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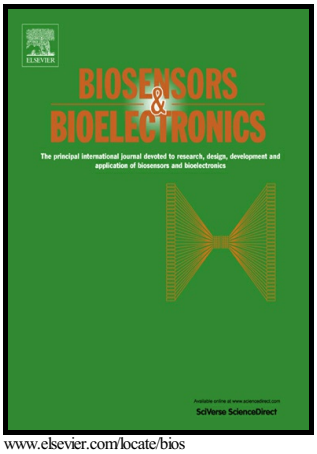

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Fully integrated ready-to-use paper-based electrochemical biosensor to detect nerve agents

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Abstract

Paper-based microfluidic devices are gaining large popularity because of their uncontested advantages of simplicity, cost-effectiveness, limited necessity of laboratory infrastructure and skilled personnel. Moreover, these devices require only small volumes of reagents and samples, provide rapid analysis, and are portable and disposable. Their combination with electrochemical detection offers additional benefits of high sensitivity, selectivity, simplicity of instrumentation, portability, and low cost of the total system. Herein, we present the first example of an integrated paper-based screen-printed electrochemical biosensor device able to quantify nerve agents. The principle of this approach is based on dual electrochemical measurements, in parallel, of butyrylcholinesterase (BChE) enzyme activity towards butyrylthiocholine with and without exposure to contaminated samples. The sensitivity of this device is largely improved using a carbon black/Prussian Blue nanocomposite as a working electrode modifier. The proposed device allows an entirely reagent-free analysis. A strip of a nitrocellulose membrane, that contains the substrate, is integrated with a paper-based test area that holds a screen-printed electrode and BChE. Paraoxon, chosen as nerve agent simulant, is linearly detected down to 3 µg/L. The use of extremely affordable manufacturing techniques provides a rapid, sensitive, reproducible, and inexpensive tool

for *in situ* assessment of nerve agent contamination. This represents a powerful approach for use by non-specialists, that can be easily broadened to other (bio)systems.

1.Introduction

Chemical and biological weapons of mass-destruction represent a serious threat for humankind. Therefore, there is a deep urge to quickly monitor a suspected area. Although many technologies guarantee a very high level of sensitivity and specificity, sometimes they lack portability or easiness. Methods such as mass spectrometry (Ringer 2013), chromatography (Black et al., 1994), infrared spectroscopy (Ewing and Lerner, 2001) and fluorescence (Dale and Rebek, 2006), despite their outstanding performance, display some limitations including the need of laboratory set-up, skilled personnel, sample treatment, and the high cost of instrumentations. These operative drawbacks highlighted lab on a chip technology as an anchor point towards the establishment of fully integrated, user-friendly, and laboratory-free procedures for fast and in situ analysis. The main advantage of similar technology resides in the integration of different approaches based on the use of electrochemical/optical detection, microfabrication, paper as active substrate, as well as novel and attractive (nano/micro/meta) materials (Cummins et al., 2016). These strategies have drawn the attention of most scientists dealing with the development of new fully integrated platforms for autonomous (multi)analysis (Gubala et al., 2011; Luppa et al., 2011; Vashist et al., 2015; Wang, 2006). For example, the Whitesides group reported the exploitation of paper as active support to develop many naked-eye colorimetric assays based on the observation of colour change (Martinez et al., 2009; Pollock et al., 2012; Vella et al., 2012). Microfabrication facilities as well as the establishment of novel materials allowed to create a plethora of *ad hoc* tools for high sensitive, low cost, and in field analysis (Cinti et al., 2015; Green et al., 2016; Medina-Sánchez et al., 2012; Rackus et al., 2015). The first crucial example of an electrochemical paper-based microfluidic device was reported by Dungchai et al. (2009). The use of photolithography allowed them to make hydrophobic areas on filter paper, where reagents (i.e. enzymes) were loaded. Electrodes were screen-printed in order to detect glucose, lactate, and uric acid in biological samples without using additional chemicals.

According to the literature, there is a plenty of novel conceived lab on a chip devices; however, two relevant points should be considered in the development of such sensing systems: i) easiness of use for end-users, and ii) affordability of the device manufacturing.

Currently, the electrochemical detection of nerve agents at lab on a chip level requires several steps during the measurement, including for example addition of reagents. For instance, we have recently

developed a lab on a chip using an electrochemical cholinesterase biosensor embedded into a miniaturized prototype able to sample air, apply a potential, register a current, and give an alarm (e.g. light-signal) in the case of nerve agent presence at μg/L levels (Arduini et al., 2012). However, each measurement needed the addition of enzymatic substrate into the device.

The research group headed by the Du group developed a test strip based on magnetic Fe_3O_4-TiO_2 nanoparticle-based immunosensing to evaluate human exposure to nerve agents (Ge et al., 2013). Although the authors integrated this test strip into an *ad hoc* polymeric case, the evaluation of nerve agent poisoning was carried out by cutting the test area with the captured immunocomplex, placing this onto screen-printed electrode (SPE), and pipetting on the electrode a solution containing thionine and hydrogen peroxide (electrochemical signal amplifiers).

Drechsel et al. (2015) tried to solve the problem of easiness of the measurement by assembling a commercially available acetylcholinesterase biosensor to a prototype composed by an AutoDip reagent pre-storage cartridge integrated with an external vertical actuator. Although the portable lab on a chip platform allowed effortless chlorpyrifos-oxon detection into apple samples, this system required sophisticated fabrication facilities for device development.

Tan et al. (2014) described the design of a microfluidic chip for the detection of Sarin. Yet, the need of sophisticated instrumentation to fabricate the microchip, i.e. CO_2 laser machining system, hydraulic press, and the use of fresh reagents and external pumps to perform the measurements, disregards a reagent free measurement and the affordability of the device manufacturing, as well.

In this context, we took advantage of paper to develop a totally affordable, reagentless, and user-friendly electrochemical device to detect nerve agents. Due to its formidable properties, the "old known" paper is leading the development of innovative and cost-effective all-in-one devices capable to perform multiple tasks such as the storage of the reagents, purification of the sample, and the flow of the samples and reagents without the need of the external pump (Hu et al., 2014; Parolo and Merkoçi, 2013; Yetisen et al, 2013; Xia et al., 2016). With respect to microfluidic devices obtained by using polymeric scaffold, paper offers the enormous advantage to be abundant everywhere without the need of expensive facilities (e.g. photolithography) to fabricate microfluidic platform.

In this overall scenario, we reported the first example of a fully-integrated paper-based electrochemical biosensor able to detect nerve agents exploiting screen-printing technology to print the electrochemical cell on paper as well as wax printing for paper based microfluidics. This biosensor was based on the capability of nerve agents to irreversibly inhibit the butyrylcholinesterase enzyme (BChE) which is able to hydrolyse the butyrylcholine, yielding butyric acid and choline as products. These compounds are not suitable for electrochemical

measurements, thus butyrylthiocholine was used as a substrate being able to provide an electroactive by-product, thiocholine instead of choline, that can be electrochemically detected (Butyrylthiocholine + BChE → Butyric acid + Thiocholine). We exploited ferric hexacyanoferrate, better known as Prussian Blue (PB), for thiocholine detection since it is able to electrocatalyse the oxidization of thiol-containing compounds at low applied potential, overcoming fouling processes occurring at bare carbon electrode (White et al., 2002). In this work, we used a nanocomposite constituted of carbon black (CB)/PB nanoparticles since carbon black acts as a nucleation site for the growth of PB nanoparticles (diameter of 19 ± 3 nm) conferring valuable electrochemical performances (Cinti et al., 2014). In order to develop the fully-integrated device, we exploited: i) a strip of nitrocellulose impregnated with butyrylthiocholine, ii) an electrode printed on waxed-paper test area, iii) a test area infused with a buffered solution and butyrylcholinesterase, and iv) a commercial adhesive tape to attach the nitrocellulose to the test area. This first completely reagentless and disposable lab on a chip platform was exploited to assess the presence of nerve agents. This novel, one-shot, portable lab on a chip was successfully evaluated using standard solutions as well as environmental samples, establishing the high accuracy of the proposed platform.

2. Experimental

2.1. Reagents and equipments

Potassium ferricyanide, ferric chloride, potassium hydroxide, hydrochloric acid (37%), potassium dihydrogen phosphate, butyrylcholinesterase (BChE) from equine serum, butyrylthiocholine chloride and paraoxon (nerve agent simulant) were purchased from Sigma Aldrich, USA. Commercial carbon black (CB) N220 powder was received as a kind gift from Cabot Corporation, Italy. For the interference studies, atomic adsorption solutions of Arsenic and Copper were purchased from Fluka, atomic adsorption solutions of Cadmium and Mercury, ammonium nitrate, and potassium chloride were purchased from Sigma Aldrich. Cyclic voltammetry and chronoamperometry measurements were carried out using a portable PalmSens Instrument (PalmSens, Netherlands) in connection with a laptop.

2.2 Production of Carbon Black/Prussian Blue nanocomposite powder

As reported in our previous paper (Cinti et al., 2014), 1 g of CB powder was suspended in a 10-mL solution containing 0.1 M potassium ferricyanide prepared in 0.01 M HCl. After 5 min of stirring, 10 mL of 0.1 M ferric chloride prepared in 0.01 M HCl was added, and the resulting suspension was stirred for 10 more minutes. CB with adsorbed PB, referred in this text as CB/PBNPs, was collected by centrifugation and washed with 0.01 M HCl (5 cycles). The precipitate was collected and dried in an oven at 100°C for 1.5 h. The obtained CB/PBNPs powder was mixed (ca. 15 min) with the graphite-conductive ink in order to obtain a 5%(w/w) modified ink. The nano-sized dimension of the synthesized CB/PBNPs nanocomposite was confirmed by SEM analysis (Supporting Information, Figure S1). An average diameter of 28 ± 7 nm was obtained by analyzing the statistical distribution of nanocomposite. This value is in agreement with our previous work (Cinti et al., 2014).

2.3. Fabrication and assembling of the paper-based platform

Firstly, a semi-circular pattern was designed with a drawing software (Adobe illustrator) and it was consequently printed onto a 25 mm x 10 mm filter paper (67 g/m^2, Cordenons, Italy) by using an office wax printer (ColorQube 8580, Xerox, USA) creating the hydrophobic pattern. The wax printed paper was then cured in the oven at 100°C for 2 min, allowing the wax to penetrate the paper creating a hydrophilic area surrounded by a hydrophobic barrier. A three-electrode system was screen-printed onto the hydrophilic area by using a screen printer (245 DEK, UK). Ag/AgCl ink (Electrodag 477 SS) was used as the pseudo-reference electrode, while, the working (4 mm diameter) and counter electrodes were obtained by printing carbon ink (Electrodag 421) containing 5% (w/w) CB/PBNPs powder. All the conductive-inks were purchased from Acheson, Italy. After the electrodes were screen-printed, the hydrophilic test area was firstly drop cast with 5 µL of a 0.05 M phosphate buffered solution (pH 8) and, when it dried, a 2-µL butyrylcholinesterase solution was drop cast over the same test area. Meanwhile, a Predator® membrane (Pall Corporation, USA), 25 mm x 2 mm, was dipped in a solution containing butyrylthiocholine chloride for 20 min and it was allowed to dry at room temperature in the dark. After both the Predator® membrane and filter paper were dried, they were assembled together with an adhesive tape (Fig. 1A). We have estimated the cost of a single paper-based SPE to be approximately equal to 0.018 euro (much cheaper than the commercial SPEs, i.e DropSens, with a cost of about 2 euros per device). In Supporting Information, we have provided the detailed costs of materials utilized in producing a paper-based SPE. Furthermore, Figure S2 displays the dimensions of the final device.

2.4. Measurements of nerve agent simulant using the paper-based platform

Because we correlated the concentration of nerve agents with the decrease of the BChE-biosensor response upon inhibited enzyme, the quantification of nerve agent simulant was conducted by involving the use of two platforms, comparing the non-inhibited (blank) to the inhibited chronoamperometric signal, respectively I_A and I_B. The blank signal was obtained by pipetting 10 µL of distilled water on the edge of the Predator® membrane where butyrylthiocholine chloride was physically adsorbed (Fig. 1B). Water flow caused butyrylthiocholine chloride to move toward the test area (Fig. 1C). When the flowing butyrylthiocholine chloride reached the intersection between Predator® membrane and waxed-filter paper, it began to wet the testing area where BChE was adsorbed and the electrodes were printed (Fig. 1D). When the liquid arrived to the testing area (ca. 30 s), 2 min were waited to allow the enzymatic reaction to occur, producing the electrochemically detectable by-product thiocholine (Fig. 1E). In order to measure inhibitor concentration, the testing area was firstly pipetted with 5 µL of a water-based solution, which contained the nerve agent simulant. After the enzyme was inhibited, the current signal was acquired by following the same procedure explained previously for the blank-measurement (Fig. 1B, C, D) revealing a reduction of the recorded signal due to the decrease of enzyme activity (Fig. 1F). All the measurements were carried out by using a portable potentiostat and a laptop as reported in Figure S3 (Supporting Information). The chronoamperometric signal was recorded after 100 s. The inhibition percentage was evaluated as a relative decay of the biosensor response by using the equation (1):

(1) $$I\% = (I_A\text{-}I_B/I_A) \times 100$$

(Fig. 1)

3. Results and discussion

3.1. Electrochemical characterization of CB/PBNPs-paper-based SPE

Nerve agent detection was carried out by exploiting their capability to irreversibly inhibit BChE, thus affecting the enzymatic reaction (2):

(2) **Butyrylthiocholine + BChE → Butyric acid + Thiocholine**

To evaluate this inhibition, the electroactive enzymatic by-product (Thiocholine) was quantified in the absence and in the presence of a nerve agent simulant using SPE modified with PB (Ricci et al., 2004; Ricci and Palleschi, 2005). However, PB is not the unique mediator to detect thiocholine. For instance, cobalt phthalocyanine (CoPc) is an interesting mediator that has been largely utilized for this purpose (Wring et al., 1990; Alonso et al., 2012; Cinti et al., 2016a). Although CoPc is a valuable electrochemical mediator towards thiols detection, we directly compared the analytical performance towards thiocholine by using SPEs modified with PB and CoPC highlighting a higher stability and sensitivity of PB with respect to CoPc (Arduini et al., 2006). The need of the most sensitive platform is linked to the fact that paper network hinders the diffusion of ions. In our recent work, we found that the diffusion coefficient of a model electroactive analyte (i.e. ferricyanide) decreases 3-fold when paper replaces plastic in SPE fabrication (Cinti et al., 2016b). We chose PB in form of nanoparticles for its enhanced surface-to-volume ratio and increased electrochemical performance associated with the use of nanomaterials. In addition, we observed that CB provides an ideal substrate to grow PBNPs, obtaining a CB/PBNPs nanocomposite as a smart working electrode modifier for hydrogen peroxide detection (Cinti et al., 2014). Although we already electrochemically characterized CB/PBNPs nanocomposite drop cast on SPE, in the present work, the paper-based SPE was modified by mechanically mixing CB/PBNPs and the conductive graphite-based ink (5% (w/w)). The use of CB/PBNPs in the ink is due to the use of filter paper as substrate to print screen-printed. Indeed, if a paper-based SPE is used, we distinguish two electrode surfaces: one in contact with the paper (bottom), the other exposed to air (top). When paper is wetted by the sample, the electrochemical signal arises from the SPE surface that is in physical contact with paper, and then with the solution to analyze (Supporting Information, Figure S4). Using this configuration, the usually employed drop casting modification is not effective because there is no interaction between the solution and the SPE exposed to the air (eventually modified). Since it is known in the literature that a substantial difference exists between electrodes modified by drop-casting or ink-mixing (Ricci et al., 2003), we characterised this novel sensor using CB/PBNPs by cyclic voltammetric experiments, carried out in phosphate buffer solution (pH 7.4) at different scan rate values (from 25 to 1000 mV/s).

(Fig. 2)

As displayed in Fig. 2A a couple of reversible peaks can be observed for CB/PBNPs-SPE. These redox peaks correspond to the Fe(II)/Fe(III) couple; more specifically, the cathodic peak is due to the reduction of PB to form Prussian white (PW) while the anodic peak is related to PW oxidized back to PB (Karyakin, 2001). As displayed in the upper inset of Fig. 2A, the peak current intensities, both cathodic and anodic, increased linearly with *v* up to 500 mV/s, indicating a redox process confined by the surface (Compton and Banks, 2011). The lower inset of Fig. 2A displays the peak-to-peak separation, which appeared roughly constant for scan rates lower than 500 mV/s, while at higher scan rates a wider separation was observed, indicating the limitations arising from charge transfer kinetics (Laviron, 1979). In Fig. 2B the cyclic voltammograms using bare SPE and CB/PBNPs-SPE in absence and in presence of enzymatic product thiocholine was reported. By using a bare SPE, only a small increase of the oxidative current was observed at the potential close to 0.4 V. The electrocatalytic behavior of PBNPs toward thiocholine oxidation was highlighted by an increase of the anodic peak and a decrease of the intensity of the cathodic peak during the voltammetric scan. In details, the presence of the enzymatic product thiocholine allowed an increase in the concentration of PW at the surface of working electrode, resulting in an increase of the anodic peak current. On the contrary, the cathodic peak current was proportional to PB at the surface of working electrode, which decreased in presence of thiocholine. The modification of SPE with CB/PBNPs nanocomposite thus led to the detection of the enzymatically produced thiocholine at low potential (around 0.2 V) with improved sensitivity. This demonstrated that the modification of the paper-based SPE with CB/PBNPs directly in the ink allowed to create an effective thiocholine sensitive platform suitable for the production of cholinesterase biosensors.

3.2. Optimization of the experimental parameters towards biosensor realization

Once the effectiveness of CB/PBNPs was established, we optimized the operating conditions, i.e. pH, applied potential, enzyme concentration, and reaction time, in order to provide a sensitive, repeatable, and time-saving platform. Chronoamperometric studies, reported in Fig. 3, were performed using a 5 mM butyrylthiocholine solution, directly pipetted onto the paper testing area where the electrodes were printed and BChE adsorbed.

(Fig. 3)

As shown in Fig. 3A, the pH effect on enzymatic activity was evaluated in the interval ranging from 7 to 8, being the values of 7 (Parsajoo and Kauffmann, 2013), 7.4 (Zhang et al., 2015; Fang et al., 2016), and 8 (Shulga and Kirchhoff, 2007; Hossain et al., 2009) usually reported in literature. The data achieved demonstrated that pH values in the considered range did not produce significant differences in terms of both sensitivity and repeatability, thus further studies were carried out at pH 8. Furthermore, the optimal applied potential was investigated from 0.2 to 0.4 (vs. Ag/AgCl). As displayed in Fig. 3B, a potential of 0.3 V represents the best compromise between the sensitivity and repeatability. Despite 0.4 V provided a higher current, a potential of 0.3 V gave better relative standard deviation of 10.5 % in comparison with the relative standard deviation of 12.7 % obtained by applying 0.4 V.

Since the inhibition of BChE by nerve agent is irreversible, the sensitivity increased with the decrease of enzyme units (Arduini and Amine, 2014), thus 35 mU was chosen as the minimum enzyme amount to give a well measurable signal (Fig. 3C). Finally, a reaction time of 2 min was selected as the best compromise in terms of sensitivity and time of analysis (Fig. 3D).

3.3. Choice of configuration: drop or all-in-paper approach?

Once optimised the experimental parameters, two measurement configurations were compared to establish the most suitable approach for the detection of enzymatic substrate. We named these approaches as “drop” and “all-in-paper”. The “drop” approach consisted in the pipetting of butyrylthiocholine solution directly on the testing area where BChE was adsorbed, close to the CB/PBNPs-SPE. Instead, the “all-in-paper” approach was obtained by physically adsorbing butyrylthiocholine onto a nitrocellulose membrane (Predator®) connected to the testing area through an adhesive tape. In the latter case, the measurements were carried out by dropping few µL distilled water at the border of the strip, allowing the substrate to flow towards the BChE area. The response of these configurations were evaluated in function of different amount of substrate, dropped or adsorbed (Fig. 4).

(Fig. 4)

As displayed in Fig. 4 we evaluated the response in function of enzymatic substrate amount in the range comprised between 2.5 and 20 mM, observing for both configurations the same value of Michaelis-Menten constant K_M^{app}= (4.2 ± 0.5) mM (Fig. 2b). Considering that nerve agents will irreversibly inhibit BChE, the minimum amount of substrate to achieve high rate of enzymatic

activity should be selected (Amine et al., 2016), thus 10 mM butyrylthiocholine was chosen for nerve agent simulant detection. Furthermore, motivated by the possibility to develop a fully integrated device, the "all-in-one" approach was selected to determine the concentration of nerve agents.

3.4. Detection of paraoxon as nerve agent simulant

To safely evaluate the capability of nerve agent detection by using this paper-based biosensor, paraoxon was chosen as nerve agent simulant, taking into account our previous results that demonstrate its suitability for this purpose (Arduini et al., 2007).

In order to develop an inhibition-based biosensor, the response due to nerve agent presence needs to be compared with the response in absence of inhibitor using Equation 1 reported in Experimental section. To do this, two paper-based platforms were used: i) "blank" for the enzymatic activity measurement without the exposure to inhibitor, and ii) "inhibited" when the platform was employed to detect the inhibitor. In details, in the "blank" platform the enzymatic activity was evaluated by pipetting 10 μL of water at the edge of the strip where 10 mM butyrylthiocholine was adsorbed, followed by the measurement at applied potential. Whereas, to detect the inhibitor, the testing area of the "inhibited" platform was firstly wetted with a small volume, 5 μL, of the nerve agent simulant paraoxon. After 10 minutes of incubation between BChE and paraoxon (incubation time), the same procedure reported for the "blank" platform was adopted in order to record a signal related to the "inhibited" platform. Under the optimized parameters, the all-in-paper biosensor was tested with several concentrations of paraoxon in the range comprised between 5 and 100 μg/L.

(Fig. 5)

As displayed in Fig. 5, the biosensor response resulted linear up to 25 μg/L with a calibration curve described by the following equation: $y = (1.7\pm0.2)\ x + (4.4\pm1.3)$, $R^2=0.987$ where y and x indicate the current and the percentage inhibition, respectively. The detection limit, calculated as the amount of paraoxon that gave 10% of inhibition, was found equal to 3 μg/L.

However, the repeatability calculated on three different replicates with a concentration of 50 μg/L paraoxon gave a relative standard deviation of 9% (Supporting Information, Fig. S6). This achievement is highly valuable, considering the entire hand-made procedure followed to produce this biosensing platform. The detection limit found is slightly higher with respect to other works reported in literature. For instance, the group of Prof. Wang obtained a detection limit close to 0.145 μg/L by using SPEs modified with carbon nanotubes (Joshi et al., 2005), the use of SPEs

chemically modified by PB allowed to reach a detection limit of 2 μg/L (Arduini et al., 2006), and Di Tuoro et al. (2011) detected paraoxon down to 0.86 μg/L by using a carbon paste electrode. However, despite the high limit of detection achieved, our device has many relevant advantages: i) this is the first fully-integrated reagent-free electrochemical device for nerve agents, while the above mentioned methods need diverse steps such as washing, drying, and managing the substrate to perform the electrochemical detection; ii) paper acts as an "active" substrate and, in comparison with the other methods, any sample treatment, such as filtration, dilution, or pH adjustment of the real sample, is required because paper can effectively filter the sample and provide all the reagents necessary for measurement.

In order to evaluate the selectivity of this platform, several ions were tested: 5 μg/L Hg(II), 10 μg/L As(III), 100 μg/L Cu(II), 10 μg/L Cd(II), 10 mM NH_4^+, 10 mM K^+, 10 mM NO_3^-, and 10 mM Cl^-. The concentrations of these ions were chosen according to the concentrations usually found in surface water (Sliva and Williams, 2001; Muniraj et al., 2012; Yan et al., 2013; Peng et al., 2015) as well as to 2008/10 EU. As reported in Figure S5, the decrease of signal was observed only when testing paraoxon 50 μg/L, demonstrating the selectivity towards paraoxon of this platform also in the presence of ions.

Regarding the selectivity among pesticides, it is well known that BChE biosensor is able to detect both organophosphorus and carbamic pesticides, which act as irreversible inhibitors of cholinesterase enzyme, demonstrating its usefulness as a prompt alarm system for people security (Arduini et al., 2010). In addition, BChE showed a higher sensitivity towards paraoxon and chlorpyrifos-methyl oxon with respect to AChE, thus making this enzyme more selective towards paraoxon, a widely used nerve agent simulant (Arduini et al., 2006; Joshi et al., 2005).

In the security field, a powerful analytical tool should tackle the application in military field and civil sector, as well. In this scenario, we have chosen to analyze river and wastewater samples to also demonstrate the suitability of this platform for environmental monitoring of pesticides. River (Aniene, Italy) and paint industrial waste (Tover, Italy) water samples were analyzed without any treatment process, i.e. filtration, dilution, and acidification. We were able to analyze μ-volumes of unfiltered water exploiting: i) the storing capacity of filter-paper which allowed to pre-load the buffer solution, and ii) the filtering peculiarity of paper capable of avoiding impurities, i.e. powder, soil, and dust, to affect the measurements. No inhibition was observed in the real matrices, demonstrating the absence of BChE inhibitors at the detection limit level of the device. Subsequently, both samples were spiked with two levels of paraoxon, 25 and 50 μg/L, compatible with the legal limit of 100 μg/L imposed for pesticides in surface wastewaters

(www.gazzettaufficiale.it/eli/id/2006/04/14/006G0171/sg.). As reported in Table 1, the values of percentage recoveries were satisfactory for both unfiltered matrices.

(Table 1)

The values reported in Table 1 demonstrated the high accuracy of this paper-based biosensor in real matrices. Moreover, the shelf life of the paper-based platform was evaluated, considering the storage stability of BChE and butyrylthiocholine. When fabricated, the assembled platform was stored at 4 °C under vacuum. BChE adsorbed on filter paper retained its activity after 30 days. Indeed, BChE was chosen as biocomponent instead of AChE, because we have demonstrated in our previous paper that BChE-biosensor is characterized by higher storage stability, which is a key point for a future market entry of the device (Arduini and Palleschi, 2012).
Butyrylthiocholine adsorbed on nitrocellulose strip showed an appreciable stability for 15 days, with a stability decrease after 30 days (Supporting Information, Figure S6). Actually, this enzymatic substrate could be easily hydrolyzed due to its high hygroscopic character; this can be overcome by including the use of nitrogen flow before closing the reservoir that contains the biosensor under vacuum (Arduini and Palleschi, 2012).

4. Conclusion

The first example of an electrochemical device fully realized on/with paper for the assessment of nerve agent simulant (paraoxon) in real environmental sites was developed. Paper was utilized as an active substrate where reagents were stored, electrodes were printed, and real samples were analyzed. The peculiarity of different typologies of paper, including filter paper and nitrocellulose immunochromatographic paper coupled with very affordable and common facilities (wax- and screen-printing) and materials (adhesive tape), allowed to fabricate an entirely hand-made, cost-effective, and reagent-free device, that is highly useful for measurements of nerve agents directly in environmental sites. This device provided an easy and cost-effective surveillance of a contaminated site without the use of any chemicals or sample treatment at the level of the end-user, allowing the detection of paraoxon down to 3 µg/L. The successful applications in river and wastewater samples were demonstrated, confirming its dual use in military field (i.e. for nerve agent detection) and civil sector (i.e. organophosphorus pesticide detection), as well. Finally, the fabrication of this device can be easily extendible toward diverse typologies of stand-alone user-friendly sensing platforms.

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Figure Captions

Fig. 1: Schematic representation of the principle working mechanism of the lab on a chip. (A) Side-view of the fully-integrated platform. (B) Pipetting μL-drop of distilled water. (C) Flowing of butyrylthiocholine through the Predator membrane towards the testing area. (D) Crossing of butyrylthiocholine from Predator® membrane to filter paper where testing zone is. (E) Occurring of

enzymatic reaction on the testing area and detection at CB/PBNPs-SPE of the electroactive by-product, I_A. (F) Evaluation of inhibitor by measuring the residual enzymatic activity on the testing area, I_B.

Fig. 2: (A) Cyclic voltammetry experiments carried out in 0.05 M phosphate buffer solution containing 0.1 M KCl (pH 7.4) varying the scan rate in the range between 25 and 1000 mV/s. Insets: (i) Correlation between the intensities of (black) anodic and (red) cathodic current peaks vs the scan rate; (ii) Correlation between the peak-to-peak potential separation *vs* the scan rate. (B) Cyclic voltammetry experiments carried out in the same buffer solution of (A) at a scan rate of 25 mV/s using a bare SPE in absence (black dashed line) and in presence (black solid line) of 50 mU BChE and 5 mM butyrylthiocholine, and using a CB/PBNPs-SPE in buffer (blue solid line), in presence of butyrylthiocholine (green solid line) and in presence of BChE and butyrylthiocholine (red solid line).

Fig. 3: Optimization of the experimental conditions for the chronoamperometric detection of 5 mM butyrylthiocholine by using BChE biosensor. Current response was recorded after 100 s. (A) pH study: 0.05 M phosphate buffer containing 0.1 M KCl, E = 0.3 V (vs. Ag/AgCl), [BChE] = 35 mU, t = 2 min; (B) E study: 0.05 M phosphate buffer containing 0.1 M KCl (pH 8), [BChE] = 35 mU, t = 2 min; (C) BChE units study: 0.05 M phosphate buffer containing 0.1 M KCl (pH 8), E = 0.3 V (vs. Ag/AgCl), t = 2 min; (D) Reaction time study: 0.05 M phosphate buffer containing 0.1 M KCl (pH 8), E = 0.3 V (vs. Ag/AgCl), [BChE] = 35 mU.

Fig. 4: Calibration curve of butyrylthiocholine analyzing 0, 1, 2.5, 5, 10 and 20 mM obtained by using (black triangles) "drop" approach where 5 µL of substrate solution were pipetted onto the testing area and (red circles) "all-in-paper" approach where a nitrocellulose paper-strip was impregnated with substrate and 10 µL distilled water were pipetted at the edge of this strip allowing substrate moving towards testing area. Butyrylthiocholine was prepared in 0.05 M phosphate buffer in 0.1 M KCl (pH 8), 0.3 V (vs. Ag/AgCl), reaction time of 2 min and current recorded after 100 s.

Fig. 5: Chronoamperometric curves obtained analysing: (i) 0, (ii) 5, (iii) 10, (iv) 20, (v) 50 and (vi) 100 µg/L of paraoxon prepared in distilled water. Curve (vii) represents the measure of buffer without butyrylthiocholine. Inset: Calibration curve obtained by analyzing paraoxon in the range comprised between 5 and 100 µg/L prepared in distilled water. Inset: Measures were performed by inhibiting the enzyme with 5 µL of paraoxon solution for 10 min, then 10 µL distilled water were

pipetted at the edge of this strip allowing substrate (10 mM butyrylthiocholine) moving towards testing area. Experimental conditions are the same reported in caption of Fig. 4.

Figures and tables

Figure 1

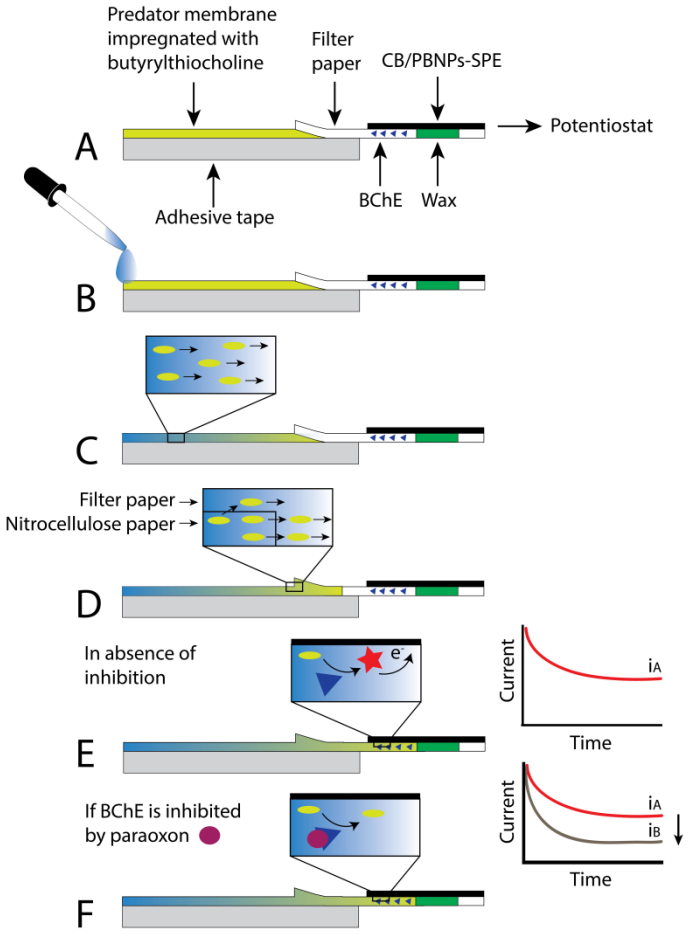


Figure 2

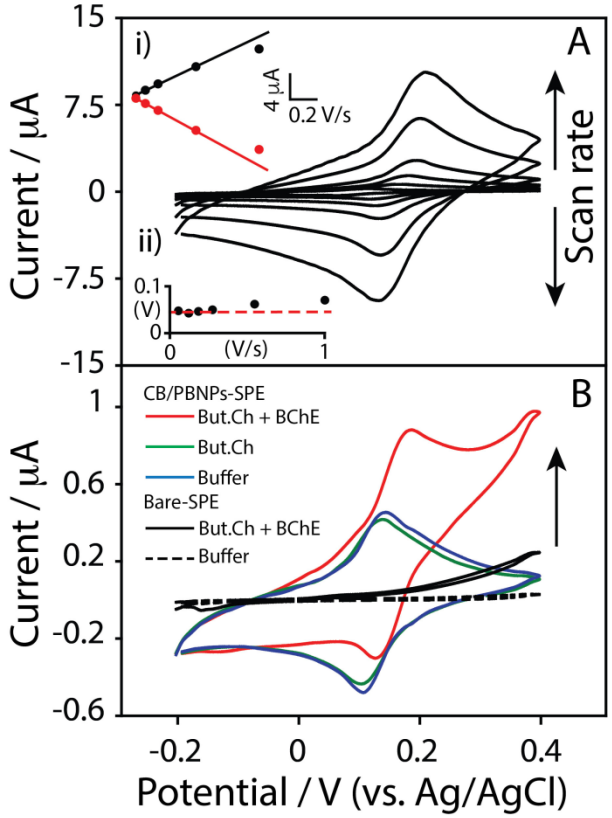


Figure 3

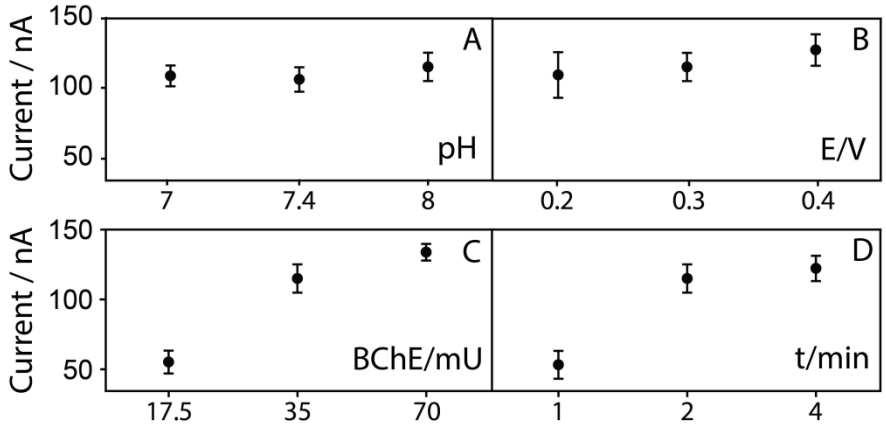


Figure 4

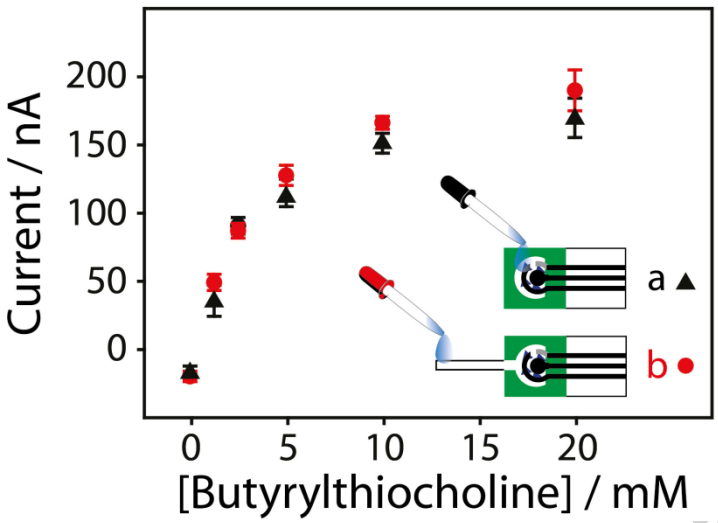
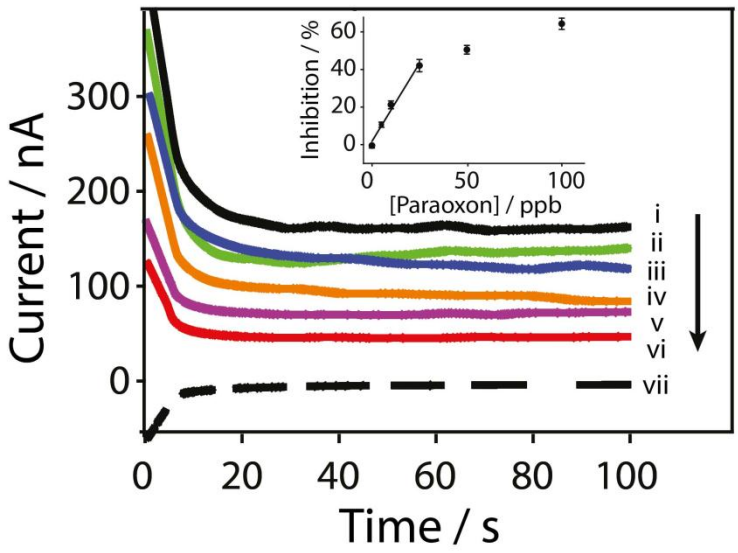


Figure 5



Graphical Abstract

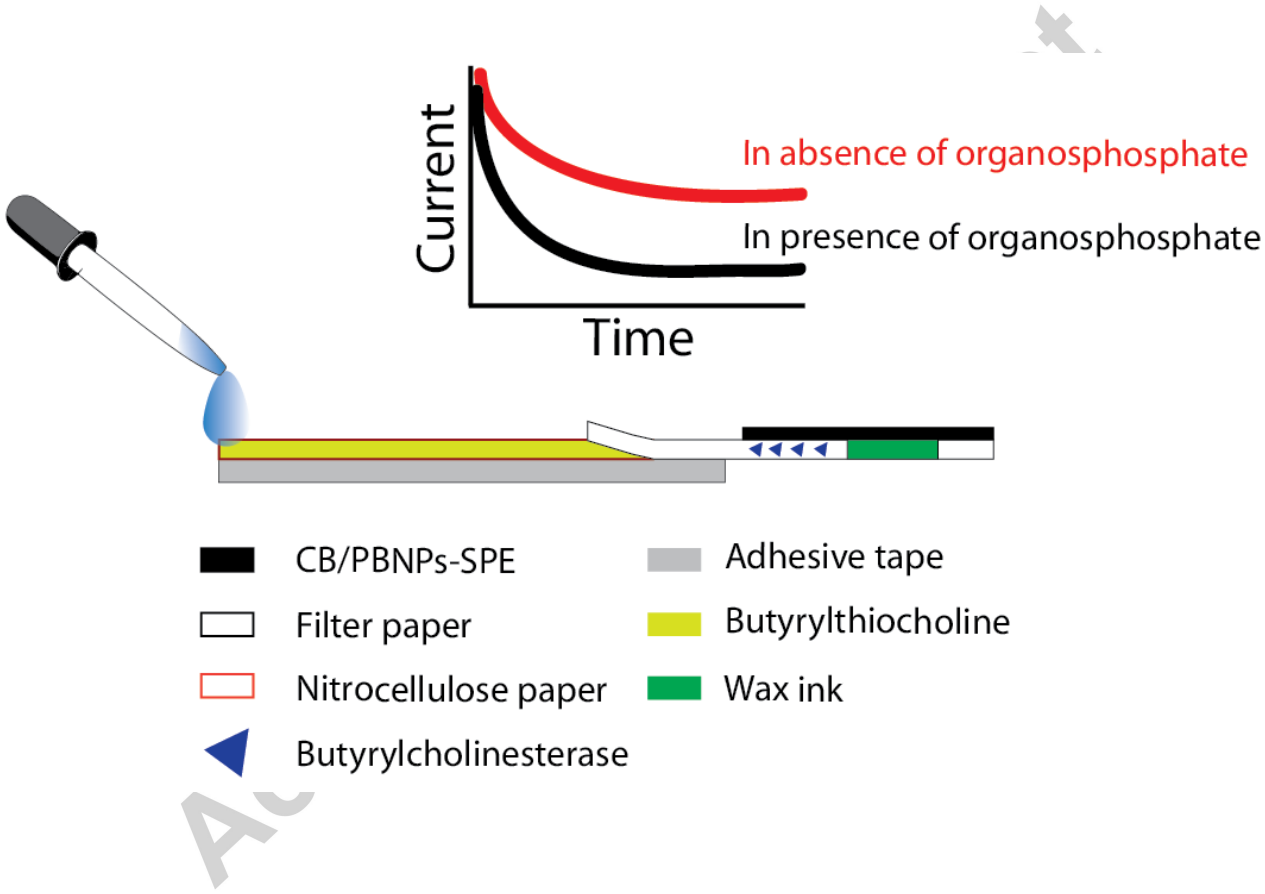


Table 1: Recoveries value obtained in untreated river water and wastewater.

Sample	**River water** (Aniene, Italy, Rome) 	**Wastewater** (ToverItalia, Italy, Rome) 
% Recovery (25 ppb spiking)	89±8	92±8
% Recovery (50 ppb spiking)	94±8	96±7

Highlights

- Fully paper-based nerve agent electrochemical biosensor
- Easy fabrication route
- Reagentless all-in-paper approach
- User-friendly and laboratory-free platform
- Concept highly extensible to others (bio)systems