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Paper-based colorimetric biosensor for antibiotics inhibiting bacterial protein synthesis

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Due to the presence of antibiotics in environmental water and their potential influence on the occurrence of antibiotic-resistant bacteria, development of a detection method suitable for the screening of environmental water for antibiotics is required. In this study, we developed a simple colorimetric paper-based biosensor based on a novel principle for the detection of antibiotics inhibiting bacterial protein synthesis, including aminoglycosides, tetracycline, chloramphenicol, and macrolides. This biosensor is based on the detection of a color change induced by β-galactosidase, which is synthesized on freeze-dried paper discs containing an *in vitro* transcription/translation system. When a water sample without antibiotics is applied to the paper discs, β-galactosidase can be synthesized, and it hydrolyzes a colorimetric substrate, resulting in a color change from yellow to purple. By contrast, in the presence of antibiotics, the color change can be hampered due to an inhibition of β-galactosidase synthesis. We investigated the effect of the incubation temperature and pH of water samples and confirmed that the paper discs showed the color change to purple in the ranges of 15–37°C and pH 6–10. We observed concentration-dependent color variations of the paper discs by the naked eye and further estimated detection limits to be 0.5, 2.1, 0.8, and 6.1 μg/mL for paromomycin, tetracycline, chloramphenicol, and erythromycin, respectively, using digitized pictures. The paper-based biosensor proved to detect 0.5 μg/mL paromomycin, spiked in real environmental water samples, by the naked eye.

[**Key words:** Resistant bacteria; Environmental water; Cell-free protein synthesis; Paper-based biosensor; Paromomycin; Tetracycline; Chloramphenicol; Erythromycin; β-Galactosidase; Chlorophenol red-β-D-galactopyranoside]

Emergence of antibiotic-resistant bacteria has been considered a global threat to human health (1). The excessive use and abuse of antibiotics have been considered to be one of the major causes of the occurrence and spread of resistant bacteria (2). Residual antibiotics in environmental water and their influence on the occurrence of antibiotic-resistant bacteria are becoming a particularly serious concern (3). A number of studies have demonstrated that the water environment is one of the most important habitats for bacteria in general and resistant bacteria in particular (4–6). The problem of the excessive use and abuse of antibiotics is much more serious in developing countries because of no or less regulation of antibiotic use (7). Therefore, the monitoring of antibiotic residues in environmental water, particularly in as many samples as possible and from various locations in developing countries, is crucial to facilitate further comprehensive studies aimed at the prevention of antibiotic resistance.

In general, antibiotics in water samples, including environmental water and wastewater, can be detected using liquid chromatography in combination with mass spectrometry (LC/MS) because of its high sensitivity and reliability (8–10). In developing countries, however, it is difficult to implement these instruments because of their high cost and complicated operation, need for well-trained personnel, and difficulty in applying them to preliminary screening. Based on these circumstances, there is a growing demand for developing a simple detection method for antibiotics in environmental water, i.e., a detection method appropriate for preliminary screening.

Biosensors have been expected to become appropriate tools for preliminary screening that could overcome the drawbacks of conventional methods. Generally, a biosensor is composed of a biological recognition element, which is combined with a suitable physicochemical transducer to convert the biological response to a measurable signal that is proportional to the concentration of the target analyte (11). More recently, patterned paper has been gaining considerable attention as a highly multiplexed platform for biosensors (12). Unique aspects of the paper platform-based biosensors are that they are low cost, easy to transport, easy to dispose, and flexible to design. A paper-based sensor was originally developed in the point-of-care clinical diagnostic field, but the paper-based sensor technology has been applied recently to other fields, including environmental analysis (13). Recently, novel paper-based sensors for a diverse range of environmental contaminants, including metals, pesticides, and bacteria, have been reported (14).

We herein describe a colorimetric paper-based biosensor based on a novel principle for the detection of one of the main antibiotic categories, i.e., antibiotics inhibiting bacterial protein synthesis,

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such as aminoglycosides, tetracycline, chloramphenicol, and macrolides. The paper-based biosensor utilizes a system proposed by Pardee et al. (15), in which reporter proteins such as the green fluorescent protein or β-galactosidase (β-GAL) are synthesized on freeze-dried paper discs containing an *in vitro* transcription/translation (IVTT) system reconstituted from purified recombinant components necessary for *Escherichia coli* translation. The concept of the paper-based biosensor is that it detects the anti-translational activity of a group of antibiotics inhibiting bacterial protein synthesis through the color change induced by β-GAL synthesis on paper discs (Fig. 1). In the absence of antibiotics, β-GAL is synthesized and hydrolyzes a colorimetric substrate, changing the color of the paper discs from yellow to purple. By contrast, in the presence of antibiotics, inhibition of the β-GAL synthesis occurs, resulting in the inhibition of the color change. This paper-based biosensor can be a basis for a visual, convenient, and simple detection method for preliminary screening of environmental water for antibiotics inhibiting bacterial protein synthesis.

MATERIALS AND METHODS

Antibiotics Paromomycin sulfate was purchased from Sigma–Aldrich (USA). Tetracycline hydrochloride, chloramphenicol, and erythromycin were purchased from Nacalai Tesque (Japan). Stock solutions were freshly prepared by dissolving antibiotics in Milli-Q water in the case of paromomycin and tetracycline. Chloramphenicol and erythromycin were dissolved in ethanol, and subsequent dilution was done with Milli-Q water.

Preparation of DNA template for IVTT reaction A DNA template for β-GAL synthesis in the IVTT reaction was prepared by polymerase chain reaction (PCR) amplification from the pET21-based plasmid containing the *LacZ* gene sequence (16) using the T7F (5′-TAATACGACTCACTATAGGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′) primer set (17) with PrimeSTAR Max DNA polymerase (Takara, Japan). The PCR fragment contains the ribosome-binding site, T7-tag sequence, *LacZ* gene under control of the T7 promoter, and T7 terminator. The resulting PCR product was purified using the QIAquick PCR purification kit (Qiagen, Germany), and DNA concentrations were determined by absorbance at 260 nm.

Preparation of IVTT reaction The IVTT system used in this study was the PURExpress *In Vitro* Protein Synthesis Kit (E6800; New England Biolabs, USA). IVTT reactions were set up on ice according to the manufacturer's instruction. In a fluorometric assay, the reaction mixture (2 μL) was composed of 0.8 μL of solution A, 0.6 μL of solution B, 100 pg of the purified PCR product containing the *LacZ* gene (DNA template), and a 10 μM fluorescent substrate, Tokyo Green-β-GAL (TG-β-GAL) (Sekisui Medical, Tokyo, Japan). In a colorimetric assay, the reaction mixture (2 μL) was composed of 0.8 μL of solution A, 0.6 μL of solution B, 2 ng of the *LacZ* DNA template, and a 0.6 mg/mL colorimetric substrate, chlorophenol red-β-D-galactopyranoside (CPRG) (Sigma–Aldrich). An IVTT reaction without the DNA template or a β-GAL substrate was used as a negative control.

IVTT reactions on paper discs Paper discs (1442-055; Whatman, UK) were prepared using a 2-mm biopsy punch (15076; Ted Pella, USA). For fluorometric assays, 2-mm paper discs were placed into PCR tubes. Then, 2 μL of the IVTT reaction mixture was spotted onto the paper discs, and the discs were flash-frozen in liquid nitrogen and freeze-dried for at least 3 h. The freeze-dried paper discs were rehydrated with 2 μL of Milli-Q water and incubated at 37°C for 2 h in a Mx3000P real-time PCR system (Stratagene, USA). For colorimetric assays, 2-mm paper discs were placed on an adhesive plate seal (DC100PCR; Nippon Genetics, Japan) in a box. The process of spotting, freezing, and freeze-drying was performed as described for fluorometric assays. Two microliters of Milli-Q water, an antibiotic solution at the concentration specified, or a specific pH solution were spotted on the paper discs for rehydration. The specific pH solutions were prepared by adding NaOH to Milli-Q water. The adhesive plate seal with paper discs was placed in a humidified microarray chamber to prevent evaporation and incubated at specified conditions. In the case of testing the effect of temperature, the paper discs were prepared in PCR tubes and incubated at specific temperatures using a thermal cycler.

Detection of responses of paper-based IVTT reactions Fluorescence from the hydrolysis product of TG-β-GAL on paper discs was monitored over time by the Mx3000P real-time PCR system (Stratagene) using the FAM channel. The paper discs containing CPRG were air-dried at 37°C for 15 min after performing IVTT reactions and their digitized pictures were obtained using a scanner (GT-9800F, Epson, Japan) at a resolution of 1600 dots per inch. Then, color changes of paper discs were quantified using ImageJ. First, images were split into red, green, and blue (RGB) channels. Then, a mean of pixel intensities was measured within each paper disc region of the green channel. The value obtained from a paper disc that did not contain the DNA template was used for background subtraction. The limit of detection (LOD) was defined as $3 \times SD/S$, where S is the slope of a linear calibration curve, and SD is the standard deviation of the mean intensity of the blank. The slope was calculated using the data for the blank and 0.5 μg/mL for paromomycin, blank and 1.0 μg/mL for chloramphenicol, and blank and 5.0 μg/mL for erythromycin. The slope for tetracycline was calculated based on a regression line from data points for the blank, 0.5 and 5.0 μg/mL.

Preparation of real environmental water samples spiked with paromomycin Real water samples were collected from three different locations, a garden stream and a pond at Osaka University (Japan) and the Hau River (Vietnam). These samples were filtered through 0.2-μm filters (Kurabo, Japan) and spiked with paromomycin to reach a final concentration of 0.5 μg/mL. Two microliters of the spiked water samples was spotted onto the paper discs with CPRG, and the resulting color change was observed by the naked eye or quantified using the image scanner and ImageJ software as described above.

RESULTS AND DISCUSSION

Confirmation of β-GAL synthesis using IVTT reaction embedded into paper discs To construct a paper-based biosensor using IVTT reactions for antibiotics inhibiting bacterial protein synthesis, we initially examined whether the reporter gene can be expressed in the IVTT system on paper discs, as previously described by Pardee et al. (15). We embedded a commercial IVTT system reconstituted from purified recombinant components necessary for *E. coli* translation, including the *LacZ* DNA template and the β-GAL substrate TG-β-GAL, onto 2-mm paper discs, and then freeze-dried the discs (Fig. 1). The IVTT system we adopted was based on the PURE system technology (18). TG-β-GAL is a non-fluorescent substrate that emits fluorescence at 510 nm with an excitation at 490 nm when hydrolyzed by β-GAL (19). After adding 2 μL of Milli-Q water, the paper-based IVTT reactions showed an increase in fluorescence intensity, while those without the *LacZ* DNA template did not

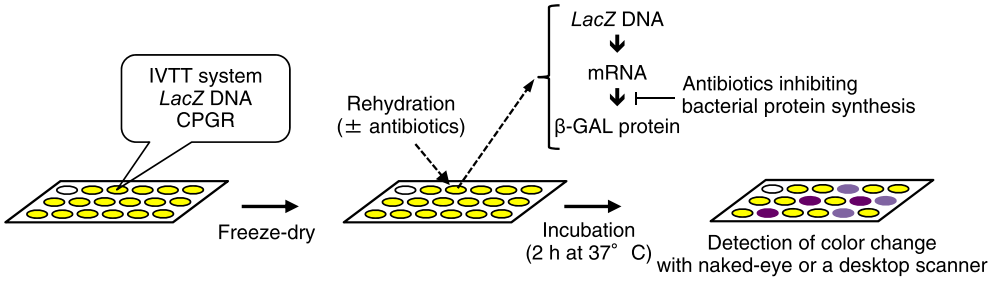

FIG. 1. Schematic of the colorimetric paper-based biosensor for the detection of antibiotics inhibiting bacterial protein synthesis. A mixture of an IVTT system, *LacZ* DNA template, and colorimetric substrate (CPRG) was embedded into paper discs, and the discs were then freeze-dried. The freeze-dried paper discs were rehydrated with water samples. In the absence of antibiotics, β-GAL is synthesized and hydrolyzes CPRG, resulting in the color change of the paper discs from yellow to purple. In the presence of antibiotics, β-GAL synthesis is inhibited, resulting in an inhibition of the color change. The color change was evaluated by the naked eye or using a desktop scanner with an image processing and analysis program (ImageJ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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