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A Novel Chemo-Enzymatic Cascade for Smart Detection of Nitro- and Halogenated Phenols

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Abstract: The flavin-dependent monooxygenase, HadA, catalyzes the dehalogenation and denitration of the toxicants, nitro- and halogenated phenols, to benzoquinone. The HadA reaction can be applied in one-pot reactions towards the *de novo* synthesis of D-luciferin by coupling with D-Cys condensation. D-luciferin, a valuable chemical widely used in biomedical applications, can be used as a substrate for the reaction of firefly luciferase to generate bioluminescence. As nitro- and halogenated phenols are key indicators of human overexposure to pesticides commonly used worldwide and indicators of pesticide contamination, the technology provides a sensitive and convenient tool for improved biomedical and environmental detection at ppb sensitivity in biological samples without the requirement for any pre-treatment. This newly developed methodology provides the dual-pronged advantage of waste biodetoxification to produce a valuable chemical as well as a smart detection tool for environmental and biomedical detection.

Urban development and use of chemicals in various industries are major factors for chemical contamination in environment worldwide. Biocatalysis provides powerful and sustainable technology for conversion of toxicants to less toxic products which, in some cases, may lead to economical advantage.[1] Halogenated and nitro-aromatic compounds are widely used in household and industrial settings including dyes, plasticizers, explosives, pharmaceuticals, flame retardants, disinfectants, chemical-warfare agents, pesticides and herbicides.[2] Spillage of nitrophenols (NPs) and halogenated phenols (HPs) in manufacturing areas, and their long-term accumulation in the environment as a result of pesticide and herbicide degradation,[3] has long been recognized as causing adverse effects in humans and wildlife. Consumption of food contaminated with pesticides/herbicides containing NP and HP substituents can lead to acute and severe diseases such as cancer.[4,5]

Dehalogenases are in the redox (EC 1.14) and hydrolase (EC 3.8) classes of enzymes. They are powerful biocatalysts for detoxifying toxic environmental pollutants[6] and their applications in industries have been demonstrated.[7] In this work, we have developed a new waste biodetoxification concept based on the reaction of HadA monooxygenase, a dechlorinating flavin-dependent monooxygenase, that can catalyze halide and nitro group elimination from NPs and HPs to generate benzoquinone (BQ).[8] The enzyme is encoded by the *hadA* gene located in the *had* operon which is responsible for chlorophenol degradation in *Ralstonia pickettii*.

It was previously reported that chemical condensation of BQ and D-cysteine (D-Cys) can result in D-luciferin, although with a very low yield (~ 0.3%).[9] We thus linked this enzymatic and chemical condensation to turn toxicants into D-luciferin *via* one-pot chemo-enzymatic reaction. The current price of D-luciferin is 206 USD per mg (Sigma) with a total market size of 18 billion USD annually.[10] D-luciferin is also widely used in biomedical research[11] and biodetection[12]; more than 2420 publications in 2018 reported the use of D-luciferin in their experiments (Supporting Information, Figure S1). As D-luciferin formation can be further applied to generate bioluminescence signals by firefly luciferase (Fluc), the chemo-enzymatic cascade developed also offers additional value in providing detection technology as an integrative biodetoxification-biosynthesis platform for biodetection of NP and HP, which are metabolites and biomarkers of pesticides/herbicides (Figure 1). Bioluminescence is a powerful detection technology because it provides high signals with low non-specific background and is widely used as a diagnostic tool.[13]

We first established an enzymatic cascade of HadA to convert 4-nitrophenol (4-NP, **2a**), 4-fluorophenol (4-FP, **2b**), 4-chlorophenol (4-CP, **2c**), 4-bromophenol (4-BrP, **2d**) and 4-iodophenol (4-IP, **2e**) into BQ. As HadA requires constant generation of reduced FAD ($FADH^-$) in addition to other co-substrates, molecular oxygen and NP or HP (Figure 2 A), two

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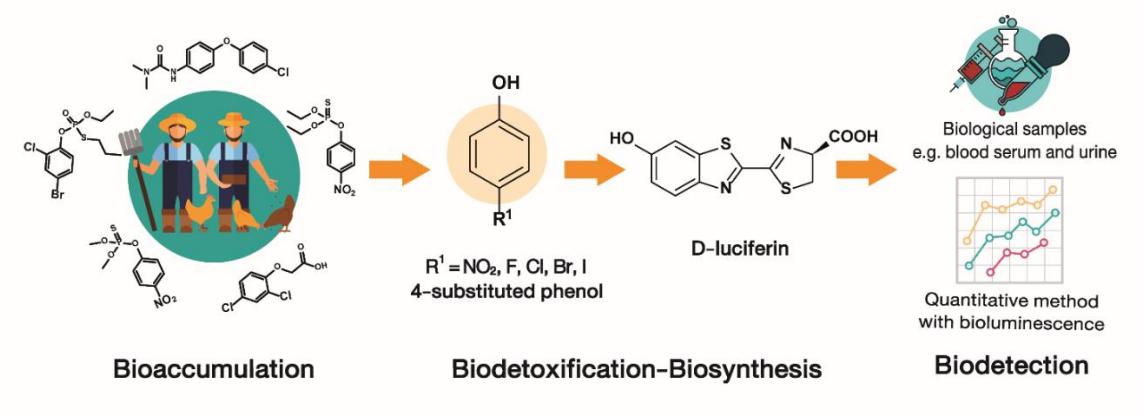

Figure 1. The concept of integrative enzymatic-bioluminescence technology for convenient and rapid measurement of halogenated and nitro- phenols for biomedical and environmental detection applications.

additional enzymatic reactions, flavin reduction and NADH regeneration are required to supply $FADH^-$ in Figure 2 B.[8] This biodetoxification cascade was quite efficient, as results indicated that all 4-NP (**2a**) and 4-HPs (**2b-2e**) could be converted to BQ with 100% yield within 180 min (Figure 2 C and Supporting Information, Figure S3 A-E). When the biodetoxification cascade was carried out in the presence of D-Cys, the resulting BQ from the HadA reaction reacted with D-Cys to form a compound with an *m/z* value of 281.0063 (Figure 2 G), which is the same as the mass spectrometry (MS) profiles of the standard D-luciferin. The luciferin product from HadA chemo-enzymatic cascades was purified and analyzed by ^{1}H NMR spectroscopy (data not shown). All results indicated that the compound obtained from the chemo-enzymatic cascade of HadA, C_1, G6PD and D-Cys was indeed D-luciferin (Figure 2 G, Supporting Information, Table S1, Figure S4), confirming that the method developed can be used to generate D-luciferin from 4-NP and 4-HPs.

We also tested whether the D-luciferin formed was indeed in the correct configuration. The enzymatic cascade of 4-NP and 4-HPs conversion to BQ and then D-luciferin was performed and purified Fluc was added. The results indicated that the D-luciferin synthesized from the HadA chemo-enzymatic cascade gave a bioluminescence peak at 560 nm (yellow-green light) (Figure 2 D and Supporting Information, Figure S4), which is the same bioluminescence characteristics as the reaction of standard D-luciferin and Fluc (Figure 2 D). Only D-luciferin could be used as a substrate for Fluc to generate luminescence, while L-luciferin could not generate any luminescence (Supporting Information, Figure S4).

In addition to the substrates and product, the LC-ESI-QTOF-MS results also detected a compound with an *m/z* = 180.0115, consistent with the MS profiles of 6-hydroxybenzothiazole-2-carbaldehyde (intermediate for D-luciferin synthesis, (Figure 2 F). This result was different from the data reported for chemical condensation in which various intermediates including *S*-(2,5-dihydroxyphenyl) cysteine, 6-hydroxybenzothiazole-2-carbaldehyde, and 2-(6′ -hydroxy-2′ -benzothiazolyl)-2-thiazolidine-4-carboxylic acid were detected.[9] The detection of 6-hydroxybenzothiazole-2-carbaldehyde as an intermediate also shed light into the mechanism of luciferin formation (Figure 2 F, Supporting information, Figure S5), that previously could not be clearly explained.

In order to increase yield of product formation, we optimized the yield of D-luciferin obtained from the chemo-enzymatic cascade by adjusting the concentrations of D-Cys and other reagents related to the HadA reaction. Results (Supporting information, Figure S6) showed that the optimum ratio of D-Cys to 4-NP was 10:1. The best yield of D-luciferin (4.6 %) (Table 1) could be obtained when using 10 μM NAD^+ (Supplementary Fig. S7), 4 μM FAD (Supplementary Fig. S8) and 50 μM HadA (Supplementary Fig. S9).

We further optimized the yield of D-luciferin synthesis using a different approach by increasing BQ stability. BQ is unstable and can form polymerized products in the presence of oxidants, radicals, or hydrogen peroxide (H_2O_2).[14] As the free reduced FAD generated by C_1 can react with oxygen to generate H_2O_2 and HadA also has an uncoupling path to generate H_2O_2 [8,14], H_2O_2 is

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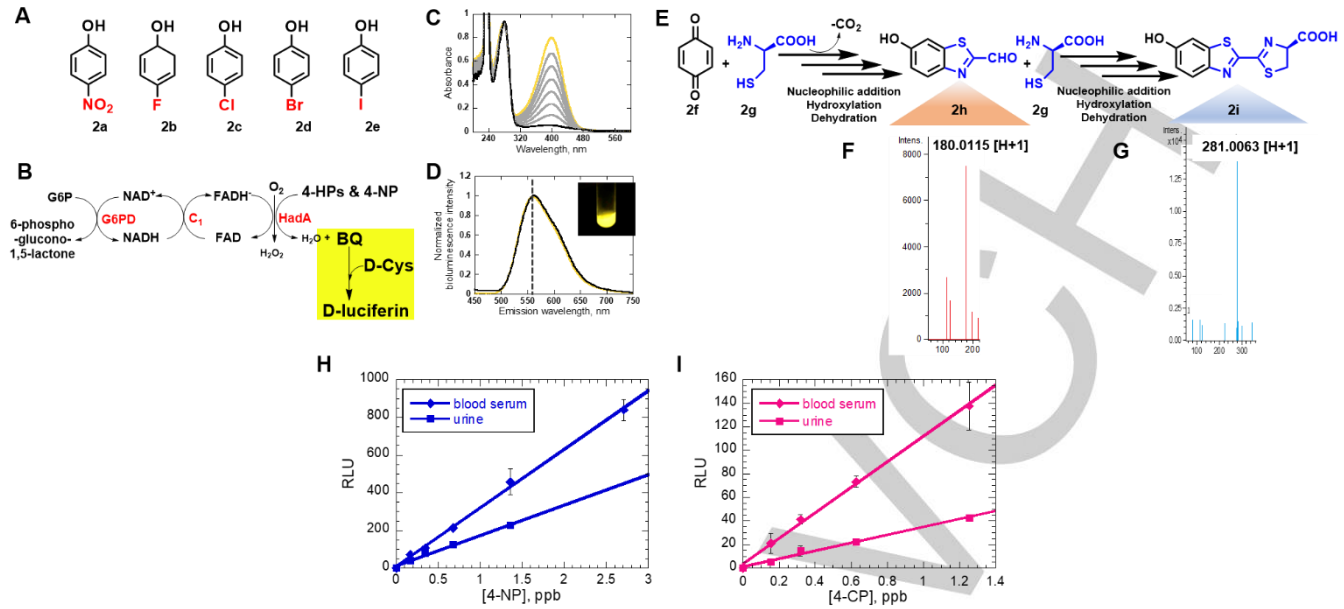

Figure 2. Bioconversion of hazardous phenols to D-luciferin. **A**, structures of phenol with 4-position substituents, including 4-NP (**2a**), 4-FP (**2b**), 4-CP (**2c**), 4-BrP (**2d**), 4-IP (**2e**). **B**, Overall reaction of the enzymatic cascades of biodetoxification of hazardous phenols to D-luciferin. **C**, The HadA biodetoxification of 4-NP (**2a**): the depletion of **2a** was monitored at 400 nm after the reaction (yellow line) started and finished after 180 min (black line). **D**, Bioluminescence due to the Fluc reaction with D-luciferin produced from hazardous phenol substrates converted by the HadA reaction. D-luciferin synthesized from the developed technology (black line) compared to commercially sourced D-luciferin (yellow line). The bioluminescence of both reactions show the same peak at 560 nm. **E,** Chemical species detected from the condensation of BQ and 2 molecules of D-Cys using LC-ESI-QTOF-MS. D-luciferin (**2i**) was produced from the condensation of BQ (**2f**) and D-Cys (**2g**), generating the 6-hydroxybenzothiazole-2-carbaldehyde intermediate, (**2h**) as an intermediate. 2h reacts with another molecule of D-Cys via nucleophillic addition, hydroxylation, and dehydration reactions to generate D-luciferin as the final product. **F**, the mass spectrum of **2h** shows an *m/z* of 180.0115. **G**, The mass spectrum of **2i** shows an *m/z* of 281.0063. Biomedical detection of organophosphate and organochlorine pesticide biomarkers in biological samples. **H**, linear plots of 4-NP (**2a**) detected in blood serum and urine samples. **I**, linear plots of 4-CP (**2c**) detected in blood serum and urine samples.

thus formed as a side product from the enzymatic cascade (Fig. 2B). Therefore, reactive oxygen species (ROS) scavengers such as catalase or superoxide dismutase (SOD) were added to destroy H_2O_2 and ROS to improve BQ stability and the yield of D-luciferin synthesis. Results in Table 1 and Supporting Information, Figure S10-12 clearly show that the addition of these ROS destroying enzymes indeed increased the yield of D-luciferin synthesis significantly by up to 15.8 % maximally in the presence of SOD.

The final parameter to increase the yield of D-luciferin formation was pH adjustment. The results (Table1, Supporting Information, Figure S13) indicated that the best yield of D-luciferin formation could be obtained at pH 8.0 (Table 1). Under this condition, the yield of D-luciferin synthesis was significantly improved to 21%, which is 70-fold greater than the method using chemical condensation of BQ and D-Cys. Although the reactions after the step of BQ formation (those shown in Fig. 2E) are not catalyzed by HadA, the chemo-enzymatic cascade of HadA can generate D-luciferin with a much greater yield than the reaction starting with a high concentration of BQ. This might be due to the ability of HadA to generate BQ constantly and slowly, preventing accumulation of BQ which may result in a low yield of D-luciferin. With this high yield of D-luciferin synthesis, the chemo-enzymatic cascade can be coupled to the Fluc reaction to create a sensitive detection technology for measurement of 4-NP and 4-HPs.

To convert 4-NP and 4-HPs to D-luciferin and then to produce luminescence signals, the chemo-enzymatic cascade to convert various concentrations of 4-NP, 4-CP, 4-FP, 4-BP and 4-IP to D-luciferin were carried out in the presence of Fluc. We also explored whether pH and the presence of other reagents such as Coenzyme A (CoA), DTT, EDTA and glycerol could enhance bioluminescence signals. CoA and DTT were added to reduce the formation of dehydroluciferyl-AMP, a side product which can inhibit light emission of Fluc.[15] Addition of glycerol is known to prolong Fluc stability and bioluminescence.[15] EDTA was added to remove trace heavy metals which may cause inhibition of activity.[15] The results (Supporting Information, Figure S14 and S15 A-E) indicated that addition of 0.05 mM CoA, 4 mM DTT, 0.5% (v/v) glycerol, 62 µM EDTA gave the highest luminescence signal, 2-fold greater than the reaction without any additives.

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Table 1. Bioconversion of 4-NP and 4-CP to D-luciferin was coupled to Fluc to generate luminescence in different matrix samples

Bioconversion				Biodetection		
Substrate	Additive	% Conversion to BQ	% Yield of D-luciferin	Toxicant	LOD, ppb*	LOQ, ppb*
BQ+ D-Cys [a]	(-)	N.D.	0.30 (ref [20])	4-NP	0.04	0.14
4-NP [a]	(-)	100	4.60 ± 0.02	4-NP (serum) [c]	0.04	0.12
4-NP [a]	Catalase	100	9.74 ± 0.01	4-NP (urine) [d]	0.05	0.17
4-NP [a]	SOD [b]	100	15.80 ± 0.11	4-CP	0.16	0.53
4-NP	Catalase + SOD [b]	100	7.31 ± 0.11	4-CP (serum) [c]	0.19	0.63
4-NP	SOD [b]	100	21.00 ± 0.11	4-CP (urine) [d]	0.12	0.40

All of the reactions were performed in 100 mM HEPES-NaOH pH 8.0. (-) No additive added. N.D. is not determined. [a] The reaction was performed in 100 mM HEPES-NaOH pH 7.5. [b] SOD is superoxide dismutase. [c] Detection of pesticide biomarkers (4-NP and 4-CP) in biological samples, human serum (pH 7.62) and [d] urine (pH 6.90). *The US-EPA and ATSDR require the concentration of 4-NP and 4-CP to be less than 20 ppb and 0.3 ppb, respectively, to certify that environmental and biological samples contain safe levels of 4-NP and 4-CP.

Furthermore, the enzymatic-bioluminescence cascade showed good linearity over a large range of concentrations (Supporting Information, Figure S16 A-E, Table S2) in the ppb region, which is the level required by the US-Environmental Protection Agency (US-EPA) and Agency for Toxic Substances and Disease Registry (ATSDR) (Supporting Information, Table S2). Therefore, the method developed should be useful for detection of these hazardous 4-NP and 4-HPs in environmental and food samples.

As NP and HP are the major metabolites that result from the intracellular xenobiotic transformation of nitroaromatic and halogenated pesticides [16], they have been routinely used as key biomarkers in urine and blood samples to determine the occupation-related health risk of workers routinely or unintentionally exposed to these compounds. Rapid and convenient tools for detection of NP and HP metabolites in biological samples would also be beneficial for measuring levels of pesticide contamination in the environment. Unfortunately, the current method of HP and NP measurement relies mostly on LC-MS or GC-MS techniques which require highly skilled analytical operators and sample pre-treatment to remove background signals.

We then further explored whether the technology could measure 4-NP and 4-HPs in samples containing high matrix background such as blood and urine without sample extraction or pretreatment. 4-NP is routinely used as a biomarker for exposure to organophosphate pesticides such as parathion and methyl parathion[16,17], while 4-CP is a biomarker of 2,4-dichlorophenoxyacetic acid (2,4-D) and chloroxuron exposure[16,18]. Various concentrations of 4-NP and 4-CP were added into (commercially purchased) serum and synthetic urine samples to mimic biological samples containing 4-NP and 4-CP, and experiments similar to those in Table 1 were carried out and luminescence measured. The results indicated that the detection assay still gave strong luminescence signals, exhibiting limits of quantification of ~0.1 ppb for 4-NP and ~0.4 ppb for 4-CP, and limits of detection of ~0.04 for 4-NP and ~0.1 for 4-CP. All measurements yielded good linearity with regression (R^2) of ~0.9900 (Figure 2 H-I, Table 1, and Supporting Information, Table S2). These detection capabilities are at the standards required by the US-EPA and ATSDR for tests for screening workers who are at risk of overexposure to aforementioned pesticides.

In conclusion, we have developed a new chemo-enzymatic cascade which can continuously convert 4-NP, and four types of 4-HPs to BQ which can then be used for *de novo* synthesis of D-luciferin. Moreover, the reaction can be coupled to Fluc to generate bioluminescence signals to detect 4-NP and 4-HPs in the range of detection required by the US-EPA and ATSDR. The method is especially valuable because it can measure the compounds directly in biological samples without the need for pretreatment to remove matrix background. Altogether, the technology reported herein offers a double-pronged advantage of biocatalysis to turn toxicants into a valuable chemical and a rapid, accurate and simple detection platform for diagnostic use in patients with exposure to pesticides or toxicant contamination in the environment through regular contact or accidental exposure. This detection assay potentially provides a technology that can save lives of developing-country farmers who cannot afford expensive diagnostic tests, and can also aid in the prevention of toxicant contamination in food and consumer products.

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Keywords: • Biocatalysis • Biorefinery • Dehalogenase • D-luciferin • Luciferase

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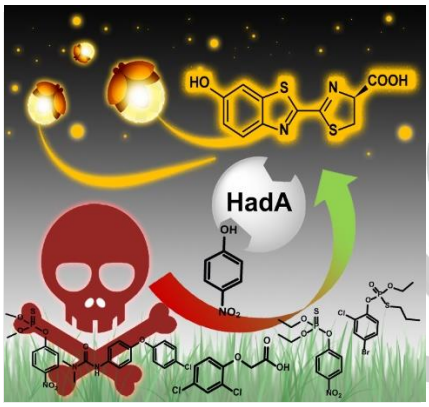

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