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RESEARCH PAPER

An optical detection module-based biosensor using fortified bacterial beads for soil toxicity assessment

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Abstract

An optical biosensor module for soil contamination assessment is presented, employing bioluminescent bacterial bioreporters encapsulated in poly-dopamine (PD)-coated alginate microbeads. The PD-coated beads displayed improved mechanical strength and stability, but somewhat delayed responses to the inducing toxicant. Using toluene as a model soil contaminant, two bioluminescent reporter strains were employed for its detection in the ambient light-blocking, temperature-controlled biosensor module. Bioluminescence of strain TV1061 (harboring an inducible *grpE::luxCDABE* fusion) increased and that of strain GC2 (harboring a constitutive *lac::luxCDABE* fusion) decreased in the presence of increasing toluene concentrations. In the former case, a maximal effect was observed in the presence of 1% toluene. This simple optical detection biosensor module may potentially be utilized for monitoring soil contamination from areas suspected of chemical pollution such petrochemical industrial zones or petrol stations.

Keywords Recombinant bioluminescent bacteria · PD coating · Soil toxicity biosensor module · BTEX · Optical sensors · Biosensors

Introduction

Soil is a very important element in an ecological system, since it provides an environment for organisms and plants. Due to rapid industrialization, however, various toxic pollutants have been thrown into the soil, and so the severity of soil contamination has been increasing globally [1, 2]. The most common soil-contaminating chemicals are known to be heavy metals, including copper, cadmium, and mercury, or organic materials, such as polychlorinated biphenyls (PCBs), phenol, and oil. Of these, the oil pollutants are becoming a serious issue in several nations [3, 4], because oil contains various toxic chemicals and they cause cross contamination to air and water as well [5]. Of particular environmental significance among the latter chemicals are volatile benzene, toluene, ethylbenzene, and xylene (BTEX), shown to cause brain and nerve damage as well as cancer in humans [6, 7]. The detection of BTEX in soil is crucial for assessing the degree of oil pollution as well as for assessing the success of remediation procedures.

While standard analytical instrumentation such as gas chromatography (GC) for BTEX analysis in soil is both highly sensitive and accurate, it fails to provide information on pollutant toxicity and bioavailability [8]. Furthermore, analytical methods are not only costly and time consuming, they also require highly sophisticated devices and skilled personnel, and are thus normally restricted to central laboratories. Therefore, there is a need for a complementary approach that allows rapid and simple sample screening, preferably on site.

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Genetically engineered bioluminescent bacteria have long been used either for overall toxicity assessment or for the detection of specific pollutants [9–11]. Such bioreporter strains can be molecularly "tailored" for specific applications by fusing a gene promoter, known to be induced by the target compound or stress condition, to reporter gene(s) the induction of which can be monitored quantitatively [12, 13]. Common reporter genes are - *lacZ*, encoding β-galactosidase [14], fluorescent protein genes such as the green fluorescent protein (GFP) [15], or prokaryotic bioluminescence genes, such as *luxCDABE*. An important advantage of the latter option is that no addition of an external substrate is required [16]. When bioluminescent reporter bacteria are exposed to the target toxicant, the entire *luxCDABE* is transcribed, generating both the luciferase enzyme (encoded by *luxAB*) and the aliphatic aldehyde that serves as its substrate aldehyde (*luxCDE*) [17]. Diverse toxicants causing toxicities of DNA damage, oxidative damage, membrane damage, and/or protein damages were shown to be detected and quantified by such stress-responsive reporter strains [18].

For the integration of live sensor cells into a functional biosensor, they often need to be interfaced with a suitable hardware platform. Such biosensors have been described for water [19] and gas [20] toxicity monitoring, prescreening of new drugs [21], the detection of endocrine-disrupting chemicals, and so on [22]. Particularly, to overcome the limitations given in using a bacterial cell solution for biosensor development, bacterial cell immobilization has been studied broadly, especially with alginate as a matrix for bacterial encapsulation, in the forms of a dip-stick biosensor [23] and the cell array chip for successful toxicity monitoring [24]. Furthermore, with the use of the electro-spraying technique, these bacterial alginate microbeads were created in a fast manner and with easy loading of bioluminescent bacteria [25]. However, the alginate microbeads used above were found to be fragile, with weak mechanical strength and easy cell leakages [26].

Therefore, in order to overcome these weak points, many researchers have proposed to make additional layers on alginate beads by coating with other materials. For example, a silica layer was formed by electrostatic attraction between poly-lysine on the alginate bead and a silica precursor [27], but the silica precursor is known to be toxic to bacteria [25]. So, a biocompatible and stable coating method is required to increase the strength of alginate beads. In this study, we have successfully developed fortified functional bacterial beads with a poly-dopamine (PD) layer, which enhanced the biocompatibility and stability of the bacterial beads in a biocompatible and stable coating technique.

Since the bacteria encapsulated in alginate beads coated by PD can not only be quickly dried out in the field, resulting in the loss of their cellular function, but also be strictly banned to be applied on the real field due to its genetically modified organism character, the microbial beads must be harbored in a working environment with controlled temperature and ambient light blocked due to relatively weak bioluminescence generated from the microbial beads. Therefore, we have successfully applied a mini-bioreactor previously developed for the soil biosensor module [10], which is ambient light blocked, temperature controlled, and water jacketed, by placing fortified functional microbeads containing different bioluminescent bacteria on the soil samples in the mini-vessel for reliable and safe bioluminescent measurement from different bioluminescent bacteria. This newly developed fortified bacterial bead-based optical detection module for soil samples can be seen in Schematic 1.

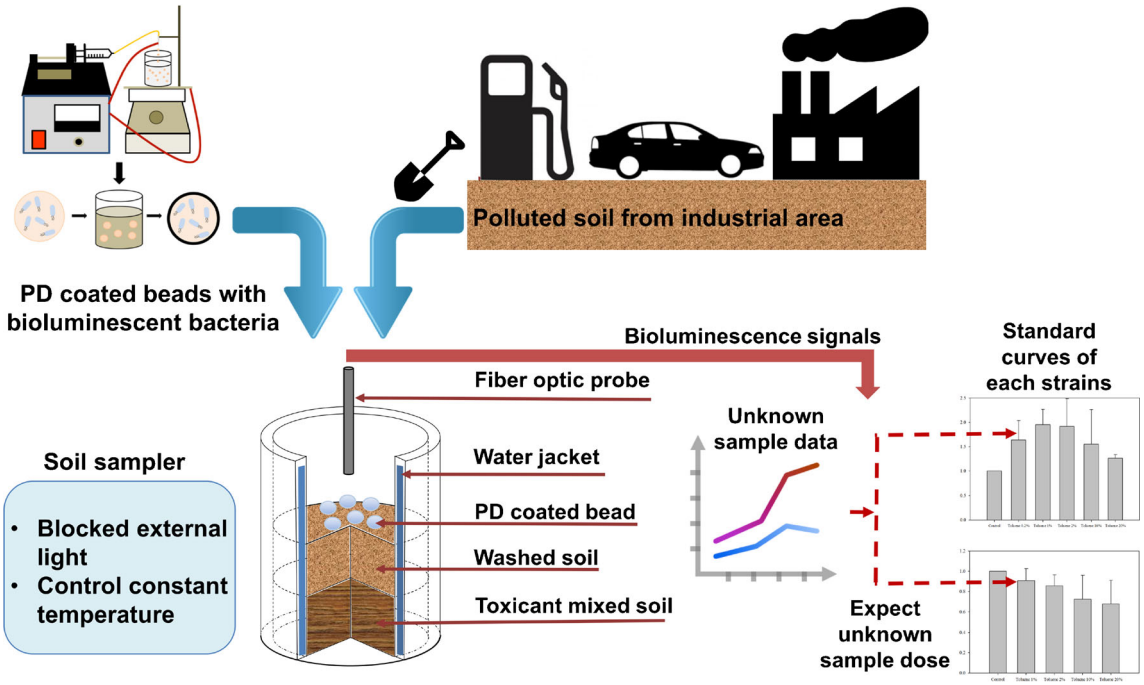
Materials and methods

Bacterial strains

The three different types of bioluminescent bacterial strains, EBSoxS, TV1061 [28], and GC2 [29], containing either *Vibrio fischeri* or *Xenorhabdus luminescens protomor::luxCDABE* genes, were used in this study. EBSoxS is an *Escherichia coli* strain, RFM443, harboring the *pBCsoxSLux* plasmid, and produces bioluminescent light (BL) in response to oxidative damage. TV1061 is an *E. coli* strain, RFM443, harboring the *pGrpELux* plasmid, specifically responsive to protein damages. GC2 is also an *E. coli* strain, RFM443, harboring the *pLacLux* plasmid. This strain generates BL constitutively, but the BL decreases when the cells are exposed to toxic chemicals or stresses. Each strain was streaked on lysogeny broth (LB) agar plates, which contain 50 μg/ml of ampicillin (Sigma), and placed for 16 h in an incubator at 37 °C. After incubation, a single colony was inoculated and grown in 1 ml LB medium, containing 50 μg/ml of ampicillin in 15-ml tubes (Corning Co.) in a shaking incubator at 37 °C and 200 rpm for 3 h. Cultured cells were poured into 20 ml of fresh LB medium, and the cells were grown in a shaking incubator until the OD value reached 2.5~3 at 600 nm [24]. After that, the cells were centrifuged at 20 °C, 3000 rpm for 30 min, and the supernatant was discarded. Three different *Escherichia coli*-based bioluminescent reporter strains employed in this study are summarized in Table 1.

Chemicals

All the chemicals including sodium alginate, calcium chloride, ampicillin, dopamine hydrochloride, tris-hydrochloride, paraquat, and toluene were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specified otherwise. Toluene was used as a model toxicant because it was one of the constituents included most substantially in oil [5]. Paraquat,



Schematic 1 This schematic shows how this PD-coated bead-based biosensor platform is prepared and used: (upper left) formation of alginate bead-contained bioluminescent bacteria using a syringe pump and power supply. Simple coating method using PD solution. (Bottom) Illustration of a mini-vessel by using PD-coated beads. (Upper right) This platform was designed for the detection of toxicants from polluted soil such as gas stations or industrial areas

known to produce superoxide radicals, was used as an oxidative stress agent [30].

Fabrication of bioluminescent bacteria-encapsulated alginate beads

Sodium alginate (Sigma) 0.2 g was prepared and dissolved into 10 ml sterilized distilled water. The bioluminescent bacteria previously centrifuged were mixed with 2.5 ml of 2% (*w*/*v*) of sodium alginate solution. And then, this mixture was placed into a 12-ml syringe coupled with a 10 cm sterilized silicon cube (KOREA ACE SCIENTIFIC CO) and a 30G needle. The mixture was pushed by a syringe pump (HARVARD APPARATUS) at 10 ml/h. The droplets were generated at the tip of the needle by the actions of gravitational and electrostatic forces. The electrostatic potential was formed by a high-voltage DC unit (model ES30P 10W, HV power supply, Florida) linked with the positive electrode being the aluminum foil on the stirrer and the negative electrode the needle and the droplets were dropped into sterilized 2% $CaCl_2$ solution. Nine kilovolts of electrostatic potential was used (upper left in Fig. 1). Alginate beads containing bioluminescent bacteria were formed by electrostatic force and a coordinate bond between alginate and Ca^{2+} after the droplets were placed in 2% $CaCl_2$ solution stirred for 1 h for hardening. After the hardening process, the beads were washed with tris-buffer (pH 8.5, 50 mM) 3 times and stored in a 50-ml tube at 4 °C to maintain bacterial viability.

Fortification of alginate beads with a poly-dopamine layer

The PD solution was prepared by mixing dopamine hydrochloride (Sigma) and 100 mM of $CaCl_2$ in a tris-HCl buffer solution (pH 8.5, 50 mM). Dopamine polymerizes spontaneously under alkaline conditions [31] (upper left in Fig. 1). The alginate beads generated as described above were added to 10 ml of PD solution in a sterilized 20-ml glass vial and shaken at 85 rpm in a shaking incubator at 25 °C for 10 min, during which a PD layer was spontaneously formed around the beads

Table 1 Bacterial sensor strains employed in this study

Strain	Gene promoter acting as sensing element	Reporter genes	Type of stress sensed	Reference
TV1061	*grpE*	*Aliivibrio fischeri luxCDABE*	Protein damage and general toxicity	[28]
EBSoxS	*soxS*	*Aliivibrio fischeri luxCDABE*	Oxidative stress	[28]
GC2	*lacZ*	*Xenorhabdus luminescens*	General toxicity (constitutive expression)	[29]

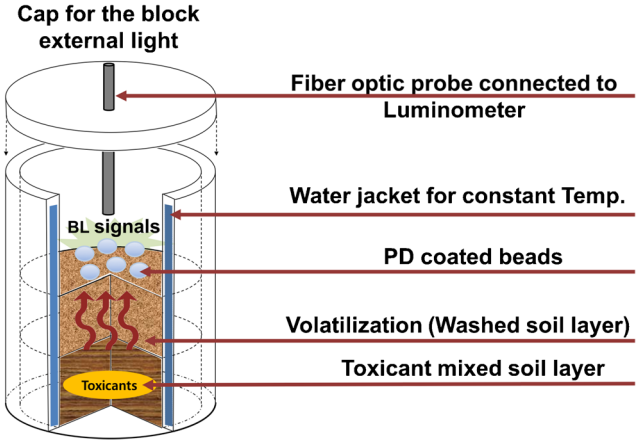


Fig. 1 The details of the optical detection biosensor module. This mini-vessel is completely blocked from the external light and water jacket equipped for maintaining a constant temperature

[32]. In this study, 5 different concentrations of PD solution for coating beads were used to confirm the increased stability of the PD-coated beads. After coating, each bead was washed by tris-buffer 3 times and stored in a 50-ml tube at 4 °C to maintain bacterial viability.

Stability test for PD-coated functional microbeads

Swelling test

Each PD-coated bead prepared was placed in the LB medium including 10 mM $CaCl_2$. This solution mixture was incubated at 37 °C, and the size of the beads was checked for 2 h at 30-min intervals. The change of bead size was observed by using a microscope-connected prove-station (MS TECH, Korea). All the stability experiments were conducted until a significant change in the parameters was measured.

Mechanical strength test

Fifty PD-coated beads were stirred (300 rpm) in a 100-ml beaker containing 50 ml of tris-buffer (50 mM) and 0.01 mg/ml sodium citrate for 3 days; the morphology and number of beads were checked after 32 h.

Cell leakage test

Fifty of the PD-coated beads were placed in the LB medium. These beads were shaken in an incubator at 25 °C and 300 rpm, and the optical density (OD) in the LB was checked every 4 h in order to investigate the cell leakage.

Instrumentation, system, and soil toxicity measurement

The optical detection module-based biosensor for soil samples is a 25-ml stainless steel cylinder [10], having a water jacket for temperature control to maintain a steady temperature at 37 °C using a water bath (JEIO TECH, Korea). This mini-vessel is also completely blocked from the ambient light for correct measurement of bioluminescence (BL) signals. The fully blocked mini-vessel was designed to place soil samples from industrial area or gas station fields for the detection of target toxicants.

Figure 1 shows the overall experimental scheme of this system. The soil sample was divided into two layers: a 1-cm-deep lower layer mixed with toluene (0~20%) was overlaid by a sterilized washed soil to a depth of 1 to 4 cm. Washed soil was prepared by washing with detergent and sterilized by autoclaving. Ten of the PD-coated beads were spread on the top washed soil layer. The bioluminescence light (BL) signal was measured by a height-adjustable fiber optic probe, positioned 10 mm above the bead layer, connected to a luminometer (Model TD-20e, Turner Design, CA) for 200 min at 20-min intervals.

Results and discussion

The mechanical strength and the stability of PD-coated alginate beads

The swelling degree of the alginate beads is an important feature in measuring the mechanical strength and the stability of microbeads. When the beads were unstable, several bonds combined within the alginate beads were weakened. For example, the alginate beads swell easily when they come into contact with monovalent ions, because Ca^{2+} in the alginate beads is substituted for the monovalent ions, and so the bond of the beads decomposes easily [33]. Accordingly, the comparison between the swelling of the alginate beads and the PD-coated beads was evaluated for 2 h incubation in the LB medium containing $CaCl_2$. In this case, 3 different PD-coated beads were prepared by using different concentrations of PD at 0.025, 0.01, and 0.005 mg/ml solutions, respectively. Figure 2 shows that as the PD concentrations for coating increased, the degree of the swelling decreased. When the concentration of PD used for coating the beads was 0.005 mg/ml, it swelled as much as the alginate beads did. The maximum ratio of the size change in percentages also shows the same trend (Fig. 2 inset). Thus, it was found that the 0.025 and 0.01 mg/ml of PD concentrations, which were appropriate for coating the beads, except 0.005 of PD, have a significant enhancement for the stability of bead swelling.

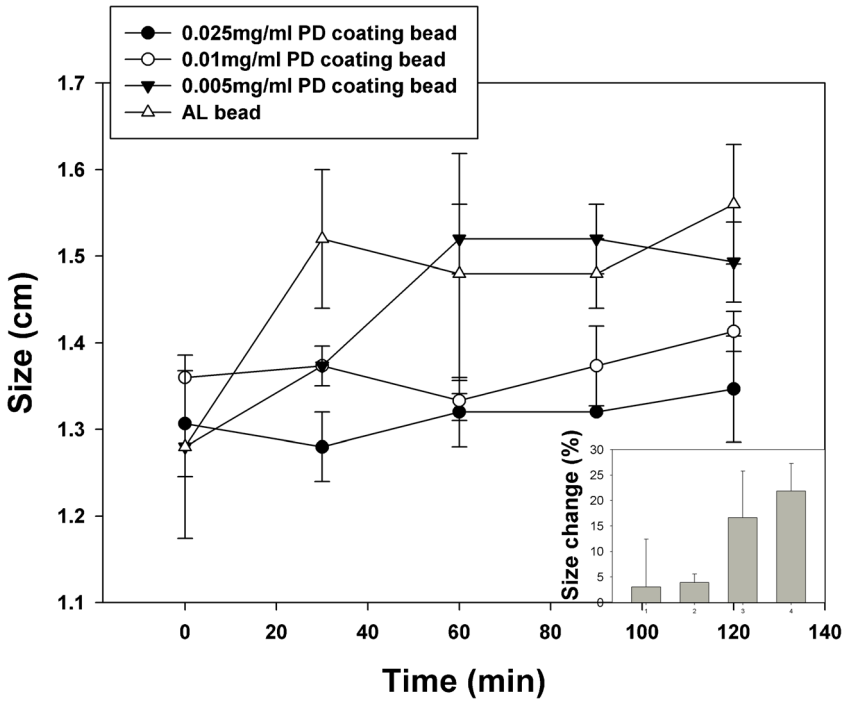


Fig. 2 Swelling effect of AL and PD-coated beads. Size change of AL and 3 different PD-coated beads according to time. Increase of maximum ratio (%) of AL and 3 different PD-coated beads after 120 min incubation in LB solution in the inset. Experiments were conducted 3 times and each point represented the average

The mechanical strength test was conducted as a continuation of the alginate bead stability test. As the PD concentrations for coating increased, the shape of the beads remained intact (see Electronic Supplementary Material (ESM) Fig. S1). Particularly, the morphology of the alginate beads began to tangle after shaking for 8 h, because the bonds formed in the alginate beads were loosened. After 32 h of shaking, the morphology of the alginate beads was unrecognizable because they were completely broken. However, even after 32 h, the PD-coated beads maintained their morphology and number. In other words, the PD-coated beads could maintain their shape in harsh conditions because they had a protective PD layer.

Cell leakage is also an important issue in these bacterial cell beads, because it could cause continuous declines in the BL signal. Accordingly, the cell leakage test was conducted through the immersion of every bead in the LB medium. In this case, the cell leakage evaluated by checking the OD of the LB medium containing an original alginate bead was about 0.45 after 24 h incubation, while the ODs of the LB media containing PD-coated beads was 0.14, 0.22, and 0.32 for the PD concentrations of 0.025, 0.01, and 0.005 used for coating, respectively (ESM Fig. S2). There was no bacterial growth in empty alginate beads without bacterial cells initially. Thus, this result indicates that the PD coating layer could prevent cell leakage by mechanically protecting the beads to some degree.

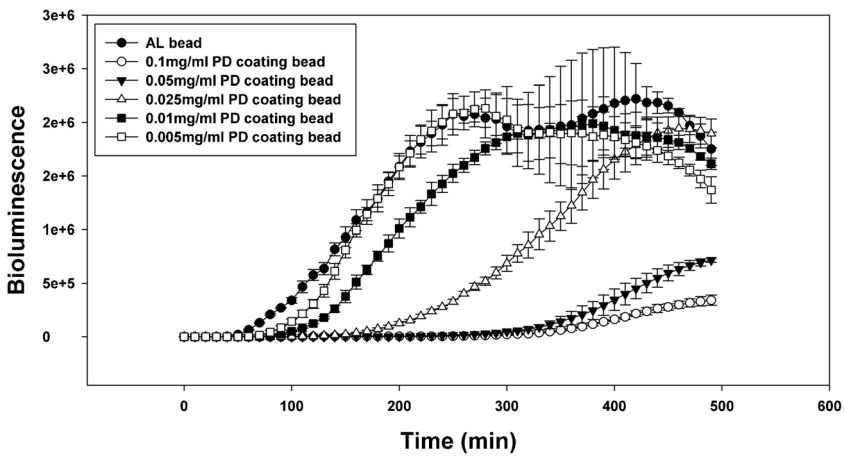


Fig. 3 Bioluminescence from AL beads and PD-coated beads containing the SoxS strain, which is induced by paraquat 25 ppm. Experiments were conducted 3 times and each point represented the average

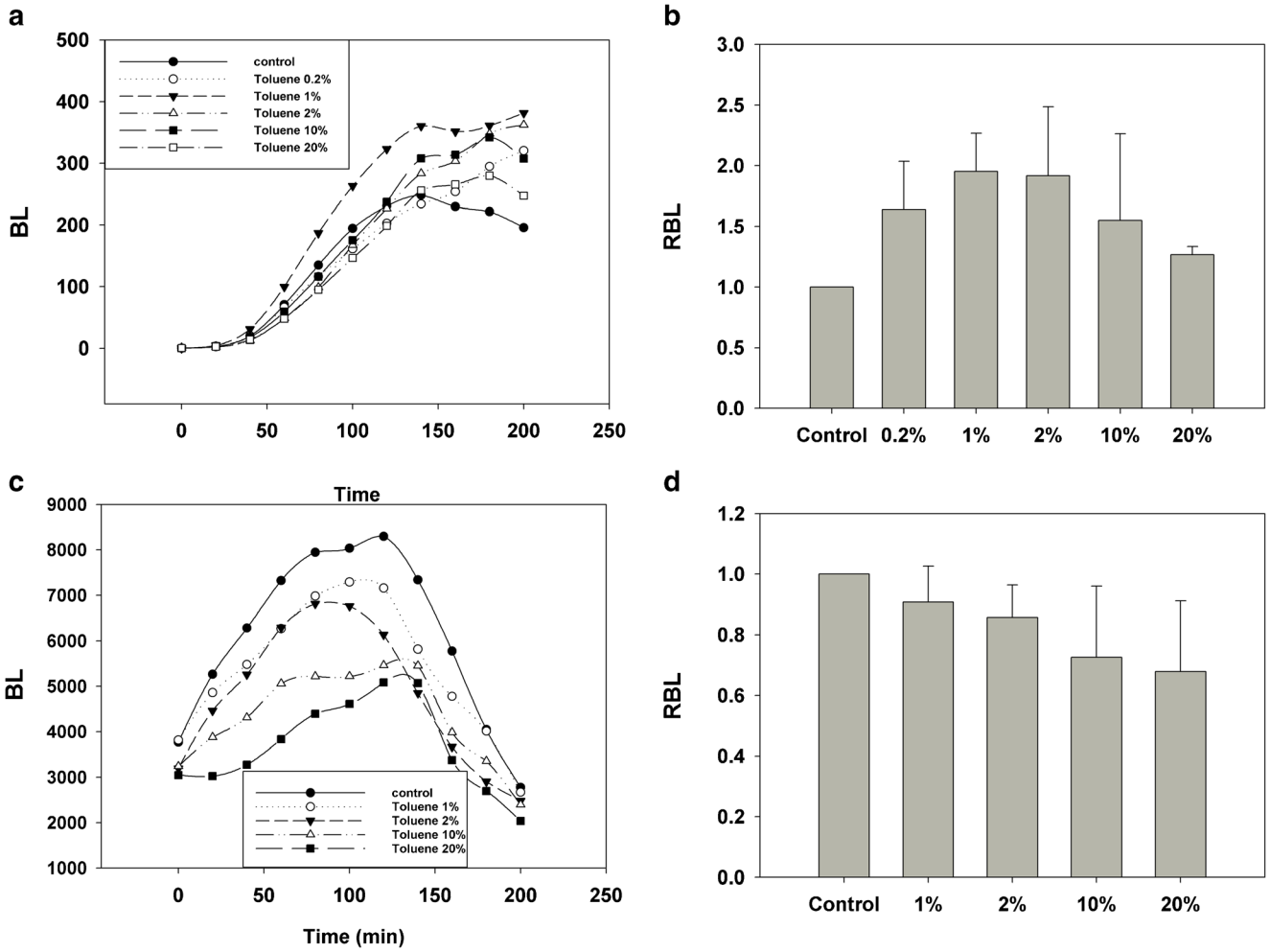


Fig. 4 Dose-dependent response of PD bead. a Dose-dependent BL signal of the PD-coated bead with the TV1061 strain induced by 5 different doses of toluene in the mini-vessel. **b** Maximum relative bioluminescence signal of the PD-coated bead with the TV1061 strain induced by 5 different doses of toluene in the mini-vessel. **c** Dose-dependent BL signal of the PD-coated bead with GC2 strain induced by 5 different doses of toluene in the mini-vessel. **d** Maximum relative bioluminescence signal of the PD-coated bead with GC2 strain induced by 5 different doses of toluene in the soil mini-vessel. Experiments were conducted 3 times and each point represented the average

The comparison of bioluminescence between the alginate microbeads and the fortified alginate microbeads with PD coating

In this study, in order to evaluate the effects of the PD coating on the alginate bacterial beads, BL signals of the PD-coated beads were compared with those of the alginate-only beads, while exposing them to toxicant. The EBSoxS strain [34] specifically responsive to the oxidative damages was immobilized in each of the PD-coated beads. Ten each of the PD-coated beads and alginate beads were loaded in 50 μl LB in a 96-well plate, respectively. After loading the beads, 50 μl paraquat (25 ppm) was added to cause the oxidative damage, and the BL signals from each well were measured for 8 h using the plate reader device. As can be seen, when the PD concentrations for coating increased, the BL signal was decreased and delayed in its response (Fig. 3). The reason why the BL signal is delayed could be the restrained bacterial growth in the PD shell-coated alginate beads [35]. In addition, when the PD concentration for coating is high, the PD shell might also absorb some of the bioluminescence light from the bacteria. Therefore, after this optimization study, the 0.01 mg/ml of PD concentration was chosen to coat the beads in following studies with soil toxicant sensing, because the BL signal from the PD-coated beads was almost the same as that of the alginate beads and the stability of the PD-coated beads was higher than that of the alginate beads at this concentration (ESM Figs. S1 and S2).

Dose-dependent response of fortified bacterial beads in the optical detection module-based biosensor for the toluene-contaminated soil

The bioluminescent bacterial biosensors have been developed for the detection of various toxicants, and these bacterial

strains were validated with many different concentrations of toxicants in our previous research [18–24]. Each stress-specific bioluminescent bacterial strain could be used for preparing standard curves for different toxicants. The responses of each stress-specific bacterial strain can be made in the same soil conditions with different dose of the toxicants as a standard curve. Then, this standard curve can be compared with the real sample data for estimating the real dose of the toxicant in the samples (schematic Fig. 1). In this study, toluene was chosen as a model chemical to represent volatile and toxic chemicals. In a dose-dependent test for the detection of soil toxicants, the TV1061 and GC2 strains were used to be immobilized in the PD-coated beads, respectively. The TV1061 strain is known to be responsive to protein damage, and so toluene is expected to cause bacterial bioluminescence responses as a target chemical. Figure 4a shows the response of this optical detection module-based biosensor with TV1061 immobilized PD-coated beads at different concentrations of toluene. As can be seen, the BL signal increased steadily in the test sample, but the BL of the negative control (without toluene) decreased after 140 min. It means the toxic effect of toluene at each different concentration used in this study can be detected in a dynamic range with this bead-based optical biosensor module platform. Figure 4b shows the trend of the maximum relative bioluminescence (RBL = test BL/control BL) with respect to the exposure time. This data shows that the BL signal was highest at the 1% of toluene, while the range of 0.2~20% of toluene could be detected using this optical detection biosensor module platform.

In the case of PD-coated beads immobilized with the GC2 strain, which has a *lac* promoter, instead of a stress-specific promoter, the bioluminescence from this bacteria strain continues to be emitted as long as the cells are alive and grow. In this case, the BL signal decreased as the concentration of toluene increased (Fig. 4c), where the toxic effect can be detected based on the reduction level of the BL signal, since the bioluminescence is decreasing when the cells are dead or less active due to the presence of the toxicant. Therefore, Fig. 4c showed a typical response from this optical detection biosensor module. The maximum RBL also followed the same trend, a gradual decrease of BL signal in response to the gradual increase of the toluene concentration (Fig. 4d). It means that the optical detection biosensor module using PD-coated beads immobilized with TV1061 and GC2 in this mini-vessel could successfully detect the toxicity of toluene-contaminated soil.

Effects of the thickness of the washed soil overspread for toluene-contaminated soil

The effect of the thickness of the washed soil spread over the contaminated soil was examined in the experiment, and the result can be seen in Fig. 5. Panel a shows the response of strain TV1061 to 1% toluene under washed soil layers of different heights. In each case, the height of the fiber optic probe was modified to be at the same distance (10 mm) above the soil surface (ESM Fig. S3). In this experiment, the PD-coated beads immobilized with TV1061 were used for the detection of toluene-contaminated soil. Figure 5 shows that the BL signal decreased as the thickness increased. The reason for this result could be the fact that volatilization of toluene was blocked by the upper soil layer, depending on its thickness. Therefore, when this optical detection module for soil toxicity measurement is used for real soil samples, thickness could be a factor effecting on bioluminescence strength. In addition, since PD was reported as a cytological compatible material [35] and stable mechanically, PD-coated bacterial alginate beads could be working successfully on the surface of the soil, without having any toxicity to the cells.

There could have been some issues about the use of this optical detection module-based biosensor for soil toxicity by using fortified PD-coated microbial beads. This system has been intended to be used in the lab or mobile vehicles, not

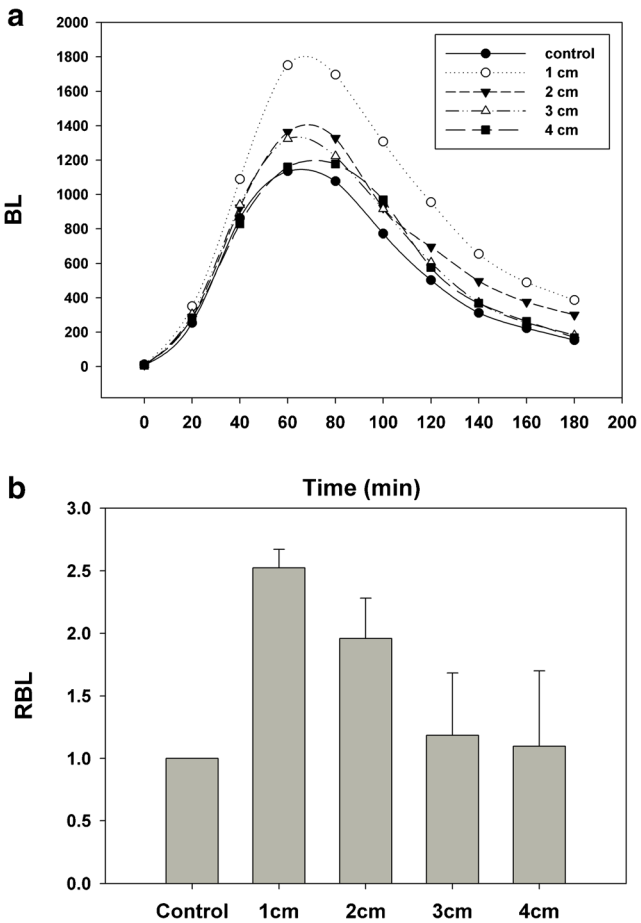


Fig. 5 Effect of the thickness of the washed soil layer on the responses of the PD-coated bead. **a** Thickness-dependent BL signal of the PD-coated bead with the TV1061 strain induced by toluene 1% in the mini-vessel. **b** Maximum thickness-dependent BL signal of the PD-coated bead with the TV1061 strain induced by toluene 1% in the mini-vessel. Experiments were conducted for 3 times and each point represented the average

spread on soil in the field, in situ, or on site, directly. For this reason, the duration of live bacteria after PD coating of alginate beads and the stability after spreading on soil have not been urgent issues in the application of this module system. In fact, the duration of live bacteria in alginate beads and the stability have already been studied previously [24, 36]. These details after PD coatings could be studied when it is used for real sample application, and optimized for each case in future.

Conclusion

In this study, we have successfully implemented a mini-bioreactor vessel, water jacketed for temperature control and with ambient light blocking, to an optical detection module-based biosensor for soil toxicity. In addition, we have also successfully developed fortified alginate beads containing bioluminescent bacteria by coating a PD layer on it, and the stability and the cell leakage were successfully improved by this PD coating. The enhanced mechanical stability of these PD-coated beads was found to be the most important factor to utilize in this bead-based optical detection biosensor module for detecting toxicities of various soil samples. By combining these two improvements, we demonstrated the application of the PD-coated bacterial beads for monitoring soil in the biosensor module. We believe that following additional optimization of the system, it will prove to be a useful tool for monitoring the pollution of contaminated soil samples and assessing their toxicity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Abbreviations PCBs, Polychlorinated biphenyls; BTEX, Benzene, toluene, ethylbenzene, and xylene; GC, Gas chromatography; GFP, Green fluorescent protein; $FMNH_2$, Reduced flavin mononucleotide; FMN, Flavin mononucleotide; PD, Poly-dopamine; LB, Lysogeny broth; OD, Optical density; BL, Bioluminescence; RBL, Relative bioluminescence

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