

# Electrochemical Biosensors for Chemical Warfare Agents

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**Abstract** Since the introduction of modern CWAs at the beginning of 20th century, there has been a continuous interest in the development of robust and reliable analytical tools for the detection of these agents, to provide early alarm in case of terroristic attacks, as well as to monitor their presence in the environment and prevent contamination. Nevertheless, some powerful analytical techniques, including chromatographic methods and mass spectrometry, may not be well suitable for field applications and fast early warning, due to the lack of portability, power requirements, long response time and expensive procedures. In this context, electrochemical (bio)sensors offer advantages in terms of high sensitivity, miniaturization, integration, low cost, and power requirements. The aim of this chapter is to highlight the important issues of electrochemical (bio)sensors for fast and cost-effective detection of CWAs in the field, considering the main advantages and limitations of this technology, and the last trends in nanotechnology, lab-on-chip, and functional materials.

**Keywords** Electrochemical (bio)sensors · Chemical warfare agents · Early warning

## 1 Introduction

Chemical warfare agents (CWAs) are highly toxic synthetic chemicals that can be dispersed in the environment as gas, liquid or aerosol or adsorbed to particles to become a powder [1].

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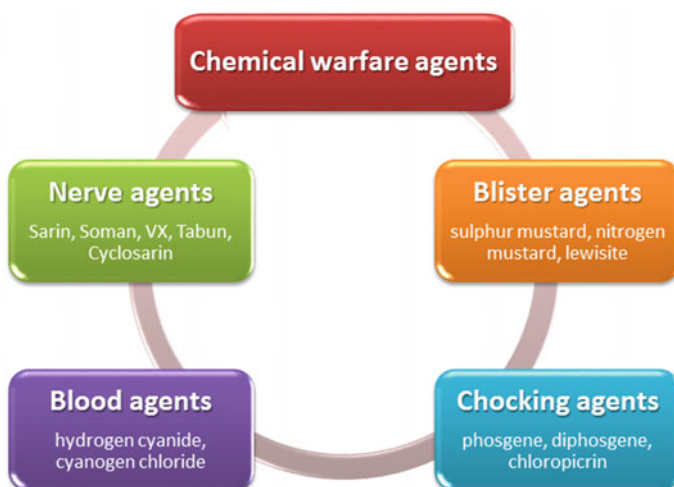
CWAs have been deliberately produced and employed in the battlefields during the 20th century with the purpose of killing or debilitating living organisms. The Chemical Weapons Convention (CWC) has classified them based on their volatility, chemical structure or the physiological effects produced on humans. Regarding the latter feature, CWAs are classified in nerve agents, blister agents or vesicants, blood agents, and choking or pulmonary agents, as reported in Fig. 1.

In addition, a special attention is devoted to toxins, which have been largely exploited as potential chemical weapons, according to the Organisation for the Prohibition of Chemical Weapons (OPCW). In Table 1, different CWA groups, their persistence, and rate of action are reported, according to OPCW.

Thanks to the knowledge of synthetic chemistry, a considerable progression in the development of chemical compounds has occurred in the past century in human conflicts (Fig. 2). This common chemical and scientific knowledge allowed an increasing sophistication of CWAs, as demonstrated by the replacement of chlorine ( $\text{Cl}_2$ ) by phosgene ( $\text{COCl}_2$ ) and then by mustard gas.

The first use of chemicals as mass destruction weapons goes back to WW I (1915), when large amounts of chlorine-containing compounds and gas warfare were released by German military forces in Belgium, causing ~91 000 deaths (~1.3 million casualties) [2]. Warfare chemicals, including phosgene, hydrogen cyanide, arsenical compounds, sulphur mustard, and lewisite have been exploited until the WW II.

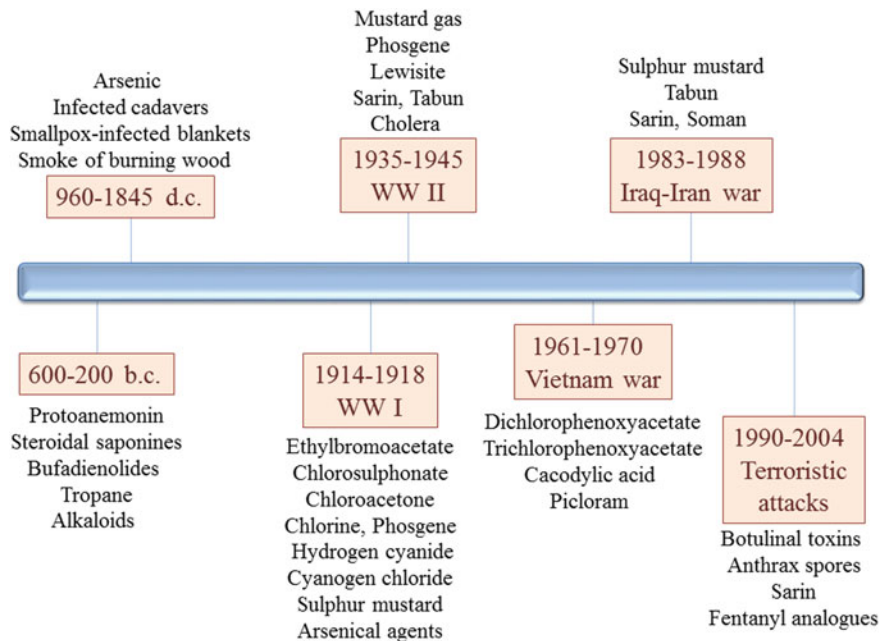
After WW II, chemical weapons were employed on a number of occasions: mustards were used during the soviet intervention into Afghan War (1978), and also used against rebels in Chad (1987); nerve agents and blister agents were used during Iran-Iraq War (1980–1988). Iraq used Sarin, hydrogen cyanide, and sulphur mustard against Iraqi Kurds. The use of chemical warfare agents, unfortunately,



**Fig. 1** CWAs classification

**Table 1** CW Agent Group, persistency and rate of action

CW agent group	Persistency	Rate of action
<i>Nerve agents</i>		
Tabun (GA)	High	Very rapid
Sarin (GB)	Low	Very rapid
Soman (GD)	Moderate	Very rapid
Cyclosarin (GE, GF)	Moderate	Rapid
VX	Very high	Very rapid
<i>Blister/vesicant agents</i>		
Sulfur mustard (H, HD)	Very high	Delayed
Nitrogen mustard (HN)	High	Delayed
Phosgene oxime (CX)	Low	Immediate
Lewisite (L)	High	Rapid
<i>Blood agents</i>		
Hydrogen cyanide (AC)	Low	Rapid
Cyanogen chloride (CK)	Low	Rapid
Arsine (SA)	Low	Delayed
<i>Choking/pulmonary agents</i>		
Chlorine (Cl)	Low	Variable
Phosgene (CG)	Low	High
Diphosgene (DP)	Low	Delayed
Chloropicrin (PS)	Moderate	Rapid



**Fig. 2** Graphical timeline of CWAs use and related dates

was also extended to the terrorist attack: the Japanese cult group AUM Shinrikyo used the Sarin gas in Tokyo subway in 1995. After the tragic events at New York (2001) and at London (2005) the possibility of CWAs being used as means of terrorism is real.

In this overall scenario, due to the high number of exposures to CWAs through different sources (e.g. recent terrorist attacks or already contaminated sites) and to their chemical broad spectrum, there is an increasing interest in the development of highly sensitive, selective, contactless, and early detection systems for low concentrations below the median lethal dose ( $LD_{50}$ : dose required to kill half of the members of a tested population).

In this context, (bio)sensor technology has a great potential to address these challenges, providing the development of tailor-made small and portable instruments, with adequate sensitivity and selectivity, reasonable cost and fast time response, and powerful ability to identify CWAs. (Bio)sensors demonstrated to possess adequate detection methodologies to (i) detect the chemical species of weapons used in terroristic attacks; (ii) measure them in exposed people to early identify the chemical contamination; (iii) monitor their presence in the environment to provide warning on contaminated sites and thus prevent contamination. The detection of CWAs is generally carried out in dedicated centralized laboratories using large and expensive instrumentations, such as gas chromatographs coupled with mass spectrometers (GC-MS). Even if these laboratory set-up methodologies demonstrated to be useful and sensitive tools to evaluate the pollution of a contaminated site, their features do not match the requirements of an early detection in case of a terroristic attack.

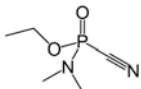
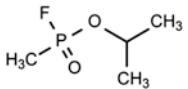
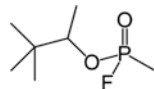
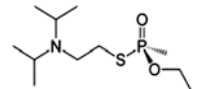
In addition, because sample collection, transport, and subsequent laboratory analysis are time and cost consuming processes, faster and cheaper analytical tools, which combine reliability and rapidity of response for the detection of lethal chemicals, are strongly required.

In this context, the rational in using electrochemical (bio)sensors resides in their special features of fast analysis, cost effectiveness, miniaturisation, simple and easy production. This sort of analytical devices can be exploited as detect-to-protect system, since they are conceived to be able to identify CWAs and provide a warning within few minutes from a chemical contamination, without user intervention. In the following paragraphs, a comprehensive description of CWAs and the main electrochemical (bio)sensors for their detection are reviewed.

## 2 Nerve Agents

Among lethal CWAs, nerve agents have had a dominant role since the WW II. Nerve agents acquired their name because they are able to irreversibly inhibit a key enzyme of nervous transmission (Cholinesterase), thus affecting the transmission of nerve impulses in the nervous system. All nerve agents belong to the group of

**Table 2** Physical-chemical properties and toxicity of some nerve agents

	Tabun	Sarin	Soman	VX
				
MW	162.1	140.1	182.2	267.4
MP (°C)	-50	-56	-42	-39
BP (°C)	247	147	167	300
V	600	17 000	3 900	10
LD <sub>50</sub>	4000	1700	300	10
LC <sub>t50</sub>	70	35	35	15

MW molecular weight, MP melting point, BP boiling point; V Volatility (mg/m<sup>3</sup> at 25 °C), LD<sub>50</sub> skin mg/individual, LC<sub>t50</sub> (mg-min/m<sup>3</sup>): lethal concentration and time (respiratory)

organophosphorus compounds (OP), and the most important nerve agents are reported in Table 2.

Among CWAs, nerve agents are preferred in the terroristic attacks because of their high toxicity and easily production; indeed, the Japanese cult group AUM Shinrikyo used Sarin gas in the terrorist attack of 1995 in Tokyo subway. For these reasons, their detection is a crucial concern in security sectors.

In literature, there are basically four different types of electrochemical (bio) sensors for nerve agent detection:

- (i) Biosensors based on cholinesterase enzyme (ChE) inhibition;
- (ii) Biosensors based on organophosphate hydrolase enzyme;
- (iii) Biosensors based on antibody use;
- (iv) Sensors based on direct electrochemical detection.

## 2.1 Biosensor Based on Cholinesterase Enzyme Inhibition

The principal biological role of acetylcholinesterase (AChE) is the termination of the nervous impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. OPs are able to inhibit this enzyme in an irreversible way; thus, measuring the enzymatic activity in absence and presence of the inhibitor, it is possible to quantify the inhibitor present in the analysed sample.

From the historical point of view, the first biosensing system based on cholinesterase enzyme inhibition was reported on Analytical Chemistry by Guilbault in 1962 [3]. This biosensing tool was constructed for the detection of nerve agents, using cholinesterase enzyme as biocomponent and a classical platinum electrode as working electrode. Since 1962, several biosensors have been developed for nerve agent detection based on cholinesterase inhibition. Searching the articles in the period 2011–2015 using as keywords “biosensor” and “cholinesterase” and

“organophosphate” on Scopus, more than 100 papers are reported, demonstrating to be a hot topic. These papers are principally focused on electrochemical biosensors for OP pesticide detection, while only few papers are focused on nerve agent compound detection such as Sarin, Soman, Tabun, and VX. This difference is due to the high level of safety required to measure nerve agents.

In this chapter, these biosensors based on cholinesterase inhibition were classified in bi-enzymatic and monoenzymatic, highlighting the biosensors specifically applied for nerve agents detection.

### 2.1.1 Bi-enzymatic Biosensor

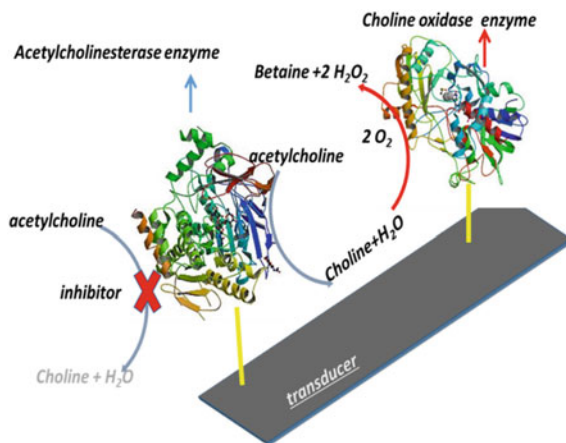
The bi-enzymatic biosensor (Fig. 3) is constructed using:

- AChE, that hydrolyses the substrate acetylcholine to choline and acetic acid;
- Choline oxidase (ChOx), that oxidises choline to betaine with  $\text{H}_2\text{O}_2$  production.

The use of ChOx is necessary in the case of amperometric biosensors because the enzymatic products of the reaction (Fig. 3, choline and acetic acid) are not electroactive. The enzymatic activity can be detected by measurement of  $\text{O}_2$  decrease using Clark’s electrode [4], or  $\text{H}_2\text{O}_2$  increase. In the latter case, the enzymatic product  $\text{H}_2\text{O}_2$  is directly measured amperometrically at around +600 mV versus Ag/AgCl using a platinum electrode [5], or exploiting: (i) redox mediators, such as ferrophthalocyanine [6] and Prussian Blue [7], and (ii) nanomaterials, in order to reduce the applied potential.

As an example, Upadhyay et al. modified a glassy carbon electrode with gold–platinum bimetallic nanoparticles for  $\text{H}_2\text{O}_2$  detection at +0.4 V in amperometric mode. This sensor was then used to assembly a biosensor immobilizing AChE/ChOx by cross-linking with glutaraldehyde [8]. The biosensor was tested with paraoxon-ethyl pesticide, showing a linear range between 100 and 500 nM,

**Fig. 3** Scheme of ChE biosensor based on bi-enzymatic approach



and also with Sarin solution, providing, as expected, an enhanced sensitivity in a linear range between 10 and 100 nM.

### 2.1.2 Monoenzymatic Biosensor

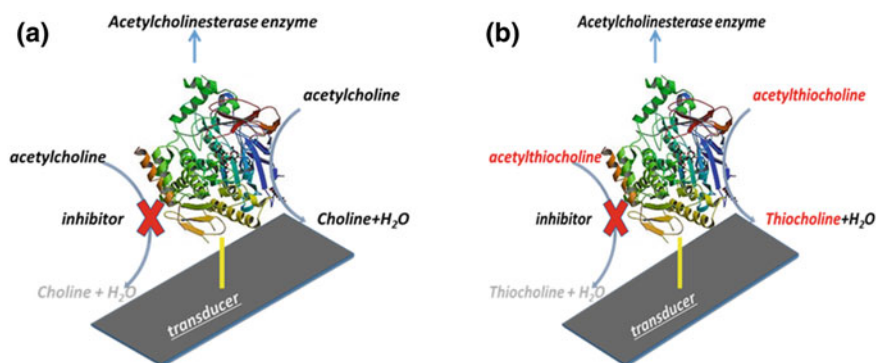
In monoenzymatic systems, the enzymatic reaction can be measured by means of different electrochemical transducers, as reported in Fig. 4:

- (i) Potentiometric: the reaction is monitored by the measurement of pH variation;
- (ii) Conductimetric: the reaction is monitored by measurement of conductivity variation;
- (iii) Amperometric: the reaction is monitored by measurement of current variation due to thiocholine oxidation; indeed, a synthetic substrate (acetylthiocholine) must be used instead of acetylcholine. This synthetic substrate is hydrolysed to acetic acid and thiocholine that is measured, being electrochemically active.

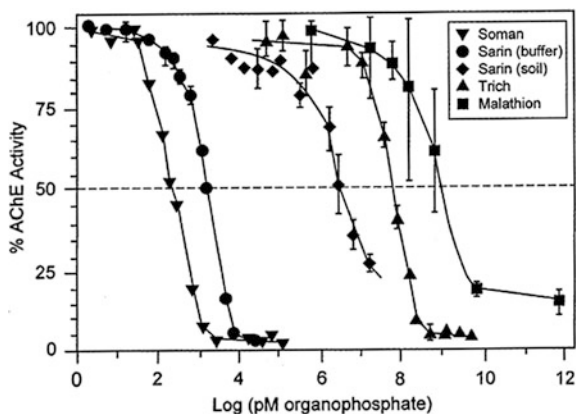
An example of potentiometric sensor is reported on Biosensor and Bioelectronic journal for the detection of OPs. In this case, a silicon-based light-addressable sensor was coupled with biotin-labeled acetylcholinesterase and streptavidin to measure both nerve agents (Sarin and Soman) and pesticides (Trichlorfon and Malathion) (Fig. 5) [9].

Examples of amperometric biosensors have been reported by Pohanka's group [10, 11] and by our group [12].

Pohanka et al. [10] exploited AChE as bioreceptors immobilized on a working electrode of printed sensor by cross-linking with glutaraldehyde and bovine serum albumin. In detail, the screen-printed electrode contains a central platinum dot shaped working electrode with a diameter of 1 mm, a platinum auxiliary and a reference electrode in silver covered with silver chloride. This electrode configuration allowed a measurement of thiocholine at an applied potential of +450 mV for



**Fig. 4** Scheme of ChE biosensor based on monoenzymatic approach using **a** potentiometric/conductimetric or **b** amperometric transducer



**Fig. 5** Calibration curves for Soman, Sarin, Trichlorfon, and Malathion in buffer, and for soil-spiked Sarin, using AChE silicon-based light-addressable potentiometric biosensor. Reprinted with permission from [9]

**Table 3** Limit of detection (LOD) for the selected nerve agents

Agent	LOD (M)
Tabun	$1.48 \times 10^{-8}$
Sarin	$5.88 \times 10^{-10}$
Soman	$1.07 \times 10^{-8}$
Cyclosarin	$9.12 \times 10^{-9}$
VX	$8.51 \times 10^{-10}$

the detection of several nerve agents (Tabun, Sarin, Soman, Cyclosarin, and VX) by AChE inhibition. Limits of detection, shown in Table 3, demonstrated the highest inhibition power of Sarin and VX, and the lowest inhibition power of Soman and Tabun.

A similar sensor was reported by the same authors on *Sensors* journal, optimising the immobilisation procedure by the use of gelatin and glutaraldehyde, and reaching lower detection limits (Sarin 7.41 pM; Soman 6.31 pM, Tabun 61.7 pM, VX 21.9 pM) [11].

Arduini et al. [12] reported the capability to detect Sarin gas by means of a portable commercial available potentiostat (PalmSens) together with the butyrylcholinesterase enzyme immobilised on a disposable graphite screen-printed electrode modified with the electrochemical mediator Prussian Blue (PB). This electrochemical mediator is able to electrocatalyse thiocholine (enzymatic product) oxidation at +200 mV as applied potential. Using the simple procedure of exposing the biosensor to the gas flow of Sarin gas, the system was capable to detect Sarin gas at  $0.1 \text{ mg/m}^3$ , with an incubation time of 30 s and a degree of inhibition of 34 %, demonstrating the high sensitivity of the biosensor.



## 2.2 Biosensor Based on Organophosphate Hydrolase

Biosensors based on the use of organophosphorus hydrolase (OPH) as bioreceptors are classified as substrate-type biosensors. Indeed, while AChE biosensor reveals OP by inhibition and the response is *inversely* proportional to the OP amount, the OPH biosensor detects OPs as a direct substrate and the response measured is *directly* proportional to the OP amount (Fig. 6).

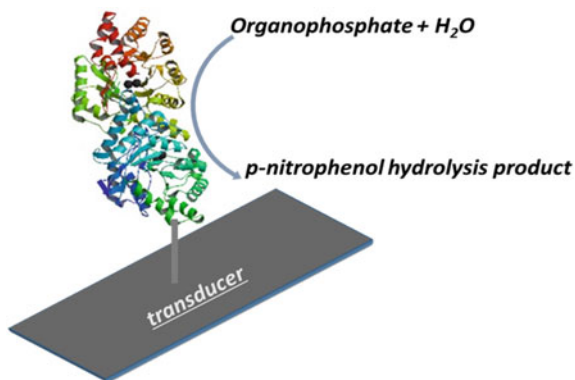
The big advantage of this biosensor relies on the direct measurement of the analyte with a faster detection, but, on other hand, its sensitivity is lower in respect to ChE biosensor.

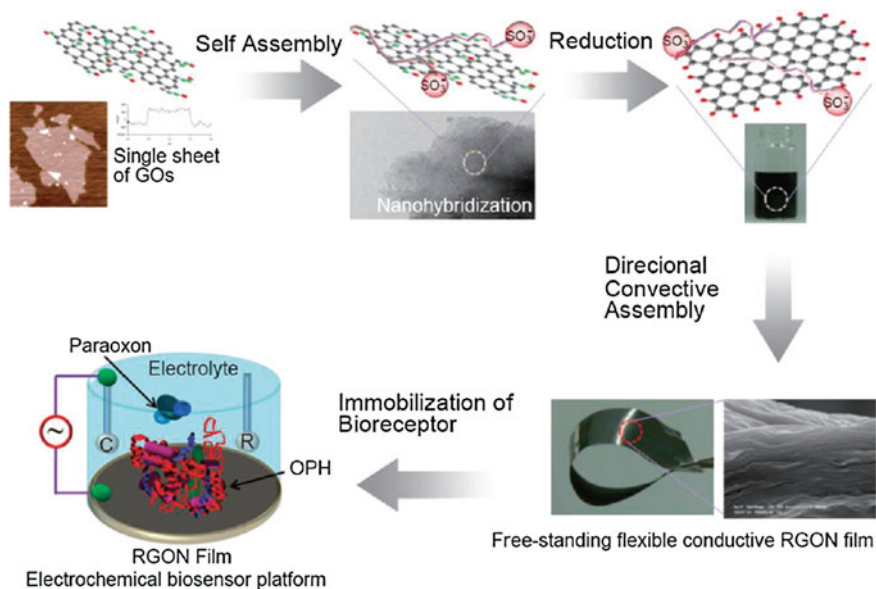
One of the first works on OPH biosensors was published in 1999 on *Analytical Chemistry* journal by Mulchandani's group. OPH was immobilised onto screen-printed carbon electrodes, and OP measurements were provided by the rapid anodic detection of the enzymatically generated *p*-nitrophenol product. This biosensor showed a linearity proportional to the concentration of the hydrolyzed paraoxon and methyl parathion substrates up to 40 and 5  $\mu\text{M}$ , with detection limits of  $9 \times 10^{-8}$  and  $7 \times 10^{-8}$  M, respectively [13]. The same group developed also a microbial biosensor using genetically engineered PNP-degrader *Moraxella* sp. displaying OPH on the cell surface, immobilised onto oxygen electrode. OPH works in tandem with a PNP oxidation machinery to degrade PNP-substituted OPs with simultaneously oxygen consuming. The amount of oxygen consumed is proportional to the analyte concentration, allowing the detection of the analyte. Under optimised conditions, the biosensor showed a detection limit of 0.1  $\mu\text{M}$  (27.5 ppb) using paraoxon as model, with an excellent selectivity against triazines, carbamates and OPs without PNP substituent [14].

A similar limit of detection (0.1  $\mu\text{M}$ ) was also reported by the OPH biosensor embedded in a flow system of Wang et al. [15] for the detection of paraoxon.

More recently, an interesting paper was published on *ACS nano* journal in 2010 by Chol et al. [16]. They reported the preparation of free-standing flexible conductive reduced graphene oxide/Nafion (RGON) hybrid films by a solution

**Fig. 6** Scheme of OPH biosensor





**Fig. 7** Illustration of a procedure to design RGON hybrids and subsequently RGON platform for application in electrochemical biosensors. Reprinted (adapted) with permission from [16]. Copyright (2010) American Chemical Society

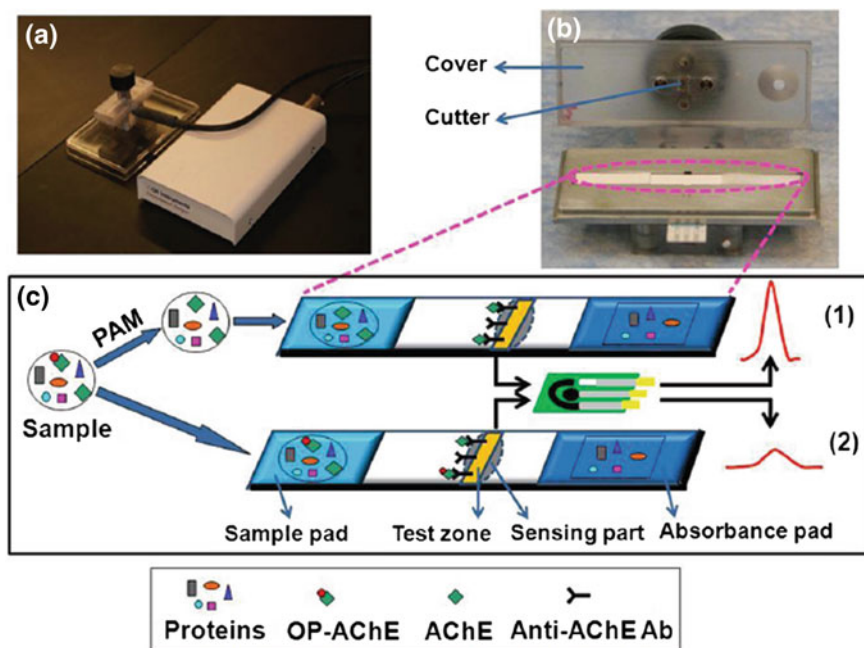
chemistry that utilizes both self-assembly and directional convective-assembly (Fig. 7). The performance characteristics of RGON were exploited immobilizing OPH and examining the amperometric response of the *p*-nitrophenol hydrolysis product at a potential of +0.85 V, reaching a detection limit of  $1.37 \times 10^{-7}$  M, and a response time of <3 s.

As illustrated in all examples reported above, the detection limit is around 0.1  $\mu$ M, which is rather higher than the one obtained using ChE biosensor (nM/pM level), confirming the higher sensitivity of ChE biosensors.

### 2.3 Biosensor Based on Antibody Use

Immunosensors are affinity biosensors based on the measurement of antibody-antigen binding. This kind of biosensors is used in security field to evaluate the exposure of human being to OPs. The mechanism of OPs poisoning involves the irreversible phosphorylation of the hydroxyl group of the serine residue in the active site of AChE, leading to its inactivation. This mechanism is exploited in the development of immunosensors for evaluating the amount of phosphorylated AChE (OP-AChE).

A crucial example of immunosensors for OP detection was described by Du et al. [17]. They developed an integrated lateral flow test strip based on an electrochemical sensor (LFTSES) for biomonitoring of OP exposure, where anti-AChE antibody are immobilised on the immunochromatographic strip (Fig. 8). Serum samples of exposed people are added on the pad, and binding of both AChE and OP-AChE to anti-AChE antibody are electrochemically evaluated adding the enzymatic substrate acetylthiocholine. The proposed immunosensors were able to provide parallel measurements of post-exposure and baseline AChE enzyme activity, and reactivation of the phosphorylated AChE was exploited to measure the total amount of AChE (including inhibited and active) which serves as a baseline (control). Therefore, the quantification of phosphorylated adduct (OP-AChE) is realized by subtracting the active AChE from the total amount of AChE. These immunosensors represents a highly interesting and promising example of point-of-care tools for the diagnosis of OP pesticide poisoning and nerve agent exposure, useful to the first responders for the rapid identification of victims in a nerve agent attack and consequently for a prompt medical treatment.



**Fig. 8** a Entire portable analytical system, b LFTSES device, and c schematic illustration of the principle of LFTSES device. Reprinted (adapted) with permission from [17]. Copyright (2012) American Chemical Society

## 2.4 Sensors Based on Direct Electrochemical Detection

Electrochemical sensors can be an alternative to electrochemical biosensors, with the advantage to overcome the use of bioreceptors and the relative problem of storage stability, even if with the drawback of a lower selectivity due to the absence of the bioreceptor.

The use of electrochemical sensors for OP detection is largely demonstrated by Liu et al. [18], which exploited a carbon paste electrode to determine OP using the adsorptive stripping voltammetric technique. They exploited the ability of OPs to be strongly adsorbed on the surface of the carbon paste transducer in order to pre-concentrate them on the surface of the working electrode. As a result, a successive desorption step allowed to obtain a peak, which height is proportional to the amount of OP detected. This sensor showed a linear range of detection between 1 and 60  $\mu\text{M}$ , using methyl parathion as organophosphate model (with 2 min of adsorption time), and a detection limit of 0.05  $\mu\text{mol/L}$  with 10 min of adsorption time.

Based on the same principle, the authors developed a similar sensor using an electrode modified with zirconia nanoparticles, exploiting the strong affinity of zirconia for the phosphoric group. The improved sensor showed a linear range over the 5–100 ng/mL (ppb) using methyl parathion as organophosphate model with 2-min of adsorption time, and a detection limit of 1 ng/mL with 10-min as adsorption time [19].

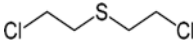
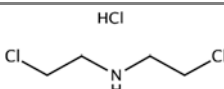
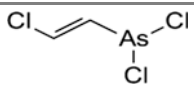
## 3 Blister Agents

Blister or mustard agents (MAs), are one of the most common CW agents, and are defined as blistering compounds owing to the similarity of the lesions caused by these substances to burns and blisters.

MAs are compounds able to generate toxic effects on living organisms, to persistently contaminate soils and water, and embrace *sulphur mustards*, such as Yperite (bis(2-chloroethyl)sulphide), and *nitrogen mustards*, such as HN1 (bis(2-chloroethyl)ethylamine), HN2 or Mustine (bis(2-chloroethyl)methylamine), and organic *arsenical Lewisite* (dichloro (2-chlorovinyl)-arsine) (Table 4). The name mustard agents arises from impure weapons-grade material, which has an odour similar to that of garlic or horseradish mustard [20]. Since 1993, CWC has promulgated several regulations, with new implementations until 1997, to prohibit the production and the use of chemical weapons, including mustard agents.

During the war between Iran and Iraq in 1979–88, Iraq used large quantities of chemical agents, leading to about 5 000 Iranian soldiers killed, 10–20 % by mustard agents, and 40 000 to 50 000 injured. However, incidents are still annually occurring in the neighbourhood of Sweden, mainly involving fishermen exposed to mustard agents brought to the surface by fishing nets. Indeed, the background is found in the dumping of chemical weapons after the WW I in waters off the Danish and Swedish coasts.

**Table 4** Physical-chemical properties and toxicity of mustard agents

	Sulfur mustard	Nitrogen mustard	Lewisite
			
MW	159.1	204.5	207.3
MP (°C)	14.5	-3.7	-18 (trans/cis mix)
BP (°C)	228 (decompose)	256 (decompose)	190 (trans/cis mix)
V	610	121	4480
LD <sub>50</sub>	100	10	30
LC <sub>t50</sub>	1000–1500	1500	1400

MW molecular weight, MP melting point; BP boiling point; V Volatility (mg/m<sup>3</sup> at 25 °C), LD<sub>50</sub> (mg/kg) lethal dose (skin), LC<sub>t50</sub> (mg-min/m<sup>3</sup>): lethal concentration and time (respiratory)

As a consequence, MAs and their degradation products, represent nowadays highly toxic environmental pollutants, being persistent in the environment for long term and causing high toxicity on biota and humans. MAs exposure may occur across skin, respiratory system, genital tract, ocular surface and gastrointestinal system, with serious acute and long term complications [21]. MAs are also known as DNA alkylating agents, being able to generate cytotoxicity, mitosis inhibition, mutagenesis, carcinogenesis, and colinomimetic effects. These mechanisms lead to final DNA damages, oxidative stress, impaired energy metabolism and consequently necrosis and cell death [22]. Several studies on battlefield victims demonstrated that exposure to mustards is a traumatic event having long-lasting effects on mental health [23].

For these reasons, MAs remain one of the highest internationally concerned issue and are receiving increasing importance regarding their decontamination and degradation, but in particular their detection in different environments water and food or in human biological fluids.

Several studies have been conducted on the development of sensing systems for the detection of mustard agents and their simulants, able to provide fast, cost-effective, and reliable analytical tools, such as fluorescence based detection kits [24, 25] and surface acoustic wave (SAW) sensors [26]. Among the different detection methodologies, electrochemical (bio)sensors are receiving several efforts in the last few years, thanks to the documented advantages of the electrochemical transduction, especially when combined with nanostructured materials. A crucial example is represented by the sensing system reported by Singh et al. [27] to detect the blister agent simulant 2-chloroethyl ethyl sulfide (CEES) by electrochemical oxidation using fast scan linear sweep voltammetry, observing oxidative currents at around +1 V. The system was based on gold electrodes modified with a nanocomposite film based on copper phthalocyanine (CuPc) and ionic liquid (RTIL) 1-butyl-3 methylpyrrolidinium bis(trifluoromethylsulfonyl) imide. The implemented sensor was able to reveal CEES in situ in a linear range between

$1.69 \times 10^{-5}$  M and  $5.07 \times 10^{-4}$  M with a LOD and LOQ value of  $1.69 \times 10^{-6}$  M and  $1.69 \times 10^{-5}$  M, respectively.

A number of ad hoc nanomaterials have been exploited by the same group Sigh et al. [28] to modify the surface of a gold electrode in order to provide the detection of mustard agent simulants by electrochemical oxidation. Indeed, they prepared a graphene oxide (GO) film, via chemical oxidation of natural graphite powder followed by microwave irradiation, to modify the surface of gold electrodes showing its excellent electrocatalytic activity to sense the mustard agent simulant thiodiglycol (TDG) with limit of detection of  $2 \times 10^{-7}$  M.

More recently, Arduini et al. [29] realised a novel electrochemical bioassay for mustard agents based on the capability of these compounds to inhibit the enzyme choline oxidase immobilized on a screen-printed electrode modified with Prussian Blue (PB) nanoparticles. The advantage in using PB nanoparticles is that a low applied potential ( $-50$  mV vs. Ag/AgCl) is employed for MA measurement, thus allowing the detection of the mustard agents free from electrochemical interferences. The proposed electrochemical bioassay allowed limits of detection of 0.45 mM for bis(2-chloroethyl)amine, 0.1 mM for 2-chloroethyl phenyl sulfide, and 7  $\mu$ M for 2-chloroethyl ethyl sulphide, showing good sensitivity and fast response, excellent premises for the development of a miniaturised sensor well suited for an alarm system in case of terrorist attacks.

## 4 Blood Agents

The name blood agent, like those of other groups of agents, derives from its effect on victims. Blood agents are distributed via the blood and generally enter the body via inhalation. They inhibit the ability of blood cells to utilise and transfer oxygen. Thus, blood agents are poisons that effectively cause the body to suffocate.

Moreover, they exert their toxic effect at the cellular level by interrupting the electron transport chain in the inner membranes of the mitochondria. Examples of blood agents include hydrogen cyanide (AC), cyanogen chloride (CK), and arsine (SA) (Table 5).

**Table 5** Physical-chemical properties and toxicity of some blood agents

	Hydrogen cyanide	Cyanogen chloride	Arsine
	H-C $\equiv$ N	N $\equiv$ C-Cl	AsH <sub>3</sub>
MW	27.03	61.47	77.95
MP (°C)	-14	-6.55	-111.2
BP (°C)	25.6	16	-62.5
V	1.000.000	Not applicable	30.900.000 (0 °C)
LD <sub>50</sub>	501	600	2.5
LCt <sub>50</sub>	2500-5000	11 000	120

MW molecular weight, MP melting point; BP boiling point; V Volatility (mg/m<sup>3</sup> at 25 °C), LD<sub>50</sub> (ppm) lethal dose (skin), LCt<sub>50</sub> (mg-min/m<sup>3</sup>): lethal concentration and time (respiratory)

*Hydrogen cyanide*, first discovered by a Swedish chemist in 1872, was used as an industrial chemical long before the comprehension of its potential as a CW agent during the WW I. French people were the first to consider it for this purpose and used shells made from this material in the battle of Somme in 1916. Cyanogen chloride was also available in plenty as a commercial product having applications as an industrial intermediate during the WW I.

Cyanide has a very high affinity for iron in the ferric ( $\text{Fe}^{+3}$ ) state. On entering the biological system, it readily reacts with trivalent iron of cytochrome oxidase (an end-chain enzyme of cellular respiration) to form a complex, thereby impairing the utilization of oxygen in the tissues. Eventually, death follows as a result of respiratory failure. The onset and intensity of symptoms depend on the concentration of inhaled toxic vapour and duration of the exposure. Symptoms of exposure to low doses of HCN are weakness, giddiness, headache, confusion and, sometimes, nausea and vomiting. Clinical signs appear only at high levels of exposure, which include fast and painful respiration, lack of coordination of movement, cardiac irregularities, hypoxic convulsions, coma and respiratory failure culminating in death. Diagnosis may be aided by characteristic odour of cyanide (bitter almond) or a faint pale-red hue of the skin.

Recent examples of electrochemical methods for cyanide detection are unexpectedly scarce, considering the huge amount of research in this area. Lindsay et al. [30] reported in their review a selection of methods mostly dating before the year 2010, based on cathodic stripping mechanism, amperometry, stripping voltammetry, and electrochemical impedance spectroscopy. Some queries require to be solved regarding repeatability and LODs, and could be answered through the use of new functional materials. Indeed, recent methods to sense cyanide, exploiting these ad hoc materials, have been reported and showed to be able to meet the requirements of the main international bodies for environmental protection, including EPA that set the maximum contaminant level for cyanide of 200  $\mu\text{g/L}$  in drinking water, or European Union and the World Health Organization that set a lower limit of 50  $\mu\text{g/L}$  in drinking water.

For example, ion selective electrodes (ISEs) are convenient and offer fast response time, but have numerous interferences such as halides, pseudohalide sulfides and various metals that are complexed by cyanide, e.g., cadmium, silver, zinc, copper, nickel and mercury [31].

In this regard, Abbaspour et al. [32] realized a chemically modified carbon paste electrode with 3,4-tetra pyridinoporphyrazinatocobalt(II) that had no response to halides, pseudohalides, or oxalate, giving an accurate potentiometric determination of spiked cyanide in spring water with a LOD of 9  $\mu\text{M}$  and a Nernstian slope of  $60 \pm 1.5$  mV/decade.

A silver doped silica nanocomposite was synthesized by Taheri et al. [33] by self-assembly of a sol-gel network and silver nanoparticles, exploiting the specific reaction of Ag nanoparticle and  $\text{CN}^-$ . Cyanide detection was determined by cyclic voltammetry technique, measuring the decrease of cathodic peaks at around +0.9 V, providing a LOD of 14 nM.

A more sensitive sensor was realized by Zacharis et al. [34] based on automated gas diffusion of HCN liberated by HCl from a 250  $\mu\text{L}$  cyanide containing sample and absorbed in a NaOH acceptor. The amperometric detection using on a silver working electrode provided a LOD ranged from 0.05 to 0.12  $\mu\text{g/L}$  depending on the number of pre-concentration cycles, while water samples spiked with 1–10  $\mu\text{g/L}$  showed recoveries of 88–112 %.

## 5 Choking or Pulmonary Agents

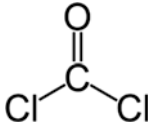
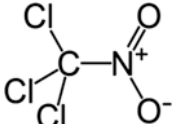
Choking or pulmonary agents are chemicals that cause severe irritation or swelling of the respiratory tract (lining of the nose, throat, and lungs). These agents function in liquid, gaseous, or aerosolized forms, operating primarily by irritating the respiratory tract and inducing swelling in these areas. Their inhalation cause burning of the throat, coughing, vomiting, headache, pain in chest, tightness in chest, and respiratory and circulatory failure.

Examples of pulmonary agents include: ammonia, bromine, chlorine, hydrogen chloride, methyl bromide, methyl isocyanate, osmium tetroxide, phosgene, diphosgene, phosgene, chloropicrin, phosphine, phosphorus, elemental, white or yellow sulfur fluoride. Some examples of pulmonary agents are reported in Table 6.

Used as chemical weapon to impede the victim's ability to breathe, these compounds have prominently figured in military conflicts, notably the US Civil War, WW I, the War in Bosnia and Herzegovina, and the Iraq War. WW I alone saw more than 70 000 cases of gas poisoning among US troops [35].

Although disulfur decafluoride and perfluoroisobutene are both the most dangerous pulmonary agents, more than 10 times with respect to other agents of this

**Table 6** Physical-chemical properties and toxicity of some choking agents

	Phosgene	Chloropicrin
		
MW	98.92	164.37
MP (°C)	-118	-69
BP (°C)	8.3	112
V	45 000	18 000
LD <sub>50</sub>	500	9.7
LCt <sub>50</sub>	570	50

MW molecular weight, MP melting point; BP boiling point; V Volatility ( $\text{mg/m}^3$  at 25 °C), LD<sub>50</sub> (ppm) lethal dose (skin), LCt<sub>50</sub> ( $\text{mg}\cdot\text{min/m}^3$ ): lethal concentration and time (respiratory)



class, *Phosgene* ( $\text{COCl}_2$ ) is the most commonly used. It is a colourless highly toxic gas, causing severe lung irritation, pulmonary oedema, asphyxia and even death at a concentration as low as 2 ppm. The first recorded use of phosgene was in 1915, and it accounted for 80 % of all chemical fatalities during WW I.

Moreover, phosgene is also a valued industrial intermediate in organic synthesis. Therefore, developing a simple and sensitive method for phosgene detection is highly desirable not only for public safety against terrorist attack but also for industrial production [36].

An effective sensor for phosgene detection was realised by Virji et al. [37], based on a novel polyaniline nanofiber composite material. They constructed, using standard photolithography, a sensor array containing 18 sensors with each sensor having 35 pairs of fingers with 20  $\mu\text{m}$  electrode gaps. The reaction mechanism was based on a nucleophilic substitution reaction of phosgene with the amine to generate an isocyanate and an acid. The acid dopes the polyaniline nanofibers increasing their conductivity by up to two orders of magnitude. With this sensor configuration, phosgene was detected well below the permissible exposure limit (0.1 ppm).

## 6 Toxins

According to OPCW, toxins are effective and specific poisons produced by living organisms, consisting of an amino acid chain which can vary in molecular weight between a couple of hundred (peptides) and one hundred thousand (proteins). Toxins are produced by numerous organisms, e.g., bacteria, fungi, algae and plants. Many of them are extremely poisonous, with a toxicity that is several orders of magnitude greater than the nerve agents. Because of the hybrid nature of toxins, they have sometimes been considered CW agents and sometimes biological agents (BW). Similarly, based on their mechanism of action, they are grouped as cardiotoxins, dermatotoxins, hepatotoxins, neurotoxins, etc. Being a heterogeneous group of compounds, they are able to interfere with biochemical processes, such as membrane function, ion transport, transmitter release and macromolecule synthesis. Human exposure to toxins can lead to serious health problems, including immunosuppression and carcinogenesis.

For these reasons, they started to attract military interest already during the first half of the last century. At that time, it was difficult to manufacture sufficiently large amounts of toxin, which caused interest to decrease. Many of the toxins considered at that time were sensitive to heat and light which made them unstable and unpractical to use. The U.S.A. ended its toxin programme in the late 1960s and destroyed its stockpile of, e.g., botulinum toxin. The Biological and Toxin Weapons Convention of 1972 prohibits the development, production and stockpiling of toxins as weapons.

The 1925 Geneva Protocol prohibition of use of chemical and bacteriological weapons also covers the use of weapons based on toxins. Since the definition of

chemical weapons includes toxins, they are also covered by the Chemical Weapons Convention.

In the late 1970s, there was a rapid development of gene technology together with biotechnology. This led to again arise the threat from toxins as CW agents. Nowadays it became possible to more easily produce greater amounts of many toxins, in some cases even synthetically. Gene technology can be used to modify the toxin genes so that the end product shows new properties as, for example, to become less sensitive to sunlight.

The scientific and commercial development has together provided increased opportunities to incorrectly utilize biotechnology for military purposes. Recent researches, for example, made it possible to “target” toxins to different body organs or structures. This new knowledge mainly emanates from civilian research into, e.g., the treatment of cancer patients.

Toxins are still considered to be less suitable for dispersal on a large scale. Nonetheless, they could be used for sabotage or in especially designed inputs, e.g., against key persons. Since toxins have low volatility, they are dispersed as aerosols and then taken up foremost through inhalation. The new microencapsulation technology, which is easy to use, makes it possible to protect unstable toxins when dispersed.

A few examples of toxins which may be used as chemical warfare agents are listed below. The trichothecenes, mycotoxins obtained from, e.g., *Fusarium* genera, were alleged in the early 1980s to have been used as CW agents in Southeast Asia (“yellow rain”), but are of no military value today.

## 6.1 *Mycotoxins*

*Botulinum* toxin is the most poisonous substance known. It is also known as agent X. Arnon et al. estimated that 1 g of this toxin, when aerosolized, could kill more than one million people. The lethal dose for a 70-kg human is estimated to be approximately 0.7 µg if inhaled or 70 µg if ingested. Botulinum toxin is produced by the bacteria *Clostridium botulinum*, which grows on poorly preserved food and causes a severe form of food-poisoning (botulism). The incubation period is between one and three days after which the victim becomes ill with stomach pains, diarrhoea, disturbances to vision, giddiness and muscular weakness. The whole body including the respiratory musculature becomes paralyzed which leads to death by suffocation within a few days.

The toxin is a protein available in seven different forms, where the most poisonous is the type A (molecular weight 150 000 Da). It is possible to vaccinate against botulism, while once the victim has become poisoned, there is no antidote. Botulinum toxin is today commercially produced and is used in treating squinting and other muscular disorders.

The literature on sensing systems for the detection of botulinum neurotoxins, based on the modes of action of these toxins (which are well-established in

literature), is very huge. Indeed, they are known to specifically cleave portions of the SNARE proteins SNAP-25 or VAMP. This interaction can be monitored by different transduction system. A central example is the study of Savage et al. [38], which reported the development of a SNAP-25 and a VAMP biosensors for detecting the activity of five botulinum neurotoxin serotypes (A–E) using electrochemical impedance spectroscopy, at concentrations as low as 25 fg/mL, in a short time-frame compared with the current standard methods of detection.

Nevertheless, the use of biosensors to measure botulinum finds many application in different fields, being the detection of botulinum essential for identifying its presence in potential cases of terrorism and food contamination, as well as in diagnosis of botulism. As an example, Chan et al. [39] described a reduced graphene oxide(rGO)/Au electrode based electrochemical biosensor for ultrasensitive detection of BoNT serotype A light chain (BoNT-LcA) protease activity in milk samples. They fabricated rGO/Au electrodes to realise a robust and biocompatible platform with enhanced electron transfer capability and large surface area for SNAP-25-GFP peptide immobilization. Once immobilised, SNAP-25-GFP peptide is specifically cut at the cleavage sites upon the addition of BoNT-LcA, consequently releasing the cut section from the electrode surface. This enzymatic activity of BoNT-LcA on SNAP-25-GFP peptide substrate was detected by monitoring the enhanced redox probe transfer rate by differential pulse voltammetry with a linear detection range up to 1 ng/mL and LOD around 8.6 pg/mL. The specificity of the biosensor reported by Chan et al. was demonstrated, as well as its feasibility in complex matrices.

A different application of botulinum biosensors is presented in the pharmaceutical field. Halliwell et al. [40] reported the development of two electrochemical bioassays for the detection of active botulinum neurotoxin in pharmaceutical samples based on gold electrodes modified with self-assembled monolayers of the SNARE protein SNAP-25, which is selectively cleaved by active botulinum neurotoxin A. The addition of the toxin to the layer was measured by cyclic voltammetry and electrochemical impedance spectroscopy, performed on the modified working electrodes to observe changes of toxin concentrations as low as 25 fg/ml, with results being obtained in less than an hour, outperforming the mouse bioassay. The presented sensing assay demonstrated to be able to replace currently standard methods based on the mouse bioassay, actually considered as the most reliable method for the detection of the active form of this toxin, but also time-consuming and expensive.

Among mycotoxins, *ricin* is a very potent toxin of plant origin, isolated from the seeds of castor oil, *Ricinus communis*. It inhibits ribosome proteins, and the toxic dose for humans is about 0.1–1.0 µg/kg, depending on the mode of administration. It was used in the famous “umbrella tip” assassination of the Bulgarian journalist. Iraq produced and weaponized several highly potent toxins such as botulinum toxin and aflatoxin.

A small number of electrochemical sensing systems have been reported in literature in the last years. A recent example is represented by the paper-based assay platform of Cunningham et al. [41] for detection of an immunological ricin chain.

The group assembled a paper platform, by simple origami paper folding, to measure the toxin by means of quantitative, electrochemical detection of silver nanoparticle labels linked to a magnetic microbead support via a ricin immunosandwich. The reported sensor demonstrated to be highly advantageous thanks to the high sensitivity (34 pM), robustness even in the presence of 100-fold excess hoax materials, cost-effectiveness (\$0.30 per assay), and speed response (9.5 min per assay).

A similar sandwich immunoassay format was described by Suresh et al. [42] for the immunosensing of ricin. In particular, they developed an amperometric immunosensor for the specific detection of *Ricinus communis* based on screen-printed electrodes modified with gold nanoparticles loaded with a multi-walled carbon nanotubes- chitosan film. Specific antibodies tagged with the enzyme alkaline phosphatase were used to convert the substrate 1-naphthyl phosphate into 1-naphthol, consequently determined with the amperometric technique and correlated with the concentration of ricin. The reported method showed high stability due to the chitosan film, short response time with good reproducibility and increased shelf life of the electrodes with immobilised antibodies. Furthermore, the optimization of composition of carbon nanotubes and gold nanoparticles was able to improve the electrochemical activity of the electrodes, which showed under optimal conditions, a wide linear response to the concentration of ricin in the range of 2.5–25 ng mL<sup>-1</sup>, a sensitive limit of detection of 2.1 ng mL<sup>-1</sup>, a relative standard deviation of 5.1 % and storage life of 32 days.

## 6.2 Phycotoxins

Many toxins are produced by marine organisms. Likewise, phycotoxins may produce undesirable effects also at low concentrations, causing intoxication syndromes.

One example is saxitoxin, which is synthesized by a type of blue-green algae (cyanobacteria). These algae provide food for different shellfish, e.g., mussels. The mussels themselves are not influenced by the poison, but human beings who later eat the mussels may become seriously ill. *Saxitoxin* attacks the nervous system and has a paralyzing effect, but causes no symptoms in the gastro-intestinal tract. The development of the illness is extremely rapid and at high doses death may occur within less than 15 min. The LD50 for man is at about 1 mg. Saxitoxin is a small molecule with a molecular weight of 370 D. It is not sensitive to heat but is destroyed by oxygen.

Several analytical methodologies have been developed for the detection of the main marine toxins, including biosensors for domoic acid, okadaic acid, and tetrodotoxin [43–50]. For instance, Micheli et al. have developed a competitive indirect test immunosensor coupled to differential pulse voltammetry for the detection of domoic acid using disposable screen-printed electrodes [44]. Domoic acid was conjugated to bovine serum albumin and was coated onto the working electrode of the SPE, followed by incubation with sample (or standard toxin) and anti- domoic antibody. An anti-goat IgG-alkaline phosphatase conjugate was used

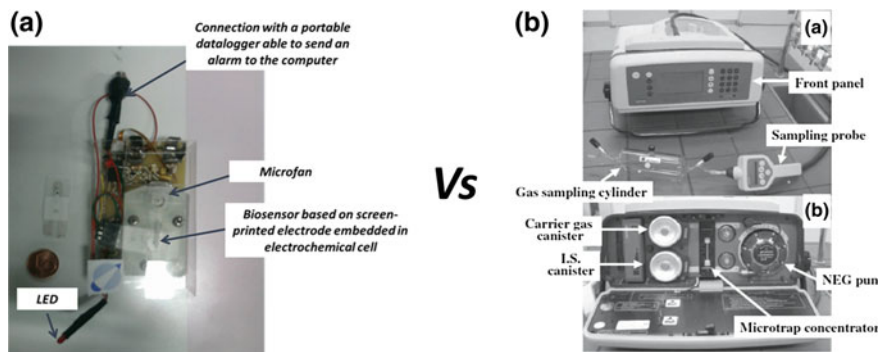
for signal measurement. The developed immunosensor is able to detect domoic with LOD equal to 5 ng/mL. For tetrodotoxin detection, a direct competitive electrochemical immunosensor was developed by Neagu et al. [46]. In the construction of this immunosensor, antigen labelled with alkaline phosphatase was prepared in order to evaluate the amount of tetrodotoxin in the sample analysed, reaching a dynamic range comprised between 2 and 50 ng/mL and LOD of 1 ng/mL. An electrochemical multi-enzymatic system based on phosphatase-2A inhibition for okadaic acid was assembled by Volpe at a [50]. The amount of okadaic acid was estimated evaluating the degree of inhibition of protein phosphatase-2A, which is proportional of the okadaic amount. The basic reaction of this system is the one catalyzed by the Phosphorylase *a* that converts glycogen and phosphate (P) into glucose-1-P, which in turn produces glucose through the use of alkaline phosphatase. The glucose is then converted by glucose oxidase into H<sub>2</sub>O<sub>2</sub> which is electrochemically oxidized at the platinum electrode inserted into a flow injection analysis, allowing for a working range of 30–250 pg/mL.

## 7 Electrochemical (Bio)Sensors for in Situ Measurement Versus Laboratory Set-up Methodologies

Having an analytical device easily used by unskilled personnel for in situ application, has led scientists to integrate the electrochemical (bio)sensors in portable and embedded apparatuses. In this context, significant examples were reported for nerve agent detection. For instance, Du et al. [51] have developed a portable set-up based on the use of screen-printed electrode modified with carbon nanotubes combined with a microflow-injection device for OP detection. This analytical system was able to assess the presence of inhibited AChE in saliva via regeneration of AChE, demonstrating the suitability for subclinical organophosphate exposure evaluation.

In the direction of automatisable systems, an interesting work was reported by the Hart's group; in this case, an array on six *engineered* acetylcholinesterase screen-printed electrodes was integrated in a novel automated instrument coupled with a neural network program. The system was successfully applied in several samples like water, food and vegetable extracts, demonstrating the possibility to cover the detection of several pesticides in a number of matrices by using an automatic and portable system [52].

The first example of lab-on-a-chip based on an electrochemical cholinesterase biosensor was developed by us using butyrylcholinesterase immobilised on screen-printed electrodes modified with Prussian Blue. In this case, the probe can be able to detect inhibitors like Sarin in gas phase using a micro fun for air sampling. The integrated miniaturized circuit is able to apply the potential, to register the current, to turn on-off the fan and eventually give an alarm switching on a led, demonstrating the readiness of the electrochemical biosensor for a rapid alarm in the case of nerve agent pollution in air [53].



**Fig. 9** **a** ChE biosensor integrated into a lab-on-a-chip. The prototype is composed of a cell in which is inserted the biosensor, a little fan able to sampling air and an electronic circuit. The circuit is able to apply the potential, to register the current, to turn on-off the fan and eventually to give an alarm by led or wireless at a personal computer through the datalogger [53] versus **b** Hapsite instrument [57]. Reprinted (adapted) with permission from [53, 57]

In order to understand the feasibility of the electrochemical biosensor use, a comparison with commercial available portable instruments is mandatory.

Various kinds of on-site nerve agent detection equipment are used by the military and police mobile teams. The portable devices are based on ion mobility [54], surface acoustic wave [55], gas chromatographs coupled with mass spectrometer (Hapsite® instrument) [56]. The commercial available systems are characterized by high selectivity and sensitivity (i.e. Hapsite), but at the same time, the instruments are difficult to further miniaturize and rather expensive. On the contrary, the lab on a chip based on cholinesterase biosensor is characterized by cost-effectiveness, easiness to use, can be easily automatized (Fig. 9) and applied for security monitoring in airports, undergrounds, etc.

In this direction, the lab on a chip based on electrochemical (bio)sensors is an useful first alarm system able to give a rapid alarm, sustaining the management of chemical risk in the case of terroristic attacks.

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