SCREEN-PRINTED ELECTROCHEMICAL IMMUNOSENSORS FOR THE DETECTION OF CANCER AND CARDIOVASCULAR BIOMARKERS

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Abstract

Electrochemical immunosensors (EIs) for the determination of disease biomarkers has attracted a wide interest due to its high sensitivity, low cost, and possible integration in compact analytical devices. The use of screen-printed electrodes (SPEs) to develop EIs contribute to the great potential they have in point of care (POC) test, since SPEs show good electrical properties and allow the reduction of the electrochemical instrumentation down to small pocket-size devices. Moreover, during the last years, SPEs have gone through significant improvements related to both their design and printing materials. Since cancer and cardiovascular diseases are the major threats of global health, there is a growing demand for develop portable, rapid, simple and inexpensive devices for the detection of these diseases. This article presents an overview about the main biomarkers of cancer and cardiovascular diseases and the EIs based on SPEs for the detection of these biomarkers.

***Keywords**: Screen-printed electrode, Electrochemical, Immunosensors, Cardiovascular biomarkers, cancer biomarkers.*

1 Introduction

A biomarker (biological marker) can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention [1]. Sensitive and selective detection of disease biomarkers is of great importance for early diagnosis, staging of disease, prediction and monitoring of clinical response to a treatment and even develop molecularly targeted therapy [1-3]. Biomarkers can be specific cells, molecules, genes, gene products, enzymes or hormones and can be measured in biological media such as tissues, cells or fluids [1,4]. To maximize the usefulness and minimize the cost and time for screening, it is advantageous that these biomarkers could be measurable in biological fluids which allow a non- or minimally invasive sample collection such as serum, urine or saliva.

During last years, the need to save time and money gaining simultaneously in efficacy, implies the decentralization of analytical operations to point-of-care (POC) system platforms (Figure 1) [5-7]. POC testing can be described as ancillary, bedside, near-patient, remote and decentralized laboratory testing, which is performed at the site of patient care [8]. POC testing can be performed in a hospital, in a doctor's office or even at home; moreover, this devices can also be very useful in resource-limited settings [8,9].

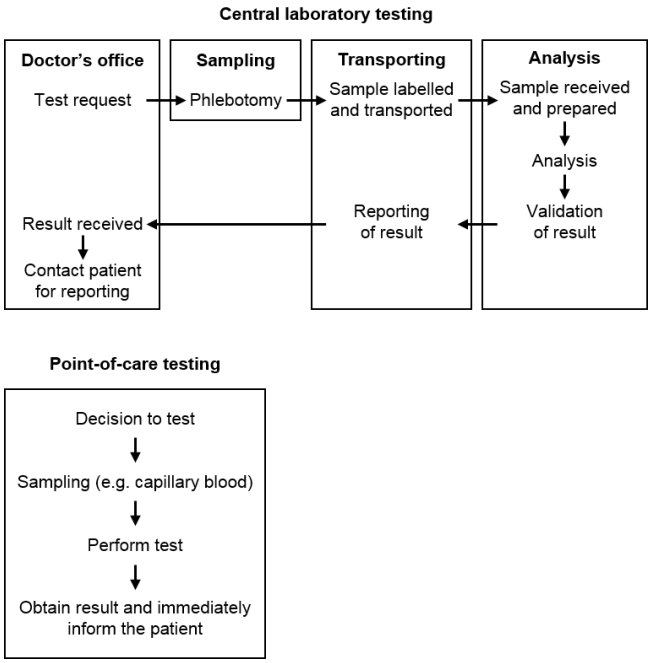

Fig. 1. Scheme simplified of the process of clinical testing using central laboratory versus POC testing. Adapted from [10].

So, the realization of POC testing requires not only fast, sensitive and selective detection, but also miniaturized, inexpensive and integrated device. Electrochemical immunosensors (EIs) with its high specificity and sensitivity, low cost, and potential of automatization and miniaturization has been a promising approach for POC testing [10,11]. Screen-printed electrodes (SPEs), with low cost and mass production, have been extensively employed for developing novel EIs providing advantages such as portability and low sample consumption [12-14]. Moreover, due to its great versatility of design, SPEs allow for development multiplexing analysis. The possibility of simultaneously determination of different analytes saving time and money has a high importance in critical clinical situations since it can discard different pathologies and conduct the patient to the correct treatment.

There are several reviews about EIs [11,12,15-20], however only a few of them are focusing on the biomarkers detected [21-23]. In addition, although the bibliography about

biosensors based on SPEs are extensive, there are few reviews about this [12,14,24,25]. This review summarizes the main biomarkers of cancer and cardiovascular diseases and recount publications about EIs based on SPEs for these biomarkers focusing on their final analytical application.

2 Electrochemical immunosensors

The International Union of Pure and Applied Chemistry (IUPAC) defines biosensor as 'a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals' (International Union of Pure and Applied Chemistry, http://goldbook.iupac.org/, 2016). The development of a biosensor was first reported by Clark and Lyons in 1962 whereby they demonstrated enzyme electrodes for glucose determination [26]. The term 'biosensor' was coined by Cammann in 1977 [27]. A biosensor has two major components: a biological detector or sensor molecule (bioelement) and a signal transducer that provides a signal that the ligand has bound to the receptor molecule [28,29].

An immunosensor is a class of biosensor that comprises an antigen or antibody species coupled to a signal transducer which detects the binding of the complementary species [29]. Regarding the detection, there are four main types of immunosensors: electrochemical (potentiometric, amperometric, conductimetric/capacitive and impedimetric), optical, microgravimetric and thermometric [30]. In relation to the immunoreactions, there are four main types of immunoassays in which an immunosensor can be based on: direct, indirect, sandwich and competitive (Figure 2). The basic principles for the assay are similar and they generally include the following steps: capture

of the analyte (usually the antigen), blocking of non-reacted surface and recognition of the analyte. Direct immunoassay is the simplest assay. It lies in immobilising the antigen to the surface and, after washing and blocking steps, a specific labelled antibody is added for detection (Figure 2A). In an indirect immunoassay the antigen is immobilized onto the surface and then it is bound to a specific antibody. Then, a labelled secondary antibody against this primary antibody is incubated for detection purposes (Figure 2B) [31]. In the sandwich assay, after antigens binds with antibodies immobilized onto the surface, labelled antibodies directed toward a second binding site of the antigen are added. Thus, the antigen is "sandwiched" between two antibodies (Figure 2C) [12,32]. In the case of competitive assays, two approaches can be followed: a first one in which immobilized antibodies react with the free antigens in competition with labelled antigens (Figure 2D) or a second one in which immobilized antigens compete with free antigens for labelled free antibodies (Figure 2E). Both these approaches are denoted as direct competitive immunoassay. The second format is generally preferred and avoid the problems related to antibody immobilization (correct orientation of the antibody and loss of affinity). It is also used when labelled primary antibodies are not available for the analyte of interest. In this case, a labelled secondary antibody is used to binding with the primary antibody for detection purposes. This format is defined as indirect competitive immunoassay (Figure 2F) [31,32]. Most of the developed immunosensors are based either on a competitive or sandwich assay. Methodologies that utilise a single recognition phase (antibody-antigen complex) suffer reduce specificity compared to dual recognition phase (sandwich complex) strategies [33]. Moreover, the sandwich format can be between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid surface [34].

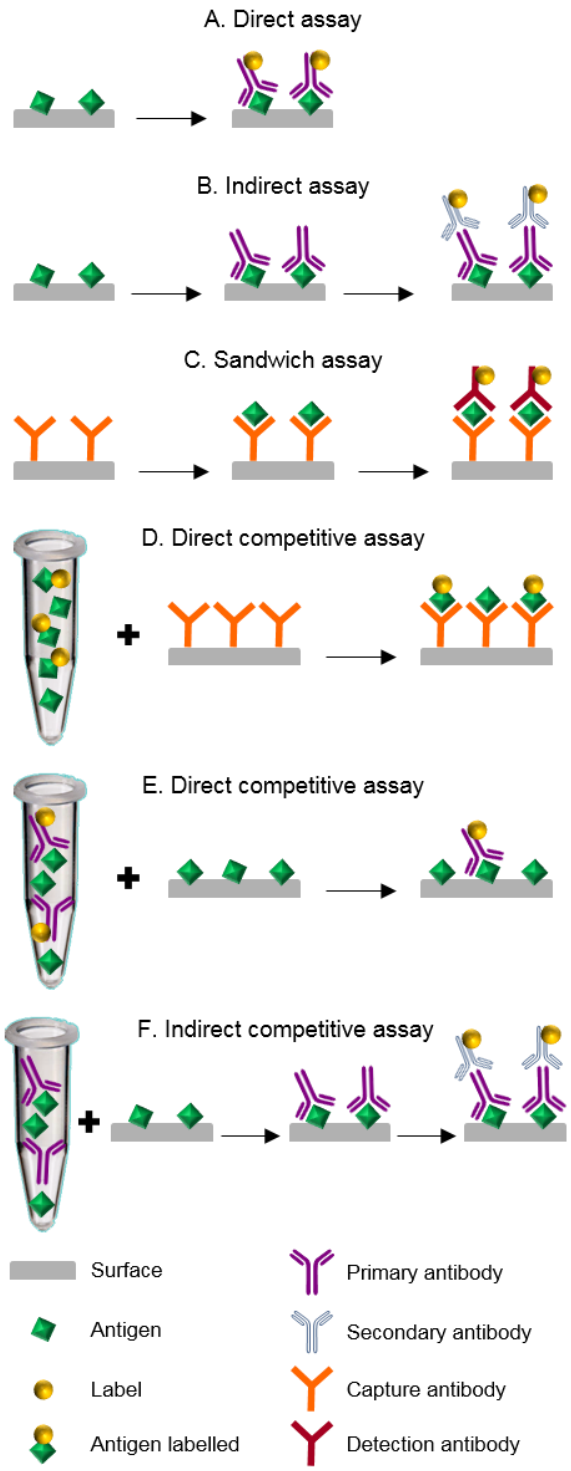

Fig. 2. Scheme of immunoassay formats.

All the immunoassay represented in Figure 2 are based on the use of a label, but the immunosensors can also be label-free. A label-free immunosensor is able to detect the physical changes during the immune complex formation, while an immunosensor based

on the use of a label measures the signal generated by the label for detect the immune complex formation allowing more sensitive and versatile detection [21]. There is a great variety of labels which can be used for electrochemical immunosensors (EIs) development such as enzymes, electroactive compounds, metal nanoparticles or Quantum Dots [11,16,17,35-37]. Although immunosensors based on the use of labels show higher sensitivity, the label-free immunosensors represent a true alternative for the development of immunosensors due to their simplicity [30]. Currently, in the literature there are great number of EIs reported for real sample analysis. The two main types of EIs used in clinical analysis are amperometric and potentiometric [38]. Impedimetric and capacitive immunosensors have started to gain interest due to their direct use to determinate the antibody-antigen interaction without the need of other reagents and the separation step, but their sensitivities are still limited [21].

3 Screen-printed electrodes

The screen-printing technology, adapted from the microelectronics industry, offers high-volume production of solid, planar, inexpensive and highly reliable electrodes; moreover, this technique holds great promise for sensors on-site monitoring [25,39]. The fabrication of screen-printed electrodes (SPEs) consists of a series of basic stages: selection of the screen which will defines the geometry and size of the SPE, selection and preparation of the inks, selection of the substrate, and printing, drying and curing steps. In summary, a SPE is fabricated by a sequential layer-by-layer deposition of an ink onto a substrate through the use of a screen or mesh which controls the film layer thickness and the geometry of the final electrode [12-14]. The substrate is commonly a solid surface made of an insulating material such as alumina, glass, ceramic, plastic, etc. and the conducting

path of the electrode usually are made of carbon ink/paste, or platinum, gold or other metal pastes. For the working electrode, the material mostly used is carbon (or modifications of carbon such as graphene, graphite, fullerene or carbon nanotubes) because it is relatively inexpensive, easy to modify and chemically inert; metals such as gold, silver or platinum are also employed but less than carbon because of their higher cost. The reference electrode material is mostly Ag/AgCl and the counter electrode usually is fabricated from the same material as the working electrode [12,14,24].

Of note, SPEs present a great versatility in the way they can be modified; these modifications give different properties to SPEs making them suitable for diverse applications. In fact, there are very few works related to the use of unmodified SPEs in the determination of interesting analytes [13,40]. SPEs can be modified by the addition of very different substances (mediators, polymers, complexing agents, metals, metal oxides, etc.) to the inks, or modifying their surface (with substances such as polymers, enzymes, metal films, etc.) [14,25,41]. Enzymes, microorganism, proteins (antibodies or antigens) and nucleic acids have commonly been employed in the construction of biosensors based on SPEs [11,24]. These biomolecules can be immobilized onto the surface of the working electrode employing a variety of immobilization strategies such as adsorption, covalent binding, entrapment, crosslinking or affinity binding [42,43]. Great advancement has been achieved modifying SPEs with nanomaterials such as metal nanoparticles or carbon nanomaterials that improve the electrochemical behaviour of the SPEs and enhanced the immobilization efficiency of biological molecules [44-48]. Moreover, the planar nature of the SPEs makes easy the modification of their surface and, through the help of an automatic dispenser, this can be done in a mass-producible way [18].

SPEs can be designed as systems of two electrodes (working and reference electrodes; known as first-generation SPEs), but usually include a three electrodes configuration (working, reference and auxiliary electrodes; known as second-generation SPEs) [14,24]. The versatility of design of the SPEs is another interesting characteristic since it allows customise the production of SPEs for special applications and multiplex analysis (Figure 3). There are many commercial sources of SPEs in different configuration (e.g. Pine Research Instrumentation, http://www.pineinst.com/echem/; BVT Technologies, http://www.bvt.cz/; Rusens Ltd., http://www.rusens.com/speng.html; DropSens, http://www.dropsens.com/; Gwent Group, http://www.gwent.org). SPEs with two or four working electrodes, arrays of eight SPEs and of 96 SPEs in a 96-well plate are commercially available making the use of these electrode even more advantageous.

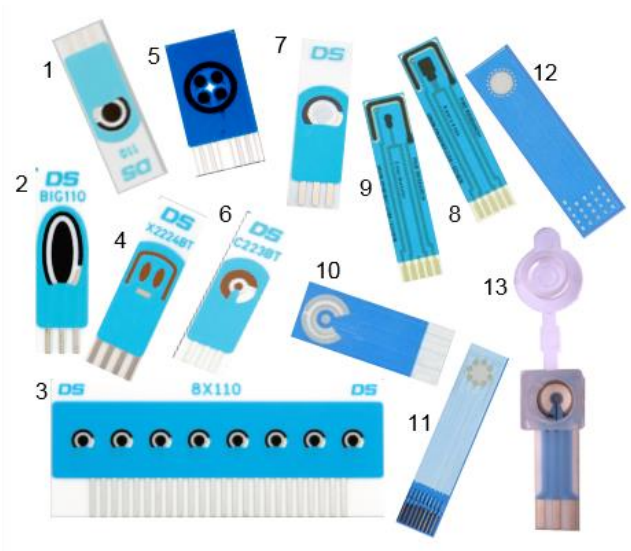

Fig. 3. Examples of commercial SPEs. Commercialised by DropSens: 1-carbon SPE, ref. DRP-110; 2-carbon SPE, ref. DRP-110BIG; 3-card with 8 carbon SPEs, ref. DRP-8X110; 4-dual gold SPEs, ref. DRP-X2224BT; 5-SPE with 4 working electrodes, ref. DRP-4W110; 6-gold SPEs, ref. DRP-223BT; 7-optically transparent SPEs, Ref. DRP-P10. Commercialised by Pine Research Instrumentation: 8,9-carbon SPE, ref. RRPE1002C, ref. RRPE1001C. Commercialised by BVT Technologies: 10-SPE with two counter electrodes, ref. AC6; 11-SPE with 8 working electrodes, ref. AC9; 12-SPE with 20 working electrodes, ref. AC10; 13-SPE with microreactor, ref. MAC.

As it has been previously mentioned, the substrate used for fabricate SPEs is commonly a rigid one, but during the last years paper have become increasingly attractive as substrate for the development of electroanalytical devices. There are many examples of SPEs based on paper or transparency to detect a wide variety of analytes [49-52]. There are examples too of SPEs fabricated on textiles or even skin-worn tattoo-based SPEs for develop wearable electrochemical devices [53-54]. This shows the great potential of the screen-printed technology for develop electroanalytical devices.

3.1 Screen-printed electrochemical immunosensors

EIs based on electrochemical detection offer several potential advantage over the more widely used spectrophotometric/fluorescence techniques, especially when sensitivity is needed [16]. Besides the sensitivity and high accuracy at low analyte concentrations, other important advantages of the electrochemical detection are its low cost, ability for miniaturization, portability, low reagent and sample consumption and the lack of interferences caused by the turbid or coloured samples [16,52].

The choice of the electrode for develop an EI is a crucial step because not only affects the cost, but also the sensitivity of the assay. Conventional electrodes, such as carbon paste or glassy carbon among others, are widely used in electrochemical laboratories because they behave very well from an electrochemical point of view [18]. But, this kind of electrodes are not well suited for develop EIs because they are not intended for single use, and often before each use their surfaces must to be regenerated consuming time and reagents. Another disadvantage of conventional electrodes to be used as transducers in EIs is that these electrodes need an external reference electrode, and often a counter electrode, so the measurement step is not too practical [18]. Moreover, they require a

quite large volume of sample for the measurement. In this context, screen-printed electrodes (SPEs) are much better suited as transducer in EIs. SPEs offer mass-production, low-cost fabrication and its miniaturized dimensions allow perform all immunological steps in a drop of few microliters of solution reducing the reagent and sample volume consumption. In addition, the decrease in the diffusion distances for the analytes to reach their surface-bound receptor partners allows shorter incubation periods and, thus, faster assays [32]. Moreover, since SPEs are disposable, they avoid others common problems of classical solid electrodes such as memory effects and tedious cleaning processes [14]. The electrochemical instrumentation used with SPEs has been reduced to small pocket-size devices which make them applicable for both personal and professional use. Thus, these electrodes have successfully been employed in the development of analytical methodologies that respond to the growing need to perform rapid *in situ* analyses [25].

4 Cancer biomarkers detection

Cancer can be defined as an abnormal and uncontrolled growth and development of normal cells beyond their natural boundaries [21,55]. Cancer can take over 200 distinct forms including lung, prostate, breast, ovarian, skin, colon, hematologic and leukaemia cancer [55]. Environmental factors (e.g. tobacco smoke, alcohol, radiation and carcinogenic chemicals), genetic factors (e.g. inherited mutations and autoimmune dysfunction) and bacterial (associated with stomach cancers) or viral infections (associated with cervical cancer) are associated with an increased risk of developing cancer [55-57]. In 2004, cancer killed 7.4 million people, and this number is estimated to

reach 12 million by 2030 [58]. Early detection and treatment of cancer increase the chance of being cured of this disease.

Existing methods of screening cancer based on cell morphology using staining and microscopy that are invasive techniques that involve taking a biopsy and then examining the tissue to identify and detect cancer cells [56]. The analysis of biomarkers in blood, urine and other body fluids (their collection is non- or minimally invasive in comparison with biopsies) is other method applied in cancer diagnosis and staging, and in monitoring response to cancer therapy. The biomarkers can also be present in or on tumour cells but the isolation of proteins from fixed tissue samples is much more difficult than those of nucleic acids, so proteins which are expressed on the cell surface are analysed by immunohistochemical assays in the fixed tumour tissue [55,59]. Due to the fact that most cancer diseases are associated with the presence of more than one tumour marker (see Table 1), multi-marker profiles (presence and concentration level; Table 2) can be essential for the early diagnosis of disease onset and be associated with the stages of tumours [55,56,59]. Cancer biomarkers can be used in several ways (Table 3) [22,57,58,60,61] :

- Diagnostic biomarker can be used for screening healthy population or high-risk individuals and assist in early detection of the disease.

- Prognostic biomarkers allow for predicting the natural course of an individual cancer assessing the malignant potential of tumours; these biomarkers guide the decision of whom to treat and how aggressively to treat.

- Predictive biomarkers can be used to monitoring the course of cancer in a patient in remission or while receiving a treatment.

- Pharmacodynamic biomarkers measure the near-term treatment effects of a drug on the tumour or on the host, and can be used to guide dose selection in the early stages of clinical development of a new anticancer drug.

Table 1. Types of cancer and tumour-associated biomarkers [22,56,59,62]. PSA: prostate specific antigen; PAP: prostatic acid phosphatase; AFP: α-fetoprotein; hCG: human chorionic gonadotropin; CAGE-1: cancer antigen gene 1; CA: cancer antigen; CEA: carcinoembryonic antigen; SCC: squamous cell carcinoma; NSE: neuron specific enolase; BTA: bladder tumour associated antigen; FDP: fibrin degradation protein; NMP22: nuclear matrix protein 22; HA: hyaluronic acid; HAase: hyaluronidase; HER2: human epidermal growth factor receptor 2.

Cancer type	Biomarkers
Prostate	PSA, PAP
Testicular	AFP, β-hCG, CAGE-1, ESO-1
Ovarian	CA125, AFP, hCG, p53, CEA
Colon and pancreatic	CEA, CA19-9, CA24-2, p53
Lung	NY-ESO-1/ESO-1, CEA, CA19-9, SCC, CYFRA21-1, NSE
Melanoma	Tyrosinase, NY-ESO-1/ESO-1
Liver	AFP, CEA
Gastric carcinoma	CA72-4, CEA, CA19-9
Esophagus carcinoma	SCC
Trophoblastic	SCC, hCG
Bladder	BTA, BAT, FDP, NMP22, HA, HAase, BLCA-4, CYFRA21-1
Breast	CA15-3, CA125, CA27-29, CEA, BRCA1, BRCA2, MUC-1, NY-BR-1, ING-1, HER2/NEU
Leukaemia	BCR, ABL, PML, BCL1, BCL2, ETO

Table 2. Normal levels for some of cancer biomarkers [62]. PSA: prostate specific antigen; hCG: human chorionic gonadotropin; AFP: α-fetoprotein; CEA: carcinoembryonic antigen; CA: cancer antigen.

Biomarker	Thresholds
tPSA	4 ng/mL
hCG	5.0 mIU/mL
AFP	10 ng/mL
CEA	3 ng/mL
CA125	35 U/mL
CA15-3	25 U/mL
CA27-29	36.4 U/mL
CA19-9	37 U/mL
CA242	20 U/mL
CA72-4	6 U/mL

Table 3. Selection of US Food and Drug Administration (FDA) approved cancer biomarkers [62,63]. AFP: α-fetoprotein; β-hCG: β-human chorionic gonadotropin; CA: cancer antigen; CEA: carcinoembryonic antigen; PSA: prostate specific antigen; HER2: human epidermal growth factor receptor 2; NMP22: nuclear matrix protein 22; FDP: fibrin degradation protein; BTA: bladder tumour associated antigen; IHC: immunohistochemistry; FISH: fluorescent *in-situ* hybridization.

Biomarker	Type	Source	Cancer type	Clinical use
AFP	Glycoprotein	Serum	Non-seminomatous testicular	Staging
β-hCG	Glycoprotein	Serum	Testicular	Staging
CA19-9	Carbohydrate	Serum	Pancreatic	Monitoring
CA125	Glycoprotein	Serum	Ovarian	Monitoring
CEA	Protein	Serum	Colon	Monitoring
tPSA	Protein	Serum	Prostate	Screening and monitoring
PSA complexed	Protein	Serum	Prostate	Screening and monitoring
fPSA (%)	Protein	Serum	Prostate	Benign prostatic hyperplasia versus cancer diagnosis
CA15-3	Glycoprotein	Serum	Breast	Monitoring
CA27-29	Glycoprotein	Serum	Breast	Monitoring
HER2/NEU	Protein (IHC)	Breast tumour	Breast	Prognosis and selection of therapy
HER2/NEU	Protein	Serum	Breast	Monitoring
HER2/NEU	DNA (FISH)	Breast tumour	Breast	Prognosis and selection of therapy
NMP22	Protein	Urine	Bladder	Screening and monitoring
Fibrin/FDP	Protein	Urine	Bladder	Monitoring
BTA	Protein	Urine	Bladder	Monitoring
High molecular weight CEA and mucin	Protein (Immunofluorescence)	Urine	Bladder	Monitoring

The first electrochemical immunosensor for tumour marker detection was reported in the late 1970s [64]. It was an amperometric sensor based on a competitive assay using catalase as label for hCG detection. Thereafter, many immunosensors for cancer biomarkers have been reported in the literature [4,14,21,22,59]. Discussion of EIs based on SPEs for some of the major cancer biomarkers is presented below (Table 4).

Table 4. Main characteristics of some EIs based on SPEs for cancer biomarkers. PSA: prostate specific antigen; IL-8: interleukin; CA: cancer antigen; CEA: carcinoembryonic antigen; HER2: human epidermal growth factor receptor 2; AFP: α-fetoprotein; SPCE: screen-printed carbon electrode; WE: working electrode; LOD: limit of detection; Ab: antibody LSV: linear sweep voltammetry; DPV: differential pulse voltammetry; CV: cyclic voltammetry; EIS: electrochemical impedance spectroscopy; SWV: square wave voltammetry; AuNPs: gold nanoparticles; AgNPs: silver nanoparticles; HRP: horseradish peroxidase; SAM: self-assembly monolayer; CNTs: carbon nanotubes; MWCNTs: multiwalled carbon nanotubes; 8; QD: quantum dots; PLA: proximity ligation assay; GOx: glucose oxidase; GO: graphene oxide; HER: hydrogen evolution reaction.

Biomarker	Methodology	Transduction technique	Sample	Concentration range	LOD	Ref.
tPSA, fPSA	Simultaneous determination in SPCE with two WEs nanostructured with AuNPs. Sandwich-type immunoassay. Ab immobilization by adsorption. Alkaline phosphatase as label.	LSV	Serum	tPSA: 1-10 ng/mL fPSA: 1-10 ng/mL	-	[65]
fPSA	Electroactive silver-mediated poly(amidoamine) dendrimer nanostructures as label. AuNPs nanostructured SPEs.	LSV	Serum	0.005-5.0 ng/mL	1.0 pg/mL	[66]
tPSA	Re-usable 8-channel SPCEs. Magnetic beads as support for a sandwich-type immunoassay using HRP as label.	Amperometry	Serum	5-100 ng/mL	1.86 ng/mL	[67]
tPSA	SPEs based on sheets of vegetable parchment. Graphene nanosheets and HRP-labelled detecting Ab functionalized with AuNPs.	LSV	Serum	2 pg/mL - 2 µg/mL	0.45 pg/mL	[68]
tPSA	Label-free. Ab immobilization by two ways tested: entrapment and affinity reaction by avidin-biotin affinity approach.	EIS	-	Entrapment: 1-10 ng/mL; Affinity: 1-10 pg/mL	Entrapment: 1 ng/mL; Affinity: 1 pg/mL	[69]
tPSA, IL-8	16 SPEs array. Detecting Ab and HRP (label) loading onto MWCNTs.	CV	-	tPSA: 5-4000 pg/mL; IL-8: 8-1000 pg/mL	tPSA: < 5 pg/mL; IL-8: 8 pg/mL	[70]
tPSA	3D origami paper SPEs. MnO_2 nanowires electrodeposited on carbon working electrode with AuNPs layer. Sandwich-type immunoassay. GOx as label.	DPV	Serum	0.005-100 ng/mL	0.0012 ng/mL	[71]
CEA	Carbon nanoparticle/poly(ethylene imine) modified SPEs. Sandwich assay. Detecting Ab labelled with CdS nanocrystal QD sensitized.	SWV	Urine	0.032-10 ng/mL	32 pg/mL	[72]

CEA	Nanosilver-doped DNA polyion complex membrane as sensing interface on thionine/Nafion-modified SPCE. Sandwich assay. AuNPs conjugated with Ab-HRP.	DPV	Serum	0.03-32 ng/mL	10 pg/mL	[73]
CEA	PLA using assembling single-stranded DNA modified AuNPs on GO modified SPCE and tow DNA-labelled antibodies.	DPV	Serum	0.01-100 ng/mL	3.9 pg/mL	[74]
CA125	Label-free. SAM formation on AuNPs modified SPE.	EIS	Serum	0-100 U/mL	6.7 U/mL	[75]
CA15-3	Sandwich assay on graphene oxide modified SPCE using peroxidase-like magnetic silica nanoparticles/graphene oxide composite as label.	DPV	Serum	0.001-200 U/mL	0.28 mU/mL	[76]
CA19-9	Sandwich assay with detecting Ab functionalized with nanogold-encapsulated poly(amidoamine) dendrimer. Signal based on HER.	Amperometry	Serum	0.01-300 U/mL	6.3 U/mL	[77]
HER2	Label-free sensor using affibody immobilized on AuNPs modified SPE as bioreceptor.	EIS	Serum	0-40 ng/mL	6.0 ng/mL	[78]
HER2	Sandwich-type immunoassay on AuNPs nanostructured SPCE. Ab immobilization by adsorption. Alkaline phosphatase as label	LSV	Serum	15-100 ng/mL	4.4 ng/mL	[79]
AFP	Sandwich-type assay on gold SPEs. Invertase as label to catalysed sucrose to glucose. Re-usable immunosensor.	Personal glucose meter	Serum	0.5-50 ng/mL	0.18 ng/mL	[80]
CEA, AFP	Sandwich assay on AuNPs CNTs-chitosan modified SPCEs with two WE. GOx as label attached on silica nanospheres.	DPV	Serum	CEA: 5.0 pg/mL - 2.0 ng/mL; AFP: 5.0 pg/mL – 1.0 ng/ML	CEA: 3.2 pg/mL; AFP: 4.0 pg/mL	[81]
CEA, AFP	Sandwich assay on prussian blue and AuNPs modified SPCEs. Glucose oxidase as label attached on antibody and AuNPs modified CNTs.	DPV	Serum	CEA: 2.5 pg/mL - 2.0 ng/mL; AFP: 2.5 pg/mL - 2.5 ng/mL	CEA: 1.4 pg/m; AFP: 2.2 pg/mL	[82]
CEA, AFP	Sandwich assay on two WE SPCE. Streptavidin-functionalized AgNPs-modified CNT to link with biotinylated detecting Ab. Signal amplification by	LSV	Serum	CEA, AFP: 0.1 pg/mL - 5.0 ng/mL	CEA: 0.093 pg/mL; AFP: 0.061 pg/mL	[83]

	AgNP-promoted deposition of Ag using a silver enhancer solution.					
CA125, CA19-9	Simultaneous detection based on competitive assay using SPCE with two WEs. Cellulose acetate membrane to immobilize thionine as mediator. HRP as label.	DPV	Serum	CA125: 0-25 U/mL; CA19-9: 0-24 U/mL	CA125: 0.4 U/mL; CA19-9: 0.2U/mL	[84]
CA15-3, CA125, CEA	Simultaneous determination in SPCE with three WEs nanostructured with AuNPs. Sandwich-type immunoassay. Alkaline phosphatase labelled antibody functionalized Au cluster with graphene for detection.	LSV	Serum	CA15-3: 0.005-50 U/mL; CA125: 0.001-100 U/mL; CEA: 0.004-200 ng/mL	CA15-3: 1.5 mU/mL; CA125: 0.34 mU/mL; CEA: 1.2 pg/mL	[85]
CA15-3, CA125, CEA	Simultaneous detection based sandwich-type assay on graphene modified SPCEs with three WE. Platinum nanoparticles as label.	DPV	Serum	CA15-3: 0.008-24 U/mL; CA125: 0.05-20 U/mL; CEA: 0.02 - 20 ng/mL	CA15-3: 1 mU/mL; CA125: 2 mU/mL; CEA: 7 pg/mL	[86]
CEA, AFP, CA125, CA15-3	Paper-based device with 8 WE. Sandwich-type assay. Radical polymerization as signal amplification.	DPV	-	CEA: 0.01-100 ng/mL; AFP: 0.01-100 ng/mL; CA125: 0.05-100 ng/mL; CA15-3: 0.05-100 ng/mL	CEA: 0.01 ng/mL; AFP: 0.01 ng/mL; CA125: 0.05 ng/mL; CA15-3: 0.05 ng/mL	[87]
CA15-3, CA125, CA19-9, CEA	AuNPs with HRP-labelled antibodies immobilized by biopolymer/sol-gel on SPCEs with 4 WE. Formation of HRP-Ab/antigen complex blocked electron transfer decreasing the signal.	DPV	Serum	CA15-3: 0.4-140 U/mL; CA125: 0.5-330 U/mL; CA19-9:0.8-190 U/mL; CEA: 0.1-44 ng/mL	CA15-3: 0.2 U/mL; CA125: 0.5 U/mL; CA19-9:0.3 U/mL; CEA: 0.1 ng/mL	[88]

4.1 Prostate specific antigen (PSA)

Prostate specific antigen (PSA) is a serine protease belonging to the human kallikrein family [89,90]. It is synthesized specifically in the epithelial cells of the prostate gland and its expression therein is regulated by the androgen receptor. Due to its high tissue specificity, PSA is one of (if not the) most widely used tumour marker [22]. It is used extensively as a biomarker to screen and diagnosis of prostate cancer, to detect recurrence

after definite therapy and to follow response to treatment in the metastatic disease setting [89]. The normal reference range for PSA is 0-4 ng/mL, but benign conditions such as benign prostatic hypertrophy, acute prostatitis and infarction may be correlated with elevated PSA levels [22]. This is the main drawback of PSA as biomarker, the lack of specificity in distinguishing prostate cancer from non-malignant prostate disease. PSA has two forms in human serum: free PSA (fPSA) and PSA complexed, being the predominant one the complex with α-1-antichymotrypsin (PSA-ACT) [90]. Total PSA (tPSA) refers to the sum of fPSA and PSA complexed, and it is used to determine some cut off. A value of tPSA above 10.0 ng/mL is regarded as positive and indicates high probability of prostate cancer; a value below 4.0 ng/mL is considered negative and indicates low probability of prostate cancer. Between 4.0 and 10.0 ng/mL the result is in the so-called "grey zone" [65,67]. fPSA is performed when the value of tPSA is in the grey zone to distinguish prostate cancer from other causes of PSA elevation considering that men with prostate cancer have elevated levels of PSA complexed and low levels of fPSA [22,91].

In 2009, a dual sensor for fPSA and tPSA using disposable commercial SPEs containing two working carbon electrodes was developed [65]. Specific antibodies for tPSA and fPSA were immobilized by physical adsorption in each working electrode previously nanostructured with gold nanoparticles (AuNPs) generated *in situ*. The immunosensor was based in a sandwich-type immunoassay performed step by step taking 3 h. The enzyme alkaline phosphatase (AP) was used as label and a mixture of 3-indoxyl phosphate disodium salt (3-IP) and silver nitrate as substrate [92]. AP hydrolyses 3-IP resulting an indoxyl intermediate which reduces the silver ions to give metallic silver (Ag^0) and indigo blue. Thus, the silver enzymatically is deposited on the electrode surface and can be detected through the redissolution peak when an anodic stripping scan is

carried out. Since the enzymatic product is metallic silver that is deposited on the electrode surface, no cross-talk between electrodes is produced and it is possible to use the same label for the detection of both analytes. This bi-sensor showed a linear range very suitable for PSA detection in real samples; it is able to detect fPSA and tPSA in the linear range 1-10 ng/mL. More recently, Pei et al. [66] developed a immunosensor for fPSA based on a sandwich-type immunoassay using SPEs nanostructured with AuNPs as transducer and a signal amplification by electroactive silver-mediated poly(amidoamine) dendrimer nanostructures for detection. In this case the assay takes 25 min and enzymes are not necessary since the silver nanoparticles can directly catalyse the reduction of H_2O_2 without the participation of bioactive enzymes. Using linear sweep voltammetry as technique for measure the analytical signal, a wide quantification range between 0.005 and 5 ng/mL was achieved. Using sheets of vegetable parchment, Yan et al. [68] fabricated stable and inexpensive carbon SPEs (SPCEs) for develop a sandwich-type immunosensor for the detection of tPSA. Using graphene nanosheets for coat the SPEs and HRP-labelled detecting antibody functionalized with AuNPs, an immunosensor with a quantification range of 2 pg/mL - 2 µg/mL was achieved. This wide linear range is possible since the graphene nanosheets increase the conductivity and the AuNPs provide a large surface area for the immobilization of the detecting antibody HRP-labelled and also enhance the electroreduction between HRP and H_2O_2 amplifying the analytical signal. The use of sheets of vegetable parchment for fabricating SPEs decreases the cost of the final sensor and, since the vegetable parchment is flammable, allow the easy and safe disposability of the immunosensor by incineration.

4.2 Carcinoembryonic Antigen (CEA)

Carcinoembryonic antigen (CEA), described in 1965, is a glycoprotein belonging to the immunoglobulin family [21]. It was among the first identified tumour antigens and is found in many carcinomas such as colon, lung, ovarian and breast cancer (Table 1) [14,72]. The clinical value of CEA detection is limited by a high false positive rate in healthy populations and by low diagnostic sensitivity and specificity, so clinical decisions regarding disease management is not based only on CEA levels [21,22]. For example, since CEA is metabolized in the liver, damage therein can elevate the CEA levels in the circulation and lead to false positive results. Moreover, CEA levels can be elevated in some patients after radiation and chemotherapy [22]. Despite these limitations, CEA is used as marker to monitor cancer recurrence after surgery and to follow patients during therapy [72].

Wu et al. [73] developed an immunosensor for CEA detection using a nanosilver-doped DNA polyion complex membrane (PIC) on the surface of the SPCEs as sensing interface. To construct this membrane, double-stranded DNA was assembled onto the surface of thionine/Nafion-modified SPCE to adsorb silver ions with positive charges and then, the silver ions were reduced to silver nanoparticles by $NaBH_4$. The capture antibody was immobilized on this surface in order to perform a sandwich-type assay using AuNPs conjugated with HRP-labelled antibody for the detection of CEA. The assay was performed in two steps taking 44 min. The use of nanosilver-doped DNA PIC membrane as immunosensing probe and HRP-anti-CEA-labelled AuNPs for signal amplification allowed to obtain a low LOD value of 10 pg/mL and a linear range of 0.03-32 ng/mL. Recently, a wider quantification range for CEA was achieved using a immunosensor based on a proximity ligation assay (PLA) [74]. The analytical signal of this sensor consisted in the electrochemical stripping of silver regulated by proximity hybridization

of single-stranded DNA. The device was prepared by assembling single-stranded DNA modified with AuNPs (ssDNA@AuNPs) on graphene oxide modified SPCE. In presence of the antigen and two DNA-labelled antibodies, the proximate complex is formed and can hybridize with the DNA assembled on the SPCE taking away the AuNPs. Thus, the silver deposition catalysed by the AuNPs decreases, and therefore the silver anodic stripping signal (Figure 4). The homogeneous proximity ligation and the hybridization of the product with the immobilized ssDNA was completed in a single step (40 min). With this strategy, a quantification range of four orders of magnitude for CEA detection was achieved (0.01 to 100 ng/mL).

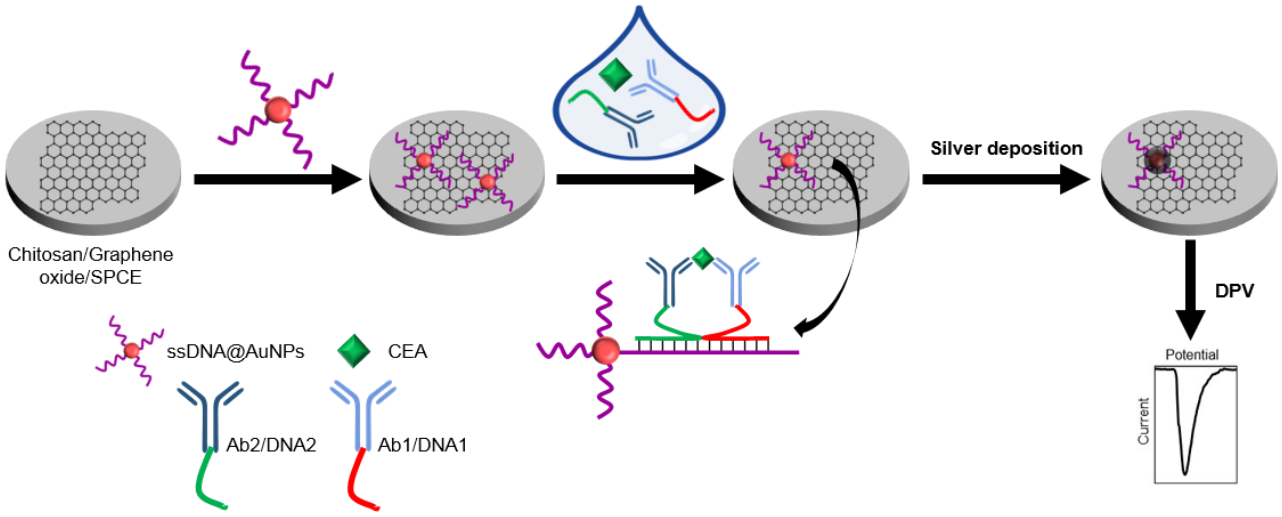

Fig. 4. Scheme of the immunosensor for CEA detection using a proximity ligation assay developed by Li et al. [74].

4.3 Others cancer biomarkers

Carbohydrate antigens, also called cancer antigens, are mainly produced in cancer cells, but rarely produced in normal tissues or benign lesions of tissues. The cancer antigens commonly detected are CA125, CA15-3, CA19-9, etc. (Table 2) [4,21,93]. Among them,

CA125 is a high molecular weight protein most commonly associated with ovarian cancer but it is also linked to uterus, cervix, pancreas, liver, colon, breast, lung and digestive cancer [55,94]. CA125 has a very low sensitivity for early stage ovarian cancer since in Stage 1, 50% of patients have normal CA125 levels [55,95]. Moreover, several non-pathological conditions such as menstruation and pregnancy can increase levels of CA125 in healthy individuals [94]. But, more than 90% of women have high levels of CA125 when the ovarian cancer is advanced, so CA125 is a valuable biomarker not only for cancer diagnosis, but also for monitoring cancer progression and treatment [14,55,95]. Normal blood levels are usually less than 35 U/mL: CA125 levels above this value is found in 1% of healthy population, 6% of patients with benign disease, 28% of patients with non-gynaecological malignancy and 82% of individuals with ovarian cancer [14,95].

CA15-3 is also an important carbohydrate antigen analysed in breast cancer patients (Table 2). It is overexpressed on the external layer of malignant glandular cells such as those seen in breast cancer [22,96]. In patients with breast cancer, CA15-3 levels increase by 10% in Stage 1, 20% in Stage 2, 40% in Stage 3 and 75% in Stage 4 of breast cancer [55]. But the diagnostic value of CA15-3 is relatively low because of an intrinsic lack of both sensitivity and specificity since high CA15-3 levels can be detected also in presence of other kinds of cancer disease such as gastric or ovarian cancers or even in presence of hepatic cirrhosis, hepatitis or hypothyroidism [96,97]. So, CA15-3 is used clinically most often to monitor patient therapy and it is considered along with tumour size, cancer stage and negative risk factors in determining treatment protocols [55]. Of note, CA27-29 is a slightly more sensitive breast cancer biomarker than CA15-3, so the US Food and Drug Administration (FDA) has approved both cancer antigens for monitoring therapy in breast cancer [22].

HER2/NEU protein belongs to the epidermal growth factor receptor (EGFR) family. This protein is amplified and/or overexpressed in approximately 20-30% of breast cancers [2]. Rising serum levels of HER2/NEU has been associated with progressive metastatic disease and poor response to therapies. In fact, HER2/NEU overexpression has been related with a poor rate of disease-free survival [21].

Taking in count that determinate the concentration of a single biomarker almost always is not enough, the possibility of determinate several cancer biomarker simultaneously is very interesting since it can provide more information about the diagnosis or evolution of the disease. Wu et al. [84] developed a multiplex immunosensor to determine CA125 and CA19-9 based on a direct competitive assay using a SPCE with two working electrodes. A cellulose acetate membrane was used to co-immobilize thionine as mediator and the two antigen on the surface of each working electrode. With two simultaneous direct competitive immunoreactions (1 h) the corresponding HRP labelled antibodies were captured on the respective electrode surface on which the immobilized thionine shuttled electrons between HRP and the electrode for enzymatic reduction of H_2O_2 to produce the analytical signal. The use of thionine as mediator immobilized on the electrodes instead of in the detection solution avoid the electrochemical "cross-talk" between the working electrodes. With this device LOD of 0.4 U/mL and 0.2 U/mL for CA125 and CA19-9 respectively were achieved (Table 4). More recently, Cui et al. [86] constructed a multiplex immunosensor for CA125, CA15-3 and CEA detection using platinum nanoparticles as label. The device is based on graphene modified SPCEs with three working electrodes where sandwich-type immunoassays (performed in two steps of 1 h) were carried out using mesoporous platinum nanoparticles labelled antibodies detection to catalyse the electro-reduction of H_2O_2 obtaining negligible cross-talk (LOD of 1 mU/mL, 2 mU/mL and 7 pg/mL for CA15-3, CA125 and CEA respectively). Another

interesting work about multiplex cancer biomarker detection is the device developed by Wu et al. [87] for CEA, AFP, CA125 and CA15-3 detection. They designed a paper-based electrochemical immunodevice with 8 carbon working electrodes screen-printed sharing the same Ag/AgCl reference and carbon counter electrodes. A sandwich-type assay was carried out on the graphene modified working electrodes. A radical polymerization reaction was used as signal amplification strategy: the antibodies detection were coupled with an initiator of the polymerization (*N*-hydroxysuccinmidyl bromoisobutyrate) and once the immunoassay is over, the polymerization is performed and then, the HRP solution was dropped onto each working electrode prior to electrochemical detection. Although the sensor is very promising since it is based on paper device and achieved a wide linear range (Table 4), it is a laborious and very long-time assay (only HRP dropping takes 10 h). In the context of portable devices development, Zhu et al. [80] developed a immunosensor based on gold SPEs for AFP detection using a personal glucose meter as signal transducer. The sensor was based on a sandwich-type assay in which the detecting antibody was labelled with the enzyme invertase. Once the reaction antibody-antigen was over, sucrose was added. Thus, in presence of invertase, the sucrose is catalysed to generate glucose and fructose, and the glucose generated is detected using the personal glucose meter. Moreover, the immunosensor can be re-usable after a regeneration step using a glycine-HCl buffer solution in order to break the antibody-antigen binding. Others similar devices using personal glucose meter (and magnetics beads) can be found in the literature for CEA and PSA detection [98,99].

5 Cardiovascular biomarkers

Cardiovascular diseases (CVDs) are the most prevalent cause of human death in both developing and developed countries [100]. According to the World Health Organization (WHO), an estimated 17.5 million (31%) of all global deaths in 2012 are related to CVDs (WHO, http://www.who.int/). CVDs are a group of disorders of the heart and blood vessels including: coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. CVDs can be caused by a quite diverse factors including genetic, age, gender and hypertension, cholesterol, diabetes, obesity and overweight, smoking and stress [15,101]. Early and quick diagnosis of CVDs is crucial not only for patient survival but also for saving a great deal of cost and time in patient treatment [102].

Myocardial infarction (MI), which is defined as the necrosis of cardiac myocytes following prolonged ischemia, is one of the most immediately life threatening forms of acute coronary syndrome (ACS) [33]. The diagnosis of AMI have been based on the WHO criteria, whereby must meet at least two of the three conditions: characteristic chest pain, diagnosis electrocardiogram (ECG) changes and elevation of the biochemical markers in their blood [102]. Although EGG is an important management tool for guiding therapy [103,104], it is a poor diagnostic test for ACS since about half of the ACS-related patients admissions in hospitals demonstrate normal or ambiguous ECG readings [102,105]. Therefore, the assessment of cardiac marker elevation is critical to make a truly informed decision on a suitable treatment [15]. The levels of such markers can give information about the type of ACS, the time of first incidence of the attack and, for certain markers, the location of the damaged cells (Figure 5) [33].

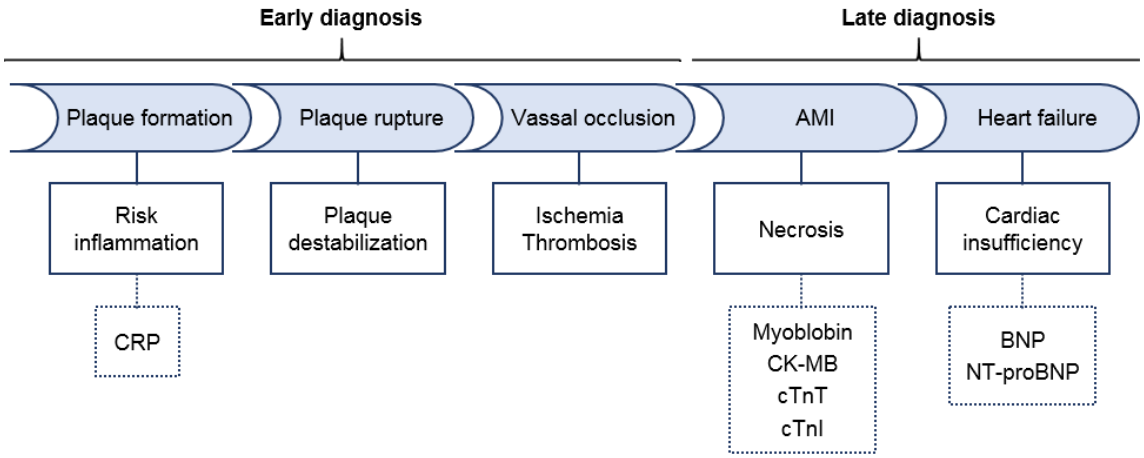

Fig. 5. Most frequently studied biomarkers in relation to the different mechanism involved in ACS. Adapted from [15,106].

The three main biomarkers for the diagnosis of AMI are: cardiac troponins (cardiac troponin T, cTnT, and cardiac troponin I, cTnI), creatine kinase MB (CK-MB) and myoglobin. Official organisms such as the European Society of Cardiology (ESC), the American Heart Association (AHA) or the International Federation of Clinical Chemistry (IFCC) define the biochemical criteria for detecting myocardial necrosis either as: (I) "a maximal concentration of cTnI or cTnT exceeding the 99th percentile of a reference control group on at least one occasion during the first 24 hours" or as (II) " a maximal value of CK-MB exceeding the 99th percentile of a reference control group on two successive samples, or a maximal value exceeding twice the upper limit of normal for the specific institution on one occasion during the first 24 hours" [106]. Cardiac troponins have been suggested by the guidelines as the preferred markers, with CK-MB as an acceptable alternative when troponins are not available.

Due to their high sensitivity and specificity, human cardiac troponins are known as the "gold standard" for diagnosis and prognosis of AMI [23]. Cardiac troponins consist of a complex of troponin C (cTnC), I (cTnI) and T (cTnT) regulating the contraction of striated

and cardiac muscle [107]. The complex dissociates with time in blood into free cTnT and I/C complex. Both cTnT and cTnI are recommended as the markers of choice because cTnC is unspecific [108]. Like CK-MB, cTnT and cTnI cannot be used as early markers because they show a similar early release kinetic following AMI in that it takes several hours for both of them to be released into circulation before being detectable [102]. However, cardiac troponins are the most specific cardiac biomarkers, and offer the widest temporal diagnostic window since their levels remain abnormal for 4-10 days after the onset of AMI with the peak concentration closely related to the infarct size [15,23]. Their cut-off levels range from 0.01 to 0.1 ng/mL for cTnI and from 0.05 to 0.1 ng/mL for cTnT [15,33,100].

Creatinine kinase (CK) is a dimeric molecule composed of two subunits (M and B) which exists in three molecular forms: MM, MB and BB. When the heart muscle dies during MI, one of the more abundant molecules released into the circulation is CK, but among the three isoenzyme forms, CK-MB offers better sensitivity and specificity compared with total CK as marker of myocardial damage. However, CK-MB diagnostic specificity is compromised when skeletal muscle is involved, such as in the case of trauma, cardiac surgery or extreme exercise. CK-MB cannot be used as an early marker because its narrow window: once released into the blood stream, CK-MB doubles its concentration within 5-6 hours after the onset of chest pain and exhibits peaks in 12-24 hours. But it can be useful for diagnosis of re-infarction and, therefore, in the evaluation of AMI. CK-MB cut-off level is defined at 10 ng/mL [15,100,102,106].

Myoglobin is a non-enzymatic protein useful in the diagnosis of AMI. Because of its small size (17.8 kDa), it is quickly released into circulation (1 hour) upon symptom onset with high sensitivity and high negative predictive value. However, myoglobin show low clinical specificity because of its abundant presence not only in myocardial but also in

skeletal muscle cells. So, injury in skeletal muscle can also increases the concentration of myoglobin. The cut-off level for myoglobin is defined in the range 70-200 ng/mL [15,23,102].

Several other cardiac biomarkers [106] have emerged such as C-reactive protein (CRP) which is an inflammatory marker and it has been the most frequently used single biomarker for CVD risk [100]. This protein is indicator of a viral and bacterial infection, however whose level can increase due to the inflammation induced by infection or injury often leading to a heart attack or stroke. The CRP level is usually less than 2 mg/L for healthy individuals, and when it is higher than 3 mg/L the person is considered at high risk of developing CVD [15,23,33,109]. B-type natriuretic peptide (BNP) and its precursor, N-terminal pro-BNP (NT-proBNP), are neurohormones synthesized primarily in arterial or ventricular myocardium, and both have shown a significant value in diagnosis and prognosis of cardiac disease [102]. Other marker to consider is the heart type fatty acid protein (HFABP), which is a stable and small protein abundantly found in the cytoplasm of myocardial cells. It is not found in the circulation under non-pathological conditions, but it is rapidly released after AMI. Thus, HFABP show potential as sensitive biomarker for early detection of AMI as well as prognosis utility in risk stratification of ACS [15,106,110]. Table 5 summarizes some of the main characteristics of most of the cardiac biomarkers.

Table 5. Main characteristics of some CVDs biomarkers [15,23,31,33,100,111]. cTnI: cardiac troponin I; cTnT: cardiac troponin T; CK-MB: creatine kinase MB; CRP: C-reactive protein; BNP: B-type natriuretic peptide; NT-proBNP: N-terminal pro-B-type natriuretic peptide; HFABP: heart type fatty acid protein.

Biomarker	CVD indicator type	MW (kDa)	Cut-off levels (ng/mL)	Specificity level	Initial elevation (h)	Time to peak (h)	Duration of elevation
cTnI	Detection of AMI	23.5	0.01-0.1	High	4-6	12-24	4-8 days
cTnT	Detection of AMI	37	0.05-0.1	High	3-6	12-24	7-14 days
Myoglobin	Early detection of AMI	18	70-200	Low	1-3	6-12	12-48 hours
CK-MB	Early detection of AMI	85	10	Medium	4-6	12-24	3-4 days
CRP	Early detection of inflammation. Cardiac risk factor	125	$<10^3$, low risk; $1x10^3$-$3x10^3$, intermediate risk; $>3x10^3$-$15x10^3$, high risk (no definitive)	Medium	4-6	12-24	3-4 days
BNP	Acute coronary syndromes. Diagnosis of heart failure. Ventricular overload	3.4	-	High	No clinical consensus	No clinical consensus	No clinical consensus
NT-proBNP	Acute coronary syndromes. Diagnosis of heart failure. Ventricular overload	8.5	0.25-2	High	No clinical consensus	No clinical consensus	No clinical consensus
HFABP	Early detection of AMI	15	6	Low	1-3	6-10	18-36 hours

5.1 EIs based on SPEs for cardiac biomarkers

During last years, many different biosensing devices have been reported for the detection of CVD biomarkers, and there are several recent reviews about sensors developed with these aim [15,31,33,100,112]. This section is focused on EIs based on SPEs for CVD biomarkers.

For cTnT detection, Silva et al. [113] developed an immunosensor using a conducting carbon silver-epoxy composite SPEs. The rigid conducting carbon polymer composite showed to be compatible to integrate streptavidin microspheres through glutaraldehyde allowing a stable immobilization of biotinylated capture antibody on the electrode surface. Using a sandwich-type assay and an anti-cTnT antibody labelled with HRP to perform the peroxidase reaction using H_2O_2 as enzyme substrate, a LOD of 0.2 ng/mL was achieved. More recently, the same author achieved a lower LOD for cTnT developing a label-free immunosensor based on amine-functionalized CNTs-SPE [114]. This device was fabricated by tightly squeezing an adhesive carbon ink containing CNTs onto a polyethylene terephthalate substrate forming a thin film. The antibody-antigen interactions at CNT-SPE surface were monitored by DPV measurements; the difference between the peak current in presence or absence of cTnT was used as analytical signal (Figure 6). The amine-functionalized CNTs incorporated into the carbon ink enabled stable measurement and oriented capture Ab immobilization, and moreover improve the electro-transfer reactions and increase the electrode surface area. The LOD achieved by this label-free device was 0.0035 ng/mL This LOD is lower than this for the immunosensor previously indicated that needed a label for cTnT detection (HRP enzyme).

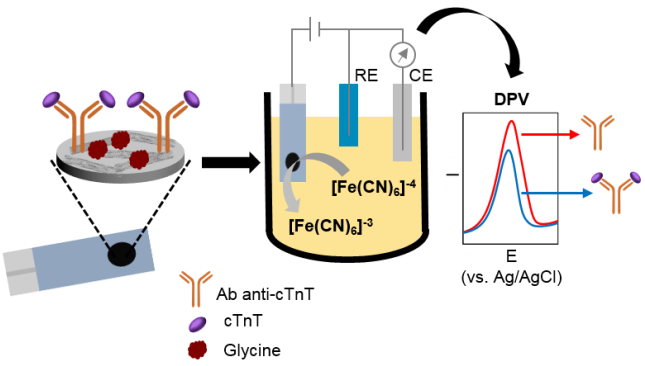

Fig. 6. Scheme of the immunosensor and the electrochemical principle of detection developed by Silva et al. [114].

For myoglobin determination there are few EIs based on SPEs. O'Regan et al. [115] developed an amperometric immunosensor for the detection of myoglobin in whole blood. It consisted on a one-step indirect sandwich-type assay using a secondary antibody labelled with alkaline phosphatase and immobilising the capture antibody by adsorption on the SPCE. The current response was measured by amperometry upon the addition of *p*-aminophenylphosphate. The quantification range achieved for myoglobin in spiked whole blood samples was 85-925 ng/mL. The simultaneous incubation of myoglobin in whole blood with the two detecting antibodies allowed to perform the assay in a shorter time than if the assay were performed step by step, maintaining the sensitivity of the sandwich assay. A wider quantification range was achieved by a label-free immunosensor developed by Suprun et al. [116]. It was based on the use of AuNPs as electrocatalysts of Fe(III)/Fe(II) electrode reaction of myoglobin. For fabricate this label-free immunosensor, the SPEs were modified with AuNPs/didodecyldimetrhylammonium bromide and the capture antibody. Once the experimental conditions were optimized, the square wave voltammetry cathodic peak of cardiac myoglobin reduction measurements give a quantification concentration range of 10-1780 ng/mL. This sensor allow direct measurement of binding events without amplification stages or cover layers of labelled antibodies, needs small sample volumes (1-2 µL) and express detection in 30 min.

Related to CRP, Gan et al. [117] developed an amperometric immunosensor for CRP determination in human serum. HRP-labelled anti-CRP antibody functionalized Fe_3O_4@Au magnetic nanoparticles were attracted to a Fe(III) phthalocyanine (FePc)/chitosan membrane-modified SPCE by an external magnetic field. After the incubation of the sensor with CRP, the access of the activity centre of the HRP to the electrode was partially inhibited leading to a linear decrease in the catalytic efficiency of the HRP to the reduction of immobilized FePc by H_2O_2 in the CRP concentration range

from 1.2 to 200 ng/mL with a LOD of 0.5 ng/mL. Moreover, the SPCE was reusable since the magnetic nanoparticles can be washed from the electrode removing the magnet, which make the basal electrode renewable for next determination by adding new modified nanoparticles on its surface. Recently, a lower LOD was achieved using a immunosensor based on bismuth citrate-modified graphite SPE [118]. This device was based on a sandwich-type assay immobilising the capture antibody by adsorption on the surface of the electrode, using a biotinylated detection antibody and streptavidin-conjugated PbS Quantum Dots (QDs). The assay was performed step by step and took nearly 3 h. The quantification of CRP was performed through acidic dissolution of the PbS QDs and anodic stripping voltammetric detection of Pb(II) released at the bismuth precursor-modified transducer. Under optimal conditions, the linear range of concentrations showed by the sensor was 0.2-100 ng/mL and the LOD was 0.05 ng/ml.

In Table 6 the main characteristics of EIs based on SPEs for the detection of cardiovascular diseases markers are summarised.

Table 6. Main characteristics of some EIs based on SPEs for CVD. cTnI: cardiac troponin I; cTnT: cardiac troponin T; CRP: C-reactive protein; HFABP: heart type fatty acid protein; MWCNTs: multiwalled carbon nanotubes; CNTs: carbon nanotubes; HRP: horseradish peroxidase; Ab: antibody; AP: alkaline phosphatase; AuNPs: gold nanoparticles; QD: quantum dots DPV: differential pulse voltammetry; ASV: anodic stripping voltammetry; SWV: square wave voltammetry.

Biomarker	Methodology	Transduction technique	Sample	Concentration range	LOD	Ref.
cTnT	Sandwich-type assay on graphite-epoxy silver SPE. Immobilization of capture Ab by integrated streptavidin microspheres	Amperometry	Serum	0.1-10 ng/mL	0.2 ng/mL	[113]
cTnT	Sandwich-type assay on SPCE modified with amino-functionalized MWCNTs. HRP as label.	Amperometry	Serum	0.02-0.32 ng/mL	0.016 ng/mL	[119]
cTnT	Label-free immunosensor based on amine-functionalised CNT-SPEs platforms.	DPV	Serum	0.0025-0.5 ng/mL	0.0035 ng/mL	[114]
cTnI	Sandwich-type assay using AP labelled detection Ab. Capture Ab immobilized by adsorption.	Amperometry	Blood	2-100 ng/mL	1-2 ng/mL	[120]
Myoglobin	One-step indirect sandwich-type assay using AP as label-Capture Ab immobilized by adsorption.	Amperometry	Whole blood	85-925 ng/mL	-	[115]
Myoglobin	Label-free immunosensor based using AuNPs as electrocatalysts of Fe(III)/Fe(II) electrode reaction of myoglobin.	SWV	Plasma	10-1780 ng/mL	10 ng/mL	[116]
CRP	HRP-labelled antibody functionalized Fe_3O_4@Au magnetic nanoparticles attracted to a Fe(III) phthalocyanine/chitosan membrane modified SPCE by an external magnetic field. After incubation, activity centre of HRP decreases linearly with CRP concentration. Reusable SPCEs.	Amperometry	Serum	1.2-200 ng/mL	0.5 ng/mL	[117]
CRP	Sandwich-type assay on bismuth citrate-modified SPE. Quantification through acidic dissolution of PbS QDs and ASV detection of Pb(II).	ASV	Serum	0.2-100 ng/mL	0.05 ng/mL	[118]
HFABP	Sandwich-type assay using AP labelled detection Ab. Capture Ab immobilized by adsorption on SPE.	Amperometry	Whole blood	4-250 ng/mL	4 ng/mL	[121]

6 Conclusions

EIs are one of the most widely used analytical techniques in the quantitative detection of biomarkers diseases due to the specific binding of antibody to its corresponding antigen. Unlike spectroscopic and chromatographic instruments, electrochemical sensors can be easily adapted for detecting a wide range of analytes while remaining inexpensive. Since SPEs show advantages such as miniaturization, mass production, customization, portability and low cost, the replacement of conventional electrodes by SPEs is making possible to explore other options in the development of EIs. Taking this into account, this review summarizes researches on biomarkers used for detecting cancer and cardiovascular diseases and on the EIs based on SPEs developed for determinate these biomarkers.

The sensitivity of biomarkers is important for detection of diseases. Thus, the choice of the antibody immobilization is a crucial step because antibody acts as the recognition element for antibody-antigen reaction, and the performance of the detection of antigen binding capacity can be improved using a proper antibody surface. Moreover, the use of nanomaterials for electrode surface modification, for signal amplification or as label, allow the improvement of the sensitivity. In addition, the great versatility of design of the SPEs offer multiplexing capability for simultaneous measurements of biomarkers.

Nonetheless, relevant and not yet totally controlled aspects such as storage and stability of EIs developed has to be improved for their use as clinical diagnosis routine tool. In one hand, the storage and transportation conditions of biosensors play an important role in their functionality and shelf life: environmental factors such as humidity, temperature and air exposure all offer potential obstacles in the functionality of biomaterials. In the other hand, the stability of proteins on the immunosensor is crucial to the feasibility of any

commercialisation prospects; a low stability break the business viability of any biosensor product. Another challenge EIs must face for its consideration as a reliable option as diagnosis or monitoring of diseases tool is its validation using real samples. This validation many times is limited since the samples used are doped samples or are not samples of real patients. Sometimes, although the samples used are real patient samples, the number of sample tested is not enough to assure a reliable validation of the immunosensor. In addition, this validation must be performed not only in terms of sensibility, selectivity and accuracy, but also of rapidity, simplicity and cost with respect to other competitive methodologies existing. Moreover, since a POC test is desirable to encapsulate all the required instrumentation in a suitable portable format, additional research efforts are needed toward the full integration of EIs in automated and miniaturized systems in order to achieved EI-based POC systems. Therefore, further efforts in immunosensor stability and validation together with continuous miniaturization and automatization of EIs are the key to the success of the use of EIs in POC testing for making clinical results available at patient bedside or physician office.

Acknowledgments

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