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Printed two-dimensional micro-ring film plate for spot assays and its functionalization by immobilized enzymes

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ABSTRACT

In this paper, we present a simple printable two-dimensional micro-ring plate for spot assays in low-resource settings. Each micro-zone on the overhead transparency contains a hydrophilic center restricted by a superhydrophobic ring. The zone can be fully wetted by a small amount of aqueous sample (0.5 μl) and can also hold a large volume up to 100 μl, offering the flexibility to handle different amount of samples. Bio-analysis capacity of the micro-ring plate was demonstrated by the Pierce 660 nm protein assay and blood typing assay. We further functionalized the plate by depositing enzymes-immobilized nanorods into micro-zones. This functionalization, for the first time, enables multi-diagnostic functions in one micro-ring as demonstrated by analysis of individual substrate of the two enzymes. The functionalized plates show a high stability under varied temperatures that mimic the changes of environment during transport. Importantly, all the assays were carried out using non-laboratory devices such as mobile phone, ensuring its low-cost application under limited resources.

1. Introduction

Qualitative and semi-quantitative spot assays are conventionally performed using polymer-molded three-dimensional multi-micro-zone plates (e.g. 96-well or 384-well plates). This type of multi-assay micro-zone plates has been an essential tool to perform various chemical and biochemical assays [1]. The importance of the plate in conducting assays has driven the development of a range of analytical instrumentations (e.g. Micro-plate reader) [1–3]. Whereas the well-established supporting instrumentations maximize the analytical power of the polymer-molded multi-assay plates for laboratory use, it makes these plates to be much less effective and less versatile for applications out of well-equipped laboratories. This view is relevant to the current human health and environmental challenges we are facing; disease screening and diagnostics in developing world, which carry 95% of world population [4], are unable to take full advantage of using multi-assay micro-well plates, since well-equipped laboratories and special supporting instrumentations are not always available in developing countries. In addition, the cost of the traditional plates as consumable products is relatively high and does match the affordability of those regions. Therefore, affordable and portable plates that do not rely on special supporting instrumentations are highly desirable to improve the availability of spot assays and diagnostics in developing countries.

The research on portable multi-assay micro-zone plates can be traced back to 1937 [5]. Yagoda fabricated two-dimensional (2D) micro-zone plates by impregnating circular hydrophobic barriers on filter paper to enclose hydrophilic zones. Aqueous samples and reagents for spot tests can be restricted in the hydrophilic porous zones rather than non-porous plastic wells. Several decades later, due to increasing interests for point-of-care test in low-resource settings, there has been a renaissance of paper-based micro-zone devices. Since 2007, much research effort has been made toward the fabrication of portable paper-based micro-zone plates to improve the affordability and remain the sensitivity of ELISA as well as other biochemical assays [1,3,6–12]. Besides that, alternative low-cost materials such as polymer film were also utilized to fabricate portable low-cost micro-zone plates [13,14]. These plates partially overcome drawbacks of paper plates, for example, the deformation of paper substrate when it needs to be repeatedly exposed to aqueous solutions during ELISA test. In our previous study [14], we have fabricated a two-dimensional 96 micro-zone plate based on cellulose-patterned polymer film by contact printing and

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demonstrated that less than 2 μl of liquid sample per micro-zone was required to perform a chemical or biochemical assay on this plate. The use of polymer film as substrate brings several advantages into spot tests: they are more processable and durable; sample penetration in the analytical zones is easily controllable, thus eliminate inter-zone leaking; assays on patterned transparent film can be analyzed using both reflection and transmission modes [13,14].

Notwithstanding these advantages, the low ability of liquid sample retention of these plates reduces their impetus for rapid diagnosis in poor areas [15]. Besides that, although the 2D plates have significant advances in packaging and transportation, the assays which need multiple diagnostic reagents and be conducted by complex processes, in fact, are not convenient for the delivery and usage. In addition, due to the low-resource settings of the poor areas, these assay plates may encounter the varied environments during the delivery. The effect of the harsh environmental conditions on the biochemical reagents functionalized on 2D plates is critical, but has not been investigated in previous studies.

In this study, we designed a simple printable 2D overhead transparency-based micro-ring plate (μRP) for spot assays in low-resource setting. The μRP can handle large volume aqueous samples for complex tests, overcoming the liquid retention problem of the paper plate. This μRP also have been utilized to identify hemagglutination reactions from the side view of aqueous sample droplets in order for the determination of blood type. We also functionalized the assay zones using immobilized enzymes [16] in order to offer needed convenience and stability for outdoor diagnostics. The stability of functionalized plates during transportation was confirmed by a series of transportation simulation experiments. The plate functionalized by immobilized enzymes provided much better deliverable stability than which functionalized by free enzymes. In addition, the functionalized μRP (F-μRP) can be directly utilized to perform single qualitative, multi-qualitative and semi-quantitative assay. Importantly, all the assays in this study were analyzed by general portable devices, such as scanner and cell phone, demonstrating the availability of μRP without support of special instrumentations.

2. Materials and methods

2.1. Materials

The polymer film used in this study was a commercial overhead transparency (Xerox). A UV curable flexographic post-print varnish (UV 412) was received from Flint Inks (Flint Group Australia). Antibodies (EpicloneTM Anti-A, Anti-B, Anti-D (IgM) FFMU Concentrate) against RBC antigens approved for human blood grouping were obtained from the Commonwealth Serum Laboratory (CSL), Australia. Chemicals: All the materials and chemicals were of analytical grade and used as received without further purification. Tetraethyl orthosilicate ($Si(OC_2H_5)4$, TEOS) (>99%), 3-glycidoxypropyl-trimethoxysilane (GOPS, 2530-83-8), hydrochloric acid (320331), ethanol amine (110167), potassium chloride (P5405), triblock copolymer pluronic p-123 (P123), enzyme horseradish peroxidase (HRP, P8125), bovine serum albumin (BSA, 05470), alkaline phosphatase (ALP, P0114), phosphate buffer saline (PBS) tablets (P4417), cellulose powder (Sigma-Cell, average particle size ¼20 mm) and Teflon powder (average particle size 35 mm) were purchased from Sigma–Aldrich (Sydney, Australia). 3,5,3′,5′-tetramethylbenzidine (TMB) (N301), nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (NBT/BCIP) (72091) and Pierce 660 nm protein concentration test reagent (22660) were purchased from Thermo Fisher Scientific, Australia. Water was obtained from a MilliQ system with the electrical resistance of 18 MΩ cm.

2.2. Fabrication of μRP (Scheme 1)

UV varnish was printed on the film using a designed printing plate. The Teflon powder was then dusted on this printed film. After UV curing, unfixed powder was removed by fan blowing.

2.3. Protein concentration test

In this test, bovine serum albumin (BSA) protein was used as model protein. BSA protein solutions in six different concentrations (0, 0.5, 1, 1.5, 2 mg/ml) were tested separately. In a typical test, 37.5 μl Pierce 660 nm protein concentration test reagent was added onto micro-ring. Then, 2.5 μl protein solution was added onto the micro-ring and mixed with the 37.5 μl reagent. The mixture was kept statically under dark environment for 5 min. Then, photo of the micro-ring was taken by smartphones and analyzed by photoshop CS5 software. The paper plate used for control experiment was fabricated by following our previous method [17].

2.4. Blood typing test

Four blood samples (A−, B+, AB+ and O−) which contain the three major antigens (antigen A, antigen B and antigen D) were chosen to demonstrate the determination of hemagglutination reaction with their correspondent antibodies by using our 2D μRP. Ten microliters of each antibody solution (Anti-A, Anti-B and Anti-D) were placed into the zoned area using a micropipette. Then, 2 μl of blood sample was mixed with three different antibody reagents respectively. After 1 min, photos were taken from the side view of the samples droplets in order to identify the occurrence of hemagglutination reaction between the blood samples with correspondent antibodies.

2.5. Functionalization of μRP

The HRP and ALP were immobilized on silica nanorods by using our previously reported methods [16]. Then, the immobilized HRP was suspended in PBS (0.1 M pH 7) and mixed with cellulose powder (the cellulose powder was used as an inexpensive material to improve image quality by providing white background and absorbing liquid in the following tests) suspension (in 0.1 M pH 7 PBS), to make the final immobilized HRP suspension (0.25, 0.5, 1.0, 1.25, 2.5, 5.0 mg/ml immobilized HRP and 40 mg/ml cellulose powder in PBS). The immobilized ALP was suspended in sodium carbonate buffer (0.1 M pH 9.5) and mixed with cellulose powder suspension (in 0.1 M pH 9.5 sodium carbonate buffer), to make the final immobilized ALP suspension (2, 4, 5, 6, 8, 10 mg/ml immobilized ALP and 40 mg/ml cellulose powder). Then, 2 μl of final immobilized HRP/ALP suspension was implanted onto micro-ring of the plate. After that, the plate was dried in fume hood for 10 min. The final immobilized HRP and ALP suspension mixture was made by mixing 1 ml 2.5 mg/ml final immobilized HRP suspension and 1 ml 4 mg/ml final immobilized ALP suspension. Then, 2 μl mixture was implanted onto micro-rings to make the multiple-functionalized plate.

2.6. Qualitative and semi-quantitative assays

For qualitative assays, micro-rings that functionalized by 1.25 mg/ml final immobilized HRP suspension, 2 mg/ml final immobilized ALP suspension and the final immobilized HRP/ALP mixture were employed. In a typical assay, 2 μl of TMB or NBT/BCIP was added into immobilized HRP or immobilized ALP functionalized