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Preparation of paper-based devices for reagentless electrochemical (bio)sensor strips

Stefano Cinti[1*], Danila Moscone[2] and Fabiana Arduini[2*]

Despite substantial advances in sensing technologies, the development, preparation, and use of self-testing devices is still confined to specialist laboratories and users. Decentralized analytical devices will enormously impact daily lives, enabling people to analyze diverse clinical, environmental, and food samples, evaluate them and make predictions to improve quality of life, particularly in remote, resource-scarce areas. In recent years, paper-based analytical tools have attracted a great deal of attention; the well-known properties of paper, such as abundance, affordability, lightness, and biodegradability, combined with features of printed electrochemical sensors, have enabled the development of sustainable devices that drive (bio)sensors beyond the state of the art. Their blindness toward colored/turbid matrices (i.e., blood, soil), their portability, and the capacity of paper to autonomously filter/purge/react with target species make such devices powerful in establishing point-of-need tools for use by non-specialists. This protocol describes the preparation of a voltammetric phosphate sensor and an amperometric nerve agent biosensor; both platforms produce quantitative measurements with currents in the range of microamperes. These printed strips comprise three electrodes (graphite for working and counter electrodes and silver/silver chloride (Ag/AgCl) for the reference electrode) and nanomodifiers (carbon black and Prussian blue) to improve their performance and specificity. Depending on analytical need, different types of paper (filter, office) and configurations (1D, 2D, 3D) can be adopted. The protocol, based on the use of cost-effective manufacturing techniques such as drop casting (to chemically modify the substrate surface) and wax/screen printing (for creating the channels and electrodes), can be completed in <1 h.

Introduction

Analytical strategies that are able to provide accurate and rapid assessment for the surveillance of health, environment, and food status are needed to achieve a better quality of life for all people. Although laboratory-bound approaches provide accurate results, the whole analytical process is still out of reach for the non-specialist—both in terms of simple interpretation and for practical application in the field. The criteria established by the World Health Organization in 2004 focused on a precise requirement: diagnostic devices, especially those for low-income countries, should be ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and delivered)[1]. The need for instrumentation for quantifying the presence of a certain target is undoubtedly the bottleneck. In fact, naked-eye approaches that are based on colorimetric methods are considered the closest, in terms of operative conditions, to non-specialists. However, some issues arise: these approaches are mainly qualitative (i.e., yes or no) and do not work that well for matrices, such as blood, that are colored or turbid. Real matrices are usually treated before the colorimetric analysis, but these actions make these methods less practical and more open to error.

To overcome these limitations, electrochemical methods can be adopted, as brilliantly demonstrated by the world's most-sold biosensor, the glucose strip[2]. The glucose-based printed biosensor alone accounts for ~85% of the global market for biosensors, being valued at ~$15.3 billion in 2015 and expected to reach $31 billion by 2024 (ref. [3]). Versatile printed electronics (sensors and displays) help to improve this technology, especially in terms of affordability. Even though the detection of glucose in whole blood does not require adjunctive tasks such as chemical management and matrix treatment, not all systems show similar simplicity. The path from the laboratory bench to the end user is often time-consuming[4].

Many commercial kits are based on multiple steps; some of them are available only at the pharmacy level because of the required experience of end users and/or specific requirements for

[1]Department of Pharmacy, University of Naples Federico II, Naples, Italy. [2]Department of Chemical Science and Technologies, University of Rome Tor Vergata, Rome, Italy. *e-mail: stefano.cinti@unina.it; fabiana.arduini@uniroma2.it

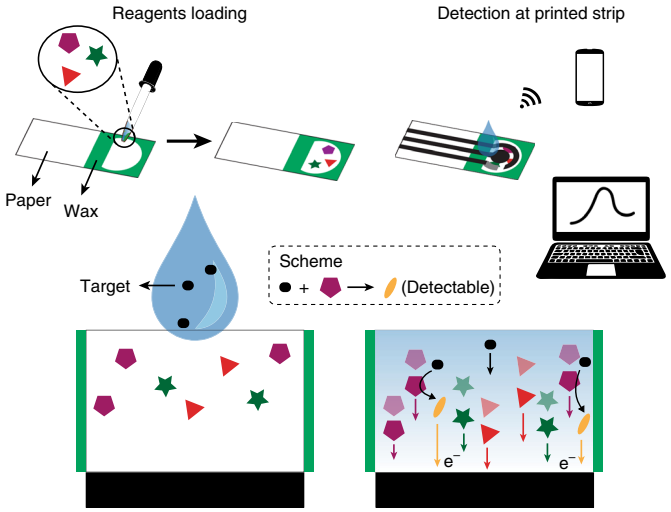

Fig. 1 | Configuration of a paper-based electrochemical device. All the reagents are preloaded onto the back of the testing area. The testing area is surrounded by a wax layer (green), where electrodes are screen-printed. When the sample, containing the target molecule, is drop-cast onto the testing area, all the reagents are dissolved and flow toward the electrodes. The device can easily be wirelessly coupled to a laptop and/or smartphone by using dedicated instruments, e.g., EmStat Blue (PalmSens), which is a small USB- and battery-powered potentiostat with integrated Bluetooth. It is ideal for mobile sensor applications (https://www.palmsens.com). Currents can be recorded and displayed on a laptop and/or smartphone by using a dedicated app, e.g., PStouch for Android devices, which can be used with all PalmSens and EmStat potentiostats. PStouch communicates with your potentiostat directly via USB or wirelessly using the PalmSens Bluetooth dongle. (e^- represents the electron(s) transfer at the electrode.)

storage. The use of paper-based substrates to develop lab-on-chip electrochemical tools could make a valuable contribution in overcoming these drawbacks, and, in fact, is widely reported in the literature[5–8]. Our group, as well as others, have reported different approaches involving cellulosic substrates to develop integrated devices for decentralized analysis.

The use of paper-based substrates to make the analytical strips is associated with lower production costs (in comparison with those printed on plastic), simpler disposal (possibility to be burned), and fewer operative tasks (especially in the case of reagent-free devices). These strips are generally fabricated using filter paper; because it is porous, the reagents can be preloaded into the device (Fig. 1).

However, the diffusion of aqueous samples within the cellulosic network of paper-based substrates represents an issue for electroanalysis; the diffusion toward the electric connections could be responsible for noise during measurements. To reduce this effect, the testing area is surrounded by a hydrophobic layer, i.e., melted wax, that prevents liquid flow toward the undesired portion of the strip[9].

We reported an approach to detect phosphate ions in environmental samples, in which the reagents were loaded into the filter paper. Because the paper does not allow the large impurities present in the matrix to enter the area where the reaction takes place, the sample does not need to be pre-treated before analysis[10]. This represents an obvious advance, considering that the gold standard method (colorimetric) requires acidification of the sample and consequent addition of ammonium molybdate, ascorbic acid, and antimony potassium tartrate, and thus suffers from refractive index errors and turbidity interferences[11]. The same approach has been also applied to the clinical field, by detecting chloride ions in sweat and serum without issues from interfering species[12].

Different papers (with different properties) can be combined in a single device, as described in our recent work to detect nerve agents in water samples. In this case, to develop an inhibition-based biosensor, the enzyme and the substrate are separately loaded onto two different paper-based substrates, i.e., filter paper and nitrocellulose (Predator membrane), to avoid undesired reactions before expected detection, and to speed up the release of the enzymatic substrate while measuring[13]. This configuration led to a clear experimental advantage for detecting enzymatic inhibitors with respect to the classic approaches. In fact, common enzymatic inhibitors are measured by following several steps: (i) measuring the enzyme activity in the absence of inhibition electrodes, (ii) allowing the inhibitor/enzyme interaction, (iii) washing and rinsing the electrochemical cell with buffer, and (iv) measuring the activity again[14]. These steps also include the addition of enzymatic substrate and pH adjustment. The unique configuration and porous structure of paper allows non-specialists to shorten this

procedure, providing a completely integrated tool that requires only a few microliters of sample, which is added to the back of the testing area; the impregnation of the testing area, where all the reagents have been preloaded, with 5–10 μL of sample allows the reagents to be completely released without generating waste when the assay is complete.

Experimental design

Choice of paper

Although the simplest approach is to use filter paper, other kinds of paper, such as office paper, can be used. Office paper can improve the sensitivity of detection by directly exposing the sample to the electrode, thus improving the diffusion of species, which is impeded by the cellulose fibers of filter paper (Supplementary Fig. 1). The structure of office paper can be used in different configurations, for instance, to exploit the electrode surface at the air interface. Although for filter paper, the sample is added to the back of the testing area, the lower porosity of office paper allows the sample (50–100 μL) to be directly cast on top of the testing area because of a slower impregnation rate of this less porous paper-based substrate. In this case, the experimental procedure is similar to the common drop analysis of plastic-based strips, with the only advantages being its low cost and sustainable nature. The quantification of alcohol content in commercial beers has confirmed the suitability of office paper to realize sustainable strips[15]. Office paper for developing paper-based devices, not only electrochemical ones, has also been reported for different applications: de Araujo and Paixao screen-printed silver ink, demonstrating the electrochemical detection of lead, chloride, and picric acid in water samples[16]; Cinti et al.[17] electrodeposited a 'green' bismuth layer onto a graphite electrode to monitor zinc ions in sweat and serum, and its colorimetric reliability was demonstrated in the determination of phenacetin in seized cocaine samples[18].

However, the way to convert paper-based substrates into sensing strips must be considered. Paper-based devices have been fabricated by using simple pencil drawing, as reported by Chagas et al., Dossi et al., and Dias et al., to develop an electrophoresis microchip for the measurement of BSA and creatinine[19], to detect vitamin B6 in food supplements[20], and to evaluate the amount of analgesics and sedation drugs in whiskey samples[21], respectively. Regarding the possibility of writing electrodes, recently, Paixao and colleagues reported the first example involving the use of a single-step CO_2 laser scribing process to pattern nanostructured electrodes on paper, providing a green solution for reagentless mass-scale production[22].

Regarding the development of such paper-based strips, a wide range of techniques have been reported including expensive (photolithography), affordable (pencil drawing, screen printing), and innovative (laser scribing) protocols.

Patterning the paper

Routes for realizing paper-based devices are many; one can choose the most appropriate approach depending on the particular requirements and facilities. For instance, paper can be patterned by using common wax printers or by using more sophisticated techniques, such as photolithography or plasma treatment, which offer higher resolution. However, the requirements for photoresists, solvents, and dedicated instrumentation, make them less suitable for 'real' cost-effective fabrication. In addition, paper-based substrates can be patterned by combining a filter paper sheet impregnated with paraffin and a native paper sheet; under the application of a pressure, a preheated metal stamp enables the thermal transfer of the paraffin to the native paper, thus forming the hydrophobic barriers. Even if this approach is low cost, it is not recommended for fabrication because of the low resolution and repeatability.

Printing electrodes

Even in the case of printing electrodes, it is possible to create the desired configuration by selecting among a wide range of techniques. If the strip's resolution must be in the millimeter range, screen printing or pencil drawing might represent the optimal choice. However, although the former allows for mass production with satisfactory reproducibility, the latter is more suitable for proof-of-concept studies than scalable processes. If micrometric strips are required, inkjet printing offers a great advantage, considering that patterns can be drawn (and re-drawn) with common software. Laser scribing, which allows pyrolyzation of the surface of paperboard to produce a conductive, porous, and non-graphite carbon material composed of graphene sheets, represents an innovative opportunity; to perform laser scribing, you need a CO_2 laser cutter system.

Customization of the testing area

Last, the customization of the testing area is strictly dependent on the nature of the modifiers. The easiest way to modify a surface is to use aqueous-based dispersions (e.g., gold nanoparticles, surfactants, and electrochemical mediators) that can be easily drop-cast and be adopted on a large scale by using automatized dispensers, i.e., BioDot (www.biodot.com). However, technical issues might arise when the modifier is not water soluble. If this is the case, the powders can be mixed directly into the electrode ink (the ink used for producing the strips). In this case, a main limitation can be the final ink rheology, which is reflected in a low homogeneity.

Affordable procedures for electrode modification, such as electroplating, the Langmuir–Blodgett technique, and dipping, have limitations related to the porosity of paper. These approaches require that the electrodes be dipped into a solution containing the modifier. Paper-based electrodes are not suitable for dipping into solution because the solvent is going to impregnate even undesired areas, not only the working electrode.

Although many methods have been reported in literature for the development of paper-based devices, we describe an easily mass-scalable and reproducible protocol by which paper-based electrochemical (bio)sensors are fabricated using low-cost manufacturing approaches such as wax-printing and screen-printing technologies. As described above, paper-based substrates have given rise to a plethora of alternative sensing tools, with applications in different fields, such as clinical, environmental, and agri-food monitoring. The goal of this protocol is to provide readers (even those who are not experts in electrochemical sensor fabrication) with a tutorial on how to fabricate the right architecture depending on their analytical needs, the target matrices, and the available facilities.

Validation of the device

When the paper-based device is finished, it must be validated. Two experimental parts need to be validated: wax patterning and screen printing. Regarding wax patterning, the final wax barriers need to prevent water from spreading within the paper out of the testing area. To validate, drop few microliters of water inside the testing area, i.e., drop 20 μL, if the testing area is 1 cm^2. Wait until the water completely evaporates; if the area outside the testing area is dry, it means the wax patterning is effective; if water diffuses out of the testing area, you need to re-wax the paper for longer time, i.e., double the curing time given in Step 2. Regarding the screen printing, you need to validate the electrical connections. Drop a few microliters of a redox probe onto the testing area, i.e., 5 mM potassium ferricyanide prepared in 0.1 M potassium chloride (KCl). Perform cyclic voltammetry in the range between −1 and +1 V. If two peaks are observed, it means the electrical connections have been screen-printed properly. If, instead, a straight line is obtained, it means the electrical connection is not right; first, check whether the connection is correct at the paper-based electrode–potentiostat interface; if it is well interfaced, you need to screen-print again.

Strengths and limitations

However, even if the fabrication of such electrochemical paper-based strips displays tremendous strengths in terms of application and simplicity in many fields, its use might be limited in regard to specific classes of matrices and in the presence of certain experimental conditions, i.e., high or low temperature. The strengths and limitations of electrochemical paper-based strips are summarized in Table 1.

Materials

Reagents

- Filter paper (67 gm^{-2}, 135-mm caliper; Cordenons)
- Office paper (Copy 2, 80 gm^{-2}; Fabriano)
- Cartridge-free ColorQube ink (Xerox, code nos. 108R00931 (cyan), 108R00932 (magenta), 108R00933 (yellow), and 108R00934 (black))
- Ag/AgCl-conductive ink (Acheson, cat. no. Electrodag 477 SS)
- Graphite conductive ink (Acheson, cat. no. Electrodag 421)
- Solvent (to wash the electrodes' masks at the end of screen-printing process; Sun Chemical, code no. 56013642)

Table 1 | Strengths and limitations of paper-based devices

Strengths	Limitations
In situ measurements	Organic-based matrices can dissolve ink of screen-printed electrodes
Miniaturization	Filter paper can have reduced sensitivity compared to traditional electroanalysis
Sustainability	Owing to the wax melting point, the working temperature is limited to 60–70 °C
All the reagents are included	To validate these methods, traditional approaches are needed, i.e., chromatography and mass spectrometry
Low cost	Unlike plastic, paper is not a flexible substrate, a severe bending can damage the electrical contacts
Paper can concentrate species, enhancing the sensitivity of the method	

- Ammonium molybdate tetrahydrate (Sigma-Aldrich, cat. no. 431346)
- Ascorbic acid (Sigma-Aldrich, cat. no. A5960)
- Antimony (III) potassium tartrate hydrate (Sigma-Aldrich, cat. no. 244791)

Phosphate sensor

- Ammonium heptamolybdate tetrahydrate (Sigma-Aldrich, cat. no. A1343)
- KCl (Sigma-Aldrich, cat. no. 746436)
- Potassium dihydrogen phosphate (Sigma-Aldrich, cat. no. 229806)
- Sulfuric acid (Sigma-Aldrich, cat. no. 339741)

Nerve agent biosensor

- Potassium ferricyanide (Sigma-Aldrich, cat. no. 244023)
- Ferric chloride (Sigma-Aldrich, cat. no. 157740)
- Potassium hydroxide (Sigma-Aldrich, cat. no. P5958)
- Hydrochloric acid (37% wt/wt; Sigma-Aldrich, cat. no. 258148)
- Potassium dihydrogen phosphate (Sigma-Aldrich, cat. no. 229806)
- KCl (Sigma-Aldrich, cat. no. 746436)
- Butyrylcholinesterase (BChE) from equine serum (Sigma-Aldrich, cat. no. C4290)
- Butyrylthiocholine chloride (Sigma-Aldrich, cat. no. B3128)
- Paraoxon-ethyl (nerve agent simulant; Sigma-Aldrich, cat. no. D9286) **! CAUTION** During the screen-printing process, including the washing steps, it is necessary to use gloves and a mask and to work under an aerated fume hood to avoid both physical contact with and the inhalation of volatile organic solvents.

Equipment

- Polyurethane-based squeegee (fabricated in-house; can be purchased at https://www.squeegeewatts.com)
- Wax printer (Xerox, model no. ColorQube 8580) or paraffin (Sigma-Aldrich, cat. no. 327204) An alternative (and even cheaper) solution to wax printing is the use of paraffin. A sheet of paper can be impregnated with paraffin and, through a hot metallic pattern, wax can be pressure-transferred to the bottom of the paper substrate[23]. Of course, this approach provides lower resolution of the channels in comparison with the use of a wax printer.
- Thermostatic oven (F.lli Galli, model no. 2100) or heating plate (Biosan, model no. MSH-300) The minimum required temperature is 100 °C. Used for waxing the paper substrate before it is screen-printed with conductive inks.
- Adhesive tape (Office Depot, cat. no. QCP-3404522)
- EmStat Blue (PalmSens) All the electrochemical measurements are carried out using portable and battery-powered instrumentation (PalmSens) connected to a laptop or to a smartphone.
- BioDot dispenser (http://www.biodot.com)
- Fan (Kooper, model no. 2408556k)
- Fibrous polymer tissue with a defined mesh (e.g., polyester 120 mesh), a very generic serigraphic polyester-based film, can be purchased from a local seller

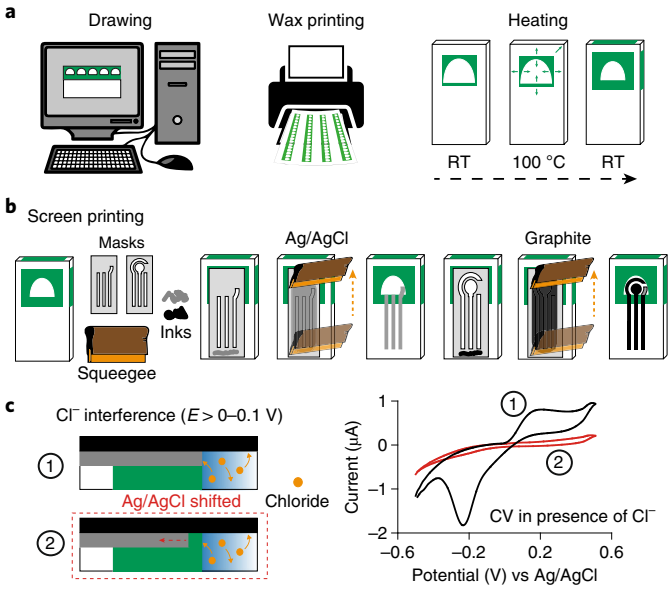

Fig. 2 | Manufacture of a paper-based electrochemical sensor. All the manufacturing steps are reported. **a**, A pattern for the testing area is drawn and wax-printed. **b**, Electrodes are screen-printed and thermally cured. **c**, If the working electrode contains some Ag-based ink, the presence of chloride ions in the working solution can affect the measurements, especially when the working potential (*E*) is >0–0.1 V (versus Ag/AgCl). To avoid the contact between Ag-based ink and the working solution, the Ag/AgCl can be screen-printed, shifting the mask/stencil. If the working solution, which contains traces of chloride ions, comes into contact with the silver strip under the working electrode (1), peaks related to the formation and dissolution of AgCl occur (black line; (1)). When the Ag/AgCl mask is shifted far from the testing area (2), the recorded voltammogram is consistent with the absence of the interference peaks (red line; (2)). RT, room temperature.

Software

- Patterns are designed with drawing software (Adobe Illustrator (https://www.adobe.com/illustrator/), Microsoft Paint (available through Microsoft Windows), or Microsoft PowerPoint (http://www.microsoft.com/powerpoint))
- PStouch for Android (PStouch, designed for tablets and phones, can be downloaded for free in the Google Play Store)

Procedure

Wax patterning the paper substrate ● Timing 5 min

1 Draw the wax pattern using common software: Paint, PowerPoint, or Adobe Illustrator (Supplementary Fig. 2).

▲ **CRITICAL STEP** The drawing software might influence the quality of the resulting wax patterning. To fabricate small channels, i.e., channels with a diameter of <1 mm, vector graphic software such as Adobe Illustrator can produce better resolution. If the size of the patterns is >1 mm, common software, i.e., Paint or PowerPoint, can be used.

2 Print a solid wax ink layer onto the selected paper substrate and heat it for a couple of minutes, depending on the substrate. When filter paper is used, 2 min at 100 °C is enough to allow the wax to penetrate the paper structure, forming a hydrophobic volume. If office paper is used as the substrate, 4 min is required to obtain the hydrophobic structure, due to the lower porosity of this latter paper in comparison with filter paper (Fig. 2a).

▲ **CRITICAL STEP** After the curing stage, wax diffuses radially. This means that the sizes of channels and/or confinements are now smaller with respect to the starting pattern. The original pattern should be larger than that expected after the curing step. The extent of this diffusion depends on both the substrate porosity and the curing time, and must be determined experimentally. For instance, if a 2-mm-wide final channel is required, a pattern with a wider channel should be designed in order to take into account that when wax melts, the thermally cured channel is going to be reduced in size in comparison to the pattern dimensions. When filter paper is cured for 2 min at 100 °C, a 10% spreading of wax is observed. The spreading

percentage depends on the type of paper, curing time, and curing temperature; it should be empirically evaluated for each experiment.

Screen-printing electrodes ● Timing 30–40 min

3 After the wax has been printed onto the paper substrate, screen-print the electrodes, using the most suitable conductive ink, depending on the particular species to be detected. Prepare the Ag electrode first. Ag/AgCl ink is used for the conductive guides for the potentiostat–working electrode communication and for the pseudo-reference electrode. Prepare a mask; this is a stencil with the shape of the electrode (Supplementary Fig. 3). An easy procedure for obtaining a fibrous polymer stencil is as follows: (i) design and print the pattern onto a paper sheet; (ii) select the appropriate fibrous polymer tissue with a defined mesh, e.g., 120 mesh has been used in the examples discussed in this protocol. The amount of printed ink depends on the mesh density; (iii) trace the pattern onto the fibrous polymer tissue; (iv) cover the area of the tissue that is not going to be printed with an insulator; and (v) let the insulator dry. Alternatively, to make an aluminum-based stencil, after the pattern has been designed, the aluminum foil can be cut with the aid of an ad hoc blade depending on the thickness' foil.

Use a squeegee to spread the conductive ink through the stencil onto the paper-based substrate as shown in Fig. 2b. The amount of conductive inks to be screen-printed depends on the type of stencil that is adopted; depending on the stencil, a fixed amount of inks will pass through, and the excess unprinted ink can be recovered and re-used.

▲ **CRITICAL STEP** This step can also be done using a screen-printing machine.

▲ **CRITICAL STEP** If the measurements are carried out by applying potentials >0.1 V (versus Ag/AgCl), and in presence of chloride ions, interference due to the oxidation of silver can affect the quantification. The occurring reaction is the following: $Ag + Cl^- \rightarrow AgCl + e^-$. This can be due to the presence of some not fully waxed spots on the paper that can allow the working solution to contact the conductive silver strips printed under the graphite working electrode. To prevent this from happening, as shown in Fig. 2c, the mask can be shifted far from the testing area during the Ag/AgCl printing process (Step 3), in order to create a tolerance wax layer between the testing area and the Ag/AgCl silver strip. As shown in Fig. 2c, (2), the edge of the Ag/AgCl ink does not coincide with the edge of the wax layer (shown in green); this configuration eliminates the direct contact between the working solution and the Ag/AgCl ink, thus avoiding silver oxidation at the working electrode (Fig. 2c).

4 After the silver-based ink is screen-printed, thermally cure it at 60 °C in an oven for ~15 min.

▲ **CRITICAL STEP** The curing temperature must always be <80–90 °C. Higher temperatures might allow the wax to melt again, reducing the testing area and affecting the printed electrodes. Depending on the physical properties of the paper substrate chosen to fabricate the device, it is possible that the wax might melt at a lower temperature. If this is the case, a suitable strategy would be to lower the curing temperature and to increase the duration, i.e., 50 °C for 20 min.

5 Prepare the working and counter electrodes, by screen-printing the graphite conductive ink as shown in Fig. 2b. Graphite-based ink must be thermally cured at the same conditions as those used for the silver-based ink, i.e., 15 min at 60 °C.

Using a modifier to enhance and/or tailor electrode performance ● Timing 10–15 min

6 Choose which modifier you are going to use. This depends on what you are trying to detect, and whether the electrodes are to be printed on filter (option A) or office (option B) paper. Table 2 shows some of the most used modifiers to be combined with electroanalytical devices. Depending on the application and on the type of paper used to fabricate the electrochemical device, the working electrode surface can be modified by following one of the two main approaches. As reported, the choice of the paper substrate influences the configuration of the electrochemical cell.

(A) **Use of filter paper**

▲ **CRITICAL** The electrochemical cell is defined by the intrinsic porosity of the filter paper.

(i) Add the modifier to the conductive ink (Fig. 3a) to develop composites[10,13,24–27], i.e., carbon black, Prussian blue/carbon black, carbon nanotubes, graphene/silver nanoparticles, and cobalt phthalocyanine, before screen-printing the electrodes as described in Steps 4 and 5.

▲ **CRITICAL STEP** When a powder is mixed together with an ink, the rheological properties of the composite should be evaluated. Some composites might not be suitable to be printed

Table 2 | Commonly used modifiers for printed electrodes' applications

Modifier	Application (used in the analysis of)
Au, AuNPs	As, Hg, Cu
Ag, Ag NPs	Halides
Bismuth film	Pb, Cd, Zn
Prussian blue	Hydrogen peroxide, thiols
Cobalt phthalocyanine	Pesticides
Graphene, carbon nanotubes, carbon black	Ascorbic acid, phenols
Conductive polymers	pH

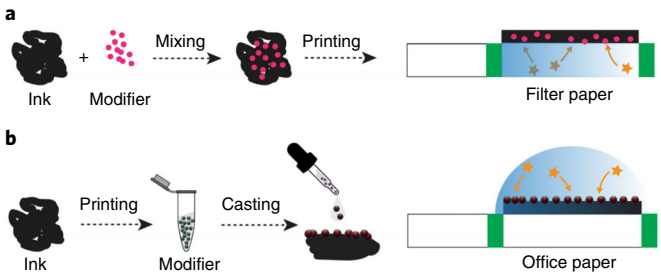

Fig. 3 | Enhancing the electrochemical properties of the paper-based devices. The electrodes can be modified with a variety of materials, depending on the required functionalization and the paper that has been chosen. **a**, When the electrodes are screen-printed onto filter paper, the modifier is directly mixed with the conductive ink. **b**, If a less porous substrate, such as office paper, is used to develop the paper-based device, the modifiers can be drop-cast on top of the electrode surface.

with a certain mask, i.e., if a polymeric mesh mask is used for screen printing, the dimension of the powder could obstruct the pores, producing a non-homogeneous printed track. Perform a repeatability study on several printed electrodes. Make ten printed electrodes and check what they look like and whether they work. Depending on the modifier, different tests are required, e.g., if a carbonaceous modifier is used, test the repeatability in the presence of 5 mM potassium ferricyanide by performing cyclic voltammetry from −1 to +1 V; calculate the relative standard deviation (RSD) relative to the anodic peak height. If the RSD appears to be ≤10%, the composite has been adequately screen-printed. If the RSD is substantially higher, the composite must be prepared ex novo by reducing the powder amount and/or the particle size.

(ii) Impregnate the testing area with a small amount of sample, i.e., 5–10 µL. As shown in Fig. 3a, when sample is added, the species come into contact with the modified electrode from the bottom.

▲ **CRITICAL STEP** Some modifiers are not soluble in the inks, especially aqueous-based modifiers, such as gold nanoparticle dispersions, which are usually prepared in aqueous media. To facilitate making a homogeneous composite in such cases, electrodes can be modified by drop casting, as detailed in option B.

? TROUBLESHOOTING

(B) **Use of office paper or waxed filter paper**

▲ **CRITICAL** The electrochemical cell is represented by a drop of sample added on top of the three electrodes (Fig. 3b).

(i) Add the modifier to the conductive ink (as in option A) or drop-cast it. In the first case, the inks are modified and subsequently screen-printed; in the second case, the electrodes are first screen-printed and then drop-cast with the modifier.

▲ **CRITICAL STEP** Drop casting is a faster procedure than ink mixing. If a modifier can be dissolved or dispersed in aqueous media, drop casting should be favored when working with office paper.

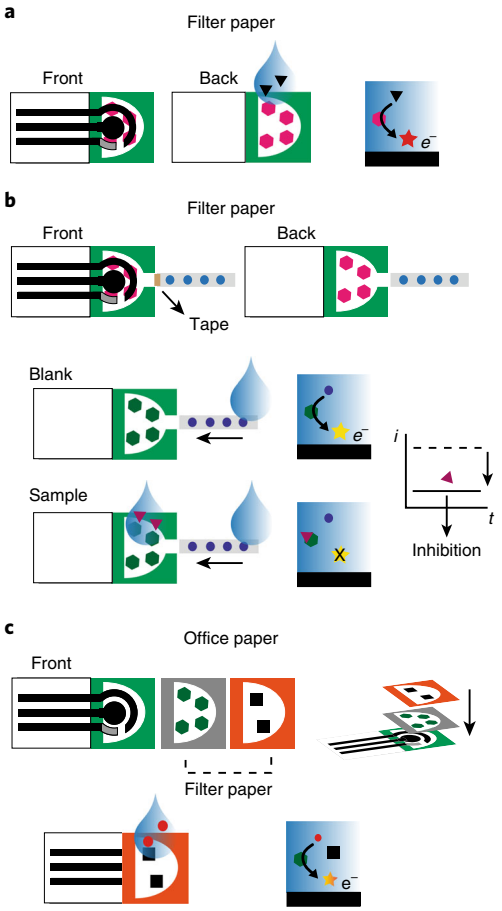

Fig. 4 | Making the paper-based electrochemical device reagentless. The real advantage of paper-based devices is their suitability as reagentless tools for non-specialized end users. **a**, The easiest way to make a paper-based electrochemical device is to load all the necessary reagents onto the testing area where the electrodes are printed. **b**, To develop an integrated inhibition-based biosensor, a possible configuration can be represented by the use of two different porous pieces physically attached with a piece of adhesive tape. The enzyme is loaded into the testing area, where the electrodes are printed, and the substrate is loaded within a specific strip. Briefly, a drop of the sample is cast where the enzyme has been loaded. If the inhibitor is present, the final current will appear decreased depending on inhibitor level. **c**, Office paper can be exploited as an alternative to filter paper when a higher sensitivity is required, and a solution is represented by the use of external porous pads loaded with all the reagents. *i*, current; *t*, time.

(ii) To perform the measurements, cover the testing area with a drop of sample, i.e., >50 µL. As shown in Fig. 3b, when sample is added, the species come into contact with the modified electrode in the area exposed to the air.
▲ **CRITICAL STEP** Standard office paper (80 gm^{-2}) can be replaced with a higher-grammage office paper, i.e., 100 gm^{-2}, or with a waxed filter paper[28]; filter paper can be easily waxed as described in Steps 1 and 2 before the electrodes are screen-printed.
? TROUBLESHOOTING

Making the strips reagentless ● Timing 5–10 min

7 To make these electrochemical paper-based devices reagentless, which means the end user is asked only to provide the sample to be analyzed, the paper must be impregnated with all the compounds necessary to carry out the analytical assay. How this is done depends on whether the device has been made with filter paper (Step 6A) or office paper (Step 6B). Depending on the application, the optimal electrode is designed, and different architectures can be adopted, as shown in Fig. 4 and described in Step 7(A (1D filter paper device), B (2D filter paper device), and C (3D origami office/filter paper)).

(A) **1D filter paper device**

▲ **CRITICAL** This approach is illustrated in Fig. 4a and should be used when direct detection of analyte is performed, i.e., phosphate detection at a carbon black–modified electrode[10], chloride detection at a silver electrode[12], or glucose detection through a glucose oxidase–mediated reaction[29].

(i) Decide which reagents will be used. If you are detecting phosphate ions at a carbon black–modified electrode, you can electrochemically detect phosphate ions by the formation of molybdate–phosphate complex in an acidic environment. The reagents are molybdate ions (to react with phosphate), sulfuric acid (to make the solution acid), and potassium chloride (to provide electrolyte for electrochemical measurement).

(ii) Prepare a solution containing all the reagents needed.

(iii) Add all the reagents to the testing area. The added volume depends on the area of testing. Wait for solvent evaporation.

▲ **CRITICAL STEP** If the reagents are thermally stable, they can be added before the electrodes are screen-printed. When bio-elements are used, i.e., enzyme, antibody, and nucleic acids, the reagents solution must be added successively to screen-printing, as described in option B.

? TROUBLESHOOTING

(B) **2D filter paper device**

▲ **CRITICAL** If an enzymatic-inhibition device is required, the enzyme and the substrate cannot be loaded in the same area as for 1D architecture. The enzymatic reaction, in the presence and in the absence of the inhibitor, must start when the sample is added; during storage, the reaction must not take place. To prevent the reaction from happening before sample is added, the enzyme and the substrate must be loaded onto different substrates that will successively come in contact. For instance, the electrode can be printed onto the filter paper impregnated with the enzyme. The substrate can be loaded onto a separate strip of filter paper or onto another porous material, i.e., nitrocellulose. This approach, called a 2D or 'plane' approach, is illustrated in Fig. 4b.

(i) Decide which reagents will be used. For example, if you are developing an inhibition-based biosensor for organophosphate detection, your reagents will be enzyme (BChE), the enzymatic substrate (butyrylthiocholine chloride), and buffer solution (phosphate buffer containing a supporting electrolyte (KCl)).

(ii) Prepare two solutions containing all the reagents needed, i.e., solution 1 (enzyme) and solution 2 (substrate), both of them dissolved in the working buffer, i.e., 0.05 M potassium dihydrogen phosphate in 0.1 M KCl (pH 8). The concentration of the enzyme (in the range of 1–10 milliunits (mU)) is chosen as the best compromise in terms of conversion rate of the enzymatic substrate (in the range of 10–50 mM) and the resulting signal in the presence of inhibition.

(iii) Cut two pieces of filter paper or another porous substrate, i.e., nitrocellulose. Their size depends on the final size of the device. You should prepare another identical device to be used to measure background signal as described in Step 7B(vii).

(iv) Add 10 μL of solution 1 to the testing area where the electrodes have been previously screen-printed (from Steps 3 to 5). Wait for solvent evaporation.

(v) Add solution 2 to the remaining piece of filter paper or nitrocellulose. Wait for solvent evaporation.

(vi) Combine the two substrates. Put them in physical contact, e.g., use a piece of adhesive tape. Add the sample after combining the two substrates.

(vii) Prepare two identical devices: one to measure a background signal in the absence of target and one to measure the signal recorded in the presence of target, i.e., inhibitor.

? TROUBLESHOOTING

(C) **3D origami office/filter paper**

▲ **CRITICAL** Office paper cannot be used to store the reagents. In this case, to make an office paper–based strip reagentless, it can be coupled to porous materials, i.e., filter paper, by using a 3D configuration. As shown in Fig. 4c, several layers of filter paper can be used to store the enzyme and the substrate. The filter pads are vertically positioned, and the sample is added to the layer on top of the configuration. After that, it flows toward the electrode area, dissolving and reacting with the reagents stored in the underlying layers.

(i) Decide which reagents will be used; they can be the same as those described in Step 7B(i).

(ii) Prepare two solutions containing all the reagents needed, i.e., solution 1 (enzyme) and solution 2 (substrate), both of them dissolved in the working buffer, i.e., 0.05 M potassium

dihydrogen phosphate in 0.1 M KCl (pH 8), if BChE is used. If another enzyme is used, its optimal buffer should be chosen.

(iii) Cut two pieces of filter paper. Their size depends on the final size of the device (suggested 1 cm^2). You should prepare another identical device to be used to measure a background signal in the absence of target and compare this with the signal recorded in the presence of target, i.e., inhibitor.

(iv) Add 10 μL of solution 1 to a piece of filter paper in the area where the electrodes have been previously screen-printed. Wait for solvent evaporation.

(v) Add solution 2 to the remaining piece of filter paper or nitrocellulose. Wait for solvent evaporation.

(vi) Assemble the two pieces of filter paper on top of the office paper–based electrode obtained in Step 5. Use a clip, so that the three pieces are in contact. The piece of filter paper containing the enzyme should be in the middle; the piece containing the substrate should be on top.

? TROUBLESHOOTING

Measuring real matrices ● Timing 1–3 min

8 Drop a small amount of the sample matrix onto the device. This can be done using a pipette, or a blood sample can be transferred directly from a finger-prick to the device,, or the device can be dipped into the solution. The matrix does not need to be treated before performing the measurements. As described in Step 7, the added sample passes through one or more filter paper layers before reaching the electrode area. The presence of the filter paper or other porous cellulosic networks is capable of blocking the gross impurities present in the sample, e.g., soil in river water or cells in whole blood. In addition, as reported in the literature, special papers can be adopted: in 2013, the Henry group adopted VF1 and VF2 blood separation membranes to isolate serum from whole blood[30].

▲ CRITICAL STEP Species radially diffuse within the cellulosic network, and sensor performance is strictly dependent on target position relative to the working electrode. When the porosity of the paper is used as the electrochemical cell, all the analyte will diffuse to the border of the testing area. The duration of a measurement should be optimized by taking into account this feature, which can limit the yield in terms of recorded current (and sensitivity).

? TROUBLESHOOTING

9 Attach the device to a suitable instrument, and measure the signal. The signal is detected by using a portable potentiostat and the most suitable technique, depending on the quantification. For instance, the detection of phosphate ions has been achieved by using cyclic voltammetry, whereas nerve agents have been detected with the use of chronoamperometry. The choice of the working potential depends on the electrode that is used for the quantification, i.e., 0.3 V (versus Ag/AgCl) is enough to detect thiols with a Prussian blue–based screen-printed electrode.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
6A(ii), 6B(ii)	Low sensitivity	Electrode area is not sufficiently high	Use carbon-based nanomaterials to improve the surface that is available for electrochemical processes
7A(iii), 7B(vii), 7C(vi)	Low repeatability	When paper is impregnated with different reagent solutions, the second solution displaces the previously loaded reagents.	Use a single solution that contains all the reagents and/or optimize the volume of reagents to load (depending on the size of the testing area)
8	Low current detected (low sensitivity)	Sample diffuses radially within the paper, far from working electrode	Reduce the duration of experiment
	The inhibition-based biosensor has low sensitivity	High concentration of immobilized enzyme	Reduce the concentration of enzyme according to the detection limit

Timing

Steps 1 and 2, wax patterning the paper substrate: 5 min
Steps 3–5, screen-printing electrodes: 30–40 min
Step 6, using a modifier to enhance and/or tailor the electrodes' performance: 10–15 min
Step 7, making the strips reagentless: 5–10 min
Steps 8 and 9, measuring real matrices: 1–3 min

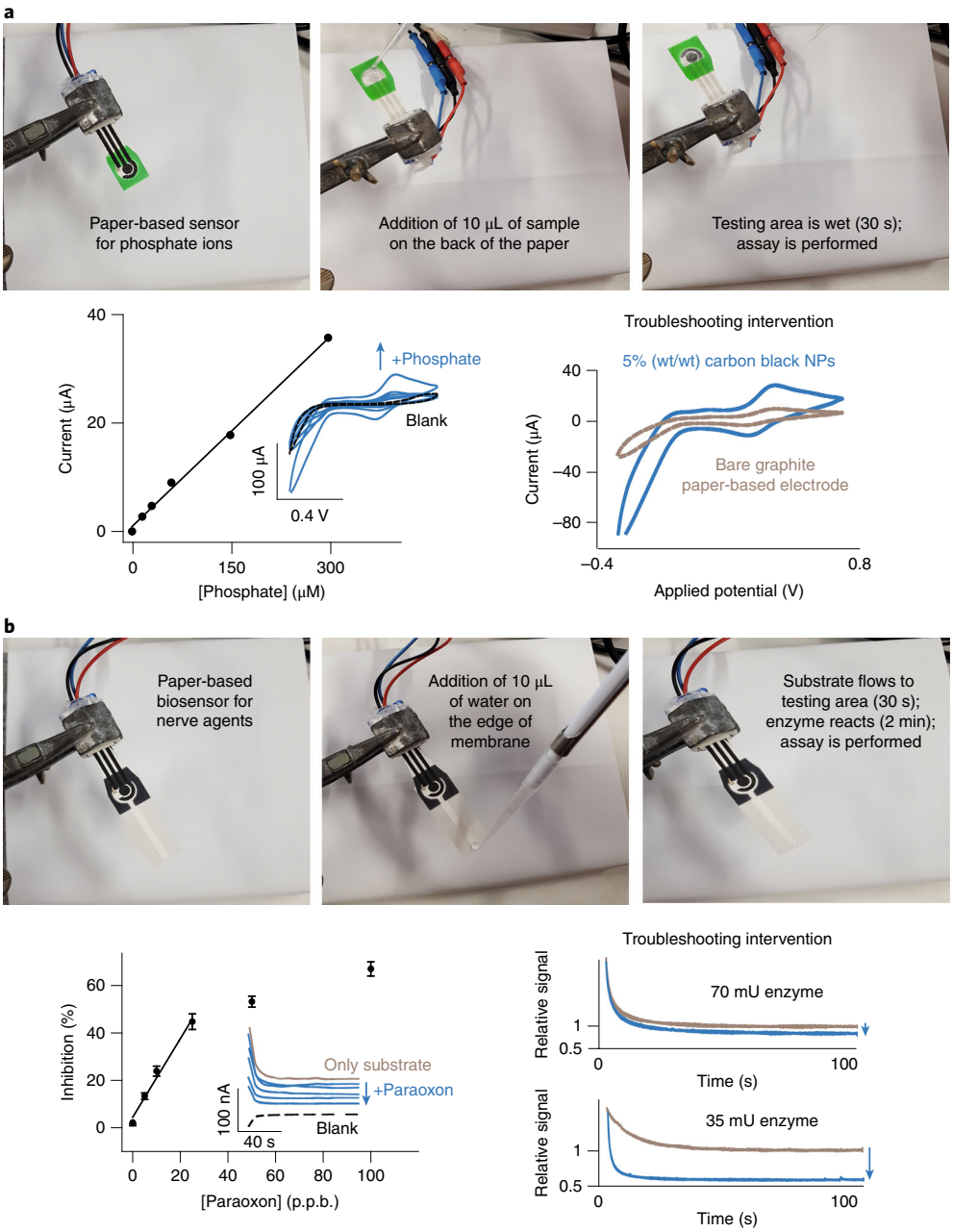

Fig. 5 | Photographs of the end products, expected results, and troubleshooting interventions. a, Photographs of the final paper-based sensor for phosphate detection (top) and a calibration curve in the micromolar range of phosphate (bottom left) obtained by cyclic voltammetry experiments. (Bottom right) Troubleshooting intervention: increase the sensitivity by modifying the electrode with nanostructured carbon black. **b**, Photographs of the final paper-based biosensor for nerve agent paraoxon detection (top), and a calibration curve (bottom left) in the parts per billion range of paraoxon obtained by chronoamperometry experiments. (Bottom right) Troubleshooting intervention: increase the sensitivity by lowering the amount of enzyme units deposited onto the paper-based testing area. NP, nanoparticle. (Brackets indicate the concentration of a species.)

Anticipated results

Phosphate ions have been detected down to the low micromolar range using an electrochemical device printed onto filter paper. In this device, the test area was impregnated with all the reagents needed to detect phosphate ions. Briefly, the paper-based device was modified with 10 μL of a solution containing 0.1 M ammonium heptamolybdate tetrahydrate, 0.1 M potassium chloride, and 0.1 M sulfuric acid. Before carrying out the measurements, water was allowed to evaporate for 10 min at room temperature (20–25 °C) with the aid of a fan. Carbon black was mixed with the graphite ink to improve the analytical performance; 0.25 g of carbon black (N220) powder was manually mixed with 4.5 g of graphite ink for 15 min, until the composite appeared homogeneous. Phosphate ions in untreated water river and industrial water were analyzed just by sampling 10 μL on the back of the paper. A delay of 30 s was applied between the addition of the sample and the start of measurement; it is necessary to dissolve the loaded reagents from the paper and to homogeneously wet the surface of the electrodes. The results are shown in Fig. 5a.

For the development of an enzymatic inhibition–based reagentless biosensor for nerve agent detection, the merging of two different mobility papers allows the production of integrated strips; as described in Step 7, an inhibition-based biosensor can be obtained by following Step 7B or Step 7C (Fig. 4b,c). In all the options, all the reagents are stored in the pieces of paper that are used to make the entire configuration. With these approaches, the tasks of the end user are limited to adding a drop containing the sample and, after an optimized delay time, recording the electrochemical signal with a potentiostat. Here, the anticipated results for a nerve agent biosensor obtained with a 2D configuration are described and shown in Fig. 5b. First, the hydrophilic test area was drop-cast with 5 μL of a 0.05 M phosphate buffered solution (pH 8). After the water evaporated, 2 μL of 17.5 U mL^{-1} BChE was drop-cast over the same test area. Subsequently, a 2 mm × 20-mm Predator membrane strip (which allows a higher liquid flow) was dipped in 1 mL of butyrylthiocholine chloride for 20 min and allowed to dry at room temperature in the dark. The two paper-based substrates were coupled with adhesive tape. The measurement was carried out by pipetting 10 μL of dH_2O onto the edge of membrane, producing the flow of the impregnated butyrylthiocholine chloride toward the detection area, where both the enzyme and electrodes had been placed. 30 s was necessary to allow the liquid to reach the testing area, and 2 min was chosen as the reaction time between enzyme and substrate. Using paraoxon as the organophosphate nerve agent simulant, BChE enzyme (35 mU) loaded onto filter paper was incubated for 10 min with the inhibitor. Successively (without any washing steps), the enzymatic substrate (butyrylthiocholine) immobilized on the Predator membrane was released toward the testing area, where it came into contact with the enzyme. Owing to the close contact between the enzyme and the inhibitor within the filter paper structure, paraoxon was detected down to 2 p.p.b. This limit of detection can be further improved by exploiting the porosity of the paper where the enzyme is stored. For instance, matrices containing 5 μL of inhibitor were created by five steps, in each of which 1 μL was added. This procedure pre-concentrates the target compound; after each addition, the evaporation of the solvent makes the matrix more concentrated, and, after the fifth step, the last microliter of matrix in contact with the enzyme contains a fivefold concentrated inhibitor. The analyses of river water and wastewaters, containing gross impurities, were carried out without any purification, thanks to the presence of the filter paper barrier. The use of paper as the substrate for making reagentless electrochemical strips strictly depends on its features. Paper is capable of storing, filtering, pre-concentrating, and making reactions happen in microenvironments.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data generated or analyzed during this study are included in this published article and its Supplementary Information files.

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Author contributions

S.C. conceived and designed the protocol. S.C., D.M., and F.A. designed, performed, and analyzed the experiments reported in the 'Anticipated results' section.

Competing interests

The authors declare no competing interests.

Additional information

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