Trends in Analytical Chemistry ■■ (2016) ■■–■■

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Electrochemical biosensors based on nanomodified screen-printed electrodes: Recent applications in clinical analysis

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ARTICLE INFO

Keywords:
Screen-printed electrodes
Nanomaterials
Enzymatic biosensors
Paper-based electrochemical sensors
Immunosensors
DNA-based sensors
Clinical analysis

ABSTRACT

This review addresses recent advances in the development of screen-printed electrode based biosensors modified with different nanomaterials such as carbon nanotubes, graphene, metallic nanoparticles as gold, silver and magnetic nanoparticles, and mediator nanoparticles (Prussian Blue, Cobalt Phthalocyanine, etc.), coupled with biological recognition elements such as enzymes, antibodies, DNA and aptamers to obtain probes with improved analytical features. Examples of clinical applications are illustrated, together with examples of paper-based electrochemical devices, of multiple detections using arrays of screen printed electrodes, and of the most recent developments in the field of wearable biosensors. Also the use of smartphones as final detectors is briefly depicted.

Contents

1. Introduction

Biosensors, i.e. analytical devices able to provide quantitative or semi-quantitative analytical information using a biological recognition element in direct spatial contact with a transduction element [1], owe their fortune to the capability to solve, in a fast and reliable way, different analytical problems in areas such as medicine, food, agriculture, environment, and security.

Among several mechanisms of transduction, the electrochemical one has received the largest credit, because of its easiness, low instrumentation cost, capability of miniaturization, and automation. In addition, the use of screen-printed electrodes (SPEs), appeared in the 1990s, largely contributed to this fortune, because of their reliability, reproducibility, mass production, and low cost.

The SPEs proved to be ductile devices, suitable to be drawn in different shapes, to be made of different materials, and suitable to

Abbreviations: Ab_2, antibody; AFP, α–fetoprotein; AGA, anti-gliadin antibodies; AgNPs, silver nanoparticles; ALP, alkaline phosphatase; AuCs/GR, Au clusters on graphene; CA 125, cancer antigen 125; CB, carbon black; CD, celiac disease; CEA, carcinoembryonic antigen; CNTs, carbon nanotubes; CRP, C-reactive protein; cTn1, cardiac troponin 1; EDCA/NHS, 3-(3-dimethylaminopropylcarbodiimide /N Hydroxysuccinimide; ELISA, Enzyme-Linked-Immunosorbent Assay; GNPs, gold nanoparticles; GR, graphene; HRP, horseradish peroxidase; MBs, magnetic beads; μPAD, microfluidic devices with colorimetric method; μPED, paper-based microfluidic devices with electrochemical detection; PB, Prussian Blue; sAA, salivary alfa-amylase; QDs, quantum dots; ROC, Receiver Operating Characteristic; sAA, salivary alpha-amylase; PPy, polypyrrole; cTnT, cardiac troponin T; DPV, Differential Pulse Voltammetry; SAM, self-assembled monolayers; SPE, screen-printed electrode; tTG, tissue transglutaminase.

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http://dx.doi.org/10.1016/j.trac.2016.01.032
0165-9936/

be modified with a variety of biological elements such as enzymes, antibodies, DNA, synthetic recognition elements and others.

Over time, their capability of further modification and miniaturization grew up, drawing the attention of biomedical and clinical industries. Up to now, several biosensors are commercially available, starting from the most widespread glucose biosensor, but also encompassing lactate, pyruvate, cholesterol, ethanol, creatinine, glutamate, choline and acetylcholine biosensors, etc., for applications not only in clinical, but also in food and environmental fields [2].

However, with the aim of improving their analytical characteristics, modifications with a variety of nanomaterials and synthetic recognition elements have been exploited with successful results in most cases. Thus, carbonaceous materials such as carbon nanotubes (CNTs), graphene (GR), carbon black (CB), metallic nanoparticles such as gold (GNPs), silver (AgNPs) and magnetic beads (MBs), and mediator nanoparticles such as Prussian Blue (PB) and Cobalt Phthalocyanine, have been used for SPEs modification, favoured also by the versatility of the screen printing technique. Nanomaterials can have the same dimensions of biological recognition elements such as DNA and proteins, and their combination could originate synergistic effects eliciting unforeseen benefits.

In this review, we would like to illustrate some examples of recent biosensors (covering the period 2012–2015) based on the use of SPEs, both as a single biosensor or in arrays of biosensors, modified with different nanomaterials and in some cases printed on paper substrates, in order to obtain cost effective sensors available also in less developed countries.

These modified sensors have been further coupled with biological recognition elements such as enzymes, antibodies, DNA and aptamers to obtain devices with improved characteristics.

The review will be limited to some clinical applications of these modified SPEs, being impossible to cover the entire field of application of these biosensors, trying to elucidate their novelty and advantages. Application to novel non-invasive matrices such as saliva will be also illustrated, and examples of paper-based electrochemical devices will be also reported, so as the most recent developments of wearable biosensors.

Moreover, the ductility of electrochemical measurements will be further proved by the possibility to assemble label free immunosensors, to perform multiple detections using arrays of SPEs, or by the use of smartphones as final detectors, exploited as self-monitors in some medical applications.

All these advancements lead screen printed electrodes to the realization of numerous Point of Care Testing (POCT), i.e. tests designed to be used at or near the site where the patient is located, and that are performed outside the facilities of clinical laboratories [3]. The benefit of POCTs are numerous, such as a rapid time of analysis, low energy use, low (or none) reagent use, very little or no sample treatment, high safety for operators, easy to use and low costs. Point-of-care applications are very useful when fast diagnostic monitoring can improve medical decision-making, such as in intensive care units, or when repetitive measurements are necessary, allowing to use them close to the patient, for example at the physician's office and to the patients' home [4]. All these characteristics perfectly match with those of SPEs, making them the most suitable devices for these types of applications.

2. Enzymatic biosensors based on screen-printed electrodes

Electrochemical enzymatic biosensors can look back over more than 40 years, beginning from the first biosensor designed by Updike and Hicks in 1967 based on the immobilization of glucose oxidase enzyme in a polyacrylamide gel on an oxygen electrode for the detection of glucose concentration in biological fluids [5], to go through the first pen-sized biosensor launched in 1987 by Medisense Inc, for self-monitoring of blood glucose by diabetic patients, and arriving at the recent innovative biosensors which exploit the new insights from research in nanotechnologies. The development of SPEs is considered a main pillar in the enzyme biosensors, since their use has moved this technology from bench laboratories to the market. Indeed, even if the first commercially glucose biosensor fabricated in 1975 by Yellow Springs Instrument Company was almost exclusively employed in clinical laboratories because of high cost of platinum electrode, the successive use of SPEs has allowed the achievement of cost-effective and hand-held biosensors for glucose monitoring and diabetes management, with the improvement of quality of life of patients and reduction of the cost of health care as well.

2.1. Nanomaterial based enzyme biosensors

The growing and exciting activities in nanomaterial development have drastically affected the research activity in the enzyme biosensor field. The capability of nanomaterials to provide an improved electrocatalytic activity and to minimise the electrode surface fouling renders their use extremely advantageous in the biosensor development. Biosensors based on SPEs modified with nanomaterials have been widely reported in literature [6,7]. The modification methods encompass the use of a drop casting techniques with nanomaterial dispersions by manual or ink-jet assisted deposition [8,9], the addition of nanomaterial in the ink during the SPE printing [10], the electrodeposition [11], the Langmuir-Blodgett film approach [12], and the deposition by means of the electrospray technique [13]. Among these methods, drop casting is the most used. Although this method is characterised by cost-effectiveness and easiness, which are good reasons for applications, it shows low homogeneity of the resulted modified-surfaces; indeed, the Langmuir-Blodgett film approach allows for a high homogenous coverage of working electrode surface, but requires more sophisticated instrumentation. Another questionable point is the different properties achieved depending on preparation methods, as in the case of graphene. To have a deeply understanding of the electrochemical behaviour of modified SPEs in function of the type of nanomaterial used, the type of graphene used as well as the modified SPEs should be characterised [14] by using SEM, X-ray photoelectron, and Raman spectroscopy, cyclic voltammetry, and electrochemical impedance spectroscopy. In this regard, we have recently performed a comparative study using SPEs modified by drop casting with carbon black, carboxylated single-walled carbon nanotubes, graphene oxide, and reduced graphene oxide. The carbon nanomaterials employed were characterized by X-ray photoelectron and Raman spectroscopy, while the modified SPEs were morphologically characterized. The electrochemical behaviour of nanomodified sensors was tested using cyclic voltammetry, amperometry, and electrochemical impedance spectroscopy to exact correlate the sensor electrochemical behaviour with the nature of the nanomaterial used, and understand how the nanomaterial affects the analytical features [15]. Indeed, the increased sensitivity, reduced detection limit, and improved stability are only some of the analytical features that are highly enhanced with the use of nanomaterials. For instance, we have recently demonstrated the ability to tune the sensitivity and detection limit of hydrogen peroxide detection, using SPEs modified with PB nanoparticles of different dimensions. This electrochemical mediator is able to selectively detect the hydrogen peroxide in presence of oxygen at potential near to zero V vs Ag/AgCl, and the dimension of nanoparticles influences its electrocatalytic properties [16]. Furthermore Prussian Blue is a mediator very stable at physiological pH and interference free from electrochemical compounds usually present in biological matrices such as blood, urine and saliva. The SPEs modified with PB layer, obtained placing a drop of mediator on a working electrode, was applied for simple and rapid analysis of uric acid in human blood

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serum [17]. The modified SPE was able to detect the hydrogen peroxide, produced by the reaction catalyzed by the Uricase enzyme immobilized directly on the modified surface of the working electrode. The application on blood sera showed a working range of 0.03–0.3 mM and a detection limit (LOD) of 0.01 mM. The recovery of uric acid in fortified serum samples was from 100 to 105% with a standard deviation <5%. To verify the accuracy of the developed biosensor, 85 blood serum samples, whose uric acid levels were independently determined by a University Hospital, were analyzed and compared with those obtained using a routine colorimetric method. Results of both methods were in agreement, showing a correlation coefficient equal to 0.981, and the Bland–Altman analysis, used to assess the quality of the regression, confirmed that the two methods were comparable. Moreover, a diagnostic test, performed by constructing the Receiver Operating Characteristic curve (ROC), was carried out in order to identify the cut-off of our method and its clinical sensitivity and specificity.

SPEs modified with PB nanoparticles using inkjet printing were employed as the basis of a cholesterol biosensor [18]. The cholesterol was assessed using cholesterol oxidase as biocomponent, measuring the enzymatic product, hydrogen peroxide, at low applied potential (0 V vs Ag/AgCl), thanks to the valuable electrochemical properties of PB nanoparticles. The uniqueness of this developed approach was the combination of the highly sensitive inkjet-printed PB nanoparticles -modified sensor with a simple low volume (4 μL) capillary-filled microfluidic chamber, obtained by an easy fabrication procedure. The application in serum demonstrated the capability of this sensor to detect cholesterol in a linear range up to 15 mM (580 mg/dL) with a LOD of 0.2 mM (8.5 mg/dL).

Composite of reduced graphene oxide and gold nanoparticles were exploited for lactate biosensor development using L-lactate dehydrogenase as biocomponent [19]. Using the applied potential of +480 mV (vs Ag/AgCl) and NAD^+ concentration of 3 mM, a calibration curve with a linear range between 0.01–5 mM was obtained, covering the normal and pathological L-lactate levels. The test performed in the artificial serum has demonstrated the L-lactate detection without urate, paracetamol and ascorbate interference. Since L-lactate can be considered as a tumor biomarker, this biosensor can be applied for early cancer biomarker detection.

Cobalt phthalocyanine nanoparticles [20] were another example of redox mediator able to detect hydrogen peroxide in presence of oxygen at low potential (+0.4 V). The modification of SPEs with this mediator in conjunction with the use of a selective membrane helped to detect different analytes in serum blood. A SPE incorporating the electrocatalyst cobalt phthalocyanine into a water-based ink formulation, has been investigated as a base transducer for a uric acid biosensor [21]. A sandwich biosensor was fabricated by first depositing cellulose acetate on this transducer, followed by Uricase and finally by a polycarbonate membrane (Fig. 1). In order to optimize the conditions for the urine analysis, several chronoamperometric measurements were carried out. The linear working range was found to be 0.015 to 0.25 mM (the former representing the detection limit), and the sensitivity was calculated to be 2.10 μA/mM, while the precision determined on unspiked urine was 5.8%. The experimentally derived values of uric acid concentrations (endogenous urine–uric acid and uric acid recovered from fortified urine) were used to calculate the mean recovery. The same design of biosensor was followed to achieve an amperometric biosensor for galactose determination in serum [22] using Galactose oxidase as biocomponent. The amperometric response of the optimized galactose biosensor was linear over the range 0.1–25 mM (LOD equal to 0.02 mM) with a sensitivity of 7.00 A mM^{-1} cm^{-2}. The response time was only 30 s. The concentration range investigated was appropriate to diagnose galactosemia (concentrations >1.1 mM in infants). Amperometric measurements were carried out in stirred solutions for the analysis of serum,

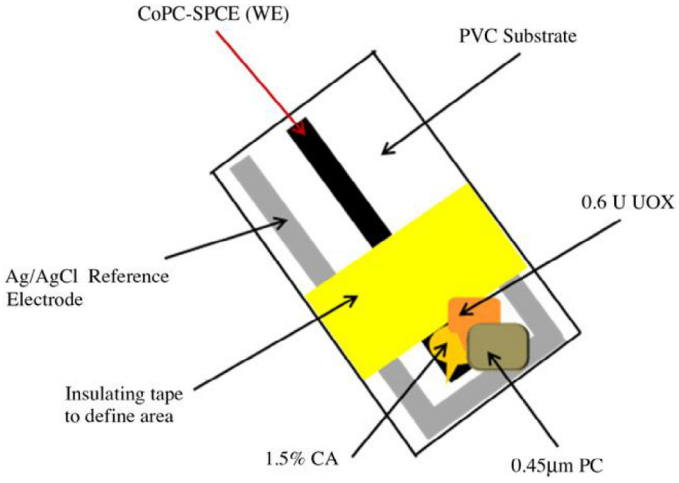

Fig. 1. Diagram of proposed sandwich biosensor strip: Cobalt phthalocyanine-SPE (CoPC–SPE) (working electrode, WE) and Ag/AgCl reference electrode; 1.5% cellulose acetate (CA); Uricase (UOX) enzyme, polycarbonate (PC) membrane over the enzyme layer [from ref. 21].

and the precision values obtained on unspiked (endogenous level of 0.153 mM) and spiked serum (1 mM added) were 1.10 and 0.11%, respectively, with a recovery of 99.9%.

2.2. Paper-based microfluidic devices

Among the cost-effective analytical tools, paper based microfluidic devices are capable of analyzing small sample volumes for multiplexed analysis, minimizing reagent consumption and analysis time, providing easy, rapid, and cost-effective analyte quantification. Furthermore, beside traditional glass and polymer based devices, the paper based can be fabricated with sustainable substrate materials, reagents can be loaded on paper allowing a reagent free detection, and the requirement, in some cases, of the pump is overcame due to the capillarity action of the paper. Many paper based microfluidic devices along with the colorimetric method (μPAD) were reported by Whiteside et al [23]; although colorimetric assays are a valuable choice, electrochemical detection provides a more versatile and quantitative methodology.

The first demonstration of electrochemical detection for paper-based microfluidic devices (μPED) was published on *Analytical Chemistry* by Henry's group [24]. The fascinating system encompasses three printed biosensors for glucose, lactate, and uric acid determination in biological samples using glucose oxidase, lactate oxidase and uricase, respectively (Fig. 2A). The content of each analyte was assessed detecting the enzymatic product hydrogen peroxide at working electrodes modified with PB, at an applied potential of 0 V vs on-chip Ag/AgCl. Values of LOD found for glucose, lactate, and uric acid were 0.21, 0.36 and 1.38 mM, respectively. In the case of glucose, the LOD achieved is lower than the value (0.5 mM) obtained by camera detection method, underscoring its competiveness in respect to the optic paper-based analytical device (μPAD) [25]. The microfluidic device was challenged in human serum, and the results gave an error comparable with that obtained by traditional tests.

A glucose quantification in artificial urine using a paper-based microfluidic devices with electrochemical detection (μPED) and glucose oxidase was reported in 2010 by Whiteside's group [26]. As highlighted by the authors, the developed μPED confines fluids in the paper channel and inhibits the convective movement of fluids, facilitating the chronoamperometric measurements (Fig. 2B). The μPED allows a glucose detection up to 22.2 mM with a LOD of

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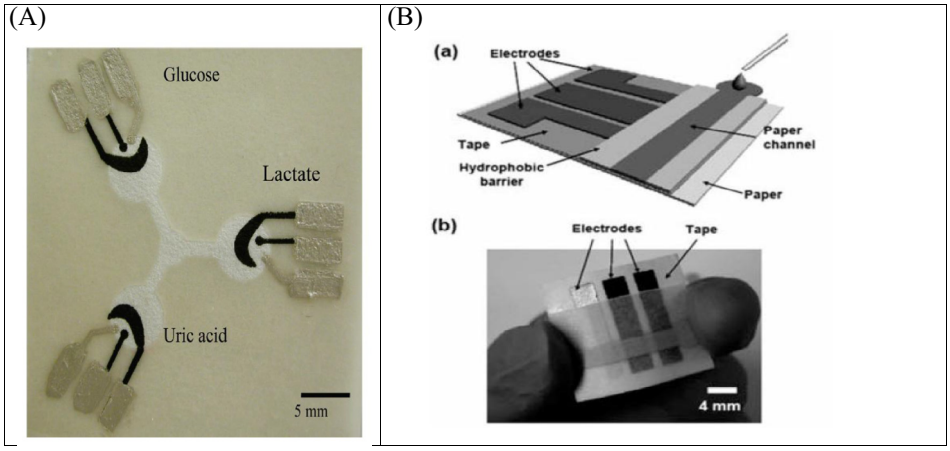

Fig. 2. A) Picture of three biosensor paper-based microfluidic devices for glucose, lactate, and uric acid. The device size is 4 cm × 4 cm [from ref. 24]; B) Schematic (a) and picture (b) of paper-based microfluidic device for glucose detection [from ref. 26].

0.22 mM, and it was successfully applied in urine, serum, and blood samples.

A paper based biosensor for cholesterol detection was recently reported [27]. The paper printed sensor was modified by electrospraying with a nanocomposite of graphene, polyvinylpyrrolidone, and polyaniline for sensitive hydrogen peroxide detection. This sensor was then used as platform for cholesterol biosensor development, immobilizing cholesterol oxidase. This biosensor detected cholesterol in a linear range comprised between 50 μM and 10 mM, with a LOD of 1 μM, and it was tested in human serum with a percentage of recovery in the range of 100–102%.

2.3. Wearable enzymatic biosensors

In the last years, several methods in clinical analysis have been explored to match the requirements of personalized health care sector. To this regard, wearable sensors have received a huge attention, since they enable a non-invasive continuous monitoring of several important parameters. An interesting work regarding lactate detection in undiluted sweat was reported by Karyakin group. Lactate is an important parameter to be detected for optimizing athlete performances in sport medicine. The quantification of lactate levels requires the collection of blood at short interval times during physical activity, because this metabolite can negatively affect the athlete performances. Even if the detection of lactate is performed using commercially available sensor strips, they require painful finger pricking, with consequent incompatibility with continuous monitoring. The developed biosensor exploited the enzymatic reaction between the lactate and the lactate oxidase immobilized on the working electrode surface for detection in undiluted sweat, using the electrochemical mediator PB (Fig. 3A). The main problem of the use of this probe in undiluted sweat relays in the high concentration of lactate (the level in human beings is usually comprised between 4 and 25 mM), which is around 10 times higher when compared with the content in blood and higher than the upper linear range of the lactate oxidase enzyme. To overcome this drawback, the authors decreased the affinity of enzyme to its substrate by using a negatively charged polyelectrolyte in the immobilization step, shielding the positive charges in the enzyme active site. The engineered lactate oxidase resulted in a higher Michaelis-Menten constant with a linear range up to 0.07 M. The developed biosensor was then integrated in a flow-through sweat collector attached to the legs or arms of human volunteers, for lactate level assessment in whole sweat. The results obtained were correlated with the values obtained in flow analysis using diluted sweat, obtaining satisfactory correlation values, and highlighting the suitability of this biosensor [28].

An innovative and very interesting approach was reported by Joseph Wang and colleagues that have developed a temporary-transfer tattoo electrochemical biosensor for continuous monitoring of lactate content in human perspiration [29]. Also in this case, the lactate oxidase was selected as biocomponent, but tetrathiafulvalene and carbon nanotubes were chosen for mediated hydrogen peroxide detection at +0.05 V vs Ag/AgCl. The tattoo sensor exhibited high sensitivity (slope equal to 10.31 μA/mM cm^2) and was highly linear up to 20 mM. The working stability is a crucial issue for in vivo sensors; for this reason, this biosensor was tested every 30 min over 8 h period using lactate concentration of 8 mM, and a good reproducibility was achieved (RSD equal to 3.6%), demonstrating its suitability for long-term epidermal use (Fig. 3B).

A first example of an easy-to-wear flexible tattoo-based epidermal diagnostic device for glucose assessment was also developed by the same Wang's group [30]. In this case, glucose biosensor was combined with reverse iontophoresis for interstitial glucose extraction. The detection of glucose was based on the use of glucose oxidase as enzyme, while the detection of the enzymatic product hydrogen peroxide was carried out by using a printed electrode prepared with PB-modified ink. The detection consisted in applying a constant current of 0.2 mA/cm^2 between two reverse-iontophoretic electrodes for 10 min, followed by the amperometric glucose response at an applied potential of –0.1 V (vs Ag/AgCl) for 5 min. This tattoo biosensor overcomes the pain associated to the finger prick required for self-testing methods, which compromises the patient compliance as well as skin irritation encountered using GlucoWatch electrochemical glucose sensor (Fig. 3C). These preliminary results showed the excellent potentiality of this innovative analytical tool for non invasive and cost-effective diabetes management.

2.4. Smart-phone sensing for enzyme testing

A significant advance compared to the present state-of-art about biosensors is represented by smartphone biosensing. Smartphones have become so ubiquitous that they are being used as virtually wearable monitors, including heart rate and activity monitoring. By taking advantage of the processing power of smartphone, peripheral non-invasive and cost-effective sensors, and wireless communication capabilities, recent efforts have been made to create various medical applications for self-monitoring. The last decade has witnessed the evolution of smartphone based biosensors for point-of-care testing [31,32]. Mass production of this kind of

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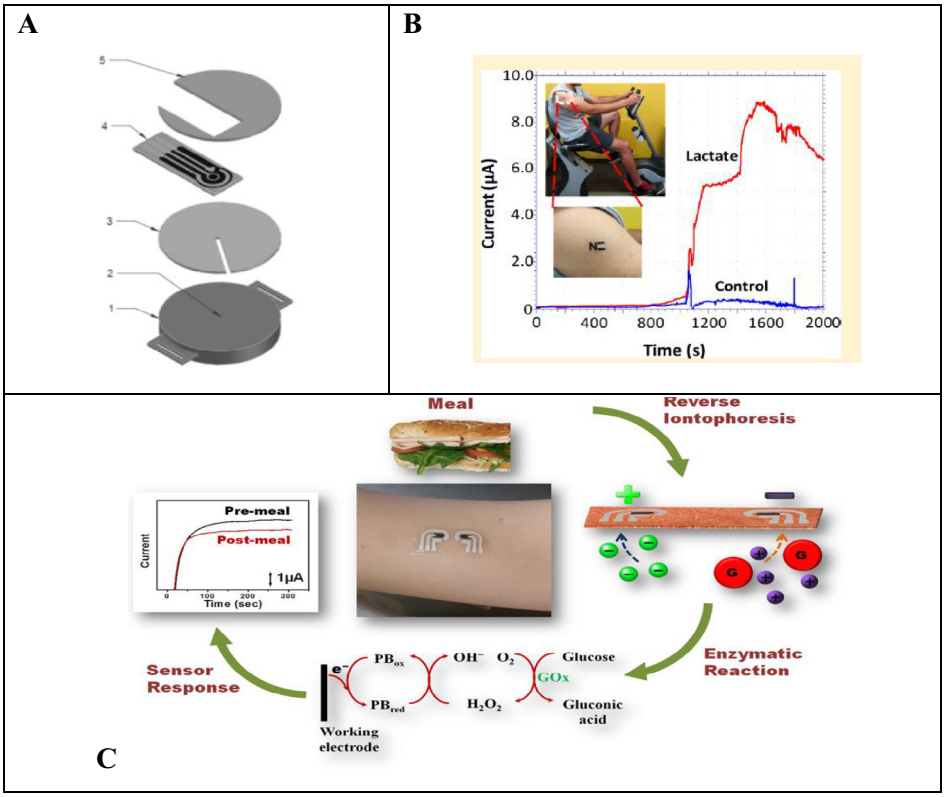

Fig. 3. A) Principal scheme of the flow-through sweat monitor used for lactate detection in undiluted sweat (1) base, (2) nozzle, (3) double side adhesive, (4) three electrode lactate biosensor, (5) polyethylenethereftalate covering [from ref. 28]. B) An "NE" lactate tattoo biosensor applied to a male volunteer's deltoid and response of the using tattoo enzyme-free (line blue) and tattoo biosensor during the exercise regimen (line red) [from ref. 29]; C) Scheme of tattoo-based platform for noninvasive glucose sensing [from ref. 30].

biosensors can take advantage of the manufacturing facilities (e.g. screen printing, laser cutting) to further reduce the cost and increase the speed of the analysis of important target molecules in human health.

One of the most recent examples, based on carbon SPE, is the smartphone-based potentiometric biosensor for quantitative detection of alfa-amylase in saliva (sAA) [33]. In this case, the enzyme was the target, and its substrate, the starch, the biocomponent. The biosensing system was composed of a sensing chip, a potentiometric reader, and an Android smartphone with an ssA-testing App installed. The sAA sensing chip (9 × 16 mm) had three layers with preloaded reagents (i.e. starch, $K_3[Fe(CN)_6]$ and NaOH). The sAA detection was carried out in different steps: the salivary sample was introduced into the reaction zone to dissolve and react with preloaded reagents (only 2.5 μL of sample were necessary). After 4 min, the sensor was pressed at the location indicated by red dot (Fig. 4), and the sample, mixed with preloaded $Fe(CN)_6^{3-}$, was pushed into the detection zone for the potentiometric measurement. In the detection zone, sAA in the sample hydrolyzed the starch to produce maltose, which reduced the electron mediator $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ under alkaline conditions. The potential shift owing to the conversion of $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ was measured by the potentiometric reader and sent to the smartphone *via* USB port. The sAA-detection App running on the smartphone converted the potential signal into sAA concentration based on a calibration curve. The potential measured was linearly correlated to the logarithm of sAA concentration from 30 U/mL to 1 kU/mL with a correlation coefficient of 0.995 (LOD = 0.12 U/mL). Since the normal range of sAA concentration is about 200–800 U/mL, this chip is applicable to analyze human saliva samples, showing the possibility to quantitatively detect sAA within 5 min, with a good agreement with commercial colorimetric kits for this enzyme (the difference in sAA concentration determined using this chip and assay kit was within 8.0%).

3. Innovative electrochemical immunosensors based on the use of SPEs

An Immunoassay is a biochemical test that detects the presence or concentration of an analyte in a sample through the highly specific molecular recognition between an antibody and epitopes of an antigen (target analyte). The key feature of all immunoassays is to produce a measurable signal in response to the binding event. The conventional Enzyme-Linked-Immunosorbent Assay (ELISA) is the most used commercially available test for clinical diagnoses and biochemical field. However ELISA is an optical approach, and as such, can suffer of several drawbacks associated with this type of measurements. These restrictions relate to potential false signals (resulting from coloured samples), sample size, rather long analysis time and difficulty of use outside the classic diagnostic laboratory [34]. In this context, electrochemical immunosensors appear as the most promising alternative to optical approach for detection of clinically important analytes. In particular, screen-printed electrodes, characterized by low-cost fabrication and large-scale production have attracted an increasing interest for the development of immunosensors. Due to the miniaturized dimensions of SPEs, all immunological steps can be performed in drop, using only a few microliters of solution, thus reducing the reagent consumption. Moreover, the decreased diffusion distances required by the analytes to reach their surface-bound receptor partners, allows shorter periods of incubation and, consequently, more rapid assays. Another advantage is that the binding event for signal generation occurs in close

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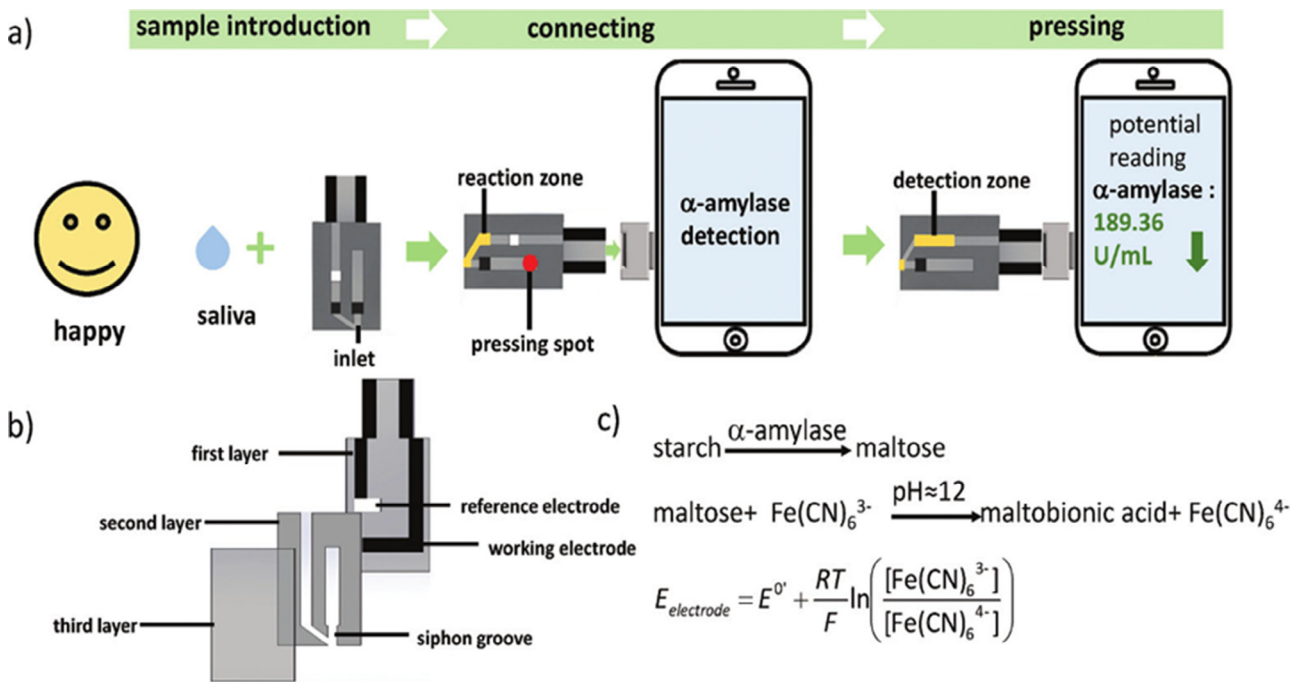

Fig. 4. Schematic illustration of (a) the sAA detection using the smartphone-based sensor for psychophysiology research and (b) the three layers of sAA sensor. The first layer is the substrate for the fabrication of the electrodes; the second layer is a double-sided adhesive tape which is patterned using a laser cutter; the third layer is a hydrophilic layer to facilitate the sample introduction by capillary action. (c) The reaction steps for the sAA detection [from ref. 33].

contact with the surface of electrochemical transducer, increasing the sensitivity of the assay [35].

Recently, novel screen-printed multichannel devices were fabricated in various architectures and employed to simultaneously run multiple detection of the same target or different targets (in a multi-array platform), overcoming the problem associated with the use of a single SPE. This paper's section will be devoted to the description of the some innovative electrochemical immunosensors for the detection of clinically important protein biomarkers. In particular, we focus our attention on label-free and no-label free electrochemical immunosensors based on the use of SPEs coupled with gold nanoparticles, carbon nanotubes, graphene, magnetic particles and quantum dots (QDs) as new strategies to improve the analytical performances of the conventional SPE-immunosensors [36].

3.1. *Label-free innovative electrochemical immunosensors*

Most electrochemical immunosensors require a label attached to an antigen or antibody to achieve an electron-transfer; during the readout, the amount of labels is detected and assumed to correspond to the number of the target analytes. However, labelling a biomolecule with various agents might influence the antigen-antibody binding efficiency [37], and the yield of the biomolecule-label coupling reaction is highly variable [38]. Thus, the use of label-free electrochemical immunosensors has become increasingly popular over the years.

Pandiaraj et al [37] have designed a label-free direct electrochemical immunosensor for the detection of cytocrome c (cyt c), a biologically important mitochondrial metalloprotein containing a prosthetic group (heme) and exploiting its reversible redox-activity. The specific monoclonal antibody was immobilized onto the surface of two nanoarchitectured sensing platforms: (i) self-assembled monolayer on GNPs electrodeposited onto the surface of a polypyrrole (PPy) matrix, previously electropolymerized on the SPE working electrode; (ii) CNTs incorporated *via* Nafion on PPy modified SPE. GNP and CNT platforms were used to enhance the direct electron transfer between the cyt c and the electrode surface, evaluated by cyclic voltammetry. Although both platforms allowed electrochemical immunosensing with high sensitivity, the overall analytical performance of the gold nanoparticle-based immunosensor (LOD = 2 nM; linear range = 2 nM–150 μM; sensitivity = 154 nA/nM) resulted better than those shown by the nanotube-based one. The Anti-cyt c/GNP/PPy based immunosensor was then applied to detect the levels of cyt c release from cell lysates of cardiomyocytes. The results obtained gave an excellent correlation with standard ELISA.

Silva et al [39] have developed a carbon nanotube screen-printed electrode for label-free detection of the cardiac troponin T (cTnT), an important marker of acute myocardial infarction. In particular, amine-functionalized CNTs were incorporated into the printing ink used to fabricate SPEs. Due to the presence of the amino groups, a non-random and stable-orientated immobilization of the specific antibody was achieved. This feature, combined with the electrochemical advantages of CNTs, allowed a rapid and sensitive detection of cTnT *via* ferrocianyde/ferricianyde redox probe, monitored by Differential Pulse Voltammetry (DPV). A gradual decrease in DPV current peaks was registered with increased cTnT concentrations. A linear response between 2.5 and 500 pg/mL was obtained with a LOD of 3.5 pg/mL, lower than any previously described immunosensor. The label free CTNs-SPE immunosensor was then applied in serum samples and the results showed good agreement with those obtained by the electrochemiluminescent immunoassay. Although further validation studies with real samples are required before its clinical use, the authors claim that immunosensor appears to be a potential tool for point-of-care acute myocardial infarction diagnostic testing.

Bhalla et al [40] deposited citrate-capped GNPs on SPEs using an innovative one-step electrochemical technique. The formation of a nanosized colloidal Au layer on SPE was characterized by cyclic voltammetry scans and impedance spectroscopy. A monoclonal antibody anti-cardiac troponin 1 (anti-cTn1) was then immobilized on the above formed Au matrix by electrostatic interaction. The

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innovation in the proposed method relies on the dual role of the colloidal GNPs as a matrix for antibody immobilization and its transduction properties. It is important to stress that to measure the interaction between cTn1 and its corresponding antibody, nor label nor redox probe were used. The authors took advantage of the highly charged nature of the antigen at physiological pH, so the binding event leads to charging of SPE surface which is detected by the capacitive component of the impedance. A LOD of 0.2 ng/mL, one order of magnitude better than that obtained with ELISA test (performed by using the same antibody), showed a good potential to diagnosis of heart failures with cardiac troponin detection in blood serum.

Ravalli et al [41] reported a label-free impedimetric immunosensor for the detection of cancer antigen 125 (CA 125), a protein associated with epithelial ovarian cancer. A self-assembled monolayer (SAM) was formed on the surface of GNPs electrodeposited on SPEs using a thiolic compound (11-mercaptoundecanoic acid), whose carbossilic groups were coupled with amino groups of anti-CA 125 antibody *via* 3-(3-dimethylaminopropyl)carbodiimide/N Hydroxysuccinimide (EDCA/NHS). Each step of the developed immunosensor was successfully characterized using cyclic voltammetry and electrochemical impedance spectroscopy, using $[Fe(CN)_6]^{4-/3-}$ as redox probe. A good linear relationship between the electron transfer resistance and CA 125 concentration in the range of 0–100 U/mL, with a LOD of 6.7 U/mL, was obtained. Experiments concerned commercial serum spiked with CA 125 at two different concentration levels were performed. The serum sample signal increased according to the CA 125 concentration, indicating that it is possible to establish a direct correlation.

3.2. No label-free innovative electrochemical immunosensors

The most common label-based electrochemical immunosensors relies on specific properties of the labels to produce a current signal for detecting a given target. In particular, the label may itself be electroactive or able to generate an electroactive product.

Yan et al [42] developed disposable electrochemical immunosensor for the detection of prostate specific antigen (PSA), in which vegetable parchment was used as substrate for SPE printing. This SPE was used to explore the fabrication of a novel, disposable and highly sensitive electro-analytical immunosensor, based on the use of GR and horseradish peroxidase (HRP)-labeled antibody (Ab_2) functionalized with gold nanoparticles (HRP-Ab_2/GNPs). GR was used to modify the sensor surface in order to increase the conductivity and stability of the immunosensor, due to its fast electron transfer and good biocompatibility. GNPs were employed not only to provide a larger surface area for the immobilization of HRP-Ab_2, but also to enhance the electroreduction between HRP and H_2O_2 to amplify the electrochemical signal, detected by linear sweep voltammetry (LSV). The as-prepared immunosensor showed a wide linear range over 6 orders of magnitude with a LOD of 0.46 pg/mL. Human serum samples were analyzed with the proposed immunosensor to detect PSA. The results, compared with the reference values obtained by commercial turbidimetric immunoassay, showed an acceptable concordance. In addition, different amounts of PSA were added into human serum sample for recovery tests, indicating good accuracy and precision.

Lai et al [43] developed a multiplexed electrochemical sandwich-type immunoassay method for simultaneous ultrasensitive detection of carcinoembryonic antigen (CEA) and α–fetoprotein (AFP). The disposable immunosensor array was prepared by covalently immobilizing capture antibodies on chitosan modified SPEs. Through a sandwich-type immunoreaction, antibodies functionalized with GNPs were captured on the immunosensor surface to induce the silver deposition (around the GNPs) from a silver enhancer solution. The deposited AgNPs were directly detected by anodic stripping analysis. The proposed multiplexed immunoassay showed wide

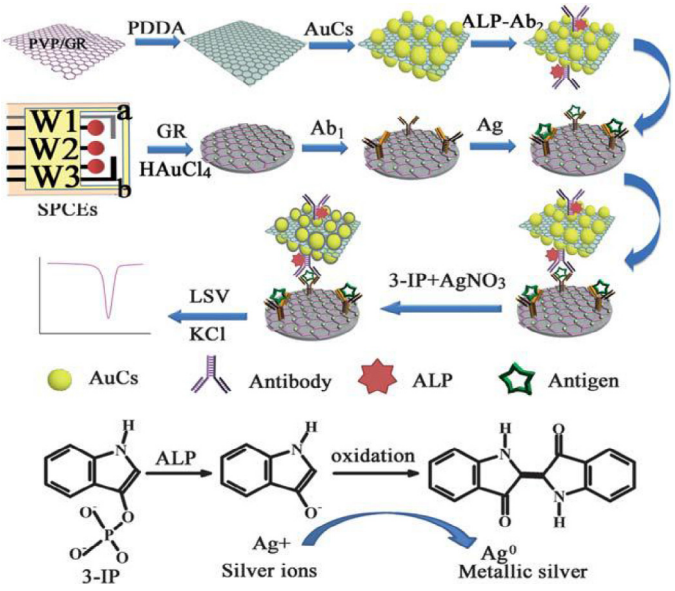

Fig. 5. Schematic representation of preparation of immunosensor array and trace tag, and detection strategy by LSV analysis of AgNPs on the immunosensor surface [from ref. 44].

linear ranges of three orders of magnitude with LODs of 3.5 and 3.9 pg/mL for CEA and AFP, respectively. The authors demonstrated the reliable detection of these tumor markers in clinical serum samples with acceptable correlation with a commercial electrochemilumiscent test.

Ge and co-workers [44] fabricated a multiplexed immunosensor platform on a carbon electrode array containing three graphite working screen-printed electrodes, for a simultaneous detection of three tumor markers of breast cancer: cancer antigens 153 and 125 (CA 153, CA 125) and CEA. GR was also used in this approach in order to modify the immunosensor surface for accelerating the electron transfer. The in situ synthesis of GNPs on GR modified electrode surface was performed to favour the immobilization of the capture antibody (Ab_1). With a sandwich-type immunoreaction, the alkaline phosphatase (ALP)-labeled antibody (Ab_2), previously immobilized on Au clusters graphene (AuCs/GR), was captured on the immunosensor surface and catalyzed the hydrolysis of 3-indoxyl phosphate in an indoxyl intermediate able to reduce silver ions, present in solution. The metallic silver deposited on the immunosensor surface, to form AgNPs, was detected by LSV. The authors have proven that when Ab_2-AuCs/GR (without ALP) was captured on the immunosensor surface a current response due to the catalysis of AuCs/GR for silver deposition was observed; this current signal greatly increased using ALP-Ab_2-AuCs/GR as label, demonstrating the synergic effect of ALP and AuCs/GR for silver deposition, amplifying the signal. Furthermore, the current response recorded using ALP-Ab_2-AuCs/GR as tracer was almost 10-fold higher than that with ALP-Ab_2, which was attributable to the signal enhancement from the high-content of the enzyme on ALP-Ab_2-AuCs/GR. The set-up of the assay is reported in Fig. 5.The group demonstrated the successfully measurements in clinical serum samples with good correlation to commercially traditional test.

Wu et al [45] first reported on the integration of a signal amplification strategy in a microfluidic paper-based electrochemical immunodevice for the multiplexed detection of cancer biomarkers (AFP, CEA, CA 125 and CA 153). In this approach, eight working electrodes, sharing one pair of counter and reference electrodes, were modified with GR/chitosan and coated with AFP, CEA, CA 125 and CA 153 capture antibodies. To perform the sandwich

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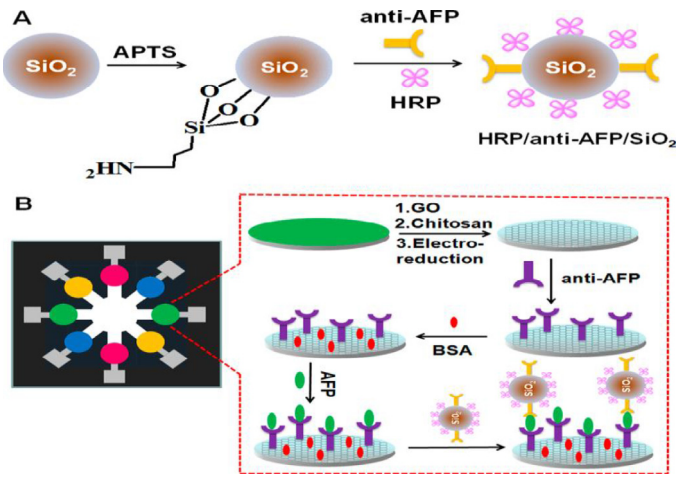

Fig. 6. A) Preparation of nanobioprobes through the coimmobilization of HRP and antibody onto monodispersed SiO_2 nanoparticles. B) Schematic representation of the fabrication and assay procedure used to prepare microfluidic paper-based electrochemical immunodevice [from Ref. 45].

immunoreactions and electrochemical detection, a sample solution containing different concentrations of AFP, CEA, CA 125 and CA 153 was added to each working electrode. Then, nanobioprobes, constructed co-immobilizing HRP and antibody on monodispersed SiO_2 nanoparticles, were added (see Fig. 6). In this manner, signal amplification was achieved through the combined use of graphene, employed to modify the immune-device surface to accelerate the electron transfer, and silica nanoparticles as tracing tags to label the antibodies. For signal detection, a mixture of *O*-phenylenediamine and H_2O_2 was added to the centre of the paper and the eight working electrodes were sequentially interrogated using DPV. This newly designed disposable microfluidic paper-based electrochemical immunodevice platform could be used to fabricate a new generation of simple, low-cost, disposable and yet recyclable, portable point-of-care diagnostic devices.

In 2012, Neves et al [46] proposed an electrochemical immunosensor for the diagnosis of celiac disease (CD) based on the voltammetric detection of human anti-gliadin antibodies (AGA) IgA and AGA IgG in serum samples. The transducer surface consisted of SPE modified with a CNT/GNP hybrid system, in which the combination of the physical and chemical properties of the individual components leads to a very useful surface for the amplification of the immunological interactions. The principle of the method is based on the interaction between gliadin, immobilized onto the nanostructured surface, and human autoantibodies directed against gliadin. This binding event was revealed by adding anti-human IgA or anti-human IgG antibodies, labelled with ALP, and a mixture of 3-indoxyl phosphate with silver ions (3-IP/Ag^+). After an appropriate incubation time, the metallic silver enzymatically deposited on the electrode surface was detected using anodic stripping voltammetry. A LOD of 9.1 and 9.0 U/mL was obtained for AGA IgA and AGA IgG, respectively. The immunosensor was then applied in human serum samples and the results obtained were supported with a commercial ELISA test.

A similar approach was used by the same authors [47] in order to fabricate other immunosensors for CD diagnosis, based on the detection of IgA and IgG autoantibodies against tissue transglutaminase (tTG) in serum samples. In this case no LODs were calculated, but a cut-off value, defined as the average peak current intensity plus three times the standard deviation obtained for 8 negative samples, was established. This value (7.3 μA and 12 μA for anti-tTG IgA and anti-tTG IgG, respectively) was used to classify serum samples as positive, negative or ambiguous.

The two immunosensors reported above [46,47] were merged, by the same authors, in a multiplexed electrochemical immunosensor for the simultaneous detection of IgA and IgG AGA and anti-tTG [48]. SPEs with two working electrodes, used for the immobilization of gliadin and tTG, were challenged in serum samples. The immunosensor results were compared with those obtained with an ELISA kit, whose recommended cut-off values were also adopted in the electrochemical measurements to classify the samples as positive, negative or ambiguous. The results showed a good agreement between the two methodologies.

Other interesting electrochemical immunosensors for the detection of anti-tTG IgA and anti-tTG IgG in human serum samples were developed by Martin-Yerga et al [49,50]. An array of 8-channel SPEs was used as transducer and modified with tTG by adsorption. Anti-human antibodies, labelled with Cd/ZnS QDs, were employed as bioreagents for the immunoassay. Cd^{2+}, released from QDs after an in situ acid attack, was detected using anodic stripping voltammetry. This sensitive technique, which provides a preconcentration of a large number of metal atoms on the electrode surface, allowed to detect 2.2 U/mL of anti-tTG IgG and 2.4 U/mL (or 2.7 U/mL depending on the methodology adopted: multistep or one step) of anti-tTG IgA. The use of different QDs could be used to create a multi-array platform for the simultaneous detection of IgA and IgG autoantibodies against both tTG and gliadin, for a more widespread diagnosis of celiac disease.

An ultrasensitive ELIME (Enzyme-Linked-Immuno-Magnetic-Electrochemical) assay for the diagnosis of CD in saliva samples that overcomes the problems related to the high viscosity and to the low concentration of anti-tTG IgA in this medium, has been developed for the first time by Adornetto et al. 2015 [51]. The system uses MBs covered with tTG, which reacts with the anti-tTG IgA antibodies present in positive saliva samples. An anti-Human IgA, conjugated with ALP enzyme, was used as label and a strip of 8 magnetized SPEs as electrochemical transducer. Compared to the previous work, optimized for serum sample analysis [52], the ELIME-assay was completely redone in order to have a system suitable for measuring anti-tTG IgA antibodies in a much more difficult matrix such as saliva. To achieve this goal, the authors focused their attention on the selection of different MBs, blocking agent and buffers used for the dilution of standards/samples and anti-human IgA-ALP, together with the evaluation of two different approaches for the immunoassay procedure. Both approaches were then applied to the blind analysis of a large number of saliva samples, whose anti-tTG IgA levels were independently determined by RIA method. The obtained results were used to perform a diagnostic test evaluation through the construction of ROC curves. From the analysis of these curves the approach reported in Fig. 7, named ELIME assay Plus (involving a pre-incubation between the anti-Human IgA-AP and saliva samples prior to the addition of MBs-tTG) showed a cut-off of 0.022 with 95% of clinical sensitivity and 96% of clinical specificity. The area under ROC curve resulted to be equal to 1, a result that classifies the test as "perfect [53]. Therefore, this innovative system based on the use of MBs, which employs saliva samples instead of serum samples could be considered an useful tool for preliminary screening diagnosis of CD in "Doctor office" or in non-hospital facilities, becoming a POCT.

An ultrasensitive amperometric magneto-immunosensor for the quantification of the human C-reactive protein (CRP), a nonspecific biomarker of inflammation and infection (often used as a predictive risk marker of cardiovascular disease in asymptomatic individuals), was developed by Pingarron's group [54]. The immunological chain, in a sandwich format, was constructed onto the surface of carboxylic-modified MBs. At the end of all immunological steps, MBs were captured by a magnet placed under the surface of a disposable gold SPE. The amperometric response was measured at –0.1 V (vs Ag/Ag Cl) upon addition of a solution containing

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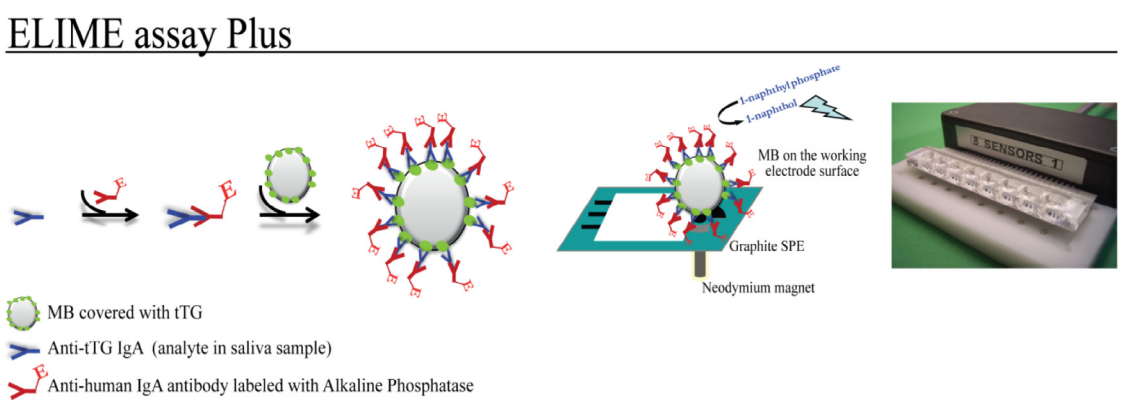

Fig. 7. Schematic representation of the ELIME assay based on MBs, as support of the immunological chain, coupled with a strip of 8 magnetized SPEs, for measuring anti-tTG IgA antibodies in saliva [from Ref. 52].

the appropriate substrates for HRP enzyme (used as label). The magneto-immunosensor possesses an excellent analytical performance achieving a LOD of 0.021 ng/mL, value well below the minimum cut-off value (1000 ng/mL) to quantify the severity of risk for cardiovascular disease, and allowing the reliable detection of CRP across the clinical relevant range in dilute blood serum.

4. DNA-based electrochemical disposable sensors

The use of disposable electrodes has also found several applications in the development of DNA-based electrochemical sensors. The possibility to identify specific DNA sequences has attracted significant attention during recent years due to its possible application in fields such as pathogen detection and genetic diseases diagnosis [55–57]. DNA electrochemical-based sensors have been thus developed and optimized for a wide variety of DNA sequences specific of certain genetic mutations, certain pathogens (i.e. E. Coli, Salmonella, etc) or specific diseases (i.e. herpes simplex virus, Epstein–Barr virus, cytomegalovirus, etc). Similarly, the use of specific DNA sequences have been used for the electrochemical detection of proteins (i.e. transcription factors or other DNA-binding proteins) and aptamers' targets (both proteins or small molecules). A complete description and updated list of examples is reported in the impressive and exhaustive review recently published by Palececk, one of the pioneers of DNA electrochemical detection [58].

Major advantages of electrochemical detection of DNA hybridization, compared to other approaches (such as optical) are the rapid response time, low cost and suitability for mass production. In this direction the use of disposable electrodes appears crucial and it is not a surprise that this type of electrochemical chips has been so widely employed for the construction of DNA-based sensors.

Obviously, the vast majority of these disposable electrodes is based on the use of gold-based ink which, although much more expensive than the common carbon-based ink, allows to achieve well ordered gold-thiol self-assembled monolayers (SAM) using DNA oligos end modified with alkane thiols. We note that despite the fact that roughness factors and surface defects of SPEs are not comparable with conventional gold rod electrodes (characterized by a very smooth surface area), the quality of SAM achieved on these disposable electrodes is still quite good and allows to reach good reproducibility and efficient electron-transfer rates [59].

We also note that one of the perks of using disposable electrodes for DNA-based sensors is the fact that electrochemical cleaning and mechanical polishing of the electrode surface is usually not needed, thus making the whole sensor production process much more immediate and easy. As an example, we note that the typical cleaning procedure for a conventional rod electrode involves first a mechanical polishing using alumina powder and then an electrochemical cleaning based on oxidation and reduction cycles in strongly acid solutions. This whole procedure usually takes about 2 hours for each electrode to be completed making the use of conventional rod gold electrode in the lab quite annoying [60]. Another advantage during the sensor production phase is represented by the possibility of using very small volumes of solution to be applied on the electrode surface. For example, for classic SPEs it is usually sufficient (when spontaneous thiol-gold linkage is used) to cast a drop of few microliters (10–20) of the thiol-labeled oligo on the surface of the gold-ink electrode to achieve well-ordered SAMs. This is usually not possible with conventional rod gold electrodes which generally require larger volumes. A caution during the use of screen-printed electrodes is to avoid that the drop of thiol-labeled oligo would be in contact with the reference electrode which is typically made of silver ink, because of the possibility to have thiol-silver reaction with the result of passivating the reference electrode surface. To avoid this, it is thus necessary to either place the drop exclusively on the surface of the gold-based working electrode or to chlorinate the silver-based ink electrode before use.

An interesting alternative to the classic SPEs for the construction of DNA-based electrochemical disposable sensors is represented by paper-based electrodes which have been recently demonstrated as advantageous for folding based electrochemical DNA sensors (Fig. 8) [61–63].

In the recent years, many examples have been proposed for the electrochemical detection of DNA and RNA sequences using disposable electrodes [64–71]. Among these, many are those based on different signal amplification procedures that allow to achieve very low detection limits [72–80]. This, however, generally requires the addition of exogenous, label containing secondary probes and, typically multi-component deposition/amplification steps.

In response to this, we and others groups developed a number of single-step, label-free electrochemical biosensors that are based on the target binding-induced folding of electrode-bound DNA probes and that are rapid, reagentless and operationally convenient [56,81]. Similar electrochemical assays have been recently proposed using different electrochemical labels and different measuring approaches using disposable electrodes [82,83].

In this regard, an important consideration should be done. The use of amplification steps is strictly required when low detection limits are needed. We have recently demonstrated, using disposable gold-based screen-printed electrodes, that electrochemical approaches based on the direct measurement of DNA hybridization, not involving any amplification steps, have physical limitations

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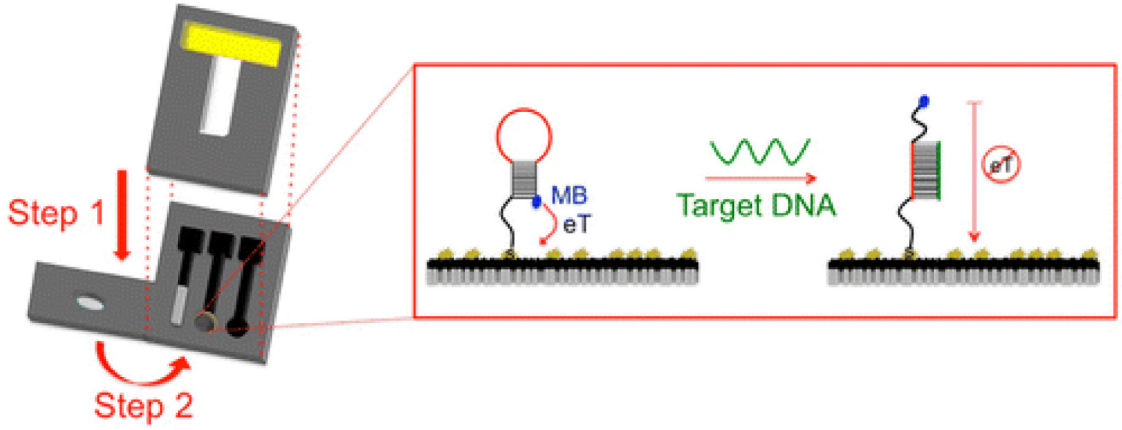

Fig. 8. Electrochemical disposable DNA-based sensors have been recently proposed using paper analytical device (PAD). The measurement of DNA sequences or aptamers' target is based on the principle of target-induced conformational switching of an aptamer linked to an electrochemical label. These paper-based sensors appear has a valuable alternative to classic screen-printed electrodes. [from ref. 61].

in their analytical performances [84]. In fact, we have demonstrated that the observed affinity of reagentless surface-based DNA biosensors is defined not by the true affinity of its probe, but instead by the effective probe concentration. So, the sensor detection limit and specificity strongly depend on the surface density of the probes, the surface area, and the volume of sample employed. Because we cannot decrease infinitively the probe surface density, this type of direct measurement will always give detection limits that cannot be much lower than few nanomolars.

We thus suggest that careful attention should be paid to the conditions under which the binding curves of electrochemical DNA-based biosensors are obtained. Moreover, we propose that the discrepancies often observed in the claimed analytical performances of similar DNA-based sensors mainly arise by the lack of understanding of the above simple physical considerations. Under normal conditions, it is unrealistic to achieve picomolar detection limits with direct electrochemical approaches that are not based on any amplification step [84].

Obviously, another important branch of DNA-based electrochemical methods is also represented by those using impedimetric measurements [85–88]. Several examples have been proposed in the last years using this type of detection coupled with SPEs. In this case, in addition to the limitations regarding the analytical performances (detection limits and specificity) seen above for direct electrochemical approaches, a careful study of the effect of matrix should be always conducted as impedimetric measurements are more prone to the effect of non-specific signals due to, for example, absorption of interfering species on the electrode surface.

One of the advantages of using DNA as recognition element is the variety of targets that can be detected. In fact, apart from the classic use of DNA as recognition element of complementary DNA or RNA sequences, DNA sequences, named aptamers, can also be selected to specifically recognize small molecules or proteins. Specific DNA sequences can also be used as convenient recognition elements of transcription factors, proteins that recognize specific double-stranded DNA-sequences and control crucial biological mechanisms such as cell proliferation and apoptosis. Finally, DNA sequences can also be used to recognize anti-DNA antibodies, important markers of several autoimmune diseases [89].

In the period considered in this review several examples have been proposed to develop electrochemical disposable sensors based on the use of DNA aptamers or DNA sequences for the detection of aptamers' targets, proteins, transcription-factors and anti-DNA antibodies [65,90–95].

Another interesting example in the vast literature of DNA-based disposable electrochemical sensors is represented by the possibility of using DNA not as a recognition element but purely as a structural scaffolding element to conjugate other recognition elements and broaden the range of possible targets detected with DNA-based sensors [96,97]. In this perspective, a neat example has been recently proposed by the group of Alexis Vallee-Belisle in collaboration with our group [98].

This approach utilizes steric hindrance effects at nanoscale level to detect large macromolecules such as proteins or antibodies (Fig. 9). The target protein, in fact, when bound to a signaling DNA strand, generates steric hindrance effects, which limits the ability of this DNA to hybridize to a surface-attached complementary strand. This steric hindrance effect has been used to achieve the multiplexed, quantitative, one-step detection of different proteins and antibodies in less than 10 minutes and directly in whole blood.

5. Conclusions

Biosensors have experienced a continuous growth since their appearance, and are expected to further growth over the next years. Although the commercialization of biosensors does not follow the advancements shown in literature, the global bio-chip market is expected to reach US$ 11.4 billion by 2018 [99]. The healthcare sector, with its requirements for *Point-of-care testing* for early diagnoses, will certainly drive the overall biosensors field, but the food safety and quality control and the environmental monitoring are areas where there is an increasing demand by people that are becoming more aware of the possible risks involved.

This review tried to illustrate the latest advances in the field of electrochemical disposable biosensors, because we are convinced that as for the glucose monitoring in diabetic patients, these devices are surely a good solution to solve problems in other fields, and/or monitoring other metabolites in which people is becoming perceptive. The rising incidences of lifestyle diseases such as cardiac ones and diabetes, the persistency of stroke as one of the leading cause of death, the increasing prevalence of infectious diseases in developing countries, strongly indicate the need of further development of new disposable biosensors suitable to be integrated in POCT devices. Adrenocorticotropic Hormone (ACTH), Endocrine testing, Gastrin, Growth Hormone, Microbiology outbreaks/epidemics, Methicillin resistant Staphilococcus aureus, Parathyroid hormone (PTH), Sepsis markers, are further potential candidates for this kind of analyses, so as current developments in POCT are

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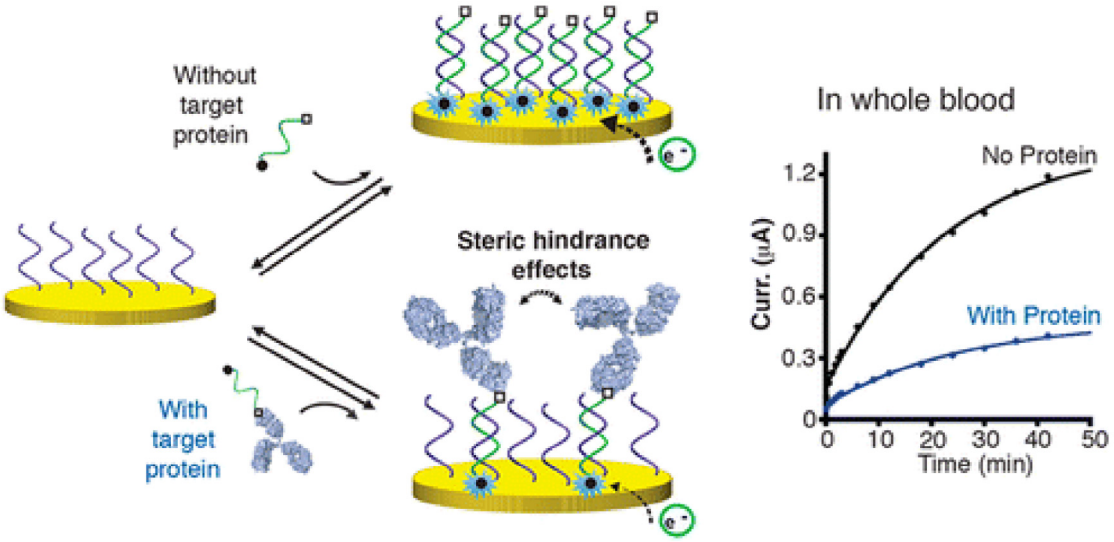

Fig. 9. Representation of an electrochemical Steric Hindrance Hybridization Assay (eSHHA) where a macromolecular target (here an antibody) can be detected by measuring the difference in hybridization efficiency between a surface-bound capturing DNA strands (purple) and a free complementary signaling DNA strand (green) labeled with a small recognition element (γ) and a signaling redox label [black circle (●)]. From ref. 98.

attempting to address the challenges of diagnosis and treatment of cancer, through the capture and analysis of circulating tumor cells (CTCs). Moreover, genomic analyses, will allow to identify several genes and their polymorphisms associated with a higher risk of different diseases [100–102].

The new discoveries in nanotechnology can be perfectly integrated with the development of these new biosensors, increasing their analytical performances and potential applications.

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