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ARTICLE

A dip-stick biosensor using bioluminescent bacteria encapsulated in color-coded alginate microbeads for detection of water toxicity†

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The use of genetically engineered bioluminescent bacteria, in which bioluminescence is induced by different modes of toxic action, represents an alternative to acute toxicity test using living aquatic organisms (plants, vertebrates, or invertebrates) in water environment. A number of these bacterial strains have been developed, but there have been no attempts to develop hand-held type of biosensor for monitoring or identification of toxicity. We report a facile dip-stick biosensor using genetically engineered bioluminescent bacteria as a new platform for classification and identification of toxicity in water environment. This dip-stick biosensor is composed of 8 different optically color-coded functional alginate beads which encapsulate different bioluminescent bacterial strain and its corresponding fluorescent microbead together. These color-coded microbeads exhibit easy identification of encapsulated microbeads since each microbead has different color code depending on the bioluminescent bacterial strain contained and improved cell-stability compared to liquid culture. This dip-stick biosensor can discriminate different mode of toxic actions (i.e. DNA damage, oxidative damage, cell-membrane damage, or protein damage) of sample water tested by dipping the stick simply into the water samples. It was found that each color-coded microbead emitted distinct bioluminescence and each dip-stick biosensor showed different bioluminescence patterns within 2 hours, depending on the toxic chemicals contained in LB medium, tap water, or river water samples. This dip-stick biosensor can, therefore, be widely and practically used in checking toxicity of water in environment primarily in situ on site, possibly indicating the status of biodiversity.

Introduction

Water in environment is what human being should always be able to use, touch and drink. If there are any toxic or hazardous compounds in source of water it would be very harmful and dangerous to human and biodiversity in environment. For that reason, water in environment and its sources should be clean and safe. However, people who live in the countries with shortage of water purification system or in a region that all water supplies have been stopped, have no choice but to use contaminated or polluted water. In addition, the increased usages of pharmaceutics, herbicides and pesticides, or man-made materials, resulting in toxic chemical discharge, are threatening the water in environment, making polluted water [1-3]. Therefore, fast and accurate toxicity sensing of water in environment including drinking water is urgent and needed, in order to protect human health and environmental biodiversity. Many studies on the discharges of toxic materials and monitoring their effects on living organism have been reported [4-6]. However, they are still suffered from a long detection time and expensive instrument. Therefore, fast and simple method to pre-screen toxicity is required.

Bioluminescent bacteria have been constructed by DNA recombination of plasmid, containing various stress promoters with *luxCDABE* gene [7-9]. Bioluminescent bacteria have been used for toxicity screening, due to their easy manipulation, fast response and low cost. Once toxic chemical induces a stress promoter inside bacteria, promoter starts the transcription of *luxCDABE*, producing luciferase which plays an important role in light emission. There are well known stress promoters which response to DNA damage, oxidative damage and so on, many

Strain names	Plasmid/Host	Damaging Stresses	Corresponding fluorescent codes (Volume Portion)	Reference
DPD2794	pRecALux/RFM443	DNA damage	Red	Vollmer et al. 1997
BBTSbmC	pJMsbmCLux/RFM443	DNA damage	Red + Green (2 : 1)	Ahn et al. 2009
EBAlkA	pCHalkALux/RFM443	DNA damage (Alkylation)	Orange	Ahn et al. 2009
EBSoxS	pBCsoxSLux/RFM443	Oxidative damage (Superoxide radical)	Green	Kim et al. 2005
DPD2511	pKatGLux/RFM443	Oxidative damage (Hydroxyl radical)	Red + Orange (2 : 1)	Belkin et al. 1996
EBHJ2	pSodALux/RFM443	Oxidative damage (response to both Hydroxyl and Superoxide radical)	No fluorescence	Lee et al. 2003
DPD2540	pFabALux/RFM443	Membrane damage	Orange + Green (1 : 1)	Choi et al.2001
TV1061	pGrpELux/RFM443	Protein damage	Red + Orange (1 : 1)	Van Dyk et al, 1995

Table 1. Stress-specific recombinant bioluminescent bacteria and their corresponding fluorescent codes.

different stress specific response strains could have been constructed [10]. According to the light emission from bacteria that have promoters induced by specific damages, the mode of toxic action of compounds can be screened. So far, our research group have been working on using bioluminescent bacteria for the detection of various toxic compounds with various platforms, such as gas sensor [11], multichannel system [12] and cell-chip with immobilization of bacteria [13, 14]. Herein, we take advantage of the intrinsic stabilization of bioluminescent bacteria in alginate microbeads and prepare color-coded alginate microbeads [14] that can be visualized and used in discrimination of bioluminescent bacteria encapsulated, thereby generating a portable dip-stick biosensor for easy monitoring and identification of toxicity in water samples for the first time compared to other biosensors developed by our group (Table S1). To demonstrate dip-stick biosensor, we encapsulated 8 different bioluminescent bacterial strains in alginate microbeads separately with different fluorescent beads to address color-coded visualization for the easy identification of each bead. We tested LB medium, tap-water, or river water samples containing toxic chemicals (single-dose or multiple-dose) to test performance of biosensor. The bioluminescence of bacterial strains used in this study was induced by different mode of toxic action such as DNA damage, oxidative damage, cell membrane damage, or protein damage [15-21]. The each color-coded alginate microbeads was set into each hole of dip-stick and bioluminescence was taken by CCD camera.

Materials and methods

Bacterial Strains and cell culture

In this study, 8 different stress-specific recombinant bioluminescent bacterial strains are used. Strains name, plasmid, host and damaging stresses characteristics are listed below in Table 1. The bacterial strains contained plasmid from either *Vibrio fischeri* or *Photorhabdus luminescens* with specific *stress promoter::luxCDABE* gene which are constructed based either on pUCD615 [22] or pDEW201 [23], respectively. DPD2794 [15], BBTSbmC and EBAlkA [16] specifically express DNA damage response, EBSoxS [17], DPD2511 [18] and EBHJ2 [19] are oxidative damage response, DPD2540 is cell membrane damage response and TV1061[20] is protein damage response. Each strain is streaked on agar plates which contain 50 μg/ml ampicillin (Sigma) and stay overnight. One colony was inoculated and grown in 1 ml of LB medium containing 50 μg/ml ampicillin in 15 ml falcon tubes in a shaking incubator at 37 °C and 250 rpm for 4 hours. Certain volume of strains in LB
medium were collected and pour into 50 ml of fresh LB medium to give 0.02 of OD value and cells are grown until they reach early stationary phase (OD=0.8). After, cells are centrifuged at 20 °C and 3000 rpm for 20 min and supernatant are discarded.

Preparation of color-coded alginate microbeads containing bioluminescent bacteria

Centrifuged bioluminescent bacteria and 2.9 ml of 2% (w/v) sodium alginate solution in distilled water were mixed and 0.1 ml of fluorescent beads (1.0 x 10^{10} beads/mL) (FluoSpheres®

Polystyrene Microspheres, 1.0 μm, Invitrogen) were added to give identification to each cell strains using red, orange and green fluorescent. Fluorescent beads used for each strain is described in Table 1. We used electrospray method to make microbeads. First, sodium alginate solution with bioluminescent bacteria and fluorescent microbeads put into 12 ml syringe which connected with 5 cm silicon tube and 30G needle. Solution in the syringe was extruded through needle by pushing force of syringe pump at 5 ml/hr and dropped into 50 ml sterilized 2% $CaCl_2$ solution on the stirrer by electrostatic and gravitational force. Electrostatic potential was generated by high voltage DC unit with connecting the positive electrode to aluminium foil on the stirrer and the negative electrode to the middle of needle. We made 7 kV of electrostatic potential to make 900~1000 μm size of bead. After forming, microbead went through hardening process that stirring in 2% CaCl2 solution for 30 min. Then, microbeads were washed with fresh LB medium 2 times and stored in 50 ml tubes at 4 °C to keep humidity until used (Fig. 1a).

Fabrication of optically coded functional microbead biosensor

Dip-stick biosensors were fabricated with transparent glass based on the computer-aided design and cut using laser engraving and a cutting machine. The size of dip-stick is 85.25 mm x 8 mm x 1.5 mm and has 10 holes that diameter is 1.25 mm and section of top to the bottom is reverse trapezoid shape so that microbeads don't escape to bottom and water samples are well merged into the hole. To sterilize each stick, we wash them with deionized water and 70% ethanol on clean benches. Then the sticks are dried under UV light for 30 min. Each sterilized stick was located on petri dish filling with 4 ml of fresh LB media. We used 100 μl pipet with tip and glass capillary connected by paraffin film to pick and place individual microbeads into the hole of stick. After fabrication dip-stick biosensors they were stored in 4 °C until used. Using an *in vivo* multispectral imaging system (Maestro 2^{TM}, Cambridge Research & Instrumentation (CRI), Inc), the fluorescence of each color-coded microbeads were taken. Three basic fluorescent: green, orange and red were excited at 505, 540 and 580 nm and the emission wavelengths were detected at 515, 560 and 605nm, respectively. From 500 to 720 nm were scanned in 10 nm bands for acquisition settings. Emission wavelength was analysed by Maestro software 2.8.0 (Cambridge Research & Instrumentation, Inc). The microbeads containing two different fluorescences are analysed based on wavelength diagram of green, orange and red fluorescence.

Test chemicals and toxicity screening

To demonstrate bioluminescence emission to each damage, 5 model chemicals were used (Mitomycin C (MMC), 1-methyl-1-nitroso-N-methylguanidine (MNNG), paraquat, hydrogen peroxide (H_2O_2) and 2,4-dicholorophenol (2,4-DCP)). The chemicals stock solutions in distilled water were diluted with fresh LB medium, tap water or river water sample (Han River in Seoul, Korea, filtered by motor filtering) for toxicity test. Before toxic chemical exposure, we took out the biosensor fabricated with microbeads from 4 °C and incubated them at 37 °C for 30 min to activate bacteria, which recover bacterial sensing capabilities. After that, biosensor was placed in square shape case (30 mm x 40 mm x 10 mm) that is made of Teflon and able to have three biosensors separately. Then, chemical solution (1 ml) was treated to be immersed for each biosensor. Biosensor was incubated in water bath at 30 °C and then placed in the dark box to take picture of bioluminescence using a cooled charge-coupled device camera (Cooled CCD camera) with constant focal plane and integration time (45 sec). Bioluminescence was taken every 15 min up to 1 hr and then every 30 min up to 2 hr after 1 hr. The bioluminescence intensity was analysed by MetaVue 5.0r6. The data were transmitted to Microsoft office EXCEL 2007 software to analyse bioluminescence induction.

Results and discussion

Bioluminescent responses of the dip-stick biosensor to toxic compound

A dip-stick biosensor was fabricated with 8 different bioluminescent bacterial microbeads which showed their own distinct stress responses. Each microbead has different fluorescent color-code to give identification and decrease the chance of confusing bioluminescent bacteria encapsulated in microbeads with the others in fabrication process. The bacterial strains were placed on the dip-stick chips (Fig. 1b) from left to right in this order: DPD2794 coded with red, BBTSbmC with red + green (2:1 volume ratio), EBAlkA with orange, EBSoxS with green, DPD2511 with red + orange (2:1 volume ratio), EBHJ2 with no fluorescence, DPD2540 with Orange + green and TV1061 with red + orange, which are responsive for general DNA damage, DNA damage cascade, alkylation DNA damage, superoxide radical oxidative damage, hydroxyl radical oxidative damage, both superoxide and hydroxyl radical oxidative damage, cell surface damage and protein damage, respectively. The 8 microbeads were distributed in the hole which has reversed trapezoid shape so that microbead cannot escape from the bottom hole of biosensor and water samples are well merged into the hole with microbeads (figure 1(a)). Using a multispectral imaging system, fluorescent codes of all microbeads were taken and analysed (figure 1(b)). Based on 3 basic spectrums (red, orange and yellow) on background, any color-coded microbeads can be identified by their own emission spectrums (figure 1(c)) and their own bacteria also can be identified based on correspondent fluorescence. Depending on chemicals bacterial responses induced by damage are

distinguishable. First, we conducted characterization of bioluminescent responses in dip-stick biosensor by spiking

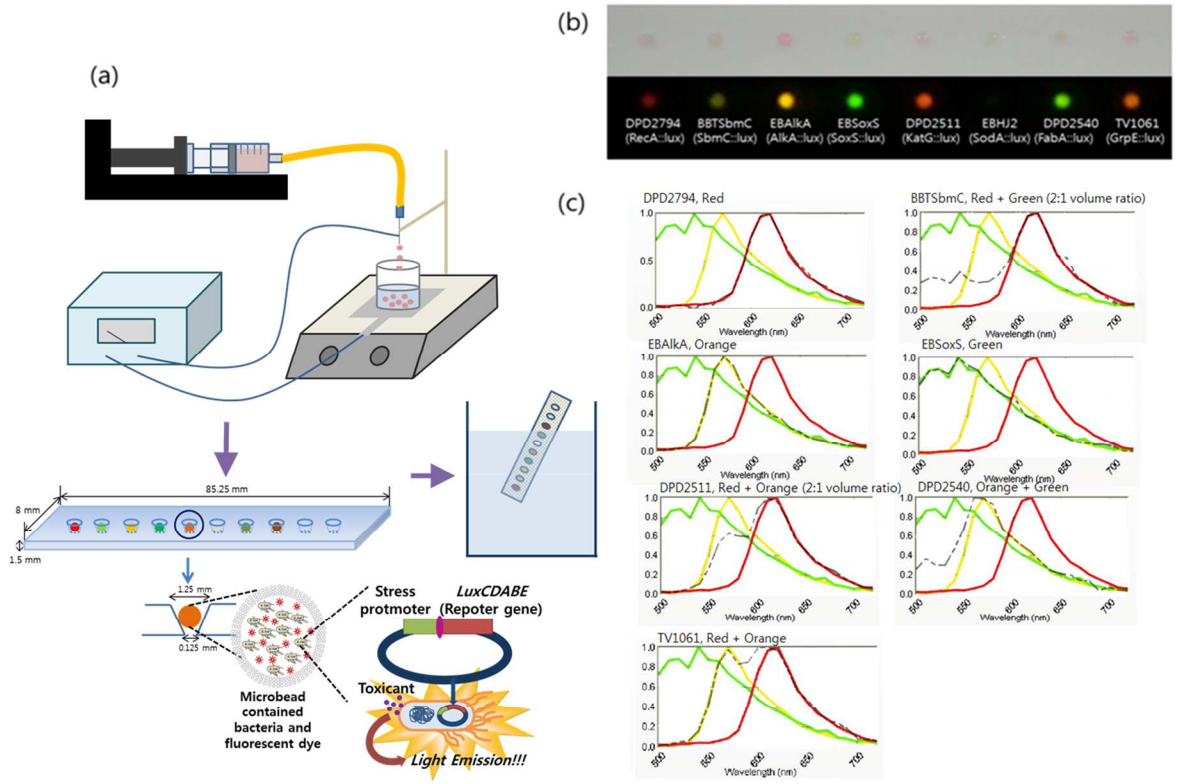


Figure 1. (a) Schematic diagram of fabricating a microbead on dip-stick biosensor and an example of usage of dip-stick biosensor. (b) Real and fluorescent picture of dip-stick biosensor fabricated with 8 different strains that are fluorescent coded. (c) Fluorescent spectrum data of microbead containing DPD2794 (Red), BBTSbmC (Red+Green (2:1 volume ratio)), EBAlkA (Orange), EBSoxS (Green), DPD2511 (Red + Orange (2:1 volume ratio)), EBHJ2 (No fluorescent), DPD2540 (Orange + Green) and TV1061 (Red + Orange) that are shown as dashed line. Three basic fluorescences (red, orange and green) are shown as red, yellow and green line, respectively

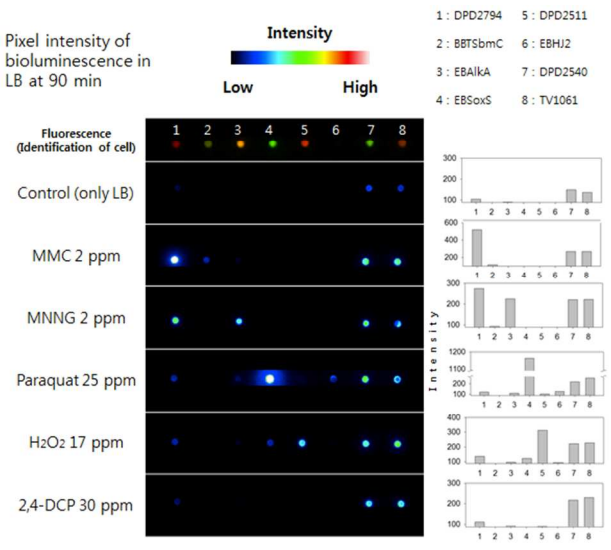


Figure 2. General characterization of bioluminescent response to 5 model chemicals.

single chemical species with various concentrations [27]. In figure 2 and S1, when treated with any individual chemicals, higher responses from DPD2540 and TV1061 were observed. This means that all chemicals treated cause cell membrane and protein damage. When MMC, antineoplastic agent which causes DNA damage by production of DNA cross-linked product and cellular SOS response [28, 29] was treated, DPD2794 showed strong response and BBTSbmC showed weak response. Also, it was hard to detect but still EBAlkA emitted light slightly. MNNG, directing methylating agents [30], caused a response from DPD2794 and EBAlkA, which means that MNNG cause general DNA damage and DNA alkylation. Paraquat generates superoxide and bacteria response to superoxide-generating agents [31], showing resistance to paraquat toxicity. Then, paraquat induced a response from EBSoxS and EBHJ2. H_2O_2, generating hydroxyl radical [32], caused a response from EBSoxS and DPD2511. They also induced DPD2794 slightly, because ROS accumulated and damage the DNA [33]. 2,4-DCP, which is phenolic compound and changes lipid-to-protein ratios of cytoplasmic and outer membrane of *Escherichia coli* [34], showed a response from DPD2540 and

TV1061. Dose-dependency of a dip-stick biosensor was shown in Figure S2 and S3. Each chemical addressed dose-dependent

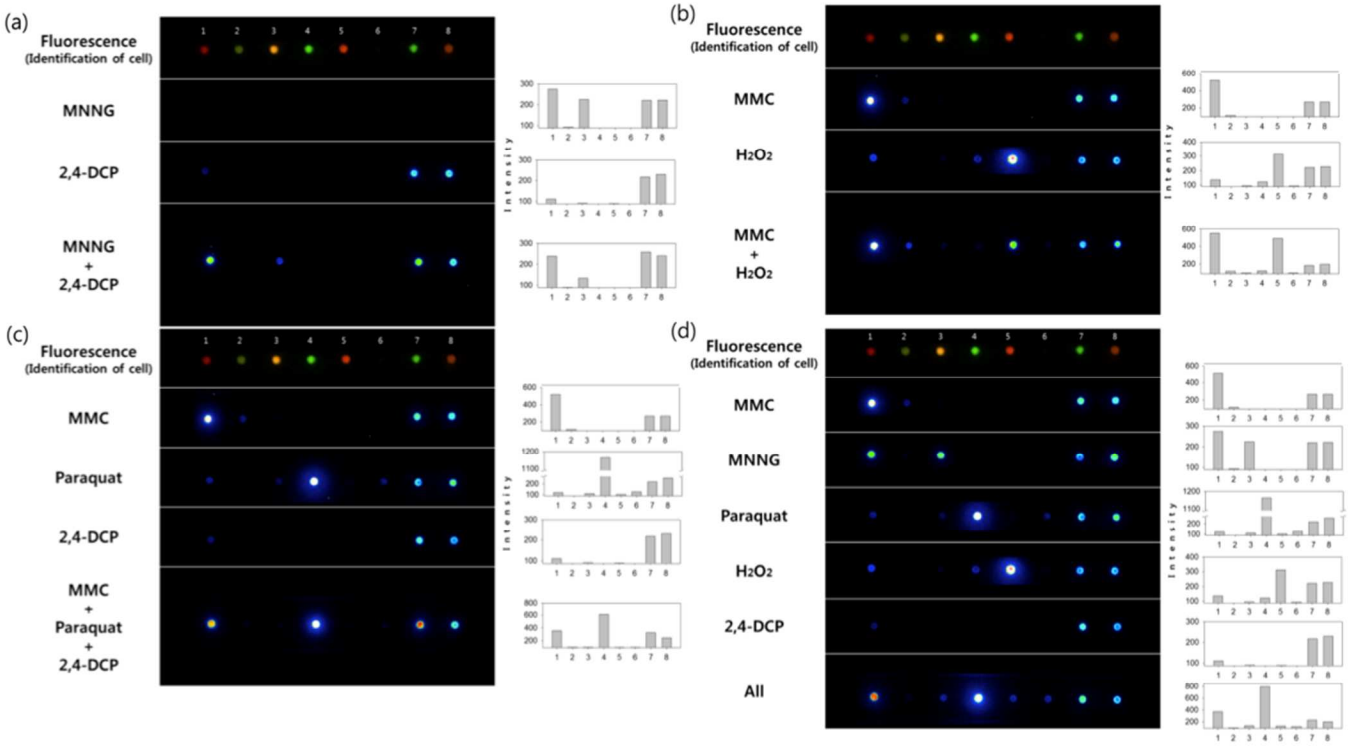


Figure 2. Bioluminescent data of Multiple-dose test using mixed chemical. (a) MMC 2 ppm and Paraquat 25 ppm (b) MNNG 2 ppm and 2,4-DCP 30 ppm (c) MMC 2 ppm, Paraquat 25 ppm and 2,4-DCP 30 ppm (d) All chemical(MMC 2 ppm, MNNG 2 ppm, Paraquat 25 ppm, H_2O_2 17 ppm and 2,4-DCP 30 ppm).

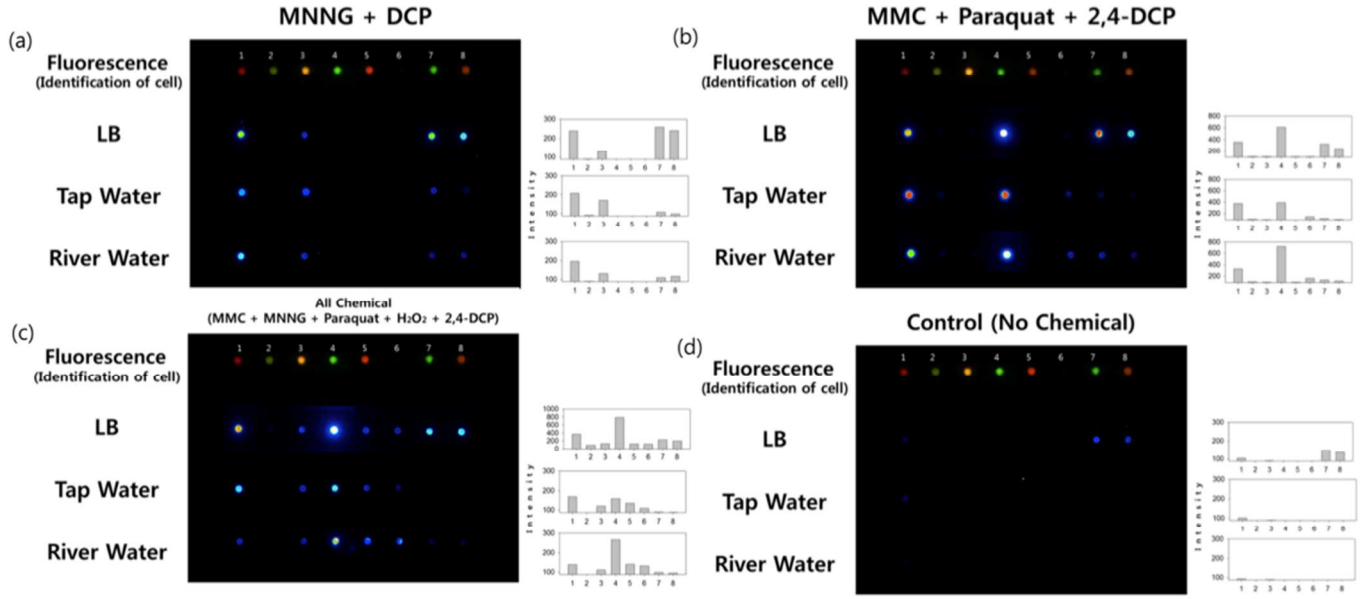
induction of bioluminescence up to critical concentration. Bioluminescence over critical concentration of chemicals was decreased because chemical toxicity was too strong to live for bacteria in microbeads.

Multiple-dose test

LB medium containing two, three or five chemicals were prepared and treated to a dip-stick biosensor (Figure 3) to investigate the bioluminescent response patterns and compare to single species dose. We used 4 different combinations of the mixed chemicals ((a) MNNG 2 ppm and 2,4-DCP 30 ppm, (b) MMC 2 ppm and Paraquat 25 ppm, (c) MMC 2 ppm, Paraquat 25 ppm and 2,4-DCP 30 ppm, and (d) MMC 2 ppm, MNNG 2 ppm, Paraquat 25 ppm, H_2O_2 17 ppm and 2,4-DCP 30 ppm) in LB. The induced bioluminescence in the combination of two and three chemicals showed the integrated patterns of individual chemical tests although their intensities were decreased (Figure 3 (a), (b), and (c)). The high loading of toxic chemicals (five chemicals mixture case) could not induce all bioluminescence appeared in individual chemical test. Most strains treated by all chemicals showed lower intensity compared to single chemical exposed one, which means that all 5 chemicals was too toxic to induce response of bacterial cells in microbeads (Figure 3(d)). In practical use, unexpected multiple pollutants may not induce the bioluminescent intensity as expected, because of high toxic strength which gives cells metabolic burden for producing bioluminescence. In that case, the test of diluted water samples may show the induction of bioluminescence intensity if the samples tested have high toxic strength [35, 36].

Spiking test in LB medium, tap water and river water sample

Chemical-spiked LB medium, tap water, or river water sample was treated on a dip-stick biosensor to see whether a dip-stick biosensor can be applied to test toxicity of tap water or river water (Figure 4). The induced patterns of bioluminescence triggered by chemicals in tap water or river water were similar with LB medium, although the intensity of bioluminescence in tap or river water samples was less than in LB. The nutrient in LB may gradually affect to bacterial cell activity inside the microbeads. These may make bacterial cells more robust during induction of stress triggered by the toxic chemicals spiked, compared to the tests performed with tap or river water. Although the intensity of bioluminescence were not same, this dip-stick biosensor is suitable for detecting toxicity of chemical in tap water or river water as describing the patterns of the



induced bioluminescence. In practical use, we considered that this dip-stick biosensor can be used as a fast pre-screening tool for that whether water samples tested have potential toxicity to be used. The specific induction of bioluminescence and variation of quantitative intensities in each microbeads to various toxic substances in water samples can give us useful information about kinds of toxicity and their strength in our hands. This type of diagnosis for environmental water could not be achievable with instrumental analysis or other biological toxicity test systems.

Figure 3. Bioluminescent response induced by chemical spiked LB medium, tap water sample and river water sample. (a) No chemical spiking, (b) MNNG 2 ppm + 2,4-DCP 30 ppm, (c) MMC 2 ppm + Paraquat 25 ppm + 2,4-DCP 30 ppm, and (d) All chemical (MMC 2 ppm, MNNG 2 ppm, Paraquat 25 ppm, H_2O_2 17 ppm and 2,4-DCP 30 ppm).

Conclusions

In this study, we successfully developed a dip-stick biosensor using 8 microbeads containing different bioluminescent bacterial strains, respectively. The size of dip-stick biosensor is 85.25 mm x 8 mm x 1.5 mm. The holes' diameter is 1.25 mm with a section that reverse trapezoid shape from top to the bottom, performing that microbeads won't be released out from the hole naturally. Each microbead has its own color-code so that it is very easy to distinguish what a bacterial strain is in its own microbead. This dip-stick biosensor is portable, and can be easily used with appropriate bioluminescent reader for detecting any toxicity of water in environment. Using this dip-stick biosensor, DNA damage, oxidative damage, cell surface damage and protein damage that response to toxic chemicals can be detected within 1 hr by measuring bioluminescence using CCD camera. This dip-stick biosensor can, therefore, be widely and practically used in checking toxicity of water in environment primarily in situ on site, possibly indicating the status of biodiversity.

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Notes and references

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