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Detection of Medium-Sized Polycyclic Aromatic Hydrocarbons via Fluorescence Energy Transfer

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Abstract

Reported herein is the use of proximity-induced non-covalent energy transfer for the detection of medium-sized polycyclic aromatic hydrocarbons (PAHs). This energy transfer occurs within the cavity of γ -cyclodextrin in various aqueous environments, including human plasma and coconut water. Highly efficient energy transfer was observed, and the efficiency of the energy transfer is independent of the concentration of γ -cyclodextrin used, demonstrating the importance of hydrophobic binding in facilitating such energy transfer. Low limits of detection were also observed for many of the PAHs investigated, which is promising for the development of fluorescence-based detection schemes.

INTRODUCTION

The accurate, sensitive, and selective detection of polycyclic aromatic hydrocarbons (PAHs) remains a crucial research objective, as many of these compounds are known or suspected carcinogens (1), environmental pollutants (2), and endocrine disruptors (3). They are known components of crude and processed oil (4), and have been found in the blood (5) and breast milk (6) of populations living in oil spill affected areas, and in seafood from the Gulf of Mexico following the Deepwater Horizon oil spill (7).

Despite the widespread prevalence and known toxicity of these compounds, current detection methods often have significant limitations, including the requirement for tedious sample preparation prior to analysis (8), or the inability to accurately distinguish structurally similar PAHs with widely disparate toxicities (9). Previous research in our group introduced a fundamentally new approach for the detection of PAHs, which relies on using the PAHs as energy donors in combination with high quantum yield fluorophore acceptors including BODIPY (compound **10**) and Rhodamine 6G (compound **11**) (Figure 1) (10–14). Energy transfer from the PAH to the fluorophore occurs when both are bound in the cavity of commercially available, non-toxic γ -cyclodextrin, leading to a new, brightly fluorescent signal in the presence of the PAH of interest (Figure 2). This energy transfer based detection

has been used in complex biological fluids (14) and with PAHs that have been extracted from crude oil samples (13).

Previous research in our group focused predominantly on lower molecular weight PAHs, including anthracene, pyrene, and benzo[*a*]pyrene, with exceptionally efficient energy transfer observed from pyrene **2** and benzo[*a*]pyrene **3** to BODIPY **10**. While benzo[*a*]pyrene is highly toxic (15), many of the lower molecular weight homologs (naphthalene, anthracene, and pyrene) have greatly reduced toxicities (16). Higher molecular weight PAHs, including benz[*a*]anthracene, benz[*b*]anthracene, benzo(b)fluoranthene, and chrysene (compounds **4–7**), are significantly more toxic to a wide variety of organisms (17,18). The detection of higher molecular weight PAHs via cyclodextrin-promoted energy transfer would have significant potential utility in PAH detection in complex environments and in environmental remediation efforts. Other known toxicants include heterocyclic aromatic compounds such as carbazole (compound **8**) (19) and environmental toxicants such as *p*-cresol (compound **9**) (20), and the ability to use cyclodextrin-based energy transfer for the detection of these classes of toxicants would provide significant operational advantages. Reported herein are the results of our efforts towards achieving these goals: detecting higher molecular weight PAHs, heteroaromatic toxicants, and low molecular weight environmental toxicants via cyclodextrin-promoted, non-covalent energy transfer.

EXPERIMENTAL

Materials and Methods

All chemicals were obtained from Sigma Aldrich Chemical Company and were used as received. Compound **10** was synthesized following literature-reported procedures (21). Human plasma was obtained from Innovative Technologies. Coconut water was obtained from CVS Pharmacy in Kingston, Rhode Island. ¹H NMR spectra were recorded on a Bruker 300 MHz spectrometer. UV-Visible spectra were recorded on an Agilent 8453 spectrometer equipped with a photodiode array detector. Fluorescence spectra were recorded on a Shimadzu RF-5301PC spectrophotofluorimeter.

Energy Transfer Experimental Procedures

For full experimental details and copies of all spectra, see the Electronic Supporting Information.

Energy transfer experiments were conducted as follows.

All analytes (**4–9**) and fluorophores (**10, 11**) were dissolved in tetrahydrofuran (THF). Small amounts of these **THF** solutions were added to a 10 mM aqueous solution of γ -cyclodextrin in phosphate buffered saline (PBS) buffered at pH 7.4. Following thorough mixing, the fluorescence emission of the solution was recorded from excitation at the analyte's excitation wavelength and from excitation at the fluorophore's excitation wavelength, and the percent energy transfer efficiencies were calculated according to Equation (1):

$$\% \text{Efficiency} = (I_{\text{DA}}/I_{\text{A}}) * 100\%, \quad (1)$$

where I_{DA} is the integrated emission of the fluorophore from analyte excitation and I_A is the integrated fluorophore emission from direct fluorophore excitation.

Two control experiments were conducted for each analyte-fluorophore combination.

1. The 10 mM γ -cyclodextrin solution was replaced with a 0 mM solution, and the same procedures were followed, to elucidate the beneficial effect of γ -cyclodextrin on promoting efficient energy transfer.
2. The fluorophore emission via excitation at the analyte's excitation wavelength (but in the absence of the analyte) was measured, and compared to the fluorophore emission via analyte excitation in the presence of the analyte. The analyte comparison ratio was calculated according to Equation (2):

$$\text{Analyte comparison} = F_A / F_{DA}, \quad (2)$$

where F_A is the fluorophore emission via analyte excitation in the absence of an analyte, and F_{DA} is the fluorophore emission via analyte excitation in the presence of the analyte. Ratios close to 1 indicate a limited contribution of the analyte, which means that the observed energy transfer peaks are likely due to the fluorophore itself having a nonzero absorbance at the analyte's excitation wavelength, rather than a result of legitimate energy transfer.

Analogous experiments were conducted in phosphate buffer (buffered at pH 7.4 but with less total salt content), coconut water, and human plasma.

Limit of Detection Experimental Procedures

Limits of detection for all analyte-fluorophore combinations were calculated by constructing calibration curves for each combination, with the analyte concentration on the X-axis and the integrated fluorophore emission on the Y-axis. The minimum analyte concentration necessary to elucidate a fluorescence response, defined as the limit of detection, was calculated from these calibration curves following literature-reported procedures (22). The limit of quantification was calculated for each combination as well, defined as the minimum concentration of analyte necessary to elucidate a quantifiable fluorescence signal response.

RESULTS AND DISCUSSION

Previous results from our research group demonstrated highly efficient cyclodextrin-promoted energy transfer for a wide range of toxicants and fluorophores, with greater than 100% energy transfer efficiencies observed in several cases (10–14). These energy transfer efficiencies were particularly efficient for pyrene and benzo[*a*]pyrene, as well as for highly toxic aromatic amines such as 2-aminofluorene (23), 2-acetylaminofluorene (23), and benzidine (24), in combination with fluorophores **10** and **11**. Attempts to extend this cyclodextrin-promoted energy transfer to analytes **4–9** resulted in highly efficient energy transfer in several cases (Table 1), in particular for analytes **4**, **6**, and **8** in combination with fluorophore **10** (Figure 3). The analyte comparison ratios for each of these cases were substantially less than 1, indicating that the observed energy transfer peaks were due to legitimate energy transfer from the analyte to the fluorophore.

A closer examination of the control experiments done in the absence of cyclodextrin indicated that for many of these analyte-fluorophore pairs, the observed energy transfer efficiencies in 10 mM cyclodextrin were unchanged or even lower than energy transfer efficiencies observed in 0 mM cyclodextrin. These surprising results point to the existence of more complex intermolecular phenomena that are occurring, rather than the previously observed ternary complex formation in γ -cyclodextrin (Figure 2).

Cases where the energy transfer efficiencies are unchanged in the presence and absence of cyclodextrin are likely a result of the donor and acceptor interacting via non-covalent, non-cyclodextrin promoted hydrophobic binding, leading to energy transfer even in the absence of cyclodextrin. For such cases, the addition of 10 mM cyclodextrin has no effect on this intermolecular hydrophobic bonding, and no changes in the energy transfer efficiencies are observed. This non-cyclodextrin promoted energy transfer requires exceptionally strong hydrophobic binding to ensure that the donor and acceptor are bound in close proximity without an external host to enforce such proximity. This strong binding is enabled by the larger size of the aromatic PAHs compared to previously studied smaller analogs (25), which increases their hydrophobic surface areas and the strength of the hydrophobic binding observed.

Cases where the energy transfer efficiencies are lower in 10 mM cyclodextrin compared to the cyclodextrin-free solution can be explained by the same non-cyclodextrin dependent hydrophobic binding and energy transfer. In these cases, however, the addition of cyclodextrin causes one of the two energy transfer partners to bind in the cyclodextrin cavity, which disrupts the close proximity of the donor and acceptor. This cyclodextrin-enforced separation of the donor and acceptor leads to a decrease in the observed energy transfer efficiencies.

Experimental evidence for the importance of strong hydrophobic binding in promoting cyclodextrin-free energy transfer comes from a direct comparison of energy transfer efficiencies in phosphate buffered saline and in phosphate buffer (without saline) (Table 2). Energy transfer efficiencies observed in phosphate buffer for compound **6** were markedly decreased compared to the energy transfer efficiencies observed in PBS. Sodium is a known kosmotropic agent (26,27), meaning that it stabilizes hydrophobic interactions such as those required for cyclodextrin-independent energy transfer (28). Because the sodium content of PBS is substantially higher than that of the phosphate buffer, hydrophobic binding is strengthened under those conditions.

Moreover, the addition of cyclodextrin had either no effect (for **6–11**) or a negative effect (for **6–10**) on the observed energy transfer efficiencies in phosphate buffer, again pointing to the significant contribution of non-cyclodextrin dependent intermolecular hydrophobic binding. Even in the absence of added sodium, the strong donor-acceptor hydrophobic binding leads to high energy transfer efficiencies.

Similar behavior was also observed in more complex fluids, including coconut water (which has been used as a plasma surrogate in emergencies) (29,30) and human plasma (31). Coconut water is known to contain a wide variety of small molecule nutrients, including

vitamins, sugars, and amino acids (32), as well as macromolecules such as proteins and lipids (33). Many of these analytes can potentially interfere with efficient non-covalent energy transfer. Plasma contains numerous lipids and proteins (34), including proteins that are known to bind highly lipophilic molecules (35). These analytes also have the potential to interfere with the desired energy transfer. Energy transfer from the PAH to the fluorophore was observed in the presence of 10 mM γ -cyclodextrin (Figure 4), but was even more pronounced in the absence of the cyclodextrin (Table 3), pointing to a scenario in which the introduction of the cyclodextrin leads to a disruption of hydrophobic binding between the donor and acceptor.

Overall, the medium-sized polycyclic aromatic hydrocarbons demonstrate efficient cyclodextrin-independent energy transfer, but demonstrate a reluctance to form ternary complexes in the γ -cyclodextrin cavity. This reluctance is likely due to the larger dimensions of these analytes (Table 4), which can preclude efficient binding in the cyclodextrin cavity. The interior diameter of γ -cyclodextrin has been reported to be between 7.5 and 8.3 Å, with a cavity height of 7.9 Å (36). Analytes 4–7 have dimensions that are comparable to or larger than the cyclodextrin cavity size, which explains their reluctance to form ternary complexes.

The ability of the analytes to undergo highly efficient cyclodextrin-independent energy transfer means that the development of detection methods based on this energy transfer is possible. The practicality of these detection methods will be greatly enhanced by the ability to detect toxicants at low concentrations (at or below recommended exposure limits). The limits of detection for each analyte-fluorophore pair were calculated, and micromolar concentration limits were determined for a number of the analytes (see ESI for more details). Most interestingly, low detection limits were also calculated for energy transfer in complex fluids such as plasma and coconut water (Table 5), highlighting the potential utility of this method for toxicant detection in complex environments.

CONCLUSION

In summary, highly efficient energy transfer has been demonstrated from a variety of medium sized polycyclic aromatic hydrocarbons to high quantum yield fluorophores. This energy transfer is independent of the concentration of cyclodextrin, and likely occurs due to the strong hydrophobic binding between the PAH energy donors and fluorophore acceptors. The hydrophobic binding is stronger in PBS than in saline-free phosphate buffer, and also occurs with moderate to high efficiencies in coconut water and human plasma. The robustness of the hydrophobic binding and the cyclodextrin-independent energy transfer leads to low detection limits for a wide variety of the analytes under investigation, highlighting the practical applications of this energy transfer in the development of turn-on detection schemes. Efforts towards the development of such detectors are in progress, and results will be reported in due course.

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Figure 1.
Structures of all analytes and fluorophores targeted for investigation.

Figure 2.
Schematic illustration of cyclodextrin-promoted energy transfer.

Figure 3.

Energy transfer in PBS observed for (A) **4–10**; (B) **6–10**; and (C) **8–10**. The black line represents analyte excitation and the grey line (red in online version) represents direct fluorophore excitation.

Figure 4.

Energy transfer from analyte **6** to fluorophore **10** in (A) coconut water with cyclodextrin; (B) coconut water without cyclodextrin; (C) plasma with cyclodextrin; and (D) plasma without cyclodextrin. The black line represents analyte excitation and the grey line (red in online version) represents direct fluorophore excitation.

Table 1Percent energy transfer efficiencies for analytes **4–9** with fluorophores **10** and **11** in PBS^a

Analyte	Fluorophore	10 mM γ -CD	0 mM γ -CD
4	10	140.2	122.0
5	10	<i>b</i>	<i>b</i>
6	10	274.3	526.6
7	10	25.1	22.4
8	10	44.8	87.5
9	10	30.2	29.3
4	11	6.4	6.6
5	11	<i>b</i>	<i>b</i>
6	11	25.8	34.1
7	11	19.4	21.1
8	11	12.0	12.9
9	11	24.2	27.9

^a All reported results are an average of at least three trials.^b Excessive overlap between the analyte and fluorophore emissions prevented efficient integration.

Table 2

A comparison of the energy transfer efficiencies in 10 mM γ -cyclodextrin dissolved in PBS and in phosphate buffer^a

Analyte	Fluorophore	ET in PBS	ET in phosphate buffer
6	10	274.3	169.6
6	11	25.8	8.2
8	10	44.8	41.8

^a All reported results are an average of at least three trials.

A comparison of energy transfer efficiencies in the presence and absence of 10 mM γ -cyclodextrin in coconut water and plasma^a

Table 3

Analyte	Fluorophore	coconut water			plasma		
		10 mM γ -CD	0 mM γ -CD	0 mM γ -CD	10 mM γ -CD	0 mM γ -CD	0 mM γ -CD
6	10	45.8	260.6		26.0		36.8
6	11	5.5	8.6		8.3		17.0
8	10	31.7	115.5		26.3		29.1

^a All reported results are an average of at least three trials.

Table 4Calculated cavity dimensions for analytes **4–9**^a

Analyte	Height	Width
4	10.7 Å	7.2 Å
5	5.0 Å	11.6 Å
6	7.2 Å	11.4 Å
7	7.2 Å	10.5 Å
8	5.3 Å	9.0 Å
9	5.0 Å	6.6 Å

^a Calculations were performed using Spartan software, semi-empirical PM3-level calculations.

Table 5

Limits of detection for selected donor-acceptor combinations

Analyte	Fluorophore	PBS	Phosphate Buffer	Coconut Water	Plasma
6	10	18.6 μ M	<i>a</i>	24.7 μ M	44.4 μ M
6	11	<i>a</i>	12.6 μ M	20.6 μ M	44.0 μ M
8	10	54.2 μ M	9.6 μ M	<i>a</i>	46.8 μ M

^a Attempts to calculate limits of detection for these analyte-fluorophore combinations led to nonsensical values; current efforts are focused on trying to understand these results.