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Glucose biosensor based on disposable electrochemical paper-based transducers fully fabricated by screen-printing

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ABSTRACT

This paper describes a new approach for the massive production of electrochemical paper-based analytical devices (ePADs). These devices are fully fabricated by screen-printing technology and consist of a lineal microfluidic channel delimited by hydrophobic walls (patterned with diluted ultraviolet screen-printing ink in chromatographic paper grade 4) and a three-electrode system (printed with carbon and/or Ag/AgCl conductive inks). The printing process was characterised and optimized for pattern each layer with only one squeeze sweep. These ePADs were used as transducers to develop a glucose biosensor. Ionic strength/pH buffering salts, electrochemical mediator (ferricyanide) and enzyme (glucose dehydrogenase FAD-dependent) were separately stored along the microfluidic channel in order to be successively dissolved and mixed after the sample dropping at the entrance. The analyses required only 10 µl and the biosensors showed good reproducibility (RSD = 6.2%, n = 10) and sensitivity (0.426 C/M cm^2), wide linear range (0.5–50 mM; r^2 = 0.999) and low limit of detection (0.33 mM). Furthermore, the new biosensor was applied for glucose determination in five commercial soft-drinks without any sample treatment before the analysis. These samples were also analysed with a commercial enzymatic-kit assay. The results indicated that both methods provide accurate results.

1. Introduction

Paper has been used as substrate in chemical analysis for a long time. However, the concept of microfluidic paper-based analytical devices (PADs) was recently introduced in 2007 (Martinez et al., 2007). In this work, the authors described a method to create well-defined, millimeter-sized channels in paper, comprising hydrophilic areas delimited by hydrophobic polymer. Two years later, Dungchai et al., introduced the electrochemical detection in PADs, beginning a new way to get analytical quantification in these devices (Dungchai et al., 2009). Since then, the research for electrochemical PADs (ePADs) has been extensive (Adkins et al., 2015; Desmet et al., 2016; Mettakoonpitak et al., 2016; Nery and Kubota, 2013).

There are several patterning methods for the effective, simple and low cost fabrication of PADs (Yong et al., 2015). However, even though screen-printing has been successfully used for massive production of disposable (bio)sensors (Fanjul-Bolado et al., 2007), there are few reports about the fabrication of (e)PADs by this technique. W. Dungchai et al. used a screen-printing machine for rubbing solid wax through a patterned screen onto filter paper. These papers were heated afterwards so that the wax melted into the substrate to form hydrophobic barriers (Dungchai et al., 2011). Other approaches dealt with polymer solutions, which were used as conventional screen-printing inks. In those cases, the solvents provided the penetration of the polymer through the paper (Sameenoi et al., 2014; Sun et al., 2015). Banks et al. also demonstrated that ePADs can be printed by serigraphy on different kinds of conventional papers (Metters et al., 2013). In that work, they addressed some problems with thick and high porous papers, such as filter papers, because the dielectric paste used for patterning the hydrophobic walls was ineffective to prevent the capillary wicking of the solution from the hydrophilic areas. Recently, our group has reported a novel method for the fully fabrication of ePADs by screen-printing (Lamas-Ardisana et al., 2017). A mixture of commercial UV-curable inkjet and screen-printing inks was used for patterning hydrophobic barriers into chromatography paper grade 1. Afterwards, a three-electrode system was screen-printed over the hydrophilic areas.

Determination of glucose is very important, especially in the fields of healthcare, clinical, food and beverage industries. In food and beverage industries, glucose quantification is required for quality controls and fermentation processes (Galant et al., 2015). Conventional methods require many time consuming steps, reagents and samples, so simple, fast and low cost methods are still needed for the determination of glucose. In this context, (e)PADs present a powerful alternative to conventional methods (Busa et al., 2016) and several ePADs for glucose

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detection have been reported in the last years (Dungchai et al., 2009, 2011; Liu et al., 2016; Nie et al., 2010a, 2010b; Rungsawang et al., 2016; Yao and Zhang, 2016).

In this work, a new approach for fabricating ePADs is presented. The dilution of a commercial UV screen-printing ink with a mixture of monomers was optimized for patterning hydrophobic walls into chromatographic paper grade 4. Subsequently, the three-electrode system was printed with carbon and Ag/AgCl conductive inks. The resulting ePADs were used as electrochemical platforms for a glucose biosensor. The microfluidic channels of the ePADs were modified at three separate points with different solutions i.e. pH/ionic strength buffering salts, ferricyanide and glucose-dehydrogenase FAD-dependent solutions. The critical parameters of the glucose biosensor were optimized and the analytical performance was determined. Finally, the ePAD biosensors were used for analysing commercial soft-drinks, without sample treatment, and the results were compared with those obtained by a commercial enzymatic-kit.

2. Experimental

2.1. Reagents, materials and apparatus

Glucose-dehydrogenase FAD-dependent (GDH) from microorganism, 500 U/mg lyophilized powder, was purchased from Sorachim S.A. Ortho-phosphoric acid 85% (w/w) and sulphuric acid 98% (w/w) were purchased from Scharlab. Other chemicals were obtained from Sigma-Aldrich. They were analytical grade and were used without any further purification. Glucose and GDH stock solutions were prepared in 0.01 M phosphate buffer pH 7.0 (PB). Glucose assay kit K-GLUHKR from Megazyme International was used for the spectrometric analyses of real samples. Whatman chromatography paper 4 from GE Healthcare Life Sciences, two conductive inks (BQ 242 carbon and 5874 silver/silver chloride, from Dupont), UV screen-printing ink (blue Ultraswitch UVSW from Maribu) and UVV6 thinner from Maribu were used for the ePAD fabrication. Other apparatus and materials are detailed in the Supplementary material.

2.2. Rheological characterization

The rheological tests were performed at 25 °C with parallel plate geometry (40 mm diameter stainless steel plane and 1 mm gap). A continuous ramp with an increasing shear rate from 1 to 1000 s^{-1} was applied during 5 min for each sample. The experimental data were fit to the power law model ($\tau = K \cdot \gamma^n$), where τ is the shear stress, γ is the shear rate, n is the flow behaviour index (or a shear-thinning index when $n < 1$) and K is the power law coefficient or consistency.

2.3. ePAD fabrication

75 ml Ultraswitch UVSW and 25 ml UVV6 were mixed and shaken manually. This mixture was rubbed onto the surface of the screen stencil with a squeegee. Only one sweep was enough for the total penetration of the ink across the chromatography paper. The patterned papers were immediately introduced into the UV oven to cure the ink. Afterwards, graphite and silver/silver chloride layers were successively printed and cured into the convection oven (120 °C, 5 min each). Graphite ink was used for the working and counter electrodes, while silver/silver chloride ink was used for the reference electrode and the conductive pads of each electrode. Fig. 1 shows the front and back sides of the ePAD. The hydrophobic walls delimited a rectangular microfluidic channel of 3 × 50 mm. The arrangement of the working electrode was perpendicular to this channel and its area just over the hydrophilic paper was 3 × 1 mm. The counter electrode was located at the end of the channel.

Fig. 1. Front and back sides of the ePAD.

2.4. ePAD characterization

The sealing tests were carried out using 10 µl of 25 mM phenol red in 0.1 M NaOH solution. For this, one single drop was carefully casted at the channel entrance (on the front printed side) and the solution was allowed to spread freely into the paper. The scanning electron micrographs were taken after sputtering 20 nm gold layers over the samples. The photos were obtained by the second electron signal at high vacuum, 20 kV accelerating voltage, 16 mm working distance and 36 spotsize.

2.5. Preparation of the glucose biosensors

The ePADs were cut to 25 mm channel length. The reagents were loaded through the back printed side. Three drops were deposited on different places. First, 1 µl of 0.5 M NaCl and 0.5 M phosphate buffer solution pH 7 was deposited at the channel entrance. Second, 1 µl of 0.5 M potassium ferricyanide was deposited at one centimetre from the channel entrance. Third, 1 µl of GDH-FAD 5 U/µl in PB was deposited at two centimetres from the channel entrance i.e. just at the back side of the working electrode. Finally, the biosensors were dried at 30 °C during 10 min.

2.6. Electrochemical measurements

The electrochemical measurements were started after 10 µl of the sample solution were dropped from a micropipette at the channel entrance. The measurement procedure included three sequential steps: 1) amperometric detection at + 0.3 V with a 100 nA current cut-off, 2) cell off during 20 s, 3) coulometric detection at + 0.3 V during 30 s. The analytical signals were the charges in the former step. The measurement of each point was repeated at least three times, using one biosensor for each repetition. The limit of detection was estimated from the IUPAC recommendation, using the $3S_b/m$ criteria (Mocak et al., 1997), where m is the slope of the linear range and S_b the standard deviation of the background (signal without glucose).

2.7. Real sample analysis

Five commercial soft drinks (Fanta zero orange, Kas zero orange, Sprite, Solan de Cabras multifruits and Pascual Bifrutas mediterraneo) were analysed in order to evaluate the biosensor applicability in real samples. The analyses performed with the biosensors were carried out without any sample treatment. The spectrophotometric analyses with the commercial enzymatic-kit required the samples dilution with PB solution, following the manufacturer instructions.

3. Results and discussion

3.1. ePAD printing and characterization

Commercial screen-printing inks are not devised for patterning hydrophobic walls into hydrophilic cellulose fibre networks. However this handicap can be overcome with the adequate selection and/or modification of the materials. We have demonstrated that UV screen-printing ink diluted with UV ink-jet inks can provide a suitable mixture for patterning hydrophobic barriers into chromatography paper grade 1 (Lamas-Ardisana et al., 2017). This procedure is successful to produce PADs by screen-printing, but the total ink penetration across this paper requires too much squeegee sweeps (five). Therefore a faster procedure was required in order to achieve a more feasible massive production of (e)PADs. In this context, our group has introduced two modifications to facilitate the ink flow through the paper. On one hand, the UV screen-printing ink (Ultraswitch UVSW) was diluted with the thinner recommended by the manufacturer (UVV6). This thinner is a mixture of monomers and, unlike the commercial ink-jet inks used before, any resin, prepolymer, (nano/micro)particle, pigment, aggregate or solid content is present. As consequence, the UVV6 was more effective because its addition allowed to tune the fluidity and the particle/colloid content of the Ultraswitch UVSW ink. On the other hand, chromatography paper grade 4 was used as substrate for printing because its pore size is the longest among the standard qualitative filter papers. Based on the manufacturer information, the particle retention at paper grade 4 is 20–25 μm while at paper grade 1 is 11 μm. This feature was taken into account to avoid or minimize the *ink-filtering* on the paper substrate. The penetration of screen-printing inks into papers generally implies the staking of their solid content at the outer fibre network, self-hindering the printing process. In order to introduce the above mentioned modifications in the printing procedure, some studies and optimizations were carried out.

First, the rheological properties of the Ultraswitch UVSW/UVV6 system were determined (Fig. S1). The undiluted Ultraswitch UVSW exhibited high consistency (4.67 Pa·s) and pseudoplastic behaviour (n = 0.853). The thinner addition modified both parameters but significant differences were observed depending on the amount used. Percentages under 15% showed a linear relationship with the consistency decrease and the flow index increase. This relation was not linear between 15% and 25% (w/w), reaching almost plateau values over this percentage i.e. k = 1.01 Pa s and n = 0.889. For this reason, only thinner percentages under 25% (w/w) were considered in subsequent studies. Fig. 2 shows the results of the sealing tests for channels printed in paper grade 4 using inks diluted with different thinner percentages. As it can be seen, the distance travelled by the dye solution inside the channel was related to the patterning of the hydrophobic barriers. The undiluted ink did not penetrate across the paper and the back side was practically unsealed, allowing the dye solution to spread out of the channel (Fig. 2A). The addition of the thinner improved the ink penetration and the paper patterning (Fig. 2B-E). Moreover, channels printed with a mixture containing 25% of the thinner, only required one squeeze sweep to provide well defined hydrophobic barriers and negligible lateral spreading of the dye solution (Fig. 2F).

Finally, the ePADs were characterised by SEM. Significant differences were observed between the printed surfaces with undiluted and 25% diluted inks. Fig. 3A and B show paper grade 4 printed with undiluted ink after one or three squeeze sweeps, respectively. As it can be seen, both surfaces were quite similar. The printed areas were easily detected because the cellulose fibres were covered under a continuous and smooth crust. This morphology would be explained by the above mentioned *ink-filtering* process and it justifies the bad results in the sealing test. Oppositely, the printed areas were hardly delimited when 25% diluted ink was used. In those cases, the cellulose fibres and pores can be observed after one (Fig. 3C) or even three squeeze sweeps (Fig. 3D). These pictures demonstrate that the ink dilution can prevent the paper clogging and, consequently, facilitate the patterning of hydrophobic walls by screen-printing. Finally, the conductive inks printed on paper presented thick deposits where the underlying fibres were not distinguished (Fig. 3E and F). These coatings would be also expected because the solid content and viscosity are relatively high in both inks.

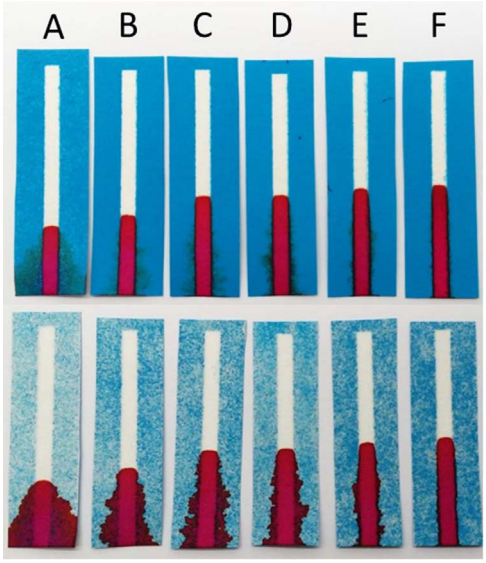

Fig. 2. Photo of channels printed with different diluted inks after the sealing test: 0, 5, 10, 15, 20, 25% (A-F). Front (up) and back (down) printed sides. Details in Section 2.4.

3.2. Biosensor optimization and characterization

Once the printing procedure was optimized, the ePADs were used for the fabrication of glucose biosensors in order to evaluate their electroanalytical performance. Glucose sensing was carried out in the presence of ferricyanide and glucose dehydrogenase FAD-dependent (Wang, 2001). In this case, one of the most classical examples of diffusional electron mediation in biosensors, ferricyanide works like an artificial mediator that shuttle electrons between the FAD redox centre and the electrode surface. Ideally, the mediator should present several characteristics in these systems, such as high reactivity with the reduced enzyme, good electrochemical properties and/or chemical stability and biocompatibility with the enzyme. The former is not a problem for the combination between ferricyanide and glucose oxidases or glucose dehydrogenases. Numerous publications have shown the long storage-stability of these glucose biosensors, even if both reagents remain mixed in dry-state (Nie et al., 2010a, 2010b; Rungsawang et al., 2016; Yao and Zhang, 2016). However, this compatibility is not always possible in biosensors because some reagents can react if they remain in contact (Forrow et al., 2005; Jayawardane et al., 2012). In those cases, (e)PADs can provide a simple solution. Hydrophilic channels can be patterned in paper, allowing to transport the sample sequentially trough several zones where different reagents are separately stored. The glucose biosensor presented here was based on this concept even though special storage conditions were not strictly required in this case. Hence the microfluidic channel was modified at three points with different solutions. The first zone was destined for buffering the pH and ionic strength. For this purpose, sodium phosphates and sodium chloride salts were deposited into the cellulose fibres by the evaporation of the corresponding buffer solution. It should be noted that the high concentration of chloride ions was useful to control the ionic strength as well as the reference potential. The second and third zones contained ferricyanide and GDH respectively. In that way, when the sample was added and flowed along the microfluidic channel by capillary action, the buffering salts, ferricyanide and GDH were successively dissolved, transported and mixed throughout the tortuous fibre

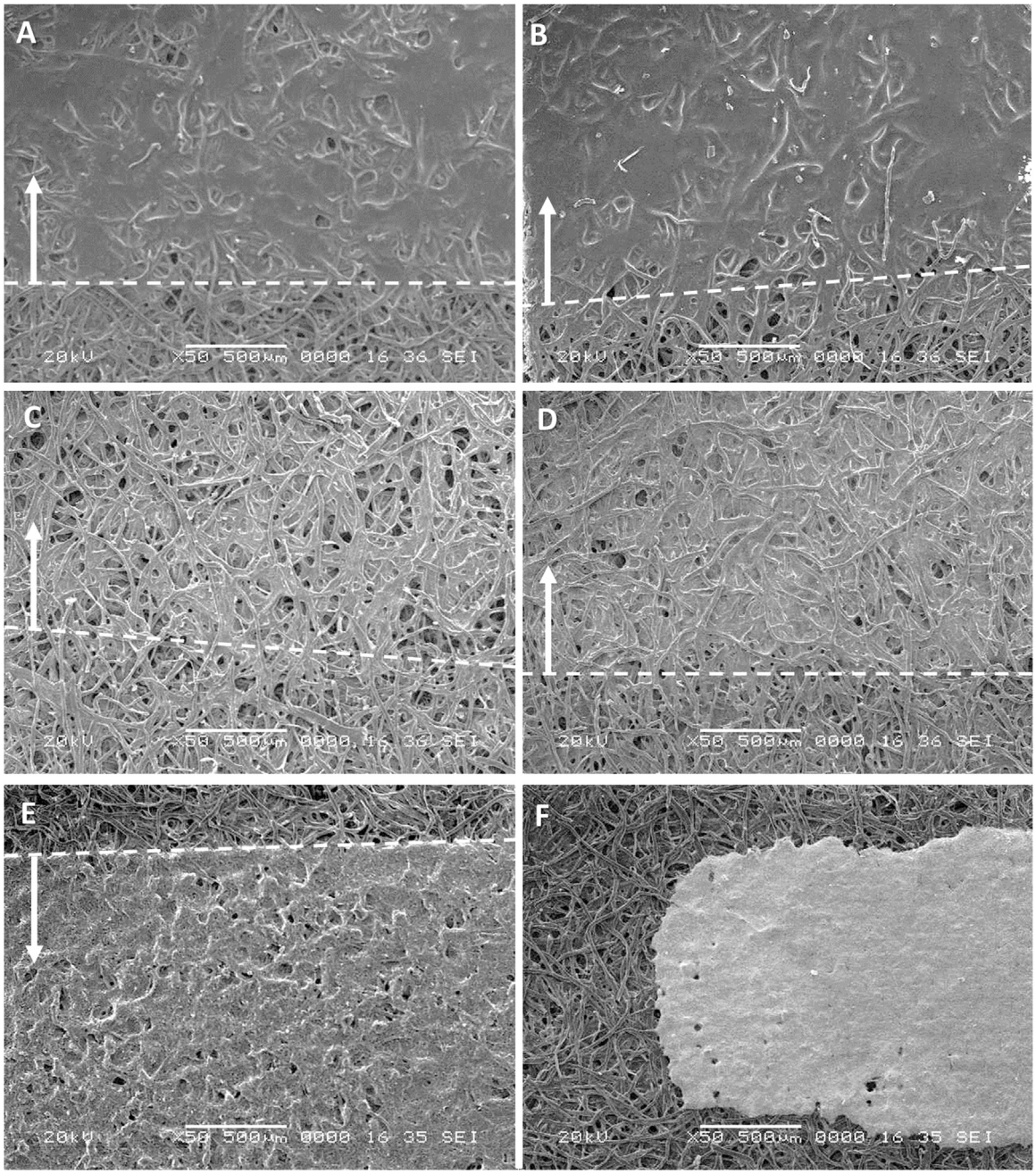

Fig. 3. Scanning electron micrographs of chromatographic paper grade 4 with undiluted UV ink (after 1 (A) or 3 (B) squeeze sweeps), 25% diluted UV ink (after 1 (C) or 3 (D) squeeze sweeps), graphite ink (E) and silver/silver chloride ink (F). Discontinuous lines and arrows indicate the limits and surfaces of the printed areas.

network, until the end of the channel. The distribution of the spot points was optimized in order to allocate the three solutions along the microchannel. The lateral spreading into the channel was similar for each case i.e. 7–8 mm with 1 µl approx. Therefore, ePADs with channels of 25 mm long were used for the glucose biosensors.

The critical parameters of the glucose biosensor were studied and optimized (details in Supporting information). The calibration curve for the optimized biosensor presented a linear relationship in the 0.5–50 mM concentration range (Fig. S3), with excellent coefficient of determination (r^2 = 0.999) and sensitivity (0.426 C/M cm^2). The limit of detection was 0.33 mM (considering ten background signals) and the repeatability was 6.2% (RSD; n = 10), calculated from signals of 10 mM glucose in PB. These analytical characteristics are comparable to other glucose biosensors based on ePADs. The limit of detection and the linear range are similar to those reported for biosensors where enzyme and ferricyanide were stored together (Nie et al., 2010a, 2010b; Rungsawang et al., 2016; Yao and Zhang, 2016). It is worth mentioning that the repeatability is slightly improved with respect to those works. It could be related to the procedure used for the electroanalytical measurements because: (i) it provides an unbiased signal when the channel is full-filled (step 1), (ii) the signal is registered after a delay time that ensures the flow-stop and the homogenization inside the channel (step 2), (iii) the analytical signal is obtained by coulometry instead of amperometry, avoiding possible mistakes related to inopportune current peaks at the registration moment (step 3).

Finally, the biosensors were tested for glucose analysis in five commercial soft drinks. Dilutions or treatments were not required and the samples were directly dropped to the biosensors. Interferences from electrochemical compounds were not advised in these samples since the charges registered with blank biosensors (without enzyme) were almost

identical to the background charges. Hence the determination was accomplished by interpolation of the corresponding charges into the calibration plot done with standards. The samples were also analysed using a commercially available spectrophotometric kit for glucose determination. The results obtained with both methods are summarized in Table S1. The application of the t-Student's test demonstrated that there were no significant differences between the values at a 0.05 significance level. Therefore, the results indicate that the ePAD biosensors have no systematic errors and can be successfully used for the determination of glucose in these soft drinks without sample dilution.

4. Conclusions

This work presents a new approach for the massive production of (e)PADs by screen-printing using commercial materials. The main technical limitation, the patterning of hydrophobic walls into the paper substrate, is overcome using chromatography paper grade 4 and diluted Ultraswitch UVSW/UVV6 (25% (w/w)). This combination minimizes the ink clogging on the paper surface and makes easier the ink penetration through the substrate. The ePADs fabricated by this procedure are suitable transducers for biosensors. Even more, it has been demonstrated here that ePADs with lineal channels can be used for storage several reagents separately in such a way that they are sequentially mixed when the sample is added. This concept was utilized to develop an enzymatic biosensor for the glucose determination in five soft drinks, without any sample treatment before the analysis. Future works would be focussed on other (bio)sensors based on similar ePAD designs, for example, lateral flow biosensors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2018.02.061.

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