CHAPTER NINETEEN

Nanoarmoring of Enzymes by Interlocking in Cellulose Fibers With Poly(Acrylic Acid)

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Methods in Enzymology, Volume 590
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2017.01.009

Abstract

A simple method for interlocking glucose oxidase (GOx) and horseradish peroxidase (HRP) in cellulose fibers using poly(acrylic acid) (PAA) as an armor around the enzyme, without any need for activation of the cellulose support, is reported here. The resulting enzyme paper is an inexpensive, stable, simple, wearable, and washable biosensor. PAA functions as a multifunctional tether to interlock the enzyme molecules around the paper fibers so that the enzymes are protected against thermal/chemical denaturation and not released from the paper when washed with a detergent. The decreased conformational entropy of the interlocked enzyme protected by the nanoarmor is likely responsible for increased enzyme stability to heat and chemical denaturants (retained $\geq$70 percent enzyme activity after washing with urea or SDS for 30 min), and the polymer protects the enzyme against inactivation by proteases, bacteria, inhibitors, etc. The kinetics of the interlocked enzyme were similar to that of the enzyme in solution. The V_{max} was 6($\pm$0.5) m*M* per minute before washing, then increased slightly to 9($\pm$1.4) m*M* per minute after washing with water. The K_m was 22($\pm$6.4 m*M*), which was slightly higher compared to GOx in solution (25–27 m*M*). Because the surface area of the paper does not limit the enzyme loading, about 20% of enzyme was successfully loaded onto the paper (0.2 g enzyme per gram of paper), and $\geq$95% of the enzyme was retained after washing. Interlocking works with other enzymes such as laccase, where $\geq$60% of the enzyme activity is retained. This novel methodology provides a low cost, simple, modular approach of achieving high enzyme loadings in ordinary filter paper, not limited by cellulose surface area, and there has been no need for complex methods of enzyme engineering or toxic methods of activation of the solid support to prepare highly active biocatalysts.

1. INTRODUCTION

Paper-based enzyme devices hold high promise for a wide range of applications such as diagnostic assays (Parolo & Merkoçi, 2012; Pelton, 2009; Yetisen, Akram, & Lowe, 2013), biosensors (Liana, Raguse, Gooding, & Chow, 2012; Martinez, Phillips, Butte, & Whitesides, 2007), and biofuel cells (Wang et al., 2014). The specific properties of paper, such as its hydrophilicity, flexibility, insolubility in water, no toxicity, and abundance make it an attractive support for enzymes (Credou & Berthelot, 2014). A serious limitation of using paper for enzyme loading, which is mostly composed of cellulose with a chemical formula of $(C_6H_{10}O_5)_n$, has been

the expensive chemical modification that is needed for the covalent linking of the enzyme to the support. This serious roadblock has been overcome by the current approach of interlocking of enzymes in the fibrous network of paper, which is novel, efficient, simple, inexpensive, and amenable to laboratories with limited resources.

Cellulose, the main component of paper, is hydrophilic, odorless, chiral, biodegradable, composed of D-glucose units, and insoluble in most organic solvents and dilute mineral acids. There are few approaches for convenient loading of enzymes onto a cellulose matrix such as physical adsorption (Sulaiman, Mokhtar, Naim, Baharuddin, & Sulaiman, 2014) and covalent crosslinking of the enzyme to the cellulose surface after chemical activation of the solid surface (Edwards, Prevost, Condon, & French, 2011). Physical adsorption is known to involve weak interfacial forces and the associated leaching of the enzyme from the surface into solution during exposure and storage. Chemical crosslinking limits enzyme loading to the accessible surface area of the matrix as well as the extent of chemical modification that has been achieved on the cellulose surface. As of now, there is no universal method for enzyme immobilization onto cellulose at high loading capacity that does not require any chemical activation of the support. We devised a simple, novel approach for the interlocking of enzymes onto cellulose fibers without any surface modification/activation of the support. In our approach, the enzyme is locked into cellulose fibers by covalent linking with a synthetic polymer such that the enzyme–polymer nanogels are directly embedded within the fibrous network (interlocking). This approach virtually eliminates long-term leaching of the enzyme and significantly enhances the stability of the interlocked enzyme. The method described here can be used for any enzyme and any polymer molecule that has the appropriate crosslinking functionalities.

1.1 The Enzymes: Glucose Oxidase and Peroxidase

Glucose oxidase (GOx, from *Asperigillus niger*) and horseradish peroxidase (HRP, from horseradish) were chosen as model systems. GOx, which catalyzes the oxidation of D-glucose to hydrogen peroxide and gluconic acid, is a widely studied enzyme for its possible usage in glucose biosensors (Oliveira, e Silva, de Souza, Martins, & Coltro, 2014; Soni & Jha, 2015; Zhang, Lyu, Ge, & Liu, 2014; Zhang et al., 2012) and biofuel cells (Wang et al., 2014). The second enzyme, HRP, then uses the peroxide produced from the GOx reaction and converts iodide to elemental iodine,

which is brown, which is produced in proportion to the concentration of glucose present in the sample (Martinez et al., 2007). Thus, the extent of brown color produced upon loading the test sample, as measured by an ordinary scanner, is then proportional to glucose present and after calibration of the sensor, one can obtain the concentration of glucose in the sample.

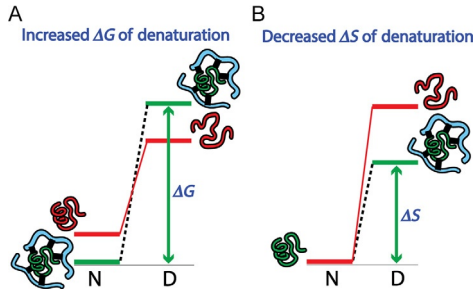
1.2 The Polymer: Poly(Acrylic Acid)

Poly(acrylic acid) (PAA, $Mw = 8000$) is a negatively charged (Lützenkirchen, van Male, Leermakers, & Sjöberg, 2011), flexible, hydrophilic polymer (Kadajji & Betageri, 2011), which is commercially available in large quantities (Buchholz, 2000). Conjugation of enzymes to PAA drastically improves enzyme stability against thermal and proteolytic degradation (Mudhivarthi et al., 2012; Riccardi et al., 2014), and these conjugates are useful for paper-based enzyme devices.

1.3 Need for Nanoarmoring of Enzymes

The stabilization of enzymes against inactivation is a major challenge that often limits their application in the laboratory or industry. There are many methods for enzyme–polymer conjugation, and one such method is the wrapping of the enzyme with a long chain synthetic polymer which can enhance enzyme stability by lowering its conformational entropy.

Stabilizing the enzyme while also maintaining enzyme activity is possible by recognizing the thermodynamics of enzyme denaturation. The native (N, folded) and denatured (D, unfolded) states are thermodynamically related via the free energy changes. In order to retain enzyme function under harsh conditions, the native state must be maintained, and the transition from native to denatured state must be inhibited. In this method, the free energy of the denatured state is increased (destabilized) and the free energy of the native state is lowered (stabilized) (Scheme 1, left). In order to increase the free energy of the denatured state, its conformational entropy is reduced by wrapping the enzyme with a synthetic polymer (right). In our case, PAA is used to wrap the enzyme and curtail the number of conformational states that the denatured state can achieve. The restricted conformational space of the denatured state is therefore, lower in free energy than a complete random coil. This is called the "entropy control" method, which has been successfully used in our laboratory to improve enzyme stability (Deshapriya & Kumar, 2013; Mudhivarthi, Bhambhani, & Kumar, 2007).

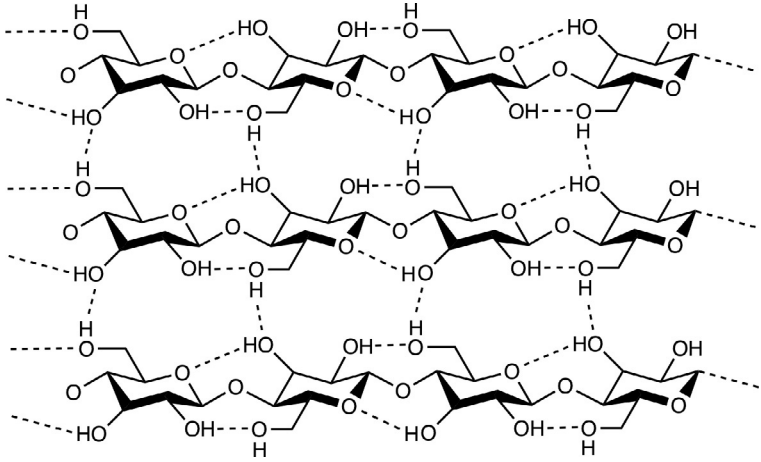


Scheme 1 (A) Enzymes can be stabilized by raising the free energy of denatured state (D) with respect that of the native state (N). (B) The increase in free energy of D may be achieved by decreasing its conformational entropy upon wrapping with a synthetic polymer.

The many available amine groups on enzymes provide a multipoint attachment by covalent linking to the polymer's carboxylic acid groups, and the covalent conjugation is readily achieved with the standard 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) chemistry, under mild conditions at room temperature. Wrapping of the enzyme with the polymer "armors" the enzyme in its native conformation, and increases the energy needed to unfold the enzyme. At the same time, the highly charged nature of the polymer prevents enzyme aggregation over time, so the enzyme remains active. A similar strategy can also be designed with a cationic polymer such as poly(L-lysine) and its amine side chains can be attached to the COOH groups on most enzymes using the carbodiimide chemistry. Because of the polymer's net charge, it is also known to protect the enzyme from proteolytic degradation and inhibit inhibition by inhibitors that have the same charge as the polymer. These nanoarmored enzymes can then function even under unfavorable conditions when compared to the unmodified enzyme, at a minimal cost, low toxicity, while also maintaining excellent activity but enhanced stability.

1.4 Rationale for Using Cellulose

Paper-based devices have been widely used in a variety of applications such as in diagnostic assays, biosensors, and biofuel cells. Paper is mostly made of cellulose fibers (Scheme 2), and cellulose is attractive due to its biodegradability, hydrophilicity, insolubility in water, flexibility, filtration capabilities, and its high abundance make it an attractive support for enzymes (Nery and Lauro, 2016). The cellulosic fibers are benign to most enzymes and most



Scheme 2 The fibers of cellulose are shown earlier where the cellulose polymer molecules are aligned and hydrogen bonds between and within the cellulose molecules hold them together.

enzymes cannot degrade, hydrolyze, or oxidize it. Cellulosic paper is also extremely inexpensive for commercial applications of bound enzymes.

1.5 Concept of Interlocking as a Novel Method for Sensor Fabrication

There are many ways of trapping enzymes in a cellulose matrix, such as covalent crosslinking or adsorption but these methods often result in severe enzyme deactivation, or loss of enzyme when subjected to washing. The method of "interlocking" enzymes in cellulose is a new approach, and it is analogous to two chain links stuck together, but not joined to one another. The only way to remove one link would be to break the link open. Similarly, the enzyme–PAA conjugate is one link, while the cellulose is the other link. The enzyme–PAA conjugate is wrapped around the nanofibers in cellulose. Together, the enzyme is "interlocked" and cannot be washed away. At the same time, the enzyme is nanoarmored by the polymer so that the enzyme's stability is enhanced as well without compromising its biological activity. Thus, two positive outcomes are achieved in one step.

1.6 General Approach to Enzyme Interlocking and Key Results

Because of the nonspecific nature of the EDC chemistry that occurs between the amine groups of the enzyme and the carboxylic acid groups of the PAA polymer, there is minimal work needed to create the nanoarmored

interlocked enzyme–PAA. Most enzymes have multiple lysine groups on their surface and PAA has a large number of carboxyl groups and it is also available commercially providing several molecular weights as desired. Large enzyme molecules would need longer PAA molecules to wrap them with, while smaller enzyme molecules might work well even with shorter PAA chains, and hence, one needs to choose an appropriate molecular weight polymer for interlocking. Typically, the polymer length can be about 10–15 times longer than the average diameter of the enzyme.

The interlocking approach via EDC chemistry can be broken down into two convenient steps: (1) activation of the polymer with EDC to prevent any enzyme–enzyme crosslinking and (2) addition of the enzyme to the activated polymer followed by deposition onto the cellulose surface to initiate interlocking. The reaction proceeds at room temperature until dry, and the enzyme paper is ready in a few minutes. This approach is also amenable to the inkjet technology as well as the 3D printing where the polymer, EDC, and enzyme solutions can be deposited on the paper in a predefined sequence and pattern. Ideally, no interactions between the cellulose fibers and the enzyme/polymer are required to interlock enzymes in paper. This is advantageous, because any such interactions might distort the enzyme's structure, and the absence of such interactions or when the interactions are very weak there is no threat to the enzyme structure. Enzymes can therefore be bound to paper without significant distortion of enzyme structure, and the bound enzyme cannot be washed away due to the "interlock" technology. Thus, the "interlock" technology and paper might provide a general protocol to bind enzymes to solid substrates by a universal approach, without loss of activity but improved stability.

Intricate structures in 2- or 3-dimensions on porous substrates can be made, where the aqueous solutions can wet the fibrous matrix. All manipulations are carried out at room temperature, using buffered solutions, desired pH and ionic strength conditions, thus preserving the native structure of the enzyme without adverse effects. The enzymes interlocked into the fibrous network of the paper are stable against washing, leaching is prevented, they remain active, and are stable against denaturants. Because the pores of the paper matrix are not blocked by this approach, the medium can be used to separate particulate material before analysis. The sample can be spotted on one side of the paper and activity assayed from the other side, so that any debris would not affect the analytical result. On the other hand, the paper matrix may also be exploited for performing any further chemistries on the sample by simply adding multiple paper layers to the sensor.

Thus, paper based, interlocked enzymes have a high potential for analytical applications. Benign, one step, simple interlocking of one or more enzymes in the cellulose matrix is reported here.

2. EQUIPMENT AND REAGENTS

2.1 General Equipment

1. *Safety*: Goggles, laboratory coat, latex, or nitrile gloves, ventilation hood. Caution when weighing out SDS (dust mask is recommended), as it is a fine powder and extremely irritating when inhaled.
2. *Waste management*: General aqueous waste receptacle.
3. Filter paper (Whatman 1 cellulose, typical 180 μm thickness, 87 g m^{-2} weight, and with 11 μm pore size, 185 mm diameter).
4. Deionized water.
5. A UV–visible spectrophotometer.
6. A benchtop mini centrifuge.
7. Microcentrifuge tubes (1.5 mL).
8. Volumetric micropipettes (20, 100, 200, and 1000 μL).
9. Beakers (100–1000 mL).
10. Stir/hot plate.
11. Analytical balance.
12. pH meter.

2.2 Enzyme Interlocking on Paper

1. Glucose oxidase (*Asperigillus niger*, GOx, EC 1.1.3.4, Sigma-Aldrich).
2. Peroxidase from horseradish (HRP, EC 1.11.1.7, Calzyme).
3. 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC, TCI America).
4. Poly(acrylic acid) (PAA, $Mw = 8000$, Sigma-Aldrich). *Tip*: Other molecular weights of PAA have been used in our laboratory, and all different samples behaved similarly. Synthesis concentrations, however, must be adjusted according to the molecular weight, as higher molecular weight polymer solutions become more viscous and these will not coat the paper evenly. The conditions of interlocking need to be adjusted to the type of enzyme used and the molecular weight of PAA, on a case-by-case basis.
5. Phosphate buffer (200 m*M*, pH 7.0).
6. Household kitchen wax paper.

7. Precision cutter.
8. Heat press.

2.3 Characterization by Gel Electrophoresis

1. Agarose (high gel temperature, USBiological Life Sciences).
2. Bromophenol blue (Sigma-Aldrich).
3. Acetic acid (Sigma-Aldrich).
4. Tris-base (Sigma-Aldrich).
5. Microwave.
6. Glycerol (Sigma-Aldrich).
7. Gel electrophoresis apparatus (Gibco Model 200, Life Technologies Inc.).
8. Brilliant blue R250 (Sigma-Aldrich).

2.4 Characterization by SDS-PAGE

1. Sodium dodecyl sulfate (Sigma-Aldrich).
2. Tris-base (Sigma-Aldrich).
3. Bromophenol blue (Sigma-Aldrich).
4. β-Mercaptoethanol (BME, Sigma-Aldrich).
5. Acrylamide (Sigma-Aldrich).
6. Glycine (Sigma-Aldrich).
7. *N′N′*-bis-methylene-acrylamide (Sigma-Aldrich).
8. Hydrochloric acid (Sigma-Aldrich).
9. Ammonium persulfate (APS, Sigma-Aldrich).
10. Isopropanol (Sigma-Aldrich).
11. Acetic acid (Sigma-Aldrich).
12. Tetramethylethylenediamine (Sigma-Aldrich).
13. Molecular weight marker (broad range protein ladder, Thermo Fischer Scientific).

2.5 Characterization by Circular Dichroism

1. A circular dichroism (CD) spectropolarimeter (JASCO model J715).
2. 0.05-cm path length quartz cuvette.

2.6 Characterization of Cellulose-Interlocked Enzymes by Laser Confocal Fluorescence Microscopy

1. 1-Ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC, TCI America).

2. Fluoresceinamine Isomer 1 (FA, Sigma-Aldrich).
3. 5(6)Carboxy-X-rhodamine *N*-succinimdyl ester (ROX, Sigma-Aldrich).
4. Acetone (Sigma-Aldrich).
5. Sodium bicarbonate (Sigma-Aldrich).
6. Dimethylsuldocide (DMSO, Sigma-Aldrich).
7. Dialysis membrane (Spectra/Por Biotech Membranes, MWCO 15K).
8. Laser confocal microscope (Nikon A1R Confocal Microscope).

2.7 Colorimetric Enzymatic Activity Assay

1. Potassium iodide (Sigma-Aldrich).
2. Glucose (Sigma-Aldrich).
3. Color scanner (Canon, CanoScan LIDE 200).
4. ImageJ image processing software (free online) with histogram package.

2.8 Enzyme Loading on Paper by Bradford Assay

1. Brilliant blue G-250 (Sigma).
2. Ethanol (Sigma-Aldrich).
3. Phosphoric acid (Sigma-Aldrich).
4. Whatman 1 filter paper, standard grade.

3. METHODS

3.1 Enzyme Conjugation to PAA and Characterization by Agarose Gel Electrophoresis

Agarose gel electrophoresis was done to verify complete conjugation of the enzymes to PAA in solution (Fig. 1A). The nanoarmored GOx–HRP–PAA (Lane 3) showed increased electrophoretic mobility toward the positive electrode when compared to GOx/HRP (Lane 1) and GOx/HRP/PAA (Lane 2) controls. The increased negative charge is due to the covalent attachment of the enzyme (NH_2 groups) to the negatively charged PAA (COOH groups), which also indicates that no unconjugated enzyme remained after the EDC reaction was complete (Scheme 3). Normally EDC reaction is 40%–50% complete, but we achieved complete conjugation because of the possible optimal ratio of the enzymes to the polymer and high EDC concentration and conditions followed. Another reason is interlocking itself is expected to be efficient because not 100% of the available carboxyls or amine groups need to react. A substantial fraction should

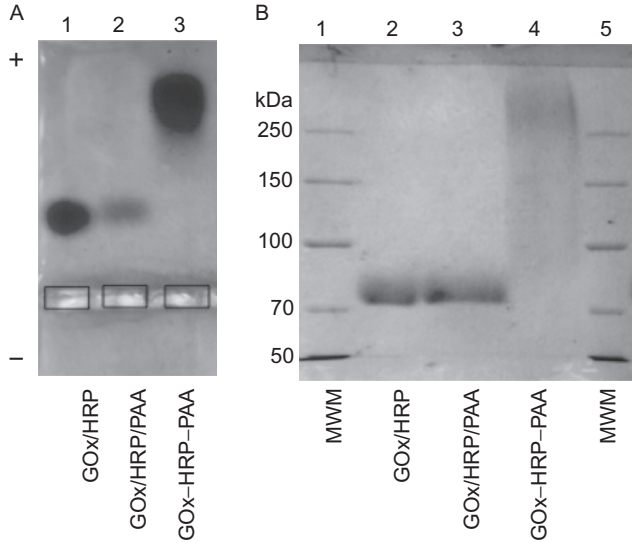
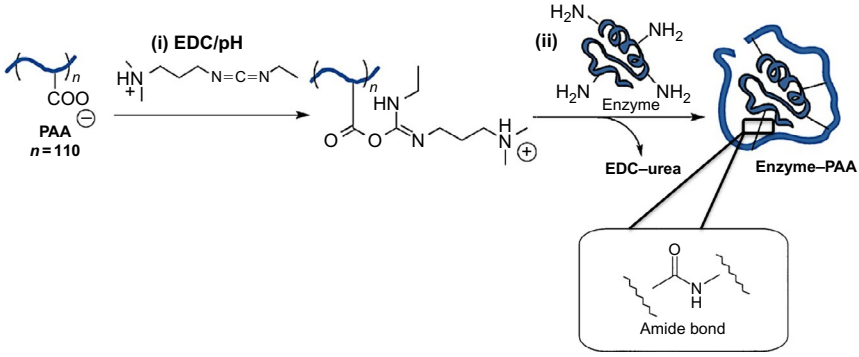


Fig. 1 (A) Agarose gel of the GOx–HRP–PAA conjugate in solution (Lane 3, concentration ratio of 16:3:10,000 μ*M* GOx:HRP:PAA), corresponding controls of GOx/HRP (Lane 1, simple mixture in solution at a concentration ratio of 16:3 μ*M* GOx:HRP) and GOx/HRP/PAA physical mixture in solution prior to EDC treatment (Lane 2). (B) SDS-PAGE of the GOx–HRP–PAA conjugate (Lane 4) shows the increased molecular weight compared to the corresponding controls of GOx/HRP (Lane 2) and GOx/HRP/PAA physical mixtures (Lane 3). Standard molecular weight markers are shown (Lanes 1 and 5). *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs"*. Chemical Communications, 52*, 2593–2596.*



Scheme 3 Chemical reaction between amine and carboxyl groups catalyzed by EDC.

react to securely interlock the components in the fibrous matrix. Thus, there is a significant margin of error that is allowed in the extent of crosslinking needed for complete interlocking of the components.

1. Prepare the agarose gel (0.125 g agarose) by microwaving a solution of agarose (0.5%, w/v) in Tris-acetate buffer (40 m*M*, pH 7.0, 25 mL) for 1 min on high setting. After microwaving, the agarose should all have dissolved.
2. Leave the agarose to cure in the gel mold for 30 min.
3. Meanwhile, prepare samples by mixing 5–6 μ*M* with loading buffer (10 μL, 50% (v/v) glycerol, and 0.1% (w/v) bromophenol blue).
4. Load 15 μL of sample in each well. Once loaded, run the agarose gel at 100 V for 30 min.
5. Stain the gel with brilliant blue R250 (0.1%, w/v) for 4 h, and then destain the gel with acetic acid (10%, v/v) overnight.

3.2 Characterization of Enzyme–PAA Conjugates by SDS-PAGE

Covalent attachment and formation of the GOx–HRP–PAA conjugate and verification of the increase in molecular weight were also confirmed by SDS-PAGE. Because of the highly crosslinked nature of the nanoarmored enzymes, the conjugate is not expected to move as freely through the gel (Fig. 1B). A 7% polyacrylamide separating gel was used with a 7% stacking gel. By comparing to the standard molecular weight markers (Lanes 1 and 5), the GOx/HRP exhibits a sharp band at ~70 kDa (Lane 2). Without the addition of EDC, the physical mixture GOx/HRP/PAA also exhibits the same molecular weight as GOx/HRP (Lane 3). It is evident that the nanoarmored GOx–HRP–PAA had a broad and high molecular weight band (Lane 4, 90–250 kDa) with no unconjugated enzyme present.

3.3 Characterization of Enzyme–PAA Conjugates by CD

CD is an excellent way of tracking overall changes in enzyme secondary and tertiary structure. One might question how the enzyme structure is affected by the covalent attachment of PAA to its surface. The ellipticity of the nanoarmored GOx–HRP–PAA in solution was found by measuring the CD spectra with a JASCO model J715 spectropolarimeter. All spectra are an average of 10 accumulations, measured with a 1 nm data pitch, and scan speed of 50 nm/min. Cuvette path lengths and sample concentrations were 0.05 cm and ~3 μ*M* (UV-CD, 190–260 nm). The GOx has a flavin adenine dinucleotide (FAD) cofactor which plays a role in the oxidation–reduction

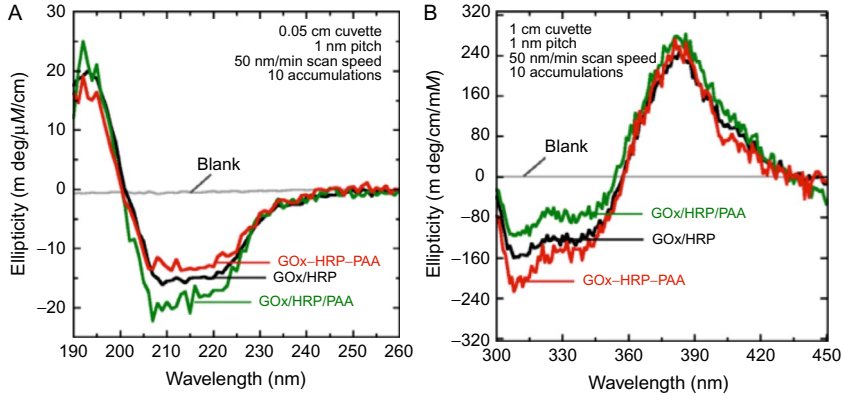


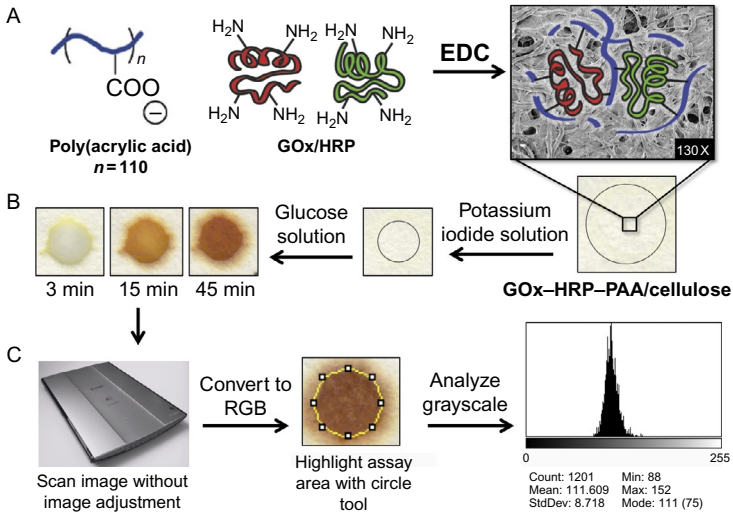
Fig. 2 (A) UV and (B) Soret circular dichroism (CD) of GOx–HRP–PAA conjugates and corresponding GOx/HRP and GOx/HRP/PAA physical mixture controls. *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs".* Chemical Communications, 52, *2593–2596.*

reaction, and its molar ellipticity was measured using 1 cm cuvette path length and ~15 μ*M* enzyme (Soret-CD, 300–450 nm). All ellipticities were normalized by dividing with path length and sample concentration. The CD spectra of all samples remained the same after conjugation to PAA (Fig. 2) with some changes in intensities. GOx–HRP–PAA in solution retained 80% ellipticity at 209 nm when compared to unmodified GOx/HRP physical mixture. The GOx/HRP/PAA control also retained 100% ellipticity. In conclusion, the spectra strongly suggest that the enzyme structure is mostly retained after nanoarmoring with PAA.

3.4 Method for Enzyme Interlocking in Paper

In this approach, circular assay "wells" were prepared on filter paper by coating with wax. The enzyme–polymer conjugate (GOx–HRP–PAA) was subsequently formed by crosslinking the carboxylate groups of the PAA to the lysine residues of GOx and HRP by the water-soluble carbodiimide, EDC (Scheme 4A). The resulting interlocked enzyme–PAA nanoarmored conjugate can be colorimetrically assayed and activity can be quantified by a color scanner and ImageJ software (Scheme 4B and C).

The significance of using a colorimetric assay was a model for a portable quick and disposable test strip, where minimal sample preparation is required in order to obtain a reading. Additionally, a colorimetric assay is useful for enzyme characterization, monitoring how the enzyme is active in cellulose,



Scheme 4 Synthesis and analysis of GOx–HRP–PAA biosensors. (A) Conjugation of the GOx and HRP to PAA is shown via EDC chemistry. The final enzyme–PAA conjugate interlocked within the filter paper (cartoon not to scale). (B) Addition of potassium iodide to the filter paper develops the color once glucose is added. (C) The resulting color intensity on the test strip is scanned and processed with ImageJ. *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs".* Chemical Communications, 52, *2593–2596.*

and how its stability is enhanced when compared to that of the enzyme in solution.

1. Cut circles (8 mm diameter) into wax paper using a precision cutter.
2. Place the cut wax paper on top of a single sheet of Whatman filter paper and apply heat press (280 °C, 20 s). The wax-coated filter paper wells have an inner diameter of ~3.5 mm. *Tip*: The well's boundary uniformity can be verified by wetting with distilled water and then observing if the water diffused into the waxed areas. The wax edges should be sharp and well defined.
3. Prepare a diluted stock solution of PAA (37 m*M*). *Tip*: Because the viscosity of PAA is very high, using a micropipette is not ideal. Instead, use a volumetric syringe to dilute the PAA. Remove any air bubbles from the syringe by pressing the plunger down quickly.
4. Prepare stock solutions of GOx ($\varepsilon_{280\ \mathrm{nm}} = 267{,}000$) and HRP ($\varepsilon_{403\ \mathrm{nm}} = 100{,}000$) each at about 16 mg/mL. Centrifuge the enzyme

solutions to remove any large impurities and obtain the enzyme concentrations using a UV–vis.

5. *Reaction mixture concentrations*: Mix PAA (10 m*M*) in phosphate buffer (200 m*M*, pH 7.0), deionized water, and EDC (100 m*M*) in a microcentrifuge tube and stir for 10 min to activate the PAA. *Tip*: EDC is hygroscopic, so it should be weighed immediately before use.
6. Premix the GOx and HRP to be added to ensure uniform distribution of enzyme in the conjugate, add the enzyme mixture to the PAA solution so that final reaction concentrations are 16 μ*M* for GOx and 3.2 μ*M* for HRP.
7. Immediately transfer 2.5 μL of the activated GOx/HRP/PAA/EDC to the wells in the wax-coated filter paper, where the crosslinking occurs.
8. *Control solutions*: Prepare samples as above (1) without EDC to create physical mixture (GOx/HRP/PAA), (2) without EDC or PAA for the adsorbed enzyme (GOx/HRP), and (3) GOx–HRP crosslinked in the absence of PAA.
9. Air dry for about 1 h at room temperature.
10. Wash the resulting GOx–HRP–PAA/cellulose and controls, three times in 25 mL of distilled water with gentle stirring to remove any unreacted PAA and EDC urea.
11. Dry for 1 h at room temperature.

3.5 Characterization of Cellulose-Interlocked Enzymes by Laser Confocal Fluorescence Microscopy

The nanoarmored enzymes interlocked in the filter paper were visualized with multichannel laser confocal microscopy. This showed the overall distribution of the enzyme and polymer in the cellulose matrix. GOx was labeled with a red dye 5(6)carboxy-X-rhodamine N succinimidyl ester (ROX–GOx) and the PAA with a green dye fluoresceinamine isomer 1 (FA–PAA). By exciting and monitoring at two different wavelengths, the area with both red (ROX–GOx) and green (FA–PAA) indicates the location of the nanoarmored enzymes in the cellulose.

The confocal microscope images show that both the enzyme (red) and the polymer (green) are localized on the cellulose fibers, indicating that the conjugate might be forming within the small intrafiber voids in the cellulose (Fig. 3A). Ambient lighting is shown in the bottom left quadrant while the overlay of all three quadrants is shown in the bottom right. The physical mixture control ROX–GOx/FA–PAA/cellulose shows a decrease

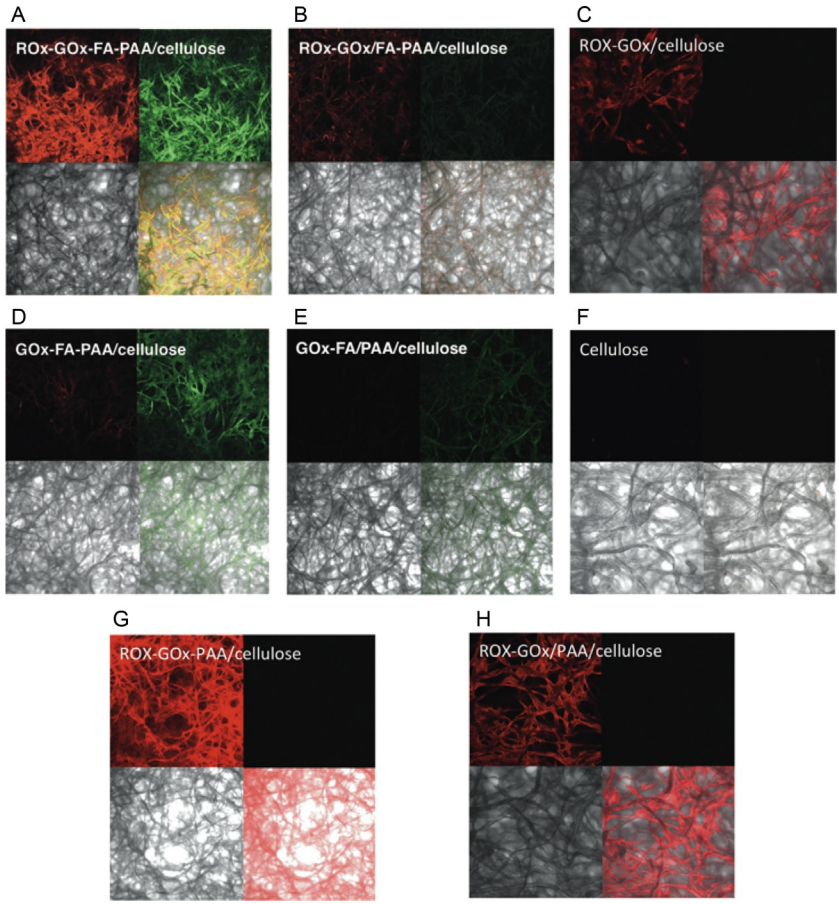


Fig. 3 Confocal fluorescence microscopy images of (A) ROX–GOx–FA–PAA/cellulose conjugate, (B) physical mixture ROX–GOx–FA–PAA/cellulose, and (C) ROX–GOx/cellulose. Additional controls include (D) the conjugate GOx–FA–PAA/cellulose, (E) physical mixture GOx/FA–PAA/cellulose, (G) conjugate ROX–GOx–PAA/cellulose, and (H) the physical mixture ROX–GOx/PAA/cellulose. (F) Bare cellulose. *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs"*. Chemical Communications, 52, *2593–2596.*

in fluorescence intensity (Fig. 3B), indicating that the enzyme and polymer are both mostly washed away. The physical adsorption of the enzyme ROX–GOx on cellulose (Fig. 3C) shows decreased fluorescence intensity compared to Fig. 3A and B, indicating that the enzyme is mostly washed off. The conjugate made with unlabeled GOx and FA–PAA (Fig. 3D) shows a high intensity of green fluorescence from the interlocked conjugate, while

the physical mixture of this same sample shows decreased green fluorescence (Fig. 3E). The bare cellulose control is shown (Fig. 3F) indicating no red or green fluorescence. The conjugate made with labeled ROX–GOx and unlabeled PAA is shown (Fig. 3F) indicating high red fluorescence from the labeled enzyme, while its corresponding physical mixture control (Fig. 3G) shows decreased fluorescence. In all cases, the physical mixtures show decreased fluorescence, indicating that the enzyme and polymer are both washing off from the cellulose paper, whereas the conjugates are retained.

3.5.1 Fluorescent Labeling of PAA Using Fluoresceinamine Isomer 1

1. Prepare a stock solution of PAA (9 m*M*) in deionized water.
2. Add EDC (1 *M*) and stirred for 10 min.
3. The FA stock can be prepared in DMSO. Add FA (10 m*M*) slowly to the PAA solution with stirring, and react for 4 h in the dark at room temperature.
4. Centrifuge the PAA solution for 30 min (10,000 rpm) to remove any unreacted solid FA.
5. Remove the pellet, and then add equal volume of acetone to the FA–PAA solution. This will cause the FA–PAA to precipitate and form a globular gel.
6. Centrifuge for 10 min (10,000 rpm).
7. Remove the supernatant containing acetone/water/unreacted dye.
8. Resuspend the FA–PAA in deionized water. Repeat acetone precipitation from Step 5 three times. This will be the purified FA–PAA stock.

3.5.2 Fluorescent Labeling of GOx With ROX

1. Prepare ROX in DMSO (10 mg/mL).
2. Add the ROX (100 μL) slowly to a solution containing GOx (20 mg/mL) in bicarbonate buffer (0.2 *M*, pH 8.3).
3. Stir for 4 h in the dark at room temperature, and then dialyze against phosphate buffer (pH 7.0, 20 m*M*) for 4 h (switch fresh buffer out every hour) to remove the unreacted fluorescent dye.

3.5.3 Laser Confocal Fluorescence Microscopy

1. Nikon A1R Confocal Microscope parameters
   a. 10 times dry objective lens.
   b. Filter paper samples are placed directly on the microscope stage.

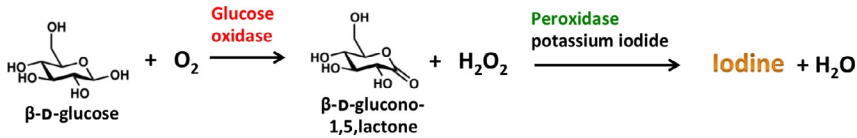
c. The FA dye is excited by a 488 nm argon laser (power level 2.4) and the fluorescence emission is monitored at 525 nm, with a PMT high voltage of 125.
d. ROX dye is excited by a 561 nm argon laser (power level 2.0) and monitored at 595 nm, with a PMT high voltage of 120.
e. The instrument pinhole was 2.17 μm.

3.6 Characterization by Colorimetric Activity Assay

An enzymatic assay using potassium iodide and glucose was used to monitor enzymatic activity and quantify enzyme retention within cellulose (Scheme 4B and C). GOx catalyzes the oxidation of β-D-glucose by molecular oxygen to form hydrogen peroxide and gluconic acid. HRP then catalyzes the oxidation of the iodide to molecular iodine, which is brownish orange colored (Scheme 5). It was quantitatively found that the brown color appears gradually and the reaction is complete after about 20–30 min (Fig. 4). A scanner is used to collect the images so that the color intensity appearance over time can be monitored using ImageJ imaging software.

Nearly 100% of the color intensity was retained for the GOx–HRP–PAA/cellulose after washing, indicating that the enzyme was retained within the paper. Respective controls (without EDC, indicated by a slash) of GOx/HRP/cellulose and GOx/HRP/PAA/cellulose physical mixtures, and GOx–HRP–PAA conjugate drop cast on cellulose 1 day after synthesis, were washed under the same conditions. After 1 day, no further conjugation could occur because of EDC hydrolysis, and no entanglement within the pores was expected. Less than 30% of the enzyme was retained in all three controls.

1. To each circular "well" add 2 μL of potassium iodide solution, prepared in deionized water (0.3 *M*). *Tip*: The potassium iodide solution can be stored at 4 °C, in the dark for at least 1 week.
2. Dry for 15 min.
3. With the scanner hooked up and ready to scan, add the glucose solution (2–40 m*M* in deionized water).



Scheme 5 Colorimetric enzyme assay by the addition of glucose oxidase and potassium iodide to the interlocked and nanoarmored enzymes–PAA conjugate.

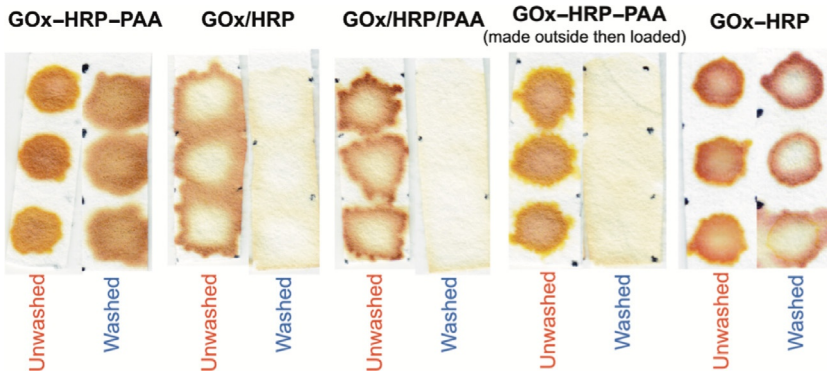


Fig. 4 Synthesis validation for GOx–HRP–PAA/cellulose conjugate interlocked with paper fibers. All samples were washed to remove any unbound material. Controls are GOx/HRP and GOx/HRP/PAA physical mixtures, GOx–HRP–PAA conjugate deposited on the filter paper 1 day after synthesis, and GOx–HRP crosslinked in the absence of PAA. *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs".* Chemical Communications, 52, *2593–2596.*

4. Scan the yellow to brown color (conversion of potassium iodide to elemental iodine) with the scanner every 2 min (600 dpi, color setting). *Tip*: The scanner may have a color adjustment setting—this should be turned off.
5. Open the resulting image with ImageJ, and convert to gray scale.
6. Highlight one "well" with the circle tool and analyze the gray scale color intensity using the histogram tool under the "analyze" dropdown.
7. Record the "mean" average color intensity and subtract this value from 255. This value increases as more iodine is produced.

3.7 Enzyme Loading on Cellulose by Bradford Assay

Because the conjugate is forming around the cellulose fibers, and the conjugate size can increase as both the enzyme and polymer concentrations increase, the cellulose surface area therefore does not limit the maximum loading of enzyme in the cellulose. Finding the enzyme loading on cellulose can be useful, as this interlocking and armoring method can easily be extended to other enzymes and other suitable supports. The loading will also indicate the extent of conjugation and how much of the enzyme washes off, which is significant to know, especially when using more expensive enzymes. Enzyme concentration can easily be quantified by using the

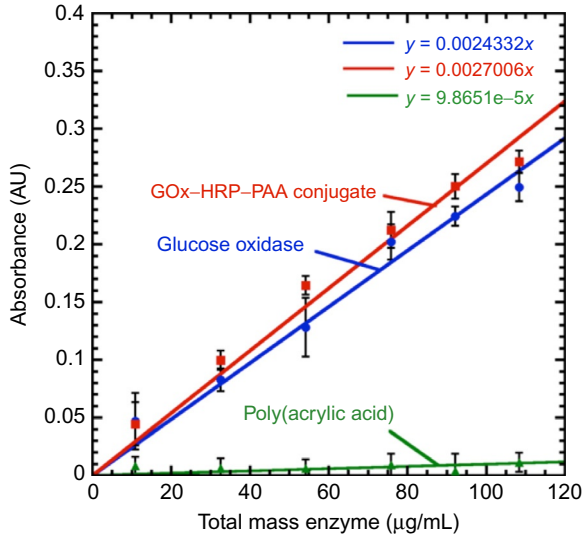


Fig. 5 Calibration graph of enzyme standards (1, 3, 5, 7, 8.5, and 10 µg enzyme in 0.1 mL) by the net absorbance at 595 nm of after adding Bradford reagent. Enzyme standards of GOx (*blue*), GOx–HRP–PAA conjugate (*red*), and control of PAA without enzyme (*green*) are shown. Net absorbance was calculated by subtracting the absorbance of buffer and reagent from the mixture of enzyme and reagent. *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs".* Chemical Communications, 52, *2593–2596.*

Bradford reagent and measuring the absorbance at 595 nm with respect to increasing enzyme concentration. A calibration graph was made for the Bradford reagent, and the unknown enzyme concentration in the washes was calculated from the calibration plot (Fig. 5).

As total enzyme mass was increased during the interlocking process, it was found that enzyme percent loading did not saturate in the range tested, confirming the system was not limited by cellulose surface area (Fig. 6A). Loading increased as more enzyme was added. The enzyme percent retention after washing also increased with increasing enzyme mass, with the highest retention for the 26 mg GOx sample that exhibited less than 1% loss of enzyme during subsequent washes (Fig. 6B). Because crosslinking probability increases as more GOx is added, high enzyme retention is achieved with more crosslinking until no more enzyme–polymer can be accommodated in the voids.

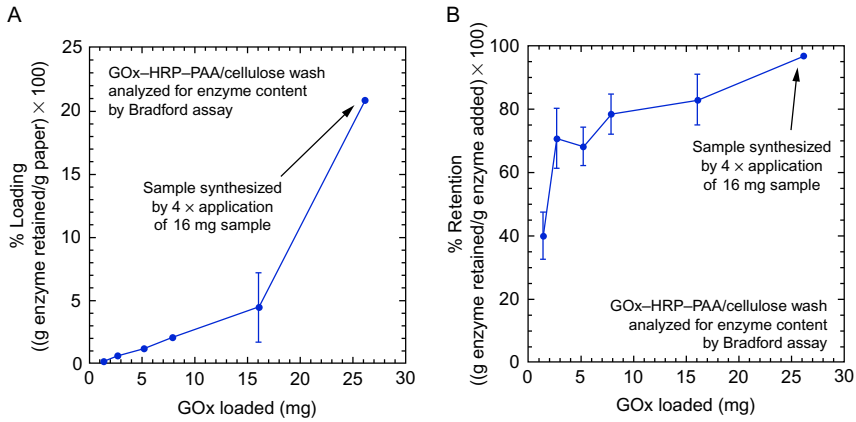


Fig. 6 (A) Percent loading of enzyme per gram of paper and (B) the percent enzyme retained within the paper after washing are shown. Error bars are provided but some are smaller than the points. *Reproduced from Riccardi, C. M., Mistri, D., Hart, O., Anuganti, M., Lin, Y., Kasi, R. M., & Kumar, C. V. (2016). Covalent interlocking of glucose oxidase and peroxidase in the voids of paper: Enzyme–polymer "spider webs".* Chemical Communications, 52*, 2593–2596.*

3.7.1 Bradford Reagent Preparation and Calibration of Enzyme Concentration

1. In a 200 mL beaker, dissolve brilliant blue G-250 (100 mg) in ethanol (95%, w/v, 50 mL). *Tip*: Make sure at this point that most of the brilliant blue is dissolved.
2. To this solution, add phosphoric acid (85%, w/v, 100 mL) and then dilute to 1 L with deionized water. Final concentrations of the reagent are 0.01% (w/v) brilliant blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.
3. Filter the solution through Whatman 1 filter paper before each use until the color is light brown (usually 2–3 times). This solution can be stored up to 6 months at 4 °C.
4. Prepare enzyme standards containing either GOx–HRP–PAA or GOx at 1, 3, 5, 7, 8.5, and 10 μg enzyme in 0.1 mL. Respective control of only PAA is made at identical PAA mass present in GOx–HRP–PAA standards.
5. To these standards, add 1 mL of Bradford reagent and mix.
6. Measure the absorbance of the solution at 595 nm 10 min after adding the reagent with respect to total enzyme concentration (Fig. 5). *Blank solution*: Phosphate buffer (20 m*M*, pH 7.0) and reagent.

3.7.2 Enzyme Loading by Bradford Assay

1. During synthesis of the GOx–HRP–PAA/cellulose, the GOx concentration was increased (16, 30, 60, 90, 180 μ*M*).
2. The EDC concentration is also increased to 1.1 *M* so that all carboxylate groups on PAA were activated.
3. Wash the GOx–HRP–PAA/cellulose samples three times in 25 mL of water for 1 h to remove any free enzyme. *Tip*: Measure the exact volume using a graduated cylinder after each wash is complete to account for slight evaporation.
4. Analyze the wash for enzyme concentration by its absorbance at 450 nm after adding the Bradford reagent. The absorbance of the wash should fall within the calibration curve, dilute as necessary.
5. Calculate the total enzyme concentration in the cellulose, taking into account volume dilutions.
6. Enzyme percent loading ((g enzyme retained/g cellulose) × 100) is calculated by dividing the mass of enzyme retained in the paper by the total mass of the cellulose, and then multiplying by 100 (Fig. 6A).
7. The percent enzyme retained (Fig. 6B) within the paper is calculated by dividing the mass of enzyme retained by the original mass of enzyme added, and then multiplying by 100. In all experiments, total filter paper weight was about 270 mg.

3.8 Porosity of the Cellulose-Interlocked Enzymes by Mercury Intrusion Porosimetry

Maintaining porosity of the cellulose paper matrix is important for minimizing low diffusion rate of the substrate to the enzymes in the matrix. In order to quantify how much the porosity changes due to interlocking, mercury intrusion porosimetry was done on the GOx–HRP–PAA/cellulose sample with the highest enzyme loading (26 mg GOx). This ensures that, even with such high loadings, the porosity is still maintained.

Four samples of GOx–HRP–PAA/cellulose and four control samples of bare cellulose were sent according to the specifications needed by Micromeritics, Georgia, USA. The measurements were conducted automatically on an AutoPore IV 9500V1.09. The samples were sealed in a penetrometer, weighed, and subjected to mercury intrusion. The GOx–HRP–PAA/cellulose had a pore volume of 0.65 mL/g, a total pore area of 14 m^2/g, a median pore diameter of 3.2 μm, and a porosity of 21%. The corresponding control of bare cellulose had a pore volume of 1.4 mL/g, a total pore area of

7.3 m^2/g, a median pore diameter of 12 μm, and a porosity of 48%. These data along with the laser confocal microscopy strongly suggest that the porosity of the cellulose is diminished but largely maintained.

3.9 Stability of the Cellulose-Interlocked Enzymes Against Denaturation

The nanoarmoring and interlocking of enzymes in cellulose is expected to stabilize the native state, and increase the enzyme's resistance against denaturation, while also giving them the advantage of being washable. Washable paper-based systems are useful in applications such as wearable sensors, or flow-through systems such as wastewater treatment. In such cases, the enzyme must remain active in harsh conditions.

Thus, the GOx–HRP–PAA/cellulose was washed in denaturants such as urea (5 *M*) or SDS (20%, w/v) for 30 min, and then rinsed three times with deionized water for an additional 30 min. It was found that the GOx–HRP–PAA/cellulose retained 103 ± (8)% of its activity after washing in urea, and 70 ± (8)% activity after washing with SDS (Fig. 7A). All other controls of GOx/HRP/cellulose (Fig. 7B) and GOx/HRP/PAA/cellulose (Fig. 7C) retained 30% or less activity after washing in these conditions. Because a 30-min cycle is a typical washing cycle, the nanoarmored and interlocked enzyme can be considered a washable system.

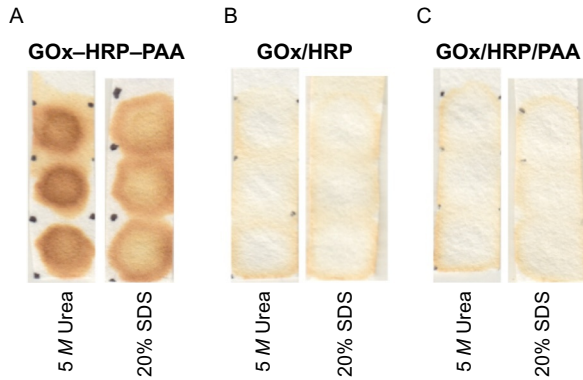


Fig. 7 Resistance to chemical denaturation of interlocked (A) GOx–HRP–PAA/cellulose and controls of (B) GOx/HRP/cellulose and (C) GOx/HRP/PAA/cellulose. The samples were all washed in urea or SDS for 30 min and then rinsed three times with distilled water for an additional 30 min.

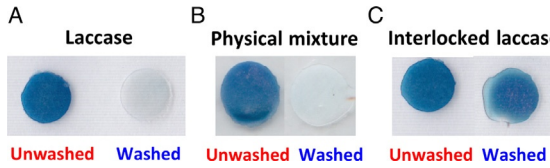


Fig. 8 Synthesis validation for (C) nanoarmored laccase interlocked with paper fibers. All samples were washed to remove any unbound material. Controls are (A) adsorbed laccase and (B) laccase physical mixtures (unpublished work).

3.10 Modular Approach for Enzyme Interlocking

Thus far, we have shown that this method of nanoarmoring by covalent conjugation to PAA improves the stability of enzymes in solution such as hemoglobin, catalase, cytochrome *c*, lysozyme, and others (Mudhivarthi et al., 2012; Riccardi, Kasi, & Kumar, 2015; Riccardi et al., 2014; Thilakarathne, Briand, Zhou, Kasi, & Kumar, 2011). The interlocking and nanoarmoring method is therefore expected to work with other enzymes and other polymers that have the required functional groups.

We recently interlocked laccase in cellulose, an enzyme that reduces a variety of phenols (Fig. 8). Even though our methods have not yet been optimized, the interlocked laccase remained in the filter paper even after extensive washing (Fig. 8C), while the respective controls of adsorbed and physical mixture of laccase was washed away (Fig. 8A and B). In an effort to enhance the stability and loading of laccase in cellulose, and because laccase only has five available lysines to conjugate to PAA, we have pre-interlocked BSA as a first coating on the filter paper, then added another layer of laccase with BSA and/or PAA. This new approach of using BSA as "packing peanuts" appears to help increase the loading and retention of laccase compared to when only PAA is used (only about 60% of the laccase activity is retained when interlocked only with PAA, but activity retention increased to greater than 60% when BSA was added. The interlocked laccase was also significantly more stable than the free enzyme (the half-life of laccase interlocked with BSA instead of PAA was $\geq$20 days, while laccase in solution at room temperature has a half-life of less than 3 days).

4. CONCLUSIONS

In conclusion, the nanoarmoring of enzymes with PAA and their interlocking within the cellulose matrix of ordinary paper is an excellent way of trapping enzymes, without the need for direct covalent attachment

or surface modification. This is the first example where the physical interlocking of the conjugate in the cellulose allows for a washable system that remains active even under strong denaturing conditions with long-term stability and reusability after washing.

ACKNOWLEDGMENTS

This work was supported by NSF EAGER award DMR-1441879 and by the University of Connecticut VPR Research Excellence award. We would like to thank K. Cole, K. Benson, and K. Kadimisetty for technical help.

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