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Increased sensitivity of 3D-*Well* enzyme-linked immunosorbent assay (ELISA) for infectious disease detection using 3D-printing fabrication technology

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Abstract. Enzyme-linked Immunosorbent Assay or ELISA -based diagnostics are considered the gold standard in the demonstration of various immunological reaction including in the measurement of antibody response to infectious diseases and to support pathogen identification with application potential in infectious disease outbreaks and individual patients' treatment and clinical care. The rapid prototyping of ELISA-based diagnostics using available 3D printing technologies provides an opportunity for a further exploration of this platform into immunodetection systems. In this study, a '3D-*Well*' was designed and fabricated using available 3D printing platforms to have an increased surface area of more than 4 times for protein-surface adsorption compared to those of 96-well plates. The ease and rapidity in designing-product development-feedback cycle offered through 3D printing platforms provided an opportunity for its rapid assessment, in which a chemical etching process was used to make the surface hydrophilic followed by validation through the diagnostic performance of ELISA for infectious disease without modifying current laboratory practices for ELISA. The higher sensitivity of the 3D-*Well* (3-folds higher) compared to the 96-well ELISA provides a potential for the expansion of this technology towards miniaturization platforms to reduce time, volume of reagents and samples needed for laboratory or field diagnosis of infectious diseases including applications in other disciplines.

Keywords: 3D printing, acrylonitrile-butadiene-styrene (ABS), chemical etching, enzyme-linked immunosorbent assay (ELISA), infectious disease surveillance

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1. Introduction

Methods for antibody labeling with enzymes, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), commonly known as Enzyme-linked Immunosorbent Assay (ELISA), is the gold standard for the demonstration of various immunologic reactions including the measurement of antibody levels in infectious diseases [1-4]. Most ELISAs are performed in 96-well microtiter plates made up of polystyrene (PS) where reliable protein adsorption methods have been responsible for the solid phase reactions [2, 4]. Although advancements have been made in traditional ELISAs such as increasing automation and the performance of multiple concurrent assays, many constraints still exists such as the need for a longer diffusion time of analytes thereby increasing the total assay duration, the need for large sample and reagent volumes and is often laboratory-based [1, 2-7].

Over the past few years, the development of newer miniaturization-driven platforms which aim to increase reaction surface-to-volume requirement and reaction kinetics through the use of microbeads [5, 8], carbon-nano tubes [2, 9], non-conventional solid-phase material such as capillary glass and other polymers [5-8, 10-12] and increased sensitivity in detection systems [2, 13-16] have improved the handling of samples, ensured high-throughput and improved sensitivity while reducing time and cost to run the assays compared to traditional ELISAs. Nonetheless, they require complex instrumentation and micro-manufacturing methods which are expensive, require skilled operation and are unparalleled in terms of the number of samples that can be tested concurrently as the traditional ELISA [2]. Hence the uptake of these newer platforms may be limited since long established institutional practices, protocols and instrumentations centered on the use of traditional ELISAs will need to be completely overhauled, which is a factor this study aimed to avoid.

The introduction of one-step fabrication technologies, based on Fused Deposition Modeling® (FDM) such as 3D printing has had a substantial impact in the development of miniaturized biomedical and biochemical detection systems such as microfluidics and LOC technologies [17]. The ease of use of various design drafting programs such computer aided design (CAD) software and the parallel development of 3D printing technologies with .STL (Standard Tessellation Language or STereoLithography) file formats as a link with 3D printers [17] has provided room for the rapid development of various prototypes and for easy and rapid design readjustment to be made following early assessments [18, 19]. These rapid cycles of designing-prototype development-assessment and feedback are important considerations in the development of biomedical devices including those with potential of use in immunoassays. The use of wax-based polymers and other thermoplastics such as acrylonitrile-butadiene-styrene (ABS) and polycarbonate (PC) among others, which are easily fabricated, modified to become more hydrophilic enhancing surface-protein adsorption and disposable due to its low cost, are important aspects in the development of ELISA-based systems [5, 11, 14,18-24]. The potential of such fabrication technologies in the development of diagnostics for infectious and other diseases and its potential impact in improving disease diagnosis, treatment, care and support, and prevention must not be excluded [25, 26].

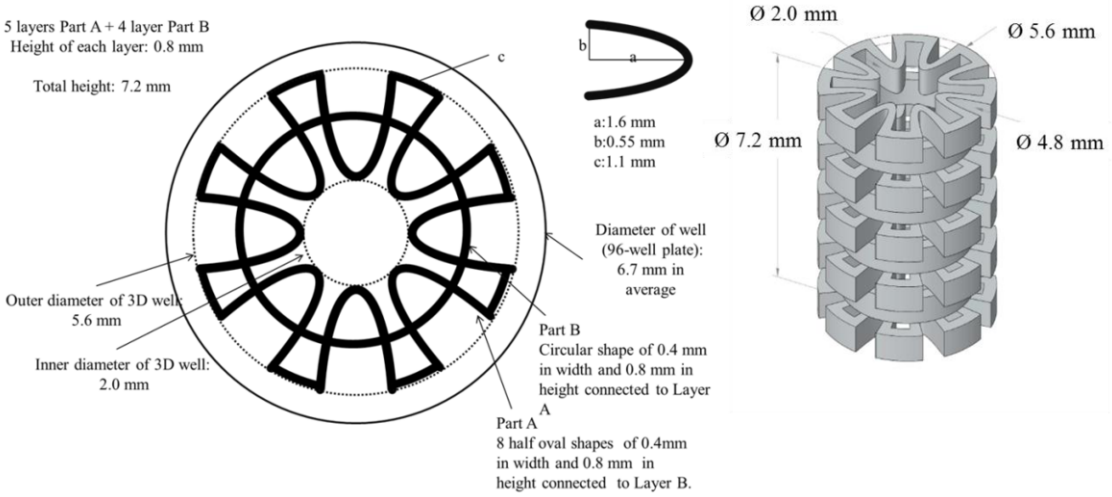
This study reports the utilization of 3D printing platforms in the development of a '3D-*Well*' composed of ABS in increasing the sensitivity of ELISA as well as its structural and physical characteristics, material and surface modification, validation in the immunological diagnosis of infectious diseases and the future expansive potential of this technology.

2. Materials and methods

2.1. 3D-Well design rationale

The aim of the design of the prototype, '3D-*Well*' used in this study was to increase the available surface for antigen-antibody binding thereby allowing a shorter diffusion distance of analytes compared to the 96-well ELISAs [Surface area: 651 mm^2 (3D-*Well*) *vs*. 151 mm^2 (96-well plate)]. In addition, to maintain the present protocols and practices for 96-well ELISAs and to allow multiple assays to be carried out at the same, the 3D-*Well* was designed to fit appropriately in the wells of the 96-well plate (Sigma-Aldrich, St. Louis, USA) used in this study. While remaining patent on either ends forming a hollow cylinder-like structure, the 3D-*Well* was designed composed of 2 parts, A and B of 2 different shapes; part A (5 layers, 8-half oval shapes in radial spokes) and B (4 layers, circular in shape) stacked up alternatively enabling the prototype to be printed in a single step. A schematic diagram with the different 3D-*Well* design parameters is shown in Figure 1. The 3D-*Well* was designed utilizing the CAD software and converted to a .STL file using the inbuilt Insight 10.2™ job processing and management software (Stratasys®, Eden Prairie, USA).

2.2. Fabrication of 3D-Well



(a) (b)

Fig. 1. 3D-*Well* Design: (a) Schematic diagram, top view (b) CAD software design.

Table 1

System configuration of Fortus 250 mc (Stratasys®, USA) printer, process parameters and materials used in this study

System Configuration	
Build envelope (XYZ):	254 × 254 × 305 mm (10 × 10 × 12 in.)
Layer thicknesses:	0.007 inch (0.178 mm)
System size/weight:	838 × 737 × 1143 mm (33 × 29 × 45 in.)
Achievable accuracy:	± 0.241 mm (± 0.0095 in.)
Material	
Build material:	ABS*plus*-P430™ (Acrylonitrile-butadiene-styrene)
Soluble release material:	*SR-30 Soluble Support* (terpolymer of methacrylic acid, styrene and butylacrylate)
Process Parameters	
Internal Temperature:	85°C
Modeling Head Temperature:	300°C
Maximum Scanning Speed:	3.6 in/s

A Fortus 250mc (Stratasys®) 3D printer, with an inbuilt 254 × 254 × 305 mm build envelope (XYZ stage) was used in the 3D printing of the prototype. This was carried out through an extruded (X-Y platform) deposition from bottom up (Z stage) of alternating semi-molten layers (300°C) of ABS*plus*-P430™ (Acrylonitrile-butadiene-styrene as build material) (Stratasys®) and *SR-30 Soluble Support* (terpolymer of methacrylic acid, styrene and butylacrylate as soluble release material) (Stratasys®), both of 0.178 mm thickness. The support material was then removed by sonication in a water bath containing a detergent solution at 30°C for 2 hours. The system specification of the Fortus 250mc 3D printer, process parameters and materials used in this study are summarized in Table 1.

2.3. ABS surface modification

The ABS surface of 3D-*Well* was modified to a hydrophilic surface by chemical etching to ensure effective protein binding capacity to its surface and the overall ELISA efficiency. Briefly, physical and other adsorbates were removed by degreasing using sonication for 3 minutes in a 50℃ in a water bath. To ensure effective chemical etching of the ABS surface, absorbates and other agents that were released after degreasing were removed by using a detergent containing solution. An etching solution of (CrO_3 0.38 g + H_2SO_4 0.37 g)/10 mL was used for chemical etching, which was carried out in a water bath at 65℃ for 3 minutes.

2.4. Sample collection and ethical statement

In an ongoing collaboration for the serological monitoring of vaccine preventable diseases in Central Vietnam, a total of 272 serum samples from patients who visited the Outpatient Department of the Hue University Hospital between March and June 2014 were screened for *Measles virus* antibodies by Immunoglobulin G (IgG) ELISA using 96-well ELISA.

Serum samples collected from all patients were carried out under informed consent. All protocols and procedures were approved by the Research and Ethical Committees for the use of human subjects of the Hue University of Medicine and Pharmacy and the Tokyo Metropolitan University.

In this study, 2 samples, serum containing *Measles virus* antibody (*Measles virus* antibody positive sample) and serum not containing *Measles virus* antibody (*Measles virus* antibody negative sample), one each respectively, were selected and used in the 3D-*Well* ELISA.

2.5. IgG ELISA

IgG ELISA was carried out using 3D-Wells with parallel testing by 96-well ELISA for validation purposes. Briefly, 3D-Wells and 96-well ELISA plates were coated with 100 µL of predetermined optimal quantity (1:4) of *Measles virus* Hemaglutination Antigen (HA) (Denka-Seiken, Tokyo, Japan) in sodium carbonate buffer at 4°C overnight. Blocking was performed with 200 µL of 0.05% Tween-20 phosphate buffer solution (PBS-T) containing 5% skimmed milk (PBST-M), followed by incubation for 1 h at room temperature (RT).

Primary antibodies consisting of 100 µL of the 2 test samples (*Measles virus* antibody positive and negative samples) were diluted in PBST-M four-folds from 1:100 to 1:6,400 and added to each well followed by incubation for 1h at RT. 100 µL of 1:1,000 HRP conjugated goat anti-Human IgG (Invitrogen, Camarillo, USA) diluted in PBST-M which served as the secondary antibody was added to each well followed by incubation for 1 h at RT. Finally, 100 µL of substrate solution containing

ABTS [2, 2' azinobis (3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The wells were incubated for 30 min at room temperature and optical density at 405 nm (OD_{405}) was measured against a reference of 490 nm using a Model 680 Microplate Reader (Bio-Rad, Hercules, USA).

Three rounds of washing were performed in between all steps with 300 µl of PBS-T per round. In the 3D-*Well* ELISA, each step was carried out by placing the 3D-*Well* in a new well of a 96-well plate. The 3D-*Well* structure was removed following incubation with the substrate solution before OD_{405} measurement.

3. Results

3.1. Physical evaluation

The 3D-*Well* consisted of 9 layers: (1) 5 8-half oval shaped layers (which included the brim at both ends) distributed evenly forming a radial spoke pattern around a central core adjoining above and/or below with (2) 4 circular shaped layers. These 9 layers form the 3D-*Well* as a hollow (open at either ends) cylindrical-like structure (closed-wall) that share a common central core. The inner diameter (central core) was measured to be 2.0 mm, the outermost diameter 5.5 mm and the total height 7.2 mm [96-well plate: well diameter: top (7.0 mm); bottom (6.2 mm) and inside depth of well: 11.4 mm]. These measurements are averages taken from three 3D-*Wells* and the error in dimensions was ± 0.1 mm, comparable to the parameters established in the 3D-*Well* designing stage. The actual 3D-*Well* prototype and the placement of the 3D-*Well* in the 96-well plate are shown in Figure 2. These results are consistent with the achievable accuracy of 3D printing platforms such as the one used in this study of ± 0.241 mm [20].

3.2. Validation

The selected samples, *Measles virus* antibody positive and negative samples, one each respectively were used for validation purposes. The sensitivity of detection of *Measles virus* antibodies in the *Measles virus* antibody positive sample was 3-folds higher on average using the 3D-*Well* at a primary anti-body dilution 1:400 (1.3-folds), 1:1,600 (2.1-folds) and 1:6,400 (2-folds) including possibly at 1:100 compared to the 96-well [OD_{405} (3D-*Well vs*. 96 well): 1:400 (3.1 *vs*. 2.4); 1:1,600 (1.9 *vs*. 0.9 and 1:6,400 (0.6 *vs*. 0.3)] while the *Measles virus* antibody negative sample showed comparable

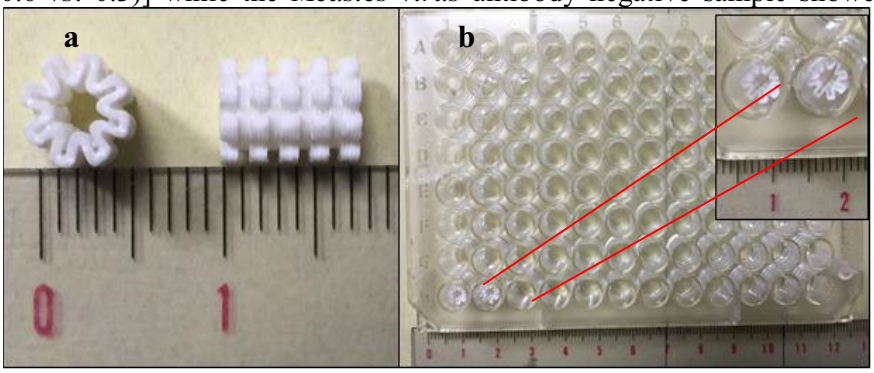
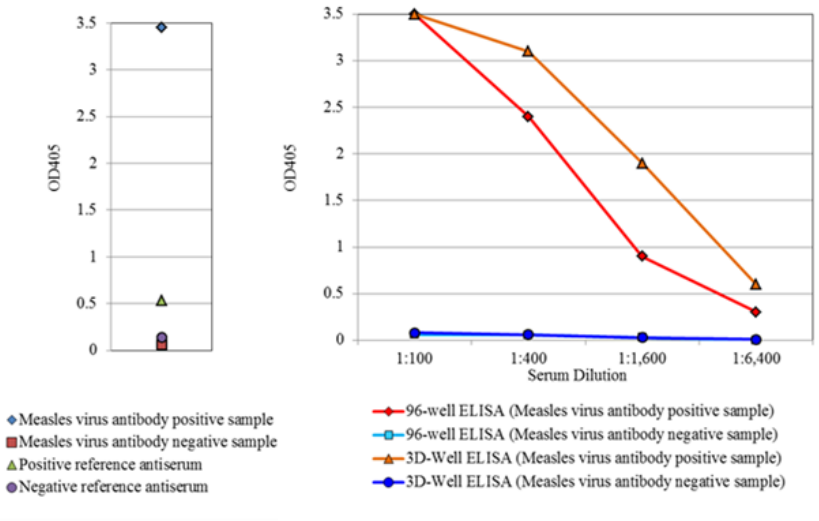


Fig. 2. 3D-*Well* prototype: (a) Photograph of the 3D-*Well* (b) Placement of 3D-*Well* in the 96-well plate.



(a) (b)

Fig. 3. Validation by IgG ELISA for *Measles virus* antibody: (a) IgG ELISA screening showing the 2 samples, *Measles* virus antibody positive and negative samples used for 3D-Well IgG ELISA validation (b) *Measles virus* antibody positive and negative samples diluted 4-folds (1:100 to 1:6,400) by 3D-*Well* and 96-well ELISA, respectively. (OD_{405}: Optical Density measured at 405nm; Results show average done in duplicates).

results between the 3D-*Well* and 96-well ELISA. These results were conclusive of the presence and absence of *Measles virus* antibodies in the test samples, respectively (Figure 3). The accuracy in the production and consistencies of the different specification achieved in the reproduction of the different 3D-*Wells* using 3D printing and the modification of ABS to a hydrophilic surface by chemical etching are important factors in the improved detection sensitivity of the 3D-*Well* ELISA. Additionally, OD_{405} was measured after 5 min incubation with the substrate solution containing ABTS in 3D-*Well* ELISA due to an observable color change. This observation further supports the increased sensitivity of the 3D-*Well* compared to the 96-well plate ELISA.

4. Discussion

The utilization of newer technologies in the development of ELISA-based diagnostics and other disease identification devices are important in addressing the unmet needs for treatment and care of individual patients and to support public health surveillance strategies [25, 26]. The rapid cycles offered by 3D printing in the designing through readily available design development software such as CAD and Insight 10.2™ job processing and management software in creating precise printing pathways to prototype development under highly accurate environments and equipment using readily available and low-cost polymer materials to allowing quick assessments and feedback, exemplified by this study, in the development of such ELISA-based and other devices are important considerations in meeting these needs and in ensuring the performance of highly sensitive assays [17, 23].

The simplicity offered by 3D printing applications in the fields of microfluidics, LOC technologies and clinical laboratory medicine diagnostics [17-19, 25, 26] and the rapid prototyping of high surface to volume devices using these platforms, such as in the development of the 3D-*Well* used in this study

allowing protein adsorption on the inner and outer wall surfaces and its fitting into 96-well plates allowed a rapid initial assessment to be performed without the need for complex instrumentations or changes made in the standard ELISA practices. The improved antibody detection sensitivity even using highly diluted samples as noted in our study provides room for further optimizations to reduce time and volume requirements, increase automation and potentially further miniaturizations of such ELISA-based diagnostics to be explored.

The modification of ABS to a hydrophilic surface by the generation of anchor sites for proteins [24], following chemical etching is important in the performance of solid phase reaction in immunoassays and in ensuring highly sensitive assays to be achieved. The utilization of numerous thermoplastics and other polymers in one-step manufacturing such as FDM technology [18] and newer 3D printing platforms such as laser-adopted stereolithography, Digital Wax Systems (DWS) which allows the use of fine materials and materials with different chemical potentially providing opportunities for the use of more hydrophilic materials to be used to develop miniaturized vessels for ELISAs in the future.

The impact of newer platforms based on improved micromanufacturing technologies in the development of ELISA-based systems for the rapid and accurate pathogen identification and serological diagnosis of infectious diseases such as in surveillance strategies during disease outbreaks or in the treatment and care of individual patients and its potential to reduce cost and time and increase portability are crucial factors especially in resource-constraint settings [8, 25, 26]. Additionally, the flexibility offered through 3D printing platforms in the cycles of designing-product development-feedback including addressing the improvements and commercialization of various prototypes will provide future opportunities to be explored in the different disciplines that share generic principles and applications such as in the fields of health, food industry, environmental, chemistry, biomedical and engineering.

5. Conclusion

The ease, rapidity and flexibility using readily available 3D printing platforms and polymer materials in the rapid prototyping of the 3D-*Well* with a higher reaction surface area compared to a 96-well provided an opportunity for a rapid assessment of the diagnostic performance of a 3D-*Well* ELISA in the serological diagnosis of infectious diseases. The fabrication of this high surface-to-volume 3D-*Well* prototype and methods to enhanced protein adsorption to its surface by chemical etching increased the detection sensitivity of the 3D-*Well* by at least 3-folds higher compared to the 96-well ELISA. This study provides an opportunity for the expansion of this platform towards further miniaturization to reduce the cost, time and volume of reagents needed in the immunological diagnosis for infectious diseases and its application in other disciplines.

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