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One-step three-dimensional printing of enzyme/substrate–incorporated devices for glucose testing

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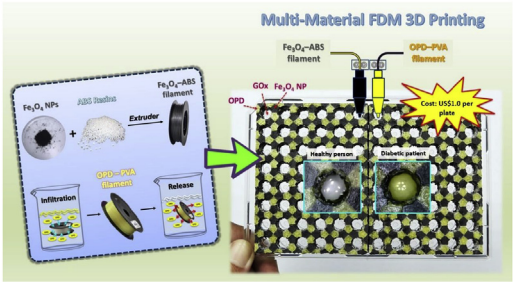
HIGHLIGHTS

- Fe_3O_4 NP–incorporated ABS and OPD–infiltrated PVA were respectively optimized.
- Enzyme/substrate–incorporated multi-well plates were one-step fabricated by 3DP.
- Well diluted samples were analyzed simply after directly loading into these devices.
- The method's detection limits reached 2.8 μM for H_2O_2 and 5.0 μM for glucose.
- These devices were highly applicable for rapid glucose screening in clinical samples.

GRAPHICAL ABSTRACT



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ABSTRACT

To substantially simplify the fabrication of analytical devices for rapid screening tests, in this study we employed multi-material fused deposition modeling-type three-dimensional printing (3DP) and two functionalized thermoplastic filaments—acrylonitrile butadiene styrene (ABS) incorporating peroxidase-mimicking iron oxide (Fe_3O_4) nanoparticles and polyvinyl alcohol (PVA) infiltrated with the chromogenic substrate *o*-phenylenediamine (OPD)—for the one-step manufacture of enzyme/substrate–incorporated multi-well plates. Upon contact with samples, these fabricated devices (i) released the chromogenic substrate OPD into the solution, (ii) efficiently catalyzed the oxidation of OPD mediated by the peroxidase substrate H_2O_2, (iii) enabled assays of those substances availably oxidized by their specific oxidases to generate H_2O_2, and (iv) facilitated colorimetric observation by the naked eye or through absorbance measurements after loading into a microplate reader. With glucose oxidase immobilized in each well, samples appropriately diluted could be directly loaded for derivatizing and analyzing glucose without adding any other reagents. After assay optimization, the limits of detection reached as low as 2.8 μM for H_2O_2 and 5.0 μM for glucose; the method's applicability was illustrated in terms of determining glucose concentrations in urine, serum, and plasma samples. These 3D-printed peroxidase mimic/chromogenic substrate–incorporated multi-well plates appear to be highly suitable for rapid and high-throughput screening of glucose in clinical samples. We demonstrate that adequate functionalization of raw materials for 3DP can contribute to the development of novel multifunctional devices with many potential practical applications.

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1. Introduction

Devices allowing simple and rapid screening tests are preferable for preliminary diagnosis in resource-limited areas or when timely equipment is unavailable [1–4]. Conventional testing devices are mostly configured with a series of two-dimensional microfluidic components, and the construction of which relies on delicate fabrication processes and skills [4–7]. Recently, additive manufacturing (three-dimensional printing; 3DP) technologies have enabled rapid laboratory-scale customization of 3D objects for designed experiments [7–17]. For example, Jue et al. used a material-jetting 3D printer to rapidly prototype a single-use, disposable interlock meter–mix device for sample-to-device metering and lysing of clinical urine samples [18]. Chan et al. developed a toolkit for actuating fluid within 3D-printed chips with torque or rotary-actuated pumps and valves, thereby allowing measurements of protein levels in spiked artificial urine samples [19]. Paterson et al. presented a 3D-printed smartphone-based photoluminescence imaging platform employing luminescent nanophosphor reporters for the highly sensitive detection of human chorionic gonadotropin levels in a lateral flow assay [20]. Thus, 3DP technologies are accelerating the technical innovations required to develop diverse screening devices, due to their great capability to simplify the manufacturing of designed multilayer devices [4–7].

Although 3DP technologies are convenient for users to manufacture devices automatically, the printing materials are inevitably limited by the manufacturers of 3D printers. Strategies are increasingly being developed to functionalize the raw printing materials or their printed devices with other physicochemical properties, thereby ensuring additional applications [21–36]. For example, Mandon et al. modified the resin and demonstrated that stereolithographic 3DP was compatible with the production of 3D biosensing components comprising enzyme [glucose oxidase (GOx) and peroxidase]-entrapped hydrogels for enzyme-based assays [31]. Compared with liquid stereolithographic resins, the thermoplastics for fused deposition modeling (FDM)–type 3DP are more difficult to functionalize because these substances incorporated into printing thermoplastics must be tolerant of a high-temperature (up to 250 °C) extrusion environment [24,29–31,34].

Fortunately, many nanomaterials (NMs) possess intrinsic enzyme-like activities (e.g., peroxidase, catalase, oxidase, superoxide dismutase), and they can be highly stable, activity-tunable, and commercially available in large quantities [37–40]. Most importantly, unlike natural enzymes, NMs with enzyme-like characteristics are suitable for incorporation into thermoplastics because they should maintain their enzyme-like catalytic activities after passing through the hot extrusion nozzle. Iron oxide nanoparticles (NPs) have been incorporated into polylactic acid filaments for fabrication of peroxidase-active multi-well plates, suggesting the feasibility of introducing enzyme-like NMs into FDM-type 3D printers for the manufacture of analytical devices [34].

To simplify the manufacture of low-cost testing devices was the major goal of this study. A dual-head FDM-type 3D printer and two functionalized thermoplastic filaments—acrylonitrile butadiene styrene (ABS) incorporating peroxidase-mimicking NMs and polyvinyl alcohol (PVA) infiltrated with the chromogenic substrate *o*-phenylenediamine (OPD)—were employed within a one-step 3DP process to manufacture multi-well plates displaying both peroxidase and chromogenic activities. Upon contacting a sample solution, these fabricated enzyme/substrate–incorporated multi-well plates could catalyze the oxidation of their released OPD in the presence of peroxidase substrate hydrogen peroxide (H_2O_2), allowing colorimetric visualization by the naked eye or through measurements of the sample's absorbance after loading into a conventional microplate reader. When coupling with glucose oxidase (GOx), glucose, which was oxidized to generate H_2O_2, could also be further analyzed. After optimizing the incorporation of the peroxidase-mimicking NMs, the infiltration and release of OPD, the immobilization of GOx, the design of the multi-well plates, and the assay conditions, the analytical applicability of this method was verified through analyses of glucose concentrations in urine, serum, and plasma samples. These 3D-printed enzyme/substrate–incorporated multi-well plates appear to be very promising systems for glucose testing and routine analyses of glucose concentrations in clinical samples. Also, it appears that suitably functionalized printing materials can allow 3DP technologies to fabricate smart diagnostic devices for rapid screening applications.

2. Experimental section

2.1. Chemicals

H_2O_2 (31642), D-(+)-glucose (G7528), GOx (G2133, from *Aspergillus niger*), peroxidase from horseradish (HRP; P8250), D-(+)-trehalose dihydrate (T0167), ammonium acetate (A7330), diethyl ether (32203), iron(III) oxide nanopowder [Fe_2O_3; 544884; <50 nm (BET); 50–245 $m^2 g^{-1}$], copper(II) oxide nanopowder [544868; <50 nm (TEM); 29 $m^2 g^{-1}$], cobalt(II,III) oxide nanopowder [637052; <50 nm (TEM); 40–70 $m^2 g^{-1}$], and silver nanopowder (576832; <100 nm; 5 $m^2 g^{-1}$) were purchased from Sigma–Aldrich. Iron(II,III) oxide nanopowder (Fe_3O_4; 47141; 50–100 nm; 20–50 $m^2 g^{-1}$) and OPD (A11946) were purchased from Alfa Aesar. Acetone (9006) and chloroform (9180) were purchased from J. T. Baker. Ethanol (95%) was purchased from Echo Chemical. Water for preparation of chemical solutions was purified through a Milli-Q Integral water purification system (Merck Millipore). POLYLAC® ABS resins (PA-747) were obtained from Chi Mei Corporation (US$0.7 per kg); PVA filaments (1.75 mm) were obtained from FLMT (US$75.9 per kg).

2.2. Fabrication of enzyme/substrate–incorporated multi-well plate

The peroxidase-active thermoplastic filaments (1.75 mm i.d.) were manufactured by introducing mixtures of peroxidase-mimicking NMs (5 g kg^{-1}) with ABS resins into a filament maker (Filabot Original, Triex® LLC) with an extrusion temperature of 180 °C. Immersion of bare PVA filaments in an OPD solution (400 mM, ethanol as a solvent; 36 h) was performed to infiltrate the chromogenic substrates into the filaments. These two treated filaments were preserved in a plastic air bag, purged with dry N_2, and stored at 4 °C. To quantify the infiltrated OPD in terms of its absorbance, an assay buffer solution (750 mM ammonium acetate, pH 5) in which a 1-cm piece of PVA filament had dissolved totally was treated with H_2O_2 (2 mM) and HRP (0.2 U mL^{-1}) to completely oxidize the released OPD.

A 48-well plate 3D object (well dimensions: 0.6 cm i.d. × 0.9 cm height; volume: 254 μL) lacking a bottom supporting plate was designed using the software SolidWorks 2013 (Dassault Systèmes). The designed 48-well plate was fabricated using a dual-head FDM-type 3D printer (DS4, double extruder, L2D) by stacking the two functionalized filaments (i) layer-by-layer (LbL), (ii) side-by-side (SbS), and (iii) layer-by-layer/side-by-side (LbL/SbS). The printer was operated with two 0.4-mm copper nozzles heated at 260 °C for the ABS filaments and 230 °C for the PVA filaments, under the printing mode of a hexagonal infill pattern with a *z*-axis (layer) thickness of 0.1 mm. These fabricated plates were manually glued onto a transparent polystyrene microplate with epoxy adhesive; each well was accurately aligned with the detector's light path to

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