Cyclodextrins as Hosts for the Array-Based Detection of Persistent Organic Pollutants in Complex Media

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CYCLODEXTRINS AS HOSTS FOR THE ARRAY-BASED DETECTION OF PERSISTENT ORGANIC POLLUTANTS IN COMPLEX MEDIA

BY

NICOLE COOK SERIO

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CHEMISTRY

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OF

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ABSTRACT

The ability to quickly determine the nature of small-molecule toxicants after an anthropogenic event would greatly benefit first responders and medical personnel. Current detection methods, while elegant, require several separation and purification steps before the samples can be submitted for analysis, which can be a time-consuming process. There is a crucial knowledge gap that exists as a result. Reported herein is the use of a non-toxic, commercially-available molecule, cyclodextrin, to rapidly isolate and detect the toxic components involved in a spill event that would address this knowledge gap. This cyclodextrin-based scheme would work as a compliment to established analysis procedures by establishing a rapid, high-throughput procedure that can be used to quickly scan samples to determine the nature of the compounds involved in a spill event. This would provide first responders with the information they need to develop an effective response in a timely manner, and samples would still be sent for more intense analysis using standardized procedures, such as those set by the Environmental Protection Agency (EPA) to confirm the results and quantify them. Similarly, this method can be used by medical personnel to quickly analyze samples from patients to determine if their symptoms are a result of a spill event.

Cyclodextrins enable the identification of toxicants proximity-induced interactions between a toxicant and high-quantum yield fluorophore. Cyclodextrins have hydrophobic cores and hydrophilic surfaces, and both the toxicant and fluorophore use the cyclodextrin as a scaffold, forcing them in close proximity to one another. Once the toxicant and fluorophore are closely associated, gamma-
cyclodextrin, the primary cyclodextrin derivative of interest in this work, facilitates proximity-induced energy transfer from the toxicant donor to the fluorophore acceptor. Energy transfer to and emission from the fluorophore occurs upon excitation of the toxicant, and the resulting emission spectra is unique to each fluorophore-toxicant combination. These unique signals can lead to the array-based detection of the toxicant as they act as photophysical “fingerprints” for the toxicant.

The cyclodextrin-based scheme discussed herein offers a number of operational advantages. First, this scheme is well-suited for high-throughput screening as fluorescence measurements are fast to obtain and samples require little pretreatment before analysis (usually a simple dilution is all that is needed). Second, detection occurs successfully in multiple complex matrices, including seawater, oiled samples, and human plasma, breast milk, and urine. As such, this method can be useful to a variety of spill scenarios, and assist medical personnel. Third, cyclodextrins can effectively remove some of the most toxic components from oil spills, helping to solve many oil-spill related problems and enabling a tandem extraction-detection system. Taken together, this work has significant applications for public health, environmental remediation, and disaster response and relief.

The first manuscript, “Efficient detection of polycyclic aromatic hydrocarbons and polychlorinated biphenyls via three-component energy transfer,” describes the energy transfer efficiencies from polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) to high-quantum yield fluorophores using proximity-induced non-covalent energy transfer. This energy transfer is efficient even with fluorescent PAHs and less fluorescent PCBs. The low limits of detection and
potential for selective detection using array-based systems, combined with the straightforward experimental setup, is the basis for using such a system to detect small molecule toxicants. This manuscript was published in the journal *Chemical Communications* in 2013.

The second manuscript, “Array-based detection of persistent organic pollutants via cyclodextrin promoted energy transfer,” focuses on applying the findings from the previous manuscript to the development of an array-based detection scheme. In this work, γ-cyclodextrin promotes proximity-induced, non-covalent energy transfer from an aromatic pollutant (analyte) to a high quantum yield fluorophore. Through the use of three different fluorophores, a sensor array that successfully classified all 30 analytes with 100% accuracy and identified unknown analytes with 96% accuracy was developed. This detection scheme was also able to identify 92% of analytes successfully in human urine. This manuscript was accepted on June 12, 2015 by the journal *Chemical Communications* for publication.

The third manuscript, “Cyclodextrin-enhanced extraction and energy transfer of carcinogens in complex oil environments,” uses γ-cyclodextrin to achieve two tandem, high-impact functions: (a) the extraction of PAHs from various oil samples into aqueous solution, and (b) the promotion of energy transfer from the extracted PAHs to a high-quantum-yield fluorophore. The extraction proceeded in moderate to good efficiencies, and the energy transfer promoted a new, brightly fluorescent signal in aqueous solution. Such a dual-function system (extraction followed by energy transfer) can be used in the environmental detection and cleanup of oil-spill-related
carcinogens. This manuscript was published in the journal *ACS Applied Materials and Interfaces* in 2013.

The fourth manuscript, “Efficient extraction and detection of aromatic toxicants from crude oil and tar balls using multiple cyclodextrin derivatives,” reports the efficient extraction of aromatic analytes from crude oil and tar balls using various cyclodextrin derivatives. Cyclodextrins are known to bind hydrophobic guests in their hydrophobic interiors, and they were able to extract aromatic analytes from the oil layer to the aqueous layer. Methyl-β-cyclodextrin and β-cyclodextrin were the most efficient at analyte extraction while γ-cyclodextrin was most efficient at promoting energy transfer. Cyclodextrins are can be used for tandem analyte extraction and detection in oil samples, with up to 86% efficient energy transfer observed in the presence of γ-cyclodextrin compared to 50% in the absence of cyclodextrin for oil spill oil extraction. This manuscript was published in the journal *Marine Pollution Bulletin* in 2015.

The fifth manuscript, “Cyclodextrin-promoted energy transfer for broadly applicable small-molecule detection,” reports energy transfer from small-molecule toxicants to organic fluorophores for a broad range of toxicants in complex biological media. The media include human plasma, coconut water (which has been used as a plasma surrogate in emergency situations), and human breast milk. This energy transfer proceeded in moderate to good efficiencies. Because this energy transfer is a generally applicable phenomenon, it has significant potential in the development of new turn-on detection schemes. This manuscript was published in the journal *Supramolecular Chemistry* in 2014.
The sixth manuscript, “Investigating fundamental intermolecular interactions in gamma-cyclodextrin host-guest complexes,” focuses on the mechanisms that underlie association complex formation using gamma cyclodextrin hosts. Binding behavior in such complexes is driven by hydrogen bonding, \( \pi-\pi \) stacking, Van der Waals forces, and the hydrophobic effect. However, because of the disparate structures of the small-molecule toxicants that have been investigated, the overall contribution of each of these forces vary between structures. Hydrogen bonding was found to be a major contributor to association complex formation. This manuscript is currently in preparation for submission to the journal *Environmental Science: Water Research and Technology*.

The seventh manuscript, “Fluorescence-based detection of environmental toxicants and toxicant metabolites in urine,” focuses on the detection of parent polycyclic aromatic hydrocarbons (PAHs) and several of their oxidized daughter derivatives. PAHs are rapidly metabolized in the body, and detecting their metabolites is important for medical personnel in assessing an individuals’ exposure to such pollutants. In this work, samples from a non-smoker and habitual smoker were studied to assess changes in analyte response. Efficient energy transfer (and thus toxicant detection) was observed in both cases. This manuscript is currently in preparation for submission to the journal *Environmental Science and Technology*. 
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Thank you to all of my friends for being there for me throughout this experience. I am so thankful for each of you.

This work is dedicated to all of the amazing people in my life. Thank you for your love and support.
PREFACE

The dissertation of my research has been presented in manuscript format according to guidelines of the graduate school of the University of Rhode Island. The complete dissertation is divided into seven manuscripts. The first manuscript (Chapter 1) was published in Chemical Communications in 2013 with authors N. Serio, K. Miller, and M. Levine. The second manuscript (Chapter 2) is under review, also at Chemical Communications, with authors N. Serio, D. Moyano, V. Rotello, and M. Levine. The third manuscript (Chapter 3) was published in ACS Applied Materials and Interfaces in 2013 with authors N. Serio, C. Chanthalyama, L. Prignano, and M. Levine. The fourth manuscript (Chapter 4) was published in Marine Pollution Bulletin in 2015 with authors N. Serio and M. Levine. The fifth manuscript (Chapter 5) was published in 2014 in Supramolecular Chemistry with authors N. Serio, C. Chanthalyama, L. Prignano, and M. Levine. The sixth manuscript (Chapter 6) will be submitted to Environmental Science: Water Research and Technology with authors N. Serio, M. Verderame, and M. Levine. The seventh manuscript (Chapter 7) will be submitted to Environmental Science and Technology with authors N. Serio, L. Gareau, J. Roque, and M. Levine.
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CHAPTER 1
Published in Chemical Communications, 2013, 49, 4821-4823

Efficient Detection of Polycyclic Aromatic Hydrocarbons
and Polychlorinated Biphenyls via Three-Component
Energy Transfer

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**ABSTRACT**

Reported herein is the detection of highly toxic polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) via proximity-induced non-covalent energy transfer. This energy transfer occurs in the cavity of \( \gamma \)-cyclodextrin, and is efficient even with the most toxic PAHs and least fluorescent PCBs. The low limits of detection and potential for selective detection using array-based systems, combined with the straightforward experimental setup, make this new detection method particularly promising.

**INTRODUCTION**

Polycyclic aromatic hydrocarbons (PAHs)\(^1\) and polychlorinated biphenyls (PCBs) are two of the ten most toxic classes of compounds according to the Center for Disease Control’s ranking in 2011;\(^2\) as such, the development of sensitive and selective detection methods remains a top priority. PAHs are formed from the incomplete combustion of petroleum, and their presence has been detected in human blood and breast milk,\(^3\) and in Gulf water seafood following the Gulf of Mexico oil spill.\(^4\) Some examples of PAHs and the FDA-recommended concentration limits of PAHs\(^1\) in seafood are shown in Figure 1.\(^5\)

---

\(^1\) Because 1 and 4 share the same molecular weight, they cannot be fully separated. Thus, the recommended limit of 1 is the combined limit for these two PAHs.
PCBs were historically used as refrigerator coolants and in a variety of manufacturing products.\textsuperscript{6} Although the use of PCBs was banned in the United States in 1979,\textsuperscript{7} their atmospheric stability means that PCBs still persist in the environment.\textsuperscript{8} Some examples of PCBs are shown in Figure 1; the FDA-recommended concentration limits for PCBs in food ranges from 0.2–3.0 parts per million (ppm).\textsuperscript{9}

![Chemical structures of PAHs and PCBs](image)

**Figure 1.** PAHs and PCBs used as energy donors, together with the FDA-recommended concentration limits for PAHs in parts per million (ppm).

Current methods for the detection of PAHs and PCBs generally rely on separation using chromatography, followed by detection via mass spectrometry (for PAHs\textsuperscript{10} and PCBs\textsuperscript{11}) or fluorescence spectroscopy (for PAHs). The development of new methods for the detection of these compounds remains a high priority, especially if such methods have improved sensitivity and/or selectivity.

We previously reported that energy transfer occurs between anthracene and a squaraine fluorophore inside the cavity of $\gamma$-cyclodextrin, with up to 35% energy transfer observed from anthracene excitation compared to direct squaraine excitation.\textsuperscript{12} The energy transfer efficiency is defined as:

\[
\text{\% Efficiency} = \left(\frac{I_{BA}}{I_D}\right) \cdot 100\% \tag{1}
\]
where IDA is the integrated emission of the fluorophore from PAH excitation and ID is the integrated fluorophore emission from direct excitation.

Although examples of energy transfer with covalently-modified cyclodextrins have been reported,\textsuperscript{13} non-covalent energy transfer inside cyclodextrin cavities is much less developed,\textsuperscript{14} even though such energy transfer is substantially easier to tune and optimize.\textsuperscript{15}

**MATERIALS AND METHODS**

Reported herein is the development of a widely applicable non-covalent energy transfer system between PAH and PCB energy donors and fluorophore acceptors. These fluorophores (Figure 2) were chosen because of their high quantum yields,\textsuperscript{16} and established use in a variety of sensing schemes.\textsuperscript{17} Compound 8 is commercially available, and compounds 9 and 10 were synthesized following known procedures.\textsuperscript{18}

![Figure 2](image.png)

**Figure 2.** Structures of the fluorophores investigated.

Energy transfer from the analytes to the fluorophores in the presence of cyclodextrin was measured by mixing the analyte and fluorophore in a $\gamma$-cyclodextrin solution in phosphate-buffered saline (PBS), which should generate a ternary complex. The complex was then excited near the absorbance maximum of the analyte and near the maximum of the fluorophore, and energy transfer efficiencies were calculated.

Control experiments were also done in which the fluorophore was excited at the analyte’s excitation wavelength in the absence of any analyte, to determine
whether peaks previously identified as energy transfer peaks might be due to fluorophore emission from excitation at a wavelength where it has non-zero absorbance.

The results of these experiments were quantified as “fluorophore emission ratios,” defined as the integrated fluorophore emission in the absence of an analyte divided by the integrated fluorophore emission in the presence of the analyte (Table 1).

<table>
<thead>
<tr>
<th>Compound 8</th>
<th>Compound 9</th>
<th>Compound 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>0.99</td>
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</tr>
<tr>
<td>Compound 2</td>
<td>0.20</td>
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<td>Compound 3</td>
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<td>0.09</td>
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<tr>
<td>Compound 7</td>
<td>1.89</td>
<td>1.78</td>
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</table>

Defined as the ratio of fluorophore emission via indirect excitation in the absence of the analyte to fluorophore emission via indirect excitation in the presence of the analyte. Any values between 0.95 and 1.05 indicate that any presumed energy transfer is merely a result of the fluorophore absorbing a non-trivial amount of energy via the “indirect” excitation pathway.

RESULTS AND DISCUSSION

These experiments revealed that some of the analyte–fluorophore pairs that have a significant fluorophore peak from analyte excitation actually have the same fluorophore peak in the absence of analyte (fluorophore emission ratio between 0.95 and 1.05). For several cases, however, the fluorophore emission ratios were significantly higher than 1 (indicating that the analyte actually quenches fluorophore emission), and in other cases the ratio was significantly less than 1 (indicating that the
desired energy transfer is observed). The maximum energy transfer efficiencies for all 
analyte–fluorophore combinations that demonstrate energy transfer are shown in Table 
2.

<table>
<thead>
<tr>
<th>Compound</th>
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<th>Compound 9</th>
<th>Compound 10</th>
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</thead>
<tbody>
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<tr>
<td>Compound 4</td>
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</tr>
<tr>
<td>Compound 6</td>
<td>7.8%</td>
<td>9.2%</td>
<td>c</td>
</tr>
<tr>
<td>Compound 7</td>
<td>b</td>
<td>8.6%</td>
<td>b</td>
</tr>
</tbody>
</table>

*a* Excessive overlap between the analyte and fluorophore prevented accurate integration. 
*b* No fluorophore peak was observed from analyte excitation. 
*c* Fluorophore emission ratios indicate no real energy transfer is occurring (emission ratios between 0.95 and 1.05).

Although anthracene 1 does not undergo significant energy transfer with 
fluorophores 8 and 9 (as measured by the fluorophore emission ratios), the highly 
toxic PAHs 2 and 3 demonstrate significant energy transfer. Benzo[a]pyrene 3 acted as 
an energy donor with fluorophores 8 and 9 (and to a limited extent with squaraine 10). 
The energy transfer peaks with compounds 8 and 10 are clearly visible at 558 nm and 
659 nm, respectively (Figure 3). Control experiments also demonstrated the necessity 
of γ-cyclodextrin for energy transfer, as in the absence of cyclodextrin only 3% energy 
transfer was observed for benzo[a]pyrene with compound 8 (compared to 10% in the 
presence of 10mM γ-cyclodextrin). The detection of benzo[a]pyrene is particularly 
crucial, due to its low recommended concentration limit (0.132 ppm) and high 
carcinogenicity.
Figure 3. Energy transfer observed with compound 3 as an energy donor with (a) compound 8 and (b) compound 10 as energy acceptors. The fluorophore emission maxima are shown in each case (360 nm excitation; 10 mM γ-cyclodextrin, 31.7 μM compound 3; 8.35 μM compound 8; 53.0 μM compound 10).

The formation of ternary complexes with compound 2 as an analyte can be measured by a decrease in the excimer emission in the presence of increasing amounts of fluorophore (Figure 4). Using pyrene 2 as an energy donor with compound 9 as an energy acceptor resulted in the sequential displacement of one molecule of pyrene from the γ-cyclodextrin cavity and a concomitant decrease in the pyrene excimer emission to 41% of its initial value (Figure 4). Both compounds 8 and 10 also acted as competent energy acceptors, with 6% and 19% energy transfer observed, respectively.

Figure 4. Decreased excimer emission of compound 2 in the presence of increasing amounts of fluorophore 9 (360 nm excitation; 10 mM γ-cyclodextrin; 39.6 μM compound 2).
Analytes 4–7 had significant interactions with fluorophores 8 and 9, as measured by their fluorophore emission ratios. For fluorophore 8, introduction of analytes 4–7 led to a decrease in the fluorophore emission via low wavelength excitation compared to what is observed in the absence of the analyte (resulting in fluorophore emission ratios greater than 1). The nature of this interaction is not fully elucidated at this point, but nonetheless has the potential to contribute to array-based detection of toxic analytes (see below).

In order for this energy transfer to be practical for the detection of toxic analytes, it needs to be both sensitive and selective. The sensitivity of this method was determined by quantifying the limits of detection for all analyte–fluorophore combinations, and the results are shown in Table 3. The limits of detection are defined as the amount of analyte necessary to observe a signal that is distinguishable from the baseline (see Supporting Information for details). The limits of detection for compounds 2 and 5 are below the FDA-recommended concentration limits, thus providing a useful mechanism for the detection of these highly toxic analytes.

| Table 3. Limits of detection for all analytes with fluorophores 8-10 (all values given in parts per million (ppm)) |
|---|---|---|---|---|---|---|---|
| &nbsp; | 1  | 2  | 3  | 4  | 5  | 6  | 7  |
| 8   | &nbsp; | 5.9 | 104 | 83 | 32 | 12 | a  |
| 9   | a   | a   | 61  | 55 | 32 | a  | 9.8|
| 10  | a   | a   | 31  | 43 | b  | b  | b  |

*a Efforts to calculate limits of detection led to nonsensical values in these cases. Current efforts are focused on solving this problem. b Limits of detection were not calculated in these cases because no energy transfer was observed.*

Selectivity in the detection of toxic PAHs and PCBs can be accomplished using array-based detection. Such detection systems have also been referred to as
“chemical noses,” and have been used successfully by a number of research groups.\textsuperscript{21} Array-based detection generally requires exposure of an analyte to a receptor array. Statistical analyses of the resulting array of signals then lead to the selective detection of particular analytes.

Preliminary efforts towards developing an array-based detection system have yielded promising results. Using the three different fluorophores (compounds 8–10) in combination with 10 mM $\gamma$-cyclodextrin, each analyte (PAH or PCB) displayed qualitatively different fluorescence patterns when excited at 365 nm (Figure 5). Qualitatively different fluorescent responses were observed even in cases where the fluorophore emission ratios indicate some degree of fluorophore quenching from introduction of the analyte. The fact that each vertical column looks different means that each analyte has a different pattern of responses with the fluorophores investigated. Efforts to translate this qualitative observation into a quantitative, selective detection system are underway.

\textbf{Figure 5.} Photograph of a preliminary array using 10 mM $\gamma$-cyclodextrin (excitation at 365 nm with a hand-held TLC lamp).
CONCLUSION

In summary, reported herein is the development of highly efficient non-covalent energy transfer in γ-cyclodextrin cavities between toxic energy donors and fluorescent energy acceptors. This energy transfer has a number of advantages compared to previously-developed systems, including: (a) high sensitivity (as low as 5.9 ppm for compound 2); (b) ease of tunability; and (c) widespread applicability to two classes of highly toxic compounds. The development of a full array-based detection system, and a detailed investigation of the energy transfer mechanism, are underway and the results will be reported in due course.

This research was funded in part by a grant from the Gulf of Mexico Research Initiative (GOMRI).

Notes and References


Supporting Information

Efficient Detection of Polycyclic Aromatic Hydrocarbons and Polychlorinated Biphenyls via Three-Component Energy Transfer

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Chemical Company and used as received, unless otherwise noted. ¹H NMR spectra were obtained using a Bruker 300 MHz spectrometer. UV-Visible spectra were obtained using an Agilent 8453 spectrometer equipped with a photodiode array detector. Fluorescence spectra were obtained using a Shimadzu RF-5301PC spectrophotofluorimeter.

SYNTHESES OF FLUOROPHORES

The synthesis of BODIPY 9 was performed according to literature procedures.


Reaction 1:

Procedure: 2.0 grams of 11-bromoundecanoic acid S1 (7.54 mmol, 1.0 eq.) was combined with 2 drops of N,N-dimethylformamide in 40 mL of dichloromethane. 1.0
gram of oxalyl chloride $S_2$ (7.88 mmol, 1.05 eq.) was dissolved in 5.0 mL of dichloromethane and added dropwise. The reaction mixture was stirred for one hour, then the crude mixture was concentrated on the rotary evaporator and dried on a vacuum overnight to remove any unreacted oxalyl chloride. The resulting acid chloride $S_3$ was dissolved in 50 mL of dichloromethane. 0.772 mL of 2,4-dimethylpyrrole $S_4$ (7.50 mmol, 0.99 eq.) was dissolved in 5.0 mL of dichloromethane and added to the reaction mixture. The resulting reaction mixture was heated to reflux for 3 hours under a nitrogen atmosphere, during which time the mixture became a dark red color. After three hours, the reaction mixture was cooled to room temperature and solvent was removed on the rotary evaporator until approximately 5.0 mL of the dichloromethane solution remained. 200 mL of $n$-hexanes were added to the flask, and the mixture was cooled overnight in the freezer at -20 °C. The hexanes were decanted from the insoluble oil and precipitate. The resulting crude product was dissolved in 75 mL of toluene and heated to 80 °C. 1.0 mL of triethylamine (7.17 mmol, 0.95 eq.) was added and the solution immediately turned light yellow. 1.0 mL of boron trifluoride etherate (8.10 mmol, 1.07 eq.) was then added and the reaction mixture was stirred at 80 °C for 30 minutes, during which time the color of the mixture darkened and became fluorescent. The reaction mixture was cooled to room temperature, and the product was extracted 3 times with brine (50 mL each time). The organic layer was dried over sodium sulfate, filtered, and concentrated. The crude product was purified by flash chromatography (1:1 dichloromethane: hexanes) to yield the desired product in 28% yield (comparable to the literature-reported 24% yield).

**Reaction 2:**
Procedure: Compound S5 (0.968 g, 2.07 mmol, 1.0 eq.) and compound S6 (0.27 grams, 2.36 mmol, 1.14 eq.) were dissolved in 50 mL of acetone. The reaction mixture was heated to reflux for two hours. After two hours, the reaction mixture was cooled to room temperature, acetone was removed, and the crude solid was re-dissolved in dichloromethane and washed with water. The organic extract was dried over sodium sulfate, filtered and concentrated, to yield compound S7 in 97% yield (0.932 grams).

Reaction 3:

Procedure: Compound S7 (0.932 grams, 2.01 mmol, 1.0 eq.) was dissolved in 150 mL of anhydrous ethanol that was purged with nitrogen. Potassium carbonate was added, and the reaction mixture was warmed to 30 °C. The reaction mixture was stirred under nitrogen for 4 hours at 30 °C. The contents of the flask were poured over 40 mL of aqueous saturated ammonium chloride, at which point the solution turned bright orange. The product was extracted with dichloromethane and washed several times with water. The organic layer was dried over sodium sulfate, filtered, and concentrated. The product was purified via flash chromatography (1:1 dichloromethane: hexanes) to yield compound 9 in 76% yield (674 mg).

The synthesis of squaraine 10 was performed according to literature procedures:

**Reaction 1:**

![Chemical structure](attachment:image)

Procedure: Compound **S8** (0.912 g, 4.58 mmol, 1.0 eq.) and compound **S10** (2.16 g, 10.08 mmol, 2.2 eq.) were dissolved in 15 mL acetonitrile. Compound **S9** (1.42 mL, 9.62 mmol, 2.1 eq.) was added, and the reaction mixture was heated to reflux for 20 hours, at which time additional portions of compounds **S9** and **S10** were added and the reaction mixture was heated to reflux for another five hours. The reaction mixture was then cooled to room temperature. The solids were filtered and washed with ethyl acetate. The filtrate was then washed with brine, dried over magnesium sulfate, filtered and concentrated. Flash chromatography with 10% ethyl acetate in hexanes yielded compound **S11** in 72% yield (1.41 g). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 7.426-7.309 (m, 5 H), 7.105 (t, J = 8.4 Hz, 1 H), 6.39 (dd, J = 2 Hz, J = 8 Hz, 1 H), 6.249-6.202 (m, 2 H), 5.019 (s, 2 H), 3.989 (s, 4 H), 1.480 (s, 18 H).

**Reaction 2:**

![Chemical structure](attachment:image)
**Procedure:** Compound S11 (0.877 mmol, 1.0 eq., 375 mg) was dissolved in 37 mL of ethanol. 10% palladium on carbon (516 mg) was added, followed by cyclohexene (compound S12, 102 mmol, 116 eq., 10.32 mL). The reaction mixture was heated to reflux for two hours. The reaction mixture was then cooled to room temperature, and filtered through celite to remove the palladium. The filtrate was concentrated, and purified by flash chromatography (10% ethyl acetate in hexanes) to yield compound S13 (296 mg, quantitative yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 7.038$ (t, J = 8.4 Hz, 1 H), 6.237-6.141 (m, 2 H), 6.087 (t, J = 2.4 Hz, 1 H), 3.977 (s, 4 H), 1.438 (s, 18 H).

**Reaction 3:**

![Chemical structure](image)

**Procedure:** Compound S13 (0.877 mmol, 2.0 eq, 296 mg) was dissolved in 8 mL of benzene and 8 mL of $n$-butanol. Compound S14 (0.439 mmol, 1.0 eq, 50 mg) was added, and the reaction mixture was equipped with a Dean-Stark trap and condenser, and heated to reflux for 24 hours. After 24 hours, the reaction mixture was cooled to room temperature and concentrated to yield compound 10 directly. $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.07$ (d, J = 9.2 Hz, 0.1 H), 7.92 (d, J = 9.2 Hz, 0.9 H), 6.24 (d, J = 9.2 Hz, 2 H), 6.069 (s, 2 H), 3.970 (s, 8 H), 1.444 (s, 36 H). ESI-MS: 753.33 (m), 775.31 (m+Na$^+$). FTIR (KBr pellet, cm$^{-1}$): 3439 (m), 1738 (m), 1613 (s), 1383 (m), 1147 (s), 810 (m).

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CONTROL EXPERIMENTS

These experiments were designed to determine the emission of the fluorophores from excitation at various wavelengths (in the absence of the analyte) and compare it to the emission of fluorophores at the same wavelengths in the presence of the analyte. This will determine whether an observed “energy transfer” peak may simply be a result of exciting the fluorophore at a wavelength where it has non-zero absorbance. These experiments were conducted as follows:

(a) The fluorophore was mixed with $\gamma$-cyclodextrin and excited at the excitation wavelength of the analyte (but in the absence of any analyte); and

(b) the fluorophore and analyte were both mixed in $\gamma$-cyclodextrin and excited at analyte excitation wavelength.

The fluorophore emission that resulted from excitation at the analyte wavelength in the absence of the analyte was compared to the fluorophore emission from excitation at the analyte wavelength in the presence of the analyte. The ratio of these two emissions, shown as “ratio of fluorophore emissions” in the tables below, is defined as:

Fluorophore emission via low wavelength excitation in the absence of an analyte/fluorophore emission via low wavelength excitation in the presence of the analyte.

This was used to determine what fraction of that peak was a result of legitimate energy transfer rather than simple excitation of the fluorophore at a wavelength where it has non-zero absorbance.

All of these experiments were done with 1.5 nm excitation slit width and 1.5 nm emission slit width.
### S1a. Anthracene (1) – Rhodamine (8)

<table>
<thead>
<tr>
<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
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</tr>
<tr>
<td>2 mM</td>
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<tr>
<td>10 mM</td>
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### S1b. Anthracene (1) – BODIPY (9)

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### S1d. Pyrene (2) – BODIPY (9)

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### S1e. Benzo[a]pyrene (3) – Rhodamine (8)

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### S1h. Phenanthrene (4) – BODIPY (9)

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<tr>
<td>8 mM</td>
<td>1.09</td>
</tr>
<tr>
<td>9 mM</td>
<td>0.99</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.95</td>
</tr>
</tbody>
</table>
S1i. Fluorene (5) – Rhodamine (8)

<table>
<thead>
<tr>
<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>1.76</td>
</tr>
<tr>
<td>2 mM</td>
<td>1.65</td>
</tr>
<tr>
<td>3 mM</td>
<td>1.60</td>
</tr>
<tr>
<td>4 mM</td>
<td>1.72</td>
</tr>
<tr>
<td>5 mM</td>
<td>1.66</td>
</tr>
<tr>
<td>6 mM</td>
<td>1.41</td>
</tr>
<tr>
<td>7 mM</td>
<td>1.04</td>
</tr>
<tr>
<td>8 mM</td>
<td>1.57</td>
</tr>
<tr>
<td>9 mM</td>
<td>2.46</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.73</td>
</tr>
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</table>

S1j. Fluorene (5) – BODIPY (9)

<table>
<thead>
<tr>
<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>0.71</td>
</tr>
<tr>
<td>2 mM</td>
<td>0.73</td>
</tr>
<tr>
<td>3 mM</td>
<td>0.67</td>
</tr>
<tr>
<td>4 mM</td>
<td>0.71</td>
</tr>
<tr>
<td>5 mM</td>
<td>0.63</td>
</tr>
<tr>
<td>6 mM</td>
<td>0.63</td>
</tr>
<tr>
<td>7 mM</td>
<td>0.57</td>
</tr>
<tr>
<td>8 mM</td>
<td>0.61</td>
</tr>
<tr>
<td>9 mM</td>
<td>0.44</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.60</td>
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</table>

S1k. 4,4′-dichlorobiphenyl (6) – Rhodamine (8)

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<thead>
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<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>1.18</td>
</tr>
<tr>
<td>2 mM</td>
<td>1.07</td>
</tr>
<tr>
<td>3 mM</td>
<td>1.09</td>
</tr>
<tr>
<td>4 mM</td>
<td>1.14</td>
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<tr>
<td>5 mM</td>
<td>1.19</td>
</tr>
<tr>
<td>6 mM</td>
<td>1.02</td>
</tr>
<tr>
<td>7 mM</td>
<td>0.81</td>
</tr>
<tr>
<td>8 mM</td>
<td>1.14</td>
</tr>
<tr>
<td>9 mM</td>
<td>1.28</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.10</td>
</tr>
</tbody>
</table>

S1l. 4,4′-dichlorobiphenyl (6) – BODIPY (9)

<table>
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<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>1.10</td>
</tr>
<tr>
<td>2 mM</td>
<td>1.01</td>
</tr>
<tr>
<td>3 mM</td>
<td>1.04</td>
</tr>
<tr>
<td>4 mM</td>
<td>1.03</td>
</tr>
<tr>
<td>5 mM</td>
<td>1.05</td>
</tr>
<tr>
<td>6 mM</td>
<td>0.99</td>
</tr>
<tr>
<td>7 mM</td>
<td>1.02</td>
</tr>
<tr>
<td>8 mM</td>
<td>1.06</td>
</tr>
<tr>
<td>9 mM</td>
<td>0.98</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.05</td>
</tr>
</tbody>
</table>

S1m. PCB29 (7) – Rhodamine (8)

<table>
<thead>
<tr>
<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>1.13</td>
</tr>
<tr>
<td>2 mM</td>
<td>1.12</td>
</tr>
<tr>
<td>3 mM</td>
<td>1.11</td>
</tr>
<tr>
<td>4 mM</td>
<td>1.22</td>
</tr>
<tr>
<td>5 mM</td>
<td>1.19</td>
</tr>
<tr>
<td>6 mM</td>
<td>0.99</td>
</tr>
<tr>
<td>7 mM</td>
<td>1.02</td>
</tr>
<tr>
<td>8 mM</td>
<td>1.19</td>
</tr>
<tr>
<td>9 mM</td>
<td>1.32</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.11</td>
</tr>
</tbody>
</table>

S1n. PCB29 (7) – BODIPY (9)

<table>
<thead>
<tr>
<th>[γ-cyclodextrin]</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>1.13</td>
</tr>
<tr>
<td>2 mM</td>
<td>1.11</td>
</tr>
<tr>
<td>3 mM</td>
<td>1.05</td>
</tr>
<tr>
<td>4 mM</td>
<td>1.04</td>
</tr>
<tr>
<td>5 mM</td>
<td>1.10</td>
</tr>
<tr>
<td>6 mM</td>
<td>1.05</td>
</tr>
<tr>
<td>7 mM</td>
<td>1.01</td>
</tr>
<tr>
<td>8 mM</td>
<td>1.07</td>
</tr>
<tr>
<td>9 mM</td>
<td>1.09</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.05</td>
</tr>
</tbody>
</table>

**Tables S1a-S1n.** Ratios of fluorophore emission.
Based on these results, we can divide the analyte-fluorophore pairs into three categories:

(a) Fluorophore emission ratios close to 1. These indicate that there is no significant interaction between the analyte and the fluorophore, and that the fluorophore peak from excitation at the analyte wavelength is merely due to the fluorophore absorbance at that wavelength. Pairs that fall into this category:

Anthracene (1) – Rhodamine (8)
Anthracene (1) – BODIPY (9)
Phenanthrene (4) – BODIPY (9)
4,4’-dichlorobiphenyl (6) – Rhodamine (8)
4,4’-dichlorobiphenyl (6) – BODIPY (9)
PCB29 (7) – Rhodamine (8)
PCB29 (7) – BODIPY (9)

(b) Fluorophore emission ratios higher than 1. In these cases, the presence of the analyte leads to a decrease in the fluorophore emission, indicating that there is some interaction between the small molecules but that it does not result in energy transfer. Pairs that fall into this category:

Phenanthrene (4) – Rhodamine (8)
Fluorene (5) – Rhodamine (8)

(c) Fluorophore emission ratios less than 1. In these cases, energy transfer from the analyte to the fluorophore occurs, resulting in amplified fluorophore emission from analyte excitation.
Pyrene (2) – Rhodamine (8)
Pyrene (2) – BODIPY (9)
Benzo[a]pyrene (3) – Rhodamine (8)
Benzo[a]pyrene (3) – BODIPY (9)
Fluorene (5) – BODIPY (9)

DETAILS FOR ENERGY TRANSFER EXPERIMENTS

All energy transfer efficiencies were calculated using Equation 1:

\[
\% \text{ Efficiency} = (I_{DA}/I_D) \times 100\% \quad (1)
\]

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

All fluorescence emissions were integrated using Origin 8.5, and were integrated vs. wavenumber on the X-axis.

General procedure for energy transfer experiments:

\(\gamma\)-cyclodextrin hydrate (CAS: 91464-90-3) was obtained from Sigma-Aldrich, and dissolved in phosphate buffered saline (PBS) at pH 7.4 at a 10 mM concentration. Serial dilutions were then performed to yield solutions with 1, 2, 3, 4, 5, 6, 7, 8, and 9 mM \(\gamma\)-cyclodextrin in PBS.

All analytes were dissolved at a concentration of 1 mg/mL in tetrahydrofuran (THF): Anthracene, pyrene, benzo[a]pyrene, phenanthrene, fluorene, 4,4’-dichlorobiphenyl, PCB29, and PCB77.
Fluorophore solutions were made as follows:

Rhodamine 8: 0.1 mg/mL in THF

BODIPY 9: 0.1 mg/mL in THF

Squaraine 10: 1 mg/mL in THF

Note: Squaraine trials were predominantly performed on a different spectrometer: a Photon Technology International (PTI) instrument, with lamp model number LPS-220B. Slit widths for this fluorimeter were 2 nm excitation slit width and 2 nm emission slit widths. Detection was done at a right angle to the excitation. As a result of the different machine, a 1 mg/mL solution of squaraine was necessary to achieve a visible fluorescent signal.

2.5 mL of the cyclodextrin solution was transferred to a quartz cuvette, and 20 µL of the analyte solution was added via micropipette. The absorbance and fluorescence spectra of the solution were recorded. The fluorophore was then added sequentially in 20 µL increments (up to 100 µL), and the absorbance and fluorescence spectra were recorded after each addition. The final concentrations of each analyte and fluorophore are shown in the tables below:

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Amount added</th>
<th>Final analyte concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 µL</td>
<td>44.9 µM</td>
</tr>
<tr>
<td>2</td>
<td>20 µL</td>
<td>39.6 µM</td>
</tr>
<tr>
<td>3</td>
<td>20 µL</td>
<td>31.7 µM</td>
</tr>
<tr>
<td>4</td>
<td>20 µL</td>
<td>44.9 µM</td>
</tr>
<tr>
<td>5</td>
<td>20 µL</td>
<td>48.1 µM</td>
</tr>
<tr>
<td>6</td>
<td>20 µL</td>
<td>35.9 µM</td>
</tr>
<tr>
<td>7</td>
<td>20 µL</td>
<td>31.3 µM</td>
</tr>
</tbody>
</table>

Table S2. Final analyte concentrations.
<table>
<thead>
<tr>
<th>Compound number</th>
<th>Amount added</th>
<th>Final fluorophore concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>20 µL</td>
<td>1.7 µM</td>
</tr>
<tr>
<td></td>
<td>40 µL</td>
<td>3.3 µM</td>
</tr>
<tr>
<td></td>
<td>60 µL</td>
<td>5.0 µM</td>
</tr>
<tr>
<td></td>
<td>80 µL</td>
<td>6.7 µM</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>8.4 µM</td>
</tr>
<tr>
<td>9</td>
<td>20 µL</td>
<td>1.9 µM</td>
</tr>
<tr>
<td></td>
<td>40 µL</td>
<td>3.8 µM</td>
</tr>
<tr>
<td></td>
<td>60 µL</td>
<td>5.7 µM</td>
</tr>
<tr>
<td></td>
<td>80 µL</td>
<td>7.6 µM</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>9.5 µM</td>
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<tr>
<td>10</td>
<td>20 µL</td>
<td>10.6 µM</td>
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<tr>
<td></td>
<td>40 µL</td>
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<td>31.8 µM</td>
</tr>
<tr>
<td></td>
<td>80 µL</td>
<td>42.4 µM</td>
</tr>
<tr>
<td></td>
<td>100 µL</td>
<td>53.0 µM</td>
</tr>
</tbody>
</table>

**Table S3.** Final fluorophore concentrations.

For each combination, two fluorescence spectra were recorded: the fluorescence from excitation of the analyte (PAH or PCB) and the fluorescence spectra from excitation of the fluorophore. The excitation wavelengths were chosen to be as close as possible to the maximum wavelength of absorption, without significantly truncating the emission spectrum. Excitation wavelengths are recorded below:

- **Anthracene 1:** 360 nm excitation; emission spectrum recorded from 370 nm – 700 nm
- **Pyrene 2:** 360 nm excitation; emission spectrum recorded from 370 nm – 700 nm
- **Benzo[a]pyrene 3:** 360 nm excitation; emission spectrum recorded from 370 nm – 700 nm
- **Phenanthrene 4:** 290 nm excitation; emission spectrum recorded from 300 nm – 550 nm
- **Fluorene 5:** 270 nm excitation; emission spectrum recorded from 280 nm – 570 nm
4,4’-dichlorobiphenyl 6: 233 nm excitation; emission spectrum recorded from 243 nm – 600 nm
PCB29 7: 233 nm excitation; emission spectrum recorded from 243 nm – 600 nm
Rhodamine 8: 520 nm excitation; emission spectrum recorded from 530 nm – 800 nm
BODIPY 9: 460 nm excitation; emission spectrum recorded from 470 nm – 800 nm
Squaraine 10: 620 nm excitation; emission spectrum recorded from 630 nm – 800 nm

EXPERIMENTS WITH UNFUNCTIONALIZED BODIPY 11

The synthesis of BODIPY 11 was performed according to literature procedures.


Control experiments with BODIPY 11 (with 10 mM γ-cyclodextrin and different analytes):

In these experiments, BODIPY 11 was excited at 360 nm in the presence and absence of analyte. These results are quantified in Table S1, where the ratio of fluorophore emission is defined as:

Fluorophore emission via low wavelength excitation in the absence of an analyte/
fluorophore emission via low wavelength excitation in the presence of the analyte.
Values close to 1 indicate that the analyte does not affect the fluorophore emission, and that no energy transfer is occurring between the analyte and fluorophore.

Energy transfer percentage is defined as:

\[
\text{Efficiency} = \left( \frac{I_{DA}}{I_D} \right) \times 100\%
\]  
(1)

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

<table>
<thead>
<tr>
<th></th>
<th>Ratio of fluorophore emission</th>
<th>Energy transfer percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>anthracene (1)</td>
<td>1.02</td>
<td>70.7</td>
</tr>
<tr>
<td>benzo[a]pyrene (2)</td>
<td>0.17</td>
<td>397</td>
</tr>
<tr>
<td>4,4'-dichlorobiphenyl (6)</td>
<td>0.98</td>
<td>40.2</td>
</tr>
</tbody>
</table>

**Table S4:** Results using BODIPY 11 as a fluorophore in energy transfer schemes.

**SUMMARY FIGURES FOR BODIPY 11 WITH ANALYTES 1, 2, and 6:**

All experiments were done at a 1.5 nm excitation slit width and 1.5 nm emission slit width.

**Figure S1.** Control experiments exciting at 360 nm in the absence (black) and presence (red) of the analyte.

**Figure S2.** Energy transfer experiments with BODIPY 11 (with 10 mM \( \gamma \)-cyclodextrin and different analytes). Red line is the excitation of the analyte-BODIPY mixture at 460 nm. Black line is excitation of the analyte-BODIPY mixture at 360 nm.
CONCLUSION This BODIPY behaves like the thiol-functionalized BODIPY 9, indicating that the thiol functionality does not interfere with the fluorophore functionality. Like BODIPY 9, control experiments indicate no significant energy transfer for the anthracene analyte. Significant energy transfer was observed for benzo[a]pyrene. No significant energy transfer was observed for 4,4’-dichlorobiphenyl at these slit widths.

EXPERIMENTAL DETAILS FOR LIMIT OF DETECTION EXPERIMENTS

The limit of detection (LOD) is defined as the lowest concentration of analyte at which a signal can be detected. The limit of quantification is defined at the lowest concentration of analyte that can be accurately quantified.

To determine the limit of detection (LOD) and limit of quantification (LOQ), each fluorophore-analyte combination was examined in the following manner:

1. 2.5 mL of 10 mM γ-cyclodextrin in phosphate-buffered saline (PBS) was measured into a cuvette and 100 μL of a fluorophore solution in THF was added. The solution was excited at the analyte’s excitation wavelength and the fluorescence emission spectrum was recorded. Four repeat measurements were made for the fluorescence emission spectra.

**Figure S3.** Zoomed-in figures of energy transfer with BODIPY 11.
2. 20 μL of a 1 mg/mL analyte solution in THF was added to the cuvette and the solution was again excited at the analyte excitation wavelength. Four repeat measurements were taken.

3. Step 2 was repeated for 40 μL of analyte, 60 μL of analyte, 80 μL of analyte, and 100 μL of analyte. In each case, the solution was excited at the analyte excitation wavelength and the fluorescence emission spectrum was recorded four times.

4. All fluorescence emission spectra were integrated vs. wavenumber, and we generated calibration curves with the analyte concentration on the X-axis (in mM) and the integrated fluorophore emission on the Y-axis. The curve was then fitted to a straight line and an equation for the line was determined.

5. For each case, the fluorophore with γ-cyclodextrin (before any analyte was added) was also excited at the excitation wavelength for the analyte, and the fluorescence emission spectrum was recorded (as per step 1). These measurements are referred to as the “blank.”

6. The limit of the blank is defined according to the following equation:

\[ LoB_{LOD} = m_{blank} + 3(SD_{blank}) \]

Where m is the mean of the blank integrations and SD is the standard deviation.

7. The limit of the blank was then entered into the equation determined in step 4 (for the y value), and the corresponding X value was determined. This value provided the LOD in μM, which was converted into parts per million (ppm) to better compare with FDA and EPA recommended concentration limits.
8. The limit of quantification (LOQ) was calculated in a similar way to the limit of detection. First, the limit of the blank for quantification was determined according to the following equation:

\[ \text{LoB}_{\text{LOQ}} = m_{\text{blank}} + 10(SD_{\text{blank}}) \]

This value was entered into the equation determined in step 4 (for the y value), and the corresponding X value was determined to be the limit of quantification in mM. This LOQ was then converted into parts per million (ppm).

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Analyte</th>
<th>Equation</th>
<th>(R^2)</th>
<th>Limit of Detection (ppm)</th>
<th>Limit of Quantification (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1</td>
<td>(y = (4E6)x + (1.28E5))</td>
<td>0.968</td>
<td>(a)</td>
<td>8.11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(y = (2E7)x - (4.58E5))</td>
<td>0.9479</td>
<td>5.86</td>
<td>7.77</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(y = (-7E6)x + (3E6))</td>
<td>0.9212</td>
<td>103.77</td>
<td>96.95</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(y = (-2E6)x + (1E6))</td>
<td>0.6441</td>
<td>83.40</td>
<td>83.30</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>(y = (-2E7)x + (4E6))</td>
<td>0.8448</td>
<td>32.36</td>
<td>32.31</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(y = (5E6)x - (3.73E4))</td>
<td>0.9076</td>
<td>11.74</td>
<td>12.36</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>(y = (2E6)x + (1.61E5))</td>
<td>0.9498</td>
<td>(a)</td>
<td>(a)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(y = (1E7)x + (1.65E5))</td>
<td>0.9687</td>
<td>(a)</td>
<td>(a)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(y = (-4E6)x + (1E6))</td>
<td>0.9709</td>
<td>61.42</td>
<td>61.32</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(y = (-3E6)x + (1E6))</td>
<td>0.8962</td>
<td>55.25</td>
<td>54.51</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>(y = (-2E7)x + (4E6))</td>
<td>0.9059</td>
<td>32.11</td>
<td>31.83</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(y = (4E6)x + (8.90E5))</td>
<td>0.8142</td>
<td>(a)</td>
<td>(a)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>(y = (5.5E5)x + (6.11E4))</td>
<td>0.9548</td>
<td>9.80</td>
<td>12.90</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>(y = (2E6)x + (4.90E4))</td>
<td>0.9917</td>
<td>(a)</td>
<td>(a)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(y = (9E6)x + (3.56E5))</td>
<td>0.9152</td>
<td>(a)</td>
<td>(a)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>(y = (-8E6)x + (1E6))</td>
<td>0.869</td>
<td>31.09</td>
<td>31.08</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>(y = (-3E6)x + (7.89E5))</td>
<td>0.9093</td>
<td>42.73</td>
<td>42.64</td>
</tr>
</tbody>
</table>

**Table S5.** Summary Table for LOD experiments. *a* Attempts to calculate the LOD using these methods resulted in nonsensical values. Current efforts are focused on solving this problem.
S4a. Anthracene (1) – Rhodamine (8)

S4b. Anthracene (1) – BODIPY (9)

S4c. Anthracene (1) – Squaraine (10)

S4d. Pyrene (2) – Rhodamine (8)

S4e. Pyrene (2) – BODIPY (9)

S4f. Pyrene (2) – Squaraine (10)
S4g. Benzo[a]pyrene (3) – Rhodamine (8)

S4h. Benzo[a]pyrene (3) – BODIPY (9)

S4i. Benzo[a]pyrene (3) – Squaraine (10)

S4j. Phenanthrene (4) – Rhodamine (8)

S4k. Phenanthrene (4) – BODIPY (9)

S4l. Phenanthrene (4) – Squaraine (10)

S4m. Fluorene (5) – Rhodamine (8)
**S4n.** Fluorene (5) - BODIPY (9)

**S4p.** 4,4'-dichlorobiphenyl (6)-BODIPY (9)

**S4o.** 4,4'-dichlorobiphenyl (6) - Rhodamine (8)

**S4q.** PCB29 (7) - BODIPY (9)

**Figures S4a-S4q.** Summary graphs for all LOD experiments.
% ENERGY TRANSFER EFFICIENCES FOR ALL ANALYTE-FLUOROPHORE COMBINATIONS:

The highest energy transfer efficiencies are highlighted in bold in each table.

S6a. Anthracene (1) – Rhodamine (8):

<table>
<thead>
<tr>
<th>100 µL dye</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM γ-CD</td>
<td>8.6</td>
</tr>
<tr>
<td>2 mM γ-CD</td>
<td>8.6</td>
</tr>
<tr>
<td>3 mM γ-CD</td>
<td>8.7</td>
</tr>
<tr>
<td>4 mM γ-CD</td>
<td>8.9</td>
</tr>
<tr>
<td>5 mM γ-CD</td>
<td>8.9</td>
</tr>
<tr>
<td>6 mM γ-CD</td>
<td>9.3</td>
</tr>
<tr>
<td>7 mM γ-CD</td>
<td>9.3</td>
</tr>
<tr>
<td>8 mM γ-CD</td>
<td>9.7</td>
</tr>
<tr>
<td>9 mM γ-CD</td>
<td>9.3</td>
</tr>
<tr>
<td>10 mM γ-CD</td>
<td>9.1</td>
</tr>
</tbody>
</table>

S6b. Anthracene (1) – BODIPY (9):

<table>
<thead>
<tr>
<th>100 µL dye</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM γ-CD</td>
<td>45.5</td>
</tr>
<tr>
<td>2 mM γ-CD</td>
<td>57.4</td>
</tr>
<tr>
<td>3 mM γ-CD</td>
<td>46.8</td>
</tr>
<tr>
<td>4 mM γ-CD</td>
<td>42.5</td>
</tr>
<tr>
<td>5 mM γ-CD</td>
<td>46.0</td>
</tr>
<tr>
<td>6 mM γ-CD</td>
<td>71.6</td>
</tr>
<tr>
<td>7 mM γ-CD</td>
<td>59.5</td>
</tr>
<tr>
<td>8 mM γ-CD</td>
<td>37.0</td>
</tr>
<tr>
<td>9 mM γ-CD</td>
<td>45.4</td>
</tr>
<tr>
<td>10 mM γ-CD</td>
<td>34.1</td>
</tr>
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</table>


S6c. Pyrene (2) – Rhodamine (8):

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>0 mM γ-CD</td>
<td>3.4</td>
</tr>
<tr>
<td>1 mM γ-CD</td>
<td>3.5</td>
</tr>
<tr>
<td>2 mM γ-CD</td>
<td>4.9</td>
</tr>
<tr>
<td>3 mM γ-CD</td>
<td>5.8</td>
</tr>
<tr>
<td>4 mM γ-CD</td>
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<td>6 mM γ-CD</td>
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<td>8 mM γ-CD</td>
<td>5.5</td>
</tr>
<tr>
<td>9 mM γ-CD</td>
<td>5.3</td>
</tr>
<tr>
<td>10 mM γ-CD</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Pyrene (2) – BODIPY (9): No tables because the fluorophore emission overlaps significantly with the pyrene excimer emission (see composite figures)

**S6d.** Benzo\([a]\)pyrene (3) – Rhodamine (8):

<table>
<thead>
<tr>
<th>100 µL</th>
<th>1 mM γ-CD</th>
<th>2 mM γ-CD</th>
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<th>5 mM γ-CD</th>
<th>6 mM γ-CD</th>
<th>7 mM γ-CD</th>
<th>8 mM γ-CD</th>
<th>9 mM γ-CD</th>
<th>10 mM γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.4</td>
<td>5.4</td>
<td>7.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.5</td>
<td>9.0</td>
<td>8.8</td>
<td>9.6</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Benzo\([a]\)pyrene (3) – BODIPY (9): Excessive overlap between the benzo\([a]\)pyrene excimer emission and the BODIPY emission.

**S6e.** Benzo\([a]\)pyrene (3) – Squaraine (10):

<table>
<thead>
<tr>
<th>100 µL dye</th>
<th>1 mM γ-CD</th>
<th>2 mM γ-CD</th>
<th>3 mM γ-CD</th>
<th>4 mM γ-CD</th>
<th>5 mM γ-CD</th>
<th>6 mM γ-CD</th>
<th>7 mM γ-CD</th>
<th>8 mM γ-CD</th>
<th>9 mM γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.5</td>
<td>12.1</td>
<td>16.6</td>
<td>14.4</td>
<td>12.3</td>
<td>12.0</td>
<td>22.4</td>
<td>24.1</td>
<td>27.4</td>
</tr>
</tbody>
</table>

**S6f.** Phenanthrene (4) – Rhodamine (8):

<table>
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<tr>
<th>100 µL dye</th>
<th>0 mM γ-CD</th>
<th>1 mM γ-CD</th>
<th>2 mM γ-CD</th>
<th>3 mM γ-CD</th>
<th>4 mM γ-CD</th>
<th>5 mM γ-CD</th>
<th>6 mM γ-CD</th>
<th>7 mM γ-CD</th>
<th>8 mM γ-CD</th>
<th>9 mM γ-CD</th>
<th>10 mM γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.2</td>
<td>4.8</td>
<td>4.0</td>
<td>4.0</td>
<td>3.9</td>
<td>4.2</td>
<td>4.2</td>
<td>3.5</td>
<td>3.6</td>
<td>5.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>
S6g. Phenanthrene (4) – BODIPY (9):

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Energy Transfer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.7</td>
</tr>
<tr>
<td>2</td>
<td>17.2</td>
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<tr>
<td>3</td>
<td>13.8</td>
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<td>12.4</td>
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<td>5</td>
<td>10.1</td>
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<td>9.1</td>
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<td>7</td>
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<td>8.5</td>
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<tr>
<td>9</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Phenanthrene (4) – Squaraine (10): Preliminary experiments (1, 5, and 10 mM \(\gamma\)-cyclodextrin) indicate no energy transfer.

S6h. Fluorene (5) – Rhodamine (8):

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Energy Transfer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
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<tr>
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<td>3.5</td>
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<td>2.9</td>
</tr>
<tr>
<td>6</td>
<td>3.3</td>
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<tr>
<td>7</td>
<td>2.8</td>
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</tr>
<tr>
<td>9</td>
<td>10.1</td>
</tr>
<tr>
<td>10</td>
<td>3.2</td>
</tr>
</tbody>
</table>

S6i. Fluorene (5) – BODIPY (9):

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Energy Transfer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>6.1</td>
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<tr>
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<td>7.6</td>
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<tr>
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<td>7.9</td>
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</tr>
<tr>
<td>9</td>
<td>9.3</td>
</tr>
<tr>
<td>10</td>
<td>16.4</td>
</tr>
</tbody>
</table>

Fluorene (5) – Squaraine (10): Preliminary experiments (1, 5, and 10 mM \(\gamma\)-cyclodextrin) indicate no energy transfer.
**S6j. 4,4’-Dichlorobiphenyl (6) – Rhodamine (8):**

<table>
<thead>
<tr>
<th>100 µL dye</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM γ-CD</td>
<td>7.8</td>
</tr>
<tr>
<td>2 mM γ-CD</td>
<td>7.6</td>
</tr>
<tr>
<td>3 mM γ-CD</td>
<td>6.4</td>
</tr>
<tr>
<td>4 mM γ-CD</td>
<td>7.2</td>
</tr>
<tr>
<td>5 mM γ-CD</td>
<td>7.9</td>
</tr>
<tr>
<td>6 mM γ-CD</td>
<td>7.0</td>
</tr>
<tr>
<td>7 mM γ-CD</td>
<td>7.2</td>
</tr>
<tr>
<td>8 mM γ-CD</td>
<td>7.4</td>
</tr>
<tr>
<td>9 mM γ-CD</td>
<td>7.0</td>
</tr>
<tr>
<td>10 mM γ-CD</td>
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</tr>
</tbody>
</table>

**S6k. 4,4’-Dichlorobiphenyl (6) – BODIPY (9):**

<table>
<thead>
<tr>
<th>100 µL dye</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>1 mM γ-CD</td>
<td>7.8</td>
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<tr>
<td>2 mM γ-CD</td>
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<tr>
<td>3 mM γ-CD</td>
<td>8.1</td>
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<td>4 mM γ-CD</td>
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<td>8.6</td>
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<td>8.5</td>
</tr>
<tr>
<td>7 mM γ-CD</td>
<td>9</td>
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<tr>
<td>8 mM γ-CD</td>
<td>8.4</td>
</tr>
<tr>
<td>9 mM γ-CD</td>
<td>9.2</td>
</tr>
<tr>
<td>10 mM γ-CD</td>
<td>9.2</td>
</tr>
</tbody>
</table>

4,4’-Dichlorobiphenyl (6) – Squaraine (10): Preliminary results indicate no energy transfer is observed.

PCB 29 (7) – Rhodamine (8): Preliminary results indicate that no energy transfer is observed.

**S6l. PCB 29 (7) – BODIPY (9):**

<table>
<thead>
<tr>
<th>100 µL dye</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM γ-CD</td>
<td>7.3</td>
</tr>
<tr>
<td>2 mM γ-CD</td>
<td>6.9</td>
</tr>
<tr>
<td>3 mM γ-CD</td>
<td>7.0</td>
</tr>
<tr>
<td>4 mM γ-CD</td>
<td>6.6</td>
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<tr>
<td>5 mM γ-CD</td>
<td>7.4</td>
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<td>8 mM γ-CD</td>
<td>7.4</td>
</tr>
<tr>
<td>9 mM γ-CD</td>
<td>8.0</td>
</tr>
<tr>
<td>10 mM γ-CD</td>
<td>8.6</td>
</tr>
</tbody>
</table>
PCB 29 (7) – Squaraine (10): Preliminary results indicate no energy transfer is observed.

**Table S6a-S6l.** Energy transfer efficiencies for all combinations.

**SUMMARY DATA FOR HIGHER SLIT WIDTHS**

For a few cases where the control experiments showed fluorophore emission ratios near 1, we conducted additional control experiments with 3 nm excitation slit width and 3 nm emission slit widths, to ensure that the fluorophore emission was accurately detected, as at the higher slit width the full emission peak could be observed. The fluorophore emission ratios are shown in the table below, and 10 mM γ-cyclodextrin was used in each case.

<table>
<thead>
<tr>
<th>fluorophore</th>
<th>analyte</th>
<th>Ratio of fluorophore emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>6</td>
<td>1.99</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>1.89</td>
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<td>10</td>
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<td>0.91</td>
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<td>11</td>
<td>6</td>
<td>1.35</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>1.25</td>
</tr>
</tbody>
</table>

**Table S7.** Fluorophore emission ratios at higher slit widths.
CHAPTER 2
Accepted for Publication in Chemical Communications,

Array-Based Detection of Persistent Organic Pollutants via Cyclodextrin Promoted Energy Transfer

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†Department of Chemistry, University of Massachusetts Amherst, Amherst, MA, USA

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ABSTRACT

We report herein the selective array-based detection of 30 persistent organic pollutants via cyclodextrin-promoted energy transfer. The use of three fluorophores enabled the development of an array that classified 30 analytes with 100% accuracy and identified unknown analytes with 96% accuracy, as well as identifying 92% of analytes in urine.

INTRODUCTION

Many anthropogenic events, such as oil spills and chemical leaks, release a diverse suite of organic chemicals en masse into the environment. These persistent organic pollutants (POPs) remain in the environment for extended periods of time, and have significant environmental and health consequences both in the short- and long-term, to humans, animals, and plants living in disaster-affected areas. Widespread and long-term environmental consequences occur because of the persistent nature of organic pollutants in the environment, which enables many toxicants to affect areas beyond the immediate contamination site. Health consequences from pollution occur via the exposure of individuals to the complex mixture of released toxicants. Both the unknown consequences of individuals’ exposure to toxicant mixtures and the persistence and mobility of such toxicants and toxicant metabolites in the environment can make the effective monitoring and treatment of individuals living in disaster areas particularly difficult.
The ability to rapidly, sensitively, and selectively identify the compound(s) involved in an anthropogenic contamination event is crucial information for first responders. In the case of an oil spill, such as 1989’s Exxon Valdez and 2010’s Deepwater Horizon spills, the compounds involved in the contamination event included numerous polycyclic aromatic hydrocarbons (PAHs) and heterocyclic hydrocarbons. There are also contamination events in which the pollutant(s) are not initially known, including the Love Canal incident in 1978 (ultimately determined to involve a complex mixture of pesticides and organochlorines), and West Virginia’s Elk River chemical spill in 2014 involving 4-methylcyclohexylmethanol and a mixture of glycol ethers (PPH), in which the full extent of the spill and chemicals involved was not initially disclosed.

These four anthropogenic disasters highlight the need for a sensing platform that can detect a wide variety of POPs with sensitivity, selectivity, generality, and rapidity. Such a detection scheme would fill a crucial knowledge gap for first responders, who currently need to wait for time-consuming laboratory tests to accurately classify the nature of the pollutants. It would work in conjunction with current methods, by allowing first responders to screen numerous samples to rapidly understand the nature of the pollutants involved and the extent of the event so that they can begin an effective response. Previous research in our groups has demonstrated that cyclodextrin-promoted energy transfer can be used for the detection of a wide range of aromatic toxicants, and that array-based detection enables the sensitive, selective, and accurate
identification of a wide variety of analytes. We present herein the design, execution, and evaluation of an extremely accurate array-based detection system for aromatic POPs based on cyclodextrin-promoted energy transfer from the POPs to high quantum yield fluorophores.

$\gamma$-Cyclodextrin promoted energy transfer uses $\gamma$-cyclodextrin as a supramolecular scaffold that enforces close proximity between the aromatic analyte energy donor and high quantum yield fluorophore acceptor. Once bound in close proximity, excitation of the donor results in energy transfer to and emission from the fluorophore, generating a unique highly emissive fluorophore signal (Figure 1). Because each fluorophore-analyte combination yields a distinct signal, statistical analyses of the response patterns of multiple fluorophores in cyclodextrin to a single analyte identifies a unique “fingerprint” for each analyte of interest.

Figure 1. Illustration of $\gamma$-cyclodextrin promoted energy transfer, wherein the analyte acts as an energy donor to a high quantum yield fluorophore acceptor.

The thirty analytes targeted for this study were chosen to cover a wide range of compound classes (Chart 1) that are highly toxic and identified as hazardous by multiple monitoring agencies, including the Stockholm Convention, the
Environmental Protection Agency (EPA), and the International Agency for Research on Cancer (IARC). Three high quantum yield fluorophores were chosen as energy acceptors (31-33).

Chart 1. Structures of all analytes (1-30) and fluorophores (31-33) under investigation.

Analytes 1-14 are PAH and PAH metabolites, and have been found in the blood and breast milk of individuals living in polluted areas, with many of them known or suspected carcinogens. PCBs (15-18) cause neurotoxicity and endocrine disruption, and many of them are known or suspected carcinogens. Many aromatic pesticides (19-22) are suspected carcinogens, and others are designated as EPA Priority Pollutants. Compounds 23 and 24 are known carcinogens and endocrine disruptors, and compound 25 is a widely used additive with suspected endocrine disrupting effects. Brominated flame retardants (26 and 27) are a class of pollutants that has been investigated for
possible toxicity.\textsuperscript{18} Compound 28 is classified by the IARC as Group 1 carcinogen, has been linked to bladder and lung cancer,\textsuperscript{19} and is an EPA Priority Pollutant. Compound 29 is an amine derivative of biphenyl and has been linked to bladder cancer.\textsuperscript{20} Compound 30 was chosen for its structural similarity to 28, to assay the array’s ability to distinguish such structural variations.

For each analyte-fluorophore pair, the integrated emission of the fluorophore from excitation near the analyte’s absorption maximum was quantified and defined as the “fluorescence response.” These responses were then evaluated using linear discriminant analysis (LDA), a well-established statistical analysis tool for array-based detection systems (Figure 2).\textsuperscript{21}

![Figure 2. General illustration of LDA analysis to identify unknowns. By comparing the unique signals generated by unknowns and comparing them to known samples, LDA can correctly identify the analyte(s) present.](image)

LDA was successful in classifying all 30 analytes with 100\% accuracy via jackknifed classification analysis (JCA), which eliminates any potential bias in the array.\textsuperscript{22} The array was also 96\% successful in identifying unknown samples from the training set correctly (115/120 correct identifications). These results represent a substantially larger substrate scope than many literature-reported arrays,\textsuperscript{23} and a success rate in line with or better than literature reports of analogous systems.\textsuperscript{24}
The array was divided into two sections to more clearly analyze the relationships between the analytes: (1) PAHs and PAH metabolites; and (2) PCBs, endocrine disruptors, pesticides, biphenyls and flame retardants (Figure 3).

Figure S1 demonstrates that all but five of the PAHs are clustered together. The five outliers are compounds 5, 7, 9, 10, and 13; many of these are structurally related to benzo[a]pyrene and are highly fluorescent analytes (which leads to a stronger emission signal). Figure 3A shows the remaining PAHs, and highlights other key structural relationships: Anthracene 1 and two of its metabolites, compounds 2 and 3, cluster together in the array but generate well-separated signals. Fluorene 11 and three derivatives, 12, 13, and 14 also appear in the same region, but again demonstrate good separation. Similarly, carbazole 12 and partly saturated analogue 13 are close together but still well separated.

Figure 3B shows the LDA plot with biphenyl-type analytes. Structural relationships can clearly be seen, for example: chlorinated compounds with similar structures cluster together, including compounds 19 and 20, and compounds 15-18, although within each cluster each compound generates a unique signal; benzidine 28 and its derivative 30 are grouped together, although structurally related 29 is not; brominated compounds 21, 26, and 27 are closely related on the LDA plot; and bisphenol A 25 and its brominated derivative 26 appear in the same region on the LDA plot.
Overall, every one of the 30 analytes generates a unique signal on the LDA plot, with analytes with structural similarities grouped in a similar area. The array successfully identified 115 out of 120 cases of unknowns for a 96% accuracy. For those analytes that appear to have overlap in the Figure 3 plots, their successful differentiation occurs in the third score, along the Z-axis (details shown in the ESI). It is important to note that LDA identifies the axis of greatest differentiation. A low score for one of the axes does not directly translate into “small feature changes” dictating differentiation, but can instead be a reflection of particularly strong differentiation across other axes. For our studies the ellipsoids provide a better qualitative measure of the degree of differentiation.

Figure 3. LDA score plots of (A) PAHs; and (B) All biphenyl-type analytes.
This sensor platform uses γ-cyclodextrin as a supramolecular host that promotes proximity-induced non-covalent interactions between the POP of interest and a high quantum yield fluorophore. For most of the POPs, this interaction occurs via energy transfer, in which excitation of the analyte results in energy transfer to and emission from the fluorophore. However even weakly photoactive analytes (i.e. compounds 21, 22, and 27) modulate the fluorescence emission of the acceptor via proximity-induced fluorescence modulation, and these changes in fluorescence are sufficient to enable accurate array-based detection. In all cases, these proximity-induced interactions rely on a multitude of non-covalent interactions to bring the molecules in close proximity, including π-π stacking, Van der Waals forces, hydrophobic binding, and electrostatic interactions. These interactions guide the response of each analyte when paired with three fluorophores, and give rise to a distinct pattern that can be deciphered via LDA analysis (Figure 4).

**Figure 4.** Proximity-induced interactions between the analyte and fluorophore give rise to a new fluorescence signal via energy transfer or fluorescence modulation.

Two critical control experiments were performed. In the first experiment, an array was generated in the absence of any analyte, using γ-cyclodextrin and the three fluorophores. The blank samples excited at 300 nm and 360 nm were
correctly classified as blank samples, whereas samples excited at 250 nm and 400 nm were misclassified as PCBs or DDT, respectively. These results indicate that there is a relatively weak response between these chlorinated compounds and the sensor platform.

A second control experiment was performed where the array was generated without γ-cyclodextrin. Ten analytes (6, 8, 11, 14, 17, 18, 19, 20, 28, and 30) were used for this experiment and the results are reported in Table S11 of the Supporting Information. LDA was able to differentiate between the analytes with 53% accuracy via JCA, in stark contrast to the results achieved with a 10 mM γ-cyclodextrin (100% differentiation). Additionally, the scale of responses in this control array is vastly different, with benzo[a]pyrene showing much less differentiation from the other analytes in the absence of γ-cyclodextrin compared to its response in the presence of cyclodextrin. This experiment highlights the integral role the γ-cyclodextrin has in successfully differentiating between analytes, by acting as a supramolecular scaffold that enforces close proximity and the necessary intermolecular orientations to enable efficient POP-fluorophore interactions.

The potential utility of this array-based detection scheme was demonstrated through detection of POPs in a complex matrix, human urine. This array was generated in a 1:1 v/v mixture of urine and γ-cyclodextrin, and fifteen analytes were used (1, 2, 3, 5, 6, 7, 8, 12, 13, 16, 17, 18, 19, 20, 22). The array was able to successfully classify the analytes with 93% accuracy via JCA.
(Figure 5). Furthermore, the array was also able to correctly identify 55 out of 60 unknown analytes.

Notably, many of the general trends that were observed in the buffer array were also observed in urine. For example, benzo[a]pyrene 6, pyrene 5, 9,10-dihydrobenzo[a]pyrene-7,8H-one 8, and 7-methylbenzo[a]pyrene 7 are all well-separated from the other analytes and are plotted in the same general area in both arrays (compare to Figure 3A). Similarly, compounds 19 and 20 are also well separated from the other analytes and score in the same general region in both matrices. Lastly, the other structurally similar analytes cluster together: PCBs 16, 17, and 18; carbazole 12 and tetrahydrocarbazole 13; and compounds 1-3. The fact that similar trends can be seen in both matrices clearly indicates that the association that occurs between the γ-cyclodextrin host and guest molecules is specific for each analyte-fluorophore combination and occurs similarly in both matrices.

In conclusion, we have developed an array-based strategy to detect a wide variety of POPs in both simple (phosphate-buffered saline) and complex (urine) environments. This work has shown that individual analytes can be identified with exceptional accuracy, highlighting the ability of this detection scheme to provide specific information that will be useful for first responders. The success of this array relies on strong non-covalent interactions between a toxicant donor, fluorophore acceptor, and cyclodextrin host to achieve efficient proximity-induced energy transfer, and the cyclodextrin host is crucial to ensure association between the toxicant and fluorophore. This method is expected to be
generally applicable for multiple classes of aromatic analytes in a range of complex environments. Applications of this array-based sensor for POP detection in real-world matrices is currently underway, and results of these and other investigations in our laboratories will be reported in due course.

**Figure 5.** LDA score plots of analytes in a urine matrix.

**ACKNOWLEDGEMENTS**

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(CA185435), and in the Rotello group by the National Institutes of Health
(GM077173).

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    http://www.cancer.org/cancer/cancercauses/othercarcinogens/generalinformati
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about car

known-and-pro

able-human-car

ogens?
sitearea=PED


Supporting Information

Array-Based Detection of Persistent Organic Pollutants via Cyclodextrin Promoted Energy Transfer

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Chemical Company and used as received. Urine samples were provided by an anonymous donor and used without any pre-treatment. $^1$H NMR spectra were obtained using a Bruker 300 MHz spectrometer. Fluorescence spectra were obtained using a BioTek Synergy Mx Multi-Mode Microplate Reader at 25°C, with the following settings:

(a) Optics: Top
(b) Gain: 100
(c) Read height: 8 mm
(d) Read speed: Normal
(e) Measured data points at 10 nm increments

All spectra were integrated versus wavenumber on the X-axis using OriginPro software. The microplates used were black FLUOTRACTM 200, 96W Microplates, and were purchased from Greiner Bio-One. Array analysis was performed using SYSTAT 13 statistical computing software with the following settings:

(a) Classical Discriminant Analysis
(b) Grouping Variable: Analytes
(c) Predictors: Bodipy, Rhodamine 6G, Coumarin 6
(d) Long-Range Statistics: Mahal

ARRAY PROCEDURES

General Procedure – Sample Preparation

The following stock solutions were made:
10 mM γ-cyclodextrin in phosphate buffered saline (PBS) at pH 7.4

1 mg/mL of each analyte (1-30) in THF

0.1 mg/mL of each fluorophore (31-33) in THF

Two samples were prepared for each analyte-fluorophore combination: one served as the sample for the training set, and the other served as the unknown. For each sample, 2.5 mL of 10 mM γ-cyclodextrin, 100 μL of fluorophore solution, and 20 μL of analyte solution were added to a vial and vigorously shaken by hand for approximately 30 seconds. The sample remained on a rotary mixer until use to ensure thorough mixing. A 96 well microplate was divided as follows: (a) the first four rows were used for the training array and the remaining four rows were used for the unknowns; and (b) the columns were divided into three sections, one for each of the three dyes. Into each well was pipetted 100 μL of the sample solution, and each solution was repeated four times (i.e. each solution was pipetted into four separate wells) to ensure that the results obtained were reproducible.

General Procedure – Fluorescence Studies

A BioTek Synergy Mx Multi-Mode Microplate Reader was used to generate the fluorescence data for the array. Each analyte-fluorophore combination was excited at the analyte excitation wavelength (see table below) and the emission was recorded: (a) Fluorophore 31 samples: 470-620 nm; (b) Fluorophore 32 samples: 500-700 nm; (c) Fluorophore 33 samples: 450-700 nm. The fluorescence of the analyte was integrated with respect to wavenumber using OriginPro software.
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Table S1. Excitation wavelengths used for each analyte.
CLASSIFICATION ANALYSIS FOR BUFFER ARRAY

Figure S1. LDA score plot for all analytes.

Figure S2. LDA score plot for all biphenyl-like analytes.

Figure S3. LDA score plot for all PAH analytes.
Table S2. Jackknifed classification matrix.

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Table S3. Cumulative Proportion of Total Dispersion values.

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Table S5. All integration values used for the training set (“Array Integrations”) and unknowns (“Unknown Integrations”).
CONTROL EXPERIMENTS

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*General Procedure – Sample Preparation*

Two samples were prepared for each fluorophore: one served as the sample for the training set, and the other served as the unknown. For each sample, 2.5 mL of 10 mM $\gamma$-cyclodextrin and 100 $\mu$L of fluorophore were added to a vial and vigorously shaken by hand for approximately 30 seconds. The sample remained on a rotary mixer until use to ensure thorough mixing. A 96 well microplate was used, and into each well was pipetted 100 $\mu$L of the sample solution, and each solution was repeated four times (*i.e.* each solution was pipetted into four separate wells) to ensure data reproducibility.

*General Procedure – Fluorescence Studies*

A BioTek Synergy Mx Multi-Mode Microplate Reader was used to generate the fluorescence data for the array. The samples were excited at one of four excitation wavelengths: 250, 300, 360, and 400 nm. The emission of each was recorded as follows: (a) Fluorophore 31 samples: 470-620 nm; (b) Fluorophore 32 samples: 500-700 nm; (c) Fluorophore 33 samples: 450-700 nm. The fluorescence emission was integrated with respect to wavenumber using OriginPro software.

CONTROL 2: 0 mM $\gamma$-Cyclodextrin

*General Procedure – Sample Preparation*

Two samples were prepared for each analyte-fluorophore combination: one served as the sample for the training set, and the other served as the unknown. For each sample, 2.5 mL of 0 mM $\gamma$-cyclodextrin (pure PBS), 20 $\mu$L of analyte, and 100 $\mu$L of fluorophore were added to a vial and vigorously shaken by hand for approximately 30
seconds. The sample remained on a rotary mixer until use to ensure thorough mixing. A 96W microplate was used, and into each well was pipetted 100 μL of the sample solution, and each solution was repeated four times (i.e., each solution was pipetted into four separate wells) to ensure data reproducibility.

**General Procedure – Fluorescence Studies**

A BioTek Synergy Mx Multi-Mode Microplate Reader was used to generate the fluorescence data for the array. The samples were excited at the excitation of the analyte (see Table S1). The emission of each was recorded: (a) Fluorophore 31 samples: 470-620 nm; (b) Fluorophore 32 samples: 500-700 nm; (c) Fluorophore 33 samples: 450-700 nm. The fluorescence emission was integrated with respect to wavenumber using OriginPro software.

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**Table S6.** Integration values for the training set (“Knowns”) and unknowns.
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Table S8. Jackknifed classification matrix summary.
Table S9. Cumulative Proportion of Total Dispersion values.

Figure S4. LDA score plot for selected analytes (all analytes were used to generate the array; select analytes are shown here for more clarity).

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**Table S10.** Classifications of all analytes (“Analyte ID”), including classifications of unknowns (“Unknown Classification”)
CONTROL 2: 0 mM γ-Cyclodextrin

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Table S11. Jackknifed classification matrix for 0 mM γ-Cyclodextrin array.

Figure S5. LDA score plot for 0 mM γ-Cyclodextrin array.

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Table S12. Cumulative Proportion of Total Dispersion.

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<td>LDA Score values for an array generated in 0 mM γ-cyclodextrin.</td>
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**Table S13.** LDA Score values for an array generated in 0 mM γ-cyclodextrin.

**URINE EXPERIMENTS PROCEDURE**

**General Procedure – Sample Preparation**

Two samples were prepared for each fluorophore: one served as the sample for the training set, and the other served as the unknown. For each sample, 1.25 mL of 10 mM γ-cyclodextrin and 1.25 mL of urine were combined and mixed in a vial. Then, 100 μL
of fluorophore was added and vigorously shaken by hand for approximately 30 seconds. The sample remained on a rotary mixer until use to ensure thorough mixing. A 96 well microplate was used, and into each well was pipetted 100 μL of the sample solution, and each solution was repeated four times (i.e. each solution was pipetted into four separate wells) to ensure data reproducibility.

**General Procedure – Fluorescence Studies**

A BioTek Synergy Mx Multi-Mode Microplate Reader was used to generate the fluorescence data for the array. The samples were excited at the excitation wavelength of the analyte under investigation. The emission of each was recorded: (a) Fluorophore 31 samples: 470-620 nm; (b) Fluorophore 32 samples: 500-700 nm; (c) Fluorophore 33 samples: 450-700 nm. The fluorescence emission was integrated with respect to wavenumber using OriginPro software.

**URINE ARRAY INTEGRATIONS**

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Table S14. Integration data for all analytes tested in a 1:1 v/v matrix of urine and 10 mM γ-cyclodextrin.
**URINE ARRAY CLASSIFICATION ANALYSIS**

![LDA Score plot of 15 analytes tested in a 1:1 v/v matrix of urine and 10 mM γ-cyclodextrin.](image)

**Figure S6.** LDA Score plot of 15 analytes tested in a 1:1 v/v matrix of urine and 10 mM γ-cyclodextrin.

**Table S15.** Jackknifed classification matrix of 15 analytes tested in a 1:1 v/v matrix of urine and 10 mM γ-cyclodextrin.

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**Table S16.** Cumulative proportion of total dispersion values.

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Table S17. LDA Score values for each analyte (“Analyte ID”) and the unknown classification identities (“Unknown Classification”).

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CHAPTER 3
Published in ACS Applied Materials & Interfaces, 2013, 22, 11951-11957

Cyclodextrin-Enhanced Extraction and Energy Transfer of Carcinogens in Complex Oil Environments

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ABSTRACT

Reported herein is the use of γ-cyclodextrin for two tandem functions: (a) the extraction of carcinogenic polycyclic aromatic hydrocarbons (PAHs) from oil samples into aqueous solution, and (b) the promotion of highly efficient energy transfer from the newly extracted PAHs to a high-quantum-yield fluorophore. The extraction proceeded in moderate to good efficiencies, and the resulting cyclodextrin-promoted energy transfer led to a new, brightly fluorescent signal in aqueous solution. The resulting dual-function system (extraction followed by energy transfer) has significant relevance in the environmental detection and cleanup of oil-spill-related carcinogens.

INTRODUCTION

Significant oil spills in recent years¹ have highlighted a number of pressing medical²,³ and environmental⁴,⁵ problems associated with oil spill cleanup,⁶ post-incident monitoring of toxicants,⁷ and the prevention of future oil spills. Such problems include the long-term environmental persistence of highly toxic polycyclic aromatic hydrocarbons (PAHs) (including the known carcinogen benzo[a]pyrene),⁸,⁹ and the accumulation of PAHs at various points in the food chain.¹⁰-¹³

Methods for removing PAHs from the environment include (a) the biodegradation of PAHs into less toxic products,¹⁴ (b) the sequestration of PAHs by applying chemical reagents such as surfactants¹⁵ or cyclodextrins,¹⁶-¹⁸ (c) the segregation of PAHs from contaminated air using aerosol filters; and (d) the removal
of PAHs from contaminated solutions using molecularly imprinted polymers\textsuperscript{19} or covalently-immobilized anthracene sensors.\textsuperscript{20}

Once the PAHs have been separated from the environment, accurately identifying them usually requires multiple steps, including (a) isolating a mixture of toxicants from a crude environmental sample; (b) separating the small-molecule toxicants by chromatography; and (c) identifying the PAHs based on their molecular weights, fluorescence spectra, or chromatographic retention times.\textsuperscript{21} An efficient system that can both isolate PAHs from complex environments and accurately identify the PAHs has not yet been reported.

\(\gamma\)-cyclodextrin is a potential candidate for the tandem isolation and identification of PAHs. In addition to its well-established ability to bind PAHs,\textsuperscript{22-23} we recently reported that \(\gamma\)-cyclodextrin promotes highly efficient energy transfer from PAHs to a series of small-molecule fluorophores.\textsuperscript{24-26} This energy transfer occurs as a result of the enforced proximity of the donor and acceptor when bound simultaneously in the \(\gamma\)-cyclodextrin cavity\textsuperscript{27,28} and is efficient for a broad range of substrates in complex biological media. Thus, a scheme involving \(\gamma\)-cyclodextrin can simultaneously sequester PAHs from complex media and facilitate energy transfer to a fluorophore within the sample, thus providing key information: that the sample of interest contains potentially toxic PAHs and will require further analysis and decontamination.

Reported herein is the successful implementation of a \(\gamma\)-cyclodextrin-based system to accomplish these two key functions: (a) extracting PAHs from complex oils and binding them with moderate to good efficiencies; and (b) promoting non-covalent,
proximity-induced energy transfer from the isolated PAHs to a high quantum yield BODIPY fluorophore. The oils used in these investigations (vacuum pump oil, motor oil, vegetable oil, and cod liver oil) contain varying levels of PAH contaminants: from no known PAHs in cod liver oil,\textsuperscript{29,30} to small amounts of PAHs in several types of vegetable oil,\textsuperscript{31,32} and large quantities of PAHs in used motor oil.\textsuperscript{33} These ‘innate’ PAH amounts were detected by measuring the energy transfer efficiencies from ‘undoped’ oil samples to the fluorophore. Samples were separately ‘doped’ with small amounts of concentrated PAH solutions, which adds to the innate PAHs found in the oils and allows for a robust PAH-to-fluorophore energy transfer signal. In addition to investigating the ability of a buffered solution of $\gamma$-cyclodextrin to extract and bind toxic PAHs, we also investigated an “oil-spill-like scenario”: cyclodextrin was dissolved in Narragansett Bay seawater where it was still able to extract PAHs with moderate efficiencies from motor oil samples.

This system of extraction followed by energy transfer has a number of advantages compared to previously-reported methods for the detection of PAHs, including the ability to easily modulate the fluorescence signal generated from the energy transfer via judicious choice of fluorophore. Results reported herein used BODIPY-based fluorophore 6; however, a simple replacement of this fluorophore with other known structures will lead to a fluorescence emission signal at a different wavelength. The ability to use a variety of fluorophores with different emission maxima will allow for the facile development of an array-based detection system.\textsuperscript{34} In such a system, each analyte will interact differently with a set of fluorophores bound in cyclodextrin. Statistical analysis of the resulting response patterns will enable the
selective detection of highly toxic PAHs, which is an exciting application of the results reported herein. Overall, this dual-function system has significant potential applications for the isolation and detection of carcinogenic PAHs in complex, real-world environments.

EXPERIMENTAL PROCEDURES

Materials and methods: Four oils were analyzed: Crisco soybean oil, Fisherbrand 19 mechanical pump fluid oil, Pennzoil SAE-5W30 motor oil, and CVS Brand Cod Liver Oil. Compounds 1-5 were obtained from Sigma-Aldrich chemical company (Chart 1) and used as received, and compound 6 was synthesized following literature-reported procedures (Chart 1). Fluorescence measurements were recorded on a Shimadzu RF 5301 spectrophotometer (1.5 nm excitation slit width and 1.5 nm emission slit width). All spectra were integrated vs. wavenumber on the X-axis using OriginPro software.

PAH extraction experiments were conducted as follows: For vegetable oil, cod liver oil, and pump oil: 2.5 mL of the oil sample was mixed with 20 µL of a 1 mg/mL solution of each analyte (1-5) in tetrahydrofuran (THF). This oil mixture was then added to 2.5 mL of an aqueous solution: either 10 mM of γ-cyclodextrin in phosphate buffered saline (PBS) or 0 mM solution of γ-cyclodextrin in PBS (control). The oil and water mixture was vigorously shaken by hand (for approximately 1 minute) to allow thorough mixing, and the layers were separated by allowing the vial to sit undisturbed for 16-24 hours. The analyte in each layer was detected by fluorescence spectroscopy: excitation of the analyte near its absorption maximum (compounds 1-3; 360 nm excitation; compound 4: 270 nm excitation; compound 5:
290 nm excitation), followed by integration of the fluorescence emission spectrum of the analyte vs. wavenumber on the X-axis (using OriginPro software).

![Chemical structures of various compounds](image)

**Chart 1**: Polycyclic aromatic hydrocarbons (1-5) and fluorophores (6-7) investigated.

The comparison of the analyte in each layer was quantified according to Equation 1:

\[
\text{Analyte comparison} = \frac{I_{\text{aqueous}}}{I_{\text{oil}}} \quad (\text{Eq. 1})
\]

where \(I_{\text{aqueous}}\) = the integrated emission of the analyte in the aqueous layer, and \(I_{\text{oil}}\) = the integrated emission of the analyte in the oil layer.

Modification for motor oil experiments: Due to difficulties in achieving a full separation of the motor oil from the aqueous layer, the motor oil was first diluted with an equal amount of \(n\)-hexanes (1.25 mL of motor oil and 1.25 mL of \(n\)-hexanes). This diluted mixture was further mixed with 20 µL of the analyte solution in THF, followed by addition to 2.5 mL of the aqueous layer (either 10 mM or 0 mM of \(\gamma\)-cyclodextrin in PBS). Seawater-based experiments were conducted by mixing the motor oil/hexane mixture with cyclodextrin dissolved in Narragansett Bay seawater, followed by separation of the layers and analysis via fluorescence spectroscopy.

**Energy transfer experiments were conducted as follows**: 100 µL of compound 6 (0.1 mg/mL in THF), 20 µL of the analyte of interest (1.0 mg/mL in
THF), 2.5 mL of the oil of interest (cod liver oil, pump oil, or vegetable oil), and 2.5 mL of the aqueous solution (either 0 mM γ-cyclodextrin or 10 mM γ-cyclodextrin in PBS) were combined in a vial. The layers were shaken to allow thorough mixing, left undisturbed for 16-24 hours, and the aqueous and oil layers were then separated. Each layer was excited at both the excitation wavelength of the PAH (270 nm, 290 nm or 360 nm) and at the excitation wavelength of compound 6 (460 nm). The energy transfer efficiency is defined according to Equation 2:

\[
\text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\% \quad \text{(Eq 2)}
\]

Where \(I_{DA}\) is the integration of the fluorophore emission from analyte excitation and \(I_A\) is the integrated fluorophore emission from direct excitation. An illustration of such energy transfer for a generic donor-acceptor pair is shown in Figure 1.

![Figure 1](image.png)

**Figure 1.** Illustration of energy transfer efficiency for a generic donor-acceptor pair.

Energy transfer from the oil directly was also measured by omitting the analyte from the procedure detailed above. After the aqueous and oil layers were separated, the energy transfer in the oil layer was quantified by exciting the oil at the analyte excitation wavelengths (270 nm, 290 nm, and 360 nm) but in the absence of any analyte, and by exciting the mixture at the fluorophore’s excitation wavelength. The fluorophore emission via indirect excitation was compared to the fluorophore emission
via direct excitation to determine the energy transfer efficiencies.

Modification for motor oil experiments: The motor oil was diluted with an equal volume of \( n \)-hexanes (1.25 mL of each), followed by addition of the fluorophore, analyte, and aqueous solution (either PBS or seawater with 10 mM \( \gamma \)-cyclodextrin (or controls without \( \gamma \)-cyclodextrin)). All subsequent steps were conducted according to the procedure detailed above.

**Control experiments were conducted as follows:** Compound 6 was excited at the excitation wavelength of the analyte (270 nm, 290 nm, and 360 nm) in the absence of the analyte and in the presence of the analyte. A “control ratio” was defined according to Equation 3:

\[
\text{Control ratio} = \frac{I_{\text{fluorophore-analyte}}}{I_{\text{fluorophore-control}}} \tag{Eq 3}
\]

Where \( I_{\text{fluorophore-analyte}} \) is the integration of the fluorophore emission in the presence of the analyte; and \( I_{\text{fluorophore-control}} \) is the ratio of the fluorophore emission in the absence of the analyte. Ratios greater than 1.05 were taken to represent cases of legitimate energy transfer. Ratios close to 1 indicated that no significant energy transfer was occurring, and that the existence of a fluorophore peak via analyte excitation was merely a result of the fluorophore having a non-zero absorbance at that particular wavelength. These control ratios were measured in both the oil layer and aqueous layer (full results are reported in the ESI).

**RESULTS AND DISCUSSION**

The two functions of this cyclodextrin-based system (extraction and energy transfer) will be discussed individually:

1. *Extraction of PAHs using \( \gamma \)-cyclodextrin.* To measure the ability of \( \gamma \)-cyclodextrin to
extract PAHs from complex oils into an aqueous environment, oil samples were doped with small amounts of PAH analytes (compounds 1-5). The PAH-doped samples were then mixed with an equal volume of an aqueous solution (either 10 mM or 0 mM of γ-cyclodextrin), and the amount of analyte in the aqueous layer was quantified. The amount of analyte extracted with a 10 mM γ-cyclodextrin solution was compared to the amount extracted with a 0 mM γ-cyclodextrin control solution, and the result defined as an “Enhancement factor” (EF) according to Equation 4:

\[
EF = \frac{\text{Analyte comparison with 10 mM } \gamma\text{-cyclodextrin}}{\text{analyte comparison with 0 mM } \gamma\text{-cyclodextrin}} \quad \text{(Eq. 4)}
\]

The analyte comparisons were quantified by exciting the doped oil samples at the analyte’s excitation wavelength and integrating the analyte’s emission peaks, both in the presence and absence of cyclodextrin.

Table 1 highlights some significant differences in the ability of the 10 mM γ-cyclodextrin solutions to extract analytes from complex oils. These enhancement factors are based on a complicated interplay of factors, including: (a) the binding

<table>
<thead>
<tr>
<th>Compound #</th>
<th>E. F. in motor oil</th>
<th>E. F. in vegetable oil</th>
<th>E. F. in pump oil</th>
<th>E. F. in cod liver oil</th>
<th>E. F. in motor oil-seawater</th>
</tr>
</thead>
<tbody>
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<td>0.99 ± 0.53</td>
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<td>0.97 ± 0.03</td>
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<td>2</td>
<td>2.11 ± 0.36</td>
<td>0.89 ± 0.39</td>
<td>1.03 ± 0.59</td>
<td>1.11 ± 0.05</td>
<td>0.92 ± 0.07</td>
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<td>b</td>
<td>0.99 ± 0.17</td>
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<td>1.02 ± 0.34</td>
<td>1.15 ± 0.06</td>
<td>1.01 ± 0.06</td>
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</table>

\(a\) All data represents an average of at least five trials
\(b\) The low quantum yield of this analyte prevented accurate identification

Table 1 highlights some significant differences in the ability of the 10 mM γ-cyclodextrin solutions to extract analytes from complex oils. These enhancement factors are based on a complicated interplay of factors, including: (a) the binding
constant of the analytes in $\gamma$-cyclodextrin; (b) the physicochemical properties of the complex oils; and (c) the solubilities of the analytes in oil compared to water. These factors can also be explained by the partition coefficients for each of these analytes in oil and water; however, these coefficients are calculated in octane, which is a very simple oil compared to the more complex nature of the oils investigated in this work. The potential contributions of each of these factors are discussed in turn:

1a. $\gamma$-Cyclodextrin binding constants. Binding affinities of analytes 1-5 are shown in Table 2. The fact that all binding constants are similar (the largest value is only 1.3 times the smallest value) indicates that the differences in binding are unlikely to be responsible for the differential behavior of the analytes in the oil extraction experiments.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Literature-reported binding constants in $\gamma$-cyclodextrin</th>
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<td>1</td>
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<td>2</td>
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<td>3</td>
<td>294 M$^{-1}$</td>
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<td>258 M$^{-1}$</td>
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<tr>
<td>5</td>
<td>332 M$^{-1}$</td>
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</table>

$a$ The binding constant of benzo[a]pyrene in $\gamma$-cyclodextrin was not reported in the literature; attempts to calculate the binding constant directly using the Benesi-Hildebrand method were unsuccessful, likely due to a complex equilibrium between binary and ternary complexes.

1b. The identity of the oil. The extraction efficiencies varied greatly depending on the particular oil. For motor oil extractions with cyclodextrin-doped PBS, the 10 mM $\gamma$-cyclodextrin layer contained substantially more PAH analyte compared to the 0 mM $\gamma$-cyclodextrin control for all analytes. Two examples of the high enhancement factors in motor oil extraction experiments are shown in Figure 2, for analytes 1 (Figure 2A) and
Figure 2. A comparison of the amount of analyte extracted from motor oil with 10 mM γ-cyclodextrin in PBS and 0 mM γ-cyclodextrin in PBS for (A) compound 2; and (B) compound 5. The black line represents the analyte extracted with 10 mM γ-cyclodextrin and the grey line represents the analyte extracted with 0 mM γ-cyclodextrin.

For vacuum pump oil, vegetable oil, and cod liver oil, the enhancement factors for all analyte-oil combinations were much closer to 1, indicating limited contributions by γ-cyclodextrin to PAH extractions. These results contrast with a recent report that showed enhanced extraction efficiencies using hydroxypropyl-β-cyclodextrin to remove PAHs from contaminated soil. The difference between these reported results and the relatively modest efficiencies reported herein is likely a result of the increased binding affinities of the PAHs in hydroxypropyl-β-cyclodextrin compared to their more modest affinities in γ-cyclodextrin (Table 2).

Interestingly, the motor oil-seawater series demonstrated different behavior than the motor oil-PBS series, with lower enhancement factors for all seawater cases (and enhancement factors less than 1 for analytes 1-3). The fact that the enhancement factors for analytes 4 and 5 are greater than 1 is likely a result of their increased solubility in water compared to compounds 1-3. Reasons for this atypical behavior in motor oil-seawater extractions may be related to the particular properties of the
seawater, including the presence of surfactants and the high salt content.

(a) Surfactants: Sea water is known to contain high concentrations of surfactants. These surfactants can form micelles that bind the PAH donor and the BODIPY acceptor in the hydrophobic interior, thereby interfering with the ability of the cyclodextrin to form the necessary ternary complexes. In addition, surfactants contain a hydrophobic tail that can bind in the cyclodextrin cavity, forming an inclusion complex with the cyclodextrin that can hinder PAH binding.

(b) High salt concentration: The high salinity of sea water can also affect the ability of the cyclodextrin to form ternary complexes and promote energy transfer. This complex formation is largely driven by hydrophobic binding, which is known to depend heavily on salt concentration. Preliminary experiments using a phosphate buffer without saline (but under otherwise identical conditions) indicated that substantially more analyte was extracted into γ-cyclodextrin dissolved in phosphate buffer (saline-free) compared to γ-cyclodextrin dissolved in sea water (for example, the analyte comparison for pyrene is 0.34 in seawater compared to 0.75 in phosphate buffer). The high salinity of sea water is thus expected to lead to a further decrease in the hydrophobic binding necessary for cyclodextrin-promoted energy transfer.

1c. Solubility of analytes in oil and aqueous layers. The solubilities of PAHs 1-5 vary widely, with compounds 4 and 5 having markedly higher aqueous solubilities compared to compounds 1-3. This increased solubility had no measurable effect on the observed enhancement factors for most extraction series (motor oil, vegetable oil, pump oil, and cod liver oil). However, the seawater-motor oil extractions demonstrated greater enhancement factors for analytes 4 and 5 compared to analytes
1-3. These results demonstrate that the solubility of the analytes can facilitate the cyclodextrin-promoted extraction and binding.

2. Energy transfer from PAHs to fluorophore 6. The extraction of PAHs into the aqueous layer proceeded with moderate efficiencies in most cases. Even in cases of low extraction efficiencies, many of the analytes underwent efficient energy transfer to the highly fluorescent energy acceptor 6. The results are summarized in Table 3, and the results of energy transfer from a sample analyte (compound 2) to fluorophore 6 are shown in Figure 3.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Motor oil</th>
<th>Vegetable oil</th>
<th>Pump oil</th>
<th>Cod liver oil</th>
<th>Motor oil-seawater</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>71 ± 1%</td>
<td>32 ± 6%</td>
<td>35 ± 0.1%</td>
<td>31 ± 2%</td>
<td>72 ± 4%</td>
</tr>
<tr>
<td>2</td>
<td>72 ± 2%</td>
<td>29 ± 0.1%</td>
<td>34 ± 2%</td>
<td>32 ± 2%</td>
<td>72 ± 2%</td>
</tr>
<tr>
<td>3</td>
<td>71 ± 1%</td>
<td>33 ± 5%</td>
<td>35 ± 3%</td>
<td>33 ± 2%</td>
<td>69 ± 4%</td>
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<tr>
<td>4</td>
<td>45 ± 8%</td>
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<tr>
<td>5</td>
<td>18 ± 5%</td>
<td>b</td>
<td>31 ± 4%</td>
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<td>b</td>
</tr>
</tbody>
</table>

*a* All data represents an average of at least five trials

*b* No energy transfer was observed

**Figure 3.** Examples of energy transfer from analyte 2 to fluorophore 6 in 10 mM γ-cyclodextrin in PBS extracted from: (A) motor oil; (B) vegetable oil; (C) pump oil;
(D) cod liver oil; and (E) in 10 mM \( \gamma \)-cyclodextrin in seawater extracted from motor oil. The black line represents analyte excitation and the grey line represents direct fluorophore excitation.

The efficient detection of benzo[a]pyrene 2 is particularly important due to its high toxicity and known carcinogenicity.\textsuperscript{45,46} The results summarized in Figure 3 demonstrate that benzo[a]pyrene can participate efficiently in extraction and energy transfer across a broad range of complex oils. There are a number of other aspects of this energy transfer that merit discussion.

2a. Quantifying fluorophore partitioning. Energy transfer occurred in the aqueous layer despite the fact that the majority of compound 6 remained in the oil layer (motor oil: 87 \( \pm \) 3%; vegetable oil: 94 \( \pm \) 3%; pump oil: 93 \( \pm \) 6%; cod liver oil: 86 \( \pm \) 12%; motor oil-seawater: 87 \( \pm \) 3%). In all cases, the energy transfer in 10 mM \( \gamma \)-cyclodextrin was substantially more efficient compared to the energy transfer in the oil layer (see ESI for details), despite the limited amount of fluorophore in the aqueous environment.

2b. Energy transfer comparisons. Figure 4 shows comparisons of the emission spectra for analytes 1-5 that were extracted from motor oil, in the absence and presence of fluorophore 6. This figure highlights a key advantage of non-covalent energy transfer, which is the ability to achieve a bright fluorescent signal at a targeted wavelength. The direct fluorescence emission of analytes 1-5 in the extracted aqueous layer is relatively weak, because the majority of the analyte remained in the oil layer. Nonetheless, efficient energy transfer occurred for analytes 1, 2, 3, and 5 to fluorophore 6, resulting in a strong fluorescent signal at 522 nm. Moreover, the fluorescence emission maxima of analytes 1-5 occur in a spectral region that is likely to have significant interference
from other analytes. Effectively shifting the fluorescence emission signal to 522 nm provides a facile way to eliminate undesired spectral interference.

![Figure 4](image)

**Figure 4.** Comparison of the fluorescence emission spectra of analytes in 10 mM γ-cyclodextrin that was extracted from motor oil in the presence and absence of fluorophore 6. (A) Compound 1; (B) compound 2; (C) compound 3; (D) compound 4; and (E) compound 5. The black line shows the emission spectra in the absence of the fluorophore and the grey line shows the emission spectra in the presence of the fluorophore. [Note that the emission spectrum of Figure 4D has been digitally altered to remove the double harmonic peak at twice the excitation wavelength; a copy of the unaltered spectrum is shown in the Supporting Information].

2c. **Innate energy transfer from the oils.** In addition to measuring energy transfer efficiencies with analyte-doped samples, the direct energy transfer of the undoped oils to fluorophore 6 was measured. These experiments were conducted by adding the fluorophore to the oil-water mixture (in the absence of the analyte), followed by separating the layers. Energy transfer efficiencies were measured in the oil layers by exciting the oil at both the analyte excitation wavelength and at the fluorophore excitation wavelength.

The results of these experiments are summarized in Table 4, and indicate some degree of energy transfer for all oils investigated. This energy transfer was most
efficient for motor oil, vegetable oil, and pump oil (with 360 nm excitation), and least efficient for cod liver oil. This data is consistent with literature reports of some degree of PAH contamination in motor oil, vegetable oil, and pump oil, and no PAHs in cod liver oil,\textsuperscript{29-33} and supports the idea that PAHs in the actual oils participate in cyclodextrin-promoted energy transfer.

Table 4. Energy transfer efficiencies from the oil samples directly to fluorophore 6

<table>
<thead>
<tr>
<th>Excitation wavelength</th>
<th>Motor oil</th>
<th>Vegetable oil</th>
<th>Pump oil</th>
<th>Cod liver oil</th>
<th>Motor oil-seawater</th>
</tr>
</thead>
<tbody>
<tr>
<td>360 nm</td>
<td>20 ± 3%</td>
<td>18 ± 2%</td>
<td>21 ± 3%</td>
<td>7 ± 1%</td>
<td>18 ± 3%</td>
</tr>
<tr>
<td>270 nm</td>
<td>8 ± 3%</td>
<td>b</td>
<td>4 ± 1%</td>
<td>5 ± 1%</td>
<td>8 ± 2%</td>
</tr>
<tr>
<td>290 nm</td>
<td>9 ± 1%</td>
<td>b</td>
<td>5 ± 1%</td>
<td>5 ± 1%</td>
<td>8 ± 2%</td>
</tr>
</tbody>
</table>

\textit{a} All values represent an average of at least five trials
\textit{b} No energy transfer was observed

2d. Control experiments. To ensure that the fluorophore peak defined as energy transfer was a result of actual energy transfer from the analyte to the fluorophore (rather than a result of the fluorophore having a non-zero absorbance at the analyte excitation wavelength), the fluorophore was excited at the excitation wavelength of the analyte (270 nm, 290 nm, and 360 nm) in the presence of the analyte and in the absence of the analyte. The “control ratio” is defined in Equation 3 (above). The results of these experiments are summarized in Table 5.

Table 5. Control ratios for all analytes in the aqueous layer (10 mM $\gamma$-cyclodextrin)$^a$

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Motor oil</th>
<th>Vegetable oil</th>
<th>Pump oil</th>
<th>Cod liver oil</th>
<th>Motor oil-seawater</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.14 ± 0.11</td>
<td>0.95 ± 0.07</td>
<td>1.07 ± 0.07</td>
<td>1.03 ± 0.02</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>1.32 ± 0.58</td>
<td>1.04 ± 0.05</td>
<td>1.05 ± 0.06</td>
<td>1.08 ± 0.07</td>
<td>1.06 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>1.31 ± 0.58</td>
<td>1.80 ± 0.47</td>
<td>1.06 ± 0.03</td>
<td>0.98 ± 0.12</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>b</td>
<td>b</td>
<td>1.00 ± 0.41</td>
<td>1.05 ± 0.02</td>
<td>1.07 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>1.17 ± 0.17</td>
<td>0.99 ± 0.13</td>
<td>1.02 ± 0.02</td>
<td>1.05 ± 0.04</td>
<td>1.02 ± 0.07</td>
</tr>
</tbody>
</table>

\textit{a} All values represent an average of at least five trials
\textit{b} No energy transfer peak was observed
These ratios can be divided into three distinct categories: (1) Ratios between 0.95 and 1.05 indicate that the fluorophore emission from indirect excitation is fundamentally unchanged in the presence or absence of analyte, which indicates that no real energy transfer is occurring; (2) ratios greater than 1.05 indicate that legitimate energy transfer is occurring, because the fluorophore integration is markedly increased in the presence of the analyte; and (c) ratios less than 0.95 indicate that the addition of the analyte leads to fluorescence quenching.

Most of the aqueous extracts demonstrated legitimate energy transfer, especially for highly fluorescent (and toxic) analytes 2 and 3. Analytes 4 and 5 demonstrated less interaction with the fluorophore (as indicated by more control ratios between 0.95 and 1.05). This limited interaction is likely a result of the blue-shifted analytes (4 and 5) having less spectral overlap with fluorophore 6 and therefore reduced energy transfer efficiencies. Some degree of spectral overlap is generally understood to be a prerequisite for efficient donor-acceptor interactions and successful energy transfer.47

2e. Extension to other fluorophores. Another key advantage to non-covalent, proximity-induced energy transfer is its modular nature, which allows for the facile tuning of the fluorescence emission signal through judicious choice of fluorophore. Preliminary investigations towards that end focused on the use of commercially available Rhodamine 6G (compound 7, Chart 1). Three examples are shown in Figure 5, where energy transfer occurred from analytes 1, 2, and 3 to fluorophore 7 in 12% efficiency for each case.
Figure 5. Energy transfer from analytes to fluorophore 7 after extraction from vegetable oil. (A) Compound 1; (B) compound 2; and (C) compound 3. The black line represents analyte excitation and the grey line represents direct fluorophore excitation.

The ability to use multiple fluorophores as energy acceptors allows for the possibility of array-based detection based on such energy transfer. In such a system, the pattern of interactions of each analyte with an array of fluorophores in cyclodextrin will provide a unique identifier for each analyte. Exposure of the array to an unknown analyte, followed by statistical analysis and pattern matching, will lead to the accurate identification of the unknown. Analogous array-based detection systems have been used for a number of key applications.48,49

SUMMARY

In summary, these experiments report the use of γ-cyclodextrin for two sequential functions: extraction of carcinogenic analytes from a variety of commercially available oils to an aqueous solution, followed by energy transfer from the analytes to a high quantum yield BODIPY fluorophore. The extraction of analytes into the aqueous layer proceeded with moderate efficiencies, depending on the particular analyte and oil investigated. Even in cases where the extraction efficiency was only modest, good to excellent energy transfer was observed from the newly extracted analyte to fluorophore 6. This multi-step system of extraction followed by
efficient energy transfer can have significant applications in the development of turn-on detection systems for oil-spill related carcinogens. Efforts towards this goal are in progress, and results will be reported in due course.

**Funding Sources**

This research was funded by a grant from the Gulf of Mexico Research Initiative (GOMRI) and by a Proposal Development Grant from the URI Council for Research.

**Notes and References**


37. Fluorophore 6 was synthesized in the laboratory for a project on fluorescently-tagged polyamines; results with this fluorophore have been shown to be indistinguishable from an unfunctionalized BODIPY; see reference 24.


Supporting Information

Cyclodextrin-Enhanced Extraction and Energy Transfer of Carcinogens in Complex Oil Environments

Materials and Methods
Vacuum pump oil (Fisherbrand19 mechanical pump fluid) was obtained from Fisher Chemical Company. Crisco pure vegetable oil, Pennzoil motor oil, and CVS-brand cod liver oil were obtained from local retailers. Seawater was obtained from the Narragansett Bay in Rhode Island. All PAHs were obtained from Sigma-Aldrich chemical company (Chart 1). BODIPY fluorophore 6 was synthesized following literature-reported procedures. UV-Visible spectra were recorded on an Agilent 8453 spectrometer. Fluorescence measurements were recorded on a Shimadzu RF 5301 spectrophotometer with slit widths of 1.5 nm excitation and 1.5 nm emission slit widths. All fluorescence spectra were integrated vs. wavenumber on the X-axis, using OriginPro Version 8.6.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Final analyte concentration</th>
<th>Excitation wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.4 μM</td>
<td>360 nm</td>
</tr>
<tr>
<td>2</td>
<td>15.9 μM</td>
<td>360 nm</td>
</tr>
<tr>
<td>3</td>
<td>19.8 μM</td>
<td>360 nm</td>
</tr>
<tr>
<td>4</td>
<td>24.1 μM</td>
<td>270 nm</td>
</tr>
<tr>
<td>5</td>
<td>22.4 μM</td>
<td>290 nm</td>
</tr>
</tbody>
</table>

Table S1. Final analyte concentrations and excitation wavelengths.

Oil Extraction Experimental Details
Sample preparation: Samples for vegetable oil, vacuum pump oil, and cod liver oil were prepared as follows: 20 μL of PAHs 1-5 (1 mg/mL in THF) were added to 2.5 mL of oil in a vial. The contents were vigorously shaken by hand for approximately 1 minute. 2.5 mL of γ-cyclodextrin (10 mM in phosphate buffered saline (PBS)) was
added to the vial and the contents were once again shaken by hand for approximately 1 minute. The sample was allowed to sit undisturbed for 16-24 hours to ensure that the layers were fully separated. The aqueous layer was removed via pipette and placed in a new vial for analysis.

Samples for motor oil extraction experiments were prepared as follows: 1.25 mL of motor oil was added to 1.25 mL of \( n \)-hexanes. 20 μL of PAHs 1-5 (1 mg/mL in THF) were added to the oil-hexane mixture in a vial. The contents were vigorously shaken by hand for approximately 1 minute. 2.5 mL of \( \gamma \)-cyclodextrin (10 mM in aqueous solution (either PBS or Narragansett Bay seawater)) was added to the vial and the contents were once again shaken. The sample was allowed to sit undisturbed for 16-24 hours to ensure that the layers were fully separated. The aqueous layer was removed via pipette and placed in a new vial for analysis.

**Control sample preparation:** The same procedures were followed as above, but instead of using 10 mM \( \gamma \)-cyclodextrin, a PBS solution without \( \gamma \)-cyclodextrin, or a seawater solution without cyclodextrin, was added.

**Fluorescence analysis for samples and controls:** Each layer was excited at an excitation wavelength near the absorption maximum for the PAH analytes (360 nm for compounds 1-3; 270 nm for compound 4, 290 nm for compound 5). The fluorescence emission spectra of the PAHs were integrated with respect to wavenumber on the X-axis. Equation 1 was used to quantify how much analyte was extracted into the aqueous layer:

\[
\text{Analyte comparison} = \frac{I_{\text{aqueous}}}{I_{\text{oil}}} \quad \text{(Eq. 1)};
\]
Where \( I_{\text{aqueous}} \) = the integrated emission of the analyte in the aqueous layer, and \( I_{\text{oil}} \) = the integrated emission of the analyte in the oil layer.

All experiments were repeated at least twice, and the values reported are averages of the results.

**Energy Transfer Experimental Details**

After the extraction experiments were performed, the oil layer and aqueous layer were recombined in a vial. 100 µL of fluorophore 6 (0.1 mg/mL in THF; final concentration = 4.75 µM) was added to the oil-water mixture, and the contents of the vial were vigorously shaken by hand for approximately 1 minute to ensure thorough mixing. The layers were separated and each layer was excited at two different wavelengths: (a) the excitation wavelength of the PAH (see Table 1); and (b) 460 nm, which is the excitation wavelength necessary to excite fluorophore 6 directly.

The fluorophore emission was integrated with respect to wavenumber on the X-axis, and the energy transfer efficiencies were calculated as in Equation 2:

\[
\text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\% \tag{Eq 2}
\]

Where \( I_{DA} \) is the integration of the fluorophore from analyte excitation and \( I_A \) is the integrated fluorophore emission from direct excitation.

Energy transfer efficiencies from the oil itself (without doping with a PAH analyte) were also conducted by following the above procedures precisely, except for eliminating the analyte. Each oil layer was excited at the analyte’s excitation wavelength (but in the absence of the analyte) to determine the innate energy donor capabilities of the oil samples.
All experiments were repeated 5-6 times, and the values reported are averages of the results.

### Summary Tables of all Energy Transfer Experiments

The energy transfer from analytes 1-5 to fluorophore 6 was quantified according to Equation 2:

\[
\text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\% \quad (\text{Eq 2})
\]

Where \(I_{DA}\) is the integration of the fluorophore from analyte excitation and \(I_A\) is the integrated fluorophore emission from direct excitation.

Energy transfer was measured in both the aqueous layer and oil layer for all samples. Energy transfer was also measured from the oil to the fluorophore, without spiking the oil layer with a particular analyte (called “Energy transfer from the oil layer” in Table S3, below).

**Table S2**: Energy transfer in the aqueous layer (PBS solution in all cases except for the seawater trials)

<table>
<thead>
<tr>
<th>compound</th>
<th>motor oil</th>
<th>vegetable oil</th>
<th>pump oil</th>
<th>fish oil</th>
<th>seawater-motor oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
<td>10 mM γ-CD</td>
</tr>
<tr>
<td>1</td>
<td>70.82 ± 0.87</td>
<td>96.34 ± 1.11</td>
<td>32.35 ± 0.87</td>
<td>54.97 ± 13.05</td>
<td>31.20 ± 2.26</td>
</tr>
<tr>
<td>2</td>
<td>71.89 ± 1.76</td>
<td>84.15 ± 2.40</td>
<td>28.50 ± 0.14</td>
<td>61.97 ± 2.39</td>
<td>53.13 ± 10.52</td>
</tr>
<tr>
<td>3</td>
<td>71.18 ± 1.00</td>
<td>78.68 ± 13.27</td>
<td>33.32 ± 4.78</td>
<td>51.97 ± 11.89</td>
<td>51.15 ± 15.69</td>
</tr>
<tr>
<td>4</td>
<td>45.45 ± 8.16</td>
<td>no ET</td>
<td>no ET</td>
<td>no ET</td>
<td>12.45 ± 11.24</td>
</tr>
<tr>
<td>5</td>
<td>17.63 ± 4.08</td>
<td>no ET</td>
<td>no ET</td>
<td>60.77 ± 16.75</td>
<td>31.40 ± 5.34</td>
</tr>
</tbody>
</table>

* a – no energy transfer was observed.

**Table S3**: Energy transfer in the oil layer

<table>
<thead>
<tr>
<th>compound</th>
<th>motor oil</th>
<th>vegetable oil</th>
<th>pump oil</th>
<th>fish oil</th>
<th>seawater-motor oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
<td>10 mM γ-CD</td>
</tr>
<tr>
<td>1</td>
<td>14.57 ± 3.43</td>
<td>13.66 ± 2.31</td>
<td>18.66 ± 1.49</td>
<td>20.15 ± 0.31</td>
<td>19.88 ± 2.19</td>
</tr>
<tr>
<td>2</td>
<td>7.17 ± 3.52</td>
<td>9.15 ± 2.53</td>
<td>13.04 ± 1.93</td>
<td>14.11 ± 1.22</td>
<td>14.99 ± 0.96</td>
</tr>
<tr>
<td>3</td>
<td>12.99 ± 3.71</td>
<td>12.35 ± 2.53</td>
<td>17.67 ± 1.56</td>
<td>19.06 ± 0.75</td>
<td>21.43 ± 2.64</td>
</tr>
<tr>
<td>4</td>
<td>3.33 ± 1.97</td>
<td>no ET</td>
<td>no ET</td>
<td>3.65 ± 1.02</td>
<td>3.94 ± 0.91</td>
</tr>
<tr>
<td>5</td>
<td>51.38 ± 0.18</td>
<td>4.49 ± 1.49</td>
<td>2.81 ± 0.82</td>
<td>3.94 ± 0.96</td>
<td>4.23 ± 2.05</td>
</tr>
</tbody>
</table>

* a – no energy transfer was observed.

**Table S4**: Energy transfer from the oil layer

<table>
<thead>
<tr>
<th>compound</th>
<th>motor oil</th>
<th>vegetable oil</th>
<th>pump oil</th>
<th>fish oil</th>
<th>seawater-motor oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
<td>10 mM γ-CD</td>
</tr>
<tr>
<td>1</td>
<td>20.34 ± 3.18</td>
<td>13.68 ± 3.03</td>
<td>18.10 ± 1.59</td>
<td>20.07 ± 0.86</td>
<td>21.43 ± 3.49</td>
</tr>
<tr>
<td>2</td>
<td>20.37 ± 3.10</td>
<td>13.68 ± 3.03</td>
<td>18.10 ± 1.59</td>
<td>20.07 ± 0.86</td>
<td>21.43 ± 3.49</td>
</tr>
<tr>
<td>3</td>
<td>20.37 ± 3.10</td>
<td>13.68 ± 3.03</td>
<td>18.10 ± 1.59</td>
<td>20.07 ± 0.86</td>
<td>21.43 ± 3.49</td>
</tr>
<tr>
<td>4</td>
<td>8.11 ± 3.18</td>
<td>no ET</td>
<td>no ET</td>
<td>4.3 ± 0.9</td>
<td>3.36 ± 2.58</td>
</tr>
<tr>
<td>5</td>
<td>9.09 ± 1.12</td>
<td>4.25 ± 1.59</td>
<td>2.84 ± 0.89</td>
<td>5.0 ± 3.1</td>
<td>3.86 ± 2.99</td>
</tr>
</tbody>
</table>
Summary Tables for Control Experiments

Control experiments were also performed, wherein the fluorophore in each layer was excited at the excitation wavelength of the analyte in the presence and absence of the analyte. The “control ratio” is defined as the fluorophore emission via indirect excitation in the presence of the analyte to the fluorophore emission via indirect excitation in the absence of the analyte. This ratio provides a measure of whether the observed fluorophore peak is a result of legitimate energy transfer or merely a result of the fluorophore having a non-zero absorbance at the excitation wavelength of the analyte.

Control ratios between 0.95 and 1.05 are defined as “non-legitimate energy transfer,” meaning that the fluorophore peak is relatively equivalent in the presence and absence of the analyte.

Control ratios greater than 1.05 represent cases of legitimate energy transfer.

Control ratios less than 0.95 represent cases where the fluorophore emission was quenched in the presence of the analyte.

Control ratios were measured for both the aqueous and oil layers for each sample.

Table S5: Control ratios of aqueous samples

<table>
<thead>
<tr>
<th>compound</th>
<th>motor oil</th>
<th>vegetable oil</th>
<th>pump oil</th>
<th>fish oil</th>
<th>seawater-motor oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.14 ± 0.11</td>
<td>1.03 ± 0.12</td>
<td>0.98 ± 0.07</td>
<td>1.07 ± 0.03</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>1.32 ± 0.58</td>
<td>1.07 ± 0.06</td>
<td>1.04 ± 0.05</td>
<td>1.24 ± 0.45</td>
<td>1.05 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>1.31 ± 0.58</td>
<td>1.10 ± 0.08</td>
<td>1.80 ± 0.47</td>
<td>0.99 ± 0.08</td>
<td>1.06 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>1.00 ± 0.50</td>
<td>1.02 ± 0.45</td>
<td>1.05 ± 0.03</td>
<td>1.00 ± 0.52</td>
<td>1.06 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>1.17 ± 0.17</td>
<td>1.06 ± 0.04</td>
<td>0.99 ± 0.13</td>
<td>1.03 ± 0.03</td>
<td>1.02 ± 0.02</td>
</tr>
</tbody>
</table>

Table S6: Control ratios of oil samples

<table>
<thead>
<tr>
<th>compound</th>
<th>motor oil</th>
<th>vegetable oil</th>
<th>pump oil</th>
<th>fish oil</th>
<th>seawater-motor oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.63 ± 0.45</td>
<td>1.17 ± 0.07</td>
<td>0.90 ± 0.09</td>
<td>0.92 ± 0.11</td>
<td>1.11 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>1.09 ± 0.38</td>
<td>0.84 ± 0.15</td>
<td>0.81 ± 0.13</td>
<td>0.65 ± 0.12</td>
<td>0.80 ± 0.13</td>
</tr>
<tr>
<td>3</td>
<td>1.47 ± 0.50</td>
<td>1.02 ± 0.03</td>
<td>1.08 ± 0.09</td>
<td>1.11 ± 0.11</td>
<td>1.14 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>0.92 ± 0.23</td>
<td>0.76 ± 0.34</td>
<td>0.73 ± 0.40</td>
<td>1.03 ± 0.33</td>
<td>1.02 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.98 ± 0.05</td>
<td>1.54 ± 1.18</td>
<td>0.96 ± 0.39</td>
<td>1.02 ± 0.01</td>
<td>1.04 ± 0.03</td>
</tr>
</tbody>
</table>
Figure S1. Unaltered spectrum of Figure 4d
CHAPTER 4

Efficient Extraction and Detection of Aromatic Toxicants from Crude Oil and Tar Balls

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ABSTRACT

Herein we report the efficient extraction of aromatic analytes from crude oil and tar balls using multiple cyclodextrin derivatives. The known propensity of the cyclodextrins to bind hydrophobic guests in their hydrophobic interiors enhanced the extraction of aromatic analytes from the oil layer to the aqueous layer, with methyl-β-cyclodextrin and β-cyclodextrin providing the most significant enhancement in extraction efficiencies of aromatic toxicants (69% aromatic toxicants in aqueous layer in the presence of methyl-β-cyclodextrin compared to 47% in cyclodextrin-free solution for tar ball oil extraction), and provide optimal tunability for developing efficient extraction systems. The cyclodextrin derivatives also promoted efficient energy transfer in the aqueous solutions, with up to 86% efficient energy transfer observed in the presence of γ-cyclodextrin compared to 50% in the absence of cyclodextrin for oil spill oil extraction. Together, this dual function extraction followed by detection system has potential in the development of environmental remediation systems.

INTRODUCTION

Anthropogenic oil spills such as the Deepwater Horizon oil spill of 2010 highlight a number of unsolved problems in the areas of oil spill cleanup and remediation, efficient detection of oil-spill related toxicants in complex
environments, and the monitoring and understanding of long-term effects of oil spills on complex ecosystems. Current methods used for the cleanup of oil spills include skimming or booning of the oil, burning oil on the surface of the water, applying chemical dispersants to facilitate oil dispersion, and introducing oil-eating bacteria for environmental bioremediation. Many of these methods suffer from potentially serious drawbacks, including the environmental damage from oil burning, the unknown toxicity of many dispersants, and the long-term disruption to the ecosystem from the introduction of non-native oil-eating bacteria. In recognition of these problems, newer environmentally-friendly cleanup methods have been developed by several research groups, including the synthesis of new hydrophobic materials, including thermally reduced graphene, a sponge, and porous materials.

We have developed a new approach for the cleanup of oil spills in marine environments that focuses on the removal of aromatic toxicants such as polycyclic aromatic hydrocarbons (PAHs). The removal of PAHs is particularly important because many of these compounds are known carcinogens or pro-carcinogens, including the Class I carcinogen benzo[a]pyrene (Chart 1, compound 3). This approach uses commercially available, non-toxic γ-cyclodextrin to bind PAHs and extract them from complex oils. Following the extraction, the PAHs are detected using cyclodextrin-promoted energy transfer to a high quantum yield fluorophore (compound 4); analogous energy transfer has already been established as an efficient method for toxicant detection in multiple complex environments. Other research groups have also reported the use of cyclodextrin derivatives to extract PAHs from complex environments, including from contaminated soil and river sediments.
In practice, our approach uses cyclodextrin for the tandem extraction and detection of PAHs from contaminated samples by using the cyclodextrin as a filter. For example, a contaminated water sample would be passed through the cyclodextrin filter. The efficiency of PAH removal can then be monitored by taking random samples from the filtered water sample and monitoring its fluorescence, where decreasing fluorescence indicates successful PAH extraction. This could also be done on sediment samples in accordance with previously published preparation methods.\textsuperscript{23-25} A dual-function system such as this could greatly aid environmental clean-up efforts.

Previous research in our group focused on the use of $\gamma$-cyclodextrin for the extraction and detection of PAHs from motor oil, vegetable oil, and vacuum pump oil. Shortcomings of this method included the moderate extraction efficiencies observed using $\gamma$-cyclodextrin, as well as the use of commercially available oils rather than oils that had been collected from contaminated marine environments. Oil collected from oil spills (termed “oil spill oil”) is more complex than the commercially available oils previously investigated, with a broad distribution of alkanes, aromatic compounds, and insoluble polymeric components.\textsuperscript{26,27} These oils also contain many oxidized PAH derivatives as a result of the exposure of the oil to oxygen-rich environments.\textsuperscript{28} Some crude oil spontaneously forms tar balls, which are oil-containing spheres formed from both oil spills as well as from naturally occurring oil sources.\textsuperscript{29} The degradation and oxidation of toxicants in tar balls has been shown to differ from that of toxicants found in bulk oil samples.\textsuperscript{30}
Reported herein is the use of a wide variety of cyclodextrin derivatives (α-cyclodextrin, β-cyclodextrin, methyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin (2-HPCD), and γ-cyclodextrin) to extract and detect aromatic toxicants from motor oil, oil spill oil, and tar balls. The extraction and detection efficiencies depend both on the identity of the oil and on the cyclodextrin host. The aromatic small molecules extracted with cyclodextrin include highly toxic PAHs, polar oxidized PAH metabolites, and a variety of other toxicants that have been found in such complex matrices. The ability of cyclodextrin to extract multiple classes of toxicants simultaneously provides a significant operational advantage in the environmental remediation of polluted marine environments.

MATERIALS AND METHODS

Materials and Methods. Three oil samples were analyzed: Pennzoil SAE-5W30 motor oil, oil collected from an oil spill site (collected in Louisiana, April 2012), and tar ball oil (collected in Alabama, November 2013). Polycyclic aromatic hydrocarbons (PAHs) 1-3 were purchased from Sigma Aldrich Company and were used as received (Chart 1). These PAHs were intentionally doped into the complex oil samples for the ‘doped oil experiments’ to measure the ability of cyclodextrins to extract and detect doped PAHs. Highly fluorescent compound 4 was synthesized following literature-reported procedures, and was used in the energy transfer experiments as a high quantum yield energy acceptor. Spectra/Por® 2 Dialysis membranes (Flat Width 45 mm, MWCO 12-14 kD) were purchased from Fisher Scientific and rinsed in deionized water for 15 to 20 minutes, in accordance with the product instructions. Fluorescence measurements were recorded on a Shimadzu RF5301 spectrophotofluorimeter, with a
1.5 nm excitation slit width and a 1.5 nm emission slit width. All spectra were integrated versus wavenumber on the X-axis using OriginPro software, version 9.1.

Chart 1. Structures of the analytes (1-3) and fluorophore 4 under investigation

Preparing motor oil, tar ball oil, and oil spill oil for analysis. The motor oil was diluted with an equal volume of \( n \)-hexanes (1.25 mL of motor oil and 1.25 mL of \( n \)-hexanes). To prepare the oil spill oil, the oil was diluted in a 1:4 ratio with \( n \)-hexanes (0.625 mL of oil spill oil and 1.875 mL of \( n \)-hexanes). The tar balls were prepared by placing a tar ball (weighing \(~1.50\) g) in a mortar and pestle and breaking it up mechanically. Then, 5 mL of hexanes was added and the tar balls were mixed into the hexanes solution. The solution was then placed in a dialysis bag and placed in a beaker with approximately 400 mL of \( n \)-octane. The sample was allowed to dialyze for 3 days until the octane turned brown in color. After this time, the bag was removed and the resulting octane solution was centrifuged at 3000 rpm for 10 minutes. The brown solution was then decanted and stored as the tar ball extract solution. For each experiment, 2.5 mL of this stock solution was used.

PAH extraction techniques. 2.5 mL of each oil sample (motor oil, oil spill oil, tar ball extract) was mixed with 20 \( \mu \)L of a 1 mg/mL solution of each analyte (1-3) in tetrahydrofuran (THF), or with 20 \( \mu \)L of pure THF (undoped sample). The samples
were vigorously shaken by hand for 1 minute, and the oil mixtures were then added to
a 2.5 mL aqueous solution of either a 10 mM in phosphate buffered saline (PBS)
cyclodextrin derivative (α-cyclodextrin, β-cyclodextrin, methyl-β-cyclodextrin, 2-
hydroxypropyl-β-cyclodextrin (2-HPCD), and γ-cyclodextrin) or a 0 mM cyclodextrin
solution in PBS (control). The mixture was vigorously shaken by hand for 1 minute to
ensure thorough mixing. The layers were allowed to sit undisturbed for 16-24 hours.
The layers were separated and the analytes in each layer, both the doped analytes (1-3)
and the undoped samples, were detected by fluorescence spectroscopy with 360 nm
excitation. The analyte fluorescence emission spectrum was integrated versus
wavenumber on the X axis (using OriginPro 9.1 software). The amount of analyte in
each layer was quantified as an “analyte comparison” and calculated according to
Equation 1:

\[
\text{Analyte comparison} = \frac{I_{\text{aqueous}}}{I_{\text{aqueous}} + I_{\text{oil}}} \times 100\% \quad \text{(Eq. 1)}
\]

where \(I_{\text{aqueous}}\) is the integrated emission of the analyte in the aqueous layer and \(I_{\text{oil}}\) is
the integrated emission of the analyte in the oil layer.

**Energy transfer detection techniques.** To a 2.5 mL solution of oil was added 100 μL
of compound 4 (0.1 mg/mL in THF), 20 μL of the analyte of interest (1.0 mg/mL in
THF) or 20 μL of pure THF (“undoped”), and 2.5 mL of aqueous solution (10 mM or
0 mM cyclodextrin derivative solution in PBS). The layers were vigorously shaken in
a vial for 1 minute and the layers were allowed to separate for 16-24 hours. The layers
were separated and each layer was excited at two wavelengths: the analyte excitation
wavelength (360 nm) and the fluorophore excitation wavelength (460 nm). Each
fluorescence emission spectrum was integrated versus wavenumber on the X axis (using OriginPro 9.1 software). The efficiency of the energy transfer from the analytes to the fluorophore was calculated according to Equation 2:

\[
\text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\% \quad \text{(Eq. 2)}
\]

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

RESULTS AND DISCUSSION

PAHs found in oil collected from environmental oil spills have undergone substantial oxidation to a variety of highly polar, oxidized products, including quinones, phenols, and other oxidized species.\(^{33}\) Consistent with these reports, when the oil spill oil was mixed with an aqueous buffer solution (0 mM cyclodextrin), it demonstrated a high concentration of photophysically active compounds partitioning into the aqueous buffer solution (Figure 1B). Water soluble photophysically active compounds extracted from oils are likely to be oxidized PAH metabolites or other water soluble aromatic moieties, a hypothesis that is supported by ample literature precedent.\(^{34-36}\) In contrast, only a negligible concentration of photophysically active compounds partitioned from the motor oil into a cyclodextrin-free aqueous layer, reflecting the lower degree of polar fluorescent metabolites found in that oil (Figure 1A). The oil-water partitioning of tar balls was intermediate between the oil spill oil and the motor oil, with 46% of the overall fluorescence found in the aqueous buffer layer (Figure 1C). The differential behavior of tar balls compared to oil spill oil can be explained by the different composition of the tar balls – they are enriched in heavier
components, such as asphaltenes, that are insoluble in water.\textsuperscript{37,38} The PAHs found in the tar ball’s interior are also somewhat protected from oxidation due to their limited interaction with the oxygen-rich environment, whereas the PAHs in oil spill oil are more susceptible to oxidation.\textsuperscript{39}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Analyte comparisons in buffer-oil mixtures for (A) motor oil; (B) oil spill oil; and (C) tar ball oil. The black line represents fluorescence of the aqueous layer and the grey line represents fluorescence of the oil layer. All samples were excited at 360 nm.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{lccc}
\hline
\textbf{Cyclodextrin derivative} & \textbf{Motor oil} & \textbf{Oil spill oil} & \textbf{Tar ball oil} \\
\hline
PBS & 24.0\% & 67.2\% & 46.8\% \\
\(\alpha\)-cyclodextrin & 5.9\% & 59.4\% & 48.6\% \\
\(\beta\)-cyclodextrin & 10.3\% & 71.9\% & 44.6\% \\
Me-\(\beta\)-cyclodextrin & 4.7\% & 71.7\% & 69.3\% \\
2-HPCD & 33.6\% & 37.2\% & 65.2\% \\
\(\gamma\)-cyclodextrin & 33.4\% & 50.9\% & 53.7\% \\
\hline
\end{tabular}
\caption{Percentage of analyte found in the aqueous layer of oil-buffer solutions with a variety of cyclodextrin derivatives\textsuperscript{a}}
\end{table}

\textsuperscript{a} All analyte comparisons were calculated using Equation 1, with undoped oil samples. All reported results represent an average of at least 3 trials.

The addition of cyclodextrin to the aqueous solutions has the potential to alter this partitioning between the aqueous and oil layers, because cyclodextrins have been shown to bind PAHs and other aromatic analytes with high efficiencies.\textsuperscript{40,41} For the
motor oil-buffer solutions, the addition of γ-cyclodextrin and 2-HPCD led to a substantial increase in the amount of photophysically active compounds extracted into the aqueous layer (from 24.0% in PBS to 33.6% and 34% for 2-HPCD and γ-cyclodextrin respectively), which is consistent with the known ability of these cyclodextrins to bind PAHs. Other cyclodextrin derivatives, including β-cyclodextrin, methyl-β-cyclodextrin, and α-cyclodextrin, have cavity sizes that are too small to bind many PAHs, and their addition had no effect on the oil-water fluorescence ratios (Table 1).

For the oil spill oil-aqueous mixtures, the addition of both 2-HPCD and γ-cyclodextrin increased the fluorescence of both the oil layer and the aqueous layer. However, the fluorescence of the oil layer increased to a much greater extent (6.95-fold) compared to that of the aqueous layer (2.42-fold increase) (Figure 2), leading to an overall decrease in the percentage of fluorescently active compounds found in the aqueous layer. These fluorescence increases can be explained by the cyclodextrin binding a variety of PAHs and PAH metabolites in both the aqueous and oil phases; in each case, binding of the fluorescent small molecules leads to a noticeable fluorescence increase through the elimination of non-radiative decay pathways. \(^42\)

![Figure 2. Changes in the fluorescence in oil spill oil-buffer solutions with the addition of various cyclodextrins in the (A) aqueous layer; and (B) oil layer. The black line shows the fluorescence in a PBS-oil solution (no cyclodextrin), the blue line shows the](image)
fluorescence in a γ-cyclodextrin-oil solution, and the red line shows the fluorescence in a 2-HPCD-oil solution. These results are representative results of 3 independent trials.

For the tar ball oil-buffer mixtures, the addition of all cyclodextrin derivatives led to modest enhancements in the fluorescence ratios of the aqueous layer, with the exception of β-cyclodextrin which showed no change in the extraction efficiencies. These results indicate that the cyclodextrins are moderately effective in extracting photophysically active analytes from the crude tar ball solution. The larger cyclodextrins likely extract PAHs via hydrophobic encapsulation of the hydrophobic PAHs, analogous to what is observed with motor oil samples and what we reported in our previous publication. However, the addition of the smaller cyclodextrins also led to an increase in the percentage of fluorescence found in the aqueous layer, even though such cyclodextrins lack sufficient steric bulk to encapsulate PAHs in their hydrophobic cavities. These cyclodextrins are likely effecting fluorescence increases by binding polar PAH analytes via hydrogen bond formation; this hydrogen bonding allows analytes that are too large to bind in the cyclodextrin interior to associate with the cyclodextrins, thereby enabling enhanced extraction into the aqueous layer.

Following the efficient extraction of PAHs from a variety of complex oils using cyclodextrin derivatives, the ability of the newly extracted PAHs to participate in cyclodextrin-promoted energy transfer in the aqueous layer was assayed. This energy transfer requires that fluorophore 4 partition efficiently into the aqueous layer. The percentage of fluorophore emission in the aqueous layer was measured for all oil-cyclodextrin combinations, and found to be particularly efficient for methyl-β-cyclodextrin containing solutions (Figure 3). This high efficiency points to a high
degree of steric and electronic compatibility between methyl-β-cyclodextrin and fluorophore 4. Notably, some degree of fluorescence emission from fluorophore 4 was found in the aqueous layer for all oil-cyclodextrin combinations, indicating the potential for efficient energy transfer in all cases.

Figure 3. Fluorophore 4 emission in aqueous-oil mixtures for (A) motor oil; (B) oil spill oil; and (C) tar ball oil. The black line represents fluorescence of the aqueous layer without cyclodextrin and the grey line represents fluorescence of the aqueous layer with 10 mM of methyl-β-cyclodextrin. All samples were excited at 460 nm.

Energy transfer in the aqueous layer was measured for all cyclodextrin-oil combinations, and some key results are summarized in Tables 2-5.

| Table 2. Energy transfer efficiencies in the undoped aqueous extracts$^a$ |
|-----------------|-----------------|-----------------|-----------------|
| Cyclodextrin derivative | Motor oil | Oil spill oil | Tar ball oil |
| PBS              | $b$          | 50.0%          | 23.9%          |
| α-cyclodextrin   | 36.8%        | 51.8%          | 33.3%          |
| β-cyclodextrin   | 45.9%        | 29.5%          | 20.4%          |
| Me-β-cyclodextrin| 35.9%        | 24.1%          | 31.6%          |
| 2-HPCD           | 74.4%        | 85.7%          | 34.5%          |
| γ-cyclodextrin   | 73.0%        | 86.4%          | 28.5%          |

$^a$ All values represent an average of at least 3 trials

$^b$ No energy transfer peak was observed

| Table 3. Energy transfer efficiencies in the aqueous extracts doped with analyte 1$^a$ |
|-----------------|-----------------|-----------------|-----------------|
| Cyclodextrin derivative | Motor oil | Oil spill oil | Tar ball oil |
| PBS              | 9.0%          | 78.7%          | 24.8%          |

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α-cyclodextrin $b$ 30.2% 32.5%
β-cyclodextrin 46.2% 34.4% 23.3%
Me-β-cyclodextrin 38.7% 26.1% 29.5%
2-HPCD $b$ 80.1% 26.6%
γ-cyclodextrin 71.0% 77.2% 28.1%

*a* All values represent an average of at least 3 trials

*b* No energy transfer peak was observed

**Table 4.** Energy transfer efficiencies in the aqueous extracts doped with analyte 2$^a$

<table>
<thead>
<tr>
<th>Cyclodextrin derivative</th>
<th>Motor oil</th>
<th>Oil spill oil</th>
<th>Tar ball oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>80.5%</td>
<td>68.8%</td>
<td>26.2%</td>
</tr>
<tr>
<td>α-cyclodextrin</td>
<td>57.7%</td>
<td>28.3%</td>
<td>32.9%</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>49.2%</td>
<td>34.2%</td>
<td>23.6%</td>
</tr>
<tr>
<td>Me-β-cyclodextrin</td>
<td>38.1%</td>
<td>28.2%</td>
<td>29.7%</td>
</tr>
<tr>
<td>2-HPCD</td>
<td>85.4%</td>
<td>73.8%</td>
<td>27.1%</td>
</tr>
<tr>
<td>γ-cyclodextrin</td>
<td>71.0%</td>
<td>80.1%</td>
<td>29.3%</td>
</tr>
</tbody>
</table>

*a* All values represent an average of at least 3 trials

**Table 5.** Energy transfer efficiencies in the aqueous extracts doped with analyte 3$^a$

<table>
<thead>
<tr>
<th>Cyclodextrin derivative</th>
<th>Motor oil</th>
<th>Oil spill oil</th>
<th>Tar ball oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>54.6%</td>
<td>62.6%</td>
<td>29.7%</td>
</tr>
<tr>
<td>α-cyclodextrin</td>
<td>64.6%</td>
<td>38.1%</td>
<td>30.5%</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>68.3%</td>
<td>32.1%</td>
<td>24.7%</td>
</tr>
<tr>
<td>Me-β-cyclodextrin</td>
<td>38.7%</td>
<td>23.5%</td>
<td>31.6%</td>
</tr>
<tr>
<td>2-HPCD</td>
<td>85.6%</td>
<td>69.5%</td>
<td>28.6%</td>
</tr>
<tr>
<td>γ-cyclodextrin</td>
<td>72.0%</td>
<td>99.4%</td>
<td>30.5%</td>
</tr>
</tbody>
</table>

*a* All values represent an average of at least 3 trials

For oil spill oil, the observed energy transfer efficiency with undoped samples in the absence of any cyclodextrin was fairly high, and the addition of β-cyclodextrin and methyl-β-cyclodextrin led to decreases in the observed energy transfer efficiencies (energy transfer efficiencies of 30% and 24% for β-cyclodextrin and methyl-β-cyclodextrin, respectively, compared to 50% in the absence of any cyclodextrin) (Table 2). The addition of larger cyclodextrins (*i.e.* 2-HPCD and γ-cyclodextrin)
caused a substantial enhancement in the observed affinities. The large degree of cyclodextrin-free energy transfer is consistent with our previously reported results that showed cyclodextrin-free association in many complex environments. In these aqueous extracts, PAH metabolites likely associate with fluorophore 4 via a combination of hydrophobic binding (between the aromatic portions of the metabolites and the aromatic moieties of the fluorophore) and hydrogen bonding (between the hydroxyl and carbonyl moieties of the metabolites and the thiol and charged portions of the fluorophore); this close association is responsible for the observed cyclodextrin-free energy transfer.

For oil collected from tar balls, a modest energy transfer efficiency in the cyclodextrin-free solution was observed in undoped samples, and this efficiency was somewhat enhanced by the addition of most cyclodextrin derivatives by 8-10 percentage points (Table 2), with only β-cyclodextrin leading to a slight decrease in the energy transfer efficiencies. The most likely explanation for this scenario is that cyclodextrins facilitate the association of the aromatic toxicants with fluorophore 4. This facilitated association can either occur via the formation of a ternary complex in the cyclodextrin cavity (as has been demonstrated for γ-cyclodextrin20-22, 44,45 and 2-HPCD46,47), or via association of one of the two energy transfer partners outside the cyclodextrin cavity (a more likely scenario for the smaller cyclodextrin derivatives).

In aqueous extracts from motor oil, the degree of cyclodextrin-free energy transfer varied depending on the identity of the doped analyte, with analytes 2 and 3 demonstrating substantially higher degrees of cyclodextrin-free energy transfer compared to analyte 1. This is consistent with our previously reported results that
demonstrated that analytes with large hydrophobic surface areas are most likely to engage in cyclodextrin-free association and cyclodextrin-independent energy transfer.\textsuperscript{19} The energy transfer efficiencies were most improved by the addition of 2-HPCD and $\gamma$-cyclodextrin, with 73\% and 74\% efficiencies observed using $\gamma$-cyclodextrin and 2-HPCD, respectively. These results are consistent with the known ability of these cyclodextrins to form ternary complexes that promote proximity-induced energy transfer.\textsuperscript{48}

The results in Table 2 highlight the ability of cyclodextrin to remove aromatic toxicants from both oil spill oil and tar ball oil. These experiments, conducted without doping a particular PAH into the complex mixture, involve the cyclodextrins extracting a wide range of toxicants from the complex oils, including PAHs, PAH metabolites, and other aromatic moieties. Overall, the results reported herein highlight the potential of cyclodextrin derivatives to promote the efficient extraction of small-molecule toxicants from oil spills, as well as their subsequent detection via energy transfer to a high quantum yield fluorophore. This system has a number of notable advantages, including:

(1) In contrast to our previously reported results that demonstrated modest extraction efficiencies using $\gamma$-cyclodextrin to extract PAHs from motor oil, vegetable oil, and vacuum pump oil, we report herein substantially improved extraction efficiencies using a variety of cyclodextrin derivatives to extract aromatic toxicants from oil spill oil and tar ball oil, with up to 72\% of the aromatic toxicants found in the cyclodextrin-containing aqueous layer, compared to our previously reported best of 34\% aromatic analytes in $\gamma$-cyclodextrin-containing aqueous layer extracted from motor oil. Oil
collected directly from oil spill sites and oil isolated from tar balls have different physicochemical profiles compared to motor oil, vegetable oil, and vacuum pump oil, as a result of the weathering process that promotes substantial oxidation of the aromatic toxicants. Environmental remediation of oil spill oil and tar ball oil from polluted marine environments is substantially more relevant for environmental disaster efforts than the remediation of commercially available oils, and the results reported herein indicate that using a variety of cyclodextrin derivatives enables the efficient extraction of toxicants from these complex oils.

(2) The cyclodextrin-based extraction followed by detection system reported herein provides a rapid method to remove toxicants from oil spills and to confirm that photophysically active analytes were removed via fluorescence energy transfer, which is a useful tool in disaster response efforts. In many oil spill situations, the precise identification of each toxicants is less crucial than the ability to remove as many toxicants as possible as quickly as possible and confirm such removal. Using cyclodextrin derivatives to enhance the extraction of photophysically active compounds from the oil layer to the aqueous layer, as demonstrated herein, provides a practical method for such environmental detoxification, and monitoring the overall fluorescence of the extracted analytes provides a rapid method to assay the efficacy of such detoxification procedures.

CONCLUSION

In conclusion, the results reported herein demonstrate that cyclodextrin-based systems can be used for the efficient extraction and detection of aromatic toxicants from real-world oil samples collected at the sites of oil spills. The system uses a
number of commercially-available, non-toxic cyclodextrin derivatives to optimize extraction and detection procedures for each oil sample investigated, and demonstrate that our previously-reported results are generally applicable for the cleanup of oil-contaminated marine environments. These results also pointed to the potential of using multiple cyclodextrins simultaneously for the cleanup of a single oil system, with the cyclodextrins that are optimal for extraction of PAHs, binding of the fluorophore, and promotion of efficient energy transfer combined into a single high-performing, multi-cyclodextrin system. Research in this direction is currently underway in our group, and the results to date support this idea. The full results will be reported in due course.

**Funding Sources**

This research was supported by a grant from the Gulf of Mexico Research Initiative.

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Supporting Information

Efficient Extraction and Detection of Aromatic Toxicants

from Crude Oil and Tar Balls

MATERIALS AND METHODS

Three oil samples were analyzed: Pennzoil SAE-5W30 motor oil, oil collected from an oil spill site (collected in Louisiana, April 2012), and tar ball oil (collected in Alabama, November 2013). Compounds 1-3 were purchased from Sigma Aldrich Co. and used as received. Spectra/Por® 2 Dialysis membranes (Flat Width 45 mm, MWCO 12-14 kD) was purchased from Fisher Scientific and rinsed in deionized water for 15 to 20 minutes, following the product instructions. Fluorescence measurements were recorded on a Shimadzu RF5301 spectrophotofluorimeter, with a 1.5 nm excitation slit width and a 1.5 nm emission slit width. All spectra were integrated versus wavenumber on the X-axis.

EXPERIMENTAL DETAILS FOR OIL PREPARATION

The motor oil was first diluted with an equal volume of \(n\)-hexanes (1.25 mL of motor oil and 1.25 mL of \(n\)-hexanes). To prepare the oil spill oil, the oil was diluted in a 1:4 ratio with \(n\)-hexanes (0.625 mL of oil spill oil and 1.875 mL of \(n\)-hexanes). The tar balls were prepared by placing a tar ball in a mortar and pestle and breaking them up. Then, ~5 mL of hexanes was added and the tar balls were mixed once more. Next, the solution was placed in a dialysis bag and placed in a beaker with ~400 mL \(n\)-octane. The sample was allowed to dialyze for 3 days until the octane turned brown in color. After this time, the bag was removed and the resulting octane/tar ball solution was
centrifuged at 3000 rpm for 10 minutes. The brown solution was then decanted and stored as the tar ball extract solution. For each experiment performed, 2.5 mL of this stock solution was used.

EXPERIMENTAL DETAILS FOR PAH EXTRACTION EXPERIMENTS

For all experiments, 2.5 mL of the oil sample was mixed with 20 μL of a 1 mg/mL solution of each analyte (1-3) in tetrahydrofuran (THF) or 20 μL of THF (undoped sample). The samples were vigorously shaken by hand for 1 minute, and the oil mixtures were then added to a 2.5 mL aqueous solution of either a 10 mM phosphate buffered saline (PBS) cyclodextrin derivative (α-cyclodextrin, β-cyclodextrin, methyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, and γ-cyclodextrin) or a 0 mM cyclodextrin solution in PBS (control). The mixture was vigorously shaken by hand for 1 minute to ensure thorough mixing. The layers were allowed to separate overnight (16-24 hours). The layers were separated and the analytes in each layer were detected by fluorescence spectroscopy with 360 nm excitation. The analyte fluorescence emission spectrum was integrated versus wavenumber on the X axis (using OriginPro 9.1 software). The analyte comparisons of each layer were quantified according to the following equation:

\[
\text{Analyte comparison} = \frac{I_{\text{aqueous}}}{I_{\text{aqueous}} + I_{\text{oil}}} \times 100\% \\
\]

(Eq. 1)

where \(I_{\text{aqueous}}\) is the integrated emission of the analyte in the aqueous layer and \(I_{\text{oil}}\) is the integrated emission of the analyte in the oil layer.
EXPERIMENTAL DETAILS FOR ENERGY TRANSFER EXPERIMENTS

To a 2.5 mL solution of oil was added 100 µL of compound 4 (0.1 mg/mL in THF), 20 µL of the analyte of interest (1.0 mg/mL in THF) or 20 µL of THF (for the undoped sample) and 2.5 mL of aqueous solution (10 mM or 0 mM cyclodextrin derivative solution in PBS). The layers were vigorously shaken in a vial for 1 minute and the layers were allowed to separate for 16-24 hours. The layers were separated and each layer was excited at two wavelengths: the analyte excitation wavelength (360 nm) and the fluorophore excitation wavelength (460 nm). Each fluorescence emission spectrum was integrated versus wavenumber on the X axis (using OriginPro 9.1 software). The resulting energy transfer efficiency can be quantified according to the following equation:

\[
\text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\% \quad \text{(Eq. 2)}
\]

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

EXPERIMENTAL DETAILS FOR CONTROL EXPERIMENTS

Control experiments were conducted wherein the fluorophore was excited at the excitation wavelength of the analyte both in the absence and presence of the analyte. A control ratio is defined as in Equation 3:

\[
\text{Control ratio} = \frac{I_{\text{fluorophore-analyte}}}{I_{\text{fluorophore-control}}} \quad \text{(Eq. 3)}
\]

where \(I_{\text{fluorophore-analyte}}\) is the integration of the fluorophore emission in the presence of the analyte and \(I_{\text{fluorophore-control}}\) is the ratio of the fluorophore emission in the absence of the analyte.
SUMMARY TABLES

Analyte comparisons

All analyte comparisons were calculated according to Equation 1. The results represent an average of at least 3 trials.

### Tables S1a-S1c. Analyte comparisons from doped samples in different oils.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.0</td>
<td>6.7</td>
<td>13.3</td>
<td>5.6</td>
<td>29.8</td>
<td>24.0</td>
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<tr>
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<td>1.4</td>
<td>4.7</td>
<td>11.2</td>
<td>9.8</td>
<td>35.4</td>
<td>23.7</td>
</tr>
<tr>
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<td>5.9</td>
<td>14.3</td>
<td>7.0</td>
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<td>36.3</td>
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</tbody>
</table>

### Tables S1b. Oil spill oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.5</td>
<td>66.3</td>
<td>76.4</td>
<td>70.0</td>
<td>31.3</td>
<td>36.8</td>
</tr>
<tr>
<td>2</td>
<td>61.8</td>
<td>61.6</td>
<td>82.0</td>
<td>73.6</td>
<td>39.6</td>
<td>46.1</td>
</tr>
<tr>
<td>3</td>
<td>60.7</td>
<td>62.2</td>
<td>76.3</td>
<td>73.2</td>
<td>15.6</td>
<td>17.2</td>
</tr>
</tbody>
</table>

### Tables S1c. Tar ball oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>56.3</td>
<td>67.9</td>
<td>60.9</td>
<td>53.4</td>
</tr>
<tr>
<td>2</td>
<td>47.3</td>
<td>47.9</td>
<td>57.4</td>
<td>64.5</td>
<td>61.7</td>
<td>56.3</td>
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<td>57.4</td>
<td>71.5</td>
<td>67.2</td>
<td>59.4</td>
</tr>
</tbody>
</table>

### Table S2. Undoped analyte comparisons (from samples that had no added analyte).
Fluorophore comparisons

All fluorophore comparisons were calculated according to the following equation:

Fluorophore Comparison = $\frac{I_{\text{aq}}}{(I_{\text{aq}} + I_{\text{oil}})} \times 100$

where $I_{\text{aq}}$ is the integrated fluorescence emission of the fluorophore in the aqueous layer from 460 nm excitation, and $I_{\text{oil}}$ is the integrated fluorescence emission of the fluorophore in the oil layer from 460 nm excitation.

All results represent an average of at least 3 trials.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>3.3</td>
<td>16.6</td>
<td>7</td>
<td>7</td>
<td>8.9</td>
<td>5.1</td>
</tr>
</tbody>
</table>

S3a. Motor oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>70.4</td>
<td>68.6</td>
<td>85.8</td>
<td>92.8</td>
<td>8.1</td>
<td>6.8</td>
</tr>
</tbody>
</table>

S3b. Oil spill oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>23.2</td>
<td>17.9</td>
<td>35.7</td>
<td>29.7</td>
<td>29.8</td>
<td>25.7</td>
</tr>
</tbody>
</table>

S3c. Tar ball oil.

Tables S3a-S3c. Fluorophore comparisons in different oils.

Energy transfer in the aqueous layer

Energy transfer efficiencies in the aqueous extracts were quantified according to Equation 2. All results represent an average of at least 3 trials.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>a</td>
<td>50.6</td>
<td>46.2</td>
<td>38.7</td>
<td>a</td>
<td>71.0</td>
</tr>
<tr>
<td>2</td>
<td>80.5</td>
<td>57.7</td>
<td>49.2</td>
<td>38.1</td>
<td>85.4</td>
<td>71.0</td>
</tr>
<tr>
<td>3</td>
<td>54.6</td>
<td>64.6</td>
<td>68.3</td>
<td>38.7</td>
<td>85.6</td>
<td>72.0</td>
</tr>
</tbody>
</table>

a No energy transfer peak was observed

S4a. Motor oil.
Tables S4a-S4c. Aqueous layer energy transfer in doped oil samples.

### S4b. Oil spill oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPDCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>78.7</td>
<td>30.2</td>
<td>34.4</td>
<td>26.1</td>
<td>80.1</td>
<td>77.2</td>
</tr>
<tr>
<td>2</td>
<td>68.8</td>
<td>28.3</td>
<td>34.2</td>
<td>28.2</td>
<td>73.8</td>
<td>80.1</td>
</tr>
<tr>
<td>3</td>
<td>62.6</td>
<td>38.1</td>
<td>32.1</td>
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<td>69.5</td>
<td>99.4</td>
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</tbody>
</table>

### S4c. Tar ball oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPDCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.8</td>
<td>32.5</td>
<td>23.3</td>
<td>29.5</td>
<td>26.6</td>
<td>28.1</td>
</tr>
<tr>
<td>2</td>
<td>26.2</td>
<td>32.9</td>
<td>23.6</td>
<td>29.7</td>
<td>27.1</td>
<td>29.3</td>
</tr>
<tr>
<td>3</td>
<td>29.7</td>
<td>30.5</td>
<td>24.7</td>
<td>31.6</td>
<td>28.6</td>
<td>30.3</td>
</tr>
</tbody>
</table>

*α* No energy transfer peak was observed

Table S5. Energy transfer in the aqueous layer of undoped samples.

<table>
<thead>
<tr>
<th>Cyclodextrin derivative</th>
<th>Motor oil</th>
<th>Oil spill oil</th>
<th>Tar ball oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td><em>α</em></td>
<td>50.0</td>
<td>23.9</td>
</tr>
<tr>
<td>α-cyclodextrin</td>
<td>36.8</td>
<td>51.8</td>
<td>33.3</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>45.9</td>
<td>29.5</td>
<td>20.4</td>
</tr>
<tr>
<td>Me-β-cyclodextrin</td>
<td>35.9</td>
<td>24.1</td>
<td>31.6</td>
</tr>
<tr>
<td>2-HPCD</td>
<td>74.4</td>
<td>85.7</td>
<td>34.5</td>
</tr>
<tr>
<td>γ-cyclodextrin</td>
<td>73.0</td>
<td>86.4</td>
<td>28.5</td>
</tr>
</tbody>
</table>

Energy transfer in the oil layer

Energy transfer efficiencies in the oil extracts were quantified according to Equation 2.

All results represent an average of at least 3 trials.

### S6a. Motor oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPDCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.9</td>
<td>8.3</td>
<td>8.3</td>
<td>8.7</td>
<td>14.9</td>
<td>21.2</td>
</tr>
<tr>
<td>2</td>
<td>7.3</td>
<td>10.3</td>
<td>9.6</td>
<td>8.8</td>
<td>12.9</td>
<td>20.0</td>
</tr>
<tr>
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<td>5.1</td>
<td>9.0</td>
<td>7.9</td>
<td>7.3</td>
<td>12.2</td>
<td>10.2</td>
</tr>
</tbody>
</table>

### S6b. Oil spill oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPDCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>a</em></td>
<td><em>a</em></td>
<td><em>a</em></td>
<td><em>a</em></td>
<td>20.3</td>
<td>19.3</td>
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<tr>
<td>2</td>
<td><em>a</em></td>
<td><em>a</em></td>
<td><em>a</em></td>
<td><em>a</em></td>
<td>19.2</td>
<td>23.7</td>
</tr>
<tr>
<td>3</td>
<td><em>a</em></td>
<td><em>a</em></td>
<td><em>a</em></td>
<td><em>a</em></td>
<td>12.5</td>
<td>10.2</td>
</tr>
</tbody>
</table>
S6c. Tar ball oil.

**Tables S6a-S6c.** Oil layer energy transfer in doped oil samples.

<table>
<thead>
<tr>
<th>Cyclodextrin derivative</th>
<th>Motor oil</th>
<th>Oil spill oil</th>
<th>Tar ball oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>14.6</td>
<td><em>a</em></td>
<td>5.2</td>
</tr>
<tr>
<td>α-cyclodextrin</td>
<td>51.8</td>
<td><em>a</em></td>
<td>5.8</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>9.5</td>
<td><em>a</em></td>
<td>15.5</td>
</tr>
<tr>
<td>Me-β-cyclodextrin</td>
<td>8.6</td>
<td><em>a</em></td>
<td>6.5</td>
</tr>
<tr>
<td>2-HPCD</td>
<td>11.1</td>
<td>21.0</td>
<td>8.4</td>
</tr>
<tr>
<td>γ-cyclodextrin</td>
<td>14.2</td>
<td>20.8</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

**Table S7.** Energy transfer in the oil layer of undoped samples.

**Control ratios for aqueous extracts**

Control ratios for all aqueous extracts were calculated according to Equation 3. All results represent an average of at least 3 trials.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.80</td>
<td>2.20</td>
<td>1.40</td>
<td>1.50</td>
<td><em>a</em></td>
<td>0.96</td>
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<tr>
<td>2</td>
<td>1.27</td>
<td>2.40</td>
<td>1.60</td>
<td>1.30</td>
<td><em>a</em></td>
<td>2.51</td>
</tr>
<tr>
<td>3</td>
<td>1.17</td>
<td>2.00</td>
<td>1.30</td>
<td>1.20</td>
<td><em>a</em></td>
<td>2.50</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

**S8a.** Motor oil.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.06</td>
<td>0.59</td>
<td>1.17</td>
<td>0.98</td>
<td>1.06</td>
<td>0.96</td>
</tr>
<tr>
<td>2</td>
<td>0.94</td>
<td>0.74</td>
<td>0.59</td>
<td>1.02</td>
<td>0.94</td>
<td>0.96</td>
</tr>
<tr>
<td>3</td>
<td>1.01</td>
<td>0.64</td>
<td>0.95</td>
<td>0.93</td>
<td>0.97</td>
<td>0.97</td>
</tr>
</tbody>
</table>

**S8b.** Oil spill oil.

<table>
<thead>
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<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.06</td>
<td>0.93</td>
<td>1.06</td>
<td>0.88</td>
<td>0.70</td>
<td>0.96</td>
</tr>
<tr>
<td>2</td>
<td>1.09</td>
<td>0.97</td>
<td>1.00</td>
<td>0.91</td>
<td>0.73</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>1.16</td>
<td>0.83</td>
<td>0.85</td>
<td>0.89</td>
<td>0.72</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**S8c.** Tar ball oil.

**Tables S8a-S8c.** Control ratios for aqueous extracts in doped samples.
Control ratios for oil extracts

Control ratios for all oil extracts were calculated according to Equation 3. All results represent an average of at least 3 trials.

<table>
<thead>
<tr>
<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.03</td>
<td>0.70</td>
<td>1.10</td>
<td>0.90</td>
<td>a</td>
<td>0.87</td>
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<tr>
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<td>0.98</td>
<td>0.60</td>
<td>1.00</td>
<td>1.20</td>
<td>a</td>
<td>0.63</td>
</tr>
<tr>
<td>3</td>
<td>1.12</td>
<td>0.70</td>
<td>1.10</td>
<td>1.10</td>
<td>a</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

S9a. Motor oil.

<table>
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<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
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<td>a</td>
<td>a</td>
<td>a</td>
<td>1.04</td>
<td>0.59</td>
</tr>
<tr>
<td>2</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>0.54</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>1.16</td>
<td>0.73</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

S9b. Oil spill oil.

<table>
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<th>compound</th>
<th>PBS</th>
<th>α-CD</th>
<th>β-CD</th>
<th>Me-β-CD</th>
<th>2-HPCD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.99</td>
<td>1.16</td>
<td>1.35</td>
<td>1.29</td>
<td>0.94</td>
<td>0.99</td>
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<tr>
<td>2</td>
<td>1.01</td>
<td>1.18</td>
<td>2.98</td>
<td>1.23</td>
<td>0.94</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>1.07</td>
<td>1.25</td>
<td>1.27</td>
<td>1.16</td>
<td>1.03</td>
<td>1.08</td>
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</tbody>
</table>

S9c. Tar ball oil.

Tables S9a-S9c. Control ratios for oil extracts in doped samples.
CHAPTER 5
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Cyclodextrin-Promoted Energy Transfer for Broadly Applicable Small-Molecule Detection

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ABSTRACT

Reported herein is the development of non-covalent, proximity-induced energy transfer from small-molecule toxicants to organic fluorophores bound in the cavity of γ-cyclodextrin. This energy transfer occurs with exceptional efficiency for a broad range of toxicants in complex biological media, and is largely independent of the spectral overlap between the donor and acceptor. This generally applicable phenomenon has significant potential in the development of new turn-on detection schemes.

INTRODUCTION

The accurate detection of small-molecule organic toxicants in complex environments has significant implications for public health. Such toxicants are potentially significant contributors to human disease,\textsuperscript{1-3} and are found in food supplies,\textsuperscript{4-6} water supplies,\textsuperscript{7} and in commercial products.\textsuperscript{8} Current methods for the detection of these chemical toxicants generally require multiple steps: (a) extraction of the toxicants from the environment,\textsuperscript{9} (b) purification of the toxicants via high-performance liquid chromatography\textsuperscript{10} or gas chromatography,\textsuperscript{11} and (c) detection of the toxicants by mass spectrometry\textsuperscript{12} or fluorescence spectroscopy.\textsuperscript{13} Such detection methods are limited in their ability to distinguish toxicants with identical molecular weights or similar fluorescence spectra.
Small-molecule toxicants can also be detected through fluorescence energy transfer-based methods. Such fluorescence energy transfer, which has been used extensively for biomolecule detection, often requires significant spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor to achieve efficient energy transfer (i.e. a Förster-type mechanism). This overlap ultimately compromises the sensitivity of the system, as even in the absence of the target analyte there is residual donor emission. Efficient energy transfer that is independent of the spectral overlap (i.e. a Dexter-type mechanism) has the potential to lead to improved sensitivities in fluorescent detection schemes.

Figure 1. Schematic illustration of cyclodextrin-promoted energy transfer from organic toxicants to fluorophore acceptors.

Reported herein is a highly efficient, practical approach for small-molecule detection: using the small molecules directly as energy donors in a non-covalent, macrocycle-promoted energy transfer scheme. In such a scheme, both the toxicant and the fluorophore are bound in the interior of γ-cyclodextrin (Figure 1). The enforced proximity of the two molecules allows for non-covalent energy transfer to occur, with excitation of the toxicant (energy donor) resulting in energy transfer to and emission from the fluorophore (energy acceptor). The energy transfer is independent
of the spectral overlap between the donor and the acceptor, and has the potential to lead to improved sensitivities in turn-on detection schemes.

![Chemical structures](image)

**Figure 2.** Known and suspected toxicants investigated as energy donors.

We recently reported that cyclodextrin-promoted energy transfer occurred from polycyclic aromatic hydrocarbons (PAHs) (compounds 1-5, Figure 2) and polychlorinated biphenyls (PCBs) (compounds 14-19, Figure 2) to three fluorophores (two of which are shown in Figure 3).22-24 Proximity-induced energy transfer between the analytes and the fluorophores occurred in the cavity of γ-cyclodextrin, resulting in up to 35% energy transfer efficiencies.
Figure 3. Fluorophores investigated as energy acceptors.

Reported herein is a substantial expansion of this preliminary report to include (a) a wide range of small-molecule toxicants as energy donors (Figure 2);25 (b) energy transfer efficiencies as high as 100%; and (c) examples of successful energy transfer in complex media: coconut water, plasma,26 breast milk,27 and seawater. The general and highly efficient energy transfer reported herein highlights the robust nature of this phenomenon and the strength of the intermolecular interactions that allow for such energy transfer to occur.

RESULTS AND DISCUSSION

The full chart of examined energy donors is shown in Figure 2. This chart contains several compounds that have been classified as known carcinogens (Group 1) according to the International Agency for Research on Cancer (IARC) (compounds 3, 6-10),28 as well as a variety of other toxicants.29-32 These structures also contain a wide variety of functional groups, steric bulk, and photophysical properties, which allows us to probe the donor features necessary for efficient energy transfer.

Energy transfer experiments were conducted by mixing the analyte and fluorophore in a 10 mM γ-cyclodextrin solution in phosphate-buffered saline (PBS), coconut water, seawater, human plasma, or human breast milk. The resulting solution was excited near the analyte’s absorption maximum (defined as “analyte excitation”)

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and near the fluorophore’s absorption maximum (defined as “fluorophore excitation”). The energy transfer efficiencies were calculated according to Equation 1:

\[
\text{% Energy Transfer} = \frac{I_{DA}}{I_A} \times 100\%
\]  

(1)

where \(I_{DA}\) is defined as the integrated fluorophore emission from indirect excitation and \(I_A\) is the integrated fluorophore emission from direct excitation. A graphical depiction of \(I_{DA}\) and \(I_A\) is shown in Figure 4.

![Graphical depiction of \(I_{DA}/I_A\) for a generic donor-acceptor.](image)

**Figure 4.** Graphical illustration of \(I_{DA}/I_A\) for a generic donor-acceptor.

Control experiments were also conducted to determine whether the observed fluorophore peaks from analyte excitation were due to legitimate energy transfer rather than a result of the fluorophore having non-zero absorption at the excitation wavelength of the analyte. In these experiments, the fluorophore was mixed with cyclodextrin and excited at the excitation wavelength of the analyte (but in the absence of any analyte). That fluorophore emission was compared to the emission of the fluorophore via analyte excitation in the presence of the analyte. The ratio of these two emissions, defined as the “Fluorophore ratio” was calculated according to Equation 2:

\[
\text{Fluorophore ratio} = \frac{I_{\text{fluorophore-control}}}{I_{\text{fluorophore-analyte}}}
\]  

(2)

Where \(I_{\text{fluorophore-analyte}}\) is the integration of the fluorophore emission in the presence of the analyte; and \(I_{\text{fluorophore-control}}\) is the integration of the fluorophore emission in the
absence of the analyte. Fluorophore ratios substantially less than 1 indicate that the fluorophore emission increases with analyte addition as a result of energy transfer.

The final concentrations of the toxicants were somewhat higher than literature-reported concentrations of contaminated biological samples,\textsuperscript{33-35} although such literature reports vary widely depending on the toxicant identity, biological fluid, and sample population. Full results for all donor-acceptor combinations in all media are reported in the Supporting Information. Particularly exciting results were found using energy donors 7, 8, 11 and 12 with acceptor 20.

\textbf{Figure 5.} Energy transfer in PBS from (a) compound 7, (b) compound 8, (c) compound 11, and (d) compound 12 to fluorophore 20. The black line represents analyte excitation and the grey line represents direct fluorophore excitation.

\textit{In Phosphate-Buffered Saline (PBS):}

The energy transfer from analytes 7, 8, 11 and 12 to BODIPY 20 in 10 mM $\gamma$-cyclodextrin in PBS was exceptionally efficient, with greater than 100% efficiencies observed in all cases (Figure 5). Control experiments with 0 mM $\gamma$-cyclodextrin in PBS showed substantially less energy transfer than the 10 mM $\gamma$-cyclodextrin solution (Table 1), highlighting the beneficial role of $\gamma$-cyclodextrin in promoting energy
transfer.

**Table 1.** Selected energy transfer efficiencies in PBS.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>In 10 mM cyclodextrin (%)</th>
<th>In 0 mM cyclodextrin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>20</td>
<td>121</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>107</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>168</td>
<td>32</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>119</td>
<td>27</td>
</tr>
</tbody>
</table>

*In coconut water:*

The composition of coconut water is remarkably similar to that of human plasma, and it has been used as a plasma surrogate during emergencies. Analytes 7, 8, 11 and 12 demonstrated efficient energy transfer in 10 mM γ-cyclodextrin dissolved in coconut water (Table 2), albeit with diminished efficiencies compared to energy transfer in pure PBS.

**Table 2.** Selected energy transfer efficiencies in complex media

<table>
<thead>
<tr>
<th>Donor</th>
<th>In coconut water</th>
<th>In plasma</th>
<th>In breast milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM CD (%)</td>
<td>0 mM CD (%)</td>
<td>10 mM CD (%)</td>
</tr>
<tr>
<td>7</td>
<td>29</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>29</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>39</td>
<td>17</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>26</td>
<td>18</td>
<td>19</td>
</tr>
</tbody>
</table>

Note: CD, γ-cyclodextrin

*a*Fluorophore 20 used as the energy acceptor in all cases

*In biological media*

The ability to achieve cyclodextrin-promoted energy transfer in biological media can provide significant benefit for the detection of toxicants. Efficient energy
transfer from compounds 7, 8, 11 and 12 to fluorophore 20 occurred in both human plasma samples and human breast milk samples that were doped with 10 mM γ-cyclodextrin (Table 2).

*Energy transfer in seawater:*

The detection of toxic oil components in seawater has significant applications in the aftermath of environmental disasters such as the Deepwater Horizon oil spill of 2010\textsuperscript{38} and the Colorado floods of 2013.\textsuperscript{39} Such components include PAHs 1-5, which we have previously shown can participate in energy transfer in purified PBS solution.\textsuperscript{23} Cyclodextrin-promoted energy transfer using these donors occurred in seawater taken from Narragansett Bay (Rhode Island), with fluorophore 20 as an energy acceptor. All PAHs (1-5) exhibited some degree of energy transfer to fluorophore 20 (Figure 6) under these conditions.

**Figure 6.** Energy transfer in seawater to fluorophore 20 from (a) analyte 1, (b) analyte 2, (c) analyte 3, (d) analyte 4, and (e) analyte 5. The black line represents analyte excitation and the grey line represents direct fluorophore excitation.

For all complex fluids, the energy transfer efficiencies were somewhat lower than the efficiencies in pure PBS. These results are not surprising, considering the complex nature of coconut water,\textsuperscript{40} human plasma,\textsuperscript{41-44} and breast milk,\textsuperscript{45,46} and the
high salt content and complex nature of seawater.\textsuperscript{47,48} That $\gamma$-cyclodextrin-promoted energy transfer from carcinogens to the fluorophores occurred successfully in such complex environments highlights the robust nature of this detection method and the underlying enabling supramolecular interactions.

In contrast to the results obtained in PBS solution, where cyclodextrin clearly promotes efficient energy transfer, many of the analyte-fluorophore pairs in complex media demonstrate equivalent or even greater energy transfer efficiencies in the absence of $\gamma$-cyclodextrin compared to the efficiencies in the presence of cyclodextrin. These results are likely due to two possible phenomena:

(a) For cases where the energy transfer efficiencies are roughly equivalent in the presence and absence of cyclodextrin, it is likely that the donor and acceptor associate without cyclodextrin due to the hydrophobic effect.\textsuperscript{49} This association leads to energy transfer efficiencies that are essentially identical regardless of the cyclodextrin concentration. Previous research in our laboratory has shown some degree of cyclodextrin-free association as well.\textsuperscript{23}

(b) For cases where the energy transfer efficiencies are lower in the presence of cyclodextrin, the cyclodextrin might bind one of the two small-molecules selectively, thus removing it from the proximity of the second molecule. This removal of one of the energy transfer partners lowers the observed energy transfer efficiencies.

\textit{Comparison to Published Methods:}

The ability to detect toxicants via non-covalent energy transfer has a number of advantages compared to previously-reported methods, including the ability to tune the emission signal of a single analyte throughout the spectral region through choosing a
variety of fluorophores. To achieve this “tuning” ability, preliminary experiments were conducted using a third fluorophore: commercially available coumarin 6 (compound 22) as a fluorescent energy acceptor with selected analytes (10 mM γ-cyclodextrin, PBS solution) as energy donors. Good energy transfer efficiencies were observed for many cases (Table 3), and in most cases the energy transfer efficiencies were substantially higher in the presence of γ-cyclodextrin compared to in its absence.

<table>
<thead>
<tr>
<th>Donor</th>
<th>10 mM CD (%)</th>
<th>0 mM CD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>38</td>
</tr>
<tr>
<td>11</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
<td>39</td>
</tr>
</tbody>
</table>

Note: CD, γ-cyclodextrin

Moreover, the use of multiple fluorophores allows for the tuning of the fluorescence signal from a single analyte. For this experiment, analyte 12 was mixed with fluorophores 20, 21, and 22 in three vials (in 10 mM γ-cyclodextrin in PBS). Excitation of each solution at 320 nm (the excitation wavelength of the analyte) resulted in three distinct fluorophore signals at 515, 530, and 555 nm for fluorophores 20, 22, and 21, respectively (Figure 7). This tuning of the toxicant signal via judicious choice of fluorophore provides maximum flexibility in developing toxicant detection schemes.
One key challenge of this method compared to published methods for toxicant detection is the difficulty in obtaining quantitative data through non-covalent energy transfer. Preliminary experiments have demonstrated that the fluorescence signal obtained via energy transfer is not proportional to the concentration of the analyte; this is line with literature reports that demonstrate a complicated relationship between fluorescence energy transfer signals and the concentration of the donor and acceptor. This relationship is affected by a multitude of other intermolecular interactions, including donor-donor interactions, fluorophore dimerization and aggregation, and undesired fluorophore self-quenching.

**General discussion:**

There are a number of factors that determine whether a particular analyte participates efficiently in cyclodextrin-promoted energy transfer, and the results reported herein provide crucial information towards deconvoluting some of these factors. High energy transfer efficiencies occur in cases where the analyte-fluorophore

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**Figure 7.** A comparison of the fluorophore emission peak from toxicant 12 to fluorophores 20-22 in 10 mM γ-cyclodextrin in PBS.
pairs (a) form ternary complexes in the cyclodextrin cavity with high affinities and (b) participate in proximity-induced energy transfer. The binding affinities in cyclodextrin are determined by the molecules’ steric and electronic characters, and the participation in energy transfer schemes is determined by steric and electronic complementarity between the donor and acceptor, molecular orientations of the two guests, and the degree of spectral overlap with the fluorophore acceptor.

The analytes that demonstrated highly efficient energy transfer in the various media included compounds 7, 8, 11, and 12 (discussed herein) as well as compounds 1-3 (reported in previous publications). The fact that compounds 11 and 12 were efficient energy donors compared to compound 5 is likely due to the presence of the nitrogen substituents, which either enhance the electron donating ability of the analyte and/or provide favourable electrostatic interactions with the highly polarized fluorophore acceptors. Directly comparing the absorbance spectra, fluorescence spectra, and quantum yields of compounds 5, 11, and 12 indicate similar photophysical properties for the three compounds, which rules out spectral overlap as a substantial contributing factor.

The success of compound 7 compared to structurally similar compound 6 may be a result of additional amino group enabling compound 7 to form more electrostatic interactions or to bind in cyclodextrin with higher affinities. The similarities in the spectral properties of compounds 6 and 7 again rule out spectral overlap as a significant factor. The fact that the photophysical properties of the toxicant energy donors play only a limited role in determining energy transfer efficiencies strongly
supports our hypothesis that proximity-induced energy transfer in the cyclodextrin cavity occurs via a Dexter-type, direct orbital overlap mechanism.

One of the most surprising results was the successful use of compound 8 as an energy donor in combination with fluorophore acceptors. Compound 8 has been used as a fluorescence quencher of other small molecules,\textsuperscript{63,64} and is only weakly fluorescent. Nonetheless, the weak photophysical activity (455 nm emission maximum from 340 nm excitation) was sufficient for it to participate in proximity-induced energy transfer. The free hydroxyl groups of the molecule likely allow for the formation of hydrogen bonds to the highly polarized fluorophore acceptors. Comparing the results obtained with compound 8 to those of compound 10 (which was relatively inefficient as an energy donor) highlight possible steric constraints (compound 10 is substantially larger than compound 8) and functional group requirements (compound 10 lacks the free hydroxyl moieties) that are necessary for cyclodextrin-promoted energy transfer.

CONCLUSION

In conclusion, highly efficient energy transfer from a variety of organic toxicants occurred to multiple fluorophore acceptors when bound in the cavity of $\gamma$-cyclodextrin. The fact that this approach is successful in many environments with a variety of analytes is very beneficial. The robust nature of this approach leaves a wide range of opportunities to expand the scope of the analytes that can be detected, as well as the environments that they can be detected in. Indeed, the only requirement is that the analyte be (at least) weakly fluorescent. Furthermore, sample preparation is simple compared to current methods, as most media simply require dilution with PBS.
The fact that γ-cyclodextrin can bind analytes within its cavity in complex environments means that it can simultaneously isolate the analytes and promote energy transfer so that the analytes can be reliably identified. This method is a significant contribution to the facile and reliable detection of toxic analytes. The ability to tune the emission signal for a particular analyte by varying the choice of fluorophore provides substantial flexibility, and can be used in the development of array-based detection schemes. The development of such an array is currently under investigation, and results of these and other experiments will be reported in due course.

EXPERIMENTAL SECTION

All chemicals were obtained from Sigma-Aldrich chemical company or Fisher Scientific and used as received. BODIPY fluorophore 20 was synthesized following literature-reported procedures. Human plasma was obtained from Innovative Technologies. Human breast milk was obtained from an anonymous donor. Seawater was obtained from the Narragansett Beach in Rhode Island. Coconut water (VitaCoco 100% Pure Coconut Water) was obtained from CVS Pharmacy.

The human plasma, seawater, and coconut water were used as received. The breast milk was prepared by separating all solids via filtration and centrifugation, followed by dilution with phosphate-buffered saline (PBS). UV-Visible spectra were recorded on an Agilent 8453 spectrometer. Fluorescence measurements were recorded on a Shimadzu RF 5301 spectrophotometer with slit widths of 1.5 nm excitation and 1.5 nm emission slit widths. All fluorescence spectra were integrated vs. wavenumber on the X-axis, using OriginPro Version 8.6.
The energy transfer experiments were conducted as follows: 2.5 mL of a 10 mM solution of γ-cyclodextrin dissolved in the fluid of interest (PBS, coconut water, Narragansett Bay seawater, human plasma, or human breast milk) were measured into a cuvette. 20 µL of the analyte (1 mg/mL) and 100 µL of the fluorophore (0.1 mg/mL) were added. After thorough mixing, the solution was excited at two wavelengths: near the analyte’s absorption maximum (defined as “analyte excitation”) and near the fluorophore’s absorption maximum (defined as “fluorophore excitation”). The energy transfer efficiencies were calculated according to Equation 1:

\[
\% \text{ Energy Transfer} = \frac{I_{DA}}{I_A} \times 100\%
\]  

(1)

where \(I_{DA}\) is defined as the integrated fluorophore emission from indirect excitation and \(I_A\) is the integrated fluorophore emission from direct excitation. A graphical depiction of \(I_{DA}\) and \(I_A\) is shown in Figure 4. Experiments were also conducted where 0 mM of γ cyclodextrin were used for each fluid, analyte, and fluorophore combination, in place of the 10 mM cyclodextrin solution.

Control experiments were conducted as follows: (a) The fluorophore was mixed with γ-cyclodextrin and excited at the excitation wavelength of the analyte (but in the absence of any analyte); and (b) the fluorophore and analyte were both mixed in γ-cyclodextrin and excited at analyte excitation wavelength. The fluorophore emission that resulted from excitation at the analyte wavelength in the absence of the analyte was compared to the fluorophore emission from excitation at the analyte wavelength in the presence of the analyte. The ratio of these two emissions, shown as the “Fluorophore ratio” was calculated according to Equation 2:

\[
\text{Fluorophore ratio} = \frac{I_{\text{fluorophore-control}}}{I_{\text{fluorophore-analyte}}}
\]

(2)
Where $I_{\text{fluorophore-analyte}}$ is the integration of the fluorophore emission in the presence of the analyte; and $I_{\text{fluorophore-control}}$ is the integration of the fluorophore emission in the absence of the analyte. Full tables of energy transfer efficiencies for all analyte-fluorophore combinations and summary figures of all analyte-fluorophore combinations are shown in the Supplementary Material.

ACKNOWLEDGEMENTS

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Supporting Information

Cyclodextrin-Promoted Energy Transfer for Broadly Applicable Small-Molecule Detection

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich chemical company or Fisher Scientific and used as received. BODIPY fluorophore 20 was synthesized following literature-reported procedures. Human plasma was obtained from Innovative Technologies. Human breast milk was obtained from an anonymous donor. Seawater was obtained from the Narragansett Beach in Rhode Island. Coconut water (VitaCoco 100% Pure Coconut Water) was obtained from CVS Pharmacy. UV-Visible spectra were recorded on an Agilent 8453 spectrometer. Fluorescence measurements were recorded on a Shimadzu RF 5301 spectrophotometer with slit widths of 1.5 nm excitation and 1.5 nm emission slit widths. All fluorescence spectra were integrated vs. wavenumber on the X-axis, using OriginPro Version 8.6.

DETAILS FOR ENERGY TRANSFER EXPERIMENTS

All energy transfer efficiencies were calculated using Equation 1:

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

(1)

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

All fluorescence emissions were integrated using Origin 8.5, and were integrated vs. wavenumber on the X-axis.

GENERAL PROCEDURE FOR ENERGY TRANSFER EXPERIMENTS:
\( \gamma \)-cyclodextrin hydrate (CAS: 91464-90-3) was obtained from Sigma-Aldrich, and dissolved in phosphate buffered saline (PBS) at pH 7.4 at a 10 mM concentration.

All analytes were dissolved at a concentration of 1 mg/mL in tetrahydrofuran (THF).

Fluorophore solutions were made as follows:

- **BODIPY 20**: 0.1 mg/mL in THF
- **Rhodamine 21**: 0.1 mg/mL in THF

2.5 mL of the cyclodextrin solution was transferred to a quartz cuvette, and 20 µL of the analyte solution was added via micropipette. The absorbance and fluorescence spectra of the solution were recorded. The fluorophore was then added (100 µL), and the absorbance and fluorescence spectra were recorded. The final concentrations of each analyte and fluorophore are shown in Table S1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Final concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43.2</td>
</tr>
<tr>
<td>2</td>
<td>38.0</td>
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<tr>
<td>3</td>
<td>30.5</td>
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<td>4</td>
<td>43.2</td>
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<td>20</td>
<td>9.1</td>
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<tr>
<td>21</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**Table S1**: Final concentrations of all analytes and fluorophores used for energy transfer investigations
For each combination, two fluorescence spectra were recorded: the fluorescence from excitation of the analyte and the fluorescence spectra from excitation of the fluorophore. The excitation wavelengths were chosen to be as close as possible to the maximum wavelength of absorption, without significantly truncating the emission spectrum. Excitation wavelengths are recorded below in Table S2:

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Analyte</th>
<th>Excitation</th>
<th>Recorded Emission Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anthracene</td>
<td>360 nm</td>
<td>370 nm - 700 nm</td>
</tr>
<tr>
<td>2</td>
<td>Pyrene</td>
<td>360 nm</td>
<td>370 nm - 700 nm</td>
</tr>
<tr>
<td>3</td>
<td>Benzo[a]pyrene</td>
<td>360 nm</td>
<td>370 nm - 700 nm</td>
</tr>
<tr>
<td>4</td>
<td>Phenanthrene</td>
<td>290 nm</td>
<td>300 nm – 570 nm</td>
</tr>
<tr>
<td>5</td>
<td>Fluorene</td>
<td>270 nm</td>
<td>280 nm – 570 nm</td>
</tr>
<tr>
<td>6</td>
<td>4-Aminobiphenyl</td>
<td>290 nm</td>
<td>300 nm – 700 nm</td>
</tr>
<tr>
<td>7</td>
<td>Benzidine</td>
<td>365 nm</td>
<td>375 nm – 700 nm</td>
</tr>
<tr>
<td>8</td>
<td>Diethylstilbestrol</td>
<td>340 nm</td>
<td>350 nm – 700 nm</td>
</tr>
<tr>
<td>9</td>
<td>4,4’-Methylene-bis(2-chloroaniline)</td>
<td>270 nm</td>
<td>280 nm – 700 nm</td>
</tr>
<tr>
<td>10</td>
<td>Tamoxifen</td>
<td>260 nm</td>
<td>270 nm – 700 nm</td>
</tr>
<tr>
<td>11</td>
<td>2-Aminofluorene</td>
<td>350 nm</td>
<td>360 nm – 700 nm</td>
</tr>
<tr>
<td>12</td>
<td>2-Acetylaminofluorene</td>
<td>320 nm</td>
<td>320 nm – 700 nm</td>
</tr>
<tr>
<td>13</td>
<td>Bisphenol A</td>
<td>250 nm</td>
<td>260 nm – 700 nm</td>
</tr>
<tr>
<td>14</td>
<td>4,4’-Dichlorobiphenyl</td>
<td>233 nm</td>
<td>243 nm – 600 nm</td>
</tr>
<tr>
<td>15</td>
<td>PCB29</td>
<td>233 nm</td>
<td>243 nm – 600 nm</td>
</tr>
<tr>
<td>16</td>
<td>PCB3</td>
<td>233 nm</td>
<td>243 nm – 600 nm</td>
</tr>
<tr>
<td>17</td>
<td>PCB52</td>
<td>233 nm</td>
<td>243 nm – 600 nm</td>
</tr>
<tr>
<td>18</td>
<td>PCB77</td>
<td>233 nm</td>
<td>243 nm – 600 nm</td>
</tr>
<tr>
<td>19</td>
<td>PCB209</td>
<td>233 nm</td>
<td>243 nm – 600 nm</td>
</tr>
<tr>
<td>20</td>
<td>BODIPY</td>
<td>460 nm</td>
<td>470 nm – 800 nm</td>
</tr>
<tr>
<td>21</td>
<td>Rhodamine 6G</td>
<td>490 nm</td>
<td>500 nm – 800 nm</td>
</tr>
</tbody>
</table>

Table S2: The excitation and emission ranges used for each compound.

DETAILS FOR CONTROL EXPERIMENTS

These experiments were designed to determine the emission of the fluorophores from excitation at various wavelengths (in the absence of the analyte) and compare it to the emission of fluorophores at the same wavelengths in the presence of the analyte. This will determine whether an observed “energy transfer” peak may simply be a result of exciting the fluorophore at a wavelength where it has non-zero absorbance. These experiments were conducted as follows:
(a) The fluorophore was mixed with $\gamma$-cyclodextrin and excited at the excitation wavelength of the analyte (but in the absence of any analyte); and

(b) The fluorophore and analyte were both mixed in $\gamma$-cyclodextrin and excited at the analyte excitation wavelength.

The fluorophore emission that resulted from excitation at the analyte wavelength in the absence of the analyte was compared to the fluorophore emission from excitation at the analyte wavelength in the presence of the analyte. The ratio of these two emissions, shown as “ratio of fluorophore emissions” is defined as:

Fluorophore emission via low wavelength excitation in the absence of an analyte/fluorophore emission via low wavelength excitation in the presence of the analyte.

This was used to determine what fraction of the peak was a result of legitimate energy transfer rather than simple excitation of the fluorophore at a wavelength where it has non-zero absorbance.

EXPERIMENTAL DETAILS FOR PLASMA PREPARATION

A human plasma sample was obtained from Innovative Technologies, with the following specifications:

Normal Single Donor Human Plasma: 50 mL; Anticoagulant: Heparin; Race: Caucasian; Age: 18-25; Gender: Male

The plasma was used as received. The following stock solutions were also prepared: 10 mM $\gamma$-cyclodextrin in phosphate buffered saline (PBS), 0.1 mg/mL in THF of fluorophores 20 and 21, and 1 mg/mL solutions of all analytes.
A blank sample was first analyzed consisting of 1.25 mL of plasma, 1.25 mL of 10 mM γ-cyclodextrin, and 100 µL of either fluorophore 20 or 21. The sample was excited at the analyte excitation wavelength and the dye excitation wavelength. Then, 20 µL of analyte was added and the sample was re-excited at both wavelengths. This procedure was repeated for each fluorophore-analyte combination.

For each analyte, a control sample was also analyzed following the procedure outlined above, with 0 mM γ-cyclodextrin (pure PBS) in place of 10 mM γ-cyclodextrin.

EXPERIMENTAL DETAILS FOR BREAST MILK PREPARATION

Breast milk was collected from a single donor and frozen until used. The breast milk was allowed to sit in a warm water bath at 30°C until thawed. Then, the breast milk was cooled to room temperature and allowed to sit at room temperature overnight. The sample separated into a clear aqueous layer and an opaque layer with solids. The aqueous layer was carefully removed via pipette. The aqueous layer was then filtered via syringe and centrifuged for 15 minutes at 6500 rpm. The aqueous layer was then removed via pipette as some solids remained on the outside of the centrifuge tube.

For each trial, 625 µL of breast milk was added to 1.875 mL (for a total volume of 2.5 mL) of PBS (0 mM γ-cyclodextrin) or 10 mM γ-cyclodextrin, depending on the experiment.
### Table S3. Summary tables for energy transfer experiments in PBS.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluorophore</th>
<th>Control Ratio</th>
<th>ET Percentage</th>
<th>Control Ratio</th>
<th>ET Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>20</td>
<td>1.11</td>
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</tr>
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<td>1.00</td>
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<td>20</td>
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### Table S4. Summary tables for energy transfer experiments in coconut water.

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<td>Control Ratio</td>
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Table S5. Summary tables for energy transfer experiments in human plasma.

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</thead>
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<td>Control Ratio</td>
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<table>
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</thead>
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Table S6. Summary tables for energy transfer experiments in human breast milk.

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<td>7.1</td>
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Table S7. Summary tables for energy transfer experiments in seawater.

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</thead>
<tbody>
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<td></td>
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<td>Control Ratio</td>
<td>ET percentage</td>
</tr>
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Table S8. Summary tables for energy transfer experiments in PBS with 22.

<table>
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<th>10 mM γ-CD</th>
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</tr>
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<tbody>
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<td></td>
<td></td>
<td>Control Ratio</td>
<td>ET Percentage</td>
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<td>1</td>
<td>22</td>
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</table>

*Excimer emission of the analyte obscured accurate quantification of the fluorophore peak.*

Table S7. Summary tables for energy transfer experiments in seawater.

Table S8. Summary tables for energy transfer experiments in PBS with 22.
CHAPTER 6
To be Submitted to *Environmental Science: Water Research and Technology*,
Investigating Fundamental Intermolecular Interactions in Gamma-
Cycodextrin Host-Guest Complexes

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Kingston, Rhode Island 02881, USA
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Manuscript 6

Investigating Fundamental Intermolecular Interactions in Gamma-Cyclodextrin Host-Guest Complexes

ABSTRACT

We have developed an array-based detection method that uses γ-cyclodextrin as a supramolecular scaffold for a small-molecule toxicant and a fluorophore to promote proximity-induced energy transfer. Because γ-cyclodextrin holds these two guest molecules in close proximity, energy transfer from the analyte to the fluorophore can occur. What remained to be investigated was the geometry of the cyclodextrin-analyte-fluorophore complexes, as multiple potential binding geometries can occur, several of which would lead to the desired high energy transfer efficiencies. Binding constants for a variety of guest molecules were calculated using fluorescence spectroscopy, and energy transfer experiments were performed under a variety of temperature, solvent, and ionic strength conditions to probe the fundamental non-covalent interactions. The results of these experiments and the information the experiments yield about non-covalent intermolecular interactions are reported herein.

Introduction

Cyclodextrins are widely-used supramolecular hosts, as their hydrophobic interiors and hydrophilic exteriors allow them to form inclusion complexes with a variety of small molecule guests. The non-covalent interactions that promote guest: host complex formation include π-π stacking, Van der Waals forces, hydrophobic binding, and electrostatic interactions. The binding affinities of small molecules in cyclodextrin cavities and the overall stability of the resulting inclusion complexes are
determined by the electronic and steric character of the guest molecule.\textsuperscript{5} The mechanisms that govern association complex formation are exceedingly complex and often difficult to predict and fully characterize, and numerous investigations studying complex formation in cyclodextrin hosts have been reported in the literature.\textsuperscript{6}

\textbf{Figure 1.} Illustration of proximity-induced energy transfer.

Cyclodextrins can be used for the detection of small-molecule analytes using fluorescence energy transfer, by enforcing close-proximity of the analytes and a high-quantum yield fluorophore (Figure 1). Upon excitation of the analyte, energy transfer from the analyte donor to the fluorophore acceptor occurs and a new fluorescence response is observed, which can be used to identify the toxicant. We have reported the sensitive and selective detection of numerous small-molecule analytes (toxicants) using this detection scheme, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, and endocrine disruptors;\textsuperscript{7} we have also used this system for the extraction and detection of polycyclic aromatic hydrocarbons (PAHs) from complex oils as part of oil spill remediation efforts.\textsuperscript{8}

For several of the analytes investigated, the analyte-fluorophore interactions are more accurately characterized as proximity-induced fluorescence modulation rather than proximity-induced energy transfer. The term fluorescence modulation is
defined as a change in emission of the fluorophore as a result of the analyte’s presence. These changes can still be used to define a characteristic response of the analyte-fluorophore combination, and can still be used for the efficient and selective detection of a particular analyte.

Due to the large scope of analytes that we have investigated (Chart 1), with a large variety of steric, electronic, and structural features, it is likely that the structures of the cyclodextrin-based host-guest complexes vary significantly. In some cases, the small molecules may associate near the cyclodextrin host rather than in the host cavity; in these cases, efficient proximity-induced energy transfer is still a likely outcome due to the enforced proximity between the donor and acceptor. The mechanisms that govern the formation of inclusion complexes (wherein the small molecule is bound in the cyclodextrin cavity) or association complexes (wherein the small molecule is held near the cyclodextrin cavity) had not previously been explored, despite the fact that such mechanisms are expected to vary widely depending on the structural features of the small molecules, the nature of the host and fluorophore, and the experimental conditions (including solvent and temperature). We report herein an investigation into precisely this objective.

**Experimental Section**

All chemicals were obtained from Sigma-Aldrich chemical company or Fisher Scientific and used as received, including compounds 1-30 (Chart 1), 32 and 33. Compound 31 (Chart 2) was synthesized following literature-reported procedures. Fluorescence measurements were recorded on a Shimadzu RF 5301 spectrophotometer with slit widths of 1.5 nm excitation and 1.5 nm emission slit widths. All fluorescence
spectra were integrated vs. wavenumber on the X-axis, using OriginPro Version 8.6. Ultrapure water was collected an 18MΩ·cm Millipore Synergy UV. For the temperature studies, a Fisher Scientific Isotemp 6200 R20 was used to control the temperature and the spectrophotometer was equipped with a single constant-temperature cell holder.

Chart 1. Analytes under investigation.

Chart 2. Fluorophores used in this study.

Energy transfer efficiencies were used to determine how much energy was being transferred from the photophysically active toxicant donor to the fluorophore acceptor. These efficiencies were calculated according to Equation 1:
% Energy Transfer = \frac{I_{DA}}{I_A} \times 100\% \tag{1}

where $I_{DA}$ is defined as the integrated fluorophore emission from indirect excitation and $I_A$ is the integrated fluorophore emission from direct excitation.

A control experiment was also performed to ensure that the desired energy transfer was actually occurring, rather than being a result of non-zero absorbance of the fluorophore at the toxicant excitation wavelength, which would also lead to an apparent energy transfer peak. The ratio of these two emissions, shown as the “Control ratio” was calculated according to Equation 2:

$$\text{Control ratio} = \frac{I_{\text{fluorophore-control}}}{I_{\text{fluorophore-analyte}}} \tag{2}$$

Where $I_{\text{fluorophore-analyte}}$ is the integration of the fluorophore emission in the presence of the analyte; and $I_{\text{fluorophore-control}}$ is the integration of the fluorophore emission in the absence of the analyte. For ratios <0.95, legitimate energy transfer was occurring; for ratios between 0.95-1.05, the observed fluorescence response was the result of nonzero absorbance of the fluorophore at analyte excitation; and for ratios >1.05, fluorescence quenching was occurring.

**Results and Discussion**

The binding constants of 1-33 in gamma-cyclodextrin were determined using the Benesi-Hildebrand method\textsuperscript{11}. Selected toxicant-fluorophore combinations were then subjected to further experimentation, which include varying the temperature of the system, varying the ionic strength of the solvent (through the addition of sodium chloride and guanidinium hydrochloride), and studying mixed aqueous-ethanol solvent systems. Each of these experiments will be discussed in turn.
**Binding constants.** Binding constants were determined by keeping the concentration of the small molecule constant, and measuring the molecule’s fluorescence emission in the presence of varying concentrations of γ-cyclodextrin. The fluorescence of the analyte was integrated with respect to wavenumber. The data was then fitted to a Benesi-Hildebrand equation for a 1:1 complex and the apparent binding constant was determined from the linear fit equation. The linear fit equation was determined by plotting 1/[macrocycle] (in M\(^{-1}\)) on the X-axis and 1/integrated analyte emission on the Y-axis. The results of these experiments are tabulated in Table 1, and R\(^2\) values greater than 0.70 were considered to be reasonable linear fits. Several aspects of this data that merit discussion.

Non-covalent macrocycle complexes arise from binding affinity between the host and guest, and the contributions of hydrophobic interactions, hydrogen bonding, steric interactions, and electrostatic complementarity between the guest and the cyclodextrin host dictates the strength of this affinity.\(^{12}\) In addition, high-energy water, resulting from unfavorable interactions between water and the hydrophobic cyclodextrin interior, occupy the cyclodextrin cavity.\(^{13}\) Inclusion of the guest depends often depends on the capability of the guest to displace this water, which provides an important driving force for complexation.\(^{14}\) In general, inclusion complexation is hindered by (1) the guest being too large for the cavity, (2) a large polar region on the analyte (resulting in partial inclusion), and (3) the guest being too small, where it can readily pass through the cyclodextrin cavity. However, binding outside the cyclodextrin cavity has been demonstrated for a variety of small molecule analytes.\(^{15}\)
so analytes which cannot form classical inclusion complexes can still associate with the cyclodextrin.

Lastly, using a Benesi-Hildebrand plot assumes that the fluorescence of the analyte increases with increasing cyclodextrin concentration, which is due to the decrease in radiative decay pathways available to the analyte to relax down to the ground state by greatly hindering its degrees of rotation. Therefore, it is understood that many of the analytes used in this study will not form classical inclusion complexes; rather, the analytes and fluorophores form association complexes with γ-cyclodextrin by using it as a scaffold through hydrogen bonding and π-π stacking. This explains the poor linear fit for some of the analytes.

The negative binding constants shown in Table 1 are most likely the result of the cyclodextrin sequestering the analyte such that the fluorescence decreases, resulting in a negative binding constant. Negative binding constants have been described in the literature previously, and guest displacement aligns with the findings of previous reports.

The analytes that showed the best fit are 12, 6, 21, 2, 7, 14, 3, and 10, and two fluorophores, 31 and 33. Previously, we determined that compound 6 compliments the dimensions of the cyclodextrin cavity and take full advantage of both Van der Waals interactions and the hydrophobic effect, which is corroborated by a good linear fit in the Benesi-Hildebrand plot. The negative binding constant could be due to one of two factors: (1) decreased excimer emission as the analyte molecules are displaced from one another, in increasing cyclodextrin concentration, and (2) highly emissive aggregates form without the cyclodextrin, and when the cyclodextrin binds to a single
guest the aggregates are disrupted. In both cases, an overall decrease in fluorescence is observed and a negative binding constant can be calculated.

Compound 7 has both a good linear fit and positive binding constant of 2000 M⁻¹. As compounds 6 and 7 differ only in a methyl group, it is likely that the methyl group of 7 does not allow strong excimer formation to occur as it does in 6, likely because the methyl group disrupts the completely planar structure of the polycyclic aromatic hydrocarbon. Because the size of 7 still compliments the cavity, strong 1:1 association complexes likely form. Compounds 2 and 3 demonstrate both good linear fits and positive binding constants (5000M⁻¹ and 430M⁻¹, respectively). These analytes use the hydrophobic effect and hydrogen bonding between the hydroxyl moieties of the cyclodextrin ring and their hydrogen bonding sites (both have ketones, and 3 has hydroxyl groups in addition) to achieve strong host-guest binding affinities. It is interesting to note the magnitude of difference between the binding constants of these analytes. Compound 3 is likely to have stronger affinity for the exterior of the cyclodextrin, and likely only slightly enters the cavity through its unsubstituted ring, which leads to a lower binding constant. Compounds 10 and 14 display both good linear fit and positive binding constants, and can be attributed to 10 having sufficient surface area to create strong Van der Waals interactions in the cavity and 14 having distinct hydrophobic and hydrophilic structures, allowing it to penetrate the cavity and be “anchored” in place through hydrogen bonding. Compounds 21, 31, and 33 have good linear fit but negative binding constants, which indicates that their size (and ability to hydrogen bond) allows them to form complexes, and the negative binding constant could be due to one of the two factors previously described.
There are a number of potential complexes that can be formed between the small molecule analytes and gamma cyclodextrin, including 1:1, 1:2, 2:1 and 2:2 guest-host complexes, and many of these stoichiometries often occur simultaneously. Moreover, there are a number of potentially co-occurring geometries, including ones with the analyte fully inside the gamma cyclodextrin cavity and those in which the analyte is associated outside of the cavity. As such, it is not surprising that many of the analytes do not show strong linear relationships, as the Benesi-Hildebrand plots are predicated on certain assumptions, including complete inclusion of the guest by the host, and that the concentration of the guest in the matrix is equivalent to the total guest concentration. These assumptions do not hold for all analytes, and can make accurately determining a binding constant difficult.19

Attempts were made to fit the data to a 1:2 guest: host complex (see Table S2 in the Supporting Information), but overall the fits were stronger for 1:1 complexation. Taken together, this data indicates that the mechanisms behind such a dynamic guest: host system is challenging to fully understand, and why definitive binding constants are difficult to obtain for a particular guest-host system.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear Fit Equation</th>
<th>R²</th>
<th>Binding Constant (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>y = -5E-10x + 2E-06</td>
<td>0.95</td>
<td>-4000.00</td>
</tr>
<tr>
<td>6</td>
<td>y = 5E-10x + 9E-07</td>
<td>0.92</td>
<td>1800.00</td>
</tr>
<tr>
<td>21</td>
<td>y = -7E-09x + 0.0001</td>
<td>0.91</td>
<td>-14285.71</td>
</tr>
<tr>
<td>2</td>
<td>y = 6E-09x + 3E-05</td>
<td>0.86</td>
<td>5000.00</td>
</tr>
<tr>
<td>7</td>
<td>y = 4E-09x + 8E-06</td>
<td>0.85</td>
<td>2000.00</td>
</tr>
<tr>
<td>33</td>
<td>y = -1E-08x + 4E-05</td>
<td>0.80</td>
<td>-4000.00</td>
</tr>
<tr>
<td>14</td>
<td>y = 5E-09x + 3E-05</td>
<td>0.79</td>
<td>6000.00</td>
</tr>
<tr>
<td>3</td>
<td>y = 7E-08x + 3E-05</td>
<td>0.78</td>
<td>428.57</td>
</tr>
<tr>
<td>31</td>
<td>y = -3E-09x + 2E-05</td>
<td>0.77</td>
<td>-6666.67</td>
</tr>
<tr>
<td>10</td>
<td>y = 1E-09x + 7E-05</td>
<td>0.73</td>
<td>70000.00</td>
</tr>
</tbody>
</table>
Analytes with binding constants above 0.70 were considered to have good linear fit.

Effect of temperature on energy transfer efficiencies. Energy transfer experiments were conducted with compounds 6, 8, 11, 12, 28, and 29 as energy donors and compound 31 as an energy acceptor, the temperature was varied from 5°C to 80°C, and the results are reported in Table 2. Control ratios confirm that these energy transfer efficiencies are a result of legitimate energy transfer rather than a result of exciting the fluorophore at a wavelength where it has non-zero absorbance (Table 3). For each analyte, energy transfer efficiencies decreased with increasing temperature. This is likely due to hydrogen bond disruption, which decreases the stability of the complex and in turn decreases energy transfer efficiency. In general, host-guest inclusion complexes are less stable with increased temperature.20
The results of Table 2 add further evidence to this observation. Compounds 28 and 29, which differ only in an additional amine group in 28, show the greatest decrease in energy transfer, with ~70% reduction in energy transfer efficiency between 5°C and 80°C. These compounds do not have a large hydrophobic surface area compared to other analytes (for example, compound 6); therefore, these analytes most likely rely on hydrogen bonding for complexation and therefore show the greatest sensitivity to temperature variation.

Compound 12 differs from 11 in that it has an amine group. These compounds demonstrate similar reductions in energy transfer efficiency between 5°C and 80°C (12: ~59%; 11: ~58%). Thus they share similar complexation dynamics, and the hydrogen bonding site offered by 12 hampers only the energy transfer efficiency. The complexes for both become less stable at higher temperatures, but hydrogen bonding is not a major contributor.

### Table 2. Results of energy transfer experiments at different temperatures with Compound 31

<table>
<thead>
<tr>
<th>Analyte</th>
<th>5 °C</th>
<th>20 °C</th>
<th>35 °C</th>
<th>50 °C</th>
<th>65 °C</th>
<th>80 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 6</td>
<td>1473.7%</td>
<td>324.3%</td>
<td>560.1%</td>
<td>548.3%</td>
<td>333.7%</td>
<td>790.3%</td>
</tr>
<tr>
<td>Compound 8</td>
<td>99.3%</td>
<td>30.9%</td>
<td>71.2%</td>
<td>67.2%</td>
<td>69.1%</td>
<td>55.6%</td>
</tr>
<tr>
<td>Compound 11</td>
<td>370.4%</td>
<td>79.0%</td>
<td>112.3%</td>
<td>110.9%</td>
<td>145.7%</td>
<td>153.7%</td>
</tr>
<tr>
<td>Compound 12</td>
<td>37.3%</td>
<td>26.4%</td>
<td>56.8%</td>
<td>50.9%</td>
<td>37.5%</td>
<td>15.2%</td>
</tr>
<tr>
<td>Compound 28</td>
<td>46.6%</td>
<td>21.7%</td>
<td>25.0%</td>
<td>15.8%</td>
<td>23.4%</td>
<td>12.4%</td>
</tr>
<tr>
<td>Compound 29</td>
<td>183.8%</td>
<td>28.6%</td>
<td>72.5%</td>
<td>59.0%</td>
<td>49.7%</td>
<td>54.3%</td>
</tr>
</tbody>
</table>

All experiments conducted in 10 mM γ-cyclodextrin in PBS. Energy transfer efficiencies calculated as in Equation 1.
Compounds 6 and 8 indicate the least sensitivity to temperature changes, with ~46% reduction and ~44% reduction in energy transfer efficiencies respectively. Complexes with these compounds most likely rely on hydrophobic interactions, and previous work indicates that hydrophobic interactions are largely unhindered by an increase in temperature. The fact that the stability of inclusion complexes decreases with increasing temperature explains the decreased energy transfer efficiencies.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>5 °C</th>
<th>20 °C</th>
<th>35 °C</th>
<th>50 °C</th>
<th>65 °C</th>
<th>80 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 6</td>
<td>0.02</td>
<td>0.07</td>
<td>0.08</td>
<td>0.05</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Compound 8</td>
<td>0.38</td>
<td>0.77</td>
<td>0.52</td>
<td>0.36</td>
<td>0.29</td>
<td>0.28</td>
</tr>
<tr>
<td>Compound 11</td>
<td>0.25</td>
<td>0.33</td>
<td>0.41</td>
<td>0.47</td>
<td>0.23</td>
<td>0.19</td>
</tr>
<tr>
<td>Compound 12</td>
<td>0.77</td>
<td>0.80</td>
<td>0.59</td>
<td>0.82</td>
<td>0.40</td>
<td>0.76</td>
</tr>
<tr>
<td>Compound 28</td>
<td>0.94</td>
<td>1.09</td>
<td>0.85</td>
<td>1.56</td>
<td>0.44</td>
<td>0.59</td>
</tr>
<tr>
<td>Compound 29</td>
<td>0.51</td>
<td>0.82</td>
<td>0.57</td>
<td>0.69</td>
<td>0.50</td>
<td>0.46</td>
</tr>
</tbody>
</table>

All experiments conducted in 10 mM γ-cyclodextrin in PBS. Control ratios defined as in Equation 2.

Interestingly, the energy transfer efficiency of 11 is an order of magnitude greater than 12 (for example, 370% for 11 and 37% for 12 at 5°C), and 6 is 1-2 orders of magnitude higher than 8 (for example, 1474% for 6 and 99% for 8 at 5°C) could be due to the greater capability of 6 and 11 to π-π stack with 31, resulting in more efficient proximity-induced energy transfer. In other words, these compounds are held in closer proximity to the fluorophore than 8 and 12. This shows that 6 and 11 penetrate the cyclodextrin cavity to a greater extent (6 can be fully encapsulated), so their inclusion complexes are stable and rigid, allowing 31 to have maximum contact with these structures and resulting in higher energy transfer efficiencies.
Lastly, the fact that the energy transfer efficiencies are significantly higher at 5°C than 80°C is particularly significant to our work. Many of the analytes shown in Chart 1 are weakly fluorescent, and by extension participate only weakly in non-covalent energy transfer. Detection experiments are carried out at room temperature, but by simply changing the temperature (which is a facile adjustment) the sensitivity of our method can be greatly enhanced, resulting in a wider range of analytes that can be detected by this method and improved sensitivity in detection.

Effect of salt addition. Salts have been known to influence inclusion complexation by a variety of pathways, which include hydrogen bond disruption, analyte association, and ternary complex formation. They also influence the hydrophobic effect, which is the propensity of nonpolar molecules to aggregate in aqueous solution to exclude water as much as possible. Salts such as sodium chloride tend to increase the hydrophobic effect (as they make it difficult for molecules to move into the bulk water), while salts like guanidinium chloride tend to decrease the hydrophobic effect (as they make it easier for molecules to move into the bulk water), and were thus used for these studies. The results of energy transfer experiments with these salts are indicated in Table 4. Interestingly, only compound 6, and to a much less extent compound 11 and 29, showed any real difference in energy transfer efficiency, including in pure water with no salt content. The remaining analytes that showed real energy transfer (Table 5), compounds 8 and 23, show negligible differences in efficiency with either salt.

Compound 11 showed a slight decrease in energy transfer efficiency with NaCl. This salt decreases the solubility of nonpolar molecules causing them to “salt-
out" via aggregation. Structurally, compounds 6 and 11 are unsubstituted PAHs and have large hydrophobic surface area. The fact that 6 shows a sharp decrease in energy transfer efficiency with this salt is consistent with aggregation. When fluorescent compounds aggregate, quenching is observed, and resulting in lower energy transfer efficiencies. Conversely, compound 29 shows slightly improved energy transfer efficiency with NaCl, as this compound has a hydrogen bonding group and smaller hydrophobic surface. Because of this, the hydrogen bonding feature of this analyte facilitates improved energy transfer efficiency. This phenomena is likely the reason that compounds 3 and 19 show real energy transfer when this salt is present (rather than a result of merely exciting the fluorophore at a wavelength where it has non-zero absorbance), as they do not in pure water or guanidinium chloride (mostly just due to fluorophore excitation directly).

**Table 4. Results of energy transfer experiments in the presence of salt additives with Compound 31**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Pure water</th>
<th>Guanadinium Chloride</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 2</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Compound 3</td>
<td>a</td>
<td>a</td>
<td>24.1%</td>
</tr>
<tr>
<td>Compound 6</td>
<td>319.9%</td>
<td>513.5%</td>
<td>200.2%</td>
</tr>
<tr>
<td>Compound 8</td>
<td>27.0%</td>
<td>27.3%</td>
<td>28.7%</td>
</tr>
<tr>
<td>Compound 11</td>
<td>56.7%</td>
<td>55.7%</td>
<td>50.2%</td>
</tr>
<tr>
<td>Compound 12</td>
<td>a</td>
<td>23.7%</td>
<td>a</td>
</tr>
<tr>
<td>Compound 16</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Compound 17</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Compound 19</td>
<td>a</td>
<td>a</td>
<td>10.0%</td>
</tr>
<tr>
<td>Compound 20</td>
<td>a</td>
<td>9.6%</td>
<td>a</td>
</tr>
<tr>
<td>Compound 23</td>
<td>20.7%</td>
<td>20.7%</td>
<td>22.6%</td>
</tr>
<tr>
<td>Compound</td>
<td>Energy Transfer Efficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>21.3% 19.2% 22.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>21.3% 19.2% 22.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>23.3% 24.3% 29.5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiments conducted with: 10 mM $\gamma$-cyclodextrin in ultrapure water (control); or a 10 mM $\gamma$-cyclodextrin solution made with a 1M salt solution in ultrapure water

*a* No real energy transfer is occurring

Energy transfer efficiencies calculated as in Equation 1.

Compound 6 showed a substantial increase in energy transfer efficiency in guanidinium chloride. This salt increases the solubility of nonpolar molecules (known as “salting in”) in water by decreasing surface tension, allowing the nonpolar molecules to move into the bulk water more easily. The loss of hydrophobicity greatly increases the energy transfer efficiency from 320% in water to 514% in this salt, yet there is no difference between the control ratios (0.08 and 0.07, respectively). The fact that the energy transfer efficiency is much stronger in the presence of this salt is interesting. Previous research suggests that guanidinium chloride does not have an effect on the structure of water nor does it bind to cyclodextrin, but it does bind to the hydrophobic surface of the guest molecule, and stabilizes the analyte in water.\(^\text{23}\) This is happening concurrently to the fluorophore 31, which could explain why the control ratios are essentially the same; in other words, the analyte and fluorophore are still forming complexes with $\gamma$-cyclodextrin. Sterics may also play a role in this; compound 6 shows strong size complementarity with the cyclodextrin cavity, and therefore has strong Van der Waals forces acting on it. This means that inclusion complex formation is highly favored for this analyte. However, the increased energy transfer efficiencies is likely a consequence of these molecules being able to better associate with one another. While they are associating similarly in space, they are able to do so such that
γ-cyclodextrin holds them in much closer proximity to one another, which results in the dramatic increase of energy transfer efficiency.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Pure water</th>
<th>Guinadinium Chloride</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 2</td>
<td>0.99</td>
<td>1.00</td>
<td>0.96</td>
</tr>
<tr>
<td>Compound 3</td>
<td>1.01</td>
<td>1.01</td>
<td>0.91</td>
</tr>
<tr>
<td>Compound 6</td>
<td>0.08</td>
<td>0.07</td>
<td>0.11</td>
</tr>
<tr>
<td>Compound 8</td>
<td>0.76</td>
<td>0.78</td>
<td>0.70</td>
</tr>
<tr>
<td>Compound 11</td>
<td>0.32</td>
<td>0.35</td>
<td>0.36</td>
</tr>
<tr>
<td>Compound 12</td>
<td>0.95</td>
<td>0.81</td>
<td>1.01</td>
</tr>
<tr>
<td>Compound 16</td>
<td>1.04</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>Compound 17</td>
<td>1.02</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Compound 19</td>
<td>0.97</td>
<td>1.01</td>
<td>0.92</td>
</tr>
<tr>
<td>Compound 20</td>
<td>1.05</td>
<td>0.93</td>
<td>1.01</td>
</tr>
<tr>
<td>Compound 23</td>
<td>0.94</td>
<td>0.90</td>
<td>0.89</td>
</tr>
<tr>
<td>Compound 24</td>
<td>0.95</td>
<td>1.01</td>
<td>1.02</td>
</tr>
<tr>
<td>Compound 28</td>
<td>1.15</td>
<td>1.12</td>
<td>1.07</td>
</tr>
<tr>
<td>Compound 29</td>
<td>0.8</td>
<td>0.82</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Experiments conducted with: 10 mM γ-cyclodextrin in ultrapure water (control); or a 10 mM γ-cyclodextrin solution made with a 1M salt solution in ultrapure water. Control ratios defined as in Equation 2.

Effect of ethanol addition. Cyclodextrins have hydrophilic surfaces which are bonded to water, and this ordered structure can be disrupted upon addition of an alcohol. Table 6 reports the results of energy transfer experiments conducted in the absence and presence of ethanol (1:1 volume ratio with PBS), and Table 7 reports the control ratios for these experiments. The hydroxyl moiety of the alcohol form hydrogen bonds to the hydroxyl groups of the cyclodextrin cavity, and the
hydrophobic portion of the alcohol enters the cavity. The result is an enhanced hydrophobic environment, and the hydrophobic effect is experienced strongly by small-molecule analytes. Furthermore, depending on the size of the analyte and the alcohol used, the alcohol may help the analyte fit more comfortably in the cyclodextrin cavity via formation of a ternary complex.24

When looking at the control ratios in Table 7, in most cases the control ratios are markedly decreased, representing increased fluorescence of the fluorophore in the presence of the analyte. However, for analytes that participate in energy transfer in the absence of ethanol, the energy transfer efficiencies are greatly diminished. It is interesting to note that in all cases where ethanol is present, true energy transfer occurs, while in its absence, compounds 2, 3, 8, 17, 19, 23, and 24 show no “legitimate” energy transfer. This may be due to ternary complex formation in the γ-cyclodextrin cavity, and ethanol allows the molecules to better compliment the cavity so a binding event occurs and the analyte can now participate in energy transfer. However, the efficiencies are modest. This could be due to the excess ethanol in solution: because hydrogen bonds are being disrupted, the analyte and fluorophore are being held in such a way that efficient energy transfer does not occur.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Absence of ethanol</th>
<th>Presence of ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 2</td>
<td>a</td>
<td>22.6%</td>
</tr>
<tr>
<td>Compound 3</td>
<td>a</td>
<td>8.3%</td>
</tr>
<tr>
<td>Compound 6</td>
<td>324.3%</td>
<td>16.7%</td>
</tr>
<tr>
<td>Compound 8</td>
<td>a</td>
<td>19.7%</td>
</tr>
<tr>
<td>Compound 11</td>
<td>30.9%</td>
<td>21.5%</td>
</tr>
<tr>
<td>Compound</td>
<td>Absence of ethanol</td>
<td>Presence of ethanol</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>12</td>
<td>26.4%</td>
<td>19.8%</td>
</tr>
<tr>
<td>16</td>
<td>79.0%</td>
<td>5.3%</td>
</tr>
<tr>
<td>17</td>
<td>a</td>
<td>7.8%</td>
</tr>
<tr>
<td>19</td>
<td>a</td>
<td>8.2%</td>
</tr>
<tr>
<td>20</td>
<td>33.3%</td>
<td>4.8%</td>
</tr>
<tr>
<td>23</td>
<td>a</td>
<td>8.2%</td>
</tr>
<tr>
<td>24</td>
<td>a</td>
<td>25.8%</td>
</tr>
<tr>
<td>28</td>
<td>21.7%</td>
<td>21.3%</td>
</tr>
<tr>
<td>29</td>
<td>28.6%</td>
<td>11.1%</td>
</tr>
</tbody>
</table>

Experiments conducted with 10 mM γ-cyclodextrin in PBS and mixed 1:1 v/v with ethanol.

*a* No real energy transfer is occurring.

Energy transfer efficiencies calculated as in Equation 1.

Again, compound 6 is an interesting case. It decreases in efficiency from 324% without ethanol to 17% with ethanol, a substantial loss, while the control ratio is essentially unchanged (0.07 to 0.08, respectively). This result seems to support the fact that inclusion complex formation is highly favorable for this analyte, and ethanol simply disrupts efficient energy transfer while having no real effect on the inclusion complex formation.

**Table 7.** Control ratios for ethanol experiments

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Absence of ethanol</th>
<th>Presence of ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.97</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>1.02</td>
<td>0.07</td>
</tr>
<tr>
<td>6</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>8</td>
<td>0.98</td>
<td>0.07</td>
</tr>
<tr>
<td>11</td>
<td>0.77</td>
<td>0.06</td>
</tr>
<tr>
<td>12</td>
<td>0.80</td>
<td>0.07</td>
</tr>
<tr>
<td>16</td>
<td>0.33</td>
<td>0.29</td>
</tr>
<tr>
<td>Compound</td>
<td>Ratio 1</td>
<td>Ratio 2</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>17</td>
<td>1.05</td>
<td>0.59</td>
</tr>
<tr>
<td>19</td>
<td>0.99</td>
<td>0.09</td>
</tr>
<tr>
<td>20</td>
<td>1.06</td>
<td>0.75</td>
</tr>
<tr>
<td>23</td>
<td>0.98</td>
<td>0.09</td>
</tr>
<tr>
<td>24</td>
<td>0.99</td>
<td>0.75</td>
</tr>
<tr>
<td>28</td>
<td>1.09</td>
<td>0.06</td>
</tr>
<tr>
<td>29</td>
<td>0.82</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Experiments conducted with 10 mM γ-cyclodextrin in PBS and mixed 1:1 v/v with ethanol. Control ratios defined as in Equation 2.

Conclusion

Non-covalent interactions between a small-molecule guest and γ-cyclodextrin are exceedingly complex. To complement the cyclodextrin cavity, guests must possess at least some of the following characteristics: (1) favorable hydrophobic interactions; (2) attractive Van der Waals forces; (3) favorable thermodynamics for the expulsion of high-energy water; (4) favorable geometry of the guest; and (5) ability to form strong hydrogen bonds. However, even if the guest cannot be encapsulated by the cyclodextrin cavity, the guest can still form an association complex with γ-cyclodextrin that is capable of facilitating energy transfer or fluorescence modulation. In this work we have explored the effect of temperature, salt addition, and ethanol addition to probe these factors. Because these analytes vary in their hydrophobic and hydrophilic structures, they are able to associate with γ-cyclodextrin by a variety of mechanisms, guided primarily by hydrogen bonding and the hydrophobic effect. Interestingly, hydrogen bonding was found to have a leading role in complex formation over the hydrophobic effect, which is in contrast to our previous hypothesis
that the hydrophobic effect would be dominant. There is much intricacy behind non-covalent interactions in general, and the interactions associated with complex formation in γ-cyclodextrin in particular.

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**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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**REFERENCES**


Supporting Information

Understanding Association Complex Formation between Cyclodextrins, Fluorophores, and Small-Molecule Toxicants

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich chemical company or Fisher Scientific and used as received, including compounds 1-30, 32 and 33. Compound 31 (Chart 2) was synthesized following literature-reported procedures.


Fluorescence measurements were recorded on a Shimadzu RF 5301 spectrophotometer with slit widths of 1.5 nm excitation and 1.5 nm emission slit widths. All fluorescence spectra were integrated vs. wavenumber on the X-axis, using OriginPro Version 8.6. Ultrapure water was collected an 18MΩ·cm Millipore Synergy UV. For the temperature studies, a Fisher Scientific Isotemp 6200 R20 was used to control the temperature and the spectrophotometer was equipped with a single constant-temperature cell holder.

General energy transfer procedure. All energy transfer experiments were conducted as follows: 2.5 mL of a 10 mM solution of γ-cyclodextrin dissolved in an aqueous solution (see below) was measured into a cuvette. 20 µL of the analyte (1 mg/mL) and 100 µL of the fluorophore (0.1 mg/mL) were added. After thorough mixing, the solution was excited at two wavelengths: near the analyte’s absorption maximum (defined as “analyte excitation”) and near the fluorophore’s absorption maximum (defined as “fluorophore excitation”). See Table S1 for these values. Three repeat
measurements were taken at each wavelength. The energy transfer efficiencies were calculated according to Equation 1:

\[
\text{% Energy Transfer} = \frac{I_{DA}}{I_A} \times 100\% \tag{1}
\]

where \( I_{DA} \) is defined as the integrated fluorophore emission from indirect excitation and \( I_A \) is the integrated fluorophore emission from direct excitation.

**Control ratio.** Control experiments were conducted as follows: (a) The fluorophore was mixed with γ-cyclodextrin and excited at the excitation wavelength of the analyte (but in the absence of any analyte); and (b) the fluorophore and analyte were both mixed in γ-cyclodextrin and excited at analyte excitation wavelength. The fluorophore emission that resulted from excitation at the analyte wavelength in the absence of the analyte was compared to the fluorophore emission from excitation at the analyte wavelength in the presence of the analyte. The ratio of these two emissions, shown as the “Control ratio” was calculated according to Equation 2:

\[
\text{Control ratio} = \frac{I_{\text{fluorophore-control}}}{I_{\text{fluorophore-analyte}}} \tag{2}
\]

Where \( I_{\text{fluorophore-analyte}} \) is the integration of the fluorophore emission in the presence of the analyte; and \( I_{\text{fluorophore-control}} \) is the integration of the fluorophore emission in the absence of the analyte. For ratios <0.95, legitimate energy transfer was occurring; for ratios between 0.95-1.05, the observed fluorescence response was the result of exciting the fluorophore at wavelength where it has non-zero absorbance; and for ratios >1.05, fluorescence quenching was occurring.

**Cyclodextrin solutions.** For each experiment, 10 mM γ-cyclodextrin solutions were prepared and the energy transfer experiments modified as follows:
1. Salt effects. Sodium chloride and guinadinium hydrochloride were used to investigate salt effects, and 1M stock solutions of each salt were prepared in ultrapure water collected from a 18MΩ·cm Millipore Synergy UV. A 10 mM \( \gamma \)-cyclodextrin solution was then prepared using these salt solutions. A control experiment was also performed with a 0 mM \( \gamma \)-cyclodextrin solution (absence of \( \gamma \)-cyclodextrin, just ultrapure water). Energy transfer experiments were then conducted using the above procedures.

2. Ethanol effects. A 10 mM \( \gamma \)-cyclodextrin solution was prepared in phosphate buffered saline (PBS). For the experiments, 1.25 mL of \( \gamma \)-cyclodextrin and 1.25 mL of ethanol was used in the cuvette (1:1 v/v). Energy transfer experiments were then conducted using the above procedures.

3. Temperature studies. A 10 mM \( \gamma \)-cyclodextrin solution was prepared in PBS. Energy transfer experiments were then conducted using the above procedures at the following temperatures: 5°C, 20°C, 35°C, 50°C, 65°C, and 80°C. The temperature control system used indicated when the desired temperature was reached, and each sample was allowed to sit in the unit for ~1 minute before the fluorescence emission spectrum was collected. This was done to ensure the sample was at the correct temperature.

4. Binding constants. The following concentrations of \( \gamma \)-cyclodextrin solutions were made in PBS: 1 mM, 3 mM, 5 mM, 7 mM, and 10 mM. The next section outlines the procedure for these experiments.

**Binding constant determination.** The binding of each analyte and fluorophore in \( \gamma \)-cyclodextrin was determined by recording the fluorescence emission spectrum of the
analyte in the presence of increasing amounts of γ-cyclodextrin. 2.5 mL of a 1 mM, 3 mM, 5 mM, 7 mM, or 10 mM solution of γ-cyclodextrin dissolved in PBS was measured into a cuvette. 20 µL of the analyte (1 mg/mL in THF) and 100 µL of the fluorophore (0.1 mg/mL in THF) were added, and the solution was excited at the small molecule’s excitation wavelength (see Table S1). The fluorescence of the analyte was integrated with respect to wavenumber. The resulting data was plotted using a Benesi-Hildebrand plot, with 1/[macrocycle] (in M⁻¹) on the X-axis and 1/integrated analyte emission on the Y-axis. Linear fits were obtained for 1:1 and 1:2 complexes. The binding constant was calculated by dividing the y-intercept of the linear fit by the slope of the line.
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*Table S1.* Excitation wavelengths used for each analyte.
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<th>Analyte</th>
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<th>Binding Constant (M$^{-1}$)</th>
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**Table S2.** Binding constants for a 1:2 guest: host complex.
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<th>Error</th>
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Table S3. Temperature results with error to Compound 31.
### Table S4. Pure water results with error to Compound 31.

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<th>Error</th>
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<tr>
<td>23</td>
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<td>0.94</td>
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<tr>
<td>8</td>
<td>27</td>
<td>±0.1</td>
<td>0.76</td>
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<td>11</td>
<td>56.7</td>
<td>±0.6</td>
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<tr>
<td>17</td>
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<td>±0.7</td>
<td>1.02</td>
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<td>3</td>
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<td>±0.01</td>
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<td>±0.00</td>
</tr>
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<td>20</td>
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<td>±0.1</td>
<td>1.05</td>
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### Table S5. Guinadinium chloride results with error to Compound 31.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Energy Transfer (%)</th>
<th>Error</th>
<th>Control Ratio</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>24.3</td>
<td>±0.0</td>
<td>0.82</td>
<td>±0.00</td>
</tr>
<tr>
<td>2</td>
<td>21.6</td>
<td>±0.2</td>
<td>1.00</td>
<td>±0.01</td>
</tr>
<tr>
<td>28</td>
<td>19.2</td>
<td>±0.1</td>
<td>1.12</td>
<td>±0.01</td>
</tr>
<tr>
<td>6</td>
<td>513.5</td>
<td>±0.8</td>
<td>0.07</td>
<td>±0.00</td>
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<tr>
<td>12</td>
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<td>±0.1</td>
<td>0.81</td>
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<td>1.01</td>
<td>±0.00</td>
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<tr>
<td>23</td>
<td>20.7</td>
<td>±0.2</td>
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<td>±0.1</td>
<td>0.78</td>
<td>±0.04</td>
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<td>11</td>
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<td>±0.9</td>
<td>0.35</td>
<td>±0.00</td>
</tr>
<tr>
<td>16</td>
<td>45.3</td>
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<td>±0.00</td>
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<td>±0.01</td>
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<td>1.01</td>
<td>±0.01</td>
</tr>
<tr>
<td>24</td>
<td>23.3</td>
<td>±0.1</td>
<td>1.01</td>
<td>±0.01</td>
</tr>
<tr>
<td>20</td>
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<td>0.93</td>
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### Table S6. Sodium chloride results with error to Compound 31.

<table>
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<th>Energy Transfer (%)</th>
<th>Error</th>
<th>Control Ratio</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
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<td>±0.3</td>
<td>0.79</td>
<td>±0.01</td>
</tr>
<tr>
<td>2</td>
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<td>±0.1</td>
<td>0.96</td>
<td>±0.01</td>
</tr>
<tr>
<td>28</td>
<td>22.4</td>
<td>±0.1</td>
<td>1.07</td>
<td>±0.00</td>
</tr>
<tr>
<td>6</td>
<td>200.2</td>
<td>±4.0</td>
<td>0.11</td>
<td>±0.00</td>
</tr>
<tr>
<td>12</td>
<td>10.4</td>
<td>±0.0</td>
<td>1.01</td>
<td>±0.00</td>
</tr>
<tr>
<td>19</td>
<td>10.0</td>
<td>±0.0</td>
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<td>±0.01</td>
</tr>
<tr>
<td>23</td>
<td>22.6</td>
<td>±0.1</td>
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<td>±0.00</td>
</tr>
<tr>
<td>8</td>
<td>28.7</td>
<td>±0.2</td>
<td>0.70</td>
<td>±0.01</td>
</tr>
<tr>
<td>11</td>
<td>50.2</td>
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<td>±0.00</td>
</tr>
<tr>
<td>16</td>
<td>49.9</td>
<td>±0.2</td>
<td>1.01</td>
<td>±0.00</td>
</tr>
<tr>
<td>17</td>
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<td>±0.5</td>
<td>1.00</td>
<td>±0.01</td>
</tr>
<tr>
<td>3</td>
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<td>±0.3</td>
<td>0.91</td>
<td>±0.00</td>
</tr>
<tr>
<td>24</td>
<td>28.9</td>
<td>±0.1</td>
<td>1.02</td>
<td>±0.01</td>
</tr>
<tr>
<td>20</td>
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<td>±0.1</td>
<td>1.01</td>
<td>±0.01</td>
</tr>
</tbody>
</table>

### Table S7. Absence of ethanol results with error to Compound 31.

<table>
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<th>Analyte</th>
<th>Control Ratio</th>
<th>Error</th>
<th>Energy Transfer (%)</th>
<th>Error</th>
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</thead>
<tbody>
<tr>
<td>29</td>
<td>0.82</td>
<td>± 0.01</td>
<td>28.6</td>
<td>± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>0.97</td>
<td>± 0.01</td>
<td>23.3</td>
<td>± 0.2</td>
</tr>
<tr>
<td>28</td>
<td>1.09</td>
<td>± 0.00</td>
<td>21.7</td>
<td>± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>0.07</td>
<td>± 0.00</td>
<td>324.3</td>
<td>± 2.3</td>
</tr>
<tr>
<td>12</td>
<td>0.80</td>
<td>± 0.00</td>
<td>26.4</td>
<td>± 0.0</td>
</tr>
<tr>
<td>20</td>
<td>0.99</td>
<td>± 0.00</td>
<td>10.7</td>
<td>± 0.0</td>
</tr>
<tr>
<td>19</td>
<td>0.98</td>
<td>± 0.00</td>
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<td>± 0.0</td>
</tr>
<tr>
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</tr>
<tr>
<td>8</td>
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<td>± 0.5</td>
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<td>± 0.7</td>
</tr>
<tr>
<td>16</td>
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<td>± 0.01</td>
<td>65.7</td>
<td>± 0.8</td>
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<tr>
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<td>± 0.00</td>
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<td>± 0.1</td>
</tr>
<tr>
<td>24</td>
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<td>± 0.01</td>
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<td>± 0.7</td>
</tr>
<tr>
<td>Analyte</td>
<td>Control Ratio</td>
<td>Error</td>
<td>Energy Transfer (%)</td>
<td>Error</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>-------</td>
<td>---------------------</td>
<td>-------</td>
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<td>± 0.00</td>
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<td>± 0.4</td>
</tr>
<tr>
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<td>± 0.00</td>
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<td>± 0.1</td>
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<td>± 0.00</td>
<td>19.8</td>
<td>± 0.1</td>
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<td>0.09</td>
<td>± 0.00</td>
<td>8.2</td>
<td>± 0.0</td>
</tr>
<tr>
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<td>0.09</td>
<td>± 0.00</td>
<td>8.2</td>
<td>± 0.1</td>
</tr>
<tr>
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<td>0.07</td>
<td>± 0.00</td>
<td>19.7</td>
<td>± 0.1</td>
</tr>
<tr>
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<td>0.06</td>
<td>± 0.00</td>
<td>21.5</td>
<td>± 0.1</td>
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<td>± 0.1</td>
</tr>
<tr>
<td>24</td>
<td>0.75</td>
<td>± 0.00</td>
<td>4.8</td>
<td>± 0.0</td>
</tr>
</tbody>
</table>

Table S8. Ethanol results with error to Compound 31.
CHAPTER 7
To be Submitted to *Environmental Science and Technology*,

Fluorescence-based detection of environmental toxicants and toxicant metabolites in urine

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Department of Chemistry, University of Rhode Island, Kingston, RI, USA

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Department of Chemistry
University of Rhode Island
Kingston, Rhode Island 02881, USA
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Manuscript 7

Fluorescence-Based Detection of Environmental Toxicants and Toxicant Metabolites in Urine

ABSTRACT

In the wake of anthropogenic releases of toxicants (for example, polycyclic aromatic hydrocarbons (PAHs)), the rapid, sensitive, and selective detection of such environmental pollutants is of great importance to first responders. Many anthropogenic events affect the local population; therefore, the detection of both the parent compound and the numerous metabolites is essential to aid medical personnel in assessing an individuals’ exposure to environmental pollutants. Reported herein is the successful development of such a system using a cyclodextrin host, wherein both the toxicant of interest and a high quantum yield fluorophore are bound within the cavity of the cyclodextrin, and detection occurs via energy transfer from the toxicant to the fluorophore. In this study, samples from a non-smoker and habitual smoker were used to assess differences in analyte response. Efficient energy transfer (and thus toxicant detection) was observed in all cases.

INTRODUCTION

The occurrence, prevalence, and persistence of polycyclic aromatic hydrocarbons (PAHs) in the environment is a significant public health concern,\(^1,2\) as many of these compounds are known or suspected carcinogens,\(^3\) mutagens,\(^4\) and teratogens. Medium-sized PAHs such as compounds 2, 3, 4 and 7 (Chart 1) are of particular concern due to their high toxicity\(^5\) (\textit{i.e.} benzo[a]pyrene 4 is a Group 1 carcinogen).\(^6\) PAHs are often formed from the incomplete combustion of petroleum,
and have been found in environments surrounding the sites of major oil spills,\textsuperscript{7-9} as well as in the blood,\textsuperscript{10} breast milk,\textsuperscript{11} and urine\textsuperscript{12} of inhabitants living in affected areas.

Current methods for PAH detection in complex environments generally use chromatographic separation\textsuperscript{13} (such as gas chromatography\textsuperscript{14} or liquid chromatography\textsuperscript{15}) to separate complex mixtures of compounds, followed by detection of each compound by mass spectrometry.\textsuperscript{16,17} While such methods are highly sensitive for individual environmental toxicants, they lack broad applicability for multiple classes of toxicants in multiple complex environments.\textsuperscript{18} The requirement for time-consuming and often costly separation procedures prior to accurate identification further limits the broad applicability of these approaches, as well as the potential development of high-throughput assays. Because such contaminated environments are often rapidly changing (\textit{i.e.} in a fast-moving stream or at the site of a rapidly spreading oil spill),\textsuperscript{19-20} the requirement for sensitive, selective, and rapid detection methods of PAHs and other environmental toxicants is crucial.

We recently developed a fundamentally new fluorescence-based approach for the detection of aromatic toxicants, by using the aromatic toxicants directly as energy donors in combination with a variety of high-quantum yield fluorophore acceptors.\textsuperscript{21-27} In this approach, energy transfer from the toxicants to the fluorophores occurs when both are bound in the cavity of a cyclodextrin host, and the fluorophore emission via toxicant excitation is used as a highly sensitive and easily tunable read-out signal. This approach has proven to be general for multiple classes of aromatic toxicants in multiple complex environments, including seawater, coconut water, and human plasma and breast milk.\textsuperscript{21}
The utility of fluorescence-based detection of toxicants would be markedly enhanced by the ability to detect environmental toxicants in human urine, as such detection would enable medical professionals to rapidly collect samples and evaluate individuals’ exposure. Moreover, because many aromatic toxicants undergo rapid \textit{in vivo} oxidation, an ideal detection strategy would be able to detect highly polar oxygenated metabolites as well as unmodified non-polar toxicants. Reported herein is the successful development of such a detection strategy: the use of cyclodextrin-promoted, proximity-induced fluorescence energy transfer to detect a wide variety of aromatic toxicants and toxicant metabolites in human urine, and the ability of such a method to distinguish urine samples collected from a habitual smoker and from a non-smoker.

\textbf{Chart 1.} All analytes (1-15) and fluorophores (16,17) targeted for investigation
EXPERIMENTAL SECTION

For full experimental details, see the Electronic Supporting Information. Compounds 1-15 and 17 were purchased from Sigma Aldrich Chemical Company and used as received (Chart 1). Compound 16 was synthesized following literature-reported procedures.30

Urine samples were collected from two anonymous volunteers: one of whom is a habitual smoker (ca. 25 cigarettes/day) and one who has never smoked. The fluorescence emission of the urine samples was measured by excitation at a wide variety of wavelengths to compare the amounts of photophysically active compounds present in both a smoker’s urine and a non-smoker’s urine. Urine samples were then diluted with a 10 mM \(\gamma\)-cyclodextrin solution or a 0 mM \(\gamma\)-cyclodextrin solution prepared in phosphate buffered saline (PBS). Small amounts of fluorophores 16 and 17 were added to the urine samples, and energy transfer efficiencies were determined by exciting the urine at a variety of analyte excitation wavelengths and at the fluorophores’ excitation wavelength, and calculating the efficiencies according to Equation 1:

\[
\text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\%
\]

(Eq 1)

Where \(I_{DA}\) is the integration of the fluorophore emission from analyte excitation and \(I_A\) is the integrated fluorophore emission from direct excitation.

Small amounts of analytes 1-15 were also added to the urine-cyclodextrin-fluorophore samples, and energy transfer efficiencies in the analyte-doped samples were calculated following the same procedures.
Limits of detection for each analyte-fluorophore combination were calculated following literature-reported procedures to construct calibration curves with the analyte concentration on the X-axis and the fluorophore emission via energy transfer on the Y-axis. These curves were then used to determine the minimum analyte concentration necessary to elucidate a detectable and quantifiable fluorescence response.

RESULTS AND DISCUSSION

In the absence of any added analyte or fluorophore, the urine samples displayed excitation wavelength-dependent fluorescence profiles, with the ratio of non-smoker urine fluorescence emission to smoker urine fluorescence emission depicted in Figure 1a. Excitation of the samples below 310 nm resulted in higher fluorescence emission signals for the smoker’s urine compared to that of the non-smoker’s (Figure 1b), whereas sample excitation above 310 nm resulted in higher fluorescence emission of the non-smoker’s urine (Figure 1c). The high level of complexity and vast structural diversity of the human urine metabolome means that it is difficult to definitively assign fluorescence emission peaks to particular chemicals or classes of chemicals; however, the high fluorescence emission of the smoker’s urine from low wavelength excitation is in accordance with literature reports of elevated arylamine levels in the urine collected from individuals exposed to cigarette smoke. Metabolites of smaller PAHs (i.e. 2-naphthol, a known biomarker of cigarette smoke exposure) also have emission maxima in this spectral region.
Figure 1. (A) Ratio of the non-smoker urine fluorescence to smoker urine fluorescence as a function of excitation wavelength; (B) Comparison of the fluorescence emission spectra from 270 nm excitation; (C) Comparison of the fluorescence emission spectra from 350 nm excitation. The black line represents the non-smoker urine emission and the red line represents the smoker urine emission.

The addition of fluorophore 16 or 17 to urine-cyclodextrin mixtures led to efficient energy transfer (Figure 2), with reasonable energy transfer efficiencies observed in the non-smoker urine (with analyte excitation at 360 nm and fluorophore excitation at 460 or 490 nm). These energy transfer efficiencies were even higher in the smoker urine samples under identical conditions, and are likely due to the higher concentrations of innate toxicants present in such samples. These toxicants, which are excited using 360 nm excitation, are able to efficiently participate in the cyclodextrin-promoted energy transfer to fluorophores 16 and 17.

Doping of the urine samples with toxicants 1-15 resulted in urine-cyclodextrin solutions with micromolar concentrations of these analytes. These concentrations are intentionally higher than those reported in undoped urine samples (typically picomolar range)\textsuperscript{38,39} to ensure that the effect of each toxicant and toxicant metabolite can be independently studied and quantified. Selected results of these energy transfer experiments are summarized in Tables 1 and 2, and full tables of all data are shown in the Electronic Supporting Information.
Figure 2. Energy transfer efficiencies in undoped urine samples. The black line represents emission from 360 nm excitation and the red line represents emission from fluorophore excitation (460 or 490 nm). (A) 4-16 in non-smoker urine; (B) 4-17 in non-smoker urine; (C) 4-16 in smoker urine; and (D) 4-17 in smoker urine.

Table 1. Selected energy transfer efficiencies in analyte-doped samples to fluorophore 16

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fluorophore</th>
<th>Non-Smoker Urine</th>
<th>Smoker Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>42.9</td>
<td>32.8</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>b</td>
<td>b</td>
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<td>3</td>
<td>16</td>
<td>144.3</td>
<td>91.1</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>32.3</td>
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</tr>
<tr>
<td>11</td>
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<td>36.9</td>
<td>32.6</td>
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<td>12</td>
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</tr>
<tr>
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<td>16</td>
<td>32.5</td>
<td>b</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>39.8</td>
<td>19.9</td>
</tr>
</tbody>
</table>

\(a\) All reported results represent the average of 4 trials

\(b\) Excessive overlap between the analyte and fluorophore emission prevented accurate integration
### Table 2. Selected energy transfer efficiencies in analyte-doped samples to fluorophore 17<sup>a</sup>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fluorophore</th>
<th>Non-Smoker Urine</th>
<th>Smoker Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mM γ-CD</td>
<td>0 mM γ-CD</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>4.3</td>
<td>4.0</td>
</tr>
<tr>
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<td>13</td>
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</tr>
<tr>
<td>15</td>
<td>17</td>
<td>14.4</td>
<td>1.5</td>
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</tbody>
</table>

<sup>a</sup> All reported results represent the average of 4 trials

Several aspects of this data merit further discussion. We have already established that cyclodextrin-promoted energy transfer works efficiently for a variety of non-polar PAH energy donors, in combination with fluorophores 16 and 17 as energy acceptors. This energy transfer is a result of the hydrophobic binding of the analytes in the cyclodextrin cavity together with the fluorophore, resulting in enforced close proximity between the donor and acceptor and high energy transfer efficiencies. For several of the larger sized PAHs (i.e. 2, 3, 7) we observed significant cyclodextrin-free association, which requires hydrophobic association of those compounds with the aromatic fluorophores to promote efficient energy transfer.

In contrast to these previously targeted non-polar analytes, metabolites 10-13 are oxygenated and highly polar, and are formed in vivo from cytochrome P450-mediated oxidation of PAHs. The extensive oxygenation decreases the hydrophobic character of the analytes; nonetheless, in all cases these PAH metabolites...
participated efficiently in cyclodextrin-promoted energy transfer to fluorophores 16 and 17 (Figure 3). The energy transfer efficiencies for most of these analyte-fluorophore combinations are comparable in the smoker and non-smoker urine samples, as the innate toxicant differences are likely insignificant compared to the concentrations of analytes added to the samples.

Figure 3. Energy transfer efficiencies in doped urine samples. The black line represents emission from analyte excitation and the red line represents emission from fluorophore excitation. (A) 10-16 in non-smoker urine; (B) 11-16 in non-smoker urine; (C) 10-16 in smoker urine; and (D) 11-16 in smoker urine.

The successful demonstration of cyclodextrin-promoted energy transfer from oxidized PAH metabolites is likely a result of the metabolites forming hydrogen bonds to the cyclodextrin host structure. The importance of hydrogen bond formation in promoting cyclodextrin-binding has been well-documented in the literature; as metabolites 10-13 contain both hydrogen bond accepting carbonyl groups and hydrogen bond donating hydroxyl groups, they are able to form strong and multi-point hydrogen bonds to facilitate successful complex formation. This hypothesis is further
supported by data that showed a marked increase in the energy transfer efficiencies in the presence of 10 mM γ-cyclodextrin compared to energy transfer efficiencies observed in a 0 mM solution (Tables 1 and 2); our previous work on hydrophobically-driven binding and energy transfer resulted in significant degrees of cyclodextrin-free hydrophobic association between the donor and acceptor, often resulting in high energy transfer efficiencies in cyclodextrin-free solutions.

The practical utility of this detection method for monitoring individuals’ exposure levels to aromatic toxicants requires general applicability for multiple classes of toxicants, rapid detection of the toxicants, selective detection of a particular toxicant, and sensitive detection of low toxicant concentrations. This method has widespread generality for detecting non-polar toxicants, polar toxicant metabolites, and heteroaromatic compounds (i.e. compound 15), and the fluorescence energy transfer provides a rapid read-out signal. Selective detection of a particular toxicant or toxicant metabolite can be accomplished using array-based detection schemes with statistical analyses of toxicant response patterns; preliminary studies indicate that arrays constructed in these systems provide excellent selectivity in toxicant identification.

The sensitivity of this detection method was determined by calculating the limits of detection (minimal sample concentration that will generate a distinguishable signal) and limits of quantification (minimal sample concentration that will generate a quantifiable signal) for all analyte-fluorophore combinations in the non-smoker urine samples, and selected examples are highlighted in Table 3. Both the aromatic toxicants and the oxidized toxicant metabolites can be quantified at micromolar concentrations;
current efforts are focused on lowering these detection limits even further to provide optimal sensitivity in toxicant and toxicant metabolite detection.

Table 3. Limits of quantification for select analyte-fluorophore combinations

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fluorophore</th>
<th>Limit of Quantification (μM)</th>
<th>Fluorophore</th>
<th>Limit of Quantification (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>27.1</td>
<td>17</td>
<td>a</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>b</td>
<td>17</td>
<td>53.7</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>72.3</td>
<td>17</td>
<td>3.6</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>b</td>
<td>17</td>
<td>153.5</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>60.0</td>
<td>17</td>
<td>b</td>
</tr>
</tbody>
</table>

a A poor linear fit was obtained
b Attempts to calculate limits of quantification in this instance led to nonsensical values; current efforts are focused on understanding these results.

CONCLUSION

In conclusion, there are an exceptionally large number of environmental toxicants that individuals are exposed to throughout their lifetimes. These toxicants are found in the environment as a result of anthropogenic oil spills and chemical leaks, through highly polluting manufacturing and waste disposal processes, and through the use of a large number of commercial products. The development of new toxicant detection methods, such as those reported herein, addresses a pressing public health need, and provides a crucial tool for environmental researchers, medical professionals, and first responders working on toxicant exposure-related problems.

ACKNOWLEDGMENT

This work is funded by grant 1R21CA185435-01 from the National Cancer Institute, by a grant from the Rhode Island Foundation, and by a grant from the Gulf of Mexico Research Initiative.
REFERENCES


10. Perez, C.; Velando, A.; Munilla, I.; Lopez-Alonso, M.; Oro, D. Monitoring polycyclic aromatic hydrocarbon pollution in the marine environment after the


Supporting Information

Fluorescence-Based Detection of Environmental Toxicants and Toxicant Metabolites in Urine

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Chemical Company and used as received, unless otherwise noted. UV-Visible spectra were obtained using an Agilent 8453 spectrometer equipped with a photodiode array detector. Fluorescence spectra were obtained using a Shimadzu RF-5301PC spectrophotofluorimeter.

Urine samples were collected from two anonymous donors – one donor who smoked approximately 25 cigarettes per day, and one donor who has never smoked.

ANALYTE DETAILS

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Excitation wavelength</th>
<th>Final concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>360 nm</td>
<td>43.2</td>
</tr>
<tr>
<td>2</td>
<td>360 nm</td>
<td>33.7</td>
</tr>
<tr>
<td>3</td>
<td>380 nm</td>
<td>30.5</td>
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<tr>
<td>4</td>
<td>360 nm</td>
<td>30.5</td>
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<tr>
<td>5</td>
<td>360 nm</td>
<td>38.0</td>
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<tr>
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<td>290 nm</td>
<td>43.2</td>
</tr>
<tr>
<td>7</td>
<td>325 nm</td>
<td>33.7</td>
</tr>
<tr>
<td>8</td>
<td>340 nm</td>
<td>44.4</td>
</tr>
<tr>
<td>9</td>
<td>360 nm</td>
<td>28.9</td>
</tr>
<tr>
<td>10</td>
<td>360 nm</td>
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<td>11</td>
<td>360 nm</td>
<td>28.5</td>
</tr>
<tr>
<td>12</td>
<td>360 nm</td>
<td>32.0</td>
</tr>
<tr>
<td>13</td>
<td>360 nm</td>
<td>39.6</td>
</tr>
<tr>
<td>14</td>
<td>277 nm</td>
<td>71.1</td>
</tr>
<tr>
<td>15</td>
<td>340 nm</td>
<td>46.0</td>
</tr>
</tbody>
</table>

Table S1. Analyte details, including excitation wavelength and final concentrations.
ENERGY TRANSFER EXPERIMENTAL DETAILS

Undoped urine samples: Smoker urine (collected from a donor who smoked approximately 25 cigarettes per day) and non-smoker urine (collected from a donor who never smoked) were diluted with a 10 mM solution of γ-cyclodextrin in phosphate buffered saline (PBS) (1:1 v/v). 100 µL of fluorophore 16 or 17 (0.1 mg/mL in THF) was added to the urine-cyclodextrin mixture, and the contents of the vial were vigorously shaken by hand for approximately 1 minute to ensure thorough mixing. The resulting solution was excited at the excitation wavelength of each analyte and at the excitation wavelength of the fluorophore, and the energy transfer efficiencies were calculated according to Equation 1:

\[ \text{Energy transfer efficiency} = \frac{I_{DA}}{I_A} \times 100\% \]

(Eq 1)

Where \( I_{DA} \) is the integration of the fluorophore emission from analyte excitation and \( I_A \) is the integrated fluorophore emission from direct excitation. Control experiments were also conducted in which the 10 mM γ-cyclodextrin solution was replaced with a 0 mM solution, and the same procedure was followed.

Doped urine samples: 20 µL of analytes 1-15 (1 mg/mL solution in THF) and 100 µL of fluorophore 16 or 17 were added to a 1:1 mixture of urine and a 10 mM γ-cyclodextrin solution. After thorough mixing, the resulting solution was excited at the excitation wavelength of each analyte and at the excitation wavelength of the fluorophore, and the “doped” energy transfer efficiencies were calculated as in Equation 1.
**Control experiments**: Control experiments were also conducted in which the 10 mM γ-cyclodextrin solution was replaced with a 0 mM solution, and the same procedure was followed.

All experiments were repeated 4 times, and the values reported are averages of the results.

**ANALYTE COMPARISON EXPERIMENTAL DETAILS**

These experiments were designed to determine the emission of the fluorophores from excitation at various wavelengths (in the absence of the analyte) and compare it to the emission of fluorophores at the same wavelengths in the presence of the analyte. These experiments were conducted as follows: (a) The fluorophore was mixed with γ-cyclodextrin and urine and excited at the excitation wavelength of the analyte (but in the absence of any additional analyte); and (b) the doped analyte was added to the cyclodextrin-urine mixture and excited at the analyte excitation wavelength. The fluorophore emission that resulted from excitation at the analyte wavelength in the absence of the analyte was compared to the fluorophore emission from excitation at the analyte wavelength in the presence of the analyte. The ratio of these two emissions is defined as: Fluorophore emission via low wavelength excitation in the absence of an analyte/ fluorophore emission via low wavelength excitation in the presence of the analyte.

**EXPERIMENTAL DETAILS FOR LIMIT OF DETECTION EXPERIMENTS**

The limit of detection (LOD) is defined as the lowest concentration of analyte at which a signal can be detected. The limit of quantification is defined at the lowest
concentration of analyte that can be accurately quantified. These experiments were conducted following literature-reported procedures.


To determine the limit of detection (LOD) and limit of quantification (LOQ), each fluorophore-analyte combination was examined in the following manner:

1. 2.5 mL of 10 mM γ-cyclodextrin in phosphate-buffered saline (PBS) was measured into a cuvette and 100 μL of a fluorophore solution in THF was added. The solution was excited at the analyte’s excitation wavelength (see table of wavelengths below) and the fluorescence emission spectrum was recorded. Six repeat measurements were made for the fluorescence emission spectra.

2. 20 μL of a 1 mg/mL analyte solution in THF was added to the cuvette and the solution was again excited at the analyte excitation wavelength. Six repeat measurements were taken.

3. Step 2 was repeated for 40 μL of analyte, 60 μL of analyte, 80 μL of analyte, and 100 μL of analyte. In each case, the solution was excited at the analyte excitation wavelength and the fluorescence emission spectrum was recorded four times.

4. All fluorescence emission spectra were integrated vs. wavenumber, and we generated calibration curves with the analyte concentration on the X-axis (in μM) and the integrated fluorophore emission on the Y-axis. The curve was then fitted to a straight line and an equation for the line was determined.
5. For each case, the fluorophore with γ-cyclodextrin (before any analyte was added) was also excited at the excitation wavelength for the analyte, and the fluorescence emission spectrum was recorded (as per step 1). These measurements are referred to as the “blank.”

6. The limit of the blank is defined according to the following equation:

\[ \text{LoB}_{\text{LOD}} = \bar{m}_{\text{blank}} + 3(\text{SD}_{\text{blank}}) \]

Where \( \bar{m} \) is the mean of the blank integrations and \( \text{SD} \) is the standard deviation.

7. The limit of the blank was then entered into the equation determined in step 4 (for the \( y \) value), and the corresponding \( X \) value was determined. This value provided the LOD in \( \mu \text{M} \).

8. The limit of quantification (LOQ) was calculated in a similar way to the limit of detection. First, the limit of the blank for quantification was determined according to the following equation:

\[ \text{LoB}_{\text{LOQ}} = \bar{m}_{\text{blank}} + 10(\text{SD}_{\text{blank}}) \]

This value was entered into the equation determined in step 4 (for the \( y \) value), and the corresponding \( X \) value was determined to be the limit of quantification in \( \mu \text{M} \).

**SUMMARY TABLES**

Fluorescence of urine samples was determined in the absence of any additional analyte or fluorophore. These values were determined by exciting the urine samples at a variety of excitation wavelengths, and integrating the resulting fluorescence emission vs. wavenumber on the X-axis. The ratio of the non-smoker urine fluorescence emission to smoker urine fluorescence emission was calculated, and the results are summarized in the following table:
<table>
<thead>
<tr>
<th>Excitation wavelength</th>
<th>Ratio of non-smoker/smoker emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>233 nm</td>
<td>0.97 ± 0.00</td>
</tr>
<tr>
<td>250 nm</td>
<td>0.93 ± 0.00</td>
</tr>
<tr>
<td>260 nm</td>
<td>0.84 ± 0.01</td>
</tr>
<tr>
<td>270 nm</td>
<td>0.75 ± 0.00</td>
</tr>
<tr>
<td>277 nm</td>
<td>0.70 ± 0.00</td>
</tr>
<tr>
<td>290 nm</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>310 nm</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>320 nm</td>
<td>0.81 ± 0.01</td>
</tr>
<tr>
<td>325 nm</td>
<td>1.05 ± 0.01</td>
</tr>
<tr>
<td>340nm</td>
<td>1.74 ± 0.01</td>
</tr>
<tr>
<td>350nm</td>
<td>2.53 ± 0.01</td>
</tr>
<tr>
<td>360nm</td>
<td>2.89 ± 0.01</td>
</tr>
<tr>
<td>365nm</td>
<td>3.15 ± 0.00</td>
</tr>
<tr>
<td>370nm</td>
<td>3.02 ± 0.00</td>
</tr>
<tr>
<td>380nm</td>
<td>2.93 ± 0.01</td>
</tr>
<tr>
<td>385 nm</td>
<td>2.80 ± 0.01</td>
</tr>
<tr>
<td>420nm</td>
<td>2.07 ± 0.02</td>
</tr>
<tr>
<td>440 nm</td>
<td>1.88 ± 0.01</td>
</tr>
<tr>
<td>460nm</td>
<td>1.82 ± 0.03</td>
</tr>
<tr>
<td>490nm</td>
<td>1.59 ± 0.01</td>
</tr>
<tr>
<td>620nm</td>
<td>0.99 ± 0.00</td>
</tr>
</tbody>
</table>

**Table S2.** Fluorescence of urine samples determined in the absence of any additional analyte or fluorophore.
No energy transfer peak was observed.

Excess overlap between the analyte and fluorophore emission prevented accurate integration.

**Table S3.** Energy transfer efficiencies in doped urine samples with 10 mM γ-cyclodextrin.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluophore</th>
<th>regular urine</th>
<th>smoker urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>42.9 ± 0.6</td>
<td>36.1 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>38.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>144.3 ± 2.8</td>
<td>63.7 ± 2.0</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>106.9 ± 5.9</td>
<td>46.3 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>30.5 ± 0.3</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>73.2 ± 0.2</td>
<td>23.6 ± 0.2</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>50.5 ± 0.1</td>
<td>49.0 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>32.3 ± 0.1</td>
<td>51.1 ± 0.2</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>36.9 ± 0.1</td>
<td>36.6 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>30.9 ± 0.3</td>
<td>30.5 ± 0.1</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>32.5 ± 0.0</td>
<td>24.5 ± 0.7</td>
</tr>
<tr>
<td>14</td>
<td>16</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>39.8 ± 0.5</td>
<td>41.2 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>4.3 ± 0.0</td>
<td>10.2 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>12.2 ± 0.1</td>
<td>13.2 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>8.4 ± 0.1</td>
<td>19.7 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>b</td>
<td>23.4 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>47.7 ± 0.3</td>
<td>13.5 ± 0.1</td>
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<tr>
<td>6</td>
<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>2.1 ± 0.0</td>
<td>3.4 ± 0.0</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>12.8 ± 0.0</td>
<td>7.4 ± 0.0</td>
</tr>
<tr>
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<td>17</td>
<td>31.0 ± 0.1</td>
<td>13.9 ± 0.1</td>
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<td>17</td>
<td>5.1 ± 0.0</td>
<td>10.4 ± 0.0</td>
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<td>17</td>
<td>17.6 ± 0.0</td>
<td>6.9 ± 0.1</td>
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<td>17</td>
<td>11.8 ± 0.0</td>
<td>9.0 ± 0.0</td>
</tr>
<tr>
<td>13</td>
<td>17</td>
<td>15.3 ± 0.0</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>14.4 ± 0.1</td>
<td>6.9 ± 0.1</td>
</tr>
</tbody>
</table>
Table S4. Energy transfer efficiencies in doped urine samples with 0 mM γ-cyclodextrin.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluorophore</th>
<th>regular urine</th>
<th>smoker urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>32.8 ± 0.9</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
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<td>3</td>
<td>16</td>
<td>91.1 ± 18.0</td>
<td>97.8 ± 3.8</td>
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<td>16</td>
<td>34.5 ± 3.1</td>
<td>41.2 ± 0.1</td>
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<td>16</td>
<td>102.2 ± 6.4</td>
<td>41.0 ± 0.2</td>
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<td>16</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>a</td>
<td>9.8 ± 0.0</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>43.5 ± 0.3</td>
<td>51.1 ± 0.1</td>
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<tr>
<td>10</td>
<td>16</td>
<td>b</td>
<td>51.5 ± 0.4</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>32.6 ± 0.4</td>
<td>36.4 ± 0.3</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>13</td>
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<td>a</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>19.9 ± 0.2</td>
<td>66.9 ± 0.7</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>4.0 ± 0.0</td>
<td>5.8 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>8.7 ± 0.0</td>
<td>13.4 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>15.7 ± 0.1</td>
<td>6.9 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>6.9 ± 0.0</td>
<td>b</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>4.7 ± 0.0</td>
<td>9.0 ± 0.0</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>a</td>
<td>2.5 ± 0.0</td>
</tr>
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<td>17</td>
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<td>8.2 ± 0.3</td>
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<td>6.3 ± 0.0</td>
<td>10.5 ± 0.1</td>
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<td>5.9 ± 0.0</td>
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<td>17</td>
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<td>6.7 ± 0.0</td>
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<td>17</td>
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<td>7.8 ± 0.0</td>
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<tr>
<td>14</td>
<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>1.5 ± 0.0</td>
<td>5.6 ± 0.0</td>
</tr>
</tbody>
</table>
No energy transfer peak was observed.

Excess overlap between the analyte and fluorophore emission prevented accurate integration.

Table S5. Energy transfer efficiencies in undoped samples with 10 mM γ-cyclodextrin.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluorophore</th>
<th>non-smoker urine</th>
<th>smoker urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>39.1 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>13.8 ± 0.1</td>
<td>38.2 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>35.9 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>38.2 ± 0.4</td>
</tr>
<tr>
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<td>16</td>
<td>12.1 ± 0.1</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>12.1 ± 0.1</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>37.8 ± 0.4</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>34.9 ± 0.4</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>34.4 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>23.1 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>12.1 ± 0.1</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>12.1 ± 0.1</td>
<td>8.7 ± 0.1</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>38.2 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>38.2 ± 0.1</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>15.0 ± 0.1</td>
<td>34.9 ± 0.4</td>
</tr>
</tbody>
</table>

a No energy transfer peak was observed

b Excess overlap between the analyte and fluorophore emission prevented accurate integration
No energy transfer peak was observed

Excess overlap between the analyte and fluorophore emission prevented accurate integration

Table S6. Energy transfer efficiencies in undoped samples with 0 mM γ-cyclodextrin.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluorophore</th>
<th>non-smoker urine</th>
<th>smoker urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>41.5 ± 1.2</td>
<td>39.8 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>31.7 ± 0.7</td>
<td>39.1 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>35.6 ± 0.7</td>
<td>37.2 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>35.6 ± 0.7</td>
<td>b</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>11.5 ± 0.1</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>34.9 ± 0.3</td>
<td>37.8 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>38.3 ± 0.1</td>
<td>51.7 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>30.8 ± 0.2</td>
<td>36.0 ± 0.5</td>
</tr>
<tr>
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<td>16</td>
<td>32.1 ± 0.1</td>
<td>48.9 ± 0.4</td>
</tr>
<tr>
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<td>16</td>
<td>27.6 ± 0.1</td>
<td>22.1 ± 0.2</td>
</tr>
<tr>
<td>14</td>
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<td>a</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>3.9 ± 0.0</td>
<td>5.8 ± 0.0</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>10.5 ± 0.0</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>5.0 ± 0.0</td>
<td>b</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>4.5 ± 0.0</td>
<td>9.0 ± 0.0</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>2.8 ± 0.0</td>
<td>2.5 ± 0.0</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>2.8 ± 0.0</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>5.4 ± 0.0</td>
<td>8.8 ± 0.0</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>5.5 ± 0.0</td>
<td>6.0 ± 0.0</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>4.2 ± 0.0</td>
<td>9.8 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>3.7 ± 0.0</td>
<td>6.6 ± 0.0</td>
</tr>
<tr>
<td>13</td>
<td>17</td>
<td>5.3 ± 0.1</td>
<td>7.6 ± 0.0</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>1.4 ± 0.0</td>
<td>5.6 ± 0.0</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

*b* Excess overlap between the analyte and fluorophore emission prevented accurate integration
A no energy transfer peak was observed.

Excess overlap between the analyte and fluorophore emission prevented accurate integration.

**Table S7.** Analyte comparison ratios with 10 mM γ-cyclodextrin.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluorophore</th>
<th>regular urine</th>
<th>smoker urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>1.00 ± 0.01</td>
<td>1.03 ± 0.00</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td><em>b</em></td>
<td>0.94 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>0.14 ± 0.00</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td><em>b</em></td>
<td><em>b</em></td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td><em>a</em></td>
<td><em>a</em></td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>0.63 ± 0.01</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>0.96 ± 0.01</td>
<td><em>b</em></td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>0.55 ± 0.00</td>
<td>0.78 ± 0.00</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>0.84 ± 0.00</td>
<td>1.04 ± 0.00</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>0.70 ± 0.00</td>
<td>0.91 ± 0.00</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>0.88 ± 0.00</td>
<td>1.10 ± 0.01</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>0.80 ± 0.00</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
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<td>16</td>
<td><em>a</em></td>
<td><em>a</em></td>
</tr>
<tr>
<td>14</td>
<td>16</td>
<td><em>b</em></td>
<td><em>b</em></td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>0.98 ± 0.01</td>
<td>0.57 ± 0.00</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>1.15 ± 0.01</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td><em>b</em></td>
<td><em>b</em></td>
</tr>
<tr>
<td>18</td>
<td>17</td>
<td>0.69 ± 0.01</td>
<td><em>b</em></td>
</tr>
<tr>
<td>19</td>
<td>17</td>
<td><em>b</em></td>
<td>0.75 ± 0.01</td>
</tr>
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<td>17</td>
<td><em>a</em></td>
<td><em>a</em></td>
</tr>
<tr>
<td>21</td>
<td>17</td>
<td>0.97 ± 0.02</td>
<td>1.02 ± 0.01</td>
</tr>
<tr>
<td>22</td>
<td>17</td>
<td>1.02 ± 0.02</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>23</td>
<td>17</td>
<td>0.52 ± 0.00</td>
<td>0.74 ± 0.00</td>
</tr>
<tr>
<td>24</td>
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<td>1.01 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>25</td>
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<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>26</td>
<td>17</td>
<td>1.02 ± 0.00</td>
<td>1.04 ± 0.01</td>
</tr>
<tr>
<td>27</td>
<td>17</td>
<td>1.05 ± 0.01</td>
<td>0.99 ± 0.02</td>
</tr>
<tr>
<td>28</td>
<td>17</td>
<td><em>a</em></td>
<td><em>a</em></td>
</tr>
<tr>
<td>29</td>
<td>17</td>
<td>0.92 ± 0.01</td>
<td>0.99 ± 0.02</td>
</tr>
</tbody>
</table>
No energy transfer peak was observed

Excess overlap between the analyte and fluorophore emission prevented accurate integration

Table S8. Analyte comparison ratios with 0 mM γ-cyclodextrin.

<table>
<thead>
<tr>
<th>analyte</th>
<th>fluorophore</th>
<th>regular urine</th>
<th>smoker urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>0.97 ± 0.03</td>
<td>0.97 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>0.30 ± 0.05</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>0.86 ± 0.04</td>
<td>1.01 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>0.50 ± 0.01</td>
<td>b</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>a</td>
<td>1.01 ± 0.01</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>0.92 ± 0.00</td>
<td>0.78 ± 0.00</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>b</td>
<td>1.02 ± 0.01</td>
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<td>16</td>
<td>b</td>
<td>1.02 ± 0.00</td>
</tr>
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<td>16</td>
<td>b</td>
<td>1.13 ± 0.01</td>
</tr>
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<td>16</td>
<td>b</td>
<td>1.06 ± 0.01</td>
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<td>16</td>
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<td>a</td>
</tr>
<tr>
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<td>16</td>
<td>0.47 ± 0.01</td>
<td>0.36 ± 0.00</td>
</tr>
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<td>17</td>
<td>1.00 ± 0.01</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>0.74 ± 0.01</td>
<td>b</td>
</tr>
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<td>17</td>
<td>0.71 ± 0.01</td>
<td>0.87 ± 0.01</td>
</tr>
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<td>0.98 ± 0.00</td>
</tr>
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<td>17</td>
<td>a</td>
<td>a</td>
</tr>
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<td>17</td>
<td>a</td>
<td>1.07 ± 0.01</td>
</tr>
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<td>7</td>
<td>17</td>
<td>1.00 ± 0.01</td>
<td>0.99 ± 0.02</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>0.87 ± 0.01</td>
<td>0.85 ± 0.00</td>
</tr>
<tr>
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<td>17</td>
<td>1.01 ± 0.00</td>
<td>1.01 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>1.11 ± 0.01</td>
<td>1.04 ± 0.01</td>
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<tr>
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<td>17</td>
<td>1.08 ± 0.01</td>
<td>b</td>
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<td>17</td>
<td>0.98 ± 0.02</td>
<td>1.02 ± 0.00</td>
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<td>17</td>
<td>a</td>
<td>a</td>
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<td>17</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>0.93 ± 0.02</td>
<td>1.01 ± 0.00</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

*b* Excess overlap between the analyte and fluorophore emission prevented accurate integration
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fluorophore</th>
<th>Equation</th>
<th>$R^2$</th>
<th>LOD (µM)</th>
<th>LOQ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>$y = 11.535x + 26749$</td>
<td>0.7245</td>
<td>8.2</td>
<td>61.0</td>
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<td>0.9351</td>
<td>-70.3</td>
<td>36.3</td>
</tr>
<tr>
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<td>16</td>
<td>$y = 2646.7x + 83966$</td>
<td>0.9786</td>
<td>-22.4</td>
<td>33.0</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>$y = 1225.7x + 384543$</td>
<td>0.4399</td>
<td>-291.8</td>
<td>-18.3</td>
</tr>
<tr>
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<td>16</td>
<td>$y = 2622.3x + 35119$</td>
<td>0.9902</td>
<td>-3.2</td>
<td>42.7</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>$y = 2646.7x + 83966$</td>
<td>0.9786</td>
<td>-22.4</td>
<td>33.0</td>
</tr>
<tr>
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<td>16</td>
<td>$y = 269.03x + 25462$</td>
<td>0.9274</td>
<td>-15.0</td>
<td>37.7</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
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<td>0.4336</td>
<td>37.9</td>
<td>-81.4</td>
</tr>
<tr>
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<td>16</td>
<td>$y = 378.12x + 43115$</td>
<td>0.9393</td>
<td>-43.0</td>
<td>20.9</td>
</tr>
<tr>
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<td>16</td>
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<td>10.4</td>
</tr>
<tr>
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<td>16</td>
<td>$y = 38.009x + 31503$</td>
<td>0.9841</td>
<td>-122.6</td>
<td>50.8</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>$y = 1172.5x + 177247$</td>
<td>0.7162</td>
<td>-127.9</td>
<td>-2.2</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>$y = 819.93x + 67624$</td>
<td>0.9101</td>
<td>-49.2</td>
<td>17.7</td>
</tr>
<tr>
<td>14</td>
<td>16</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>$y = 280.66x + 47883$</td>
<td>0.9267</td>
<td>-73.4</td>
<td>18.0</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>$y = 262.41x + 13084$</td>
<td>0.9614</td>
<td>57.7</td>
<td>68.2</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>$y = 280.66x + 47883$</td>
<td>0.9267</td>
<td>-73.4</td>
<td>18.0</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
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<tr>
<td>6</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
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<tr>
<td>9</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>13</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>$y = 3.746x + 21416$</td>
<td>0.8716</td>
<td>306.8</td>
<td>236.3</td>
</tr>
</tbody>
</table>

*a* No energy transfer peak was observed

*b* A poor linear fit was observed

*c* Efforts to calculate LODs in these cases led to nonsensical values. Current efforts are focused on understanding these cases

**Table S9.** Limit of detection summary table.
SUMMARY FIGURES FOR ALL LOD EXPERIMENTS

S1a. Analyte 1 – Fluorophore 16

S1b. Analyte 2 – Fluorophore 16

S1c. Analyte 3 – Fluorophore 16

S1d. Analyte 4 – Fluorophore 16

S1e. Analyte 5 – Fluorophore 16

S1f. Analyte 7 – Fluorophore 16

Analyte 6 – Fluorophore 16

No energy transfer peak was observed
S1g. Analyte 8 – Fluorophore 16

\[ y = -30.097x + 31556 \]
\[ R^2 = 0.4336 \]

S1h. Analyte 9 – Fluorophore 16

\[ y = 378.12x + 43115 \]
\[ R^2 = 0.9933 \]

S1i. Analyte 10 – Fluorophore 16

\[ y = -0.7836x + 32056 \]
\[ R^2 = 0.0566 \]

S1j. Analyte 11 – Fluorophore 16

\[ y = 123.66x + 37285 \]
\[ R^2 = 0.8463 \]

S1k. Analyte 12 – Fluorophore 16

\[ y = -70.152x + 32133 \]
\[ R^2 = 0.9823 \]

S1l. Analyte 13 – Fluorophore 16

\[ y = 38.009x + 31503 \]
\[ R^2 = 0.9841 \]

Analyte 14 – Fluorophore 16

*No energy transfer peak was observed*
S1m. Analyte 15 – Fluorophore 16

\[ y = 262.41x + 13084 \]
\[ R^2 = 0.9614 \]

S1n. Analyte 1 – Fluorophore 17

\[ y = -0.0025x + 28117 \]
\[ R^2 = 3E-6 \]

S1o. Analyte 2 – Fluorophore 17

\[ y = 43.528x + 40612 \]
\[ R^2 = 0.987 \]

S1p. Analyte 3 – Fluorophore 17

\[ y = 470.37x + 41086 \]
\[ R^2 = 0.9121 \]

S1q. Analyte 4 – Fluorophore 17

\[ y = 1172.5x + 177247 \]
\[ R^2 = 0.7162 \]

S1r. Analyte 5 – Fluorophore 17

\[ y = 819.93x + 67624 \]
\[ R^2 = 0.9101 \]
Analyte 6 – Fluorophore 17

No energy transfer peak was observed

S1s. Analyte 7 – Fluorophore 17

S1t. Analyte 8 – Fluorophore 17

S1u. Analyte 9 – Fluorophore 17

S1v. Analyte 10 – Fluorophore 17

S1w. Analyte 11 – Fluorophore 17

S1x. Analyte 12 – Fluorophore 17
S1y. Analyte 13 – Fluorophore 17

\[ y = 20.336x + 24666 \]
\[ R^2 = 0.975 \]

No energy transfer peak was observed

S1z. Analyte 15 – Fluorophore 17

\[ y = 23.709x + 23454 \]
\[ R^2 = 0.9577 \]

Figure S1a-S1z. Summary figures for LOD experiments.