

Electrochemical and Photoelectrochemical Biosensors for Biomarker Detection

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Abstract. A rapid and accurate medical diagnosis is essential in order to determine the health status of a patient. Nowadays, most of the clinical analyses are performed in specialized laboratory, which required specific instrumentation and trained personal, resulting in an increase of analysis costs and time. In this context, biosensors represent ideal tools capable to provide a specific and fast response together with low cost, easy use and portable size features. This work attempts to provide a review of the research progresses of electrochemical and photoelectrochemical biosensor platforms in clinical applications that have been published in recent years. Special emphasis will be devoted to discuss examples for breast cancer biomarker detection, because breast cancer, is considered the leading cause of cancer-related deaths worldwide in women, representing 15% of all cancer related amongst women, with a 6% mortality rate (based on overall cancer deaths). The manuscript is focused on aptamer-based biosensors, because, due to their stability and their relatively low cost, they have been successfully applied in many biosensor formats for breast cancer biomarker detection.

Keywords: Biosensors · Breast cancer · Biomarker · Aptamer

1 Introduction

Cancer represents one of the leading causes of death worldwide. There are more than 100 different types of cancer affecting different human organs. Following statistics, in Europe in 2012 the most common primary cancer sites in men were prostate, lung, colorectal and bladder. In women, breast cancer was by far the most frequently diagnosed neoplasm, followed by colorectal, lung and corpus uteri cancers. Breast cancer, represents 15% of all cancer related amongst women, with a 6% mortality rate (based on overall cancer deaths) [1].

It is now well known that early detection of cancer represents the best opportunity to increase the survival rate of patients. In this perspective the discovery and the clinical validation of cancer-related biomarkers represent an ideal tool.

In clinical analysis, a biomarker represents a bio-molecule whose concentration increases/decreases over a threshold level (named also as clinical cut-off) in a body fluids, tissue, etc. in the presence/absence of a disease. Rigorous definition was given

both by Food and Drug Administration (FDA) and European Union National Institute of Health which defined a biomarker as a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention. The biomarkers act as indicators of a normal or a pathogenic biological process. They allow assessing the pharmacological response to a therapeutic intervention. A biomarker shows a specific physical trait or a measurable biologically produced change in the body that is linked to a disease or a particular health condition” [2].

These molecules should ideally possess the following properties:

- Biomarker expression should be related to the process that causes cancer and to the type of the cancer.
- Biomarker level should be higher enough, in biological fluids or tissue, to be measured in an easy and trustworthy way.
- Biomarker concentration (increasing or decreasing) should be strictly correlated to the disease variation and to the treatment outcome.

Because proteins influence the molecular pathways in the cells and they are strictly related to disease state and they are released in biological fluids, the cancer-related proteins are one of the most studied and used biomarkers in clinical analysis [3–6].

In the cancer biomarker analysis, biosensors play an interesting role because they allow fast, accurate, sensitive, selective and low cost analysis coupled with the possibility of device miniaturization and multi-analysis detection in contrast with the conventional clinical procedure (such as ELISA test, fluorescent or chemiluminescent assays) [7–9].

In this work, we focus our attention on the development of electrochemical and photoelectrochemical biosensor for determination of breast cancer biomarkers reported in the literature in the last three years.

2 Aptamer

Aptamers are synthetic oligonucleotides sequences (both DNA, in particular ssDNA, and RNA) or peptide capable to bind a target protein with affinity and selectivity comparable of those of monoclonal antibodies. Due to their advantages respect to antibodies (such as high stability, chemical synthesis, low dimension, affinity for small molecules, etc.) aptamers were successfully applied in many fields including drug discovery and delivery, tissue bio-imaging and also as therapeutic agents and as bioreceptors in biosensor applications for clinical, environmental and food analysis [10–12].

2.1 Nucleic Acid Aptamers

Nucleic acid aptamers (both DNA and RNA), which exhibits a high affinity for a specific target, are synthesized through a process named as systematic evolution of ligands by exponential enrichment (abbreviated as SELEX) developed in 1990s by two independent research groups [13, 14].

The original process (conventional SELEX) consists in a series of binding, elution and amplification steps repeated several times (about 20) which led to the selection of a pool of aptamers with high affinity for a specific target. In particular, an initial library of 10^{15} – 10^{16} random sequences (each one composed by a 20–30 bp variable region and a 15–25 bp flanked region, used to anneal the primer during the Polymerase Chain Reaction, PCR, amplification step) is incubated, in controlled experimental conditions (pH, ionic strength, temperature, incubation time etc.), with the target molecules. After the affinity reaction, the unbound oligonucleotides are removed while the DNA sequences bound to the analyte are eluted and subsequently amplified by PCR process. The binding/elution/amplification steps are then repeated, in more stringent experimental conditions, with the new pool of oligonucleotides. For RNA aptamer selection, the initial DNA library is transcribed into a RNA library, which is incubated with the target molecules; after the elution step, selected RNA aptamers are converted again into DNA sequences, amplified by PCR, and transcribed back to RNA for the next round. In both cases, at the end, the selected aptamer are sequenced and characterized by thermodynamics and kinetic measurements in terms of affinity constant (K_a), dissociation constant (k_d) and the ratio ($K_D = K_d/K_a$) [15].

In order to overcome some drawbacks of SELEX conventional procedure (such as long working time, high operative and instrumentation costs), some variants were studied and introduced (i.e. in capillarity and micro-flow electrophoresis SELEX, magnetic beads-based SELEX or cell- and in vivo SELEX)

All these processes are generally followed by a post-modification step in which various positions of the oligonucleotide chain (i.e. 2', 3' and 5') can be functionalized with specific molecules (polyethylene glycol tag, PEG, -SH, -NH₂, biotin, sugar, etc.) for particular applications. In some cases, in order to avoid the decreasing of aptamer affinity, chemically modified DNA/RNA sequences can be directly introduced into the initial library [16].

2.2 Peptide Aptamer

Peptide aptamers have been defined by Colas et al. in 1996 [17] as combinatorial protein molecules in which a variable peptide sequence with affinity for a given target protein is displayed on an inert, constant scaffold protein. They are extremely simple molecules, selected from combinatorial libraries on the basis of their affinity to the target protein or small molecule, and, generally, expressed in bacterial cells, such as *E. coli*. [10, 17]. There are many protein scaffolds reported in the literature and they have been intensely reviewed in the past [1, 10].

Among this class of bioreceptor, Affibody[®] molecules are commercially available. The Affibody molecule is an engineered version (Z domain) of one of the five stable three- α -helix bundle domains from the immunoglobulin Fc-binding region of staphylococcal protein A. This molecule is constituted by only 58 amino acids without disulphide bonds and can therefore be produced in simpler organism such as prokaryote, rather than the animal system required in antibody synthesis. Affibodies

can include specific labels, such as fluorophores, radioactive labels and other moieties, such as biotin, which can be used to couple the affibody to surfaces or other molecules, including enzymes [18, 19].

3 Biomarkers in Breast Cancer

Some specific biomarkers are often useful for selecting the appropriate treatment options in breast cancer. Some of them are here described.

CA15.3 (also known as MUC1 from the coding gene) is a type I transmembrane glycoprotein, that is mainly overexpressed in breast and ovarian carcinomas. It possesses a molecular mass ranging from 300 to 600 kDa and consists of two subunits (a C-terminal cytoplasmic domain and an extracellular subunits containing the variable number tandem repeat, VNTR, domain). The disease status in breast and recently in ovarian cancer patients is routinely assessed by monitoring the serum levels of circulating CA15.3 protein and its elevated levels (>20 U/mL) are always associated with poor survival [20].

Human epidermal growth factor receptor 2 (HER2), also known as ErbB2, c-erbB2 or HER2/neu, is a 185 kDa protein belongs to a family composed of four structurally related members, HER1 (ErbB1, also known as EGFR), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). In particular, HER2 is a type 1 transmembrane glycoprotein which includes three distinct regions: an N-terminal extracellular domain (ECD), a single α -helix transmembrane domain (TM), and an intracellular tyrosine kinase domain. Overexpression of HER2 usually results in malignant transformation of cells accounts for $\sim 25\%$ of all breast cancer cases (clinical cut off: 15 ng/mL). Furthermore, it was also found that survival rate and recurrence probability of the tumor for HER2 positive breast cancer patients are significantly shorter than patients without HER2 overexpression. HER2 can be also found at high concentration in blood in the presence of other cancer types such as gastric, ovarian and prostate [21].

Vascular endothelial growth factor term is referred to a family of dimer glycoprotein (covalently linked by 2 disulfide bridges) which includes five members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, placenta growth factor, PGF) and their associated receptors (VEGFR-1, VEGFR-2, VEGFR-3). Homologs of VEGF protein are also discovered in the genome of some viruses (VEGF-E) or in the venom of some snakes (VEGF-F). Because VEGF-A was the first and the most studied protein (in particular related to carcinogenesis and cancer biology), often, in literature, the term VEGF strictly indicate the VEGF-A member. From biological point of view, VEGF and its receptors play a main role in the formation of cardiovascular system; in presence of a cancer process VEGF is mainly involved carcinogenesis and in tumor metastasis. VEGF concentration in blood and serum (which values is much higher than 100 pM in pathological condition) can be thus used as biomarker associated with diagnosis and prognosis of different type of cancer diseases with particular reference to the presence of metastasis processes [22].

4 Electrochemical and Photoelectrochemical Biosensor for Breast Cancer Biomarker Detection

4.1 Electrochemical-Based Biosensors

Electrochemical techniques as transduction mechanism in aptasensors development were widely known. The common electrochemical techniques used for the detection of the analyte include potentiometry, amperometry, voltammetry, conductometry and electrochemical impedance spectroscopy. Further and complete description of these electrochemical techniques can be found in Ref. [23].

Herein, some examples of electrochemical aptasensors development for breast cancer biomarkers detection using label and label-free approaches are briefly discussed.

An aptamer sandwich-based assay for MUC1 detection was reported by Florea et al. [24]. In this work, anti-MUC1 primary aptamer was immobilized on the surface of streptavidin-coated magnetic beads, followed by reaction with biotin (as blocking agent) and with MUC1 protein. Then, incubation with a biotinylated secondary anti-MUC1 aptamer and with streptavidin-alkaline phosphatase were carried out. The electrochemical detection of alpha-naphthol (obtained by the hydrolysis of alpha-naphthyl phosphate) by the use of differential pulse voltammetry (DPV) technique allowed the detection of MUC1 in the range between 0.05 and 0.28 nM MUC1 with a detection limit of 0.07 nM. The proposed aptasensor showed also a high selectivity for MUC1 protein in the presence of mucin 4 and mucin 16 (as non-specific proteins) and the ability to detect MUC1 in cancer patient serum sample.

In a recent work, the development of a label-free biosensor for HER2 cancer biomarker detection based on the use of anti-HER2 Affibody, as bioreceptor, and of gold nanoparticles (AuNPs)-modified graphite screen-printed electrode, as nanostructured electrochemical transducer, was proposed (Fig. 1) [25]. In particular, after the electrodeposition of AuNPs on the working electrode surface, the sensor was modified by the incubation with the anti-HER2 Affibody. Mixed-SAM formation (by the use of the use 6-mercapto-1-hexanol) and surface blocking step (by the use of BSA) were performed. Affinity reaction with HER2 protein was then evaluated by means of electrochemical impedance spectroscopy, EIS (linear range: 1–40 $\mu\text{g/L}$ HER2, detection limit: 6 $\mu\text{g/L}$). The proposed affibody-based biosensor showed also good response in HER2-spiked serum samples.

An aptamer-based sandwich assay for VEGF detection was reported by Ravalli et al. [26]. After the modification of the graphite working electrode surface by the use of AuNPs, the incubation with thiolated primary anti-VEGF aptamer was carried out, followed by blocking step (by the use of 6-mercapto-1-hexanol) and affinity reaction with VEGF. The assay was then completed by the addition of a biotinylated secondary anti-VEGF aptamer and by streptavidin-alkaline phosphatase. The electrochemical evaluation of alpha-naphthol (produced by the enzyme in the presence of alpha-naphthyl phosphate) allowed the construction of the calibration curve in a linear range between 40 and 250 nM with a limit of detection of 30 nM.

Recently, Baydemir et al. [27] described the use of Affibody molecule for TNF- α detection. TNF- α is an inflammatory cytokine produced by the immune system. Serum TNF- α level is elevated in some pathological states such as septic shock, graft

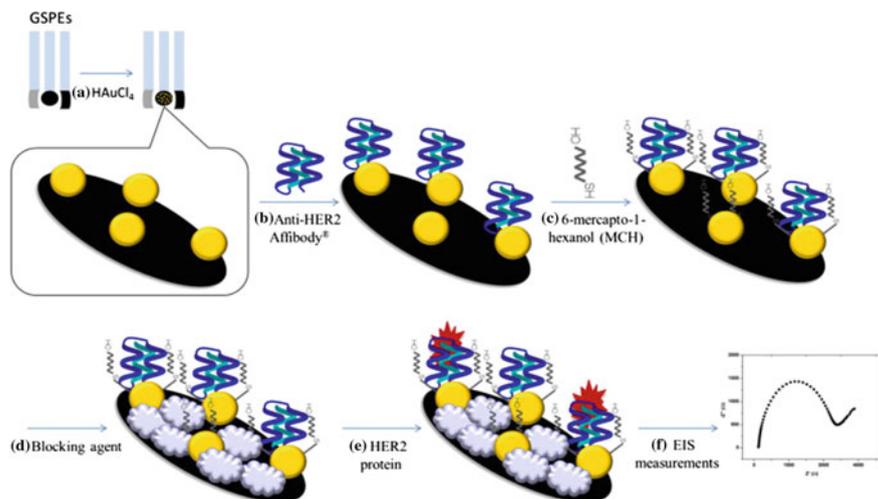


Fig. 1 Schematic representation of label-free HER2 cancer biomarker detection using anti-HER2 Affibody-modified gold nanostructured graphite screen-printed electrodes (GSPEs) (reproduced with permission from [25])

rejection, HIV infection, neurodegenerative diseases, rheumatoid arthritis and cancer. Detecting trace amount of TNF- α is, also, very important for the understanding of tumor biological processes [28]. Magnetic beads were used as support for Affibody immobilization and screen printed carbon electrodes were used as transducers. TNF- α calibration curve was performed, obtaining a detection limit of 38 pg/mL, the quantification range of 76–5000 pg/mL and RSD% 7.

4.2 Photoelectrochemical-Based Biosensors

Photoelectrochemical biosensors are based on the use of photoactive materials for the development of the transducer. The photoactive material is responsive to a light excitation, generating a photocurrent on a conductive substrate. Inorganic and organic semiconductors can be used as photoactive materials.

An anodic photocurrent occurs when the conduction electrons are transferred to the electrode, and valence holes neutralized by electrons supplied by an electron donor in solution [29]. If the conduction electrons are transferred to a solution-solubilized electron acceptor, a cathodic photocurrent is measured. The presence of an efficient electron donor/acceptor prevents the electron-hole recombination and thus increases and stabilizes the photocurrent.

Nowadays, inorganic semiconductors are mainly fabricated using nanomaterials like SnO₂, TiO₂ nanoparticles (NPs) as well as CdS, CdSe quantum dots (QDs). Frequently, these nanomaterials are assembled on a conductive substrate, such as gold, Indium-Tin-Oxide (ITO) or F-doped SnO₂ (FTO). Organic photoactive materials include small molecules such as porphyrin, phtalocyanine, and their derivatives, azo

dyes, metal complexes as well as polymers. The light excites the molecules that can react with an electron donor or an electron acceptor, producing anodic or cathodic photocurrents, respectively.

Hybrid semiconductors can be obtained by coupling two inorganic semiconductors with different band gaps or organic complexes combined with inorganic materials; in this sense improved conversion efficiency is obtained by coupling semiconductors with different band gap.

Photoelectrochemical detection offers some peculiar advantages, such as miniaturized and low cost instrumentation. Moreover, by using light for excitation and electrochemistry for detection, photoelectrochemistry can reach high level of sensitivity because of the reduced background associated with it. According to the mode of signal transduction photoelectrochemical biosensors could be classified as potentiometric or amperometric photoelectrochemical biosensors.

Recently, Tan et al. described the use of TiO₂ nanotube arrays (TiO₂ NTs), grown on a titanium foil and decorated with Au nanoparticles (AuNPs) for the detection of MUC1 (Fig. 2) [30]. AuNPs were used for improving the electrical conductivity of TiO₂ NTs and for the immobilization of the MUC1 aptamers. CdTe QDs-labeled complementary single-stranded DNAs (c-DNA@QDs) are hybridized with the MUC1 aptamer to form a TiO₂ NT/aptamer/c-DNA@QD aptasensor. In the absence of target MUC1, under the irradiation of visible light, a high photocurrent response was observed due to the light absorption of CdTe QDs and the photoinduced electron transfer from CdTe QDs to TiO₂ NTs through DNA chain. However, in the presence of MUC1, MUC1 combined with its aptamer and CdTe QDs-labeled c-DNAs left the

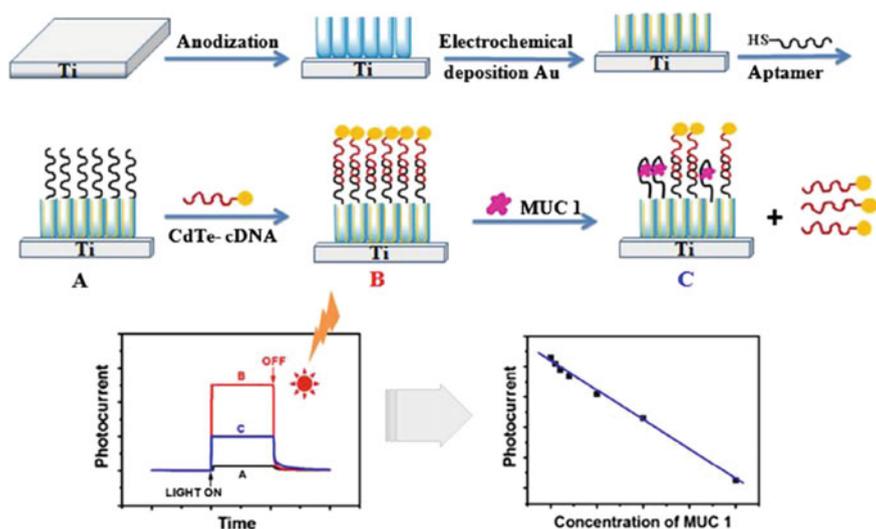


Fig. 2 Schematic representation of TiO₂ NT/aptamer/c-DNA/QD-based photoelectrochemical aptasensor for MUC1 cancer biomarker detection (reproduced from [30] with permission of The Royal Society of Chemistry)

TiO₂ NTs, leading to a decreasing of photocurrent value. Therefore, the detection of target MUC1 could be sensitively transduced via detection of the photocurrent reduction.

5 Conclusions

Nowadays, aptamers have been proposed as innovative, synthetic bioreceptors in biosensing. Within this review, different applications of both nucleic acid and peptide aptamers have been discussed in the field of breast cancer biomarker monitoring.

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