Characterizing spatial distribution of ink-jet printed horseradish peroxidase on paper substrates

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Abstract

In solid phase bioanalysis, bioagents are incorporated onto solid materials to give them the ability to detect and/or quantify specific target analytes. Previous research has shown that the enzyme horseradish peroxidase (HRP) can be printed on paper without significant inactivation using piezoelectric ink-jet technologies. However, bioanalytical performance of the resulting bioactive papers can be affected not only by the printing conditions and quantity of enzyme deposited, but also by the enzyme spatial distribution and printed enzyme stability. In this study, confocal laser scanning microscopy (CLSM) is used to characterize the in-plane and cross-sectional localized enzyme activity of HRP ink-jet printed on fibrous supports with different surface chemistry and composition. Key paper attributes that can affect the analytical performance are identified.

Introduction

Bioactive papers are envisioned as value-added fibre-based products containing an advanced biological functionality that is capable of identifying, capturing and/or inactivating harmful substances [1,2]. Some of the expected advantages of embedding sensors into the fibrous supports are: low cost, portability, easy of use and instant response [2]. Biosensing is a promising area of application for bioactive papers; amongst biomolecules, enzymes are well characterized analytical reagents that can selectively detect and/or quantify their substrates [3].

Even though it has been shown many biomolecules can be incorporated into paper to impart certain functionality [4-7] , it has also been found that most bioagents partially or completely lose biological activity if deposition and immobilization conditions such as temperature, moisture level, shear rate or pH are not optimized. The fundamental understanding of the bioagent-paper interaction as a function of immobilization strategy and the binding behaviour and the distribution of the bioagent in the support require further investigation.

Meanwhile, ink jet printing technology has unique benefits in delivering biological solutions onto the solid materials. Relatively small dispensed volumes (10-20 picoliter per drop), non contact operation, speed, and comparatively high spatial resolution are some advantages of the ink jet technology over other deposition techniques [8, 9].

Some researchers have successfully printed enzymes using ink jet technology [4,10-16]. However few of them have attempted to print on cellulosic supports. In a pioneer research, Roda et al. [4] 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 ink-jet transparency film. The authors reported that the best intensity and spatial distribution in terms of the chemiluminescent response was obtained with the permeable paper supports. The other non permeable supports produced detection problems due to enzyme washout. More recently, Martinez et al. [6, 7] have reported a method for patterning photoresist onto chromatographic paper to form defined hydrophilic areas for detection of glucose and protein in urine.

This paper aims to systematically study the performance of paper substrates as enzyme immobilization support and the efficacy of using ink jet printing technology for developing bioactive papers. Previously, we have reported a bioink formulation having HRP as main active ingredient that can be reliably ink jet printed without significant loss of enzyme activity [5]. The same HRP bioink formulation was printed on several commercial papers and their qualitative and quantitative bioanalytical performance in H_2O_2 biosensing was evaluated. It was found that the paper supports significantly affect the bioanalysis [16]. In this study, we interrogate the printed paper structure to find possible explanations for these differences. A method for characterizing the spatial arrangement of active printed HRP enzyme based on confocal laser scanning microscopy is developed. The cross-sectional and surface distribution of the active enzyme on various commercial papers, handsheets and coating layer are presented.

Methods and Materials

Bioink for fluorescence detection of H_2O_2

Amplex Red (Molecular Probes) was initially dissolved in dimethylsulfoxide (DMSO) according to the supplier instructions to obtain a 10 mM solution. A 50 U/ml (pyrogallol units) HRP enzyme and 0.12 mM Amplex Red bioink formulation was prepared in a 40mM Potassium Phosphate buffer (pH 6.8) containing 0.1 wt-% Triton X-100 as surfactant, 10 wt-% glycerol as a humectant and a 0.5 wt-% carboxymethyl cellulose as viscosity modifier. This bioink formulation can be reliably ink jet printed using the piezoelectric material deposition system.

Fibrous substrates

Table 1 summarizes the different commercial papers used as solid supports in this study.

Table 1. Paper substrates

Code	Type	General use
C	Uncoated 30% post-consumer fibre	office, printer, copiers
D	Uncoated wood free	office, laser printer
E	Uncoated color copy cover	premium color copies
F	Coated grade with calcium carbonate pigment and latex binder	premium offset prints
G	Coated ink jet grade with silica pigment	premium ink jet prints

Also, handsheets of 60 g/m² basis weight were prepared in tap water (ph=7) following Tappi test method T220 with a commercial bleached kraft softwood pulp as furnish. The pulp was initially beaten to 450ml Canadian Standard Freeness with a PFI mill and was increasingly sized using between 0 to 1.6 wt-% doses of a 1% solution cationic dispersed rosin size Ultra-pHase® 35 and a 1% solution aluminum sulfate. During the internal sizing process, the pH value and the rosin to aluminum sulfate ratio were kept constant at 4.5 and 1, respectively. Rosin was first added to the beaten pulp suspension followed by aluminum sulfate after 30s, and continuously stirred for 8min before forming the handsheets. The handsheets were air-dried and their basis weight, thickness, and degree of sizing were tested. The sizing level was measured following Tappi test method T530 using a Hercules Sizing Tester (HST).

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 the 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 from Whatman with 0.45μm 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). Three types of patterns shown in Figure 1 were printed.

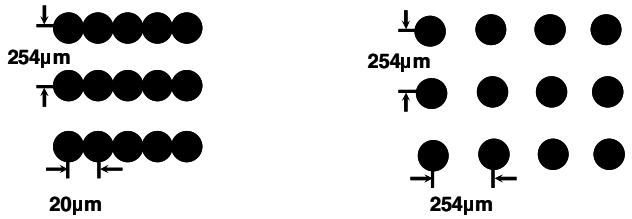

Figure 1 – *Sketch (not to scale) of the ink jet printed patterns. Lines (left) and dots (right)*

Confocal Scanning Laser Microscopy

A fluorescent probe, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine), was employed to characterize the surface and cross-sectional spatial distribution of the active HRP in the fibrous supports. HRP catalyzes the reaction of Amplex Red (non fluorescent) with H_2O_2 to produce resorufin, a red-fluorescent oxidation product with a 571nm absorption peak and a 585nm fluorescence emission maximum. The reaction has been used to detect concentrations of peroxidase in solution as low as 1 x 10^{-5} U/ml [17].

$$\underset{\text{(Non-fluorescent)}}{\text{Amplex Red}} + H_2O_2 \xrightarrow{\text{HRP}} \underset{\text{(Fluorescent)}}{\text{Resorufin}} + 2H_2O$$

The bioink formulation containing Amplex Red was printed on the paper supports under the conditions detailed before. The printed papers were exposed to a 1mM H_2O_2 solution to develop the fluorescent response. Positive and negative controls were also prepared. A sample completely soaked in the Amplex Red-containing bioink was used as the positive control. A sample completely soaked in the Amplex Red-containing bioink free from the HRP enzyme was used as the negative control.

The resulting samples were used directly for CLSM surface imaging or were embedded using SPI-Pon™ 812 Epoxy Embedding Kit, cured at 60°C for 24hs and cross-sectioned with a diamond knife in a Leica Ultramicrotome. The blocks were imaged under a 63X oil immersion objective (HC PL APO CS, NA 1.4) with a two-detection-channel laser confocal microscope Leica TCS SP2 producing two types of images simultaneously. The microscopy conditions are detailed in Table 2.

Table 2. Laser Confocal Microscopy Conditions

Excitation	Beam Splitter	Channel	Emission/ Reflection	Image
Green HeNe Laser 100% 543nm	DD 488/543	1	Red Visible Fluorescence 555nm - 700nm	Active HRP enzyme map
		2	Reflection 540nm-545nm	High scattering power particles (pigments/ fillers of paper)

Results

HRP spatial distribution in commercial papers

The cross-sectional distribution of active HRP was examined for samples C to G. Figure 2 shows the combined paper filler/pigment-enzyme distribution for a negative control, an inkjet printed sample and a positive control of the papers. High refractive index species (pigment or fillers) are shown in green and active HRP enzyme is shown in red.

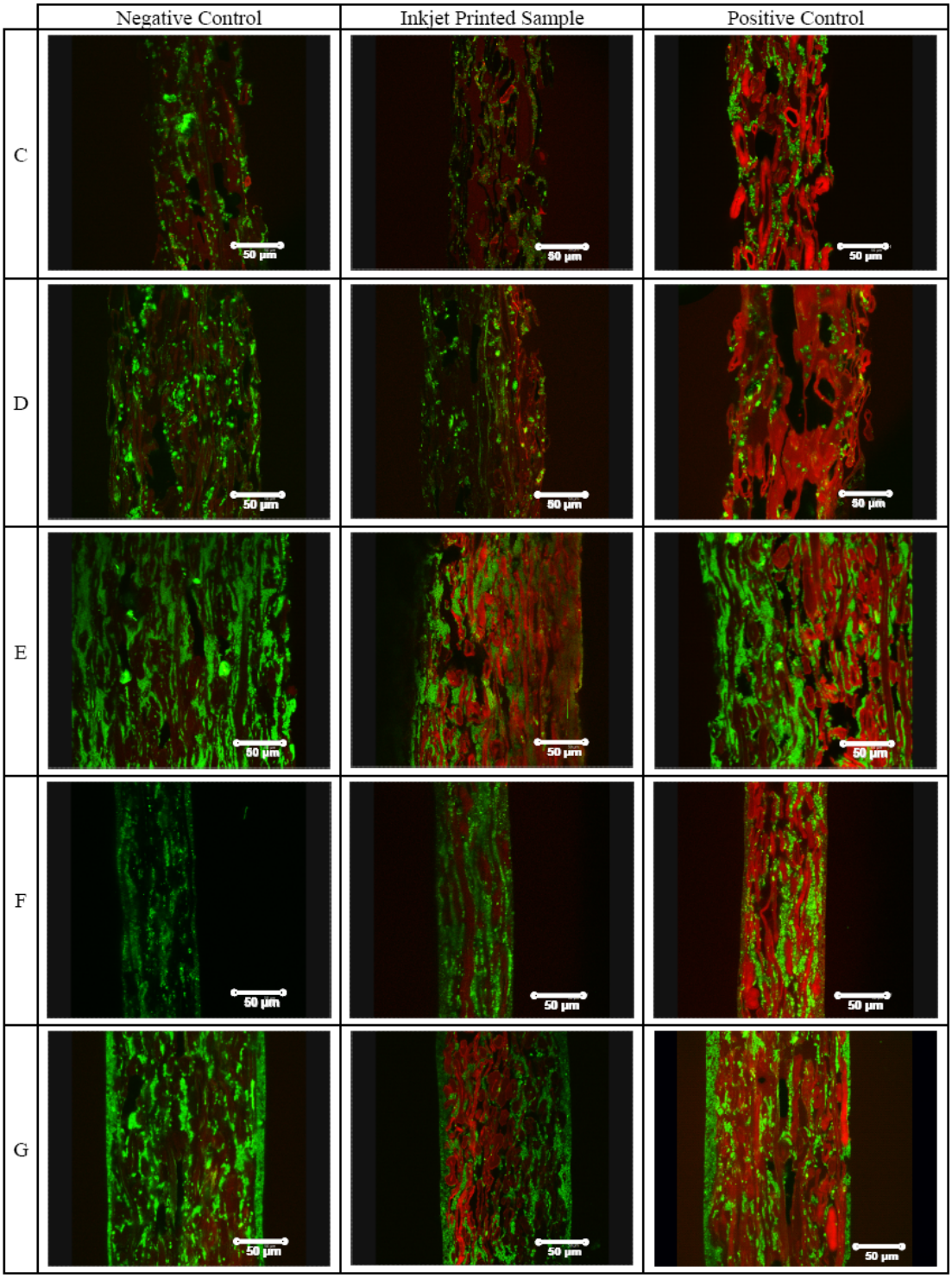

Figure 2. *Combined cross-sectional CLSM images of the active HRP enzyme (red) and pigments/fillers (green) for negative controls, ink jet printed sample and positive control of papers C to G detailed in Table 1. Scale Bar = 50µm.*

Figure 2 shows that some of the printed samples (papers D, F and G) exhibited partial penetration of the bioink in the thickness direction; whereas the other samples (C and E) are fully penetrated by the bioink. It follows that the local concentration of enzyme in the papers with partial thickness penetration of the bioink is higher than in the papers with full penetration. This observation is consistent with the enhanced bioanalytical performance observed in commercial papers with higher degree of sizing [16].

Also, within the CLSM lateral resolution (116nm/pixel), it can be observed from Figure 2 that for all the paper samples the enzyme appeared to be preferentially localized in the fibers and not in the inorganic pigment or filler domains (red and green areas do not overlap). The result is particularly evident for the two-sided ink jet grade coated paper (paper G): the coating layers and the filler do not show detectable enzyme activity. It appears that cellulosic fibers present a more suitable environment to the enzymes than the inorganic pigments and fillers.

The cell wall of swollen delignified cellulose fibers is characterized by rather monodisperse microvoids with an average size around 100nm in diameter [18]. The microvoids in the cellulose fiber wall exist while the fiber is wet and disappear as the structure dries and shrinks. The HRP molecule, on the other hand, is an ellipsoid with 6.5 nm x 5.4 nm x 4.3 nm in main dimensions [19] that can move with water as long as it does not become physically entrapped or chemically bound. Not only the microvoids in the cellulose fiber are the smaller pores available in the paper structure to entrap the enzyme, but also being dynamic pores they can lock the enzyme inside the fiber cell wall as the paper dries. In addition, cellulose has hydroxyl and carboxyl groups that can interact with some of the aminoacids sites in the enzyme surface increasing the enzyme-cellulose affinity; this point is the subject of ongoing investigations. These cellulose fiber attributes may justify the higher concentration of enzyme found in the fibers.

HRP surface and cross-sectional distribution in coated layers

To further validate differences in the enzyme distribution between inorganic pigments/fillers and fibers, a thick layer of coating (10pph SB latex and ground calcium carbonate) was printed and examined under similar experimental conditions as the paper samples.

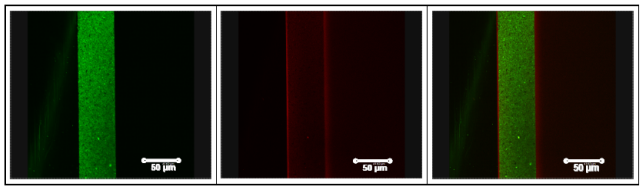

Figure 3. *CLSM cross-sectional images of thick coating layer with HRP enzyme printed on the left side and exposed to H_2O_2 solution on the right side. Left: pigment map. Center: active HRP enzyme map. Right: overlay of pigment and enzyme maps.*

Figure 3 shows the pigment (green) and enzyme (red) distributions and their combined visualization. Interestingly, the enzyme moves in the same direction as the analyte diffuses, away from the bulk of the coating mainly remaining on the surface of the coating layer. It seems that the pores in the coating structure are too big to entrap the enzyme and it can freely flow through the coating layer without significant binding.

HRP spatial distribution in handsheets with increasing internal sizing level

Figure 4 shows the surface view of active HRP enzyme distribution obtained using CLSM for a series of handheets prepared with increasing addition of sizing agent between 0 and 1.6 wt-% with respect to the dry fiber furnish and printed with Amplex Red-containing bioink.

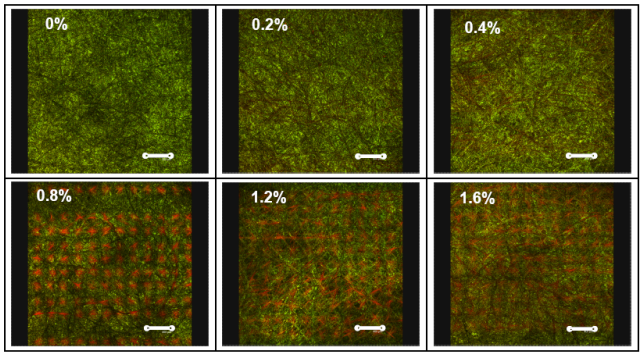

Figure 4. *Combined surface view CLSM images of the active HRP enzyme (red) and fibers (green) for handsheets increasingly sized from 0 to 1.6 wt-%. Scale Bar = 400µm.*

For sizing levels lower than 0.8 wt-% the bioink spreads all over the handsheet and not detectable red fluorescence is observed due to the very low local enzyme concentration. As the sizing level increases from 0.8 wt-% to 1.6 wt-% per each drop of bioink delivered by the printer, a printed red fluorescent circular spot is detected after exposure to the analyte.

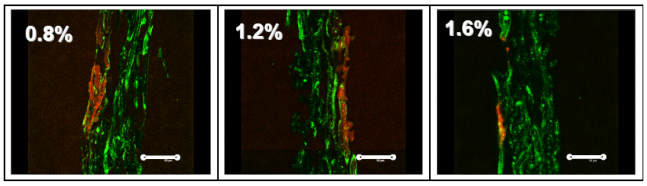

Figure 5. *Combined cross-sectional CLSM images of the active HRP enzyme (red) and fibers (green) for handsheets increasingly sized from 0.8 to 1.6 wt-%. Scale Bar = 50µm.*

Moreover, Figure 5 illustrates the bioink cros-sectional distribution for the handsheets that exhibited well-defined red-fluorescent circular printed spots. As expected, the CLSM images show a decrease in the cross-sectional bioink penetration as the sizing level increases. However, from figure 7 it also appears that oversizing the handsheet (e.g. 1.6 wt-%) does not reduce further the degree of spreading or intensifies the red fluorescent response. It appears that although minimized spreading and penetration should maximize the local enzyme concentration, the highly hydrophobic fibrous support might have partially inactivated the

HRP enzyme. The impact of support hydrophobicity on enzyme biological functionality is worthy of further research.

Conclusions

A new technique based on CLSM allowed the characterization of the active enzyme spatial distributions in naturally fluorescent paper substrates. CLSM images suggest that partial penetration of the bioink and minimum spreading favor the bionalytical response. Also, HRP enzyme preferentially locates in the fiber cell wall and not in the pigments or fillers. It appears that the microvoids in the fibers cell wall may have helped entrap the enzyme and present a more suitable microenvironment for the preservation of their biological functionality.

The impact of surface chemistry was studied by increasing the sizing level of a series of handsheets. It was confirmed that spreading and penetration can be controlled to enhance the colour response by maximizing the local active enzyme concentration. However, a limit exists to the amount of sizing agent that can be added. Oversizing the fibers can lead to partial inactivation of the enzyme and; hence, reduced bionalytical performance. This effect is currently under study.

The uncoated wood free paper (paper D) presented the best overall bioanalytical performance. The graphic arts coated papers present an improved surface for printing capable of retaining most of the ink on the surface. However, the pigment coatings seem not to contribute to the bioanalytical response favorably.

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