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BIOLUMINESCENCE INHIBITION ASSAY FOR THE DETECTION OF HYDROXYLATED POLYCHLORINATED BIPHENYLS

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Abstract

Hydroxylated polychlorinated biphenyls (OH-PCBs) are an important class of contaminants that mainly originate from polychlorinated biphenyl metabolism. They may conceivably be as dangerous and persistent as the parent compounds; most prominently, OH-PCBs are endocrine disruptors. Due to increasing evidence of the presence of OH-PCBs in the environment and in living organisms, including humans, and of their toxicity, methods of detection for OH-PCBs are needed in the environmental and medical fields. Herein we describe the development and optimization of a protein-based inhibition assay for the quantification of OH-PCBs. Specifically, the photoprotein aequorin was utilized for the detection of OH-PCBs. We hypothesized that OH-PCBs interact with aequorin and we established that OH-PCBs actually inhibit the bioluminescence of aequorin in a dose-dependent manner. We took advantage of this phenomenon to develop an assay that is capable of detecting a wide variety of OH-PCBs with a range of detection limits, the best detection limit being 11 nM for the compound 2-hydroxy-2',3,4',5',6-pentachlorobiphenyl. The viability of this system for the screening of OH-PCBs in spiked biological and environmental samples was also established. We envision the implementation of this novel bioluminescence inhibition assay as a rapid, sensitive and cost-effective method for monitoring OH-PCBs. Furthermore, to the best of our knowledge this is the first time aequorin has been employed to detect an analyte by the inhibition of its bioluminescence reaction. Hence, this strategy may prove to be a general approach for the development of a new generation of protein-based inhibition assays.

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Supporting Information. Figure S-1. Response curve for coelenterazine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Introduction

Polychlorinated biphenyls (PCBs) have long been investigated for their irreversible environmental and biological damages. PCBs are toxic and persistent chemicals that were primarily used as insulating fluids and coolants in heavy-duty electrical equipment in power plants, industries and large buildings across the United States. Among the characteristics of PCBs that made them so desirable for these uses was their extreme stability. As a result of this stability, PCBs are highly resistant to degradation in nature, which leads to their accumulation in the environment as well as bioaccumulation in living organisms. In bacteria and higher organisms, including humans, PCBs are biotransformed by cytochrome P-450 mono-oxygenases and metabolized to hydroxylated polychlorinated biphenyls (OH-PCBs) and methyl-sulfone PCBs.¹ The OH-PCBs are slightly more hydrophilic than PCBs, nevertheless, they are still slowly excreted due to selective binding to proteins and to retention in cells.² In fact, these compounds have been found in the blood of humans and wildlife.^{3–6} OH-PCBs are emerging as dangerous persistent environmental contaminants; their toxicity may be greater than that of the parent PCBs, particularly with regard to endocrine disruption. A number of OH-PCBs have been identified as endocrine disruptors.^{7–10} For example, 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl was found to be weakly estrogenic, while 4-hydroxy-2,3,3',4,5,5',6-heptachlorobiphenyl and 4-hydroxy-2,2',3,4',5,5'-hexachlorobiphenyl were found to be anti-estrogenic. The same study concluded that persistent OH-PCBs inhibit gap junction intercellular communication, which may lead to tumor promotion.⁷ Furthermore, 4-hydroxy-2,3,3',4,5,5',6-heptachlorobiphenyl exposure has been correlated with higher free thyroxine levels in newborns⁸ and 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl exposure with lower levels of thyroxine in fetal rat plasma and brain.⁹

Hydroxylated polychlorinated biphenyls have also been detected in aquatic environments, possibly being produced through aerobic biodegradation¹¹ or by reaction of the parent PCBs with hydroxyl radicals.¹² These compounds have been quantified in rain, snow and surface water with higher levels recorded in snow and surface waters near sewage treatment plants,¹³ ground waters downstream of landfills¹⁴ and in near-shore surface waters.⁵ The persistent nature of OH-PCBs in the environment causes human and animal exposure, which, along with bioaccumulation in body tissues, can create negative health effects to those exposed. Thus, improved methods for the detection of OH-PCBs in the environment should help facilitate environmental clean-up efforts and limit their adverse health effects on humans.

Traditional detection methods for OH-PCBs have been mostly based on GC-MS and LC-MS analyses,^{15,16} which require a series of sample preparation steps, and are time consuming as well as costly. Recently, our group developed a whole cell biosensing system capable of detecting several OH-PCBs and a detection limit of 1.0×10^{-8} M OH-PCBs in environmental and serum samples was achieved. This whole cell sensing system represents a more rapid and cost-effective approach to OH-PCB detection and is amenable to incorporation into portable field devices.¹⁷ While this sensing system demonstrated advantages over traditional methods, employing a protein instead of an entire cell as the sensing element has the advantage of further reducing the response times since the diffusion

of the analyte into the cell as well as activation of the cell machinery to produce the reporter molecule are eliminated. Additionally, it is easier to incorporate proteins into miniaturized devices for field analyses than it is to incorporate and maintain living cells. With these improvements in mind, we designed and developed a protein based bioluminescence inhibition assay system that could lead to a more rapid method of detection of OH-PCBs, especially for onsite screening of environmental and biological samples.

The design of this protein based inhibition assay utilizes the bioluminescent protein aequorin (AEQ) as both the recognition and reporter molecule. AEQ is a photoprotein found in the jellyfish *Aequorea victoria* which bioluminesces in the presence of calcium ions, coelenterazine and molecular oxygen. Aequorin consists of an apoprotein, apoaequorin (apoAEQ), composed of 189 amino acids that form a hydrophobic pocket, where an organic prosthetic group, coelenterazine, is non-covalently bound. AEQ has four EF hand structures, three of which bind calcium, and three triads of amino acids each consisting of tryptophan, tyrosine and histidine residues within its hydrophobic pocket that stabilize coelenterazine for bioluminescence (Figure 1).¹⁸ When calcium is added to AEQ, the protein undergoes a conformational change that leads to oxidation of coelenterazine to coelenteramide and release of CO₂ and light at 469 nm.¹⁸ We have taken advantage of this phenomenon, and studied the interaction between the photoprotein and different organic molecules to assess the binding selectivity of the amino acids within the hydrophobic pocket. We hypothesized that the three-dimensional structure resulting from the amino acids folding in space should result in the differential binding of organic molecules, and perhaps displacement or blockage of the interaction between the coelenterazine and the apoprotein, which in turn, should alter the emission of bioluminescence. Comparison of coelenterazine and the OH-PCBs showed that there are similarities in their structures and suggested the potential for the binding of OH-PCBs to the hydrophobic pocket of aequorin, which could displace coelenterazine or alter its binding to aequorin, resulting in a change in bioluminescent activity. Hence, we decided to examine the effect of the OH-PCBs on aequorin activity with the goal of exploring the potential use of aequorin in a bioluminescent inhibition assay for these compounds. Interestingly, we demonstrated that various OH-PCBs can bind to AEQ, causing a dose-dependent decrease in the bioluminescence of AEQ. We took advantage of this concentration-dependent modulation of the bioluminescence signal by the OH-PCBs to develop an inhibition assay for this family of toxic compounds. We achieved a one-phase screening assay that requires no separation steps or sample preparation, thus, it is rapid, inexpensive, simple and amenable to miniaturization and onsite testing. A unique feature of the developed inhibition assay is that AEQ plays a dual role, namely it is the recognition as well as the signal generating molecule, thus not requiring labeling or gene fusion methods to couple the recognition and reporting elements. Herein, dose-response curves for various OH-PCBs were also constructed in serum and tap water to prove the feasibility of employing our method in the detection of this family of compounds in biomedical as well as in environmental samples.

Experimental Section

Construction of Plasmid pIN4-AEQ

The apoaequorin cysteine-free mutant gene was cloned into plasmid pIN4 yielding pIN4-AEQ. The pIN4-AEQ vector was transformed into *E. coli* TOP10 cells, and the DNA was isolated for sequencing. The constructed DNA was sequenced at the University of Kentucky Advanced Genetics Technology Center (AGTC) to confirm the presence of the in frame AEQ gene in the plasmid.

Expression and Purification of AEQ

The bacterial cells were grown overnight at 37 °C, 250 rpm in 5 mL of LB broth containing 100 µg/mL ampicillin. This culture was used to inoculate 500 mL of LB broth containing 100 µg/mL ampicillin, and this was grown overnight at 37 °C, 250 rpm. The cells were harvested by centrifugation at $22,100 \times g$ at 4 °C for 15 minutes. The medium (supernatant) was saved and the expressed aequorin in the medium was precipitated by adding glacial acetic acid until the pH reached approximately 4.0. The precipitated proteins were isolated by centrifugation at $22,100 \times g$ at 4 °C for 30 minutes. The pellet was resuspended in 30 mM Tris/HCl, pH 7.5 buffer containing 2 mM EDTA (Buffer A) and the pH was adjusted to 7.5 with 5 M sodium hydroxide. This resuspended protein was gently shaken at 4 °C for 30 minutes. The suspension was centrifuged at $22,100 \times g$ at 4 °C for 15 min to remove any particulates.

The protein was purified using a BioCAD Sprint Perfusion chromatography system from PerSeptive Biosystems (Framingham, MA) with a DEAE Sepharose Fast Flow-ion exchange column (20 mm diameter $\times$ 70 mm). The column was equilibrated with Buffer A. The supernatant containing the protein was loaded, and the column was washed with three column volumes of the equilibration buffer. The protein was eluted using a gradient of 0 % (no elution buffer) to 50 % elution buffer (Buffer A containing 1 M NaCl) over ten column volumes. Five-milliliter fractions were collected. An SDS-PAGE was run to verify the fractions containing the apoprotein, which were then pooled together.

The combined fractions were further purified with a Butyl Sepharose hydrophobic interaction chromatography column (20 mm diameter $\times$ 70 mm). Ammonium sulfate was added to the combined DEAE fractions until the ammonium sulfate concentration was 1.0 M. The column was equilibrated with 20 mM Bis-Tris, pH 7.2 buffer containing 2 mM EDTA and 1 M ammonium sulfate. The protein was loaded on the column and the column was washed with three column volumes of the equilibration buffer. The column was further washed with 20% equilibration buffer, 80% elution buffer (20 mM Bis-Tris, pH 7.2, 2 mM EDTA), and then the apoaequorin was eluted using a gradient of 80 % to 100 % elution buffer over two column volumes. Five-milliliter fractions were collected. The purity of the protein was confirmed by using SDS-PAGE. The fractions containing the pure apoprotein were pooled together and dialyzed for 16 hours at 4 °C against 2 L of Buffer A using 3500 MWCO Pierce dialysis cassettes. The protein concentration was determined by the Bradford protein assay, with BSA as a standard. Protein was stored at -80 °C until use.

Effect of DMSO and OH-PCB on AEQ

The stock solution of apoaequorin protein (1.21×10^{-5} M) was diluted to 1.21×10^{-7} M in Buffer A, and incubated with a three times molar excess of coelenterazine at 4 °C for 18 h. To evaluate the DMSO effect, four samples were prepared: AEQ with a) 10% DMSO, b) 20% DMSO, c) 10% Buffer A and d) 20% Buffer A. For a), an aliquot of 180 μ L of protein was mixed with 20 μ L of DMSO and for b), an aliquot of 160 μ L of protein was mixed with 40 μ L of DMSO. The same dilutions were performed for c) and d), using Buffer A instead of DMSO. After mixing the protein with DMSO or buffer, 10 μ L of solution was added to a disposable glass tube. Bioluminescence was triggered by injecting 50 μ L of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl_2 buffer, and bioluminescence was measured on the Optocomp I luminometer (MGM Instruments, Hamden, CT). The same procedure was repeated for the model OH-PCB, 2-hydroxy-2',3',4',5',5'-pentachlorobiphenyl, (OH-PCBs and PCBs were purchased from Accustandard Inc. (New Haven, CT)) by adding the model OH-PCB dissolved in DMSO in place of the DMSO alone or buffer. OH-PCBs are a health hazard and generate hazard waste therefore they should be disposed of properly.

Coelenterazine Dose-Response Curve - EC_{50} Determination

Aliquots of 80 μ L of 1.21×10^{-7} M apoAEQ in Buffer A were added to the wells of a polystyrene microtiter plate containing 20 μ L of DMSO. Various concentrations of native coelenterazine were then added, in triplicates (final concentrations ranging from 1.0×10^{-5} M to 1.0×10^{-11} M). The samples were mixed and stored at 4 °C for 18 h. Bioluminescence was triggered by injecting 100 μ L of 100 mM Tris/HCl, pH 7.5, 100 mM CaCl_2 buffer, and bioluminescence was collected for 6-s on the FLUOstar Optima luminometer (BMG Labtech, Cary, NC).

Association Study – Determining the optimum concentration of coelenterazine

A 1.0×10^{-2} M stock solution of the 2-hydroxy-2',3',4',5',5'-pentachlorobiphenyl in DMSO was serially diluted in DMSO. Three assays were set up with different final concentrations of coelenterazine: 1.0×10^{-6} M, 6.1×10^{-8} M and 5.0×10^{-10} M. For each assay, an aliquot of 20 μ L of each concentration of 2-hydroxy-2',3',4',5',5'-pentachlorobiphenyl was added to 80 μ L of 1.21×10^{-7} M apoAEQ in Buffer A in triplicates in polystyrene microtiter plate wells. Also, a blank sample containing 20 μ L DMSO was prepared in triplicates. For each assay, the appropriate final concentration of coelenterazine was added, mixed and stored at 4 °C for 18 h. Bioluminescence was measured on the FLUOstar as described previously.

Time Study

An aliquot of 20 μ L of each concentration of 2-hydroxy-2',3',4',5',5'-pentachlorobiphenyl in DMSO was added to 80 μ L of 1.21×10^{-7} M apoAEQ in Buffer A in triplicates, for each time point, in polystyrene microtiter plate wells. Also, a blank sample containing 20 μ L DMSO was prepared in triplicates, for each time point. A final concentration of 5.0×10^{-10} M coelenterazine was added to each well, mixed and stored at 4 °C for 2, 4 and 18 h. At each time point, bioluminescence was measured on the FLUOstar as described previously.

Dose-Response Curves

The protocol was the same for all OH-PCBs studied. An aliquot of 20 μL of each concentration of OH-PCB in DMSO was added to 80 μL of 1.21×10^{-7} M apoAEQ in Buffer A in triplicates in polystyrene microtiter plate wells. Also, a blank sample containing 20 μL DMSO was prepared in triplicates. A final concentration of 5.0×10^{-10} M coelenterazine was added to each well, mixed and stored at 4 $^{\circ}\text{C}$ for 2 h. Bioluminescence was measured on the FLUOstar as described previously.

Structural Characteristics

The dihedral angles between the two rings of the OH-PCBs were calculated in Chem3D Pro12.0 using MM2 force field parameters during energy minimization. The molecular surface volumes were calculated using Connolly solvent excluded volume parameters after the energy minimization was performed. ClogP values were calculated in ChemDraw Ultra 12.0 using BioByte's Bio-loom software.

Results and Discussion

In 1961, Shimomura et al. isolated the photoprotein aequorin from the jellyfish *Aequorea victoria*,¹⁹ and since then, AEQ has been characterized and extensively studied. Employing AEQ as a reporting element has several advantages. In contrast to fluorescence, bioluminescence does not suffer from background emission as there is no need for the use of an excitation source for the photoprotein to emit light. When employing AEQ, background interference is avoided, and thus AEQ-based assays often render very sensitive detection. Furthermore, our group and that of others have demonstrated that AEQ can be detected at attomole levels.²⁰ Due to the remarkably low limits of detection achieved when using aequorin, this photoprotein has been employed in many bioanalytical applications, including, as a calcium indicator in living cells and for *in vivo* imaging,^{21,22} as a reporter in DNA hybridization assays^{23,24} and immunoassays,^{25–28} and as the signal generating molecule in molecular switch sensing systems.^{29,30} Herein, we have developed a sensitive, AEQ-based bioluminescence inhibition assay for the detection of OH-PCBs. A unique feature of this inhibition assay is that the AEQ plays a dual role, namely it is the recognition as well as the signal generating molecule. Specifically, upon incubation of apoAEQ with various OH-PCBs and coelenterazine, the amount of bioluminescence decreases as the concentration of the OH-PCB increases, making it possible to determine the concentration of OH-PCB in a sample.

The wild-type AEQ contains three cysteine residues; however, Kurose et al. prepared a cysteine-free mutant of AEQ and demonstrated that this cysteine-free variant had bioluminescent activity that was 10 to 20% greater than that of the wild-type photoprotein.³¹ In addition, the wild-type aequorin requires the presence of a reducing agent for the efficient binding of coelenterazine, while the cysteine-free aequorin does not. Given these characteristics of the cysteine-free AEQ that simplify its use and potentially increase the sensitivity of an assay employing it, we chose to use the cysteine-free aequorin in our studies. The *E. coli* system utilized for the expression of AEQ results in the release of the protein into the culture medium allowing for a simplified purification procedure. The protein

was purified to >95% purity as determined by SDS-PAGE. Characterization of the purified protein showed that AEQ had a specific activity of 4.4×10^{12} RLU/mg, a 0.59 s half-life and a 470 nm emission maximum.

The purified active AEQ was used to develop our inhibition assay for OH-PCBs. Initial experiments were performed by incubation of eight selected OH-PCBs, which ranged from one to five chlorine substituents with one or two hydroxyl groups, with apoaequorin and coelenterazine demonstrating that all these OH-PCBs caused a significant decrease in bioluminescence intensity. Since the OH-PCBs must be dissolved in DMSO, the effect of DMSO on AEQ activity was first examined. It was found that DMSO did not decrease AEQ bioluminescence activity. Out of the eight compounds studied, 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl had the most significant effect, a 99% decrease of AEQ bioluminescence activity; therefore, it was chosen as a model compound to develop and optimize our system.

In a typical inhibition assay format, the binding of a single concentration of labeled ligand is measured in the presence of various concentrations of unlabeled ligand. Here, coelenterazine takes the place of the labeled ligand, since it is needed to produce the signal and the OH-PCB would be considered the unlabeled ligand, since it may compete with the coelenterazine for binding to aequorin. For the development of this assay, three parameters, namely the concentration of apoaequorin, the concentration of coelenterazine and the incubation time were evaluated for optimized analytical performance. The apoaequorin concentration chosen was 1.21×10^{-7} M (9.68×10^{-8} M final) so that the number of protein molecules was low enough for competition to occur and there was sufficient activity for the decrease in bioluminescence to be observed in the presence of the OH-PCB. An association study was performed in order to determine the concentration of coelenterazine to be utilized in our inhibition assay. In standard competitive inhibition binding experiments, higher concentrations of labeled ligand (coelenterazine) require larger concentrations of unlabeled ligand (OH-PCB) to compete for half of the receptor binding sites. Assuming our assay performs in a competitive inhibition manner we optimized the assay to employ the least amount of coelenterazine required to get a good bioluminescent signal, so that lower concentrations of OH-PCB would be needed for competition and inhibition of bioluminescence, thus yielding better detection limits. For this, apoAEQ at a final concentration of 9.68×10^{-8} M in the presence of 20% v/v DMSO was incubated with various concentrations of coelenterazine to produce AEQ (Figure S-1). From this data, the EC_{50} was determined to be 6.18×10^{-8} M of coelenterazine. Determining the EC_{50} value gave us a basis for the concentrations of coelenterazine to evaluate in the assay development. Thus, coelenterazine concentrations at, above and below the EC_{50} value were studied in the presence of various concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl. Figure 2 shows that all three coelenterazine concentrations yield dose-response curves with the IC_{50} values for the OH-PCB decreasing as the coelenterazine concentration decreases. The IC_{50} is the half maximal inhibitory concentration, which is a measure of the effectiveness of a ligand in inhibiting a function, in this case bioluminescence, thus demonstrating the functional strength of the inhibitor. A concentration of 5.0×10^{-10} M coelenterazine was chosen as the optimal concentration

given that it produced the lowest detection limit of 3.3×10^{-8} M of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl.

Using the optimal concentrations of apoAEQ and coelenterazine, a time study was performed to determine the optimum incubation time required to produce the best response in the presence of var concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl. The data in Figure 3 demonstrates that after two, four and sixteen hours of incubation with coelenterazine, the responses to 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl are similar. Incubation periods of less than two hours did not yield significant bioluminescence. In order to reduce the overall assay time we selected an incubation time of two hours given that the detection limits are similar for all incubation periods tested.

Finally, employing 9.68×10^{-8} M (final concentration) apoaequorin, 5.0×10^{-10} M coelenterazine and two hours of incubation other OH-PCBs were evaluated and dose-response curves obtained (Table 1). The results show that the different OH-PCBs yield a variety of IC_{50} values and detection limits. The lowest detection limits were obtained with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl and 2-hydroxy-2',3,4',5',6-pentachlorobiphenyl. Conversely, 3,4-dihydroxy-3'4'-dichlorobiphenyl and 3-hydroxy-4'-chlorobiphenyl did not produce a decrease in the bioluminescence emission of AEQ. Comparisons of the structures of the OH-PCBs and their IC_{50} values can lead us to speculate about what structural features of the OH-PCBs are important for the inhibition of aequorin activity. The effectiveness of this inhibition appears to be related to the hydrophobicity, the dihedral angle between the two rings of the biphenyl and the molecular surface volume of the OH-PCB. To gain some insight into how these parameters might affect the response of aequorin to the OH-PCBs, the ClogP values, dihedral angles and molecular surface volumes of each OH-PCB were estimated using ChemBio Office Software (Table 1).

The Spearman correlation factors (statistical correlation between two variables) between the detection limits and the ClogP values, the dihedral angles and the molecular volumes were calculated, giving R values of -0.87 ($P < 0.0001$), -0.66 ($P = 0.0071$) and -0.88 ($P < 0.0001$), respectively. These data revealed that there is indeed a correlation between the detection limit for an OH-PCB and the ClogP value, the dihedral angle and the molecular volume of that OH-PCB. These three parameters all contribute to the effect of the analyte on AEQ bioluminescence, albeit to a different degree. Depending on the analyte studied, one parameter will play a more important role than another, causing the differential responses observed. In general, the data in Table 1 indicates that the non-coplanar OH-PCBs that are more hydrophobic and have a greater volume demonstrate a higher affinity for the AEQ, which results in lower detection limits. Additionally, compounds having the hydroxyl group at the *ortho* position seem to yield better responses than similar compounds with hydroxyl groups at the *para* position. Two of the three coplanar OH-PCBs tested showed no effect on AEQ, while the third, 4-hydroxy-3,3',4',5-tetrachlorobiphenyl, yielded only a small alteration of the bioluminescence signal. The ability of this compound to cause a decrease, although small, in the AEQ bioluminescence intensity, as compared to the ineffectiveness of the other two coplanar OH-PCBs, can be attributed to its higher hydrophobicity. The results obtained suggest that OH-PCBs that are non-coplanar, highly hydrophobic and occupy a larger molecular volume better inhibit aequorin activity. In this regard, we speculate that the OH-

PCBs with high endocrine-disrupting activity, i.e., 4-hydroxy-2,3,3',4',5'-pentachlorobiphenyl, 4-hydroxy-2,3,3',4',5,5',6-heptachlorobiphenyl, and 4-hydroxy-2,2',3,4',5,5'-hexachlorobiphenyl, would be suitable for this inhibition assay. These OH-PCBs are non-coplanar, highly hydrophobic, have a large molecular volume and are similar in structure to OH-PCBs, 4-hydroxy-2',3,3',4',5'-pentachlorobiphenyl and 4-hydroxy-2,2',3',5',6'-pentachlorobiphenyl, which greatly inhibited aequorin bioluminescence. In the future, if these high endocrine-disrupting activity OH-PCBs were to become available, we plan to analyze them in this system.

Since OH-PCBs are found in human blood,³⁻⁶ we aimed at demonstrating the feasibility of using this bioluminescence inhibition assay for detecting OH-PCBs in blood serum. For this, human serum samples were spiked with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl standards at various concentrations prepared in DMSO (90 μ L of serum plus 10 μ L of standard). An aliquot of 20 μ L of the spiked samples was added to 80 μ L of apoAEQ and coelenterazine and, following the optimized procedure described above, a dose-response curve was obtained (Figure 4). The detection limit for 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl in serum was calculated to be 5.02×10^{-6} M. The higher detection limit obtained in serum, compared to that obtained in buffer, can at least in part be attributed to the presence of human serum albumin (HSA). HSA is thought to bind to the OH-PCBs and therefore, would prevent the OH-PCB from interacting with the aequorin and inhibiting its bioluminescence. This is supported by the fact that a dose-response curve for 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl obtained using buffer containing HSA demonstrated a detection limit of 3.45×10^{-6} M (data not shown). It should be noted that HSA concentrations vary among individuals; therefore, for this screening assay to be employed for accurate detection of OH-PCBs in serum, the effect of various physiological and pathological HSA concentrations should be evaluated. If differences were to be observed, individual HSA levels would have to be determined in order to calibrate the response and normalize the analyte concentration.

A dose-response curve using tap water was also obtained to demonstrate the feasibility of employing this AEQ inhibition assay for detecting OH-PCBs in water samples. For this, tap water was spiked with the 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl standards at various concentrations prepared in DMSO, and a dose-response curve (Figure 4) was generated by employing the same assay conditions used for the response in buffer. The detection limit in tap water was found to be 1.32×10^{-7} M, which is comparable to the detection limit obtained in buffer, and the dose-response curve in tap water virtually overlapped to that in buffer, thus demonstrating that the matrix effect was negligible. In summary, we have demonstrated that it is feasible to detect OH-PCBs in biological and environmental samples by employing the AEQ inhibition assay that we developed. The concentrations of OH-PCBs in serum³⁻⁶ and in natural waters^{5,13,14} vary widely; therefore, there is a need for sensing systems able to detect these compounds at different concentrations in biological and environmental samples. In that regard, the rational tuning of AEQ can be employed to generate mutant AEQs with different properties and binding abilities, which would lead to shifts in the detection limits and working ranges, thus improving the analytical characteristics of the sensing system and its applicability. Furthermore, in physiological and environmental samples OH-PCBs are present in mixtures. This inhibition assay is expected

to be able to screen multiple OH-PCBs in a sample; however, it should be pointed out that the presence of multiple OH-PCBs may give complex results. It is possible that certain concomitantly present compounds decrease or enhance the ability of other compounds to inhibit aequorin bioluminescence.

The ability of other compounds to inhibit the bioluminescence of aequorin was investigated to evaluate the selectivity of the developed inhibition assay. For this, various organic compounds were dissolved in DMSO, and then tested in the above assay format. The data in Figure 5 shows that the compounds that are more hydrophilic than OH-PCBs, such as, amantadine, 3-chloro-benzoic acid and nicotine, do not decrease the bioluminescence generated by AEQ. Therefore, they do not interfere with our inhibition assay. Only a few of the hydrophobic compounds tested demonstrate some inhibition of AEQ's bioluminescence. However, the decrease in bioluminescence observed is not as pronounced as with 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl, indicating that these compounds have a low affinity for AEQ. The compounds 2,4,4'-trichlorobiphenyl, 2,2'dibromobiphenyl, pentachlorophenol, and 2',3,4,4',5-pentachlorobiphenyl produce a 41%, 43%, 34%, and 36% decrease in bioluminescence, respectively, whereas 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl at the same concentration produces a 91% decrease in bioluminescence when compared to the bioluminescence produced by AEQ in the absence of potential inhibitors. These four compounds are similar in structure to the OH-PCBs; therefore, it is reasonable to expect that AEQ would recognize them to a certain extent. Given that such compounds are also environmental pollutants, our system could be used as a screening system for a broader range of organic pollutants.

Conclusions

In conclusion, we have developed a novel AEQ-based bioluminescence inhibition assay for the detection of OH-PCBs. To the best of our knowledge this is the first time that AEQ has functioned as both the reporter and the recognition element for an analyte other than calcium. Except for calcium detection, the employment of AEQ as a reporter requires AEQ to be genetically fused or chemically conjugated to the analyte or recognition element. These steps are tedious, make the system more complex and may cause a decrease in AEQ bioluminescence activity, thus negatively affecting the attainable limits of detection. In our assay format, coelenterazine and the OH-PCB are incubated with apoAEQ and within two hours results are obtained. No separation steps are required or expensive instrumentation. Consequently, this assay can be employed as a rapid, cost-effective and easy-to-use screening method for OH-PCBs. In addition, the feasibility of detecting OH-PCBs in spiked biological and environmental samples was confirmed. It should be pointed out that rational tuning of AEQ can be accomplished to develop mutant AEQs, which would be employed in analogous bioluminescence inhibition assays leading to shifts in the detection limits and working ranges, thus improving the analytical characteristics of the sensing system and its applicability. The rational tuning of AEQ has been studied demonstrating that changing various amino acids has created mutants with different properties.^{32–34} Given that the OH-PCBs are binding in the hydrophobic pocket, altering amino acids in the pocket could change the interactions between the protein and OH-PCB, thus changing the analytical performance characteristics of the inhibition assay accordingly. Further, our findings have

demonstrated a unique approach to using AEQ as a reporter as well as a recognition element allowing for an original class of AEQ inhibition assays to be investigated. This platform could be used to screen a variety of molecules depending on their interactions with AEQ and has provided insight to interferences that can disrupt the bioluminescence of AEQ in nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

AEQ	Aequorin
OH-PCBs	Hydroxylated polychlorinated biphenyls
PCBs	Polychlorinated biphenyls
LC-MS	Liquid chromatography-Mass Spectrometry
GC-MS	Gas chromatography-Mass Spectrometry

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Figure 1.
The 3-D crystal structure of AEQ with coelenterazine in the hydrophobic pocket. Protein data bank structure 1EJ3.

Figure 2.

Dose-response curves for 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl obtained by incubating a known fixed concentration of apoAEQ (1.21×10^{-7} M) with various concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl in the presence of a given concentration of coelenterazine for each of the curves shown. The red curve corresponds to a concentration of 1.0×10^{-6} M coelenterazine, the blue to 6.1×10^{-8} M coelenterazine, the black to 5.0×10^{-10} M coelenterazine. The data points are an average of three measurements ± 1 standard deviation. Plotted in GraphPad Prism 5.0 using One Site Fit IC₅₀ non-linear fit.

Figure 3.

Time Study. The curves shown were obtained by incubating fixed concentrations of apoAEQ (1.21×10^{-7} M) and coelenterazine (5.0×10^{-10} M) with various concentrations of 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl for different times: 2 h – blue, 4 h – red, 16 h – black. The data points are an average of three measurements $\pm$ 1 standard deviation. Plotted in GraphPad Prism 5.0 using One Site Fit IC₅₀ non-linear fit.

Figure 4.

Dose-response curves for 2-hydroxy-2',3',4',5,5'-pentachlorobiphenyl obtained in buffer (red circles) as well as in serum (green triangles) and tap water (blue squares) by spiking serum or tap water with various concentrations of the aforementioned OH-PCB and adding these samples to apoaequorin and coelenterazine. The data points are an average of three measurements $\pm$ 1 standard deviation. Plotted in GraphPad Prism 5.0 using One Site Fit IC_{50} non-linear fit.

Figure 5. Selectivity studies performed by incubating a concentration of 1.21×10^{-7} M apoAEQ with 1.0×10^{-4} M concentrations of various compounds in the presence of 5.0×10^{-10} M of coelenterazine. The data points are an average of three measurements ± 1 standard deviation.

Table 1

Characteristics of the OH-PCBs evaluated in the AEQ bioluminescence inhibition assay. The coplanar OH-PCBs are in red and the non-coplanar OH-PCBs are in black. The detection limit was defined as the average signal of the blank (reaction mixture without OH-PCB) minus 3 standard deviations. The x's stand for no response. IC₅₀'s were calculated in GraphPad Prism 5.0 using One Site Fit IC₅₀ non-linear fit. The ClogP was calculated in ChemDraw Ultra. The dihedral angle and molecular surface volume were calculated in Chem3D Pro.

OH-PCB	Detection Limit, μM	IC ₅₀ , μM	ClogP	Dihedral Angle, deg	Volume, $\text{\AA}^3$
2-hydroxy-2',3,4',5',6-pentachlorobiphenyl	0.011	0.028	6.09	72	210.6
2-hydroxy-2',3',4',5',5'-pentachlorobiphenyl	0.033	0.097	6.47	63	209.3
2 hydroxy-2',3',4',5',5'-tetrachlorobiphenyl	0.34	1.33	5.51	64	194.4
4 hydroxy-2',3,3',4',5'-pentachlorobiphenyl	0.50	2.28	6.19	60	207.8
4-hydroxy-2',3',5',6'-pentachlorobiphenyl	1.23	9.77	6.39	70	213.8
4-hydroxy-2',4',6'-trichlorobiphenyl	2.50	15.4	5.09	66	180.6
4-hydroxy-2',3,4',5'-tetrachlorobiphenyl	3.30	17.5	5.72	58	193.5
4-hydroxy-3,3',4',5'-tetrachlorobiphenyl	19.0	130	5.97	39	192.5
4-hydroxy-2',5'-dichlorobiphenyl	23.0	138	4.62	59	165.2
2-hydroxy-2',4',6'-trichlorobiphenyl	34.0	155	4.79	71	182.5
4-hydroxy-2',3,5'-trichlorobiphenyl	40.0	290	4.99	57	179.1
2,3-dihydroxy-3',4'-dichlorobiphenyl	47.9	550	3.88	47	171.4
2,3-dihydroxy-2',5'-dichlorobiphenyl	132	1380	3.75	63	171.3
3,4-dihydroxy-3',4'-dichlorobiphenyl	X	X	4.18	38	169.7
3-hydroxy-4'-chlorobiphenyl	X	X	4.14	38	150.3