A Biosensor for the Detection of Acetylcholine and Diazinon

E. G. Herrera†, A. Bonini†, F. Vivaldi, B. Melai, P. Salvo, *Member, IEEE,* N. Poma, D. Santalucia, A. Kirchhain and F. Di Francesco

***Abstract*— Acetylcholine is a neurotransmitter and a neuromodulator found in the autonomic, peripheral and central nervous systems. Diazinon is a pesticide with toxic effects on humans, such as the inhibition of acetylcholine. In this paper, a biosensor is proposed for the detection of acetylcholine (range 70 – 1000 µM) and diazinon (range 0.3 – 20000 ppb). This biosensor combines a pH-sensitive layer of reduced graphene oxide functionalized with 4-aminobenzoic acid and acetylcholinesterase. This enzyme was immobilized on reduced graphene oxide and it catalyzed the conversion of acetylcholine into choline and acetic acid, locally decreasing the pH value and triggering the sensor response. The limit of detection for the acetylcholine and diazinon were 70 µM and 0.3 ppb, respectively.**

I. Introduction

Biosensors exploit the high specificity of a bioreceptor to bind selectively analytes in complex matrices [1–3]. The bioreceptor is coupled to a transducer, which provides a quantifiable signal when the bond with the target molecule occurs [4, 5]. Biosensors can be used for a wide range of applications such as medical diagnostics, drugs development, food safety, process control, environmental monitoring, defense and safety [1, 6]. In the case of electrochemical biosensors, the analyte is electroactive and the output signal is electrical. The advantages of electrochemical biosensors include low detection limits, a wide range of linear response, good stability and reproducibility [7–9].

Enzymes are widely used as biorecognition elements in biosensors due to their high specificity and sensitivity, low cost and easy handling. However, it is crucial to find the conditions that preserve the biological activity of an enzyme when it is adsorbed on the transducer [10–12]. If no specific studies on the enzyme structure are available, knowledge of the kinetic parameters that characterize the enzyme, i.e. the Michaelis–Menten constant, K_m, and the maximum rate, V_{max}, can provide valuable information on the affinity towards the substrate. Briefly, low values of K_m indicate high affinity of the enzyme for its substrate [11, 13, 14]. In humans, acetylcholinesterase (AchE) is present in the nervous tissue and in red blood cells. This enzyme catalyzes the following reaction:

$$acetylcholine + water \xrightarrow{AchE} choline + acetic\ acid \quad (1)$$

P. Salvo is with the Institute of Clinical Physiology, National Council of Research, Via Moruzzi 1, 56124, Pisa, Italy (corresponding author, phone: +39 050 3152703; e-mail: pietro.salvo@gmail.com).

E. G. Herrera, A. Bonini, B. Melai, N. Poma, D. Santalucia, F. Vivaldi, A. Kirchhain, F. Di Francesco, are with the Department of Chemistry and Industrial Chemistry, University of Pisa, Via Moruzzi 12, 56124, Pisa, Italy (e-mail: f.difrancesco@unipi.it).

†These authors equally contributed to this work.

The presence of inhibitors leads to a change in the concentration of acetylcholine (ACh), which plays a role in Alzheimer's disease and can cause involuntary muscle contractions [15–17]. Widely used as a pesticide in agriculture, diazinon (Dz) is an organophosphorus molecule that has an inhibitory effect on AChE [18–21].

This paper proposes a biosensor for the detection of ACh or Dz based on the catalytic activity of AChE. The enzyme is adsorbed a on reduced graphene oxide (rGO) layer functionalized with 4–aminobenzoic acid (4–AB). The rGO layer is known to be sensitive to changes in pH [22] and 4–AB was used to improve this sensitivity. This approach enabled the direct detection of ACh and Dz out of the inhibition and its analytic parameters agree with those reported in the literature [23, 24]. The fabrication is suitable for mass production and involves a simple manufacturing process when compared to other potentiometric biosensors developed so far [25–27].

II. Materials and Methods

A. Reagents and Instrumentation

Sodium nitrite (372109) was purchased from Carlo Erba. Sodium hydroxide (30620) was purchased from Riedel–de Haën., ascorbic acid (A7506-1KG), sodium chloride (746398), potassium phosphate monobasic (795488), dibasic potassium phosphate (795496), 4–aminobenzoic acid (100536), N– (3–Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 03449), Whatman® polyamide membrane filters (WHA10414012, pore size 0.2 µm, membrane diameter 47 mm), N-hydroxysuccinimide (NHS, 130672), Acetylcholine chloride (60311), diazinon (0000333415) and Acetylcholinesterase (43) were all purchased from Sigma Aldrich. Graphene oxide dispersion in water (concentration 4 mg/mL) was obtained from Graphenea.

The measurement of the open–circuit potential (OCP) by a potentiostat / galvanostat, (EMstat 4 by Palmsens) allowed to characterize the enzymatic activity and to determine the analytical response of the biosensor. The setup was similar to that reported in [28, 29] where a pH meter (Crison Basic 20) was used with a micro P electrode (XS sensor). All measurements were carried out at 20 °C ± 1 °C.

B. Modification of the electrode surfaces

The biosensor was fabricated on a flexible board (polyethylene terephthalate, 125 µm thick) with one working electrode (WE, graphite, diameter 2 mm) and one Ag/AgCl reference electrode (RE, about 1 mm^2) (Fig. 1). The WE was sonicated at 40 kHz for one minute to obtain a polished

978-1-5386-1311-5/19/$31.00 ©2019 IEEE

surface. The reduction of GO and a detailed description of the board are reported in [30].

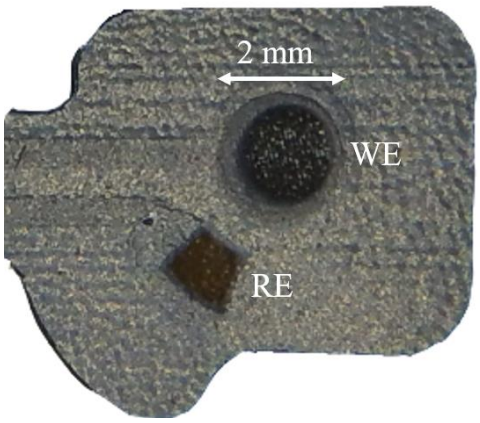

Figure 1. Detail of measurement board with an rGO working electrode (WE) and an Ag/AgCl reference electrode (RE).

After sonication, 1 µL (1 mg/mL) of an rGO dispersion functionalized with 4–AB (rGO–AB) was added and left to dry in an oven at 110 °C for 3 h. The biosensor was obtained immobilizing AChE onto the WE by the conjugation via EDC/NHS coupling of the carboxylic groups of the rGO–AB with the amino groups of the enzyme. An aliquot (5 µL) of EDC/NHS 0.4 M in PBS 0.1 M at pH 7 was manually drop–cast onto the WE and the board was kept at 4 °C for 1.5 h. The WE was then rinsed with PBS 0.1 M at pH 7. An aliquot of 2 µL containing AChE (0.5 mg/mL) in PBS 0.2 M at pH 7 was manually drop–cast onto the WE and kept at 4 °C overnight. The WE was eventually rinsed in PBS 0.1 M at pH 7 for 1 h to remove the enzymatic residues.

C. Characterization of the enzymatic activity of AChE

The change of potential between WE and RE resulting from the reaction (1) was measured over time. The ACh concentration ranged from 70 µM to 3000 µM. Blank measurements were carried out using a board without the enzyme. The Michaelis–Menten curve was fitted on data to determine the values of K_m and V_{max}. The experiments were performed in PBS 1 mM at pH 7.

D. Determination of the analytical figures of merit

ACh biosensor. A range of concentrations was selected (i.e. the range of linearity resulting from the experiment described in II.C) and linearity interval, limit of detection, and sensitivity of the sensor were determined. The detection of ACh depends on the local changes of pH induced by the acetic acid produced by (1).

Dz biosensor. The change in surface potential was measured against the addition of ACh 20 µM (procedure A). The electrode was immersed in a solution of Dz in water and procedure A was repeated for a range of concentrations of Dz ranging 0.01 to 2000 ppb.

III. Results and Discussion

A. Characterization of biosensor response and enzymatic activity

Figure 2 shows the signal when ACh was added onto the rGO-AB WE, in the absence (black) and in the presence (red) of AChE. The enzyme induced a considerably higher biosensor response, thus confirming the functionalization of rGO-AB and the preservation of enzymatic activity even once bound to the surface. The 4–AB adds oxygenated groups (e.g. carboxylic groups) to the rGO structure and improves the sensitivity towards pH allowing a covalent interaction with AChE. AChE has multiple accesses to the active site, thus the orientation is not a critical factor as it happens with most enzymes [21, 31]. Figure 2 also shows that the non-functionalized rGO WE was not suitable for sensing ACh.

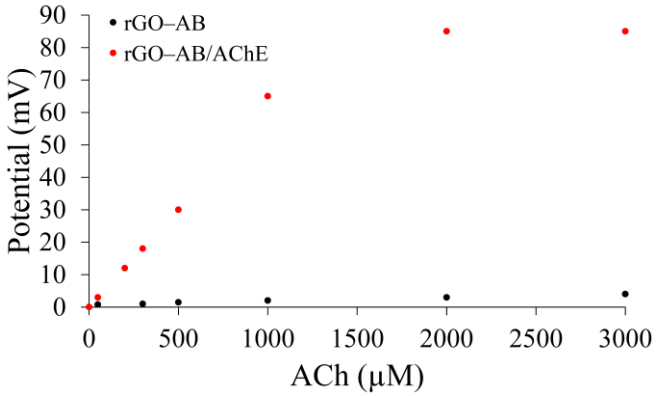

Figure 2. Response of the biosensor after the addition of ACh onto the rGO-AB WE, in the absence (black) and in the presence (red) of AChE.

Figure 3 shows the biosensor response, which is fitted quite well by a Michaelis–Menten curve (v_0 depends on the ACh concentration). Table 1 reports the fitted value for K_m and V_{max} (Adjusted R-square= 0.9738, Root-mean-square error = 4.585). The value of K_m is in the typical range for free enzymes in solution, i.e. 10^{-1} – 10^{-5} mol/L [32]; therefore, we hypothesize that the enzyme was likely to preserve its native structure and activity when immobilized onto the rGO–AB WE.

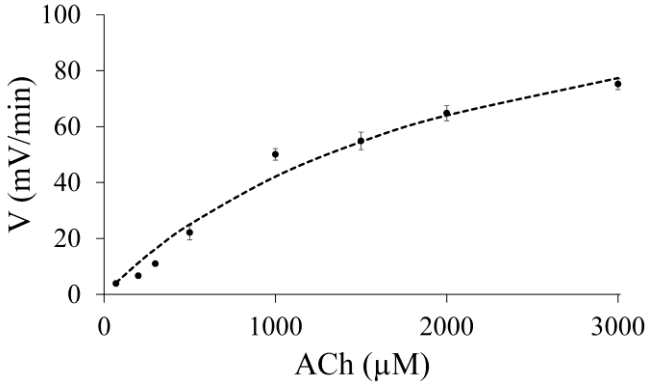

Figure 3. Biosensor response fits a Michaelis–Menten curve (kinetic rate V as a function of ACh concentration) when AChE is immobilized onto the rGO–AB working electrode.

TABLE I. KINETICS PARAMETERS FOR ADSORBED ACHE.

WE	K_m (µM)	V_{max} (mV/min)
rGO–AB	2158	133

B. *Determination of the Analytical Figures of merit for the Acetylcholine and Diazinon Biosensor*

Figure 4 shows the linear biosensor response when ACh is in the range 70 – 1000 µM. The sensitivity was 6 µV/µM and the limit of detection (LOD) 70 µM. The sensitivity is comparable with the values reported in the literature [23]. The response time was lower than 120 s for any concentration value.

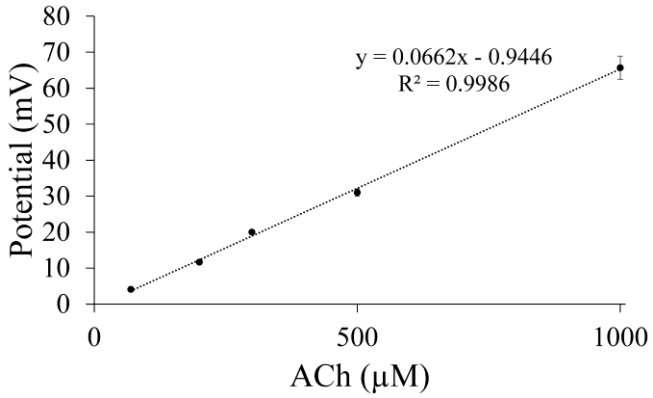

Figure 4. Calibration curve of rGO-AB/AChE in the range 70 – 1000 µM of ACh in a PBS 1 mM at pH 7. Error bars represent the standard deviation calculated from a total triplicate measurements with five biosensors (n= 15).

Table II shows the reproducibility over 1 week expressed as RSD% calculated for five biosensors at each concentration value.

TABLE II. REPRODUCIBILITY OF THE ACETYLCHOLINE BIOSENSOR.

Acetylcholine [µM]	RSD% 1 day	RSD% 7 day
70	6	9
200	5	8
300	2	9
500	3	8
1000	5	14

Figure 5 shows the percentage of inhibition (%I) obtained with rGO–AB/AChE WEs exposed to Dz. The value of %I was calculated from the following equation:

$$\%I = (\Delta V_i - \Delta V_0) / \Delta V_0 \quad (2)$$

where ΔV_i is the change of potential of the WE after exposure to Dz, and ΔV_0 is the potential change of the WE before exposure to Dz. The %I allows the indirect measurement of the enzyme concentration in a simple and fast way. Dz decreases the activity of ACh even at concentrations lower than 1 ppb. The median %I (I_{50}) was 8726 ppb, whereas the LOD was 0.3 ppb. Although not tested in real samples, this LOD is better than that reported in recent papers [33, 34].

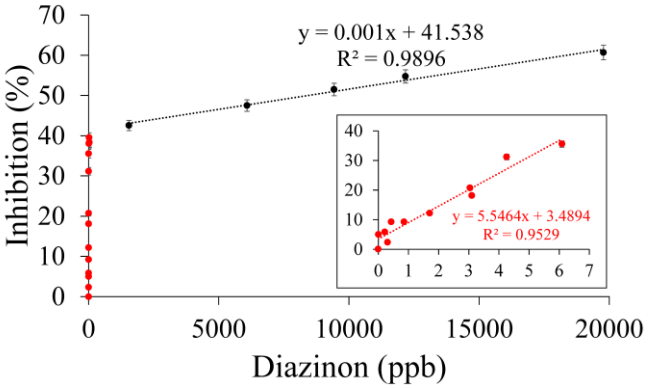

Figure 5. Percentage of AChE inhibition versus Dz concentration. The inlet shows the linearity in the range [0.3, 6] ppb.

IV. CONCLUSION

The functionalization of an rGO layer with 4–AB and AChE proved a reliable method to obtain an electrochemical biosensor for the detection of acetylcholine and diazinon. Sensitivity, reproducibility and LOD were comparable with or better than those reported in the literature. The biosensor was tested in PBS, thus future work will be focused on test with real samples, where the effects of interferents needs to be evaluated. The long-term stability (i.e. for periods longer than one week) should be tested; however, this biosensor is currently envisioned to be used as one-time disposable device for a fast measurement of acetylcholine and diazinon. A membrane to stabilize and protect the reference electrode is being tested and will be included in future versions of this biosensor.

REFERENCES

[1] A. P. F. Turner, "Biosensors: Sense and sensibility," Chem. Soc. Rev., vol. 42, no. 8, pp. 3184–3196, 2013.

[2] S. Deng, J. Lei, L. Cheng, Y. Zhang, and H. Ju, "Biosensors and Bioelectronics Amplified electrochemiluminescence of quantum dots by electrochemically reduced graphene oxide for nanobiosensing of acetylcholine," Biosens. Bioelectron., vol. 26, no. 11, pp. 4552–4558, 2011.

[3] I. Y. Sohn *et al.*, "Ph sensing characteristics and biosensing application of solution-gated reduced graphene oxide field-effect transistors," Biosens. Bioelectron., vol. 45, no. 1, pp. 70–76, 2013.

[4] C. Ispas, I. Sokolov, and S. Andreescu, "Enzyme-functionalized mesoporous silica for bioanalytical applications," Anal. Bioanal. Chem., 2009.

[5] V. Bille, D. Plainchamp, S. Lavielle, G. Chassaing, and J. Remacle, "Effect of the microenvironment on the kinetic properties of immobilized enzymes.," Eur. J. Biochem., vol. 180, pp. 41–47, 1989.

[6] M.Zarei, "Portable biosensing devices for point-of-care diagnostics: Recent developments and applications," Trends Anal. Chem., vol. 91, pp. 26–41, 2017.

[7] D. Grieshaber, R. MacKenzie, J. Vörös, and E. Reimhult, "Electrochemical biosensors - Sensor principles and architectures," Sensors, vol. 8, no. 3, pp. 1400–1458, 2008.

[8] C. Zhu, G. Yang, H. Li, D. Du, and Y. Lin, "Electrochemical sensors and biosensors based on nanomaterials and nanostructures," Anal. Chem., vol. 87, no. 1, pp. 230–249, 2015.

[9] N. X. Viet, M. Chikae, Y. Ukita, and Y. Takamura, "Enzyme-free glucose sensor based on micro-nano Dualporous gold-modified screen-printed carbon electrode," Int. J. Electrochem. Sci., vol. 13, pp. 8633–8644, 2018.

[10] J. P. Chambers, B. P. Arulanandam, L. L. Matta, A. Weis, and J. J. Valdes, "Biosensor recognition elements," Curr. Issues Mol. Biol., vol. 10, pp. 1–12, 2002.

[11] E. Herrera, J. Valdez Taubas, and C. E. Giacomelli, "D-Amino acid oxidase bio-functionalized platforms: Toward an enhanced enzymatic bio-activity," Appl. Surf. Sci., vol. 356, pp. 679–686, 2015.

[12] S. Gon and M. M. Santore, "Single component and selective competitive protein adsorption in a patchy polymer brush: opposition between steric repulsions and electrostatic attractions," Langmuir, vol. 27, no. 4, pp. 1487–1493, 2011.

[13] L. E. Valenti *et al.*, "Optimizing the Bioaffinity Interaction between His-Tag Proteins and Ni(II) Surface Sites," in ACS Proteins at Interfaces III. Chapter 2, 2012, pp. 37–53.

[14] W. Tischer and F. Wedekind, "Immobilized Enzymes: Methods and Applications," Top. Curr. Chem., vol. 200, pp. 95–126, 1999.

[15] R. V Shamagsumova, D. N. Shurpik, P. L. Padnya, I. I. Stoikov, and G. A. Evtugyn, "Acetylcholinesterase biosensor for inhibitor measurements based on glassy carbon electrode modified with carbon black and pillar[5]arene," Talanta, vol. 144, pp. 559–568, 2015.

[16] M. Pohanka and P. Dobes, "Caffeine inhibits acetylcholinesterase, but not butyrylcholinesterase," Int. J. Mol. Sci., vol. 14, no. 5, pp. 9873–9882 2013.

[17] J. Singh, K. Kour, and M. B. Jayaram, "Acetylcholinesterase inhibitors for schizophrenia," Cochrane Database Syst. Rev., vol. 1, CD007967, 2009.

[18] T. D. Lazarević-Pašti, A. M. Bondz, I. A. Pašti, S. V Mentus, and V. M. Vasic, "Electrochemical oxidation of diazinon in aqueous solutions via electrogenerated halogens – Diazinon fate and implications for its detection," J. Electroanal. Chem., vol. 692, pp. 40–45, 2013.

[19] F. Gómez, M. V. Martínez-Toledo, V. Salmerón, B. Rodelas, and J. González-López, "Influence of the insecticides profenofos and diazinon on the microbial activities of *Azospirillum brasilense*," Chemosphere, vol. 39, no. 6, pp. 945–957, 1999.

[20] K. Z. Guyton *et al.*, "Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate," Lancet Oncol., vol. 16, no. 5, pp. 490–491, 2015.

[21] S. Karanth, "Diazinon," in Encyclopedia of Toxicology: Third Edition, 2014.

[22] P. Salvo *et al.*, "Graphene-based devices for measuring pH," Sens. Actuators B Chem., vol. 256, pp. 976–991, 2018.

[23] M. Snejdarkova *et al.*, "Acetylcholinesterase sensors based on gold electrodes modified with dendrimer and polyaniline: A comparative research," Anal. Chim. Acta, vol. 514, no. 1, pp. 79–88, 2004.

[24] R. E. Gyurcsányi, Z. Vágföldi, K. Tóth, and G. Nagy, "Fast Response Potentiometric Acetylcholine Biosensor," Electroanalysis, vol. 11, no. 10–11, pp. 712–718, 1999.

[25] N. Sattarahmady, H. Heli, and A. A. Moosavi-Movahedi, "An electrochemical acetylcholine biosensor based on nanoshells of hollow nickel microspheres-carbon microparticles-Nafion nanocomposite," Biosens. Bioelectron., vol. 25, no. 10, pp. 2329–2335, Jun. 2010.

[26] S. Blanco, R. Vargas, J. Mostany, C. Borrás, and B. R. Scharifker, "A novel nickel nanowire amperometric sensor: Direct current vs. alternating current strategies for ethanol, acetaldehyde and acetylcholine detection," J. Electroanal. Chem., vol. 740, pp. 61–67, 2015.

[27] C. Bonnet, S. Andreescu, and J.-L. Marty, "Adsorption: an easy and efficient immobilisation of acetylcholinesterase on screen-printed electrodes," Anal. Chim. Acta, vol. 481, no. 2, pp. 209–211, Apr. 2003.

[28] P. Salvo *et al.*, "Temperature and pH sensitive wearable materials for Monitoring Foot Ulcers," Int. J. Nanomedicine, vol. 12, pp. 949–954, 2017.

[29] B. Melai *et al.*, "A graphene oxide pH sensor for wound monitoring," in Proceedings of the 38th Ann. Int. Conf. of the IEEE Engineering in Medicine and Biology Society (EMBC), Orlando (USA), 2016, pp. 1898–1901.

[30] P. Salvo *et al.*, "Temperature and pH sensors based on graphenic materials," Biosens. Bioelectron., vol. 91, pp. 870–877, 2017.

[31] V. Tougu, "Acetylcholinesterase: Mechanism of Catalysis and Inhibition," Curr. Med. Chem. Nerv. Syst. Agents, vol. 1, no. 2, pp. 155–170, 2001.

[32] K. B. Taylor, *Enzyme Kinetics and Mechanisms*. Dordrecht: Springer Netherlands, 2002.

[33] N. Pajooheshpour *et al.*, "Protein templated Au-Pt nanoclusters-graphene nanoribbons as a high performance sensing layer for the electrochemical determination of diazinon," Sens. Actuators B Chem., vol. 275, pp. 180–189, 2018.

[34] M. Khadem *et al.*, "Modification of Carbon Paste Electrode Based on Molecularly Imprinted Polymer for Electrochemical Determination of Diazinon in Biological and Environmental Samples," Electroanalysis, vol 29, no. 3, pp. 708–715, 2016.