. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta:\) 8.07 (d, \(J = 1.5\) Hz, 1H), 7.75 (d, \(J = 8.3\) Hz, 1H), 7.45 (dd, \(J = 8.1, 1.7\) Hz, 1H), 3.12 (s, 1H), 2.82 (s, 3H). \(^{13}\)C NMR (125 Hz, CDCl\(_3\)) \(\delta:\) 168.12, 153.21, 136.39, 128.26, 126.09, 126.08, 121.33, 119.79, 83.38, 21.08 ppm. HRMS (ESI) theoretical \([M+H]^+ = 174.0372\), found \([M+H]^+ = 174.0378\).

**5-ethynyl-3-ethyl-2-methylbenzothiazolium iodide (3).** 5-ethynyl-2-methylbenzothiazole (1 g, 5.77 mmol) was mixed with 2 ml of iodoethane in 1.5 ml of acetonitrile. The mixture was heated in microwave reactor (CEM, discover) at 110 °C for 2 h. Precipitate was filtrated and washed with diethyl ether to afford 220 mg grey powder, yield 38.6%. m.p. : 256 – 267 °C (dec.). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta:\) 8.52 (d, \(J = 1.1\) Hz, 1H), 8.47 (dd, \(J = 8.5, 1.5\) Hz, 1H), 7.85 (dd, \(J = 8.4, 1.3\) Hz, 1H), 4.75 (q, \(J = 7.2\) Hz, 2H), 4.59 (s, 1H), 3.23 (s, 3H), 1.43 (m, 3H). \(^{13}\)C NMR (125 Hz, CDCl\(_3\)) \(\delta:\) 178.89, 141.14, 131.39, 130.18, 125.65, 123.20, 120.19, 84.29, 82.36, 45.36, 17.47, 13.74 ppm. (ESI) theoretical \([M]^+ = 202.0685\), found \([M]^+ = 202.0692\).
4-((3-ethyl-6-ethynylbenzothiazol-3-ium-2-yl)methylene)-2-((3-ethylbenzothiazol-2-ylidene)methyl)-3-oxocyclobut-1-enolate (5). Compound 5 was synthesized by two continues steps. First synthesis of 3-((3-ethylbenzothiazol-2-ylidene)methyl)-4-hydroxycyclobut-3-ene-1,2-dione. 3-butoxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione (330 mg, 1.0 mmol) was dissolved in 20 ml of ethanol. After the solution was heated to reflux, 0.3 ml of 40% NaOH water solution (w/w) was added, and the reaction was kept at refluxing for 30 min before cooled to room temperature. The pH of the solution was adjusted to 3 ~ 4 using 2N HCl. Then extracted with dichloromethane two times, organic layers were combined and the solvent was removed under reduced pressure. This resulting intermediate 3-hydroxy-4-((3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione was used immediately without further purification. This intermediate and 5-ethynyl-3-ethyl-2-methylbenzothiazolium iodide (275 mg, 0.84 mmol) were dissolved in 10 ml mixed n-BuOH/pyridine solvent (7 : 3, v/v). The mixture was refluxed with a Dean-Stark apparatus for 4 h. After cooled to room temperature, diethyl ether was added and the precipitated product was collected by filtration. Further purification was carried out by flash column chromatography (silica gel) with dichloromethane/methanol (20 : 1, v/v) as eluent, providing 160 mg of blue powder. Yield: 35%, m.p. : 230 – 232 °C (dec.). 1H NMR (500 MHz, CDCl3) δ: 7.55 (t, J = 6.3, 1H), 7.35 (m, 3H), 7.22 (m, 3H), 5.88 (m, 2H), 4.14 (m, 4H), 3.15(d, 1H), 1.40 (m, 6H). 13C NMR (125 MHz, DMSO-d6) δ: 177.30, 174.77, 159.39, 157.42, 141.47, 140.95, 129.23, 128.32, 127.34, 124.62, 123.08, 121.05, 120.91, 115.33, 114.96, 113.02, 85.75, 85.36, 83.60, 82.03, 12.74, 12.45 ppm. HRMS (APCI) theoretical [M+H]+ = 457.1039, [2M+Na]+ = 935.1825, [3M+Na]+ = 1391.2791, found [M+H]+ = 457.1037, [2M+Na]+ = 935.1811, [3M+Na]+ = 1391.2744.

8-bromo-2’-deoxyadenosine (6). Synthesis of 8-bromo-2’-deoxyadenosine was carried out according to literature method. In general, 2’-deoxyadenosine (1.0 g, 3.72 mmol) was suspended in NaOAc/AcOH
buffer (40 ml, 0.9 M, pH = 4), the resulted suspension was heated strongly to make a clear solution. After cooled to room temperature, a solution of 1.15 g Br₂ in 40 ml Milli-Q water was added slowly, within two portions. The mixture was stirred at room temperature for 24 h. Saturated NaHSO₃ water solution was added to the solution, until the bromine color disappeared. Then 2N NaOH was used to adjust the pH to 8, during which procedure white precipitate formed gradually. 400 mg white powder product was obtained by filtration followed by washing with cold water. Yield: 30.4%. m.p.: 242 – 245 °C (dec.). ¹H NMR (500 MHz, DMSO-d₆) δ: 8.13 (s, 1H), 7.55 (s, 2H), 6.32 (t, 1H), 6.34 (m, 2H), 4.51 (m, 1H), 3.92 (m, 1H), 3.69 (m, 1H), 3.61 (m, 1H), 3.28 (m, 1H), 2.22 (m, 1H).

5'-O-(4,4'-dimethoxytrityl)-5-ido-2'-deoxyuridine (7). 5-ido-2'-deoxyuridine (1 g, 2.82 mmol) was dissolved in 15 ml dry pyridine and reacted with 4,4'-dimethoxytrityl chloride (DMT-Cl) (1.15 g, 3.40 mmol) for 4 h at room temperature. Afterwards, 15 ml of ice-cold water was added to the solution and the resulting mixture was extracted twice with dichloromethane. The organic layers were combined and washed with water, dried over MgSO₄. Solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography, eluted with hexane/ethyl acetate (1 : 1, v/v) to give 1.67 g product, yield 90%. m.p.: 110 – 112 °C. ¹H NMR (500 MHz, CDCl₃) δ: 9.45 (s, 1H), 8.07 (s, 1H), 7.08 to 7.42 (m, 9H), 6.77 (d, J = 8.9 Hz, 4H), 6.24 (t, J = 6.7 Hz, 1H), 4.52 (m, 1H), 4.05 (m, 1H), 3.72 (s, 6H), 3.32 (m, 2H), 2.83 (s, 1H), 2.45 (m, 1H), 2.21 (m, 1H).

Synthesis of dA-SQ. Under a nitrogen atmosphere 8-bromo-2'-deoxyadenosine (225 mg, 0.68 mmol), squaraine compound 5 (200 mg, 0.44 mmol), Pd(pph₃)₄ (50 mg, 0.044 mmol), Cul (17 mg, 0.088 mmol) were mixed in 5 ml of DMF. 200 μL DIPEA was added slowly into the reaction and stirred at room temperature for 24 h. Diethyl ether was added, and the resulted precipitate was collected by filtration. Further purification was carried out by column chromatography (silica gel), first eluted with ethyl
acetate/methanol (10 : 1) and then dichloromethane/methanol (10 : 1). 34.5 mg of purple solid was obtained, yield 11%. m.p. : 180 °C (dec.). \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \(\delta\): 8.19 (s, 1H), 7.93 (m, 2H), 7.78 (d, \(J = 1\) Hz, 1H), 7.63 (m, 3H), 7.48 (m, 2H), 7.31 (m, 1H), 6.57 (t, \(J = 7.3\) Hz, 1H), 5.87 (s, 1H), 5.76 (s, 1H), 5.41 (d, \(J = 4.4\) Hz, 1H), 5.31 (dd, \(J = 7.8, 4.4\) Hz, 1H), 4.54 (d, \(J = 2\) Hz, 1H), 4.32 (m, 4H), 3.93 (d, \(J = 2.9\) Hz, 1H), 3.7 (m, 1H), 3.52 (m, 1H), 3.17 (m, 1H), 2.30 (m, 1H), 1.30 (q, \(J = 7\) Hz, 6H). \(^{13}\)C NMR (125 MHz, DMSO-d\(_6\)) \(\delta\): 177.92, 174.19, 159.91, 156.81, 156.51, 149.05, 141.79, 140.92, 133.11, 130.89, 128.39, 127.46, 124.81, 123.37, 123.16, 120.15, 118.89, 114.66, 113.22, 94.67, 88.72, 86.04, 85.49, 79.68, 71.59, 62.60, 12.81, 12.42 ppm. HRMS (ESI) theoretical [M+H]^+ = 706.1901, [2M+H]^+ = 1411.3729, found [M+H]^+ = 706.1913, [2M+H]^+ = 1411.3710.

**Synthesis of dU-SQ.** Under a nitrogen atmosphere a mixture of 5-iodo-2'-deoxyuridine (200 mg, 0.56 mmol), squaraine compound 5 (200 mg, 0.44 mmol), Pd(pph\(_3\))\(_4\) (50 mg, 0.044 mmol), Cul (17 mg, 0.088 mmol) and 250 mg of Amberlite IRA-67 in 5 ml of DMF was stirred at room temperature for 24 h. The Amberlite IRA-67 beads were excluded by filtration first, and to the DMF solution diethyl ether was added, resulted precipitate was collected by filtration. Further purification was carried out by column chromatography (silica gel), first eluted with ethyl acetate/methanol (10 : 1) and then dichloromethane/methanol (5 : 1). 42 mg of purple blue solid was obtained, yield 14%. m.p. : 203 °C (dec.). \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \(\delta\): 11.73 (s, 1H), 8.42 (s, 1H), 7.88 (m, 2H), 7.59 (m, 2H), 7.47 (m, 1H), 7.30 (m, 2H), 6.14 (t, \(J = 6.6\) Hz, 1H), 5.83 (s, 1H), 5.76 (s, 1H), 5.29 (d, \(J = 3.9\) Hz, 1H), 5.21 (t, \(J = 4.9\) Hz, 1H), 4.34 (d, \(J = 6.8\) Hz, 2H), 4.27 (t, \(J = 4.6\) Hz, 3H), 3.83 (d, \(J = 3.4\) Hz, 1H), 3.64 (m, 2H), 2.18 (t, \(J = 6.6\) Hz, 2H), 1.28 (dt, \(J = 14.1, 6.9\) Hz, 6H). \(^{13}\)C NMR (125 MHz, DMSO-d\(_6\)) \(\delta\): 177.21, 174.76, 161.83, 159.35, 157.47, 149.86, 144.64, 141.55, 140.97, 128.89, 128.31, 127.13, 124.64, 123.11, 121.65, 114.16, 113.05, 98.40,
91.93, 88.07, 85.75, 85.42, 83.76, 70.38, 61.27, 12.74, 12.51 ppm. HRMS (ESI) theoretical \([M+H]^+ = 682.1618\), found \([M+H]^+ = 682.1622\).

**Synthesis of dU(DMT)-SQ.** Under a nitrogen atmosphere a mixture of 5’-O-(4,4’-dimethoxytrityl)-5-iodo-2’-deoxyuridine (160 mg, 0.24 mmol), squaraine compound 5 (100 mg, 0.22 mmol), Pd(ppy)\(_4\) (25 mg, 0.022 mmol), Cul (8.5 mg, 0.044 mmol) and 150 mg of Amberlite IRA-67 in 3 ml of DMF was stirred at room temperature for 24 h. The Amberlite IRA-67 beads were excluded by filtration, and then diethyl ether was added to the DMF solution, resulted precipitate was collected by filtration. Further purification was carried out by column chromatography (silica gel), elute with dichloromethane/methanol (20 : 1 to 15 : 1). 50 mg of red purple powder was obtained, yield 23%. m.p. : 215 – 218 °C. \(^1\)H NMR (500 MHz, DMSO-d\(_6\)) \(\delta\): 11.8 (s, 1H), 8.15 (s, 1H), 7.9 (d, J = 7 Hz, 1H), 7.73 (d, J = 8.2 Hz, 1H), 7.6 (d, J = 8.2 Hz, 1H), 7.45 (m, 3H), 7.29 (m, 7H), 7.16 (m, 2H), 6.84 (m, 5H), 6.16 (t, J = 6.6 Hz, 1H), 5.84 (s, 1H), 5.74 (s, 1H), 5.35 (d, J = 4.4 Hz, 1H), 4.33 (m, 3H), 4.13 (m, 2H), 3.96 (d, J = 2.9 Hz, 1H), 3.55 (s, 6H), 3.22 (m, 2H), 2.29 (m, 2H), 1.30 (t, J = 7 Hz, 3H), 1.18 (t, J = 7 Hz, 3H). \(^1\)C NMR (125 MHz, DMSO-d\(_6\)) \(\delta\): 177.24, 174.70, 161.81, 159.39, 158.51, 157.35, 149.75, 141.26, 140.96, 136.03, 135.84, 130.10, 129.36, 128.31, 128.06, 113.69, 113.22, 113.05, 98.74, 91.95, 86.52, 86.42, 85.76, 85.39, 83.18, 70.86, 64.02, 55.41, 12.73, 12.36 ppm. HRMS (ESI) theoretical \([M+H]^+ = 985.2935\), \([M+Na]^+ = 1007.2755\), found \([M+H]^+ = 985.2955\), \([M+Na]^+ = 1007.2732\).
4.7 Spectrum Appendix

$^1$H NMR of compound 1.

$^{13}$C NMR of compound 1.
MS of compound 1.

1H NMR of compound 2
$^{13}$C NMR of compound 2.

MS of compound 2.
$^1$H NMR of compound 3.

$^{13}$C NMR of compound 3.
MS of compound 3.

$^1$H NMR of compound 5.
$^{13}$C NMR of compound 5.

MS of compound 5 ($[\text{M+H}]^+=457.1039$, $[\text{2M+Na}]^+=935.1825$, $[\text{3M+Na}]^+=1391.2744$).
$^{1}$H NMR of compound 6.

$^{1}$H NMR of compound 7.
$^1$H NMR of compound dA-SQ.

$^{13}$C NMR of compound dA-SQ.
MS of compound dA-SQ ([M+H]^+=706.1913).

^1H NMR of compound dU-SQ.
$^{13}$C NMR of compound dU-SQ.

MS of compound dU-SQ ([M+H]$^+$=683.1622).
$^1$H NMR of compound dU(DMT)-SQ.

$^{13}$C NMR of compound dU(DMT)-SQ.
MS of compound \textbf{dU(DMT)-SQ} ([M+H]^+=985.2902).

### 4.8 References


(38) Lakowicz, J. R. Principles of Fluorescence Spectroscopy; Kluwer: New York, **1999**.


CHAPTER 5. WATER SOLUBLE SQUARAINÉ DYE FOR DETECTION OF MERCURY IONS

5.1 Introduction

Detection of mercury pollutions is crucial and developing selective sensors is still gathered public attentions. Mercury ions could be very toxic to organic bodies and environment, and the resulted disorders is well known, once inhaled by human body it can result permanent brain and central nervous system diseases.\textsuperscript{1,2} Plasma mass spectrometry and atomic absorption/emission spectroscopy are normally used for metal ions detection at low concentration.\textsuperscript{3,4} However, these methods always limited by expensive instruments and restrict sample preparing steps. An alternative strategy is using organic chromophores, which can lead to turn on/off absorption/emission once they interacted with heavy metal ions.\textsuperscript{5-13} In general, the ion grabbing groups in chromophore can selectively interact with metal ions, leading to optical changes of the chromophore once the complex is formed, thus exhibit a positive signal showing the presence of target ions. When organic chromophores are applied as heavy metal sensors, the response is often instant and naked eye detectable.\textsuperscript{14}

Squaraine dyes have been synthesized and modified to be used for a lot of applications in advanced materials, and squaraine based metallic ion sensors has gained growing interest.\textsuperscript{15,16} Ajayaghosh and Arunkumar developed rigid-flexible-rigid bichromophoric squaraine chemosensors and used for the detection of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} in acetonitrile.\textsuperscript{17,18} By using a scavenging strategy, Mártinez-Máñez et. al. reported fluorescence enhancement based on Hg\textsuperscript{2+} triggered squaraine dye formation in water/acetonitrile mixed solvent.\textsuperscript{19,20} And recently, Cu\textsuperscript{2+} specific recognition has been achieved using 2-picolyl modified 2,4,6-trihydroxyphenylsquaraine in HEPES/THF solvent mixture.\textsuperscript{21} However, most often
the metal ions detections were performed in organic solvent or a combination of organic solvent and water, which hampered the further application of in aqueous solutions.

N-propanesulfonate-benzo[e]indolium squaraine (SQ-SO2) showed BSA sensing properties and have been applied for imaging studies before.²² Because of its good solubility in water and potential metal ions interaction positions of sulfonate²³ and oxygen atom in squaraine ring,²⁴ we tested the cation recognition properties of SQ-SO2. Here in, we showed the application of SQ-SO2 as a naked eye colorimetric sensor for Hg²⁺ at the detection level of μM. UV-vis spectropotometric studies show a good recognition sensitivity towards Hg²⁺, while other metal ions only cause negligible absorption changes except Fe³⁺. Moreover, the absorption change caused by Hg²⁺ interaction is reversible when stronger chelates like KI and EDTA were added to the resulting solution. Further fabrication SQ-SO2 into agarose hydrogel may be interesting for application where sensitivity, portability and low cost are required.

5.2 Materials and General Techniques

Synthesis of SQ-SO2 was reported before.²² Agarose was purchased from Fisher Scientific. All other chemicals were purchased from Sigma-Aldrich and Acros and were used as received. Absorption experiments were carried out using Agilent 8453 Uv-vis spectrophotometer in 1 cm path length quartz cuvettes. Metal salt used in the sample including dehydrolized or hydrolized: LiCl, NaCl, KCl, CaCl₂, CuSO₄, ZnSO₄, MgSO₄, BaCl₂, Co(OAc)₂, Ni(OAc)₂, FeSO₄, Pb(NO₃)₂, AgNO₃, Hg(ClO₄)₂ and Fe(NO₃)₃. Water solutions were prepared using DI water at room temperature. Filter paper used for hydrogel based assay was purchased from Whatman (diameter of 125 mm).
5.3 Results and Discussion

5.3.1 Selectivity towards Mercury Ions

Studies of SQ-SO2 metal sensing behavior showed a great affinity towards Hg^{2+}. Naked eye identification of Hg^{3+} relative to other environmentally and biologically relevant metal ions is shown in Figure 5-1. When the squaraine dye was mixed with other metal ions at 1.0 mM concentration, no obvious color change occurred except for Fe^{3+}; however, when Hg^{2+} was added at 0.5 mM concentration, almost no original blue color remained. The introduction of Fe^{3+} also caused color fading of SQ, resulting in a light yellow solution, which may due to oxidation such as that proposed by Das and coworkers.\textsuperscript{23}

![Figure 5-1: Picture showing the color change upon addition of various metal ions in water solution of SQ-SO2 (5.0 μM). Concentration of Hg^{2+}: 0.5 mM; concentration of other metal ions 1.0 mM. A1-A6: no metal, Li^+, Na^+, Mg^{2+}, K^+, Ca^{2+}; B1-B6: Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}; C1-C4: Ag^+, Ba^{2+}, Fe^{3+}, and Hg^{2+}.](image)

The absorption behavior of SQ-SO2 in the presence of 0.5 mM Hg^{2+} and 1.0 mM other metal ions were carried out by UV-vis spectroscopy. Upon addition of Hg^{2+}, a decrease in the absorption band at 656 nm was observed, as shown in Figure 5-2a. In contrast, with the introduction of other metal ions (Li^+, Na^+, Mg^{2+}, K^+, Ca^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Ag^+, Ba^{2+}), at twice the concentration, only a slight drop of the maximum absorption band at 656 nm or no change at all was observed. Figure 5-2b shows the absorption intensity change profiles of 5.0 x 10^{-6} mol/L SQ-SO2 water solution at 656 nm in the presence
of 1.0 mM of each selected cation and with 0.5 mM of Hg$^{2+}$. From the comparison of absorbance at 656 nm we can clearly see the SQ-SO$_2$ dye has high selectivity to Hg$^{2+}$, at the concentration of 0.5 mM, it can cause 94% decrease of the original absorption intensity. When Fe$^{3+}$ was introduced to SQ-SO$_2$ solution, there was less intensity decrease in contrast to Hg$^{2+}$ with 90% reduction of the maximum absorption at 656 nm. At 656 nm the $I_0/I$ values of other metal ions were less than 1.2, showing a negligible change compared with Hg$^{2+}$.

![Absorbance vs Wavelength](image)

**Figure 5-2:** (a) Uv-vis absorption changes of SQ-SO$_2$ (5.0 x 10$^{-6}$ mol/L) water solution in the presence of 1.0 mM of Li$^+$, Na$^+$, Mg$^{2+}$, K$^+$, Ca$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Ag$^+$, Ba$^{2+}$, Fe$^{3+}$ and 0.5 mM of Hg$^{2+}$; (b) absorption intensity change profiles based on (a), select wavelength 656 nm.

### 5.3.2 Sensitivity towards Mercury Ions

Titration of 5.0 x 10$^{-6}$ mol/L SQ-SO$_2$ water solution with Hg$^{2+}$ was carried out to study the response of absorption intensity. **Figure 5-3a** shows the changes in absorption of an SQ-SO$_2$ aqueous solution upon addition of increasing amounts of Hg$^{2+}$. With increasing amount of Hg$^{2+}$, there was a continuous decrease in the absorption intensity at 656 nm. Relations of the absorption intensity to the mole ratio of SQ/Hg$^{2+}$ were studied, and the data is plotted in **Figure 5-3b**. The relation of the mole ratio of SQ/Hg$^{2+}$ to 656 nm
absorption intensity exhibited a linear response with an equation of \( y = 1.0061 - 0.1009x \), and the linear fitting \( R^2 = 0.99915 \). These results demonstrate, at the Hg\(^{2+}\) concentration in the range of 0 to 45 \( \mu \text{M} \), there is a linear response of the absorption intensity at 656 nm. Thus, the water soluble SQ-SO\(_2\) dye may well be a good sensor to detect and identify Hg\(^{2+}\) at low concentrations (on the \( \mu \text{M} \) level).

**Figure 5-3:** (a) Effect of Hg\(^{2+}\) on the absorption spectrum of 5.0 \( \times 10^{-6} \) mol/L SQ-SO\(_2\). (b) Plot of absorption intensity at 656 nm vs. the mole ratio of [Hg\(^{2+}\)]/[SQ].

The absorption intensity decrease of SQ-SO\(_2\) can be explained by the interaction of SQ-SO\(_2\) with Hg\(^{2+}\) ions. Two sulfonate groups in the SQ-SO\(_2\) molecular structure act as chelating groups to complex Hg\(^{2+}\),\(^{23}\) plus the negative charged oxygen atom on the squaraine four membered ring may also participate in the coordination and generate a SQ-Hg\(^{2+}\) complex.\(^{24}\) In addition, with increased addition of Hg\(^{2+}\) free SQ-SO\(_2\) was consumed and afforded increasing SQ-Hg\(^{2+}\) complex formation, leading to the reduction of the original SQ-SO\(_2\) absorption signal. After SQ-Hg\(^{2+}\) complexation was completed at a 1:10 molar ratio, stronger chelates towards Hg\(^{2+}\) were added, e.g., EDTA and iodide were introduced to the complexation, which might form stronger complexes with Hg\(^{2+}\) compared with SQ-SO\(_2\), and the spectral change is shown in **Figure 5-4**. The formation of the SQ-Hg\(^{2+}\) complex caused the decrease of blue color intensity
(absorption at 656 nm). However, the blue color recovered after KI and ethylenediamine tetracarboxylic acid (EDTA) were introduced. EDTA can bind with Hg$^{2+}$ more efficiently compared with SQ-SO$_2$, so after introduction to the SQ-Hg$^{2+}$ complex (colorless), the Hg$^{2+}$ were more likely to complex with EDTA and thus release SQ-SO$_2$ (blue color) as free ligands, consequently generating a blue color. According to the intensity of the SQ-SO$_2$ absorption, 95% of SQ-SO$_2$ was released from the SQ-Hg$^{2+}$ complex after adding addition of EDTA. When KI was added, a similar result was obtained with 70% dissociation of the SQ-Hg$^{2+}$ complex.

![Figure 5-4](image.png)

**Figure 5-4:** Absorption spectrum of 5.0 × 10^{-6} mol/L SQ-SO$_2$ water solution (black line), and after addition of 50 μM Hg$^{2+}$ (red line). Absorption recovery of SQ-Hg$^{2+}$ complex (red line) followed by addition of KI (blue line, 0.5 mol/L) and EDTA (green line, 0.1 mol/L).

The affect of Fe$^{3+}$ was examined also, even though Fe$^{3+}$ can lead to color fading of SQ-SO$_2$, much higher concentration was required in order to generate complete bleaching. Meanwhile, rather than the type complex formation incurred with SQ-Hg$^{2+}$, the color bleaching caused by Fe$^{3+}$ is likely due to redox
processes in which the color could not be recovered by addition of strong chelating groups of EDTA since it is irreversible.\textsuperscript{23}

Figure 5-5: $^1$H NMR study of SQ-Hg$^{2+}$ complex, with molar ratio of SQ-SO2/Hg$^{2+}$ increased from 1:0, 1:5, to 1:10.

$^1$H NMR studies were carried out in order to confirm the formation of complexes between the squaraine dye and the mercury ions. The spectra were recorded in DMSO-$d_6$ solution. The spectrum taken in the absence of any Hg$^{2+}$ exhibited nicely split peaks and the integration corresponded well with the structure. However, once Hg$^{2+}$ was introduced, there was an obvious shift of the peaks belonging to the conjugated squaraine system (Figure 5-5). The resonance associated with the C=C double bond, located at 5.8 ppm, disappeared while the benzoindo hydrogens shifted and broadened. The hydrogens on the C3 linkage also shifted as marked by the arrows. From 1:5 to 1:10 ratio, the spectra did not change much. So from the $^1$H NMR results, interaction between the squaraine dye and mercury ion is confirmed. The interactions were nearly complete at the 1:5 ratio, since further addition of mercury ion showed no obvious change. The
sauraine system plus the terminal sulfonate groups may be involved in the chelating procedure, as evident by the chemical shifts and splitting patterns.

5.3.3 Fabrication of SQ-SO2 Doped Hydrogel Materials

Developing new Hg\(^{2+}\) sensing materials that have the combined advantages of selectivity, sensitivity, portability, and ease of fabrication remains challenging. Using a hydrogel as fabricating materials for metal ion sensing applications has grown interest recently.\(^{25-27}\) In our studies, water soluble SQ-SO2 was doped in an agarose hydrogel, and Hg\(^{2+}\) detection was performed in a convenient fashion. In general, 2 wt% agarose was mixed with approximately 5 × 10\(^{-5}\) mol/L SQ-SO2 in hot water solution. After cooling to room temperature a blue colored hydrogel was obtained and further applied as a Hg\(^{2+}\) indicator. Different concentrations of Hg\(^{2+}\) was added to the hydrogel. The characteristic blue color of SQ-SO2 doped in the hydrogel faded after Hg\(^{2+}\) diffused, generating a SQ-Hg\(^{2+}\) complex (Figure 5-6). Identifying the reduced color by naked eye and the boundary of the yellow ring was possible.

Figure 5-6: Illustration of a SQ-doped hydrogel as an Hg\(^{2+}\) sensor (left). Photograph of 2 wt% agarose hydrogels doped with SQ-SO2 (~ 5 × 10\(^{-5}\) mol/L) followed by addition of 100 µL of different concentration of Hg\(^{2+}\) after 1 h (right): 1) no Hg\(^{2+}\), 2) 1 × 10\(^{-1}\) mol/L Hg\(^{2+}\), 3) 1 × 10\(^{-2}\) mol/L Hg\(^{2+}\), 4) 1 × 10\(^{-3}\) mol/L Hg\(^{2+}\), and 5) 1 × 10\(^{-4}\) mol/L Hg\(^{2+}\). The vials were inverted.
5.4 Summary

The mercury ion sensing properties of SQ-SO2 were examined, exhibited a naked eye detection behavior. This squaraine dye exhibited high Hg²⁺ selectivity relative to most other cations. Using ¹H NMR and absorption titration experiments, the formation of SQ-Hg²⁺ complexes along with the possible interaction positions were proposed. Further, by doping the water soluble SQ-SO2 into an agarose hydrogel, an Hg²⁺ sensor was readily fabricated, suggesting potentially advantageous properties such as selectivity, sensitivity, portability, and low cost.

5.5 References

(1) Harris, H. H.; Pickering, I. J.; George, G. N. The Chemical Form of Mercury in Fish. Science 2003, 301, 1203.


This dissertation contained the design, synthesis, and characterization of new squaraine dyes and supramolecular assemblies and their various applications. All new compounds were characterized by $^1$H NMR, $^{13}$C NMR, HRMS, absorption, and fluorescence. Utilizing the specific properties of the squaraine family, applications of these materials covered controlled aggregation and modulation of linear and nonlinear absorption, protein sensing and labeling chromophores, fluorescence viscosity sensors, and metal ion sensing. Chapter 1 provided an overview of squaraine dye synthetic methods and strategies and various applications based on this unique chromophore class. Chapter 2 focused on the aggregation applications of the squaraine family, and two squaraine compounds were designed and synthesized in order to perform aggregation under different strategies. Reversible polyelectrolyte-templated aggregation was realized, facilitating enhancement and modulation of two-photon absorption. Chapter 3 covered protein sensing and protein labeling studies using two water soluble squaraine dyes and the properties were further applied to cell level. Large enhancements in fluorescence were realized upon squaraine-protein supramolecular assembly. Chapter 4 included the synthesis of a class of squaraine dye modified with deoxynucleotides and their applications as fluorescent viscosity sensors. This afforded imaging of high viscosity regions of cells during mitosis, providing a valuable tool in the study of cellular dynamics. Chapter 5 discussed the metal ion sensing behavior of a water soluble squaraine dye, and the absorption intensity of squaraine dye shows a linear relationship with the concentration of selective heavy metal ions. Based on knowledge acquired in these studies, a hydrogel-based sensor was fabricated for the selective detection of $\text{Hg}^{2+}$ over a range of biologically- and environmentally-relevant concentrations.

The first part of this dissertation was concerned with developing the means to control $\text{J}$-aggregation of squaraine derivatives in aqueous solution. A new benzothiazolium squaraine dye $\text{SQ-Py}$ was designed and
synthesized. The structure was comprised of two pyridium pendants linked to the squaraine system, which enhanced water solubility and provided an interaction handle with aggregation-inducing reagents. We investigated the conditions for J-aggregation formation of SQ-Py. The nonfluorescent J-aggregate was triggered by 0.01 mol/L PAA-Na, resulting in a J-band that had a more intense and sharp absorption relative to the J-band formed in a high concentration of NaCl (aq), indicating that the polyelectrolyte PAA-Na served as a good template for supramolecular J-aggregate assembly. A reversible aggregation cycle was achieved by introduction polyelectrolyte and removing it from the mixed system using metal strong chelates (e.g., Ca$^{2+}$). These results demonstrate the potential use of templates to trigger and control J-aggregation in aqueous solution through interactions between an anionic polyelectrolyte and a cationic hydrophobic squaraine dye. Significantly, the two-photon absorption of each repeat unit of the SQ-Py J-aggregates was higher than the unaggregated or lower aggregated dimers, with a 23-time enhancement of the 2PA cross section at 920 nm. The controllable supramolecular assembly and J-aggregate formation provides a way to modulate the linear and nonlinear absorptivity of SQ and achieve a relatively high 2PA cross section, which is interesting for a number of potential applications (e.g., optical data storage, optical switching, and optical power limiting).

By employing a different strategy, J-aggregation was introduced on a natural polyamine backbone. An unsymmetrical squaraine dye containing a terminal carboxylic acid group was synthesized and covalently attached to a poly-l-lysine backbone via amidation. The poly-l-lysine templated J-aggregation was studied, and the result showed this optional strategy could be potentially interesting.

Future directions for research on squaraine dye J-aggregation materials may include studies outlined below. (1) Design and utilization of squaraine derivatives with stronger initial 2PA cross sections. Apply the same strategy as described above by introduction of positively charged pendants and inducing J-
aggregation by a polyelectrolyte. With the 2PA cross section enhancement properties via J-aggregation, the resulting supramolecular assembly should yield a record 2PA cross section. (2) Explore the high 2PA properties of J-aggregation in thin films in the near-IR region for other 2PA applications such as optical switching.

In the second part of this research, two squaraine dyes, SQ-SO1 and SQ-SO2 were synthesized with sulfonate pendants that provided good water solubility. SQ-SO1 and SQ-SO2 contained different conjugated systems, which modified absorption and emission wavelengths along with aggregation behavior. Through noncovalent interactions with the biopolymer BSA, both squaraine dyes exhibited large fluorescence enhancement and extended luminescence lifetime. The observed large fluorescence turn-on is believed to be due to the change of environment after strong complexation with the BSA protein. Titration experiments were carried out using displacement reagents, allowing the assessment of site selectivity. The results indicated that these squaraine dyes were binding to both site I and site II of BSA with preference to site II. Nanoparticles were later synthesized using fluorescence enhanced BSA-SQ complexes with an average particle size of ca. 100 nm. Within the nanoparticle, the squaraine dyes exhibit a reduced tendency of aggregation and increased near-IR fluorescence intensity. These BSA-SQ NPs were further applied to HCT 116 cells, affording fluorescence microscopy imaging. To track the BSA-SQ NPs following their uptake by HCT 116 cells, the lysosomal compartment of HCT 116 cells were stained with LysoSensor Green. Large amounts of the fluorescence emitted from the BSA-SQ particles overlapped with lysosomes (high colocalization agreement, confirming lysosomal selectivity). In addition to live cells, fixed cells were also used to study the BSA location sensing properties of the squaraine dye, and bright emission was collected after the staining with SQ-SO2, further demonstrating the SQ-BSA nanoparticle versatility as a fluorescent probe. These two water soluble squaraine dyes
engaged in fluorogenic sensing with BSA through noncovalent interactions, showing the potential application as a near-IR protein labeling reagent for protein tracking for both ex vivo and in vivo imaging.

Future directions for research on squaraine dye as protein sensing and labeling reagents could include studies of the following: (1) Perform target protein locating and related cancer cell curing utilizing the singlet oxygen generation properties of squaraine chromophores. (2) Extending the conjugation systems of squaraine chromophores in order to match the requirement of even longer absorption and emission wavelength to near infrared.

In chapter 4, we have demonstrated the synthesis of squaraine dye-modified deoxyadenosine and deoxyuridine compounds. Substantial effort was expended to optimize reaction conditions, e.g., by evaluating different organic bases and catalyst amounts in the reaction. These non-natural deoxynucleosides with a C-modified squaraine chromophore exhibited sharp and intensive absorption in the near-IR region, along with identical fluorescence emission at 695 nm in DMSO solution. Both absorption and emission spectra of these squaraine dye-modified deoxynucleosides show viscosity-dependent properties in glycerol/water mixed solvents. The enhancement of fluorescence under highly viscous solvent reached above 300-fold, which is the highest value reported for viscosity fluorescence enhancement in the literature thus far. This huge fluorescence enhancement in viscous solvents was attributed to a combination of effects of aggregation and rotation. Thus, these non-natural deoxynucleosides can have potential applications for studies such as near-IR nucleoside tracking and viscosity related sensors at the cellular level.

Future directions for research on squaraine dye-modified deoxynucleotides could include studies of the following: (1) by changing squaraine dye donors to aniline with four hydroxyl groups, the stability of the squaraine dyes can be greatly enhanced, once stabilized squaraine dyes are conjugated with
deoxynucleotides, deoxynucleosides oligomers can be synthesized with a DNA synthesizer. The resulting oligomer may be very interesting for studies of optical properties and aggregation behavior with DNA templating. (2) Incorporate the squaraine dye-modified deoxynucleotide into double stranded DNA structures as a fluorescent probe for the studies of DNA structure and nuclei viscosity measurements.

In chapter 5, the same benzoindolium squaraine dye with two sulfonate pendants were used as metal sensors. In this work, the two sulfonate groups not only facilitated metal sensing in aqueous solution but also involved in chelating interactions with heavy metal ions. Squaraine dye **SQ-SO2** exhibited a selective and sensitive affinity towards Hg$^{2+}$ in water solutions, while being insensitive to most other metal ions that are relevant to the environment and biology. Titration experiments were performed and the response of the concentration of Hg$^{2+}$ to the absorption intensity of **SQ-SO2** exhibited good linear fitting properties, which can be further used to determine the heavy metal ion concentration in contaminated and polluted samples.

Future directions for research on squaraine dye-based heavy metal sensing materials could include the following studies: (1) modify the squaraine chromophore with known metal chelating functional groups for specific metal ions to achieved unique metal selectivity. (2) By changing the squaraine dye donor, the absorption and emission wavelengths could be shifted to even longer wavelength. Thus, metal sensing can be performed under a broad range of wavelengths, even in the near-IR region, which is attractive for research in bioenvironments.
APPENDIX: LIST OF PUBLICATIONS DURING PH.D. THESIS


