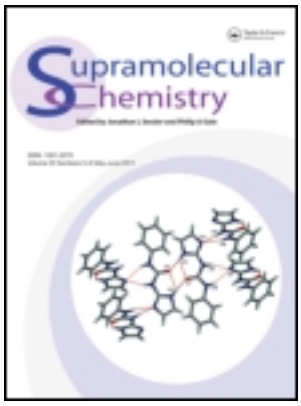

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Supramolecular assembly of a new squaraine and β-cyclodextrin for detection of thiol-containing amino acids in water

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A new unsymmetrical aniline-based squaraine (SQ2) bearing binding unit of Hg^{2+} ion was designed and synthesised. SQ2 can form 1:2 inclusion complex with β-cyclodextrin, and the resulting complex, which undergoes absorption and fluorescence bleaching upon binding Hg^{2+}, can serve as a turn-on colorimetric or fluorescent chemosensor in organic solvent-free aqueous solution for thiol-containing amino acids with high selectivity and tunable measuring range.

Keywords: squaraine; β-cyclodextrin; thiol-containing amino acids; molecular recognition

1. Introduction

Thiol-containing amino acids which are known to be the key constituents of many proteins and enzymes such as cysteine or intermediate metabolites such as homocysteine play essential roles in the biological systems (*1*, *2*). They are important indicators of a large variety of diseases such as Alzheimer's and cardiovascular diseases (*1*, *2*). For instance, the normal concentration of total cysteine (tCys) in plasma is in the range of 250–275 μM. Either over 300 μM or less than 225 μM of tCys hints a high risk of vascular diseases (*2*). Thus, it is of great value to develop rapid, sensitive and selective methods to quantitatively detect the thiol-containing amino acids. In this regard, both colorimetric and fluorescent sensors are highly pursued by virtue of their sensitive responses, inexpensive instrumentation, simple procedures and even naked-eye recognition. So far, a number of sensitive and selective chromogenic or fluorogenic probes have been ingeniously developed for sensing thiol-containing amino acids (*3–17*). However, few of them have a wide measuring range spanning from several millimolars to several hundred millimolars, the concentration regime of thiol-containing amino acids *in vivo* (*18–22*).

Very recently, we have presented an intriguing strategy to modulate the concentration window of thiol-containing amino acids that a squaraine-based chemosensor can respond (*23*). We designed and synthesised a squaraine derivative (SQ1, Scheme 1) bearing sulphur-containing binding units of Hg^{2+} ion at the two terminals of the π-conjugation scaffold. Chelation of Hg^{2+} disrupts the π conjugation and leads to absorption and fluorescence turn-off of SQ1. The thiol-containing amino acids can sequester Hg^{2+} and restore the absorption and fluorescence of SQ1. In this way, the sensing of the thiol-containing amino acids becomes a 'turn-on' manner. More importantly, the measuring range of the thiol-containing amino acids may be easily controlled by adjusting the excess amount of Hg^{2+}, because the free Hg^{2+} will be consumed first by the thiol-containing amino acids.

However, the SQ1- and Hg^{2+}-based chemosensor ensemble suffers from two drawbacks, one is the poor water solubility of SQ1 which limits the system to be utilised only in organic solvent-containing media (e.g. acetonitrile/water in 2:1 volume ratio), and the other is its low selectivity which leads to responses not only to thiol-containing amino acids but also to histidine. It has been reported that some squaraine derivatives can form host–guest inclusion complexes with β-cyclodextrin, by which the aggregation tendency of squaraines is restricted greatly, and meanwhile the water solubility of squaraines is enhanced remarkably (*24–26*). This prompted us to use β-cyclodextrin to improve our SQ1- and Hg^{2+}-based chemosensor system. Nevertheless, the two terminals of SQ1 are so large in size that the β-cyclodextrin cannot incorporate SQ1 into its cavity. Therefore, we designed and synthesised a new unsymmetrical squaraine SQ2 (Scheme 1) on the basis of SQ1, one side of which is replaced by *N*,*N*-diethylaniline that is small enough to be encapsulated into β-cyclodextrin. It was found that the SQ2–Hg^{2+}–β-cyclodextrin ensemble can discriminate thiol-containing amino acids from other amino acids including histidine in organic solvent-free aqueous solution, and the dynamic range still remains tunable as the case of SQ1–Hg^{2+}.

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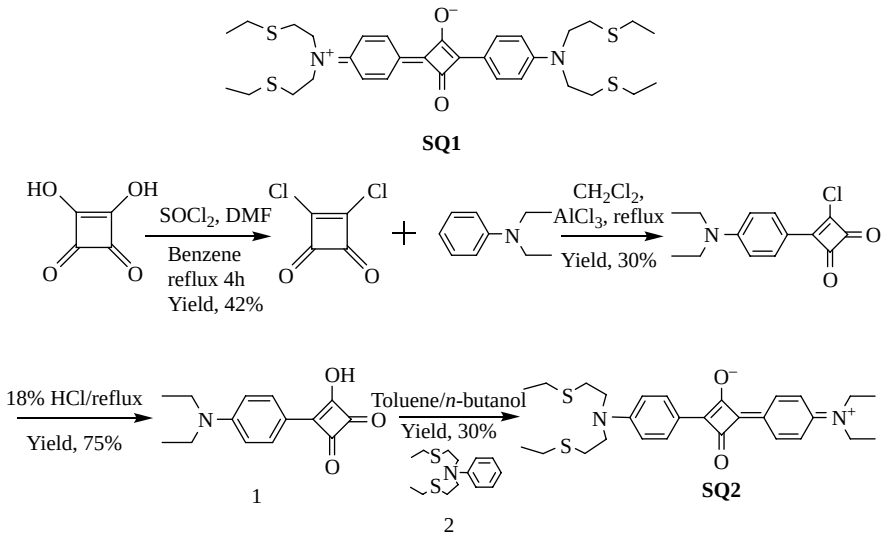

Scheme 1. The chemical structure of SQ1 and the synthetic route of SQ2.

2. Experimental section

2.1 General

Squaric acid, *N*-phenyldiethanolamine and ethanethiol were purchased from Alfa Aesar, Tianjin, China and used without any further purification. Other materials, such as *p*-toluenesulphonyl chloride, *N*,*N*-diethylaniline, aluminium chloride, thionyl chloride and solvents were purchased from Beijing Chemical Plant, Beijing, China and used as received.

^{1}H and ^{13}C NMR spectra were obtained on a Bruker DMX-400 MHz spectrophotometer. High-resolution mass spectra were carried out on a Bruker APEX IV FT_MS. UV/vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence spectra were run on a Hitachi F-4500 spectrophotometer.

2.2 Synthesis of SQ2

Compounds **1** and **2** (Scheme 1) were synthesised according to the reported literatures (*27*, *28*). SQ2 was then synthesised by the condensation of compounds **1** and 2 with azeotropic removal of water in *n*-butanol/toluene (1:1, v/v) under reflux. Melting point: 200°C (dec); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (t, 4H, $J = 9.78$ Hz), 6.82 (d, 2H, $J = 9.00$ Hz), 6.76 (d, 2H, $J = 9.00$ Hz), 3.70 (t, 4H, $J = 7.54$ Hz), 3.54 (q, 4H, $J = 7.12$ Hz), 2.79 (t, 4H, $J = 7.56$ Hz), 2.62 (q, 4H, $J = 7.40$ Hz), 1.29 (m, 12H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 190.7, 187.4, 183.3, 153.9, 152.0, 134.2, 133.0, 120.7, 119.7, 112.5, 112.2, 51.8, 45.5, 29.1, 26.6, 15.0, 12.9; HR-MS (ESI): calcd for ($C_{28}H_{36}N_2O_2S_2 + H^+$) 497.2291, found 497.2284.

3. Results and discussion

Scheme 1 shows the method that we used to synthesise SQ2. The *N*,*N*-diethylaniline-based semi-squaraine (compound **1**) and *N*,*N*-bis(2-(ethylthio)ethyl) aniline (compound **2**) were synthesised according to the literature procedures (*27*, *28*). SQ2 was then obtained by the condensation of compounds **1** and **2** in toluene/*n*-butanol (1:1, v/v) under reflux, and characterised by ^{1}H, ^{13}C NMR and high-resolution MS.

As shown in Figure 1, SQ2 shows remarkable aggregation tendency in the aqueous solution, evidenced by a very broad absorption band extending from 480 to 780 nm and a negligible fluorescence emission (also see Figure S1, available online). Upon the addition of β-cyclodextrin, the absorption band of SQ2 is narrowed and intensified gradually and finally appears as a sharp peak centred at 648 nm, a typical absorption character for classical squaraine dyes in the monomeric form (*29*). Additionally, the fluorescence emission intensity of SQ2 is increased gradually with the addition of β-cyclodextrin, in good agreement with the transformation of a squaraine dye from the aggregated form to the monomeric form (*30*, *31*). Such transformation is believed to be the result of the host–guest inclusion interaction between SQ2 and β-cyclodextrin. Such an inclusion dissociates the aggregate, protects the fluorophore from water quenching and restricts rotations of groups, all disfavouring the non-radiative decay and, therefore, favouring the fluoresence enhancement. The fluorescence quantum yields of SQ2 in different solvents were measured and presented in Table S1 (available online). SQ2 is not emissive in water, but in the presence of β-CD the fluorescence quantum yield is as high as 0.28.

The inclusion complex is consistent with 1:2 stoichiometry between SQ2 and β-cyclodextrin, and the association constant was calculated to be $1.13 \times 10^5\ M^{-2}$ using Benesi–Hildebrand method (Figure S2, available online) (*32*, *33*). The low association constant between SQ-2 and β-cyclodextrin prevents the measurement of the

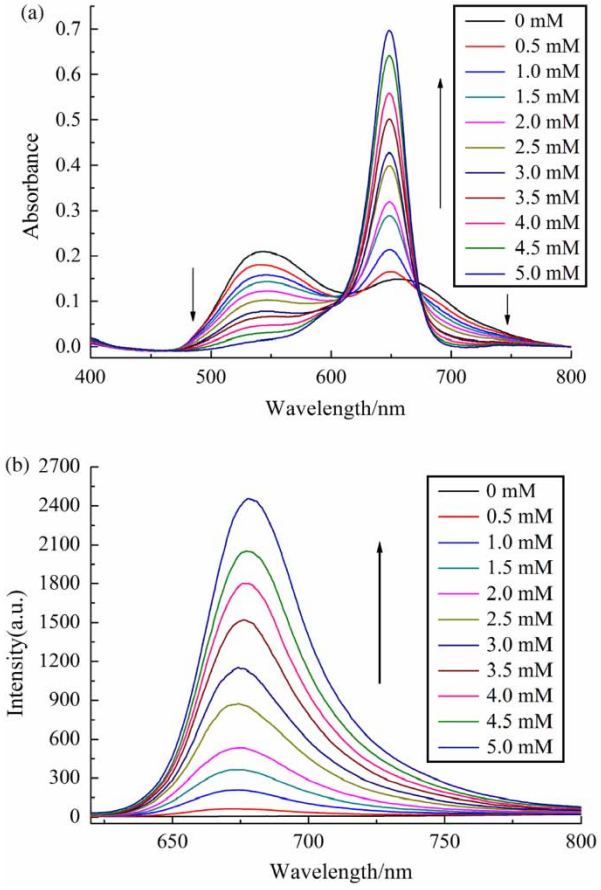

Figure 1. The absorption (a) and fluorescence (b) spectra of SQ2 (10 μM) in the aqueous solution in the presence of varied concentrations of β-cyclodextrin.

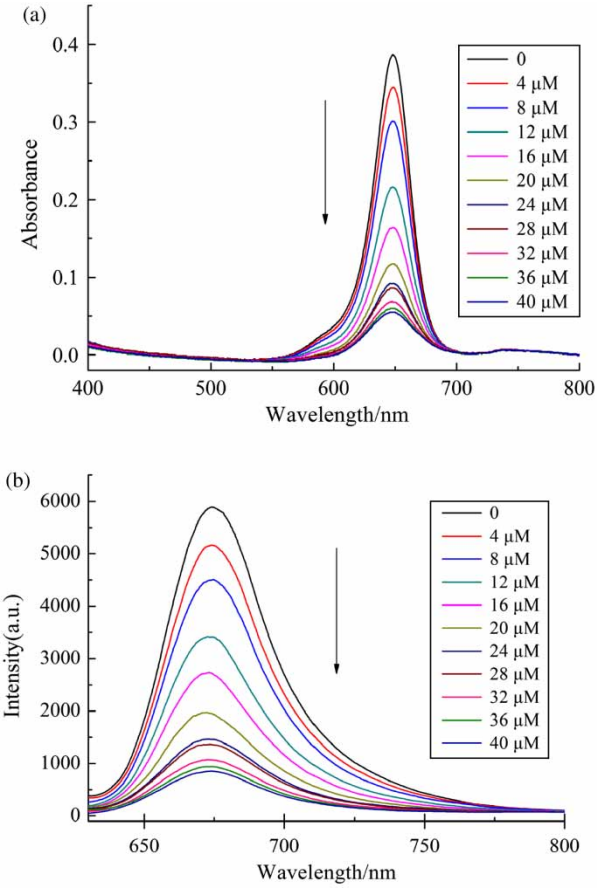

Figure 2. The absorption (a) and fluorescence (b) spectra of SQ2 (5 μM) and β-cyclodextrin (15 mM) in the aqueous solution in the presence of varied concentrations of Hg^{2+} ion.

stoichiometric ratio by Job's plot. When the total concentration of SQ2 and β-cyclodextrin is as low as 10^{-5} M, the interaction between them is too weak to be measured. When the total concentration of them is as high as 10^{-4} M, the poor solubility of SQ2 will lead to its precipitation. The low association constant also hinders the stoichiometry measurement by MALDI-TOF method.

In contrast, there are no detectable absorption and fluorescence emission responses of SQ1 towards β-cyclodextrin, suggesting that the incorporation of SQ2 in β-cyclodextrin is a threading process through the *N*,*N*-diethylaniline side, which is much smaller in size than the other side of SQ2. The optimal structure of bis[4-(diethylamino)phenyl]squaraine is obtained by Gaussian calculations as shown in Scheme S1 (available online). The distance between the two oxygen atoms in the cyclobutene ring is 4.565 Å, whereas the cavity diameter of β-cyclodextrin is 6.0–6.5 Å (*34*), supporting the threading interaction mode. Such an assembly manner not only makes SQ2 to be dissolved in organic solvent-free aqueous solution in its monomeric form, but also makes the inclusion complex to keep Hg^{2+} ion binding ability because the (ethylthio)ethyl side chains are expected to be out of the cavity of β-cyclodextrin.

The inclusion interaction between SQ2 and β-cyclodextrin is further confirmed by circular dichroism (CD) spectra (Figure S3, available online). SQ2 does not have any chiral centre and therefore CD signal. However, a sharp and intense CD band emerges at 650 nm in the presence of β-cyclodextrin, attributable to the induced CD signal of SQ2. This induced CD signal obviously results from the inclusion of SQ2 within the chiral cavity of β-cyclodextrin.

Figure 2 shows the absorption and fluorescence variations of the aqueous solution of SQ2 and β-cyclodextrin upon the addition of Hg^{2+} ion. When the concentration of Hg^{2+} reached 40 μM, both the visible absorption and the fluorescence emission were nearly bleached. Similar Hg^{2+} responses were also observed for SQ1 in acetonitrile/water (2:1) solution (*23*), attributable to the chelation of Hg^{2+} to the terminal binding unit, which diminishes the electron-donating ability of nitrogen atom and switches off the visible absorption and emission of the squaraine chromophore. The binding stoichiometry

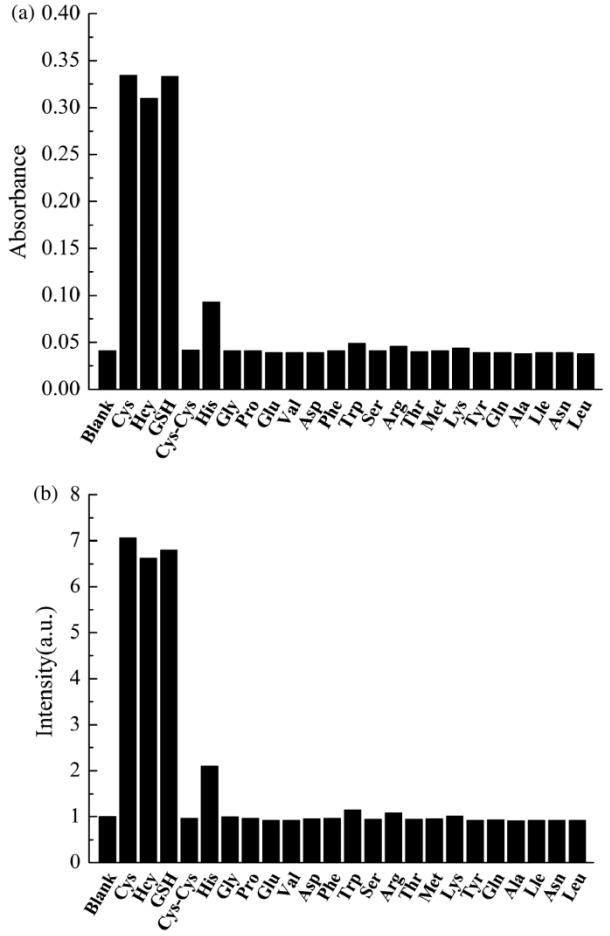

Figure 3. Absorbance at 648 nm (a) and fluorescence intensity at 675 nm (b) of the water solutions of SQ2 (5 μM), β-cyclodextrin (15 mM) and Hg^{2+} (80 μM) upon the addition of various amino acids (80 μM).

of the inclusion complex and Hg^{2+} is consistent with a 1:1 mode, and the association constant was calculated to be $3.3 \times 10^3\ M^{-1}$ using Benesi–Hildebrand method (Figure S4, available online). Several Hg^{2+} sensors based on squaraine dyes have also been reported recently (*35–37*).

Then, we examined the thiol-containing amino acid sensing ability of SQ2–Hg^{2+}–β-cyclodextrin ensemble in organic solvent-free aqueous solution. Figure 3 shows the responses of SQ2 (5 μM)–Hg^{2+} (80 μM)–β-cyclodextrin (15 mM) ensemble to various amino acids. Among the examined 23 amino acids and short peptide, only cysteine, homocysteine and GSH (glutathione) which contain thiol group can restore the visible absorption and fluorescence of SQ2 (Figure S5, available online, shows the case of cysteine). The fluorescence intensity was enhanced by about sevenfold in the presence of thiol-containing amino acids, corresponding to a recovery of 85% with respect to the fluorescence intensity before Hg^{2+} addition, whereas the colour of the solution altered from colourless to light green, discernible easily by naked eye. In our previous work, SQ1–Hg^{2+} in acetonitrile/water (2:1, v/v) can response histidine as efficiently as thiol-containing amino acids. However, for SQ2–Hg^{2+}–β-cyclodextrin in water, the sensitivity to histidine is greatly reduced, consequently the improved selectivity of SQ2–Hg^{2+}–β-cyclodextrin allows for the discrimination of thiol-containing amino acids from histidine and other amino acids.

The selectivity difference between SQ2–Hg^{2+}–β-cyclodextrin in water and SQ1–Hg^{2+} in acetonitrile/water (2:1, v/v) may originate either from the presence of β-cyclodextrin or from the change of solvent. To clarify the reason, we first investigated the interaction between histidine and β-cyclodextrin which may impede the chelation of Hg^{2+} and histidine. However, the addition of β-cyclodextrin does not lead to any absorption spectrum changes of histidine, suggesting negligible interactions between them. Then, we studied the influence of solvent on the amino acid sensing behaviour of SQ2–Hg^{2+}. As shown in Figure 4 and Figure S6 (available online), we measured the absorption recovery of SQ2–Hg^{2+} system caused by cysteine and histidine in the mixture of water and THF. When the volume ratio of water to THF is 5:5, both cysteine and histidine can efficiently restore the absorption of SQ2. With the increase in the volume ratio between water and THF, the absorption restoring capability of histidine declines remarkably, while that of cysteine decreases only a little bit. As a result, at the volume ratio of 8:2 for water and THF, the response of SQ2–Hg^{2+} is still significant to cysteine, however, negligible to histidine. These results demonstrate that the improved selectivity of SQ2–Hg^{2+}–β-cyclodextrin in water than SQ1–Hg^{2+} in acetonitrile/water (2:1, v/v) primarily results from the solvent variation.

In our previous work, we have demonstrated that the SQ1–Hg^{2+} in acetonitrile/water can detect cysteine with a tunable measuring range by simply changing the

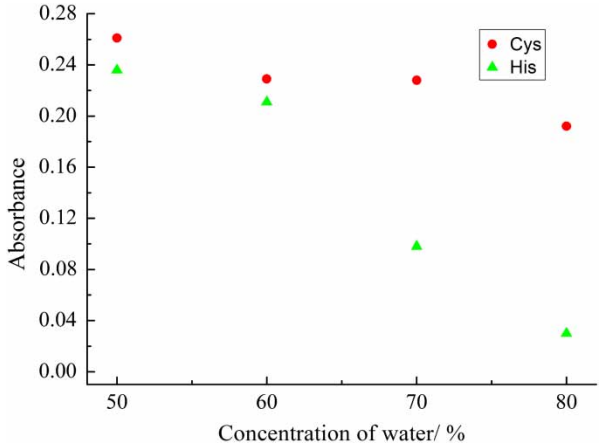

Figure 4. The absorbance values at 648 nm of SQ2 (5 μM)–Hg^{2+} (200 μM) solution in water/THF of varied volume ratio in the presence of either cysteine (200 μM) or histidine (200 μM).

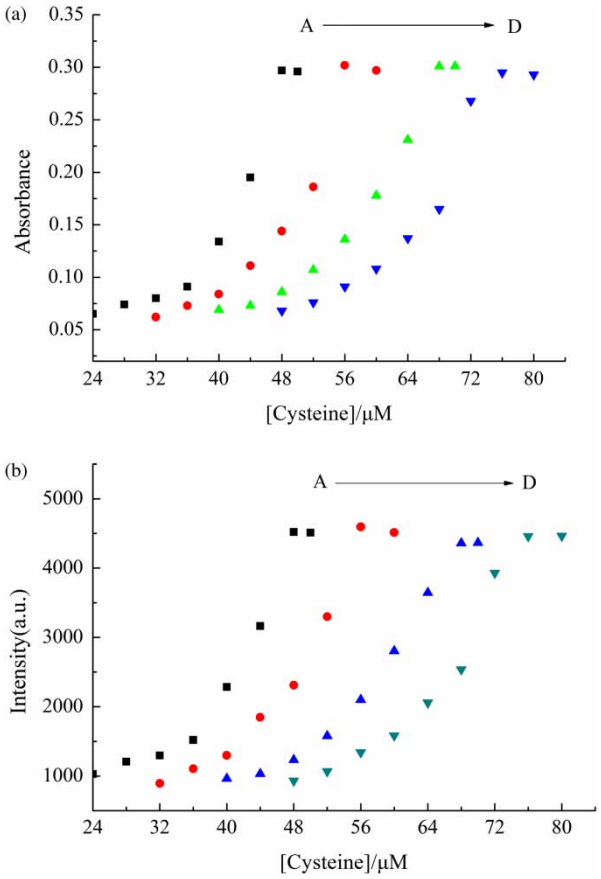

Figure 5. Absorbance at 648 nm (a) and fluorescence intensity at 675 nm (b) of aqueous solutions of SQ2 (5 μM), β-cyclodextrin (15 mM) and Hg^{2+} in the presence of varied concentrations of cysteine. The concentrations of Hg^{2+} are 50 μM (A), 60 μM (B), 70 μM (C) and 80 μM (D), respectively.

concentration of Hg^{2+}. Such an interesting feature still remains for SQ2–Hg^{2+}–β-cyclodextrin in water.

As shown in Figure 5, when the concentration of Hg^{2+} is 50 μM, the absorption and fluorescence turn-on of the system begins at 32 μM of cysteine and completes at 48 μM of cysteine. The linear detection range is from 36 to 48 μM as shown in Figure S7 (available online) (correlation coefficient = 0.996). In the case of 60 μM of Hg^{2+}, the 'turn-on' begins at 40 μM and completes at 56 μM. In a word, with the increase in the concentration of Hg^{2+}, the response window of SQ2–Hg^{2+}–β-cyclodextrin shifts to higher concentration level of cysteine. By changing the concentration of Hg^{2+} to 300 μM, the cysteine in the range of 275–290 μM, the concentrations out of the normal level (250–275 μM) of tCys in plasma, may be detected (Figure S8, available online).

4. Conclusions

In summary, the host–guest interactions between SQ2 and β-cyclodextrin are utilised to dissolve SQ2 in organic solvent-free aqueous solution in its monomeric form. As a result, SQ2–Hg^{2+}–β-cyclodextrin ensemble can sense thiol-containing amino acids not only in the aqueous solution but also with improved selectivity, allowing for discrimination of thiol-containing amino acids from all other amino acids including histidine. Furthermore, the measuring range of thiol-containing amino acids may be easily modulated by changing the concentration of Hg^{2+} ion. Further investigation to find biological benign metal ion for the substitution of Hg^{2+} is currently underway.

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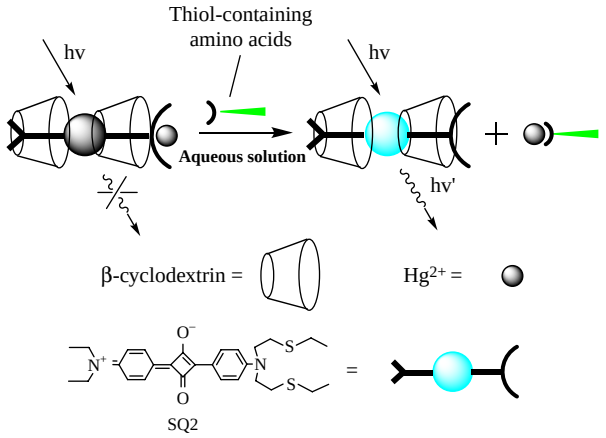

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