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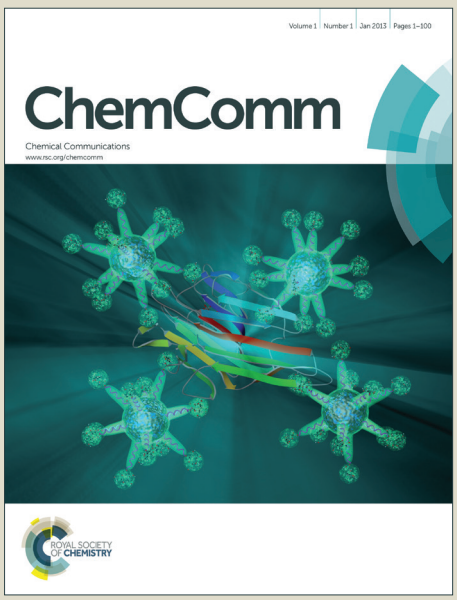

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ARTICLE TYPE

A sensitive colorimetric and fluorescent sensor based on imidazolium-functionalized squaraines for the detection of GTP and alkaline phosphatase in aqueous solution

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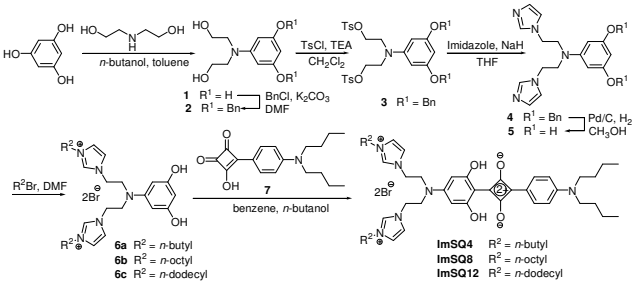
Imidazolium-functionalized squaraine ImSQ8 is synthesized as a sensitive colorimetric and fluorescent chemosensor for GTP in aqueous solution. The detection limit of GTP reaches 5.4 ppb. Its applications in the live-cell imaging and enzyme activity assay have also been demonstrated.

The guanosine-5′-triphosphate (GTP) is one vital member of nucleotides, which acts as a substrate for RNA synthesis and provides energy for some metabolic reactions.[1] Intracellular GTP levels are closely relevant to definite pathological states. Thus, it is important to monitor the concentration of GTP.[2] Sensors based on analyte-induced colour changes or fluorescent changes are particularly attractive owing to their simplicity, high sensitivity and real-time detection.[3] A number of colorimetric and/or fluorescent sensors have been designed for various nucleotides in the past decades.[4] In particular, the recognition of adenosine-5′-triphosphate (ATP) has attracted much more attention.[5] However, there are relatively few reports on chemosensors that selectively communicate with GTP.[6] Moreover, due to the poor water-solubility of many hosts, a cosolvent has to be added. In addition, many known sensors are less sensitive, and as a result, a high concentration of GTP would usually be required to enhance spectral response. Therefore, there is still a demand to develop more sensitive fluorescent sensors for the detection of GTP in aqueous solution.

Squaraines are a well-documented class of organic dyes with interesting photophysical properties such as sharp and intense absorption bands in the red to near-infrared region and high fluorescence quantum yields.[7] Moreover, because of the sensitivity to slight external stimulation, squaraine dyes have been extensively used for the detection of various biomolecules.[8] However, most squaraines display poor water-solubility, and there are no squaraine-based chemosensors for nucleoside phosphates so far. In this report, we wish to present the design, synthesis and spectroscopic study of imidazolium-functionalized squaraines that serve as a colorimetric and fluorescent sensor for the detection of GTP in aqueous solution. Imidazolium has been proven to be a potential receptor for anions with excellent water-solubility.[9] Thus, we speculated that the imdazolium unit would not only provide effective binding sites for nucleotides, but also improve the water-solubility of squaraine derivatives.

Scheme 1 shows the synthetic strategy of imidazolium-functionalized squaraines. 3,5-Dihydroxyaniline bisimidazolium derivative **6** was synthesized *via* a six-step process by using phloroglucinol as the starting material, followed by a condensation with semi-squaraine **7** to afford the target molecules with different alkyl chains. It is noteworthy that two hydroxyls on the benzene ring of **6** may be indispensable for increasing its reactivity or stabilizing the resulting squarines *via* intramolecular hydrogen bonding interaction. The bisimidazolium-containing aniline derivatives with one or no *meta*-hydroxyl group on the benzene ring could not deliver the desired squaraines (ESI†, Scheme S1).



Scheme 1 Synthetic routes of **ImSQ4**, **ImSQ8** and **ImSQ12**.

Initially, the UV/Vis spectra of **ImSQ4**, **ImSQ8** and **ImSQ12** were studied in neutral buffer (ESI†, Fig. S1). Squaraines **ImSQ4** and **ImSQ8** showed good solubility in HEPES buffer and exhibited a characteristic sharp and intense absorption band of the monomeric squaraine chromophore at around 658 nm with a broad shoulder at approximately 615 nm. It was found that increasing the length of the alkyl chain led to poor water-solutility, which may presumably be attributed to the enhancement of the dye's aggregation tendency. As a result, the dodecyl-anchored squaraine **ImSQ12** exhibited a very weak monomeric absorption band centered on 650 nm and an additional hypsochromic absorption band at around 513 nm. The fluorescense spectra of imidazolium-functionalized squaraines were also investigated upon excitation at 613 nm (ESI†, Fig. S2). **ImSQ4** and **ImSQ8** both displayed a strong emission in HEPES buffer (10 μM, pH = 7.2), but the emission of **ImSQ12** was hardly detected due to its severe aggregation behavior.

Subsequently, colorimetric responses of **ImSQ4** and **ImSQ8** to a range of anions were investigated. In the colorimetric test, no

colour change was observed during the addition of various anions into buffer solution of **ImSQ4**. In contrast, an apparent colour change from azure to navy blue was observed upon addition of GTP into the buffer solution of **ImSQ8** (Fig. 1). The other anions including ATP, PPi, GDP, GMP, phosphate, halide, acetate, bicarbonate, sulfate and nitrate ions did not lead to appreciable color changes. These observations clearly indicated that **ImSQ8** exhibited a high selectivity for naked-eye detection of GTP over other nucleotides and various anions in aqueous solution.

Fig. 1 Color changes of **ImSQ8** (10 μM) upon addition of sodium salt of various anions (1.0 equiv) in HEPES buffer (10 mM, pH = 7.2).

To further confirm the spectral changes of **ImSQ8** toward various anions, the absorption spectra of **ImSQ8** in HEPES buffer (10 mM, pH = 7.2) was studied. As shown in Fig. 2a, with increasing amounts of GTP, the absorption of monomeric **ImSQ8** at approximately 658 nm was weakened significantly and the absorption of the aggregates at approximately 557 nm was enhanced gradually. The relative ratio of absorbance of **ImSQ8** at 557 and 658 nm (A_{557}/A_{658}) increased linearly with GTP concentration at less than 8 μM.[10] The typical detection limit of GTP by this protocol was estimated to reach 5.4 ppb (1.04×10^{-8} M), which exhibited a high sensitivity.[6a,6b,11] The changes in the absorption spectra of **ImSQ8** in HEPES buffer upon addition of other anions were also studied (ESI†, Fig. S3). Fig. 2b shows the dependence of A_{557}/A_{658} on the different concentrations of various anions. It is clear that the most striking effect is observed for GTP, and its A_{557}/A_{658} value is about 2-fold of ATP.

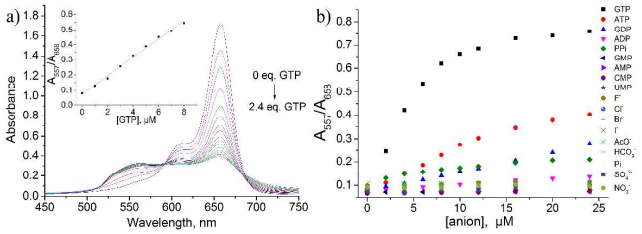


Fig. 2 (a) Absorption spectra of **ImSQ8** (10 μM) upon addition of different equivalent of GTP in HEPES buffer (10 mM, pH = 7.2). Inset: linear relationship between A_{557}/A_{658} of **ImSQ8** and the GTP concentrations. (b) The dependence of A_{557}/A_{658} of **ImSQ8** on different anions at increasing concentrations in HEPES buffer (10 mM, pH = 7.2).

Fluorescence responses of **ImSQ8** to a range of various nucleotides and anions are depicted in Fig. 3a. Upon addition of different anions (1.0 equiv), GTP induced the most remarkable fluorescence change. The addition of 2.4 equiv of GTP resulted in approximately 5.5-fold fluorescence quenching of **ImSQ8** along with an emission shift from 680 to 670 nm (ESI†, Fig. S4). Subsequently, the live-cell imaging experiment was performed *via* incubation of Bel-7402 cells with **ImSQ8** (20 μM) in a physiological saline solution containing 1% DMSO for 30 min at 37 ºC. As shown in Fig. 3b, a clear red fluorescence image was observed from fluorescence microscopy. When GTP was added and incubated for another 30 min, a significant fluorescence quenching phenomenon was observed (Fig. 3c; ESI†, Fig. S5). The experimental results revealed that **ImSQ8** would be a potentially useful reagent for detection of GTP in living biological samples.

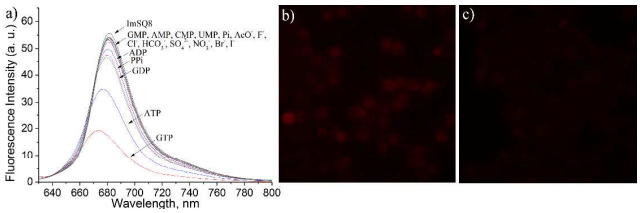


Fig. 3 (a) Fluorescence emission of **ImSQ8** (10 μM) upon addition of sodium salt of various anions (1.0 equiv) in HEPES buffer (10 mM, pH = 7.2). λ_{ex} = 613 nm. Fluorescence microscopy images of Bel-7402 cells treated with ImSQ8 (20 μM): (b) before and (c) after adding GTP.

The selectivity of **ImSQ8** for GTP over GDP and GMP enables us to explore the possibility of applications in other areas of biology. Alkaline phosphatase (ALP) is widely distributed in biological tissues.[12] The level of serum alkaline phosphatase is used as an important detection index for several diseases.[13] Given that ALP can catalyze the hydrolysis of GTP to produce GDP, GMP, guanosine and phosphate, we envisioned that **ImSQ8** may be utilized as a real-time fluorescence sensor for the detection of ALP because the hydrolytic products have almost no influence on the spectral changes of **ImSQ8**. As shown in Fig. 4, after adding ALP (133 mU mL^{-1}) into the HEPES buffer (10 mM, pH = 7.2) containing **ImSQ8** and GTP at 25 ºC, the fluorescence intensity increased gradually by prolonging the incubation time. The fluorescence intensity remained unchangeable after 18 min, which demonstrated that the ALP-catalyzed hydrolysis completed. The inset of Fig. 4 displays the variation of the fluorescence intensity of **ImSQ8** at 675 nm after incubation with different amounts of ALP (0-200 mU mL^{-1}) for different times. The corresponding calibration plot between the fluorescence intensity at 675 nm and the ALP concentration at 8 min is also shown in the inset, which can be utilized to build up a real-time analytical method to detect the enzyme activity.

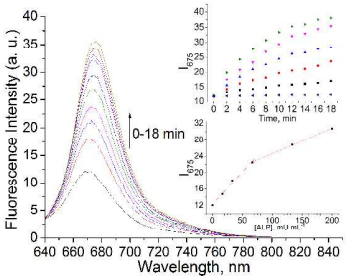


Fig. 4 Fluorescence real-time detection of the ALP-catalyzed hydrolysis of GTP (20 μM) with **ImSQ8** (10 μM). Time trace (0-18 min) of the fluorescence change of **ImSQ8** with GTP after adding ALP (133 mU mL^{-1}). Inset: (1) Time-trace plots of **ImSQ8**-GTP with 0 (◀), 17 (■), 33 (●), 67 (▲), 133 (▼), 200 (▶) mU mL^{-1} of ALP detected by the fluorescence intensity at 675 nm; (2) The calibration plot between the fluorescence intensity at 675 nm and the ALP concentration at 8 min. λ_{ex} = 613 nm.

To validate the aggregation behavior of **ImSQ8** caused by the host-guest interaction, a simple Tyndall effect experiment was carried out.[14] As illustrated in Fig. 5a, under laser irradiation (532 nm), no Tyndall phenomenon was observed for alone **ImSQ8** in HEPES buffer, whereas the addition of GTP resulted in distinct Tyndall scattering. The Tyndall phenomenon disappeared after incubation with ALP. These results demonstrated that the

aggregation of host molecules was triggered by GTP. TEM analyses were further conducted to investigate the aggregation behavior of **ImSQ8**. TEM images of **ImSQ8** in the absence and presence of GTP, dip cast on a carbon-coated copper grid revealed quite different appearance (Fig. 5b and 5c). In the absence of GTP, the diameters of most particles of **ImSQ8** were estimated to be approximately 10 nm. Upon addition of 2.0 equiv of GTP, the diameters of particles became larger up to 60-200 nm, which disclosed that the aggregate formation was remarkable. Dynamic light scattering (DLS) measurements confirmed the solution-phase aggregation behavior with the average diameters of 247.0 nm, which was in qualitative agreement with the TEM studies (ESI†, Fig. S6).

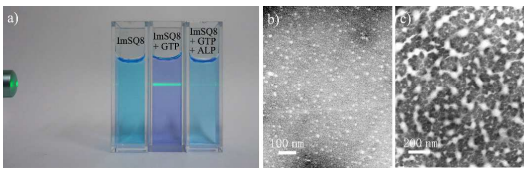


Fig. 5 (a) Photographs of **ImSQ8** (10 μM) in HEPES buffer (10 mM, pH = 7.2) before and after adding GTP (2.0 equiv) and subsequenty ALP (133 mU mL^{-1}) under laser irradiation (532 nm). TEM images of **ImSQ8** (40 μM) in HEPES buffer (10 mM, pH = 7.2) (stained with sodium phosphotungstate): (b) before and (c) after adding GTP (2.0 equiv).

The proposed model of the host-guest interaction between **ImSQ8** and GTP is illustrated in Fig. 6. Monomeric **ImSQ8** emits a red fluorescence upon excitation. In the presence of GTP, **ImSQ8** may assemble on the GTP template to form aggregates *via* electrostatic interactions between the positively charged imidazolium cations and negative triphosphate anions and/or hydrogen bonding interactions between the imidazolium C2 hydrogen and the negatively charged oxygen of triphosphate, which triggers fluorescence quenching. Upon addition of ALP, GTP is hydrolyzed gradually to GDP, GMP, guanosine and phosphate. As a result, the degree of aggregation of **ImSQ8** decreases and the fluorescence intensity is enhanced. Due to more hydrogen bonding sites on guanine than adenine, the GTP is more likely to induce aggregation *via* hydrogen bonding-driven self-assembly than that of ATP, which affords an opportunity to distinguish GTP from ATP.[15]

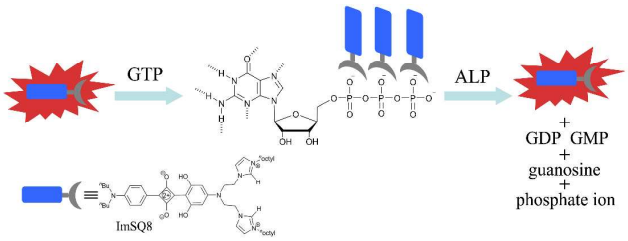


Fig. 6 The proposed model of the host-guest interaction.

In conclusion, we have developed a sensitive colorimetric and fluorescent sensor for the detection of GTP in aqueous solution. The detection limit of GTP is up to 5.4 ppb. By adjusting the length of the alkyl chain, squaraine **ImSQ8** not only exhibits good water-solubility, but also is capable of entering the cells as an imaging reagent. The selective recognition of **ImSQ8** for GTP is attributed to the aggregation-caused spectral change. The hydrolysis of GTP to GDP, GMP, guanosine and phosphate catalyzed by ALP induces fluorescence turn-on, which enables **ImSQ8** to be applied to an enzyme activity assay.

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Notes and references

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† Electronic Supplementary Information (ESI) available: detailed experimental procedures and analytical data. See DOI: 10.1039/b000000x/

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