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Staphylococcal enterotoxins (SEs) have repeatedly caused food poisoning incidents worldwide. Some of the challenges associated with food poisoning outbreaks are that traditional detection methods are expensive and require long processing times and trained technicians. Microchannel devices represent a potential detection method by which these difficulties can be overcome. In this paper, we propose that immuno-pillar devices may represent a rapid, highly sensitive, and low-cost analytical system for the simultaneous detection of staphylococcal enterotoxin types A, B, and D (SEA, SEB, and SED) in milk. To prepare milk samples simulating food contaminated with SEs, commercial milk was spiked with equal amounts of SEA, SEB, and SED. A quantitative analysis of the milk samples was performed within 15 min by using the microchannel device. The analysis required only 0.5 μL of untreated milk sample. The resultant limit of detection was 15.6 pg/mL for each SE, and the total assay time and sensitivity were markedly shorter and higher, respectively, than those for commercially available assay kits. The detection range of each enterotoxin using these devices was estimated as 15.6 pg/mL–100 ng/mL, which completely covers the SE concentrations that can lead to foodborne diseases based on the US Food and Drug Administration's criterion for the infectious SE dose for SE poisoning (1 μg SE). Using our devices, frequent assessment of food potentially contaminated with SE is possible.

Introduction

The enterotoxin produced by *Staphylococcus aureus* is one of the primary causes of food poisoning outbreaks in the world.[1, 2, 3, 4, 5] Although the number of outbreaks of enterotoxin food poisoning has steadily decreased over recent years,[6] careless handling of food and a lack of knowledge about staphylococcal enterotoxin (SE) could easily trigger a serious incident such as that experienced in 2000 in Osaka, Japan. This incident was caused by heat-sterilised fat-free milk that was exposed to room temperature for several hours. The total number of victims of this outbreak was 14780.[7]

S. aureus exists everywhere including the intestine and skin of humans. The growth temperature range of *S. aureus* is 7.2–60 °C,[8] within which the bacteria increase in number while producing enterotoxin. Cooking *S. aureus*-containing food at sufficiently high temperatures eliminates the bacteria; however, the SE is heat-resistant thus is not destroyed by cooking. Peoples' ignorance regarding the heat resistance of SE has resulted in minor as well as serious outbreaks of food poisoning. To avoid serious food poisoning outbreaks it is important to ensure that foods and raw materials are not contaminated with SE. While the most common SE detection methods, including reversed passive latex agglutination and enzyme-linked immunosorbent assays (ELISA), are highly sensitive (detection limits of 250 pg/mL[9] and 28.2 pg/mL,[10]

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respectively), they have a number of limitations including requiring long processing times (80 min or more), well-trained technicians, expensive analysis kits, specific testing for each SE ty

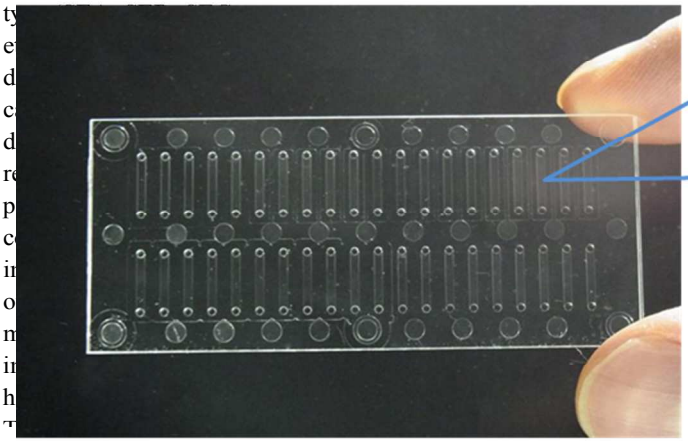

Fig. 1 Picture and schematic of the immuno-pillar device.

demonstrated immuno-pillar devices to be suitable for rapid, easy-to-use, low-cost, multiplex, and highly sensitive immunoassays. [15, 16] The immuno-pillars have a porous structure, which allows molecules such as protein biomarkers, SE, and fluorescence-labelled antibodies to penetrate into the immuno-pillars, and therefore sandwich-type immunoassays can be carried out using antibody-immobilized polystyrene beads contained within the immuno-pillars. The immuno-pillar devices represent a detection method for SE contamination that overcomes all the problems associated with current methods. Here we demonstrate the use of immuno-pillar devices for the simultaneous detection of SEA, SEB, and SED, which are the three worst factors of SE poisoning.[1]

Experimental methods and materials

Materials and reagents

Carbonate buffer (CB, pH 9.7) was prepared with sodium carbonate and sodium bicarbonate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Phosphate-buffered saline (PBS, pH 7.4) was prepared with phosphate-buffered saline powder (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Millipore water (Millipore Corporation, Billerica, MA). Polystyrene b Polysciences, Inc. (Warring styrene beads and its coeffi respectively. The polystyre ior to use. *S. aureus* strain a University Hospital and th Research Institute.[16] d from the enterotoxin g Es with more than 95% pur btain each anti-SE were isolated fro All anti-SEs antibodies were tested their spe LISA prior to use.[16] Standard, native SEs with m urity ≥3.6%; Minorakuren, Gifu, Japan) to simulate contaminated milk. AnaTag HiLyte Fluor 647 Protein Labelling Kits (AnaSpec, San Jose, CA), Alexa Fluor 488 Monoclonal Antibody Labelling Kits (Life Technologies, Carlsbad, CA), and HiLyte Fluor 555 Labelling Kits-NH_2 (Dojindo Laboratories, Kumamoto, Japan) were used to label the anti-SEA, anti-SEB, and anti–SED antibodies, respectively. This allowed us to fluorescently quantify the respective SEs by changing the optical systems and lasers. Bovine serum albumin (BSA; Blocker™ BSA) was obtained from Pierce (Rockford, IL) and a 1% (v/v) BSA solution (BP) was prepared by diluting 10% BSA with PBS. A polyethylene glycol (PEG)-based photo-crosslinkable prepolymer solution (MI-1) and a photoinitiator solution (PIR-1) were purchased from Kansai Paint Co., Ltd. (Osaka, Japan).

Construction of immuno-pillar devices

A photograph of the immuno-pillar device and a schematic of the device components are shown in Fig. 1. Forty straight, rectangular microchannels with cylindrical inlets and outlets

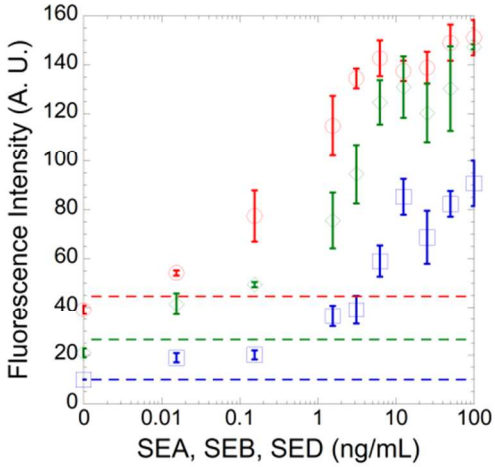

Fig. 2 Calibration curves for SEA (red), SEB (blue), and SED (green). Values and errors were calculated as averages and SDs of the fluorescence intensities of the immuno-pillars, respectively. The dashed lines represent the detection limits and were estimated as 3SD above the backgrounds.

were constructed on a cyclic olefin polymer (COP) substrate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) by injection moulding and were produced on a large scale. In each microchannel, immuno-pillars with a diameter of 200 μm were constructed on a triangular lattice with a distance of 600 μm between nearest neighbouring sites.[16] Polystyrene beads (1 μm in diameter) supporting anti-SEA, anti-SEB, or anti-SED IgY antibodies were placed together in each immuno-pillar. Anti-SEs IgY antibodies were immobilized onto polystyrene beads via hydrophobic interaction, and to prevent non-specific binding of the other proteins, blank sites between these IgY antibodies on the microbeads were blocked with 1% BSA. The immuno-pillar devices were constructed using the standard contact UV exposure technique described elsewhere,[15] and thus only a brief outline of the process is given here. The polystyrene beads supporting the anti-SEA, anti-SEB, or anti-SED antibodies were separately prepared in three independent vials after which the three suspensions were mixed at a volumetric ratio of 1:1:1. The concentration for each antibody-immobilized bead after mixing was 7×10^9 mL^{-1}. The MI-1 (200 μL), PIR-1 (10 μL), and PBS (100 μL) were added to the mixed bead suspension (100 μL) and the mixture was stirred using a vortex. The mixture was introduced into the microchannels using a pipette (PIPETMAN®, Gilson S.A.S, Villiers le Bel, France) and was irradiated with UV light (365 nm) through a photomask for about 10 seconds. The photo-crosslinkable prepolymer was polymerized in the microchannel according to the photomask pattern after which the exposed areas formed hydrogel pillars containing ~30000 beads (about 10000 beads of each antibody type). The number of beads affected the fluorescence intensity because the correlation between capture probability for antigen and the number of beads is positive. UV irradiation time influenced the beads number in the hydrogel pillars because the size of pores in the hydrogel pillars depended on the UV irradiation time. As the irradiation time was shorten, the pores enlarged and the beads leaked from the pillars. Note that the number of beads was reduced to one-third of that in the devices for single assay.[16] Finally, uncured prepolymer was removed using a suction pump and the hydrogel pillars were immersed in BP for at least 30 min until use. The manufacturing cost per immuno-pillar device including the COP substrate was less than $1.

Assay procedures

Milk samples containing three SEs (SEA, SEB, and SED) at the same concentrations were prepared. The SE concentrations used were 0.0156, 0.156, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 ng/mL and milk containing no SEs was used as a blank sample. Injection and removal of solution were carried out by using a pipette (PIPETMAN®, Gilson S.A.S, Villiers le Bel, France) and suction pump, respectively. Detailed assay procedures are as follows. The BP solution was removed from the microchannel, after which 0.5 μL of milk sample was introduced into the microchannel and incubated for 5 min. After suctioning the milk sample, washing with PBS (0.5 μL) was carried out 5 times. Then, 0.5 μL of the mixture of fluorescently labelled detection IgY antibodies specific for SEA, SEB, and SED was introduced into the microchannels and incubated for 5 min in the dark. The immuno-pillars were finally washed with PBS 5 times and immersed in BP solution. The washing step took 30 seconds. Therefore, one assay was completed within approximately 15 minutes. By changing both the optical filter and the excitation light source, three fluorescence images per immuno-pillar were captured using an inverted fluorescence microscope (LX-71, Olympus, Tokyo, Japan) equipped with an electron multiplying charge-coupled device camera (C9100-13, Hamamatsu Photonics, Shizuoka, Japan), an Ar laser (model IMA101010B0S, 488 nm, Melles Griot, Carlsbad, CA), a Nd-YAG laser (model 4611-050-1000, 532 nm, JDS Uniphase, Milpitas, CA), and a He-Ne laser (model 05-LHP-991, 633 nm, Melles Griot). Fluorescence images captured with Ar laser, Nd-YAG laser, and He-Ne laser represented the fluorescence of detection antibodies for SEB, SED, and SEA, respectively. The fluorescence intensities of the immuno-pillars were analysed with AquaCosmos software (Hamamatsu Photonics).

Results and Discussion

We performed the calibration of detector before the assay for the antigens with a series of concentrations. We adjusted the gain of the detector by using each antigen with the highest concentration. Milk samples were tested in a dose dependent manner. The resulting calibration curves for SEA, SEB, and SED are shown in Fig. 2. We performed the assay for 3 replicates per sample. The values and standard deviations (SDs) in Fig. 2 were calculated from those results. Based on the statistical theory, the limits of detection (LODs) were estimated as 3SDs above the backgrounds (negative control values) and are represented by the dashed lines in Fig. 2. The background is the sum of the auto-fluorescence of IgY-coated beads, plastic substrate, and UV curable resin. The LOD for each SE was

estimated to be 15.6 pg/mL, which is lower than not only those of the most common SE detection methods including latex agglutination[9] and ELISA[10, 16] but also those of the immuno-pillar devices for single assays of SEA and SED.[16] The inherent high sensitivity of the immuno-pillar devices is attributed to their high surface-area-to-volume (*S*-to-*V*) ratio. Let us evaluate the *S*-to-*V* ratios for the microtiter plate wells and for the immuno-pillar devices. *S* and *V* are defined by the surface area of the reaction field and the solution volume, respectively. For the microtiter plate wells, the *S*-to-*V* ratio for 50 μL of milk sample in a microtiter plate well (6.5 mm in diameter) supporting the anti-SEA, SEB, and SED antibodies is estimated to be 4.3 cm^{-1} for each SE type. On the other hand, for the case of the immuno-pillar devices, *V* is defined by the dimension of the microchannel (40 μm × 1000 μm × 6500 μm) and *S* is the sum of the surface areas of 160000 polystyrene beads in 16 pillars. Thus, the *S*-to-*V* ratio of the immuno-pillar devices is about 19 cm^{-1}, which is 5 times larger than that of the microtiter plate wells. The sensitivity improvement in comparison with the immuno-pillar devices for single assays can be explained by the relaxation of the self-quenching[17]: in the immuno-pillars, the antibody-immobilized polystyrene beads were clustered via hydrophobic interaction between the capture antibodies and in the case of the multiplex immunoassay devices, these clusters were composed of three kinds of beads supporting the anti-SEA, SEB, or SED antibodies. As a result, the distance between the same fluorescence-labelled antibodies was relatively extended in the present devices in comparison with that in the devices for single assay.

The multiplex immuno-pillar devices were found to have a wide detection range of 15.6 pg/mL–100 ng/mL for SEA, SEB, and SED. The US Food and Drug Administration's estimation of the poisonous dose of SE is 1.0 μg, which translates to the lowest acceptable intake of SE-contaminated milk (100 ng SE/mL milk) being 10 mL. According to Pfaff,[18] the average sip of water is 12.75 mL, which exceeds the lowest acceptable intake of SE-contaminated milk. The LOD (15.6 pg/mL) of the device described here is much lower than the lowest SE concentration among those have been found in major food poisoning outbreaks (380 pg/mL).[19] Contaminated food that could potentially cause a food poisoning outbreak could therefore be immediately identified using this microchannel system.

Conclusions

In this paper, we fabricated the immuno-pillar devices for simultaneous detection of SEs at very low cost and demonstrated quantitative analysis of SEs in 0.5 μL of the milk sample. Milk samples were prepared to contain SEA, SEB, and SED, which are the major factors of foodborne illness.[1] Simultaneous quantitative analysis of the milk samples with no pre-treatments (enrichment of SEs, removal of unwanted materials, etc.) was carried out using immuno-pillar devices, which were found to exhibit high sensitivity (LOD: 15.6 pg/mL), a wide dynamic range (15.6 pg/mL–100 ng/mL), as well as rapid (15 min) and simultaneous detection of SEs. The US Food and Drug administration's criterion for pathogeny of food poisoning (1 μg SE) equates to 10 mL–64.1 L of contaminated milk (100 ng/mL–15.6 pg/mL), a range that completely covers the amount of milk that people would ingest at one time. The high sensitivity and high assay speed attributed to high surface-to-volume ratio of the immuno-pillar devices. The device described here may be a powerful tool for on-site detection in food factories as well as for the identification of SE-contaminated foods in foodborne outbreaks of food poisoning.

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