Pin-based flow injection electroanalysis

Estefanía C. Rama, Agustín Costa Rama, M. Teresa Fernández-Abedul*

Departamento de Química Física y Analítica, Universidad de Oviedo, 33006, Oviedo, Spain

ABSTRACT: This work describes the use of mass-fabricated stainless-steel pins as new low-cost electrodes for a flow injection analysis (FIA) system with electrochemical detection. The pins serving as electrodes are directly punched in the tubing where solutions flow, being one of the simplest flow cells for FIA. This cell consists of a carbon ink coated pin as working electrode and two bare pins as counter and reference electrodes. The pins are able to perform at least 300 measurements. Moreover, they can be easily replaced showing good repeatability and reproducibility (RSD lower than 6% in all the cases). As a proof-of-concept, the feasibility of the system to determine glucose was evaluated by enzymatic assay using glucose oxidase, horseradish peroxidase and ferrocyanide as electron-transfer mediator. The application of this system to real food samples has shown accurate results.

Nowadays there is a widespread interest in exploring common, low-cost and disposable materials for the fabrication of sensing devices[1,2]. Electrochemical techniques result very adequate for the development of simple and small-size analytical devices due to its low cost, portability, ability for miniaturization, low sample consumption and high accuracy at low analyte concentrations[3]. Moreover, the increasing demand of information requires performing more analysis in less time. Automation, one of the main trends in analytical chemistry, together with simplification and miniaturization[4], plays an important role in this context. From its beginning in 1975 by Ruzicka and Hansen[5,6], flow injection analysis (FIA) has become a mature and important branch of contemporary analytical chemistry, so much that the number of published scientific papers exceeds 20 thousands[7,8]. This analytical technique is widely used in different fields of chemical analysis such as environmental and clinical chemistry or food and agriculture industry. FIA allows the automation of analyses decreasing human errors and analysis time, and therefore, increasing the accuracy. Moreover, it offers other advantages such as simple and flexible configuration and fast response time[9]. Electrochemical detection, especially amperometric techniques, has been widely coupled to FIA[7,10–13]. Traditionally, the three-electrode potentiostatic flow cell consists of a working electrode embedded in a polymeric block and the reference and counter electrodes are placed downstream. Regeneration of the surface of the working electrode by polishing (e.g. glassy carbon, gold disk) is common and in the case of carbon paste this could be renewed.[10] Nowadays many possibilities of wall-jet[11,12,14–16] or thin-layer[17,18] miniaturized working electrodes or whole electrochemical cells where all the three electrodes are located in the same card are possible. The most widespread methods for low-cost fabrication of these electrodes are deposition of carbon or metallic films using either thick-film (e.g. screen-printing[19]) or thin-film (e.g. sputtering or chemical vapor deposition[13]). But these technologies, in most of the cases, require stencils or masks to deposit the conductive materials on the substrate[1,20], usually made of ceramic, glass or polymers, increasing the fabrication cost and time. Alternative methods to fabricate low-cost electrodes are based on drawing with a graphite pen a specific design on the surface of paper using a graphite pen[21] or pencil[22,23]. But, all these methods do not allow modifying the setting of the electrodes in the electrochemical cell once the device is finished. Moreover, the mechanical stability of paper makes it not appropriate for the continuous flow of solution.

Recently, in a work made in collaboration with Whitesides group[24], the use of already mass-produced stainless-steel pins as electrodes have been reported. In this work, we proposed the use of pins as electrodes in paper and thread-based systems to quantify lactate in human plasma. The use of pins as electrodes makes possible developing devices with a high versatility for the disposition of the electrodes. Moreover, they are inexpensive and disposable, available nearly worldwide, highly conductive, easily storable, electrochemically stable in neutral or mildly acidic or basic aqueous solutions[25] and easily modifiable with conductive inks by simple dip and dry methodologies. We have recently demonstrated the possibility of developing enzymatic biosensors through the determination of glucose by immobilizing glucose oxidase, horseradish peroxidase and ferrocyanide as mediator on the head of a pin coated with carbon ink[26]. Apart from its head as electrode surface, the sharp tip allows drilling the substrate, either flat thin films or flexible polymers, and the shaft is useful as connection point to the potentiostat. Moreover, the shape and hardness of pins make them easy to handle and store in small-size boxes.

In the present work we develop, by the first time, a FIA system with electrochemical detection using mass-fabricated stainless-steel pins as electrodes. The pins are directly inserted in a piece of tubing where solutions flow. Therefore, this system is simpler than a conventional electrochemical flow cell and the electrodes can be easily replaced. Reference and auxiliary electrodes are located in a tubing piece downstream the working electrode. The accuracy of this system was evaluated and as a proof-of-concept, we tested its feasibility to measure the concentration of glucose, a relevant analyte in clinical and food fields. Glucose was determined by the injection of the product of the enzymatic reaction with enzymes glucose oxidase and horseradish peroxidase, using ferrocyanide as elec-

tron-transfer mediator. Ferricyanide enzymatically generated was electrochemically reduced when it passes over the head of the pin inserted in the tubing. Although the low cost of the pins (approx. \$3.5 / 400 pins) allows considering them as disposable, the flow over the pin head cleans the surface obtaining reproducible measurements over time. This allows to use only one pin for many measurements. The accuracy of the results obtained with the FIA system for glucose concentration in real food samples was evaluated by comparison with the results obtained using an enzymatic commercial kit with spectrophometric detection.

EXPERIMENTAL SECTION

Chemicals. Glucose oxidase from *Aspergillus niger* (GOx), horseradish peroxidase, Type VI-A (HRP), ferrocene carboxylic acid (FcCO$_2$H), glucose assay kit (GAGO20) and potassium ferrocyanide (K$_4$Fe(CN)$_6$) were purchased from Sigma-Aldrich. D-(+)-Glucose anhydrous was delivered by Merck. N,N-dimethylformamide (DMF) and isopropyl alcohol were purchased from VWR International. Graphite ink (C10903P14) was provided by Gwent Electronic Materials Ltd. Ultrapure water obtained from a Millipore Direct-Q^{TM} 5 purification system was used throughout this work. All other chemicals were of analytical reagent grade.

Working solutions of FcCO$_2$H and glucose, as well as solutions of GOx/HRP/ferrocyanide were prepared daily in 0.1 M phosphate buffer, pH 7.0.

Materials and apparatus. Pins (AIN265925) were purchased for Metalúrgica Folch, S.L. The pins chosen were stainless-steel pins with the following dimensions: 26-mm long, 0.59 mm of shaft diameter and 1.5 mm of head diameter. A 12-cylinder Perimax Spetec peristaltic pump (Spetec GmbH) and a six-port rotatory injection valve (model 1106, Omnifit Ltd.) were used for the flow injection system.

Chronoamperometric and voltammetric measurements were performed with a μStat 8000 potentiostat (DropSens) interfaced to a Pentium 4 2.4 GHz computer system controlled by DropView 8400 2.0 software. A home-made saturated calomel electrode (Figure S1) and a pH/mV meter purchased for Crison were used for evaluation of the potential of the pseudo-reference electrode (stainless-steel pin). All measurements were carried out at room temperature.

Pin-based FIA system. The stainless-steel pins were cleaned by sonication in isopropyl alcohol for 20 min. Two of these pins were used as reference and counter electrodes without further treatment. The working electrode was a stainless-steel pin coated with freshly prepared carbon ink. The carbon ink and the procedure used to prepare the working electrode were optimized by us in a previous work[26]. The carbon ink consisted of a mixture of graphite ink and N,N-dimethylformamide (DMF) with a mass ratio 1:1, prepared using an ultrasonic bath for 1 hour (37 kHz of frequency and 320 W of power) in order to obtain a homogeneous ink. The head of the pins was coated immersing them in the ink and leaving to dry for 15 min in an oven at 70ºC. This process was repeated 3 times but the drying time after the last immersion was 12 hours instead 15 min in order to assure complete evaporation of the solvent. After that, the pins coated with carbon ink were ready to use as working electrodes.

The flow injection system (Figure 1A) consisted of the peristaltic pump for generating a continuous flow of 0.1 M phosphate buffer pH 7.0, and an injection valve equipped with a 100-μL loop. The tube of the pump was made of PVC and its diameter was 0.889 mm. The flow rate of the carrier was 1.5 mL·min^{-1} all along the work. The flow cell consisted of three stainless-steel pins: a carbon-coated pin as working electrode (WE) and two bare pins as reference (RE) and counter (CE) electrodes. The pins were directly inserted in a piece of pump tube of PVC whose diameter was of 1.651 mm. The tube between the injector and the working electrode was also made of PVC and its diameter and length was 0.508 mm and 25 cm respectively. The insertion of the pins in the tubing was easily achieved because the thin sharp tip of the pins allows drilling easily the tubing. The procedure consists of introducing the sharp tip first into the inner part of the tubing to drill it and then pulling the pin until only the head of the pin is inside. The shaft of the pins was used to connect them with the potentiostat. Putting the pins upside down favored connecting them to the potentiostat through crocodile clips that also held the tubing system. In order to change WE without disturbing RE and CE, these pin-based electrodes were inserted in a different piece of tubing. The two pieces of tubing with the pins were interconnected using a T-connector with one of the ends sealed. A picture of the pin-based electrochemical flow cell is shown in Figure 1B. Figure 2A shows several carbon-coated pins inserted in tubing ready to use as working electrodes in the FIA system.
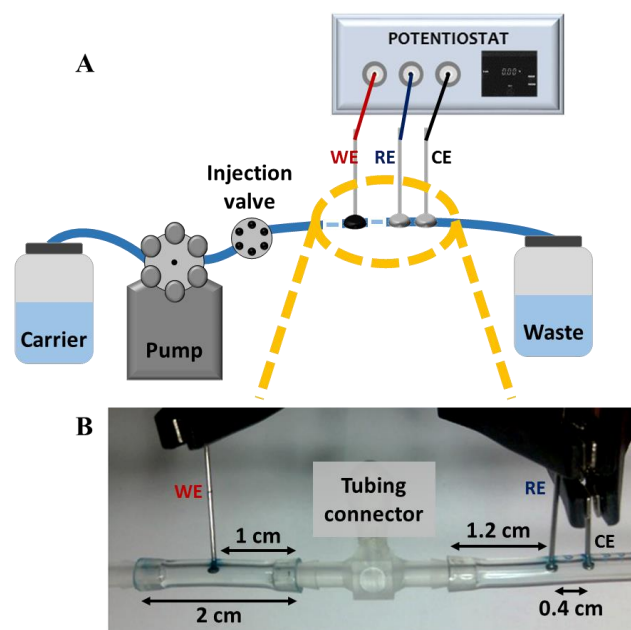

Figure 1. (A) Scheme of the pin-based FIA system. (B) Picture of the pin-based electrochemical flow cell.

Glucose determination. The procedure for measuring the concentration of glucose was as follows. First of all, a mixture of GOx, HRP and ferrocyanide (0.12 U/μL, 0.1 U/μL and 20 mM, respectively) in 0.1 M phosphate buffer pH 7.0 was prepared. Then, this mixture was added to the sample in a volume ratio sample : mixture 95% : 5%, and was left to react for 1 min before injecting into the flow system. Thus, glucose concentration was determined measuring chronoamperometrically the concentration of ferricyanide generated enzymatical-

ly (according to the reactions indicated in Figure S2). For each mole of glucose, two moles of ferrocyanide are oxidized to ferricyanide[27,28]. Applying -0.1 V *vs*. stainless-steel pseudo-

reference electrode, the ferricyanide enzymatically generated was reduced, and the current measured was proportional to the
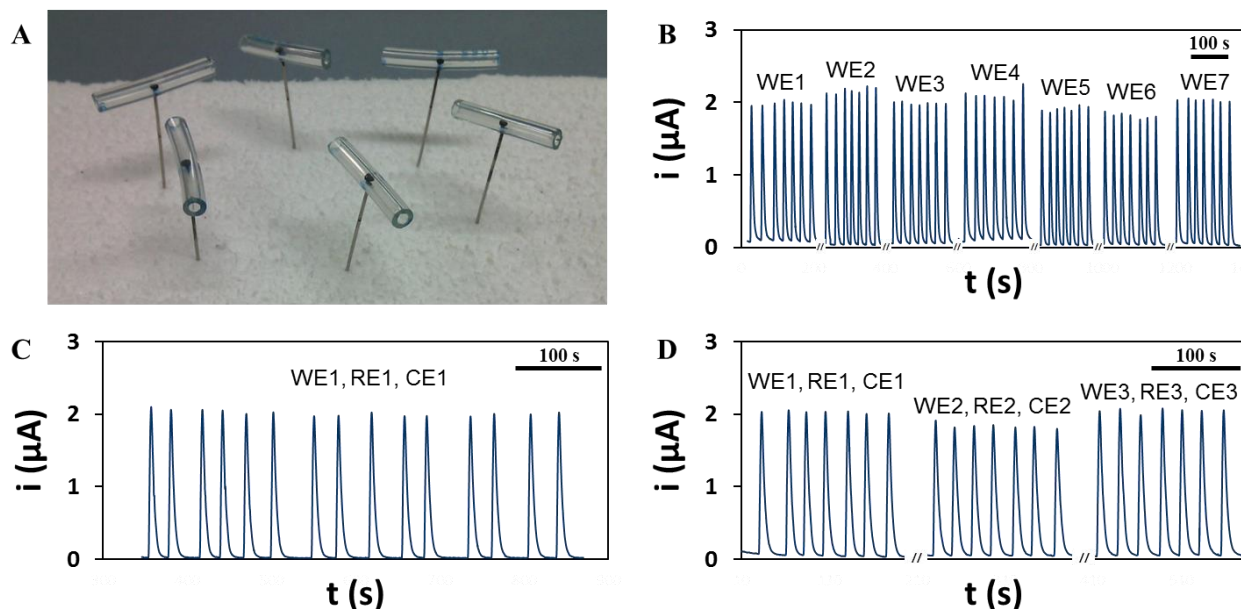

Figure 2. (A) Several pieces of pump-tubing with carbon-coated pins inserted ready to use as working electrodes in the FIA system (B, C, D). Fiagrams performed by injecting a 0.25 mM $FcCO_2H$ solution and applying +0.4 V *vs*. a stainless-steel pseudo-reference electrode using: 7 different pins as working electrodes (7 injections for each one) maintaining the same reference and counter electrodes (B), the same pins as working, reference and counter electrodes for 15 injections (C), and 3 different trios of pins serving as working, reference and counter electrodes (7 injections for each group of three) (D).

concentration of ferricyanide generated and therefore, to the concentration of glucose in the sample.

Glucose concentration was measured in two real food samples (cola beverage and orange juice). The only sample treatment needed was a dilution in 0.1 M phosphate buffer pH 7.0. In order to test the accuracy of the results obtained, the samples were also analyzed using a commercial glucose kit with spectrophotometric detection.

RESULTS AND DISCUSSION

The use of pin-based electrodes allow to develop electrochemical cells with different size and electrode distribution, allowing to place the electrodes in the way the final application demands. In this work we have designed a very simple and low-cost electrochemical flow cell based on this type of electrodes. The distribution chosen for this work was one between the multiple options that can be developed and optimized. For the sake of simplicity, all of the pins were of stainless steel but in the case of the working electrode, the head (the part of the pin that was inside the flow system) was coated with carbon ink. The tubing employed for inserting the pins was the one used for peristaltic pumps made of PVC that recovers the shape after the puncture avoiding leaking. Two different tubing pieces were employed: one for the working and another for both the reference and the counter electrodes. The low cost of the pins allows considering them as disposable. However, if they are stable enough and precise measurements are obtained (as in this case), change is not needed and the system can be employed for a long period of time or high number of meas-

urements. In any case, reference and counter electrodes are located in a tubing piece separated from this of the working electrode in order to change them independently.

Since a pseudo-reference electrode (stainless-steel pin) is employed, its stability was evaluated measuring the potential between a stainless-steel pin and a saturated calomel electrode.
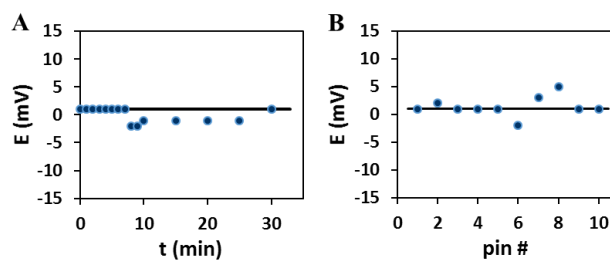

Figure 3. Variation of the potential measured between a saturated calomel electrode and (A) one pseudo-reference electrode for 30 min and (B) 10 different pseudo-reference electrodes at 5 min.

The pin was immersed in one of the arms of a U-shaped glass piece filled with a saturated KCl solution and connected to the potentiometer with a crocodile clip. As it can be seen in the scheme of Figure S1, the bottom of the other arm is filled with mercury and calomel paste and a platinum-wire electrode was introduced and connected also to the potentiometer. Figure 3 shows the variations in the potential with time. As it is shown in Figure 3A, the initial potential of the reference electrode

after immersion in the solution was 1 mV. We can consider that the reference electrode does not need a setup time (period before the potential becomes stable) and that the potential was maintained for 30 min. Over this time, potential does not drift more than 3 mV. Differences between 10 stainless-steel pins is shown in Figure 3B and was not higher than 7 mV (most of them showed the same potential, 1 mV). However, it has to be said that 5 more pins showed unstable measurements and were discarded. Therefore, it is important to check the potential of the pin serving as reference electrode before setting the system. Then, although the precision in between different pin-based reference electrodes as well as their low cost ($3.5 / 400 stainless-steel pins) allows considering them as disposable, the stability of the potential and the simplicity of the system make possible to use the same reference in several measurements. In this case all the work (hydrodynamic curve, calibration of $FcCO_2H$ and glucose determination) has been made using the same electrochemical cell (same working, reference and counter electrodes) although with the aim of checking the precision, several electrochemical cells were evaluated as commented in the following section.

Evaluation of the pin-based FIA system. We tested the performance of this pin-based FIA system using ferrocene monocarboxylic acid ($FcCO_2H$) since it is a redox probe with well-characterized electrochemical behavior. First of all, in order to know the behavior under flow conditions, a hydrodynamic curve (i vs. E curve) was recorded by injecting a 0.25 mM $FcCO_2H$ solution in a continuous flow (1.5 mL·min^{-1}) of 0.1 M phosphate buffer pH 7.0 and applying potentials comprised between 0.0 V and +0.5 V vs. a stainless-steel pseudo-reference electrode (Figure 4). Although a lower potential could be applied and this of the pseudo-reference electrode has demonstrated to be stable, in order to assure the oxidation of $FcCO_2H$, +0.4 V has been chosen for further pseu shows that, although it increases with potential, at +0.4 V it is acceptable (less than 10 nA with a signal of 100 nA for 0.01 mM $FcCO_2H$ solution).
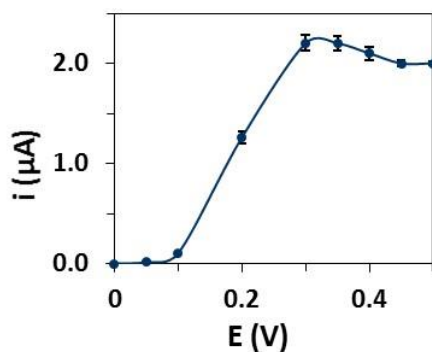

Figure 4. Hydrodynamic curve performed between potentials 0.0 V and +0.5 V vs. a stainless-steel pseudo-reference electrode injecting a 0.25 mM $FcCO_2H$ solution (1.5 mL·min^{-1} flow rate; each point is the mean of 7 injections).

Therefore, applying a potential of +0.4 V as oxidation potential and with the aim of knowing the precision of the system several studies were performed. Firstly, one carbon-coated pin acting as working electrode was tested injecting 15 times a

0.25 mM $FcCO_2H$ solution (Figure 2C). An intensity of current in the maximum of 1.99 ± 0.04 µA with an RSD of 1.8 % was obtained. The width of FIA peaks at base line was 20.4 ± 0.9 s and, therefore, the sample throughput of the system is 176 h^{-1}. In order to evaluate the precision of the system when different carbon-coated pins were used, several tubing pieces containing pin-based WEs (Figure 2B) were tested (without changing the pins serving as reference and counter electrodes).

Injections of a 0.25 mM $FcCO_2H$ solution produces a current intensity in the maximum of 1.9 ± 0.1 µA with an RSD of 5.6 % (mean of 7 injections for each one of the 7 carbon-coated pins tested; Figure 2B). Moreover, the reproducibility of the system when all the electrodes are changed was also checked. Using a 0.25 mM $FcCO_2H$ solution, the mean of the intensity of current obtained using 3 different trios of pins serving as working, reference and counter electrodes (7 injections in each case) was again 1.9 ± 0.1 µA with a RSD of 5.3 % (Figure 2C). These RSD values show the robustness of the system and the high usefulness of pins as electrodes in a flow injection analysis system.

Signals obtained by injecting different concentrations of $FcCO_2H$ were recorded and shown in Figure 5. The response of the system to $FcCO_2H$ concentration was linear between 0.01 mM and 1 mM, giving a calibration plot that follows the equation i (µA) = 7.32 [$FcCO_2H$] (mM) + 0.06 with a R^2 = 0.9990. To note, even though the small size of the working electrode, this pin-based electrode system shows a linear range of two orders of magnitude.
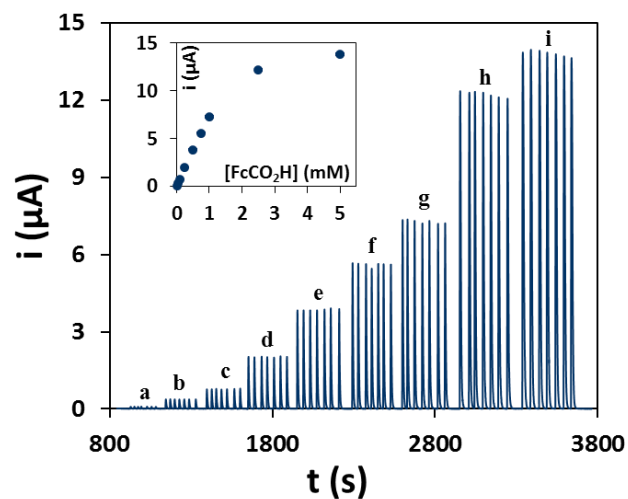

Figure 5. Fiagrams recorded by injecting the following concentrations of $FcCO_2H$ (applying +0.4 V vs. stainless-steel pseudo-reference electrode; flow rate 1.5 mL·min^{-1}): 0.01 (a), 0.05 (b), 0.10 (c), 0.25 (d), 0.50 (e), 0.75 (f), 1.00 (g), 2.50 (h) and 5.00 (i) mM. Inset: calibration curve in the plot in the $FcCO_2H$ concentration range comprised between 0.01 and 5.00 mM (each point is the mean of 7 measurements).

Apart from the linear relationship, it is worth to note that the stability and robustness of the pins as electrodes were also demonstrated since the same group of pins (working, pseudo-reference and counter electrodes) was used for more than 300 injections without loss of signal (these injections include measurements not only for ferrocene, but also for determining

glucose concentration, performed as is indicated in the following section). Moreover, it is important to note that the total cost of this pin-based system (that allows their use as disposable elements) was $0.28 (Table S1). Parameters such as the residence time of the sample in the detector (width of the peak at the baseline, 20.4 s) and dead volume of the detector (estimated from the volume of the tubing in between the electrodes after subtracting the volume of the three pinheads, 110 μL) could be varied by using a tube with different length and diameter for fabricating the cell. Another important feature of the pin-based working electrode here used is its rapid response since the appearance time of the analytical signal is *ca.* 3 s. The FIA system developed is considered as a low dispersion one employed just as a means of presenting the sample to the detector. Flow injection analysis is a mature field that offers many possibilities for fitting with different applications and then, lower volume tubing and connectors could be also employed if required.
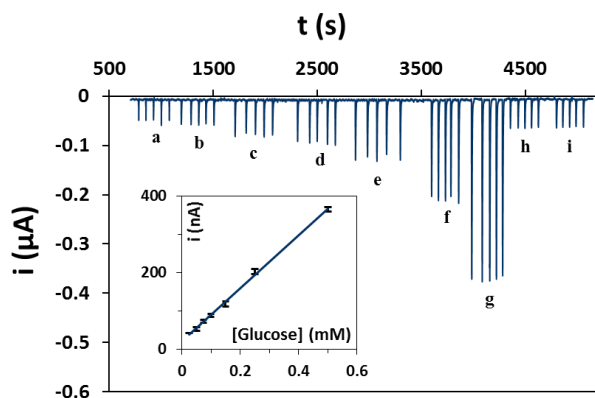

Figure 6. Fiagrams recorded for different concentrations of glucose (0.025 (a), 0.05 (b), 0.075 (c), 0.100 (d), 0.150 (e), 0.250 (f) and 0.500 mM (g)) and for the samples (orange juice (h) and cola beverage (i)) applying a potential of -0.1 V *vs.* a stainless-steel pseudo-reference electrode and a 1.5 mL·min^{-1} of flow rate. Inset: calibration plot in the glucose concentration range comprised between 0.025 and 0.500 mM (each point is the mean of 5 measurements).

Glucose determination: calibration and real sample analysis. The feasibility of the pin-based FIA system to measure the concentration of glucose, an analyte that is relevant in several fields such as clinical analysis or food industry, in real samples was evaluated. In this way, a bienzymatic assay employing glucose oxidase (GOx), horseradish peroxidase (HRP) and ferrocyanide as mediator of the electron transfer was performed off-line. The reaction takes place for 1 min and then the mixture is injected. The analytical signal is recorded at -0.1 V (see cyclic voltamogramm in Figure S4). Figure 6 shows the calibration curve for the measurements of different glucose concentrations with values ranging between 0.025 and 0.500 mM (Figure S5 shows zoomed fiagrams of some of the injections, where no relevant noise / ripples are observed). The sensitivity obtained was 694 nA·mM^{-1} with R^2 = 0.998, showing a good linearity. The limit of detection (LOD) and the limit of quantification (LOQ) calculated according to $3s_b/m$ and $10s_b/m$ criteria respectively, where m is the slope of the linear range and s_b the standard deviation of the intercept, were 0.02 and 0.05 mM respectively. This system achieved lower

LOD and LOQ for glucose detection than the pin-based glucose sensor we reported previously[26]. Moreover, when this system is compared with other FIA system for glucose determination, it presents comparable or even better analytical parameters (linear range, LOD and LOQ). For example, Samphao *et al.*[17] obtained a linear range from 0.2 to 9 mM, a LOD of 0.1 mM and a LOQ of 0.3 mM using screen-printed carbon electrodes modified with manganese oxide, where gold-decorated Fe_3O_4 nanoparticles modified with glucose oxidase were immobilized. Zhao *et al.*[29] achieved a linear range comprised between 0.1 to 2.5 mM and a LOD of 0.04 mM using the electrocatalytic oxidation of glucose at a nickel electrode.

Two real samples were analyzed using the FIA system developed and the results were compared with the values obtained using a commercial glucose kit with spectrophotometric detection. Table 1 shows the results given by both methods. The application of the Student's t-test demonstrated that there were no significant differences between the values given by the commercial kit and those obtained with our pin-based FIA system, at a 0.05 significance level. This indicates the good accuracy and precision achieved using the pins as electrodes in a FIA system.

Table 1. Determination of glucose in real samples with the proposed FIA system and with a commercial enzymatic kit with spectrophotometric detection. Data are given as average ± SD (n = 5 for the FIA system and n = 3 for the spectrophotometric kit).

Real sample	FIA system	Commercial kit
Cola beverage (g/100 mL)	3.3 ± 0.3	3.12 ± 0.03
Orange juice (g/100 mL)	3.22 ± 0.07	3.17 ± 0.03

CONCLUSIONS

Here we have demonstrated that the use of simple stainless-steel pins as electrodes set out a method to assemble and reconfigure devices according to the needs of specific applications. A conventional FIA system was combined with a new electrochemical cell, based on common low-cost stainless-steel pins for generating a highly precise and accurate analytical automatic methodology. This is the first work that shows the possibility of incorporating pin-based electrodes in a FIA system. The system developed gives precise and reproducible measurements. The pins, that can be disposable due to its low-cost and easy preparation, also show enough stability for being used in a continuous flow analysis during hundreds of measurements. They can be used as reliable electrodes and offer multiple options for developing electrochemical devices.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: Scheme of the saturated calomel electrode and of enzymatic reactions, evaluation of the noise of the baseline, cost analysis, cyclic voltammogram of ferrocyanide and zoomed fiagrams for different concentration of glucose (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*e-mail: mtfernandeza@uniovi.es;
Phone (+34) 985102968.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

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Estefanía C. Rama, Agustín Costa-García, M. Teresa Fernández-Abedul*

Departamento de Química Física y Analítica, Universidad de Oviedo, Julián
Clavería 8, 33006, Oviedo (Spain)

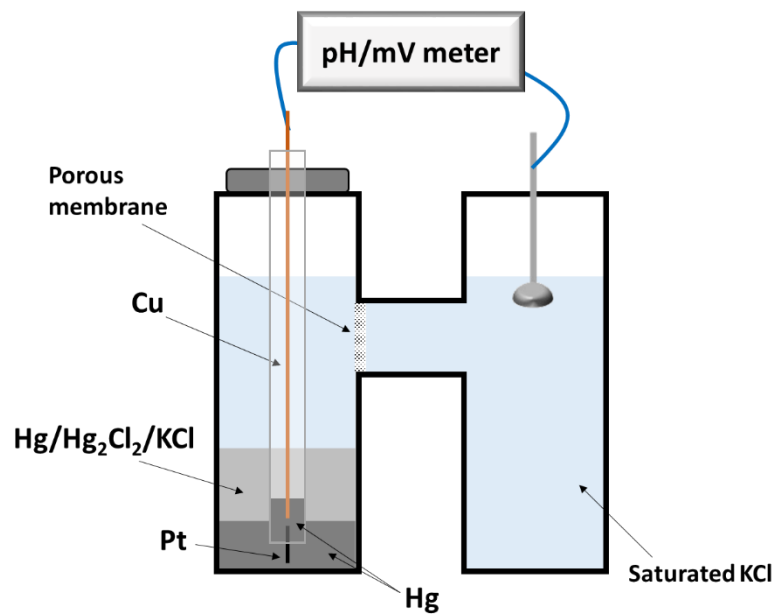*e-mail: mtfernandeza@uniovi.es

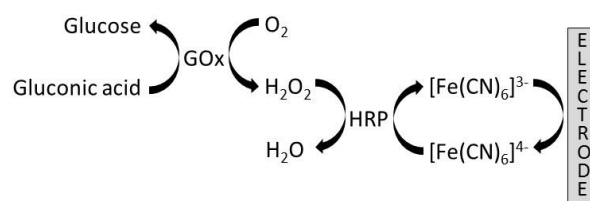## Contents

Figure S1. Scheme of the saturated calomel electrode.

Figure S2. Scheme of the enzymatic reactions involved in glucose determination.
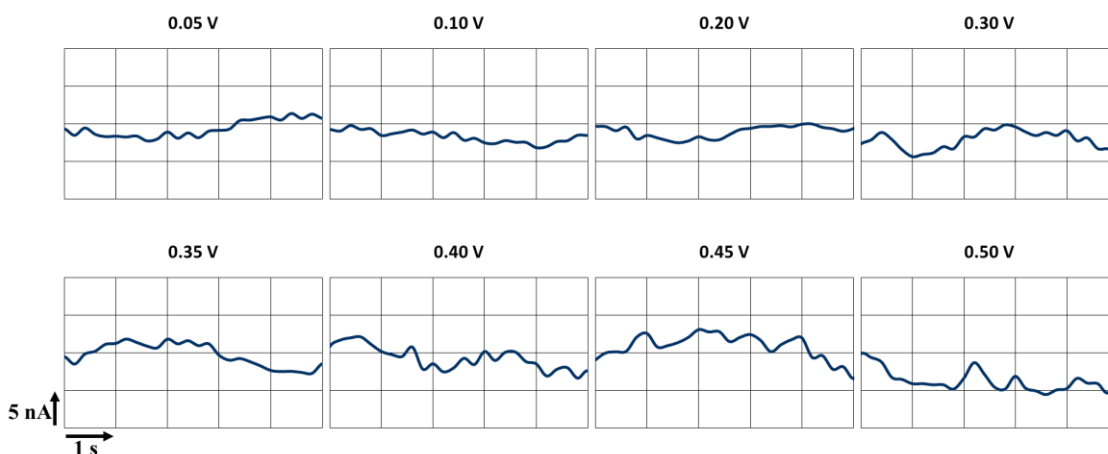
Figure S3. Baseline for different detection potentials *vs*. stainless-steel pseudoreference electrode.

COST ANALYSIS

Table S1 shows a brief analysis of cost for each three-pin system. Excluding labour and capital expenses, and the cost of the conventional instrumentation of a FIA system, the cost of preparing the final system is $0.28. To note, each system is able to perform more than 300 measurements. Moreover, the prices here considered were supplied in research quantities, but all of the materials and reagents are cheaper if they are purchased in bigger quantities.

Table S1. Cost of fabrication for each three-pin analytical system.

Item	Cost	Cost for system / $
Pins	$3.5 / 400 pins	< 0.027
Carbon ink	$32 / 50 g	< 0.049
DMF	$96 / L	< 0.006
Isopropyl alcohol	$100 / L	< 0.015
Tubing	< $30 / 10 m	< 0.18
Total cost for system with three pins		< 0.28

CYCLIC VOLTAMMOGRAM OF FERROCYANIDE

In order to determine anodic and cathodic peak potentials (*vs*. a stainless-steel pseudoreference electrode) for the ferro/ferri system and to set the most adequate potential for recording the fiagrams for glucose determination in the FIA system, a cyclic voltammogram was recorded in a 1.0 mM ferrocyanide solution in 0.1 M phosphate buffer of pH 7.0 (Figure S4). This was made using an electrochemical cell constructed by drilling the pins in a transparency sheet as is detailed in our previous work of pin-based biosensors[24]. The cyclic voltammogram (i-E curve) was recorded by dropping a 70-μL aliquot of a ferrocyanide solution covering all the three pins. The potential was scanned between -0.2 and 0.7 V at 50 mV·s^{-1}. A well-defined process is observed with a formal potential of 213 mV.

A -0.1 V potential was chosen for recording the analytical signal in the FIA system since this potential is low enough to assure the electrochemical reduction of ferricyanide.
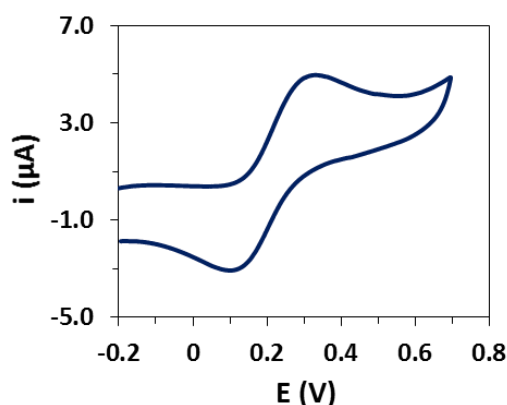

Figure S4. Cyclic voltammogram recorded in a 1 mM ferrocyanide solution in 0.1 M phosphate buffer pH 7.0 at a scan rate of 50 mV·s^{-1} in an electrochemical cell that uses a pin coated with carbon ink as working electrode and two bare pins as counter and reference electrodes.
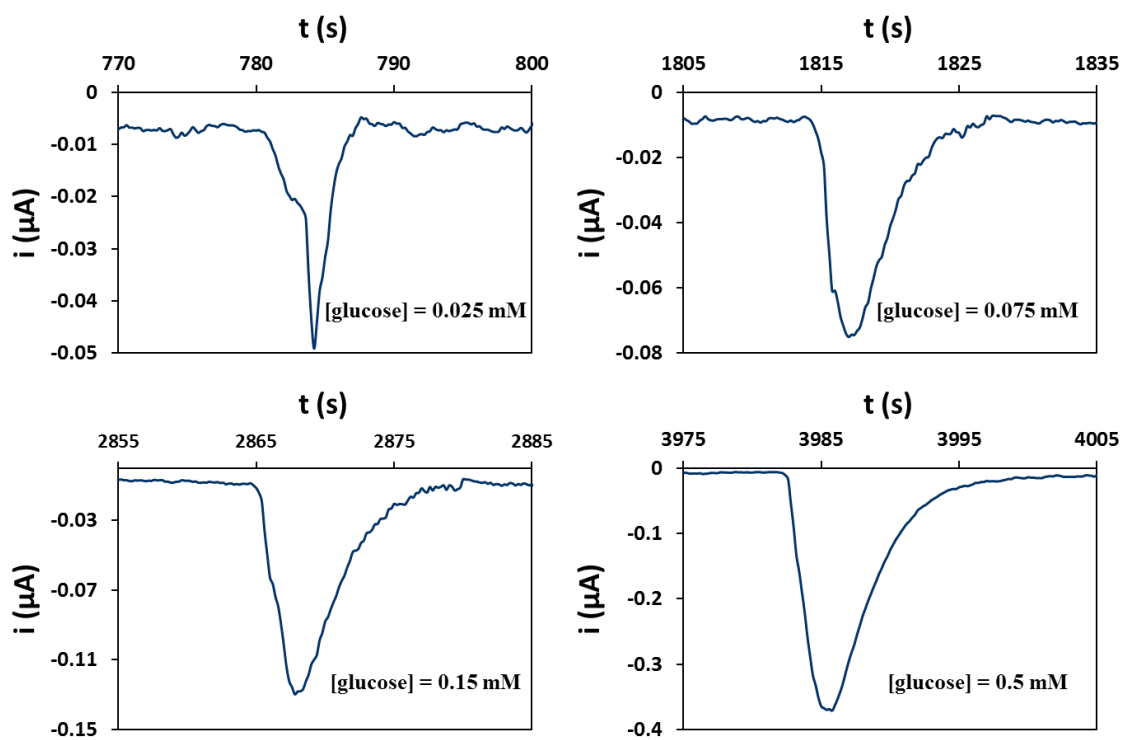
ZOOMED FIAGRAMS

Figure S5. Zoomed fiagrams (i *vs*. t) recorded for different concentrations of glucose (0.025, 0.075, 0.150 and 0.500 mM) applying a potential of -0.1 V *vs*. a stainless-steel pseudoreference electrode and a 1.5 mL·min^{-1} of flow rate.