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Fluorescent detection of polycyclic aromatic hydrocarbons in ternary cyclodextrin complexes

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Reported herein is a new method for the detection of polycyclic aromatic hydrocarbons (PAHs) which relies on energy transfer from the PAH to a near-infrared emitting squaraine fluorophore. This energy transfer occurs inside the γ-cyclodextrin cavity, with up to 35% emission observed from energy transfer compared with exciting the squaraine directly.

**Keywords:** cyclodextrin; fluorophores; fluorescence; squaraine; polycyclic aromatic hydrocarbon

The detection of polycyclic aromatic hydrocarbons (PAHs) remains an important research objective, as many of these compounds are highly toxic (1,2) and are known carcinogens (3,4). These compounds are generally formed from the incomplete combustion of petroleum, and have been found in Gulf Water seafood following the BP oil spill (5). The FDA recently published ‘levels of concern’ for these compounds in seafood, i.e. recommended concentration limits, many of which are below 1 ppm (Figure 1).

Several methods for PAH detection have been reported in the literature (6,7). Although many of these methods have been used successfully (8,9), there remains a need for new methods, especially those that can detect PAHs at sub-ppm concentrations and distinguish between structurally similar analytes that have widely disparate toxicities. For example, benzo[a]pyrene 5 has a recommended concentration limit of 0.132 ppm, whereas anthracene 1, with a similar structure, has a recommended concentration limit that is four orders of magnitude higher.

Reported herein is a new method for the detection of PAHs which relies on ternary complexes formed with the PAH, and a near-infrared emitting squaraine dye in γ-cyclodextrin, which can be easily adapted for the array-based detection of a variety of PAHs. The system utilises energy transfer from the PAH, with excitation of the PAH resulting in efficient energy transfer to and emission from the squaraine (Figure 2). The cavity of γ-cyclodextrin is sufficiently large to accommodate two small-molecule guests (10–13) and cyclodextrins are known to encapsulate PAHs (14–17). Although this binding has been used to remove PAHs from contaminated soil samples (18) and to enhance the rate of PAH degradation (19,20), it has not been used for PAH detection.

The energy transfer from the PAH to a squaraine fluorophore occurs despite the limited spectral overlap that exists between the PAH and the squaraine, which is typically a requirement for Förster resonance energy transfer (21–23). This requirement often leads to overlap between the emission spectra of the donor and the acceptor, which ultimately compromises the sensitivity of the system. Some isolated examples have demonstrated superior energy transfer with decreased spectral overlap between the donor and the acceptor (24–26). Our system represents an additional example of such non-Förster energy transfer, and has the potential to lead to the development of highly sensitive sensors for PAH detection.

The squaraines investigated herein were synthesised using literature-reported procedures (Figure 3) (27–29). The PAHs investigated include anthracene 1, pyrene 2 and benzo[a]pyrene 5. For each experiment, ternary complexes were formed by pre-mixing the PAH in a macrocyclic host and then slowly adding the squaraine fluorophore. All experiments were carried out in phosphate-buffered saline (PBS) at pH 7.2. The resulting ternary complexes were excited at 360 nm (near the PAH absorption maximum) and at 620 nm (near the squaraine absorption maximum). The efficiency of energy transfer was calculated by dividing the squaraine emission from energy transfer by the squaraine emission from direct excitation, as in Equation (1):

\[ \text{Efficiency} = \left( \frac{I_{DA}}{I_D} \right) \times 100\% \]

where \( I_{DA} \) is the integrated emission of the squaraine from PAH excitation and \( I_D \) is the integrated squaraine emission from direct excitation.

Experiments were also carried out with the reverse order of addition, i.e. where the squaraine was first added to the macrocycle, followed by PAH addition. Identical energy transfer results were obtained for these systems.
After investigating a variety of macrocyclic hosts, near-infrared emitting squaraine dyes and PAHs, we identified the optimal squaraine and macrocycle for PAH detection: \( \gamma \)-cyclodextrin in combination with squaraine 7. This combination was used to form ternary complexes with anthracene 1 and pyrene 2, with efficient energy transfer occurring in those systems.

In particular, for ternary complexes formed in 9 mM \( \gamma \)-cyclodextrin with anthracene 1 (45 \( \mu \)M) and squaraine 7 (11 \( \mu \)M), excitation of the complex at 360 nm resulted in 35% of the squaraine emission observed from direct excitation (Figure 4(a)). This energy transfer occurs despite the lack of any appreciable overlap between the emission spectrum of anthracene and the absorption spectrum of the squaraine (Figure 5). Simultaneously, anthracene emission decreased in the presence of increasing amounts of \( \gamma \)-cyclodextrin, to 61% of its initial value at 9 mM \( \gamma \)-cyclodextrin compared to its value in pure PBS (Figure 4(b)).

Due to the complete lack of spectral overlap between anthracene and squaraines, such energy transfer from anthracene to squaraine is virtually unprecedented. One literature-reported example demonstrated energy transfer from an anthracene-containing macrocycle to an encapsulated squaraine guest in a two-component system (30). An energy-minimised depiction of anthracene 1 and squaraine 7 (using an MM2 force field; Figure 6) indicates substantial overlap of the anthracene with the planar squaraine core, which can facilitate efficient energy transfer.

Tetra-acid squaraine 8 also functioned as a reasonable energy acceptor, with up to 27% squaraine emission observed via energy transfer (10 mM \( \gamma \)-cyclodextrin, 45 \( \mu \)M anthracene 1 and 15 \( \mu \)M squaraine 8). The second-best performing host, Me-\( \beta \)-cyclodextrin, only effected 13% and 8% energy transfer between anthracene 1 and squaraines 7 and 8, respectively. No other macrocyclic hosts led to significant energy transfer between anthracene 1 and any of the squaraines tested (see Supporting Information for more detail), which is likely because their small internal cavities preclude the formation of ternary complexes (i.e. 5 Å internal diameter for \( \alpha \)-cyclodextrin and 6.9 Å for \( \beta \)-cyclodextrin, compared to 8.5 Å for \( \gamma \)-cyclodextrin) (31).

Similarly, pyrene 2 was also used successfully in energy transfer schemes with squaraines 7 and 8 in \( \gamma \)-cyclodextrin. In the absence of squaraine 7, pyrene displayed an excimer peak centred at 460 nm, which is consistent with two molecules of pyrene binding in the \( \gamma \)-cyclodextrin cavity simultaneously (32). The addition of squaraine led to a decrease in the pyrene excimer band (to 13% of its initial value), with a concurrent increase in the squaraine emission due to energy transfer (Figure 7).

Up to 19% of energy transfer was observed for 11 \( \mu \)M squaraine 7 and 40 \( \mu \)M pyrene 2 in 6 mM \( \gamma \)-cyclodextrin. For squaraine 8, up to 15% energy transfer was observed.
The existence of energy transfer is taken as evidence that ternary complexes have formed inside the γ-cyclodextrin cavity, as control experiments carried out without cyclodextrin resulted in virtually non-observable energy transfer for squaraine 7 in combination with all of the PAHs tested.

Mechanism of energy transfer
The mechanism of energy transfer within the ternary complex likely occurs via a direct electronic exchange (i.e. non-Förster) mechanism as a result of the close proximity of the donor and acceptor. A key advantage to this

Figure 4. (a) Comparison of fluorescence spectra from excitation at 360 nm to excitation at 620 nm (9 mM γ-cyclodextrin, 45 μM anthracene and 11 μM squaraine 7) and (b) a comparison of the fluorescence spectra at different concentrations of γ-cyclodextrin (45 μM anthracene and 53 μM squaraine 7).

Figure 5. Graphs illustrating the lack of appreciable spectral overlap between the PAH fluorescence spectra and the squaraine absorbance spectra.

Figure 6. Energy-minimised structure of squaraine 7 and anthracene 1.
mechanism is that the squaraine fluorescent signal occurs in a ‘dark’ spectral region (free of contamination from residual anthracene and pyrene emission). The near-infrared spectral region is also free of contamination from biological analytes (33).

**Advantages compared to other systems**

Because PAHs are fluorescent, their presence can be monitored directly via fluorescence spectroscopy (34). Nonetheless, the energy-transfer system reported herein has substantial potential advantages for detection, which are as follows: (a) the signal that is generated via energy transfer occurs in the near-infrared spectral region, which is an area that has little overlap from other biological (and non-biological) analytes and (b) the system provides a straightforward way to develop an array-based system for PAH detection using a variety of squaraine–γ-cyclodextrin complexes (35). Each PAH will have a unique set of photophysical interactions with the squaraine–cyclodextrin array, which can be used to distinguish between structurally similar PAH analytes with widely disparate toxicities (36).

**Limit of detection**

By using this energy transfer system, the limit of detection of anthracene is found to be 1.1 µM (corresponding to 0.2 ppm (parts per million, mass-to-mass measurement of anthracene in the buffer solution)). The energy transfer observed for 7 ppm of anthracene, in combination with 11 µM squaraine 7 and 10 mM γ-cyclodextrin, is shown in Figure 8. These low limits of detection are below the concentration limits recommended by the FDA for anthracene.

**Conclusion**

In conclusion, efficient energy transfer has been demonstrated from anthracene and pyrene donors to a near-infrared emitting squaraine fluorophore acceptor. This energy transfer, which occurs via the formation of ternary complexes, can be used to generate a sensor array for the sensitive and selective detection of toxic PAHs. Efforts towards screening a variety of PAHs, as well as a variety of commercially available fluorophores, are currently in progress and results will be reported in due course.

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