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Electrochromic biosensors based on screen-printed Prussian Blue electrodes

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Highlights

- x We present the production of an electrochromic screen-printable paste based on Prussian Blue.

- x These blue electrodes display excellent spectroelectrochemical reversibility and high catalytic activity towards the reduction of H₂O₂.
- x These blue electrodes enable *both* the electrochemical and optical quantitative determination of hydrogen peroxide.
- x The reported blue electrodes are highly suitable transducers for the construction of oxidase-based biosensors.
- x A glucose biosensor is presented to demonstrate the spectroelectrochemical capabilities of these new electrodes.

Abstract

Prussian Blue (PB)-modified graphite screen-printed electrodes are increasingly being used in electrochemical biosensors. However, they do not allow the observation of the electrochromism of PB. This work presents the construction of PB-based, electrochromic screen-printed biosensors. Although electrically more resistive than their graphite counterparts, these new PB-based electrodes enable both the amperometric and colorimetric detection of hydrogen peroxide. This is the first time that this has been achieved using screen-printed electrodes, and we demonstrate it spectroelectrochemically on a glucose biosensor. The biosensor electrochemical performance equals that of previously reported PB/graphite electrodes, being able to detect down to 4 μ M H₂O₂ and 54 μ M glucose. At the same time, and in contrast to PB/graphite electrodes, the new PB-based electrodes afford the optical detection of these two analytes down to 1.2 μ M and 15 μ M, respectively. The dynamic ranges of the glucose biosensors obtained at the PB-based electrodes are 0.1-1 mM (amperometric) and 0.025-2.5 mM (Colorimetric), matching the physiological glucose concentration range in body fluids other than blood or serum.

Keywords: Prussian Blue electrodes; Screen-printed electrodes; Spectroelectrochemistry; Biosensors; Hydrogen peroxide detection; Glucose sensing.

1. Introduction

Prussian Blue consists of iron (II) and (III) atoms bridged by cyanide groups in a cubic crystal structure of formula Fe₄³⁺[FeII(CN)₆]₃. This composition and structure bestow Prussian Blue its characteristic colour and electrochemical properties. Prussian Blue undergoes two reversible redox

reactions, each associated with an ion exchange step and its respective colour change.

Thus, Everitt's salt, the reduced form, is colourless $K_4Fe_4^{2+}[FeII(CN)_6]_3$, while the oxidized form is yellowish and known as Berlin Green, $Fe_4^{3+}[FeIII(CN)_6]_3A$, where A represents an anion inserted in the crystal lattice to balance charge in the oxidation process. In addition to its electrochromic behavior, that makes it suitable for the fabrication of displays and smart glass, [1] Prussian Blue also displays excellent catalytic properties for the reduction of oxygen and hydrogen peroxide. Specifically, this is enabled by the high-spin Fe^{2+} atoms in the reduced form, [2] which makes Prussian Blue films more active towards H_2O_2 reduction than Pt electrodes, and only slightly less active than peroxidase enzymes. [3]

This ability to selectively catalyze the reduction of H_2O_2 has been successfully exploited in first generation oxidase-based electrochemical [4] and optical [5] biosensors. Karyakin et al. were first to demonstrate the construction of such biosensors. [3, 4] Since then, the number of reports featuring Prussian Blue as artificial peroxidase has increased continuously. [6-9] Surprisingly, despite its electrochromism and sensing capabilities, very few works report the exploitation of its electrochromism for (bio)sensing purposes, and those that do usually rely on PB electrodeposited on expensive transparent electrodes [10-14].

Table 1 provides a summary of key works featuring screen-printed electrodes (SPEs) modified with PB which are used for the amperometric detection of hydrogen peroxide for biosensing purposes, including this work.

Table 1. Comparison of different Prussian-blue screen-printed sensors and biosensors used in the amperometric determination of H₂O₂. DS stands for commercial Dropsens SPEs and GNT for electrodes fabricated with a screen-printing paste from Gwent Ltd, UK.

Electrode	Preparation of PB	Analyte	LOD	Range	Sensitivity	Reference
PB on GC	electrodeposition	Glucose	1 μ M	1 μ M-5 mM	0.18 A cm ⁻² M ⁻¹	[3]
PB/PPy on C	electrodeposition	Glucose	0.1mM	0.1-20mM	1.0-1.9 μ A cm ⁻² mM ⁻¹	[15]
PB on ITO	electrodeposition	Lactic acid	1mM	1-20mM	Unreported	[16]
PB (unspecified electrode)	electrodeposition	H ₂ O ₂	0.1 μ M	0.1 μ M-0.1 mM	0.6 A cm ⁻² M ⁻¹	[17]
		Glutamate	0.1 μ M	0.1-100 μ M	0.21 A cm ⁻² M ⁻¹	
Modified carbon paste	growth on graphite	H ₂ O ₂	0.5 μ M	0.5 μ M-5 mM	0.11 A cm ⁻² M ⁻¹	[18]
Screen-printed electrode (DS)	undisclosed	Glucose	100 μ M	0.1-10 mM	5.6x10 ⁻⁴ A cm ⁻² M ⁻¹	
		Uric acid	3 μ M	10-200 μ M	0.89 A cm ⁻² M ⁻¹	[19]
Screen-printed electrode (DS)	undisclosed	Catechol	0.4 μ M	1-90 μ M	Unreported	[20]
Screen-printed electrode (DS)	undisclosed	Paraoxon	12 μ M	5-150 μ M	Unreported	[21]
Screen-printed electrode (DS)	Screen-printed				undisclosed	
Screen-printed electrode (DS)						

0.015 A cm ⁻² M ⁻¹	. 0 5 -	[22]
5.8x10 ⁻⁴ A cm ⁻² M ⁻¹	0 .5 m	[23]
0.1 A cm ⁻² M ⁻¹	M	[24]

undisclosed

G
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0
-
3
3
.1
m
M

undisclosed

H₂O
3.6
0-

μM

electrode

2
μM
100

(GNT)

Electrode	Preparation of PB	Analyte	LOD	Range	Sensitivity	Reference
		Glucose	0.7 mM	0-100 mM	$4.8 \times 10^{-3} \text{ A cm}^{-2} \text{ M}^{-1}$	
		Lactate	1.19 mM	0-50 mM	$3.0 \times 10^{-3} \text{ A cm}^{-2} \text{ M}^{-1}$	
		Uric acid	4.6 mM	0-35 mM	$4.5 \times 10^{-4} \text{ A cm}^{-2} \text{ M}^{-1}$	
Screen-printed electrode (GNT)	undisclosed	Lactate	0.15 μM	0.15 μM -1.1 mM	$0.4 \text{ A cm}^{-2} \text{ M}^{-1}$	[25]
Carbon SPE	PB drop casted on C-SPE	Cysteine	98.9 μM	300-700 μM	$0.06 \text{ A cm}^{-2} \text{ M}^{-1}$	[26]
PB/Carbon SPE	PB incorporated to C-paste	Cysteine	67.4 μM	100-600 μM	$0.09 \text{ A cm}^{-2} \text{ M}^{-1}$	[26]
SiO ₂ -ATO/PB	Growth of particles	H ₂ O ₂	3.8 μM	0.025-5 mM	$0.07 \text{ A cm}^{-2} \text{ M}^{-1}$	This work
ITO/PB ⁽²⁾	Growth of particles	H ₂ O ₂	3.2 μM	0.01-5 mM	$0.08 \text{ A cm}^{-2} \text{ M}^{-1}$	This work
SiO ₂ -ATO/PB	Growth of particles	Glucose	54.1 μM	0.1 – 1 mM	$8.8 \times 10^{-3} \text{ A cm}^{-2} \text{ M}^{-1}$	This work
ITO/PB	Growth of particles	Glucose	70.5 μM	0.1 – 1 mM	$7.4 \times 10^{-3} \text{ A cm}^{-2} \text{ M}^{-1}$	This work

(1) SiO₂-ATO/PB: Prussian Blue was chemically grown on SiO₂ core and ATO-shell 3 μm particles.

(2) ITO/PB: Prussian Blue was chemically grown directly on 100nm ITO nanoparticles.

Regarding the preparation of PB-modified electrodes, there are three main ways:

electrodeposition, chemical growth on an electrode surface, and deposition of PB-modified graphite pastes. Of these, since it was first reported by Neff, [27] electrodeposition has been the preferred method when it comes to developing electrochromic devices and electrochemical sensors. The main advantages of electrodeposition methods are not only the selective growth on electrode surfaces alone, but also the control of growth conditions. This ultimately means

controlling device-critical features such as film thickness, roughness, porosity, and stability. [28]
Deposition methods based on potentiostatic, [17] galvanostatic, [27] cyclic, [3] and pulse
voltammetry [29] control have been reported. PB is typically electrodeposited from solutions

containing Fe(III) and ferricyanide ions. However, electrodeposition from Fe(II) and ferrocyanide is also possible and may be desirable when a less acidic pH is required to preserve the activity of enzymes or other biomolecules.[15]

Chemical growth, on the other hand, may also be used to produce Prussian Blue films, both on electrodes [26, 30, 31] and on inert surfaces [32] in many different ways. [33] However, this is not an ideal approach because it lacks the surface selectivity and much of the control that electrochemical methods enjoy.

Nevertheless, chemical synthesis of PB is used in the formulation of screen-printing materials, which is the third relevant approach that we have identified for the production of PB-modified electrodes.

Up until the mid 1990s, most electrochemical studies on Prussian Blue used electrodeposited films. However, the use of chemically synthesized Prussian Blue has been made popular through the introduction of carbon paste [34] and screen-printed electrodes [35] (SPEs). PB-modified pastes represent a straightforward way to overcome critical production issues affecting electrodeposition, such as the nature of the electrodeposition process and its relatively high cost. Screen-printing also extends the nature of possible substrates beyond conducting electrode materials, to include paper, textiles, ceramics, polymers and, given the right combination of solvents and curing conditions, nearly any material imaginable.

PB-mediated pastes have typically three key components: a conducting material, the PB itself, and a suitable binder system. The conducting material used in existing commercial products is graphite, and different resins are used as binder agents.

In this work, we have grown PB chemically over the surface of clear conducting particles, and then mixed the modified particles with a Viton® binder system to produce an electrochromic

paste. This material had been previously reported for the construction of electrochromic displays [36] but, to the best of our knowledge, this is its first demonstration in (bio)sensing. Thus, in contrast to previously reported PB/graphite-based screen-printed electrodes, which are limited to electrochemical detection only, the electrodes presented here enable both electrochemical and optical detection of H_2O_2 . The electrochemical performance of the materials presented here is comparable to graphite-based materials, but their electrochromism makes them very attractive also for the construction of optical (bio)sensors. This concept is demonstrated in the context of a glucose oxidase-based biosensor.

2. Experimental

2.1 Reagents and materials

Potassium ferrocyanide, ferrous sulfate, potassium chloride, acetic acid, glucose, bovine serum albumin (BSA), and 2-butoxyethyl acetate were purchased from Sigma-Aldrich. Glucose oxidase ($236 \text{ U} \cdot \text{mg}^{-1}$) was acquired from Sekisui Diagnostics (UK). Chitosan (M.W. 100,000 – 300,000) was purchased from Acros Organics. SiO_2 -ATO conducting particles (nominal size of $3 \mu\text{m}$) Zelec 1610-S were kindly provided by Milliken Chemical (BE). ITO conducting particles (nominal size of 5 nm), Nan Tek®, were purchased from Alfa Aesar. Viton® fluoroelastomer (GBL-600S, DuPont) was acquired from Eagle Elastomer (USA). Carbon conducting paste ref. C2030519P4 and PB-modified carbon paste ref. C2070424P2 were purchased from Gwent Electronic Materials, Ltd (UK), Electrodag PF-455B photocurable dielectric and 725A silver pastes were obtained from Henkel (ES). 0.5mm-thick PET sheets (Autostat WP20) were purchased from MacDermid (UK). PB-modified screen-printed carbon electrodes (PB-SPCEs) ref. 710 were purchased from Dropsens (ES).

All chemicals were used as received without further purification.
paste. This material had been previously reported for the construction of electrochromic displays

2.2 Instrumentation

Electrochemical and spectroelectrochemical measurements were done using a SPELEC instrument (Dropsens) controlled by DropView SPELEC software (version 3.0), installed on a PC running Windows 10. Scanning electron microscopy images were obtained at an Auriga microscope (Carl Zeiss). A Kulikke&Soffa four-point probe connected to a 3455A Digital Voltmeter (Hewlett Packard) was used to measure the sheet resistance of the fabricated materials.

2.3 Electrochromic Prussian Blue screen-printed electrodes

Prussian Blue screen-printing pastes were prepared following a previously published protocol. [36] Briefly, 20 g of conducting ITO or SiO₂-ATO particles were suspended, under vigorous stirring, in 200 mL of a 25 mM iron (II) sulfate aqueous solution. Then, 30 mL of a 60 mM ferricyanide solution were added dropwise to the stirred solution. The resulting blue particles were separated by decantation, washed with dilute HCl, and dried at 100°C.

Prussian Blue-modified conducting particles were next combined with a 15-20% Viton solution prepared in 2-butoxyethylacetate to a 5:2 pigment to binder proportion, and thoroughly mixed in a ball mill, until the resulting paste presented a honey-like texture. The particle size was measured using a 50 µm grind gauge, obtaining a maximum particle size range between 5 and 12 µm. This is extremely good considering that the starting particle size was nominally 3µm, and that no additives were used to promote particle suspension in the paste.

Due to the high electrical resistance of the printed Prussian Blue layers, it was needed to print **2.2 Instrumentation** over a conducting substrate. The simplest and most cost-effective case involved its application over a graphite screen-printed electrode. However, to enhance the colour contrast of the electrochromic change, a SiO₂-ATO layer [37] was additionally printed between the graphite and

the Prussian Blue layers. All the electrodes presented in this work have been prepared as previously reported, using a manual, home-made screen printing setup. [38]

2.4 Glucose biosensor fabrication

Prussian Blue screen-printed electrodes (PB-SPEs) were modified following a modified version of a previously reported protocol. [39] Briefly, 4 μL of a 0.5% wt. chitosan solution prepared in 0.1 M acetic acid was cast on the surface of the electrodes and allowed to dry at room temperature. The electrodes were subsequently rinsed with deionized water, and 6 μL of a 5 mg mL^{-1} GOx containing 15 mg mL^{-1} BSA were then cast and allowed to dry. The electrodes were stored at 4°C.

2.5 Electrochemical measurements

All the experiments were carried out in a 50 mM phosphate buffered solution and 0.1 M KCl, adjusted to a pH of 5.5. The selected pH ensures the stability of the Prussian Blue while keeping the activity of the oxidase enzymes on the electrode near their optimum activity. For the electrochemical characterization, the electrodes were preconditioned by cyclic voltammetry, applying 20 cycles at a scan rate of 20 mVs^{-1} , to equilibrate the crystalline structure of the Prussian Blue with the K^+ cations in the supporting electrolyte. [17] Note, however, that no preconditioning step was required for the sensing of hydrogen peroxide or glucose.

Limits of detection, LoD have been determined based on conventional $3\sigma/m$ methodology, where σ corresponds to the background signal standard deviation, and m to the slope of the linear range [40].

with the optical shutter closed. Background spectra were recorded over completely reduced

2. Spectroelectrochemical measurements

the Prussian Blue layers. All the electrodes presented in this work have been prepared as Reflectance measurements were done using DropSens-SPELEC reflectance cell, using solutions of same composition as those described in section 2.4 above. The dark spectrum was recorded

with the optical shutter closed. Background spectra were recorded over completely reduced

electrodes, which displayed the clearest colour. Unless otherwise stated, spectra were collected using an integration time of 300 ms. Data analysis was carried out using SPELEC DropView software.

3. Results and discussion

3.1 Characterization of Prussian Blue electrodes.

The electrochemical and spectroelectrochemical performance of the PB-SPEs was evaluated and compared to that of (i) electrodes fabricated with a commercial PB-Graphite paste, and (ii) to commercial PB-SPCEs. For the sake of clarity and simplicity, from here on we will refer to these as “commercial materials” and “graphite electrodes” indistinctively, and to ours as “electrochromic pastes” or “PB-electrodes”.

Figure 1 shows cyclic voltammograms recorded in supporting electrolyte. The most striking difference in the voltammetry of the different PB-modified pastes is the much higher currents observed for the blue pastes presented here, compared to those obtained from the commercial materials. One feature of ITO nanoparticles compared to SiO₂-ATO particles is that pastes with significantly higher particle to binder ratios can be formulated, so even higher PB loadings could, in principle be achieved, but the significantly higher cost of ITO nanoparticles compared to SiO₂-ATO particles should also be considered.

The cyclic voltammograms in Figure 1 suggest that commercial graphite pastes contain a much lower amount of Prussian Blue than the electrochromic pastes presented here. Also, the voltammograms in Figure 1 show that our PB-based electrodes display relatively larger background currents. Note that similarly high background currents have been observed for electrodes made with unmodified ATO particles, [37] but also on sol-gel-derived Prussian Blue-

silicate electrodes [10]. We believe there are two likely reasons for such currents; first, the electrodes, which displayed the clearest colour. Unless otherwise stated, spectra were collected

porous structure and resulting large surface area of the electrodes (see Figure S1), but also, in the present case, the mild electroactivity of indium and antimony tin-oxides, [41, 42].

FIGURE 1 AROUND HERE

The PB-electrodes presented here exhibit a superior electrochemical behavior based on their peak-to-peak separation compared to commercial materials based on graphite (Table S1). This is probably due to a better contact of the PB and the conducting material. ITO-modified nanoparticles display the smallest peak-to-peak separation, ca. 30 mV at 5 mVs^{-1} , presumably due to the better conductivity afforded by ITO nanoparticles compared to SiO_2 -ATO micro-particles. However, the much higher PB loading, arising from the massive available surface area of the nanoparticles combined with the thickness of the screen-printed layer, results in much broader peaks compared to the other materials. This peak broadness, which may be detrimental for electroanalytical applications, is due to a limitation in the rate of potassium exchange during the redox process. In fact, this limitation has been observed for all 4 electrode types under study in the form of linear plots of peak current vs. square root of scan rate (Figure S2). Such a linear dependency points at a diffusion controlled process, where the only diffusing species involved here are potassium cations. In contrast, thin film electrogenerated PB electrodes [43, 44] exhibit a surface-controlled regime as predicted by the Laviron expression. [45] The reason for this different behavior is in the thickness of the PB layer. While electrodeposited layers are typically in the range of nanometers (hundreds), screen printed layers are at least a few microns thick and can exchange larger amounts of cations. In all cases, charge densities have been calculated using the electrode geometric area, which allows for a very limited comparison as the thickness of the

PB coating on the screen-printed electrodes is unknown. Therefore, although the actual surface area of our screen-printed electrodes has not been determined, the charge density analysis of the PB peak at the different electrodes shows that our PB-based electrodes contain between 5 and 7 times more Prussian Blue than an electrodeposited layer (See Table S1).

Another important difference between graphite-PB and the SiO₂-ATO/ITO-PB-electrodes is their electrical conductivity. We found, using four-point probe measurements of test structures, that the resistivity of Prussian Blue modified particles increases by more than 5 times compared to a paste of unmodified particles at the same concentration (see Table S2). This suggests that commercial materials may have been produced simply by adding PB particles to an existing graphite paste formulation, which could also explain their smaller PB signals, and their somewhat broader peak-to-peak separation in the voltammetry.

Next, the spectroelectrochemical response of the PB-PEs was compared. The black colour of graphite in the commercial PB-modified commercial electrodes precluded the observation of any spectroelectrochemical changes, in contrast with the ITO and ATO based pastes presented here. Figure 2A and Figure 2B show cyclic voltammograms of the two types of PB-SPEs together with the derivative of their respective voltabsorptograms. A clear correlation between the electrochemical and the spectroscopic behavior of these electrodes is apparent, although a shift ca. 50 mV is observed between the two signals in ITO/PB electrodes. This shift in peak position between electrochemical and optical signals has been reported in the past and, again, is attributed to have been adversely affected.

to the mass transport limitations of K^+ exchange from the Prussian Blue lattice, and which is PB coating on the screen-printed electrodes is unknown. Therefore, although the actual surface involved in the colouration process. [34] In spite of this slight delay between the current and the colour change, neither the electrocatalytic performance of the material nor its electrochromism

have been adversely affected.

FIGURE 2 AROUND HERE

The performance and stability of the electrochromic material under a continuous switching regime was also studied. The potential of the working electrode was stepped between -0.1 and +0.4 V vs. Ag/Ag⁺ in 60-second intervals in a 0.25 mM H₂O₂ solution prepared in the supporting electrolyte. The experiment was carried out in the presence of hydrogen peroxide to simulate the conditions of an oxidase-based biosensor where hydrogen peroxide is produced. Figure 2C shows the change in absorbance as reducing and oxidizing potentials are applied. The data show that ITO/PB electrodes display a higher contrast between the oxidized and reduced states than SiO₂-ATO/PB electrodes do. This is mainly due to two reasons. First, the higher PB loading of the ITO nanoparticle-based electrodes, confirmed by the charge densities in Table 1 above, makes these electrodes show a much deeper blue colour than SiO₂-ATO based electrodes, and so when Prussian Blue is reduced into the colourless Everitt's salt or Prussian White (PW), the contrast to the pale yellow of the underlying nanoparticles is enhanced. The second reason seems to be a wetting issue of SiO₂-ATO-based electrodes. Although both types of electrodes are highly hydrophobic due to the Viton binder (see Table S3), SiO₂-ATO-PB electrodes wet more slowly than ITO based electrodes. This is likely due to differences in surface topology and roughness. Eventually, however, the electrolyte wets the entire surface, allowing the interaction between more PB particles and the solution. Although this enables the maximum contrast of the material on electrochromic switching, this is still lower than at PB-ITO nanoparticle-based electrodes.

Figure 2D shows this gradual contrast increase at SiO₂-ATO-PB electrodes, from an initial 0.5 a.u. up to roughly 0.7 a.u.

On the other hand, this effect is not observed when a polymeric layer, such as chitosan in this work, covers the electrochromic material. The chitosan coating seems to wet the electrode surface completely, reducing the hydrophobicity of the material and facilitating the electrode interaction with the test solution (see Table S3).

3.2 PB electrodes for H₂O₂ sensing

The various Prussian Blue electrodes were used in the electrochemical determination of hydrogen peroxide, and their behavior was compared. Electrodes were polarized at a reduction potential of -0.1 V vs Ag, where Prussian White is formed and the catalytic reduction of peroxide takes place.

Figure 3 shows current density as a function of peroxide concentration for the 4 electrodes tested. In all cases, a linear response is observed between 25 μ M and 5 mM H₂O₂. The amperometric sensitivities of the four electrode types are very similar, roughly 0.08 A·cm⁻²·M⁻¹, and detection limits are in the range of 1 μ M to 4 μ M (see Table S4). This is in line with other reports on PB-modified graphite electrodes (see Table 1), and indicative of the suitability of these electrodes as an artificial peroxidase for sensing applications, particularly in non-invasive detection systems.

FIGURE 3 AROUND HERE

In addition to enabling the amperometric detection of H₂O₂, these PB-based electrodes also allow optical quantification through reflectance spectroscopy. It is surprising that, whilst the

electrochemical detection of H_2O_2 at Prussian Blue electrodes is amply demonstrated and

accepted, there are much fewer examples of optical quantification of H_2O_2 at Prussian Blue electrodes, including electrodeposited Prussian Blue layers. [5, 14, 46, 47]

The electrochromic properties of the fabricated PB-SPEs were also exploited in the optical quantification of hydrogen peroxide. The measurement was carried out as follows. First, Prussian Blue was reduced to Prussian White at potential of -0.1 V vs. Ag. Once a stable background colorimetric signal was observed, after approximately 60 seconds of electrode polarization, the potentiostat was switched off, leaving the electrochemical cell at open circuit. Then, the peroxide in the solution chemically oxidized the Prussian White back to Prussian Blue, and the corresponding colour change was monitored by UV-Vis reflectance (Fig. 4A). Maximum colour contrast depends on the colour of the underlying conducting particles, which were white or pale yellow respectively. These were found to be 675 nm for SiO_2 -ATO/PB electrodes and 700 nm for ITO/PB. Figure 4B shows plots of absorbance versus time at these wavelengths, as a means to monitor the hydrogen peroxide reduction at the PW/PB surface. As the data show, the higher the H_2O_2 concentration, the faster the electrode recovers its blue colour fully. Figure 4C shows the relation between peroxide concentration and reflectance measured at 700 nm, 100 seconds after electrode depolarization. In a manner akin to coulometric sensors, which sensitivity and detection limit can be adjusted by choosing a suitable (current) integration time, here the sensor performance can be adjusted to the sample concentration simply by adjusting the integration time of the spectrophotometer and/or the sampling time after electrode depolarization (in our case 300 ms and 100 s, respectively). This provides control over the sensitivity, linear range or detection limit of the method (Table S5).

FIGURE 4 AROUND HERE

Longer experimental times result in markedly increased sensitivities, and consequently lower detection limits. Moreover, the sensor dynamic ranges also vary, so the technique can be easily adjusted to match a sample expected concentration, and thus improve the reliability of the measurement.

Note that, although dissolved oxygen is also able to oxidize Prussian White back to Prussian Blue, the rate of this reaction is considerably slower than the peroxide driven oxidation, and so its effect may be ignored and the process considered selective towards peroxide, barring cases of extremely low peroxide concentration.

When comparing the two PB-based electrochromic electrodes, ITO electrodes display higher absorbances and sensitivity values than ATO-based electrode. This may be due to the higher amount of electrochromic material present in the paste which enhances the colour contrast between the reduced and oxidized states of the material (see Table S1).

Tables S4 and S5 show that the optical method is slightly more sensitive. Controlling integration and sampling times in the spectroscopic detection lets the user modify the sensitivity of the measurement and adjust it to the specific requirements of the application. This makes this approach a powerful and versatile basis for the design of enzymatic biosensors.

3.3 Electrochromic glucose biosensor

Once the suitability of the PB-SPEs as a sensing platform for hydrogen peroxide was established, a glucose biosensor based on glucose oxidase was prepared. The enzymatic oxidation of glucose yields hydrogen peroxide which, at the PB-modified electrodes, enables the quantification of glucose both electrochemically and spectroscopically.

Figure 5a shows the amperometric Michaelis-Menten plots for glucose using the two

fabricated electrochromic PB-SPEs. The dynamic range of both biosensors is slightly shorter

than other reported biosensors (see Table 1), ranging from 0.1 to 1 mM glucose and reaching saturation above 2.5 mM. We believe that at this point the amount of hydrogen peroxide produced by the oxidase inhibits the enzymatic activity of GOx. This could be solved by improving the fabrication of the glucose biosensor, using different polymeric membranes and/or stabilizing agents to control the diffusion of the analyte through the biosensor, and to increase the operational stability of the enzyme. [48] On the other hand, the analytical performance of our *blue* biosensors in terms of detection limit and sensitivity, *ca.* 60 μM and $8 \cdot 10^{-3} \text{ A} \cdot \text{cm}^{-2} \cdot \text{M}^{-1}$ (see Table S6), is comparable to that of other reported amperometric biosensors (see Table 1).

FIGURE 5 AROUND HERE

The maximum current densities registered for the glucose biosensors can be extrapolated to the hydrogen peroxide calibration plot in Figure 3 to have an estimation of the amount of hydrogen peroxide produced in the enzymatic reaction. A value of 0.25 mM H_2O_2 is obtained, which in the spectroscopic experiment would generate an absorbance of roughly 0.1 a.u. To increase this absorbance value, and also the sensitivity of the glucose sensor, the sampling time in the spectroscopic determination was modified from 100 s to 300 s.

Figure 5b shows the spectroscopic calibration plots of the glucose biosensor for the two different PB-SPEs. As seen, no substantial differences are observed when either the amperometric or the spectroscopic detection method is used, as a similar dynamic range is achieved in all cases, reaching in both cases a plateau at concentrations higher than 2.5 mM. A slight gain in the detection limit is achieved as it decreases up to roughly 15 μM likely due to the

effect of the high background currents present in the amperometric detection that disappear when

a spectroscopic detection method is used but, all in all, the performance of both types of electrochromic electrodes is comparable, regardless of the detection method employed.

4. Conclusions and outlook

We have presented a new kind of electrochromic, Prussian Blue based screen-printed sensors suitable for spectroelectrochemical detection. To the best of our knowledge, this is the first report of a screen-printed electrochromic paste applied to sensing and biosensing.

The Prussian Blue electrochromic pigment used is prepared by chemically growing a thin PB layer on the surface of two different kinds of tin oxide-based conducting particles, namely 50-100nm indium tin oxide (ITO) nanoparticles, and 3 μ m SiO₂ particles covered by a thin (ca. 100nm) antimony tin oxide (ATO) shell. These PB-modified pigments, combined with a Viton® binder system, yield high-quality screen-printing pastes. However, the high electrical resistance of the resulting layers required printing the material on a conventional electrode surface. Also, to increase the colour contrast further, an undercoat of a similar SiO₂-ATO paste was printed underneath the blue coating and over a graphite electrode.

The electrocatalytic response of these blue pastes toward the reduction of hydrogen peroxide is in line with that of the commercial PB-modified screen-printed electrodes and materials. However, in contrast to these, the materials presented here also enable the optical detection and quantification of hydrogen peroxide in the μ M-mM range, which represents a clear advantage and opens new avenues for the design of novel multi-functional spectroelectrochemical devices.

These electrochromic sensors pose important advantages compared to both electrodeposited PB and screen-printed PB-modified graphite electrodes. They overcome electrodeposition limitations, such as (i) the “wet” nature of the process, (ii) the need to control critical electrodeposition parameters such as current and potential distribution over large area substrates,

and (iii) it replaces an intrinsically “batch” process by a “continuous” large area process, screen-printing. On the other hand, compared to currently available PB-modified graphite pastes, the materials presented here enable the use of both electrochemical and optical detection, opening new design avenues. One possible direction would be the development of multiparametric devices based on arrays of electrochromic electrodes. A smart example of this has been recently presented by Zhang and Liu where lactic acid can be quantitatively determined by the naked eye.[16]

Although in this work we have printed these electrochromic electrodes over graphite transducers, printing them over transparent electrodes would provide additional advantages, such as the possibility to observe the colour change from the back a transparent substrate. Although analysis of coloured samples, such as blood, may be more challenging, one way to keep the colour contrast would be to protect the electrochromic electrode with a white semipermeable coating or membrane. In any case, the possibility to screen-print electrochromic electrodes widely opens the range of possible applications for electrochromism in analytical chemistry , including non-invasive sensors and smart sensing tags in food packaging, and even gas sensors.

Last, this work has focused on the use of PB as model electrochrome. However, other electrochromic materials, including conducting polymers,[49, 50] which have also been used in the construction of electrochromic sensors, could also be used in the mass production of new sensors using the approach presented here to formulate related screen-printing materials.

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ACCEPTED MANUSCRIPT

Captions to Figures

Figure 1. Cyclic voltammograms obtained in supporting electrolyte for the different types of PB-SPEs. Scan rate of 5 mVs^{-1} . $\text{SiO}_2\text{-ATO/PB}$ (Black), ITO/PB (Blue), DropSens C/PB (Orange), Gwent C/PB (Green).

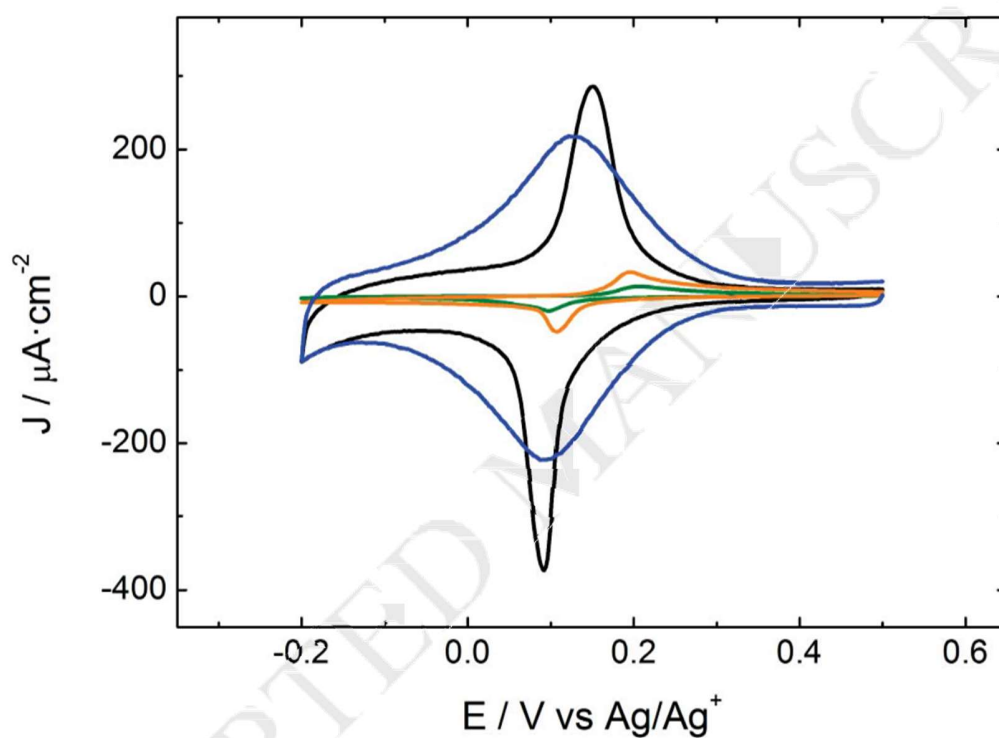


Figure 2. Cyclic voltammogram in supporting electrolyte and derivative of the voltabsortogram for a $\text{SiO}_2\text{-ATO/PB}$ (a) and ITO/PB (b) electrode. Scan rate 5 mVs^{-1} . (c) Plot of the evolution of absorbance when potential was stepped from -0.1 to $+0.4 \text{ V vs Ag/Ag}^+$ at intervals of 60s in a $0.25 \text{ mM H}_2\text{O}_2$ solution in supporting electrolyte. (d) Representation of the contrast variation of the experiment in (c) for a longer time scale.

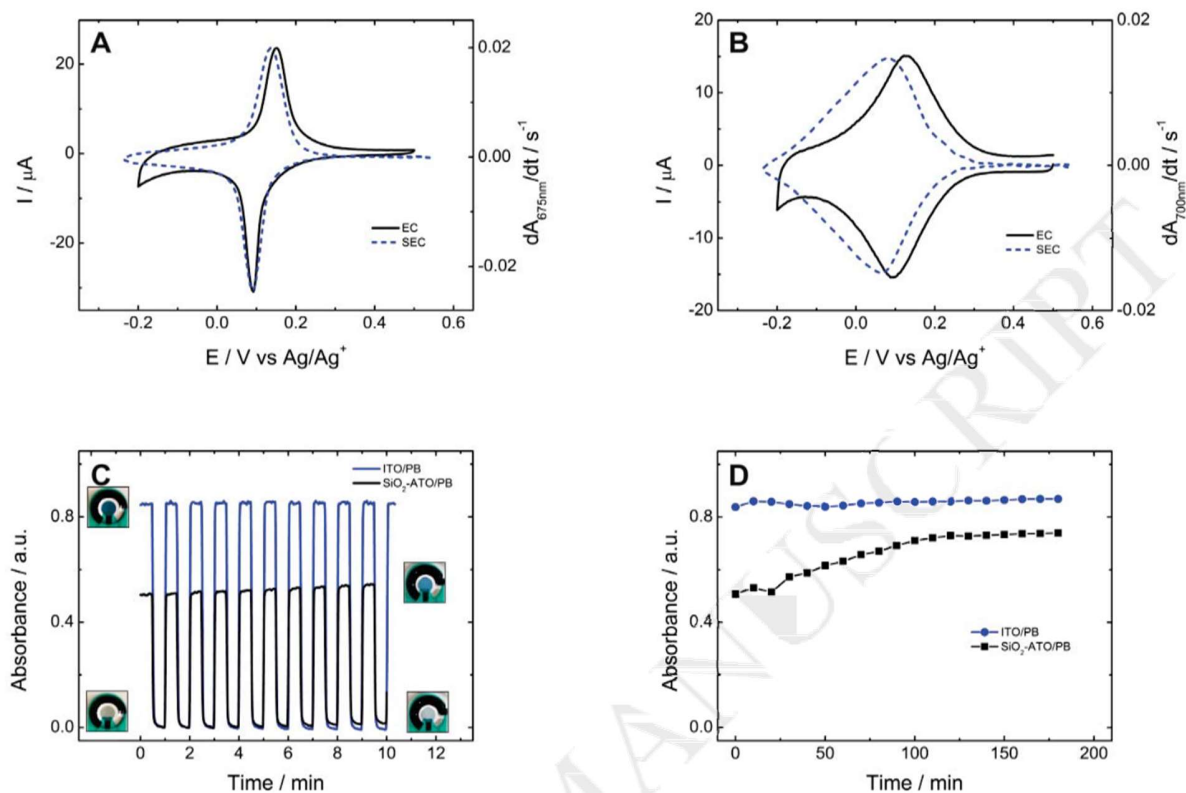


Figure 3. Plot of the current density against the hydrogen peroxide concentration. Applied potential of -0.1 V vs. Ag/Ag^+ pseudo reference electrode. DropSens C/PB (Orange triangles), ITO/PB (Blue diamonds), Gwent C/PB (Green squares), and SiO₂-ATO/PB (Black circles).

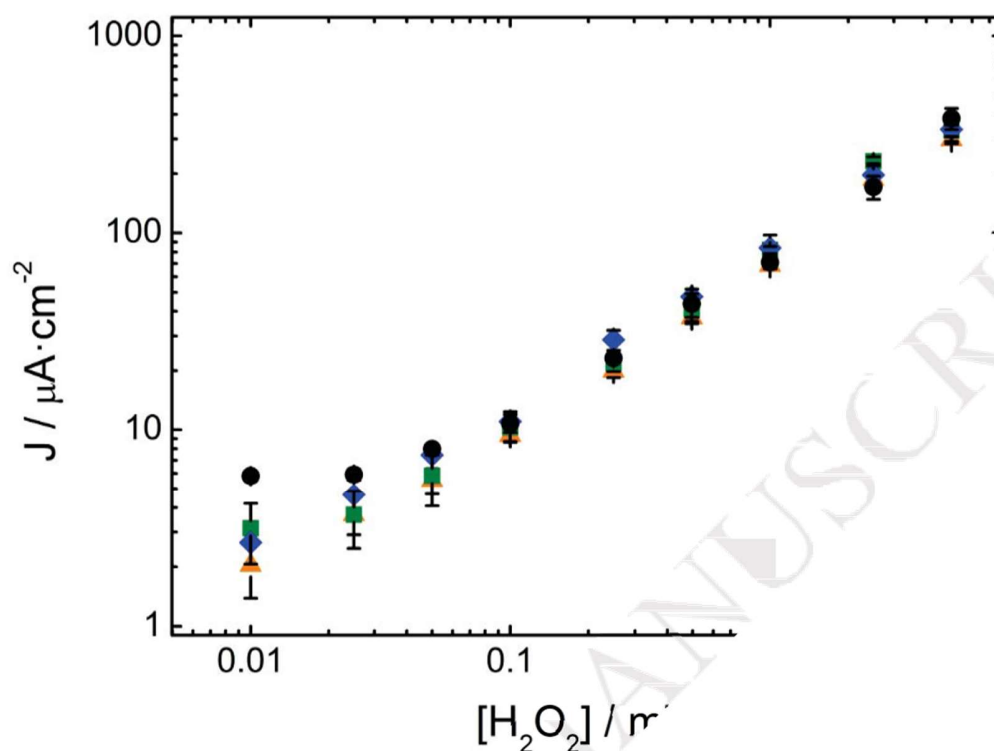


Figure 4. (a) Progress with time of the absorbance spectra for an ITO/PB electrode in a 0.25 mM H₂O₂ solution in supporting electrolyte. (b) Evolution of the absorbance measured at 700 nm with time for different hydrogen peroxide concentrations in an ITO/PB electrode (see Figure S3 for the plots in (a) and (b) obtained with a SiO₂-ATO/PB electrode). (c) Plots of the absorbance measured at 700 nm for a fixed time of 100 s in ITO/PB (Blue triangles) and SiO₂-ATO/PB (Black circles) electrodes. Error bars represent the standard deviation for three different electrodes.

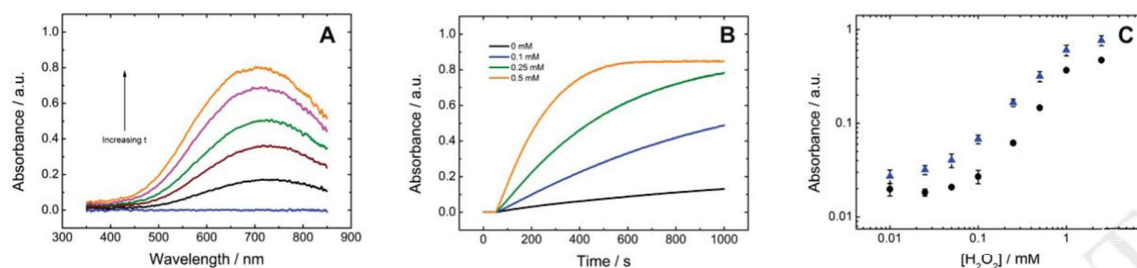
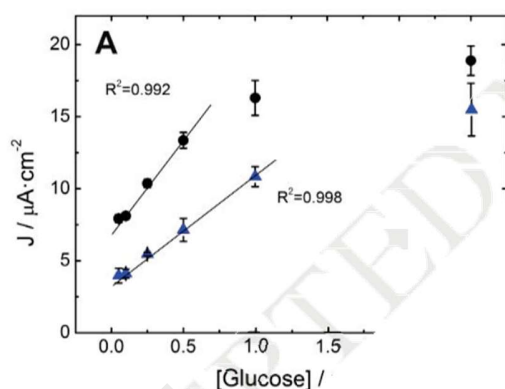


Figure 5. (a) Amperometric and (b) spectroscopic calibration curves for the glucose biosensor obtained with ITO/PB (Blue triangles) and SiO_2 -ATO/PB (Black circles) electrodes. Error bars represent the standard deviation of three different electrodes.



Captions to Tables

Table 1 Comparison of different Prussian-blue screen-printed sensors and biosensors used in the amperometric determination of H_2O_2 .