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BRIEF COMMUNICATION

Paper-based luminescence bioassay method embedding a sequence of enzymatic reactions to detect sulfonamide groups

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Abstract

Sulfonamide residue in foodstuffs and the environment is a serious global concern for their contribution to the occurrence of antibiotic-resistant bacteria, especially in developing countries. Here, we describe a novel, simple, and low-cost bioassay for sulfonamides, which has high potential versatility for use in low-resource settings. The bioassay method is based on a purpose-built luminescent assay reaction that detects sulfonamide groups. The luminescent assay reaction comprises dihydropteroate synthase, a target enzyme of sulfonamides, and luminescent pyrophosphate detection reagent, which triggers a sequence of biomolecular reactions that convert sulfonamides to emit luminescence. The novel assay detected at least six different sulfonamides with an estimated limit of detection of $<25\ ng\ ml^{-1}$ in a solution-phase assay using a microplate reader. More importantly, the luminescent assay reaction functioned even after spotting and freeze-drying on a wax pattern-printed paper platform. The paper-embedded luminescent assay reaction showed response signals to sulfadiazine within 30 min at a limit of detection similar to that of the solution-phase assay using a microplate reader. The signal could be recorded using a digital camera in the dark and required no other laboratory infrastructure, freeing the assay from the constraints of a well-fitted laboratory.

Sulfonamides (SAs) are a group of antibiotics that have been widely used to treat bacterial infections in humans and animals, and promote growth and prevent disease in livestock and aquaculture in numerous areas. Antibiotic consumption is rising globally, possibly because of increased income and demand for meat and other animal products [1, 2]. The increased and inappropriate use of antibiotics has resulted in the detection of SA residues in foodstuffs and environmental samples [3–8], which are considered a severe threat to human health mainly because of their promotion of antibiotic resistance in bacteria [9]. This is particularly prevalent in the developing countries where antibiotic regulation and control are generally less enforced. Therefore, obtaining comprehensive information on residual antibiotics and the completion of their global status is crucial, particularly in developing countries. To accomplish this, various confirmatory methods to monitor SA residues, such as chromatographic and screening methods including immunoassays or biosensors, have been developed [10, 11]. However, most of these methods require expensive instruments, reagents, well-trained personnel, or extensive sample preparation. Therefore, cheaper and simpler detection methods that are suitable for the low-resource settings of developing countries or can be performed outside a well-fitted laboratory are highly required.

Thus, this study aimed to develop a novel, simple, low-cost, and rapid bioassay method for SA detection, which has high potential versatility for use in low-resource settings. The target of SAs is dihydropteroate synthase (DHPS), which is involved in the folate biosynthetic pathway of bacteria and catalyzes the conversion of *para*-aminobenzoic acid and dihydropteridine pyrophosphate, releasing pyrophosphate (PPi, Fig. S1). SAs compete with *para*-aminobenzoic acid or

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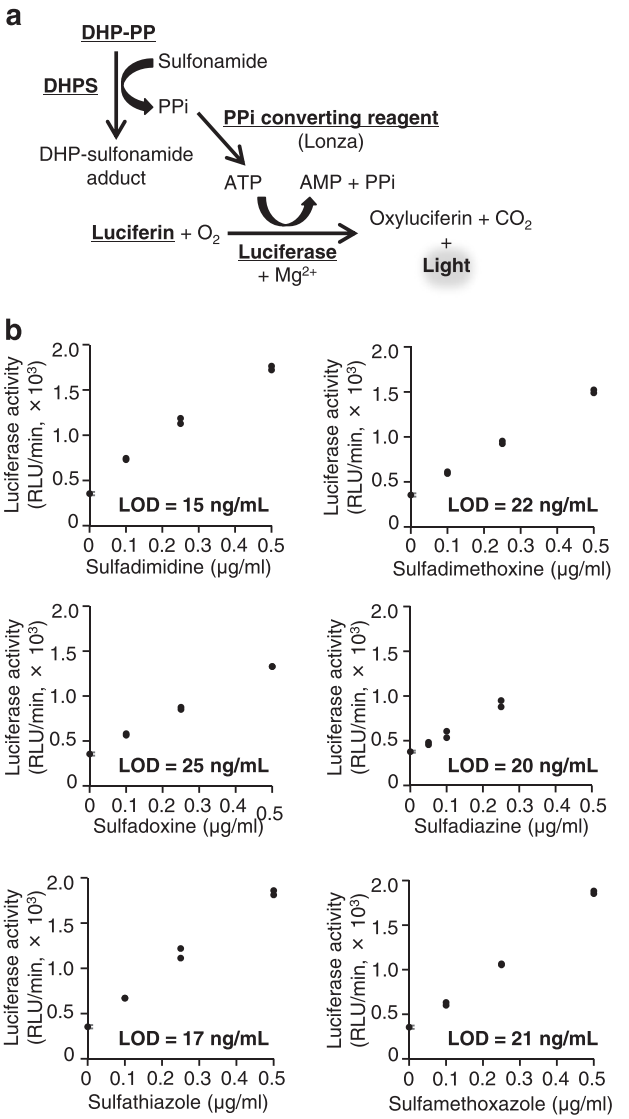

Fig. 1 Luminescence-based sensor solution for sulfonamides (LUCS) on a microplate reader platform. **a** Design and workflow of LUCS. Primary components of the LUCS are shown in bold font and underlined. **b** Response to various sulfonamides (SAs) at different concentrations. The means ± standard deviation of eight independent reaction pellets are presented for the blank sample, and the result of two independent reaction pellets are presented for SA solution

act as analogs of its substrate to produce non-functional SA-pterin adducts [12]. We speculated that the DHPS enzyme has potential as a recognition element, and therefore designed and formulated a luminescence-based sensor solution for SAs (LUCS), which comprises recombinant DHPS, dihydropteridine pyrophosphate, and a bioluminescent assay for detection of PPi (PPiLight inorganic pyrophosphate assay; Lonza, Fig. 1a). PPiLight reagent is a commercial bioluminescent assay kit, which detects PPi by enzymatically converting it to ATP (converting reagent) and the luminescence intensity is subsequently measured by an ATP-dependent luciferase (detection reagent). The working principle of the LUCS is based on the following facts (Fig. 1a): (1) SAs act as an alternative substrate of DHPS, forming PPi and (2) the resulting PPi is converted to ATP, which acts as a substrate for the luciferase-mediated conversion of luciferin to oxyluciferin, accompanied by luminescence emission proportional to the amount of PPi. We adopted luminescence as an output signal because it can be detected with high sensitivity using an inexpensive and portable digital camera, as described later.

To formulate LUCS, DHPS was prepared by recombinantly expressing *Escherichia coli* DHPS enzyme in *E. coli*, followed by purification (Fig. S2). Dihydropteridine pyrophosphate was synthesized by bioconversion from a commercial 7,8-dihydroneopterin using recombinant proteins of the two upstream enzymes of DHPS in the folate biosynthesis pathway (Figs. S1 and S2). To examine whether a solution of LUCS could react according to the design principles, lyophilized LUCS pellets prepared in microtubes were rehydrated with water or 1.0 μg ml^{-1} of sulfadiazine (SDZ) solution and monitored using a luminescent microplate reader. The result revealed that the SDZ solution remarkably increased the luminescent intensity (Fig. S3a). The response of the LUCS to SDZ was dependent on the components in the bioconversion solution and DHPS enzymes (Fig. S3b). LUCS detected at least six different SAs including SDZ (Fig. S3c) with estimated limit of detection (LOD) values <25 ng ml^{-1} (Fig. 1b). In contrast to antibody-based methods and similar to a recently published DHPS-based fluorescence polarization assay [13], the LUCS can be used to detect a group but not individual SAs. Therefore, the LUCS is likely to be suitable for class-specific detection in a single run, enabling the assay to be rapid, low sample consuming, high-throughput, and to reduce detection cost per assay. These characteristics are important factors for a tool that would be suitable for low-resource settings.

Although the solution-based LUCS has considerable potential as a bioassay method to detect SA groups, it requires an expensive luminescence microplate reader, which is not suitable for use outside a well-fitted laboratory or in low-resource settings. In addition, liquid reactions are difficult to handle outside the laboratory. There is an alternative, cheaper, and portable luminometer, but it is not suitable for multiplexed assays and requires a relatively large volume of reagents. Therefore, we next determined if the LUCS could function on a paper-based platform using a digital camera for signal readout (Fig. 2a). Recently, paper has gained considerable attention as a platform for inexpensive, low volume, flexible, portable sensors based on chemical or simple biochemical assays [14, 15], because of its versatility, high abundance, and low cost [16]. More recently, the paper-based system has been used with complex networks of biomolecular reactions, such as cell-free protein synthesis reaction [17].

In this study, the paper for embedding LUCS was prepared by printing hydrophobic wax barriers on commercial

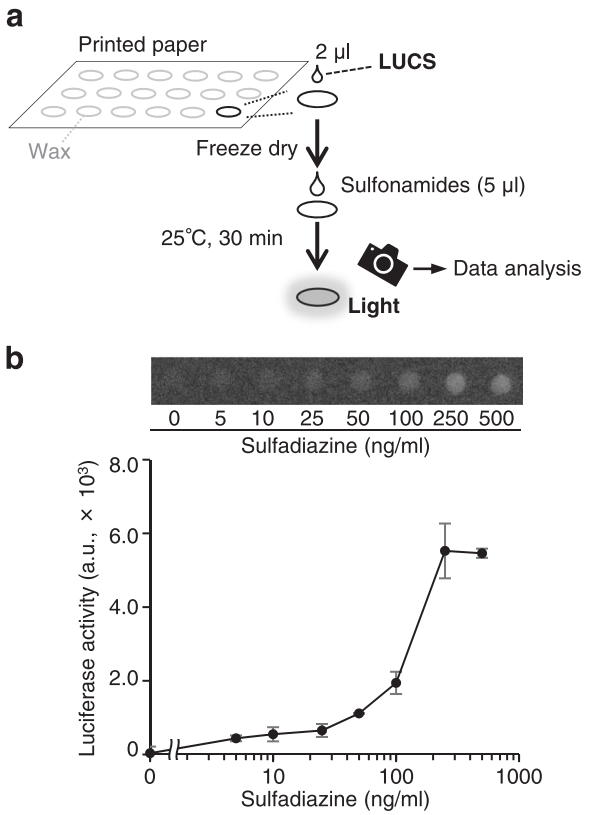

Fig. 2 Luminescence-based sensor solution for sulfonamides (LUCS)-embedded paper-based bioassay method. **a** Design and workflow of a paper-based bioassay method. **b** Response to various concentrations of sulfadiazine (SDZ). Darkfield images of LUCS-embedded paper rehydrated with various concentrations of SDZ were acquired using a digital camera at 5-min intervals, and the luciferase activities were evaluated. Data are median ± median absolute deviation of six or three independent assays for water or SDZ, respectively. A representative dark field image is shown

paper (1442-150, Whatman, UK) using a wax printer-based method [18] (Fig. S4). Briefly, 2 µl LUCS solution was spotted onto the paper in the printed wax rings and then freeze-dried (Fig. 2a) to make the paper-based LUCS easier to handle and transport. When 0.5 µg ml^{-1} SDZ solution or water was applied to the LUCS-embedded paper, luminescent spots could remarkably be detected for SDZ using a digital camera in the dark within 30 min (Fig. S5). Concentration-dependent increases in light emission were also observed for different concentrations of SDZ (0–0.5 µg ml^{-1}) using the LUCS-embedded paper and a digital camera (Fig. 2b). LOD was estimated as the concentration that generated luciferase activity (a linear slope of the increase in light emission) three times the median absolute deviation of the linear slope of increase in luminescence of a blank sample (see detail in supplementary materials and methods). An estimated LOD was 28 ± 14 ng ml^{-1}, which is similar to the levels estimated in a microplate reader-based assay (as shown in Fig. 1b). For example, Wei et al. [19] reported that maximum concentrations of ~20–200 ng ml^{-1} were frequently detected for SAs in wastewater and surface water around poultry farms in Jiangsu, China. Thus, our LUCS-embedded paper-based bioassay method has sufficient potential for detecting SAs residues in the environmental water samples. Interestingly, when real environmental water samples collected from two different locations at Osaka University, wherein SAs were not detected using LC/MS/MS (see details in supplementary materials and methods), were spiked with SDZ and subjected to the paper-based bioassay method, similar levels of estimated LOD values (24 ± 13 or 34 ± 22 ng ml^{-1}) were obtained as the results shown in Fig. 2b (Fig. S6), and percent recovery was 98% or 89%, respectively. These results indicated the potential of our method for testing real environmental water samples. Furthermore, we examined the storage stability of the LUCS-embedded paper and found that the paper showed similar estimated LOD values for SDZ (11 ± 5 or 16 ± 8 ng ml^{-1}) after 7 or 28 days of storage at 4°C, respectively. This storage stability makes our LUCS-embedded paper relatively easy to handle even outside the laboratory and easy to transport to remote locations. The details on the analysis of spiked real environmental water samples and storage stability tests are described in supplementary materials and methods.

Overall, in this study, we showed a novel concept of SA detection, the LUCS-embedded paper-based bioassay method that uses a sequence of biomolecular reactions to convert SAs to emitted luminescence. By printing hydrophobic wax rings on paper using a wax-printer (Fig. S4), embedding LUCS, and applying water samples within each of the printed wax rings on paper, this paper-based bioassay method enables users to perform multiplexed assays efficiently within 30 min using a portable digital camera with no requirement for an expensive laboratory infrastructure. In this study, photographed images were captured in the dark room of our laboratory, but we also found they could be captured outside the laboratory using a cardboard box and a blackout curtain (Fig. S7). Currently, the PPiLight kit, which contributes to the major proportion of the LUCS reagent expenses, costs less than US 5¢ for one spot on a LUCS-embedded paper. This is much cheaper than the ELISA plate kit. However, there is still a definite scope for improvement. First, further improvement of the sensitivity is required, because SAs are generally detected in foodstuffs and environmental samples in the order of picograms to nanograms per milliliters. Progress in the methods for concentrating SAs and designing sequences of enzymatic reactions embedded on paper would solve this problem. Further tests are likely to be required for foodstuffs because there is a high possibility that sample treatments, such as the solid-phase extraction, are required for sample clean-up to remove their matrix effects. Further optimization is also required for storage stability. We are currently working on establishing a LUCS-embedded paper that can be stored at

temperatures above room temperature, which will further aid its global versatility. Nevertheless, considering its ease of use and disposal, cost, assay time, and no requirement for trained personnel and laboratory infrastructure, we believe our paper-based SA bioassay method will be promising as an SA screening and monitoring tool for foodstuffs and environmental samples. Specifically, it has high potential versatility for use in the low-resource settings of developing countries, where a higher detection frequency and contaminated concentrations of antibiotic residues have been observed. Thus, our proposed bioassay method could contribute to ameliorating critical health-related situations in developing countries by reducing the incidence of resistant bacteria.

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Compliance with ethical standards

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

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