Piezoelectric ink jet printing of horseradish peroxidase on fibrous substrates

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Abstract

Solid phase bioanalysis relies on the creation of a highly selective and sensitive layer of bioagent on a solid support capable of recognizing specific target analytes. Using ink jet printing technology picoliter volumes of bioagents can be precisely patterned over large surfaces with controlled uniformity. Despite the advantages of bioanalytical methods and the flexibility of ink jet printers, producing this type of functional materials is challenging. Solid supports can affect the biological activity, the spatial distribution and the binding capacity of the bioagent causing problems during post print detection. In this study a bioink containing the enzyme horseradish peroxidase (HRP) was jetted with a piezoelectric-based ink-jet deposition system on various fibrous substrates with differences in composition, surface chemistry and pore structure. Enzyme activity in solution and after printing was tested colorimetrically using 2, 2'-azino-di-(3-ethylbenzthiazoline) 6-sulphonate (ABTS) as chromogen. It was found that the papers tested have significantly different bioanalytical response in the detection of hydrogen peroxide. The uncoated papers showed the most sensitive green color response; whereas, the uncoated wood free paper gave the broader range of measurement and the highest color intensity. In contrast, the coated papers developed lower color intensity and the cast coated paper produced a pink color response possibly due to enzyme intermediate compounds produced under conditions of substrate inhibition.

Introduction

Bioanalytical techniques exploit the superior recognition ability of biomolecules to detect the presence of specific target analytes [1]. Enzymes, in particular, have been immobilized in solid materials to selectively detect and/or quantify their substrates [2]. Furthermore, bioanalysis used in conjunction with various physico-chemical transducers has been used to develop biosensors aimed at detecting, quantifying and/or monitoring single or multiple analytes in complex sample matrices [3].

The solid support where biomolecules are immobilized plays a significant role in the performance of bioanalytical systems [4]. Studies on the effects of immobilization on enzymatic reactions have suggested that properties of the immobilization support can strongly influence enzyme activity and stability over time [5]. When restricted in spatial movement, proteins can adopt new configurations and the degree of accessibility to the active site can change. The microenvironment that surrounds the immobilized enzyme can result in different optimum for pH and temperature ranges comparing to the case when the enzyme is free to move in solution.

Among the wide range of supports available for enzyme immobilization, cellulosic fibrous materials are attractive because they offer: a porous structure with large surface area for interactions allowing larger biomolecule load, a suitable environment for biorecognition events, low cost, portability, and disposability. Cellulose and its derivatives have found widespread use as support for immobilized biomolecules [6,7]; whereas, paper has been incorporated in dry reagent chemistries either as preformed matrix or as reflective layer [8]. Recently, Kinnunen [9] defined a bioactive paper as a fibre-based product with an integrated biological component capable of identifying microbes, toxins or other harmful substances

Ink jet printing is a promising technology to incorporate biological functionalities into solid materials. Relatively small dispensed volumes (10-20 picoliter per drop), non contact operation, speed, and comparatively high spatial resolution are some advantages of ink-jet technology over other deposition techniques [10,11]. Some researchers have successfully printed enzymes using ink-jet technology. Table 1 summarizes the printing systems, bioagents and supports used by these researchers and their intended applications.

Table 1. Previous work on ink jet printing of enzymes

Ref.	Ink jet	Enzyme	Support	Application
[12]	piezo	GOD, urease	ISFET sapphire	biosensor
[13]	electrostatic	GOD	carbon electrode	biosensor
[14]	thermal	POD	papers , plastics	bioanalysis
[15]	thermal	GAL	polyester sheet	exploratory
[16]	thermal	GOD	glass coated with PEDOT-PSS polymer	biosensor
[17]	piezo flow through	GOD	Au sputtered glass slide	biosensor

Few studies have attempted to pattern enzymes on cellulosic fibrous supports. In a pioneer research, Roda et al. [14] deposited a bioink containing HRP, buffer, and surfactant using a commercial thermal ink-jet printer, on various solid supports including cellulose papers with basis weight ranging between 30 and 80g/m², cellulose filter paper, nylon sheet, photographic gelatin paper, tissue paper and in-jet transparency film. The authors reported that the best intensity and spatial distribution in the chemiluminiscent signal was obtained with the permeable paper supports. Moreover, the fast diffusion of chemiluminiscent substrate in the low basis weight paper resulted in a faster signal development. Detection problems were observed with non permeable supports produced by enzyme washout. More recently, Martinez et al. [18] have reported a method for patterning photoresist onto chromatographic paper to form defined hydrophilic areas for the enzymatic detection of glucose in urine.

No systematic study of the effect of paper support characteristics on enzyme activity and bioanalytical performance is available from the literature. Therefore, this paper aims to systematically study the performance of paper as enzyme immobilization support and the efficacy of using ink jet printing technology for developing bioactive papers. To achieve this goal a biological ink containing HRP enzyme as main active component was deposited and physically immobilized on various fibrous substrates using a piezo-based drop-on-demand ink jet printer. The impact of the paper supports on the performance of hydrogen peroxide bioanalysis was investigated.

Methods and Materials

Reagents

Horseradish peroxidase (EC 1.11.1.7, type VI, highly stabilized, 200-300 U/mg) was purchased from Sigma (Oakville, ON, Canada). A 50 U/ml (pyrogallol units) HRP enzyme and 18.2 mM ABTS solution was prepared in a 40mM Potassium Phosphate buffer (pH 6.8) containing a Triton X-100 as surfactant, glycerol as a humectant and a carboxymethyl cellulose as viscosity modifier.

Fibrous substrates

Table 2 summarizes the different paper substrates used in this study.

Table 2. Paper substrates

Code	Type	General use
A	Standard cellulose chromatography paper	chromatography
B	Uncoated 30% post-consumer fibre	office, printer, copiers
C	Uncoated wood free	office, laser printer
D	Color copy cover	premium color copies
E	Coated ink jet grade with silica pigment	premium ink jet prints
F	Cast coated	photographic quality

Printer

A material deposition system based on piezoelectric ink jet technology (Dimatix DMP 2800) located inside a room with controlled atmospheric conditions (23 ± 1°C temperature and 50 ± 2% relative humidity) according to the standard TAPPI T 402 was used. The printer includes a MEMS-based cartridge-style disposable printhead with 16 nozzles linearly spaced at 254 µm and typical drop size of 10 pl. Each cartridge has a reservoir capacity of 1.5 ml. The cartridge reservoirs were cleaned with both deionized water and buffer before filling with ink. To avoid clogging of the printhead nozzles all the liquids were pre-filtered through a nylon membrane Whatman with 0.45 µm pore size. The printer is equipped with a high speed camera (drop watcher) that allows observation of the drop formation on the printhead nozzles and jetting process. A reliable and stable operational window for microdrop generation was selected by adjusting addressable jetting parameters (firing frequency, nozzle voltage, waveform, and reservoir pressure).

Solid phase bioanalysis of H_2O_2

Bioanalyis of H_2O_2 is based on its decomposition to H_2O in the presence of a hydrogen donor, the leuco-dye, 2,2'-azino-di-(3-ethylbenzthiazoline) 6-sulphonate (ABTS), specifically catalized by the enzyme HRP [19] as follows:

$$ABTS_{(reduced)} + H_2O_2 \xrightarrow{HRP} ABTS_{(oxidized)} + 2H_2O$$

In the reduced form the chromogen is colorless and as the enzymatic reaction proceeds, a green color corresponding to the oxidized form of ABTS is developed in direct proportionality to the amount of H_2O_2 converted.

Detection

For the quantitative determination of H_2O_2 the backside of the printed paper strips was kept in contact with solutions of H_2O_2 of known concentration for 2 min after which the excess solution was removed with a bottling paper. The diffuse reflectance spectrum of the printed spots for the wavelength interval 400 to 700 nm was measured using a color reflection spectrodensitometer X-Rite 530 after 1 hour of exposure to the H_2O_2 solution to ensure that the samples were dry and there was no further colour change. The illuminant D65 and the geometry 2° was selected for the measurements. Also, a flexible thin film UV filter that allowed less than 10% transmission below 390 nanometers was used in the color measurements to remove the contribution from optical brighteners present in some of the paper samples.

Bioanalysis calibration

Kubelka Munk theory was used to linearly relate the diffuse reflectance, **R,** measured on the paper color spots and the analyte concentration, **C**. Following similar approach as in [20], the function $\mathbf{A_R = -\ln(R/R_b)}$ **vs. log C** was plotted as a calibration curve. $\mathbf{A_R}$ represents the solid phase analogous of absorbance in liquid media, **R** is the diffuse reflectance of the color spot and $\mathbf{R_b}$ is the diffuse reflectance of the blank. A printed spot exposed to deionized water was used as blank.

Results

Bioink formulation

Several additives are incorporated in comercial ink formulations for the graphic arts to optimize ink rheological parameters and to make them stable and ejectable. In the case of bioinks; however, these additives may produce inactivation or denaturation of the enzyme immediately or over time. Thus, a suitable bioink formulation has to be able to maintain the activity of the enzyme and at the same time produce stable and repeatable drops during jetting. In order to jet the bioink, the viscosity and surface tension of the bioink (initially in the range 60-65mN/m and 1-1.2cps, respectively, without additives) had to be adjusted to the optimum values (30mN/m and 5cps) as suggested by the literature [10, 21].

Previously, we have systematically studied the impact of piezoelectric ink additives, especially viscosity modifiers, on the HRP activity [22]. We found that viscosity modifiers typically used in commercial ink formulations significantly impaired HRP activity possibly due to limitations in diffusion. In contrast, a high molecular weight charged polymer increased viscosity to the

desired range for piezoelectric jetting without negatively affecting the HRP activity. Good jettability was obtained for the HRP bioink by adding a suitable surfactant, a viscosity modifier, and a humectant.

Piezoelectric ink jet printing of HRP

The bioink was printed on the fibrous substrates detailed in Table 2 using the Dimatix DMP 2800 printer. Drops with approximately 10±1ng/drop were consistently jetted at a speed in the range 7-9 m/s using a 2 KHz firing frequency, a 30V driving voltage and an ink firing temperature of 28°C. Table 3 illustrates the papers printed with the bioink after color development upon exposure to solutions of increasing H_2O_2 concentrations.

Table 3. HRP enzyme spots ink jet printed on paper substrates after color development upon exposure to increasing H_2O_2 concentrations

$[H_2O_2]$ mM	A	B	C	D	E	F
10^{-6}						
10^{-5}						
10^{-4}						
10^{-3}						
10^{-2}						
10^{-1}						
0.2						
0.3						
0.4						
0.5						
0.6						
0.7						
0.8						
0.9						

Bionalysis of H_2O_2 on fibrous substrates

Color profile

Figure 1 shows the diffuse reflectance spectra of the colored spots on different paper substrates for a fixed analyte concentration. Most of the papers exhibit a reflectance peak at 480nm, characteristic of the green color developed upon reaction. The cast coated paper, in contrast, developed a pink color with peak reflectance at 700nm. This pink color might indicate the presence of HRP intermediate compounds generated under conditions of substrate inhibition [23].

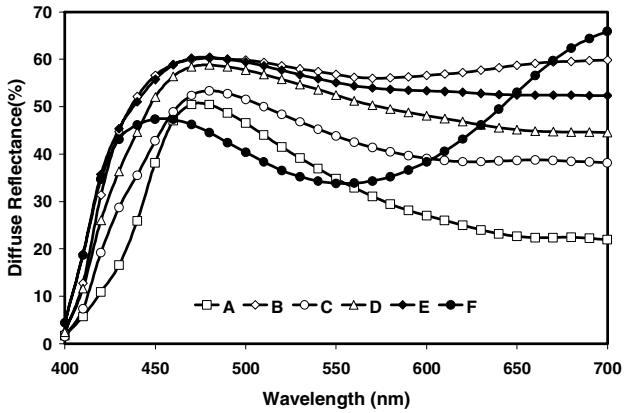
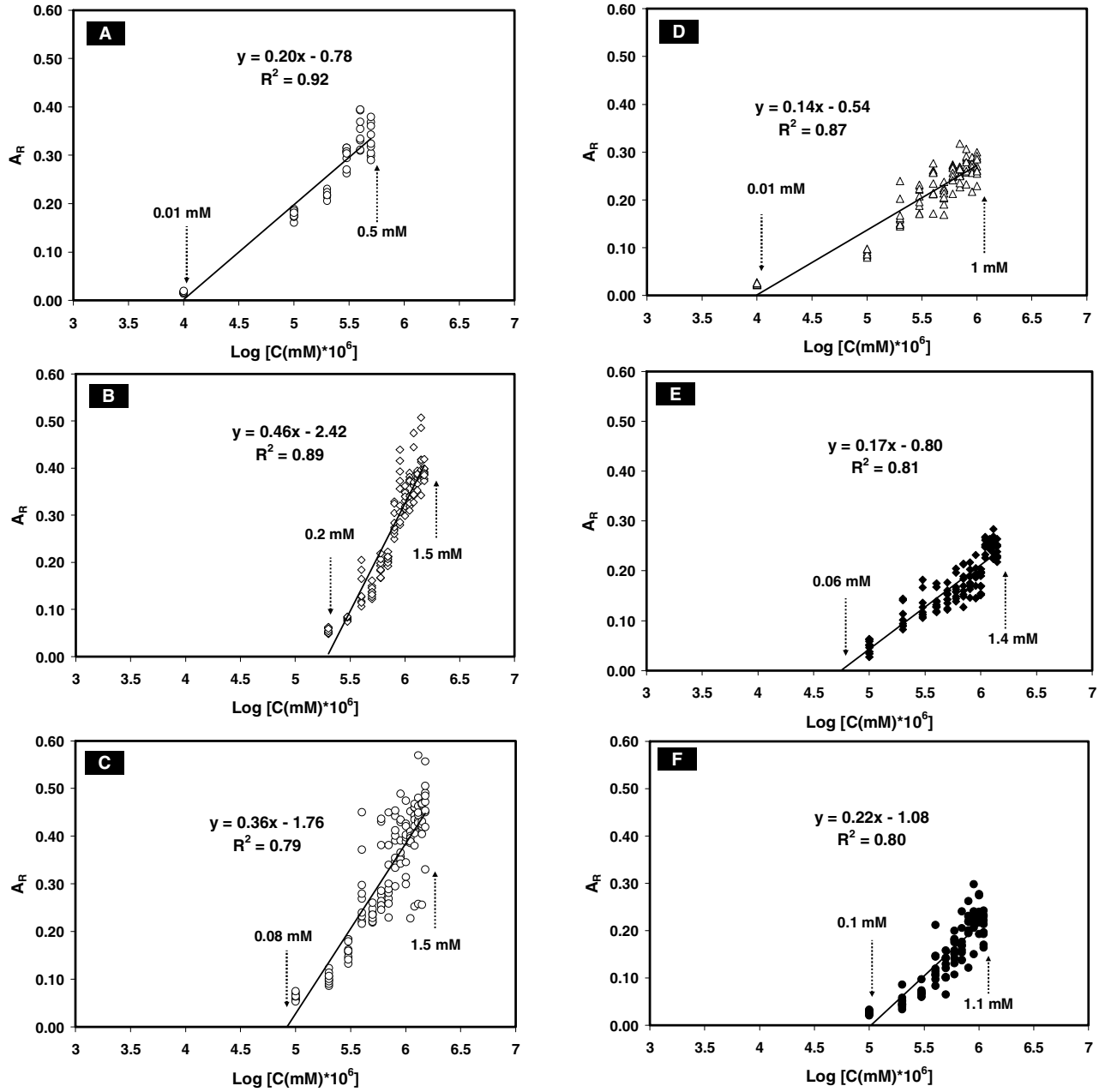


***Figure 1**. Diffuse reflectance spectra of colored spots on different paper substrates corresponding to 0.5 mM H_2O_2 solution*

Bioanalytical performance

Figures 2 (A, B, C, D and F) show the calibration plots of $\mathbf{A_R}$ **vs log C** obtained using the peak diffuse reflectance of the colored spots developed on different paper substrates when exposed to increasing analyte concentrations. Each plot includes performance indicators for quantitative bioanalysis: the *lower limit of detection* was defined as the concentration of analyte corresponding to the intercept of the calibration curve with the abscissa axis; the *upper limit of detection* was defined as the maximum concentration of analyte that allowed maximum linear correlation between $\mathbf{A_R}$ and **log C** in the calibration curve; the *range of measurement* was obtained from the difference between upper and lower limits of detection; and the *sensitivity* of the technique was represented by the slope of the calibration curve.

Papers A and D presented the lowest limit of detection (0.01mM). Uncoated papers were almost twice more sensitive than the rest of the papers tested but they differed in the intensity of the color developed upon reaction. For similar analyte concentration, the woodfree uncoated paper exhibited the highest $\mathbf{A_R}$ in the series. The broader range of measurement also pertained to the uncoated woodfree paper. For H_2O_2 bioanalysis, the degree of correlation between color signal and analyte concentration was good but not as high as values reported in the literature for chemical analysis of inorganic cations on paper [20]. The higher variability expected in bioagents as opposed to chemical reagents and printing uniformity, a consequence of both paper properties and printing parameters, could have played a role in these results.



Figures 2 (A,B,C,D,E,F). *Calibration curves of* A_R ***vs log C*** *for* H_2O_2 *bioanalysis performed on different paper substrates (as detailed in Table 2) printed with HRP bioink*

Conclusions

The enzyme HRP can be reliably printed on fibrous substrates using piezoelectric ink jet technology. It was found that papers differ significantly as solid supports for quantitative bioanalysis of H_2O_2 using the enzyme HRP and the chromogenic substrate ABTS:

- The chromatographic paper (A) enabled lower detection limits but had very narrow range of measurement and low color intensity.
- The uncoated papers (B and C) presented the more sensitive bioanalytical response. However, for comparable analyte concentrations the wood free paper developed more intense color and wider range of measurement that the uncoated paper with recycled content
- The coated papers (D and E) exhibited the lowest color intensities.

- The cast coated paper (F) had a completely different color profile possibly due to the presence of reaction conditions of substrate inhibition.

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References

[1] Zvi Liron, Avraham Bromberg and Morly Fisher. Novel Approaches in Biosensors and Rapid Diagnostic Assays. (New York: Kluwer Academic/Plenum Publishers, 2001) pg.173.

[2] R. Freitag, "Utilization of Enzyme-Substrate Interactions in Analytical Chemistry", Biomedical applications, 722(1-2):279 (1999).

[3] M. P. Byfield, R.A. Abuknesha, "Biochemical aspects of biosensors", Biosensors and Bioelectronics, 9(4-5):373-399 (1994).

[4] W. Tischer , V. Kasche "Immobilized enzymes: Crystals or carriers?", Trends in Biotechnology, 17(8):326-335 (1999).

[5] M. F. Chaplin. Enzyme technology. (New York, Cambridge University Press, 1992) pg. 80.

[6] P. Gemeiner, V. Štefuca, V. Báleš, "Biochemical engineering of biocatalysts immobilized on cellulosic materials", Enzyme and Microbial Technology, 15(7):551-566. (1993).

[7] M.D. Lilly, "Enzymes immobilized to cellulose", Methods in Enzymology, 44:46-53 (1976).

[8] B. Walter, "Fundamentals of dry reagent chemistries: The role of enzymes", Methods of biochemical analysis, 36:35-62 (1992).

[9] L. Kinnunen, "Potential of bioactive paper explored", Paperi ja Puu, 88(2):84-85+66 (2006).

[10] P. Calvert, "Inkjet printing for materials and devices", Chemistry of materials, 13(10):3299-3305 (2001).

[11] E.R. Lee. Microdrop generation. (1st edition, CRC Press, Boca Raton, 2003), pgs. 1, 15, 55, 183, 221, 231.

[12] J. Kimura, Y. Kawana, T. Kuriyama, "An Immobilized Enzyme Membrane Fabrication Method using an Ink Jet Nozzle", Biosensors, 4, 41 (1988).

[13] J.D. Newman, A.P.F. Turner, G. Marrazza, "Ink-jet printing for the fabrication of amperometric glucose biosensors", Analytica chimica acta, 262(1):13 (1992).

[14] Roda, M. Guardigli, C. Russo, P. Pasini, M. Baraldini, "Protein microdeposition using a conventional ink-jet printer", Biotechniques, 28(3):492-496 (2000).

[15] L. Setti, C. Oiana, S. Bonazzi, B. Ballarin, D. Frascaro, A. Fraleoni-Morgera, S. Giuliani, "Thermal Inkjet Technology for the Microdeposition of Biological Molecules as a Viable Route for the Realization of Biosensors", Anal. Lett., 37, 1559 (2004).

[16] L. Setti, A. Fraleoni-Morgera, B. Ballarin, A. Filippini, D. Frascaro, C. Piana, "An Amperometric Glucose Biosensor Prototype Fabricated by Thermal Inkjet Printing", Biosensors bioelectronics, 20(10):2019 (2005).

[17] F. Turcu, G. Hartwich, D. Schäfer, W. Schuhmann "Ink-jet microdispensing for the formation of gradients of immobilised enzyme activity", Macromol Rapid Commun., 26(4):325-330 (2005).

[18] A.W. Martinez, S.T. Phillips, M.J. Butte, G. M. Whitesides, "Patterned Paper as a Platform for Inexpensive, Low-Volume, Portable Bioassays", Angew. Chem. Int. Ed., 46, 1318 –1320 (2007).

[19] R.E. Childs, W.G. Bardsley, "The steady state kinetics of peroxidase with 2,2' azino di (3 ethylbenzthiazoline 6 sulphonic acid) as chromogen", Biochemical Journal, 145(1):93-103 (1975).

[20] A. Ghauch, C. Turnar, C. Fachinger, J. Rima, A. Charef, J. Suptil, M. Martin-Bouyer, "Use of diffuse reflectance spectrometry in spot test reactions for quantitative determination of cations in water", Chemosphere, 40:1327-1333(2000).

[21] H. Dong, W.W. Carr, J.F. Morris, "An experimental study of drop-on-demand drop formation", Phys. Fluids 2006, 18, art. no. 072102

[22] S. Di Risio and N.Yan," Piezoelectric Ink-Jet Printing of Horseradish Peroxidase: Effect of Ink Viscosity Modifiers on Activity", submitted to Macormolecular Rapid Communications.

[23] O. Ryan, M.R. Smyth, C. Ó Fágáin, "Horseradish peroxidase: the analyst's friend",Essays in Biochemistry, 28:129-145 (1994).

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