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Performance and penetration of laccase and ABTS inks on various printing substrates

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ABSTRACT

Introduction of an enzyme and a colour-forming reagent into paper enables the development of an authenticity indicator. The purpose of this work was to study the performance of *Trametes versicolor* laccase, TvL, and ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt, in various printing substrates when printed with inkjet. The printing substrates included pre-coated mechanical paper additionally coated with PVA, silica and latex. The focus was on the bioanalytical performance and ink penetration. The setting of the printed TvL and ABTS ink was studied visually, with optical and confocal microscopy and with a so-called tape laminating technique. Technical properties of the printing substrates and effect of the surface chemistry were discussed and related to the bioanalytical properties.

TvL activity persisted well during the printing. The best colour response was attained using the PVA-coated base paper. The film-forming ability of the PVA was found to be the main contributor to the colour reaction. The uniform, dense and non-porous PVA layer retains the ABTS and TVL molecules on top of the printing substrate. The high local ink concentration on the PVA coating layer combined with the absorptive paper substrate suggests that the PVA film acts as a filtering layer which retains TvL and ABTS molecules in the coating layer but allows most of the ink solvents to penetrate into the paper structure. TvL and ABTS molecules are also trapped in the PVA polymer network due to swelling effect of water. Electrostatic attractions between the PVA and ABTS and TvL molecules do not contribute to the colour reaction.

1. Introduction

Bioactive paper can be defined as a paper-based substrate including functionalities based on the selective reactions of biomolecules, such as enzymes and antibodies [1]. Bioactive papers have numerous application possibilities related to e.g. medical diagnostics, public health, safety, security and smart packaging [1,2]. Ultimately, bioactive paper can be harnessed to various applications such as package-integrated indicators, test strips for diagnostics, environmental and industrial applications as well as document or product authentication [2]. Paper is a good substrate for bioactive ink thanks to its low cost, biomolecule compatibility and porous structure [3,4]. However, the effect of the paper structure and chemical composition on fluid transport or biomolecule functionality is not fully understood [4].

Bioactive paper products can be fabricated by a high-speed mass manufacturing process: web forming, coating and converting and printing [2]. Printing provides a cost-efficient way of applying biomolecules onto large and flexible substrates such as paper [1,2,5,6]. The most significant aspect in printing is the influence of the printing process on the ink's biological performance. Inkjet technology as a non-contact printing method does not expose the biomolecule to high mechanical impact or contamination risks, which favours its selection as the printing method [2]. In addition, the transferred material amount can be exactly controlled [1].

Inkjet printing of aqueous inks demands distinct properties from the printing substrate: a balance between fast ink drying and ink holdout to achieve good ink density and colour saturation. Sizing materials with their film-forming ability and strong hydrophobicity promote the ink holdout, whereas paper with very open structure and weak sizing absorbs the ink rapidly. Several parameters affect the suitability of the printing substrate for inkjet printing. Hardwood and softwood content, filler amount and type, porosity, internal and surface sizing and sheet formation all influence the performance of uncoated papers. The inkjet printing quality of the

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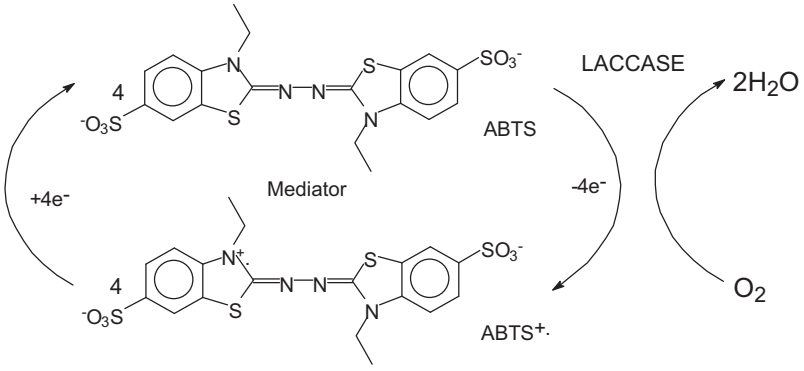

Scheme 1. Laccase-catalysed reaction with ABTS as a substrate.

coated papers depends on the pigment and binder type and their ratio, coating weight and the base sheet property [7].

Inkjet printing of biomolecules has been studied to some extent [2,8–12]. However, only relatively few studies have paid attention to the effect of the printing substrate [2,8,9] and ink penetration [12], although the importance of the printing substrate choice has been recognised [2,12,13].

It is necessary in many applications that the biomolecules are firmly attached to the paper structure. In others, this requirement is less critical. An excellent review describing the immobilisation and binding of biomolecules on paper was presented by Pelton [3]. Attachment of the bioactive element has been achieved by various means, such as the use of porous silica nanoparticles [14], modified silica particles [15], microgel [16], sol–gel [17–20] and microencapsulation [21]. The effect of polymers on the retention of active ingredients in bioactive paper has been described by Khan et al. [22] and Zhao et al. [23]. Khan et al. [22] investigated the capacities of different polymers to improve enzyme retention on paper. Three polymers, an anionic, a cationic and a neutral polymer, deposited as monolayers on the paper, were all found to be efficient at increasing the enzyme concentration on the paper and preventing leaching upon rewetting of the paper. Zhao et al. [23] spotted gold nanoparticles on poly(vinyl alcohol)-coated paper substrate for bio sensing assays, and reported that the role of PVA was probably associated with water retention by the polymer, preventing spreading and too rapid drying and helping biomolecules to maintain their function.

Di Risio emphasized the role of the cellulosic surfaces on which the biomolecules are immobilised for the bioactive paper functionality [2]. Surface chemistry and structure of the cellulosic support affect significantly how the biomolecules are placed and distributed on the surface, the attachment strength between the biomolecule and the solid, and the biomolecule stability. A cellulosic support should preserve the native structure of the biomolecule in order to provide stability to the biomolecule. It should also retain the bioactivity and reduce non-specific binding [24].

The effect of paper substrate on bioactive performance was studied by Di Risio and Yan [12]. Horseradish peroxidise, HRP, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt, ABTS, were inkjet-printed on different paper grades. Two important aspects affecting the bioanalytical response were postulated to be the ink distribution within the paper structure and the surface chemistry. Reduced penetration and spreading of the bio-ink resulted in better colour response for uncoated papers. In coated papers, HRP was located in the fibre cell wall rather than in pigments or fillers. Most probably the micro voids in the wet fibre cell wall assisted entrapping of the enzyme upon drying and offered a more favourable microenvironment for preserving the HRP biological functionality than pigments. This partly explains the poor analytical signals in the coated papers. The colour response was improved by restricting the spreading and penetration of the HRP bio-ink by adjusting the surface chemistry of the paper. The more hydrophobic coating produced a more intense colour response up to a certain degree of sizing after which the intensity decreased possibly due to partial inactivation of the HRP [12].

Roda et al. [10] reported that permeable, conventional cellulose paper supports were favourable for the intensity and spatial distribution of the chemiluminescent response of HRP. Detection problems were encountered with impermeable supports due to enzyme washout. The effect of the porosity in the inkjet printing can be clarified: small pores in the printing substrate contribute to rapid inkjet ink penetration and large pores act as the solvent storage [25]. The physical connection between the small and large pores is vital when the absorption rate and absorption volume is to be maximised [25].

This study aimed to clarify the factors behind the bioanalytical response on different printing substrates, especially from the biochemical viewpoint. The role of the printing substrate in the performance of a bioactive paper product based on inkjet-printed laccase, a multicopper phenol oxidase enzyme, was investigated. The oxidation of ABTS, which is catalysed by laccase to dark green ABTS+ radical, was the colour-forming reaction (Reaction Scheme 1). As silica is typically used as a pigment and PVA as a binder in inkjet printing papers [26], these substances together with latex were used as the paper coatings. PVA provides excellent binding strength and improves the inkjet print quality compared to typical latex binders, whereas silica reduces the ink drying time when added with the PVA compared to pure PVA coating [27].

The spreading and penetration of the inks containing laccase enzyme and its substrate, ABTS, on three different coated printing substrates as well as on pre-coated mechanical paper as reference base paper, were studied visually, microscopically (optical and confocal) as well as by using the so-called tape laminating technique. The main factors contributing to the enzyme-substrate colour-forming reaction were clarified. The optimal coating structure for the functionality of the authenticity indicator application was also investigated.

In this study ABTS and laccase were used as model substances. Ideally, the findings could also be applied to other substrates and enzymes. This concept was not optimised for detecting specific substances but rather a detection method of authenticity. As an example of a potential application ABTS could be applied by a specific pen, brush or printer on an invisible, printed pattern of laccase on an entrance ticket in order to reveal authenticity. In addition to authenticity purposes, the colour reaction could also be used for recreational, promotional and educational purposes in which the ABTS could be applied by a specific pen. Moreover laccase deposited on paper-based substrates by ink jet has other applications, such as active and intelligent materials (e.g. oxygen