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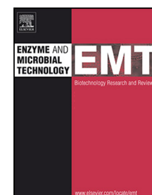

Modification of glucose oxidase for the development of biocatalytic solvent inks

Joey N. Talbert, Fei He, Kayla Seto, Sam R. Nugen, Julie M. Goddard*

Department of Food Science, University of Massachusetts, 102 Holdsworth Way, Amherst, MA 01003, USA

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ABSTRACT

Inkjet printing of enzymes onto hydrophobic polymeric material offers the potential for economical rapid deposition and patterning of biocatalysts for biosensor, microarray, and intelligent packaging applications. Non-polar solvent based inks provide simple vehicles for direct printing on these materials; however, enzymes are not readily soluble in such inks. Glucose oxidase (*Aspergillus niger*) was made soluble in toluene by hydrophobic ion pairing with didodecyldimethylammonium bromide. Following modification, single enzyme composites with a mean diameter of 12.5 nm were formed. The enzymes showed no significant change in K'_m and a 46% decrease in k'_{cat} compared to the native enzyme. Modification allowed for direct printing and patterning on PET using piezoelectric inkjet printing. Specific activity of the modified enzyme was reduced from 889×10^3 μmol/min/g to 2×10^3 μmol/min/g after printing. These results suggest that direct inkjet printing of enzymes onto hydrophobic polymers may be accomplished using enzyme modification as a means to induce solubility in solvent inks.

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1. Introduction

Inkjet printing of enzymes onto material substrates has been proposed as a means to deposit enzymes onto a surface in a rapid and controlled manner. Printing allows for precise placement of small volumes of bioactive molecules onto materials such as paper in a simple and low-cost approach [1,2]. Deposition of enzymes by printing offers the potential to be used in a number of nanobiomanufacturing applications including diagnostics, active and intelligent packaging, and microarrays [2–4]. A number of enzymes in aqueous systems have been printed using an inkjet system including glucose oxidase, acetylcholinesterase, laccase, alkaline phosphatase, and horseradish peroxidase [1,3–7]. Both piezoelectric and thermal inkjet printing methods have been employed for depositing liquid enzyme solutions [4,5,8,9]. Of these, piezoelectric is more suitable for protein printing due to the reduced exposure to heat which may denature the enzyme; however, piezoelectric printing requires higher 'ink' viscosities compared to thermal printing [9,10].

Printing of enzymes is accomplished by delivering a solution of enzyme through a print nozzle onto a desired material [10]. The ink used for delivery must meet the viscosity and surface tension necessary for deposition, as well as allow for adhesion of the solution to the material substrate. For a solution to adhere to a material substrate, the solution needs to be able to wet the material. To enable optimal wetting, the solution should have a surface tension lower than the surface energy of the material [11,12]. For enzyme applications aqueous-based inks would, ideally, be employed to allow for appropriate enzyme solubility and activity retention of the enzyme in solution. However, many polymeric materials such as polyethylene terephthalate and polyethylene, which are desirable for use in microfluidic diagnostic platforms and intelligent packaging applications, have surface energy values that are lower than the surface tension of water and do not allow for direct printing [11]. Therefore, alternative strategies to direct printing of simple aqueous solutions onto these polymeric materials must be developed. These strategies should act alone or in combination to enable deposition of the ink from the nozzle, wetting of the solution on the material substrate, enzyme solubility, activity retention of the enzyme before and after printing, and, when desirable, reusability of the enzyme.

Strategies to enhance ink compatibility with material substrate include the utilization of surfactants, modification of material surface chemistry, and the use of non-polar solvents [5,9,11,12]. Surfactants can be used to reduce the surface tension of aqueous-based solutions, but these additives, as well as the solution pH, may interfere with enzyme activity. Foaming can also occur when using surfactants in solution. Surface modification of material surfaces through oxidation or coating with hydrophilic materials can increase the surface energy of the material. However, these modifications require additional processing steps to create a compatible

* Corresponding author. Tel.: +1 413 545 2275; fax: +1 413 545 1262.
E-mail addresses: goddard@foodsci.umass.edu, joey.talbert@gmail.com (J.M. Goddard).

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platform for the ink, which can add cost and alter fluidics [8]. Additionally, the surface modification may alter retained enzymatic activity following deposition [13].

Non-polar solvent-based inks allow for direct printing on uncoated polymeric materials, can be removed by rapid evaporation, can be fine-tuned to achieve printing properties and can solubilize hydrophobic polymeric binders to yield water resistant ink. However, enzyme incorporation into non-polar solvent inks is limited by enzyme solubility. While enzymes have been shown to retain activity in non-polar solvents such as toluene and hexane, they are not soluble [14]. To enhance solubility in non-aqueous conditions, enzyme has been entrapped within reverse micelles, altered by genetic approaches, as well as modified by chemical methods including immobilization, crosslinking, PEGylation, and hydrophobic ion pairing [15–17]. Of these, hydrophobic ion pairing is of particular interest due to the ability to modify the enzyme through simple and rapid modification with limited materials. Hydrophobic ion paring relies on charged surfactants to ionically associate with charged amino acid groups or to the charged enzyme [18]. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) has been applied to modify chymotrypsin to induce solubility in isooctane [19,20]. Likewise, peroxidase, lipase, and glucose oxidase have been modified using the hydrophobic ion pairing technique [21–25]. These methods enable the production of single enzyme modifications, with no diffusion limitations that prevent substrate accessibility to the enzyme.

Glucose oxidase (EC 1.1.3.4) is an oxidoreductase enzyme that converts glucose, in the presence of oxygen, into gluconolactone with hydrogen peroxide being produced as a byproduct. The enzyme is used in applications including in-line glucose diagnostics, disposal biosensors (e.g. glucose test strips for diabetes monitoring), and oxygen scavenging packaging materials [26,27]. Glucose oxidase has been inkjet printed onto functionalized silicon oxide, indium-tin-oxide (ITO)-coated polyethylene terephthalate, ITO-coated glass, and PEDOT/PSS. To prevent loss of printed enzyme through dissolution, sensors containing the printed enzyme have been dip-coated in cellulose acetate to form a semipermeable membrane. These methods for printing of glucose oxidase do not allow for direct printing and immobilization of the enzyme—limiting the use of the enzyme for manufacturing of next-generation biocatalytic materials. Given the applicability of non-polar solvent inks for printing on polymeric materials, and the ability to induce solubility of enzymes through chemical modification, the objective of this work was to utilize hydrophobic ion pairing as a means to induce solubility of glucose oxidase in non-polar solvents to enable direct inkjet printing of biocatalytic solvent inks onto polymeric materials.

2. Materials and methods

2.1. Materials

Glucose oxidase (*Aspergillus niger*; Type X-S), didodecyldimethylammonium bromide, *o*-dianisidine dihydrochloride, Bradford reagent, and horseradish peroxidase (Type I) were obtained from Sigma. Polyethylene terephthalate (PET; 200 μm thickness) was obtained from Macmasters (Denver, CO, USA). All other reagents were obtained from Fisher Scientific (Pittsburgh, PA) and used as received.

2.2. Enzyme modification

Glucose oxidase (EC 1.1.3.4) modification was adapted from the method described by Jia [25]. Glucose oxidase (GOx) was diluted in 20 mM acetate buffer to a concentration of 650 μg of protein/mL and brought to 25 °C. Buffer pH values of 3.5, 4.5, 5.5 and 6.5 were evaluated with pH 5.5 being used for routine analysis. Protein concentration was determined using the standard BCA assay at 37 °C as described elsewhere under circular rotation at 20 rpm [28]. Didodecyldimethylammonium bromide (DDAB) was prepared in toluene and brought to 25 °C. DDAB concentrations of 1 mM, 2 mM, 3 mM, and 4 mM in toluene were tested with a DDAB concentration of 2 mM being used for routine analysis. Equal parts of the enzyme solution and the DDAB solution were added together. For routine modification, 1 mL of the enzyme solution was added dropwise to 1 mL of the DDAB solution under constant stirring. The solution was stirred at 300 rpm and 25 °C for 2 min. The emulsified solution was centrifuged at 12K × *g* for 3 min at room temperature (ca. 20 °C). The toluene layer containing the enzyme as well as the interfacial layer was removed, filtered through a 0.22 μm PTFE syringe filter, and centrifuged for an additional 3 min at 12K × *g*. The organic layer was removed from the remaining aqueous layer and stored at 4 °C until further use. Protein concentration of the organic layer was measured using the BCA assay described previously. Following completion of the BCA reaction, samples were spun at 12K × *g* for 3 min to separate toluene and enable clarification of the reaction product.

2.3. Hydrodynamic particle size

The hydrodynamic diameter of native and modified enzyme was measured using dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK). Native and modified enzymes were diluted to a concentration of 1 mg of protein/ml and read in a quartz cuvette. Native enzyme was diluted in 20 mM acetate buffer (pH 5.5) and the modified enzyme was diluted in toluene. Refractive indexes of 1.496, 1.450 and 1.330 were used for toluene, protein, and water, respectively.

2.4. Enzyme specific activity and kinetics

The specific activity of native glucose oxidase (0.05 μg of protein/ml) was measured at 35 °C and pH 5.1 (50 mM acetate buffer) using glucose as the substrate in the presence of 3 pyrogallol U/ml horseradish peroxidase, 0.15 mM *o*-dianisidine dihydrochloride, and 555 mM glucose [29]. For the modified enzyme, activity was determined using 0.1 μg of protein/ml. For kinetics, glucose concentrations of 1–64 mM were evaluated. Color change was measured at 500 nm in 15 s intervals for 6 min under constant shaking. Substrate conversion was determined from the extinction coefficient of *o*-dianisidine (7.5 mM^{-1}) and the initial rate of substrate conversion. K'_m and V'_{max} were determined by applying Michaelis–Menten kinetics and extrapolated using Graphpad Prism software (v. 5.04, Graphpad Software, La Jolla, CA). Turnover number (k'_{cat}) was calculated from a molecular weight of 160 kDa for glucose oxidase.

2.5. Piezoelectric inkjet printing

PET coupons were cleaned with isopropyl alcohol and deionized water sequentially in an ultrasonic bath for 15 min then dried with N_2 gas prior to use. A piezoelectric (drop-on-demand) inkjet printer (DMP-2831, Fuji Dimatix Corporation) with 10 pL cartridges was used for all inkjet printing processes. Biocatalytic ink was developed by diluting modified glucose oxidase in toluene to a final concentration of 100 μg/ml, and printed to a targeted protein concentration of 20 $\mu g/cm^2$ on PET. Ink droplets were produced using an ejection frequency of 23 kHz and a jetting voltage of 38 V at 28 °C. For enzyme activity measurements, drops were dispensed onto PET substrate held at 31 °C with a spacing of 5 μm. For patterning experiments, drops were dispensed onto PET with a spacing of 15 μm. A custom ejection waveform was generated for inkjet printing.

2.6. Printed enzyme activity

Strips of printed enzyme on PET (0.8 cm × 1.3 cm) were tested for protein concentration using the BCA assay at 37 °C. PET films containing printed enzyme were mounted in an Arrayit 96 well-glass bottom plate (Arrayit Corp., Sunnyvale, CA, USA). Activity was assessed using 0.15 mM *o*-dianisidine dihydrochloride, 555 mM glucose, and 3 U/ml horseradish peroxidase and measuring the initial rate of color formation across 6 min at 35 °C; pH 5.1 (50 mM acetate buffer) at 500 nm. Specific activity was compared to the native and modified enzyme at 0.05 μg/ml as described previously. Enzyme reusability was determined by washing wells containing mounted PET films with printed enzyme 3 times with 200 μL nanopure water and immediately measuring specific activity as described previously. To test for non-immobilized activity, enzyme was printed directly into a glass vial using the conditions described for printing onto PET. The enzyme was reconstituted to volume using toluene and the protein concentration verified using the BCA assay prior to measurement of enzyme activity.

2.7. Enzyme pattern development

Printed enzyme patterning on PET was realized by applying 0.5 mL of Bradford reagent dye directly onto the printed side of the material and developing the color for 15 min at room temperature (ca. 25 °C).

2.8. Surface wetting

Water and toluene contact angles on PET were measured on a Krüss DSA100 (Hamburg, Germany) goniometer equipped with a direct dosing system (DO3210, Krüss, Hamburg, Germany) at room temperature under atmospheric conditions. The tangent method 2 from the Drop Shape Analysis software version 1.91.0.2 was used for the measurement of advancing and receding contact angles using an automatic dispenser at a dispensing rate of 10 μL/min.

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