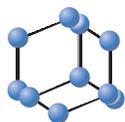


REVIEW ARTICLE

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SCIENCE

Recent Advancements in Electrochemical Biosensors for Alzheimer's Disease Biomarkers Detection

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Abstract: Background: It is estimated that the average time between the diagnosis of Alzheimer's disease (AD) and the patient's death is 5-9 years. Therefore, both the initial phase of the disease and the preclinical state can be included in the critical period in disease diagnosis. Accordingly, huge progress has recently been observed in biomarker research to identify risk factors for dementia in older people with normal cognitive functions and mild cognitive impairments.

Methods: Electrochemical biosensors are excellent analytical tools that are used in the detection of AD biomarkers as they are easy to use, portable, and can do analysis in real time.

Results: This review presents the analytical techniques currently used to determine AD biomarkers in terms of their advantages and disadvantages; the most important clinical biomarkers of AD and their role in the disease. All recently used biorecognition molecules in electrochemical biosensor development, *i.e.*, receptor protein, antibodies, aptamers and nucleic acids, are summarized for the first time. Novel electrochemical biosensors for AD biomarker detection, as ideal analytical platforms for point-of-care diagnostics, are also reviewed.

Conclusion: The article focuses on various strategies of biosensor chemical surface modifications to immobilize biorecognition molecules, enabling specific, quantitative AD biomarker detection in synthetic and clinical samples. In addition, this is the first review that presents innovative single-platform systems for simultaneous detection of multiple biomarkers and other important AD-associated biological species based on electrochemical techniques. The importance of these platforms in disease diagnosis is discussed.

Keywords: Alzheimer's disease, biomarkers detection, early diagnosis, biosensors, surface modification, multiplex assays.

1. INTRODUCTION

The morbidity of AD doubles every 5 years in people over 60 years and currently affects more than 44 million people worldwide [1-7]. It is predicted that this number will increase to 131.5 million people by 2050 [8], *i.e.*, about 1 in 85 will be affected by AD if current

trends persist without counteraction resulting from medical progress [9, 10]. The disease process can begin 20 years before the first signs of cognitive decline [11, 12]. Due to the great adaptation abilities of the human brain, the first important symptoms may appear only after the disappearance of about $\frac{3}{4}$ of the neurons. In this case, the brain exceeds the threshold of cognitive performance.

Despite medical progress and the development of scientific knowledge, AD remains an incurable disease, and its definitive diagnosis can only be made after the patient's death based on the pathophysiological

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image of the brain [13]. To date, the molecular cause of AD-causing processes has not been clearly defined and remains a contentious issue [14, 15]. According to commonly accepted criteria, the occurrence of dementia is the condition for diagnosing AD [16]. The disease develops very slowly and the resulting changes are irreversible. In some cases, significant deterioration is observed within 2-3 years, in others, the course of dementia is slower and may last over 10 years. It is known that pathological abnormalities, such as amyloid plaques, form approximately 10-20 years before the appearance of cognitive dysfunction symptoms and considerable loss of nerve cells [17]. Early diagnosis of people without cognitive impairment (*i.e.*, before the loss of neurons and synapses) would allow the use of new effective therapies in the future, giving hope for effective treatment and maintaining normal brain functions. The possibility of early detection of the disease, and thus the early use of anti-inflammatory drugs or antioxidants long before the onset of the first symptoms, can even prevent the occurrence of the disease [3].

Accordingly, great progress has recently been observed in biomarker research aimed at identifying dementia risk factors in people with normal cognitive functions [3, 5, 18]. Mass spectrometry (MS) [19], manganese-enhanced magnetic resonance imaging (MEMRI) [20], enzyme linked immunosorbent assay (ELISA) [21-23], flexible multi-analyte profiling (xMAP) [24], immunohistochemistry (IHC) [25-27], western-blot [28-30], fluorescence [31-33] and position emission tomography (PET) [34] are among techniques currently used to determine AD biomarkers. Despite the undoubted merits, the main disadvantages of these analytical methods are that they are time-consuming, relatively expensive, hardly available, require large sample volume and specialized equipment; and they are not yet adapted for point-of-care (POC) diagnostics (Table 1). The ease-of-use platforms with high sensitivity and specificity, and suitable for POC diagnostics are still critically needed.

The development of electrochemical biosensors is probably one of the most promising methods to solve some of the problems regarding sensitive, fast and cost-effective measurements [51]. Furthermore, their potential in microfabrication and portability can also be used to allow for their use in simple point-of-care devices and aid in drug-screening processes of effective therapeutic molecules for neurodegeneration associated with AD.

The other important issue related to biosensors is the proper immobilization of biorecognition molecules

on the surface of electrodes, which affects the sensitivity and specificity of biomarker determination. The immobilization procedure must maintain the molecule responsible for biorecognition close to the transducer surface while retaining its biological activity in a reproducible manner. The immobilization layer should give the biological molecule enhanced stability [52-54]. There are different strategies of electrode surface functionalization for the immobilization of biorecognition molecules on a solid support. Nonetheless, the covalent binding between these molecules and the electrode surface is one of the most widely used. Just for example, immobilization of antibodies on the glutaraldehyde (GA) layer [55] or on 3 cyanopropyltrimethoxysilane self-assembled monolayers [56] or also immobilization of thiolated antibodies on the gold surface [57] has been reported.

In recent years, the field of biosensors has been growing, and the application of nanotechnology has developed as one of the biggest opportunities to achieve higher sensitivity for biosensors [58]. At the present time, in the construction of electrochemical biosensors for biomedical applications associated with AD biomarkers detection, the use of different types of nanomaterials like porous magnetic microspheres [59], nanotubes [60], graphene oxide [61], indium tin oxide [56] and metallic nanoparticles [62] has been reported in this review.

The important challenge in electrochemical biosensors development concerns simultaneous multianalytes detection. This is extremely important in the context of detection of multiple AD biomarkers, which is fundamental for correct disease diagnosis and prognosis. Simultaneous detection of several clinically relevant biomarkers is essential for clinical applications and it is an effective solution in improving diagnostic value, while a single biomarker detection is usually not sufficient. Recently, the interest in electrochemical biosensors for simultaneous multiple biomarker detection, including AD biomarkers, has gained more attention than ever, and this issue will also be discussed in this review.

2. BIOMARKERS OF AD DIAGNOSIS

A biomarker has been defined as an objectively measurable change arising in biological environments such as human cells, tissues or body fluids. This change may describe the pathological condition or the body's response to treatment when assessing the effectiveness of pharmacological therapy [63, 64]. Compounds that are "candidates" for AD biomarkers must reflect the basic neuropathological characteristics of this disease. Biomarker determination should be performed using a quick, safe and easy diagnostic test, which allows detecting the disease in the phase before

the appearance of characteristic clinical symptoms [17].

The search for AD markers concerned the analysis of the cerebrospinal fluid since compounds that reflect

all pathological conditions can be found in the fluid that washes the diseased tissue of the central nervous system. However, it is much easier to use blood as a source of biomarkers for diagnostic purposes, because

Table 1. Techniques for AD biomarkers detection.

Technique	Advantages	Disadvantages	Refs.
IHC	The ability to simultaneously detect many antigens in hundreds of tissues. Routinely performed. Low technological requirements. Preservation of histological information.	Expensive. Time-consuming. Variable antibody reactivity. Non-specific background signal. Variability dependent on fixation procedure, staining protocol, and antibody selection.	[35-37]
ELISA	Cost-effective. Widely established. Absorbance is proportional to antigen concentration. Quantitative, very sensitive.	Time-consuming. Inefficient. Insensitive to low level markers. Non-specific interactions. False positives/negatives possible, particularly with altered/mutated antigen. High amounts of protein lysate required.	[38, 39]
MS – based technologies	No protein binding reagent required. Protein isoforms can be distinguished. Good combination of sensitivity and selectivity. Highly specific chemical information (accurate mass, characteristic fragment ions).	Expensive. Time-consuming. Strict low-pressure requirements. Require specialized equipment. Hardly available.	[38, 40-43]
MRI	High tissue contrast. Not or minimally invasive procedure. No radiation associated with imaging. No need for iodinated contrast. Superior soft tissue imaging with excellent spatial resolution.	High cost. Limited availability. Claustrophobia due to the smaller patient bore. Contraindicated in patients with some metal implants and fragments. Requires specialized equipment. Hardly available.	[44-46]
Western-blot	Separation of proteins according to molecular weight. Specific interaction of antibody and antigen can be directly visualized. Relatively simple and cost-effective method.	Low- or medium-throughput. High amounts of protein lysate required. An imbalance in any step of the procedure may skew the entire process.	[38, 47]
xMAP	Reduced cost and labour by multiplexing for complex projects. Smaller sample requirements for complex projects. Open architecture platform.	Expensive. Cross reactivity of some antibodies, which has to be avoided. Requires the optimization during initial stages in the development of new assays. Hardly available.	[48]
PET	Providing functional and biological information. May have diagnostic value detecting metastatic lesions that would have been missed on conventional imaging.	High cost. Poor spatial resolution and lesion detectability. Shielding to avoid radiation exposure. Administration by intravenous injection of radiopharmaceutical compounds.	[44, 49]
Fluorescence	Immune to light scattering. High specificity due to unique optical properties of molecules. It can measure analyte concentration in terms of fluorescence intensity and decay times. High sensitivity (especially with iodinated ligands).	Susceptible to autofluorescence. Limitations associated with photostability and loss of recognition capability.	[49, 50]

the access to the blood is very easy and the method of its collection is less invasive, which involves much less discomfort for the patient. Moreover, the detection of AD biomarkers in the blood is not an easy task. First of all, there is the so-called blood-brain barrier, hindering the transport of potential AD markers from the cerebrospinal fluid to the blood, which results in the possibility of too low blood concentrations [65]. Secondly, the blood component is a protein characterized by high abundance. For this reason, the determination of low-abundance proteins is much more difficult, especially that the concentration of proteins in the blood is higher than in the cerebrospinal fluid [66].

At present, three cerebrospinal fluid (CSF) biomarkers for AD diagnosis have been established and published worldwide: amyloid β peptides ($A\beta_{1-42}$ and $A\beta_{1-40}$), total tau protein and phosphorylated tau protein [67]. S100B protein, apolipoprotein E and glycated albumin are also major etiological AD factors [68-72], which are listed as the important biomarkers of this disease. These biomarkers with a significant role in the clinical practice of AD are presented below:

(I) Amyloid- β ($A\beta$) peptides consisting of about 40 amino acids are components of another large protein called $A\beta$ -APP peptide precursor protein. β -amyloid is

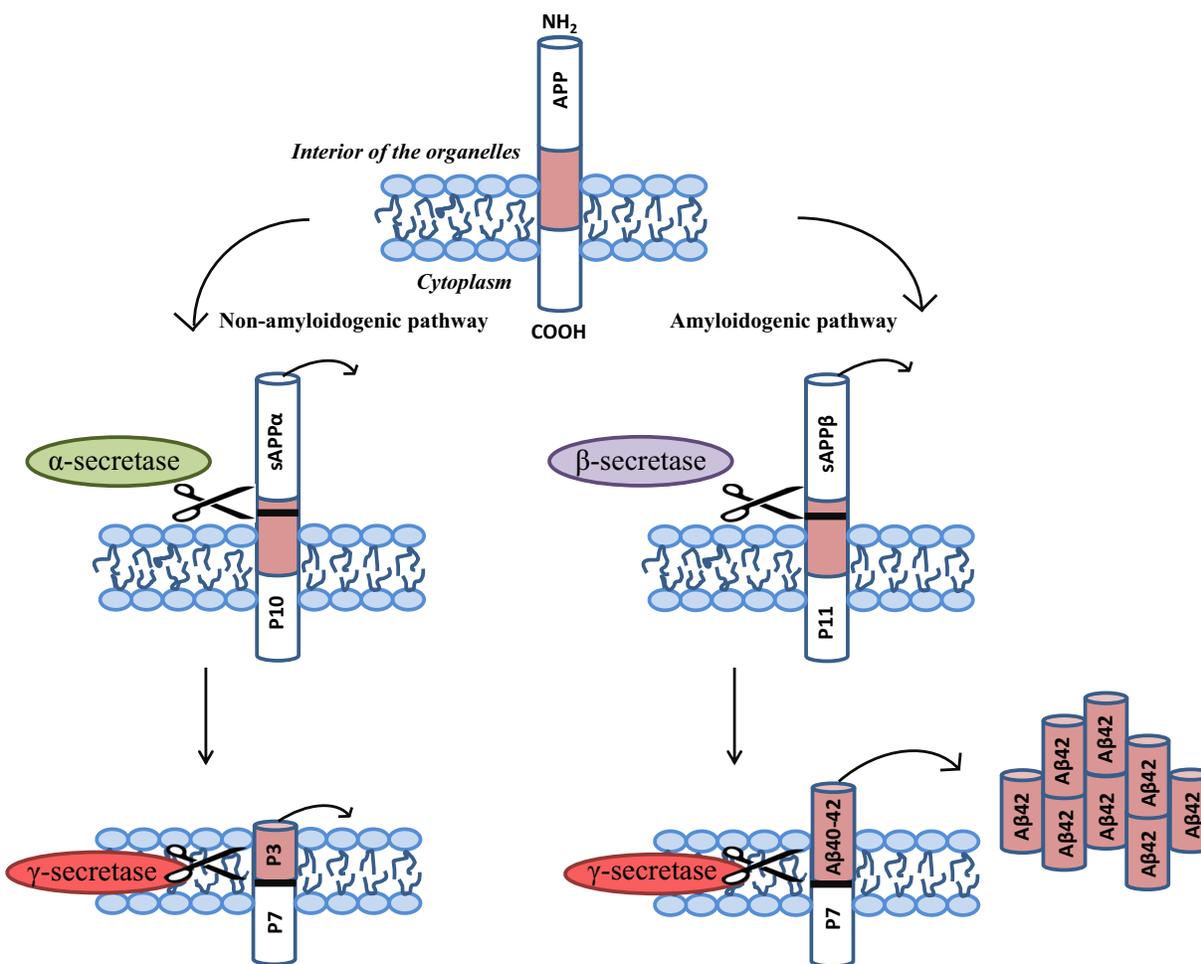


Fig. (1). A series of endoproteolytic cleavage of the APP protein leading to the formation of peptides by non-amyloidogenic and amyloidogenic pathways. In the first of them, the process takes place using α - and γ -secretase enzymes, while in the second one, β - and γ -secretase are involved. Non-amyloidogenic secretion products are soluble α -APPs peptide and non-toxic P3 and P7 peptides. Cleavage of APP with α -secretase prevents β -amyloid formation. The amyloidogenic hydrolysis products are soluble β -APPs protein and P11 peptide. The latter is further hydrolyzed by γ -secretase to release a 39-43 amino acid β -amyloid peptide because γ -secretase can cleave P11 peptide at slightly different locations. A form of β -amyloid 42 ($A\beta_{1-42}$) is also produced, but in a smaller amount [74]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

formed from the precursor protein APP by non-amyloidogenic and amyloidogenic proteolysis (Fig. 1) [73].

Each of the forms of soluble oligomers resulting from APP transformations has a different mechanism of neurotoxic action. The oligomeric forms display a neurotoxic action: di-, tri-, tetramers and higher forms have been distinguished. A β peptide dimers are involved in the initiation of the proinflammatory reaction by activating glial cells. These cells secrete agents with potential neurotoxic effects, *i.e.*, free radicals or cytokinins. Thus, dimers alone do not act directly adversely on nerve cells [75]. Medium soluble and globular forms have the ability to binding to synapses and destroy the connections between them. Their long-term neurotoxic effect is manifested by the death of nerve cells in a specific region of the hippocampus-C1 [76]. Protofibrillar forms affected the electrophysiological properties of membranes, including action potential and membrane depolarization [77]. The cause of cell death is the toxic effect of aggregated forms of A β [78]. Histopathological examination reveals outside neurons the senile plaques containing fibrillar A β [79, 80]. Elevated levels of the β -amyloid peptide can be detected even many years before the symptoms of the disease are recorded [72, 81]. From the point of view of early diagnosis, it is important to observe the dynamics of plasma changes in the level of β -amyloid peptide composed of 40 and 42 amino acids, which may enable an assessment of Alzheimer's disease risk. The physiological concentration of A β present in human plasma as well as in the cerebrospinal fluid ranges from 0.1 to 0.5 nM [82]. An increase in total A β concentration is observed in the first asymptomatic preclinical phase [83]. As the disease progresses and amyloid deposits gradually form in the brain, A β , and in particular A β_{1-42} levels, often decrease to normal. This information indicates that fundamental biochemical events relevant to AD can be monitored in blood.

(II) Another important AD biomarker is tau protein. The aggregation of tau into neurofibrillary tangles within brain tissues is associated with AD pathology. This protein is responsible for the binding and stabilization of microtubules in neuronal axons [74], a process that is inhibited when tau becomes phosphorylated. AD-related pathology proceeds in precisely designate stages, based on the conception that neurofibrillary tangles develop from an accumulation of abnormal tau. Abnormal phosphorylation is a decisive step causing the formation of tau filaments (soluble and insoluble) [84]. During the neurodegeneration process, tau undergoes hyperphosphorylation, which results in conformational changes and its dissociation from microtubules,

as well as self-association in glycates [85]. Complementary animal models indicate that tau hyperphosphorylation alone can cause neurodegeneration, leading researchers to conclude that hyperphosphorylated tau is toxic to neurons and plays an important role in AD neuropathology [86]. High levels of tau in the CSF of AD patients can reflect the intensity of neuronal damage and brain degeneration [87]. In AD, total tau (t-tau) or phosphorylated tau (p-tau) concentrations are increased compared to cognitively normal individuals [88].

(III) The brain in Alzheimer's disease is characterized by high levels of S100 family proteins, especially S100B protein – a protein that binds calcium ions [89, 90]. In the brain, S100B protein is secreted primarily in glial cells, such as astrocytes and oligodendrocytes [91, 92]. Its elevated level was found in the serum and cerebrospinal fluid of patients with AD [93, 94]. It is believed that its higher concentration plays a role in the pathogenesis of neurodegenerative processes [69, 89]. S100B induces both neurotrophic and potential neurotoxic effects, depending on its concentration in the body [95]. Nanomolar concentrations of S100B stimulate the growth of neuritis [92], while micromolar concentrations of this protein are detrimental to these structures [96]. High levels of extracellular S100B protein are detected after serious brain injuries or in the case of neurodegenerative diseases such as AD [69, 97, 98]. Controlling the S100B protein level may be useful in evaluating the severity of dementia progressing with disease development [68].

(IV) Another important AD biomarker is glycated albumin. Albumin is a protein found in the highest concentration in both cerebrospinal fluid and plasma. Albumin present in plasma is mainly produced by the liver, and a small portion of it enters the brain [99]. The process of albumin glycation results in the formation of toxic aggregates [100] and significantly inhibits the slowing of A β_{1-42} aggregation [101]. It can be speculated that glycation reduces the effect of aggregation inhibition by A β [101]. A similar mechanism has been proposed for prions that bind A β monomers and oligomers, depending on their conformational state [102, 103]. This reduction in the inhibitory effect of albumin on A β fibrillation is particularly important in the brain parenchyma, where A β aggregation plays a key role in the onset of AD. Albumin-related glycine modification affects cells on both sides of the "blood-brain barrier" and weakens the inhibition of A β filament formation, usually associated with native albumin [104]. The accumulation of pathological amounts of proteins, modified as a result of the glycation process in the patient's

brain, causes that these modifications may contribute to AD etiology. As already mentioned, the causes and pathological mechanisms of AD are not fully understood and are still under discussion [101]. However, higher levels of glycated albumin were found in the cerebrospinal fluid and plasma of Alzheimer's patients compared to the control groups [101, 105, 106].

(V) Apolipoprotein E (ApoE) is a 299 amino acid protein encoded by the *APOE* gene [107]. ApoE lipoproteins bind to several cell surface receptors to deliver lipids, as well as to hydrophobic A β peptide, which is believed to initiate toxic events leading to synaptic dysfunction and neurodegeneration in AD [108]. The apoE protein is supposed to influence AD pathogenesis through a variety of actions. It influences the innate immune system, the effects of the blood-brain barrier, for the accumulation of A β and synaptic function [109]. APOE4 alleles are correlated with cholinergic dysfunction and increased amyloid burden [110]. Several studies have demonstrated the important involvement of apoE in AD. This was first suggested by Strittmatter and Roses [10], who showed that, of the three polymorphic forms of APOE, carriers of APOE4 are more liable to develop AD. Additionally, the cognitive changes in the APOE4 carriers were demonstrated to occur several years earlier, with a dose-dependent effect [86]. However, the exact influence of APOE on AD and dementia pathophysiology is unclear. ApoE isoforms differentially regulate A β aggregation and clearance in the brain, and have distinct functions in brain lipid transport, glucose metabolism, neuronal signaling, neuroinflammation and mitochondrial function [108].

3. SENSORS

According to the IUPAC definition, a chemical sensor is a device that converts chemical information about the concentration of a particular sample component to an analytically useful signal. It consists of two parts: an analytically active layer in which the intermolecular (receptor-analyte) recognition process takes place and transducer. The chemical or physicochemical signal, generated as a result of the recognition process, is converted in the transducer section into an analytical signal. The transducer is, therefore, part of the sensor responsible for converting chemical information into an analytically useful signal [111].

The sensor should generate a repeatable analytical signal in a short time and have two characteristics: selectivity and sensitivity. The first feature determines

the ability to accurately measure a particular size by omitting the influence of other parameters and the analytically active part is responsible for it. The second one determines the sensor's predisposition to measure the smallest values of the sought-after quantity. The analytically active and transducer parts of the sensor correspond to this parameter [112].

Depending on the type of analytically active material, sensors are divided into chemical sensors and biosensors. In chemical sensors, analytically active parts are synthetic molecules (receptors) that selectively recognize analyte molecules [113]. In biosensors, the biological material is an analytically active material, most often involving antibodies, isolated enzymes, natural receptors, microorganisms, tissues, organelles, DNA, RNA or whole cells [113-115].

Biosensors are usually classified according to the type of signal transmitted, type of transducer, and are divided into electrochemical, electric, acoustic, optical and thermal/calorimetric sensors [110, 113]. Electrochemical transducers have been frequently used in biosensors for the detection of AD biomarkers due to the advantages of cost-effectiveness, easy production, and user friendliness [110]. Therefore, this review concerns biosensors based on these types of transducers (Fig. 2).

Biosensors are also often classified according to the reaction type that generates the analytical signal. Analyzing this parameter, we can distinguish between catalytic biosensors (*e.g.*, enzymatic) and affinity biosensors (receptor protein reactions – analyte, antibody – antigen, hybridization reactions) [116].

The development of biosensors began in 1962, from the first mentions of the use of biosensors in the publication of Clark and Lyons [117]. The first amperometric biosensor described there was an enzyme electrode designed for glucose determination [118]. Since then, intensive research has been underway, resulting in the development of increasingly advanced, more sensitive and reliable biosensors [119].

3.1. Intermolecular Recognition

The operation of chemical sensors is based on intermolecular recognition processes occurring at the interface between the analytically active layer and the analyzed solution. The domain dealing with these issues is supramolecular chemistry, defined by one of its founders – J. M. Lehn – as chemistry of molecular aggregates and intermolecular bonds [120].

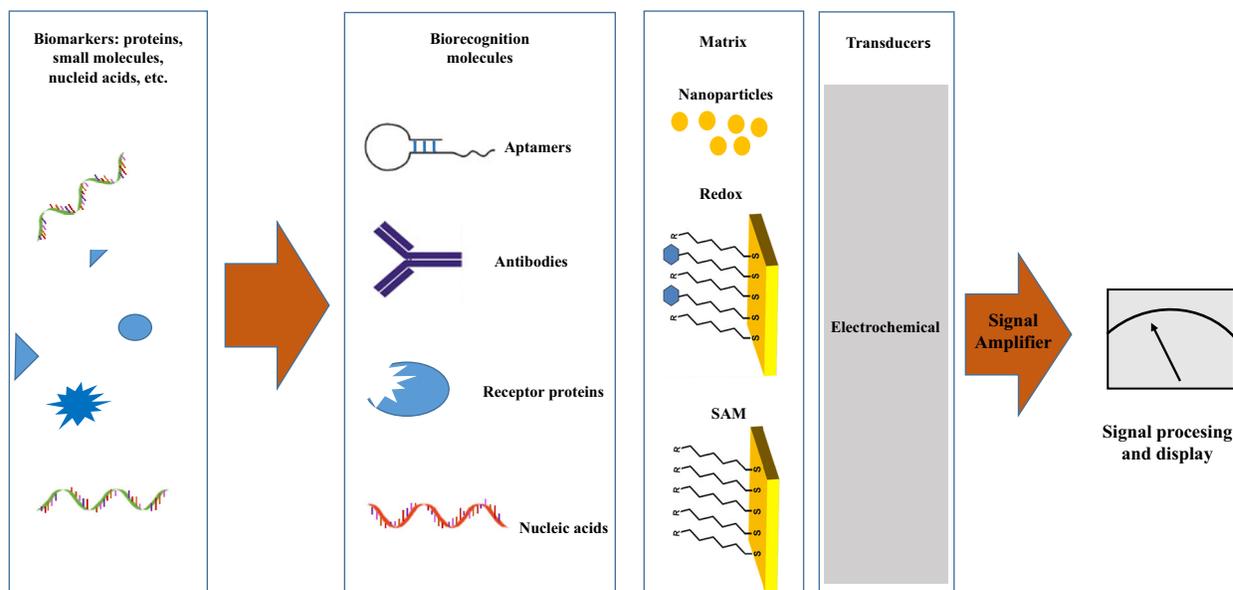


Fig. (2). Scheme of a biosensor detection strategy. Interaction between biorecognition molecule and specific biomarkers generating biorecognition signal. Transducers convert the biological recognition event into a measurable signal. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

In the studies of supramolecular systems of the “guest-host” complexes, the main areas of interest include: determination of the complex structure, type of interactions and stoichiometry of supramolecular components, as well as constant stability of supramolecular systems and kinetic parameters [121].

Most often, the “host” (receptor) is a large molecule having specific “guests” (analytics) binding sites in the structure. The role of “guest” can be played by cations, anions or more complex molecules. Selective formation of the “guest-host” complex (receptor-analyte) requires mutual complementarity of its constituting molecules, *i.e.*, achieving the appropriate geometric, spherical, energetic and electronic state. The term intermolecular recognition formulated by J. Rebek seems to be the most accurate: effective intermolecular recognition requires surfaces of complementary sizes, shapes and functions [121]. During complex formation, the analyte molecules fit into the structure of the “host” molecule as the “key to the lock”. This concept was used for the first time over a hundred years ago (in 1894) by E. Fischer and it has been functioning until now.

4. BIORECOGNITION MOLECULES

Synthetic and biological recognition molecules are at the heart of most of current bioreceptor assays. In principle, any biomolecules that have the capability of recognizing a target analyte can be used as a biorecep-

tor. Enzymes were the first recognition molecules combined in biosensor designs with broad range sensing applications. Nevertheless, other bioreceptor elements such as protein affinity systems and antibodies were introduced very shortly in the design of biosensors. Owing to the introduction of bioengineering techniques, and the complications to obtain recognition element against small size molecules, many novel biosensor recognition elements have been developed and synthesized [122]. Among the various types of biorecognition elements: receptor proteins, antibodies, aptamers and nucleic acids have been recently integrated into the biosensor designs as a popular choice for the detection of AD biomarkers, as discussed below.

4.1. Receptor Proteins

Receptor proteins are embedded in the cell membrane and span across the membrane, so that part of them is protruding out of the cell and the other part is located inside the cell. Receptor proteins are responsible for opening and closing of membrane channels for the transport of specific metabolites. These proteins are molecules characterized by a specific affinity for hormones, antibodies and other biologically active elements.

In the first years of using receptors in analytically active layers of biosensors [115], their low acquisition efficiency, relative instability, laborious isolation and long-term purification of proteins from cell mem-

branes were quite a hindrance. Despite this, receptors as recognition elements showed high affinity and binding specificity for a specific analyte. Over time, due to the development of cell recombination techniques and gene expression systems, it is now possible to obtain receptor proteins in huge quantities from the point of view of biosensors. Therefore, the receptors are successfully used as analytically active elements in the biosensitive layer. In the literature, there are several successful applications of histidine-tagged receptor proteins that act as biosensor recognition elements [123-127].

Mikula *et al.* used RAGE receptor domains to determine the presence of S100B protein in the buffer and in human plasma as well as in the presence of other potential markers for early diagnosis of Alzheimer's disease ($A\beta_{1-40}$). RAGE receptor domains were fused with poly-histidine tags. They are polypeptide elements that are introduced into the protein sequence by genetic engineering. Such a polyhistidine label consists of 2 to 10 histidines. The tag containing 6 histidines, often designated with His₆-tag or 6xHis-tag abbreviations, was patented by Roche [128]. The presence of human plasma and other AD biomarkers ($A\beta_{1-40}$) has no influence on biosensor performance. The detection limit was in the pM range and indicated that the biosensor was suitable for the determination of S100B protein in physiological samples [123, 128].

Histidine-tagged domains of RAGE receptor immobilized in biosensor monolayer were also used to determine $A\beta_{16-23}$ and $A\beta_{1-40}$ peptides [124-126]. These biosensors displayed good analytical parameters such as selectivity, sensitivity, detection limit in nM range and no effect of the human plasma matrix on the analytical signal. Considering the above arguments, the proposed biosensors could be included in the measurement tools suitable for cost-effective theranostics.

4.2. Antibodies

Antibodies (Abs), which belong to the most exquisitely designed molecules in nature, play an important role in a number of sensor devices due to their excellent target specificity and affinity. Many applications of Abs have been reported in the area of immunosensor development [129]. However, there is a huge gap between a vast number of priority analytes and a limited number of available immunosensors. Therefore, Abs engineering is a powerful tool for modifying antibodies properties. An article of Janata from 1975 is considered as the first development of an immunosensor [130]. However, it actually presents a biosensor with a receptor that is not an antibody, conc-

navalin A. Currently, immunosensors are the subject of not only many experimental articles, but also a number of reviews [131-134].

Guillozet-Bongaarts *et al.* introduced the tau-C3 monoclonal antibody specific to tau cleaved at aspartic acid 421 by caspase [135]. John Hardy *et al.* described transgenic AD mice in which $A\beta$ was significantly reduced and amyloid plaques were removed after injection of anti- $A\beta$ antibody [136]. Wang *et al.* used antibodies immobilized on the surface of a gold electrode in optimal orientation by protein G interaction as an analytically active element of an electrochemical biosensor for the detection of tau protein. The application of protein G for antibody immobilization ensured that Fab antibody binding domains were oriented away from the biosensor surface and free to react with target antigens. It increased the loading capacity of the biosensor and its sensitivity to antigens [137]. The latter biosensor was based on a microelectrode array with four gold microband electrodes and could detect the full-length of tau protein. It displayed a significantly lower quantification limit (0.03 pM) than the critical cut-off value (4.3 pM) of CSF tau protein, and this is important because recently described biosensors have a detection limit about five orders of magnitude higher than the CSF tau cut-off value, which differentiates AD cases from controls [138].

4.3. Aptamers

Aptamers are small single-stranded DNA or RNA nucleotides or peptides that exhibit high binding affinity and specificity for their targets such as metal ions and amino acids, proteins, antibodies, whole cells, bacteria or viruses [139-144]. They were first reported in 1990 by Ellington and Szostak [145], who presented RNA molecules that bind to a small organic dye. Since then, short strands of DNA or RNA that assume specific three-dimensional conformations and which are selected to target various molecules have been defined as nucleic acid aptamers. For *in vitro* selection of RNA or DNA aptamers, molecules from large populations of random sequences generated in a process known as SELEX (systematic evolution of ligands by exponential enrichment) are used. It is a combinatorial chemical technique involving the screening of specific ligands by repeated splitting and amplification rounds from a large nucleic acid library containing over a thousand different candidates [146].

Aptamers have a significant advantage in biomarker discovery over other recognition molecules due to their ability to distinguish between different modified forms and isoforms of the same protein. Moreover, it is

important that aptamers affinity can be adapted by optimizing their recognition sequence or/and manipulating binding reaction conditions, making aptamers ideal molecular recognition tools [147].

Cleavage of the amyloid precursor protein (APP) by BACE-1 (β -site APP cleaving enzyme-1) results in the production of amyloid- β ($A\beta$). Thus, this enzyme should be a valuable target for the interference of $A\beta$ production and the treatment of AD. Liang *et al.* selected A1-aptamer DNA with BACE1 binding properties of good affinity and specificity, and thus a potential of decreasing $A\beta_{40}$ and $A\beta_{42}$ production [148]. In turn, Rentmeister *et al.* selected RNA aptamers against the short cytoplasmic tail (B1-CT) of this APP cleaving enzyme from the β -site. They can specifically bind to B1-CT without affecting other essential biological activities, so that they can potentially be used to prevent or slow the onset of AD [149]. Zhou *et al.* described an electrochemical biosensor that uses $A\beta$ oligomer antibodies and a nanocomposite of gold nanoparticles with aptamer and thionine as a recognition element for determining $A\beta$ oligomers. Compared with the known $A\beta$ detection method, the aptamer-based electrochemical assay was characterized by high sensitivity due to signal amplification by high thionine load on AuNPs, and high specificity resulting from highly specific recognition of $A\beta$ oligomers with DNA aptamer antibodies. Due to these advantages, the biosensor was successfully applied to determine $A\beta$ oligomers in artificial cerebrospinal fluid samples [62]. A sensitive inkjet-printed electrochemical aptasensor has been successfully fabricated for the detection of lysozyme. Lysozyme also plays an important role as a biomarker in various disease diagnosis such as AD. Aptamers were immobilized on the working electrode in such a way that the printed ink contained the dispersion of the CNT-aptamer complex (dispersed CNT-aptamer complex). This method allows to control aptamer density as well as high-resolution patternability. The high affinity between single-stranded DNA and carbon nanotube (CNT) was used to immobilize aptamers. The biosensor was characterized by a 90 ng/mL detection limit and a reasonable shelf-life of approximately 21 days at room temperature [150].

4.4. Nucleic Acids

Nucleic acid based electrochemical biosensors have applications in diagnostics of AD. These biosensors primarily use deoxyribonucleic acid (DNA) as an oligonucleotide probe. The fundamental principle behind nucleic acid-based biosensors depends, among others, on sequence complementarity as per Chargaff's rules of

base pairing (for DNA, $A = T$, $G \equiv C$), with the exception of aptamers [151]. Additionally, the complementary strand has to be antiparallel, which is a consequence of the double helix model described by Watson and Crick [154]. The principle of aptamer based detection is more akin to antigen-antibody or receptor-ligand interactions [152, 153].

Nucleic acid based biosensors are developed by immobilization of nucleic acids (DNA, RNA, oligonucleotides) to a solid support by adsorption, covalent bonding or ionic interaction [8]. The immobilization process also aids in probe orientation and ready accessibility to target element. Hybridization based biosensors rely on the duplex formation between nucleic acids. Hybridization normally takes place between a known DNA sequence, *i.e.*, probe and an unknown counterpart *i.e.*, target DNA, but DNA-RNA and RNA-RNA hybridizations can also occur [6]. The single-strand nucleic acid can bind through electrostatic interactions, hydrophobic interactions, or their complementary shapes [151].

DNA electrochemical biosensors have been developed for the detection of apoE genotypes in PCR-amplified DNA extracted from human blood [155-157]. An electrochemical DNA biosensor using a disposable electrochemical printed chip for the detection of apoE 4 from unpurified PCR amplicons has been reported by Ahmed *et al.* [155]. Marrazza *et al.* demonstrated a DNA electrochemical biosensor for apoE genotyping coupled with PCR. This method can discriminate the six genotypes of apoE [157]. Another sensitive DNA electrochemical biosensor for detection of the apoE 4 gene has been developed by Lu *et al.* The biotinylated oligonucleotide probe with a sequence complementary to apoE 4 gene at codon 112 was immobilized on the Au electrode *via* Au-S bond. To eliminate nonspecific adsorption of the conjugates, the electrode was blocked with 6-mercaptohexanol (MCH). The immobilized biotinylated oligonucleotide probe captures complementary apoE 4 gene [158].

5. ELECTROCHEMICAL BIOSENSORS FOR AD BIOMARKERS DETECTION

5.1. Singleplex Assays

Detection of AD biomarkers has been coupled with electrochemical transducers due to their high sensitivity, specificity, ease of use and fast response to the analyte of interest. Transducers can be classified by the mode of electrochemical transduction [159]. Typically, in (bio-) electrochemistry, the test reaction either generates a measurable current (amperometric), measurable

potential or charge accumulation (potentiometric), or measurably alters the conductive properties of a medium (conductometric) between electrodes [160]. Cyclic voltammetry (CV), square wave voltammetry (SWV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) are among the most common electroanalytical techniques used for the detection of AD biomarkers [110]. Marrazza *et al.* developed new procedures for the detection of apolipoprotein E polymorphism in human blood based on coupling DNA electrochemical or piezoelectric sensors with polymerase chain reaction (PCR). The electrochemical sensor was obtained by immobilizing single-stranded oligonucleotides onto graphite screen-printed electrodes by adsorption at controlled potential [157]. Another ultrasensitive sandwich-type electrochemical immunosensor for the quantitative detection of APOE4 was designed based on fractal gold (FracAu) nanostructures and enzyme amplification. FracAu nanostructures were directly electrodeposited by hydrogen tetra-chloroaurate (HAuCl_4) on a polyelectro-

lyte modified indium tin oxide (ITO) electrode. The detection performance of the modified interface was investigated using cyclic voltammetry (CV). After functionalization with HRP-labeled APOE4 antibody, human APOE4 could be quantitatively detected based on the current response [161]. Mikula *et al.* presented an electrochemical biosensor consists a system of thiol derivative of pentetic acid (DPTA) complex with Cu(II) created on gold electrode surface for immobilization His-tagged domains of RAGE (Fig. 3). Domains of RAGE has been applied as an analytical active element for the determination of the glycosylated albumin. The analytical signals of a biosensor are generated based on the change in the electrochemical properties of the Cu(II) redox centers upon binding glycosylated albumin by His-tagged domains of RAGE. The recognition process was observed using the Osteryoung square – wave voltammetry (OSWV). The presence of 70 pM serum human albumin as well as 10 nM $\text{A}\beta_{1-40}$ and S100B protein has a slight influence on the biosensor responses [162].

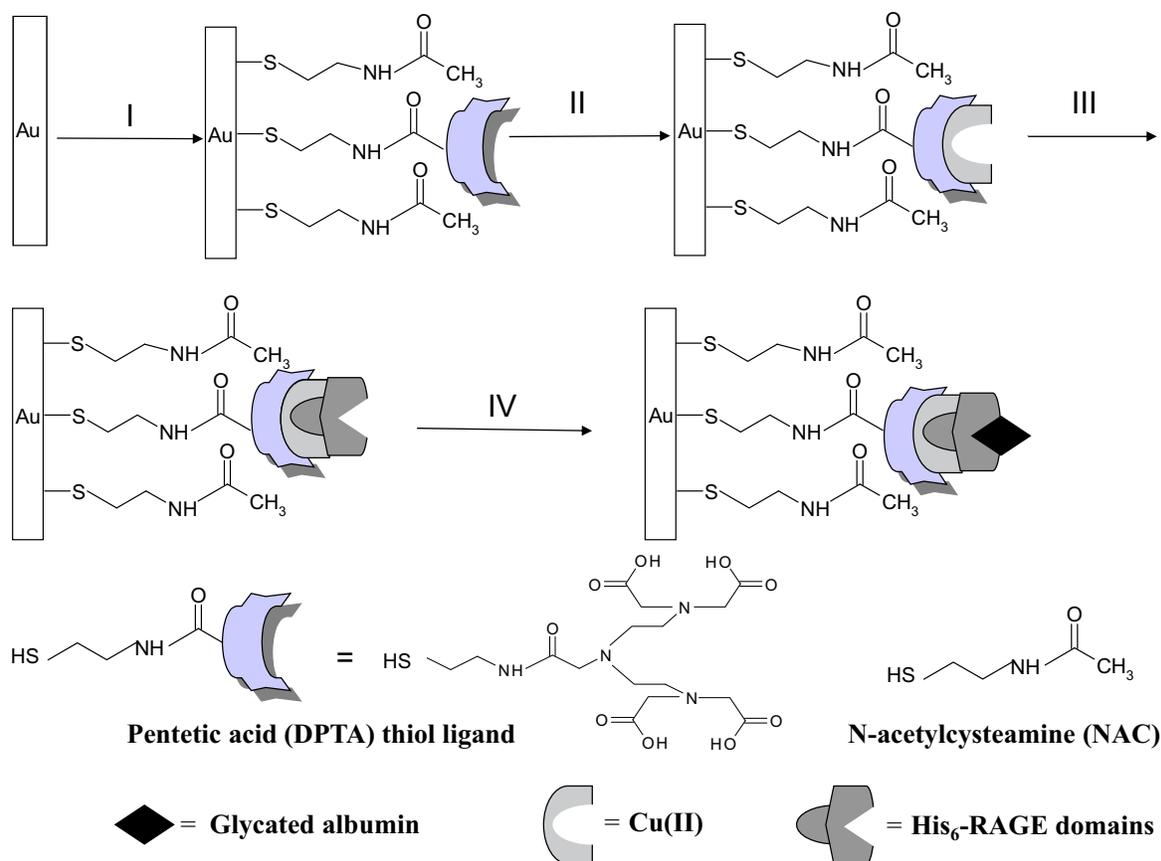


Fig. (3). Schematic representation of glycosylated albumin detection. The immobilization of His₆-RAGE domains consists of: (i) formation of a mixed layer of N-acetylcysteamine (NAC) and the thiol derivative of pentetic acid (DPTA); (ii) complexation of Cu(II) by DPTA; (iii) oriented immobilization of His₆-RAGE domains *via* coordination bonds between Cu(II) sites from the DPTA-Cu(II) complex and imidazole nitrogen atoms of a histidine tag. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

for the detection of total A β peptides. The gold nanoparticles modified with A β_{1-16} -heme were captured by a monoclonal antibody specific for the common A β N-terminus. The antibody was immobilized onto the gold electrode surface. Anchored AuNPs modified with A β_{1-16} -heme demonstrated electrocatalytic O₂ reduction. The incubation of the monoclonal antibody immobilized on the electrode surface with native A β decreased the amount of AuNPs modified with A β_{1-16} -heme linked onto the electrode, causing a decrease in the reduction current of O₂ to H₂O₂. Voltammetric responses were found to be proportional to the A β concentrations from 0.02 to 1.50 nM, and a detection limit of 10 pM was obtained [9]. Derkus *et al.* reported a novel immunosensor for the simultaneous quantification of myelin basic protein and tau protein in the cerebrospinal fluid and human serum. The immunosensor was developed based on the screen printed carbon electrode modified with graphene oxide and amine functionalized 1st generation trimethylolpropane tris[poly(propyleneglycol)] (pPG) dendrimers. A novel carrier system graphene oxide/pPG nanocomposite structure was used for the immobilization of antibodies of anti-myelin basic protein and anti-tau protein. The detection limits for immunosensor presented (0.30 nM for myelin basic protein and 0.15 nM for tau proteins) indicated its suitability for the levels required for analysis in a neuroclinic [61]. Another four-electrode electrochemical biosensor for the detection of tau protein was reported by Wang *et al.* The biosensor was based on the antibodies used as analytically active molecules immobilized on a self-assembled monolayer and protein G deposited on a gold microband electrodes surface. The assay performed by using electrochemical impedance spectroscopy was fast, very sensitive and displayed a linear response with increasing tau concentrations. The presence of human serum and bovine serum albumin has no influence on the biosensor performance. The detection limit for the full-length 2N4R tau protein was in the pM range (0.03 pM). Taking into account the above parameters, this technology could be adapted for the detection of different biomarkers to allow a multiple assay to identify AD progression in medical samples analysis [137]. Another sensitive approach utilizes the antibody-modified carbon fiber microelectrodes for *in vivo* analysis of A β concentration. Using this method, the A β concentrations in the interstitial fluid of the hippocampus of transgenic and wild-type mice with human A β were detected. The A β contained a tyrosine residue that can be oxidized at approximately 0.65 V (*vs.* Ag/AgCl) on a carbon surface. These authors used the square wave voltammetry to mea-

sure tyrosine oxidation. The antibody was immobilized on the electrode surface to achieve selectivity because there is a lot of other proteins that contain tyrosine. The authors also monitored A β half-life *in vivo*. They administered a γ -secretase inhibitor that inhibits A β generation and demonstrated that the A β levels decreased in a concentration-dependent manner after inhibitor treatment [165].

Another platform designated for electrochemical immunosensor employs indium tin oxide disposable sheets were modified using 3 cyanopropyltrimethoxysilane self-assembled monolayers as elements for precise immobilized anti-CRP antibody *via* covalent interactions without the need for any cross-linking agent. This sensitive approach allows the electrochemical detection of C-reactive protein (CRP) by analysis of charge transfer resistance changes. The proposed immunosensor was sensitive, with a detection limit of 0.455 fg mL⁻¹. Detection of CRP in human serum samples was measured by fabricated biosensor to determine the feasibility of the biosensing system for medical purposes [56]. Another miniaturized platform designated for ultrasensitive biosensor employs monoclonal amyloid-beta antibodies (mAb) as a biorecognition elements immobilized on a disc-shaped platinum/iridium (Pt/Ir) microelectrode surface. The novel approach in the modification strategy of microelectrode relied on electropolymerization by conducting free amine-containing aromatic polymer (poly (ortho-phenylenediamine) (PoPD)), followed by cross-linking with glutaraldehyde (GA) for subsequent covalent coupling of mAb on the microelectrode surface, GA created a stable 'click compound' on the microelectrode surface by covalently linking to the amine groups of hydroxylysine or lysine in the antibodies (Fig. 5). This approach improved the impedimetric detection performance of A β_{1-40} in terms of charge transfer resistance (about 400-fold difference) compared to the adsorption-based immobilization method. The real diagnostic applicability of this biosensing platform was evaluated using brain tissue lysate samples. Diagnostic performance of immunosensor was proven to be more effective than conventional ELISA in terms of lowest detection limit 4.81 pg mL⁻¹, sample volume consumption and assay simplicity [55].

Another sensitive approach utilizes the genomic DNA samples wherein enzyme-assisted electrochemical detection of apoE4 gene enables signal amplification by using ferrocene (Fc)-capped gold nanoparticles modified with streptavidin. Benefitting from amplified signal, the apoE4 gene in genomic DNAs was detected at less than 0.1 pM level. Biotinylated oligonucleotide

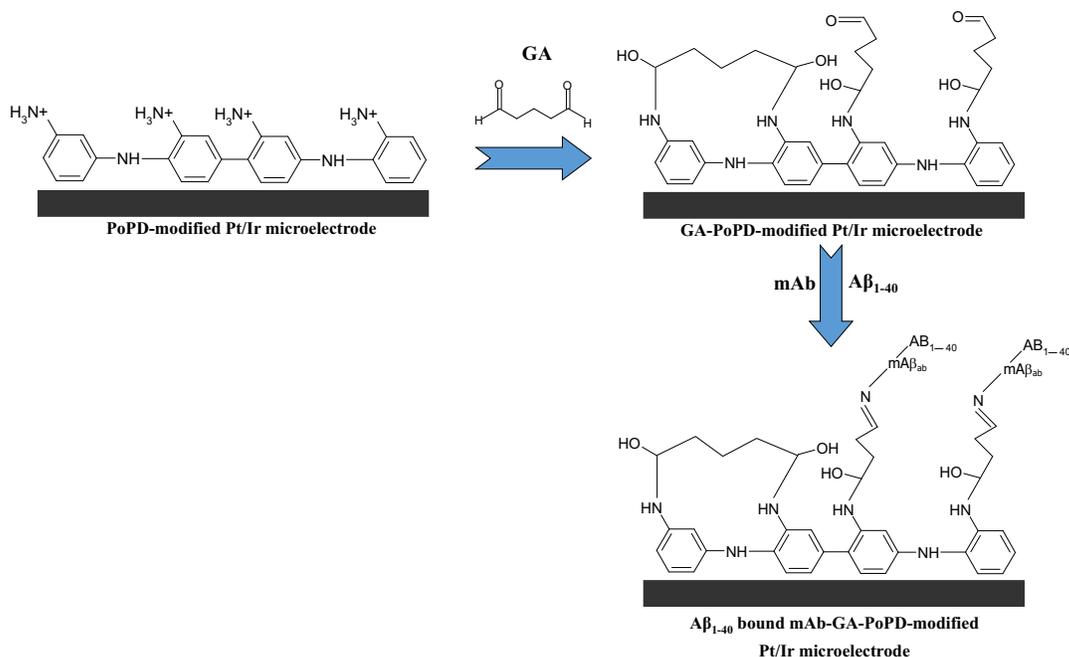


Fig. (5). Schematic representation of the reaction mechanisms occurring during the preparation of immunosensor and subsequent detection of $\text{A}\beta_{1-40}$. Adapted with permission from Ref [55]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

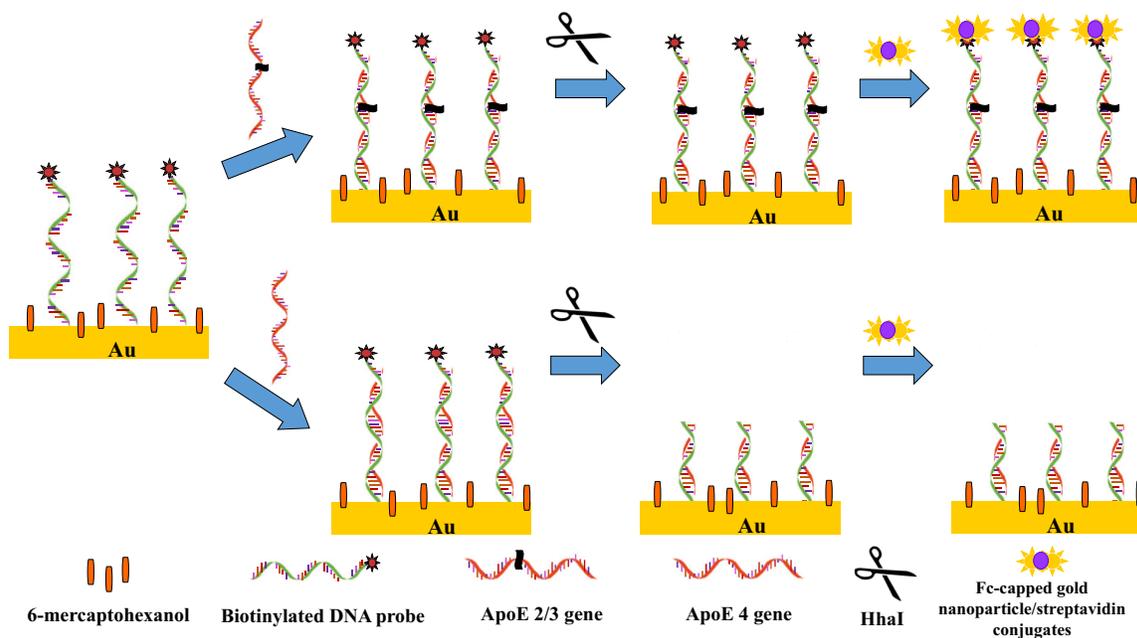


Fig. (6). Schematic of electrochemical detection of apoE 4 gene. Adapted with permission from Ref. [158]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

probes capture complementary apoE 4 gene immobilized on the gold electrodes surface. This is followed by hybridization with apoE 4 gene at codon 112 or apoE 2/3 gene with a single base mismatch relative to apoE 4 gene, enzymatic cleavage by restriction enzyme HhaI and then attachment of ferrocene (Fc) – capped

gold nanoparticle modified with streptavidin (Fig. 6). Cleavage only occurs at the complementary apoE 4 duplex, therefore this sequence can be discriminated against other apoE sequences [158].

Another extremely sensitive electrochemical aptasensor method for the quantitation of amyloid beta

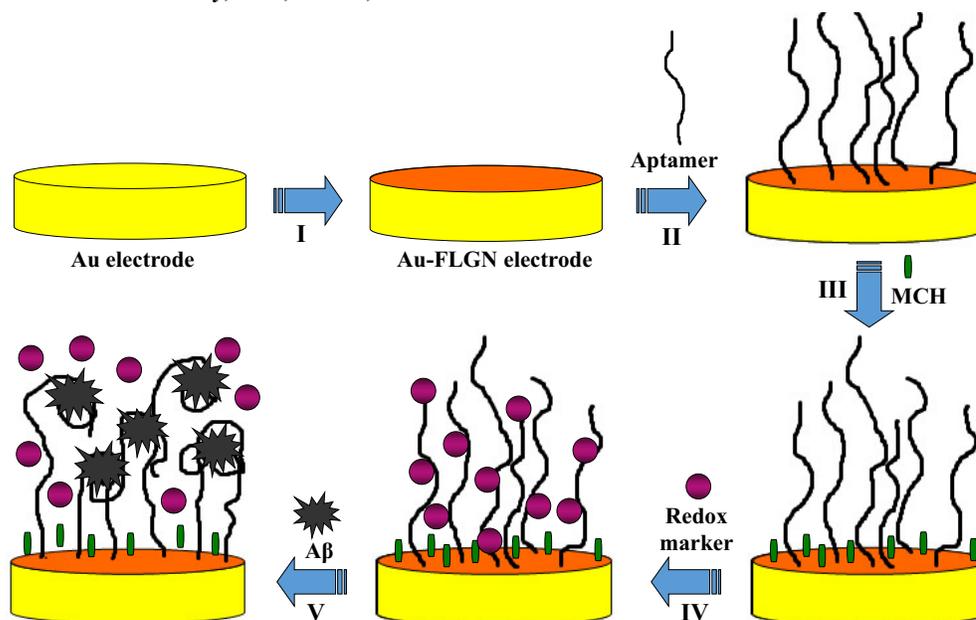


Fig. (7). Fabrication steps of the aptasensor and A β determination: I– electrodeposition of fern leaves-like gold nanostructure (FLGN), II– aptamer immobilization, III– 6-mercaptohexanol (MCH) immobilization, IV– A β incubation. Adapted with permission from Ref. [166]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Specifications of electrochemical biosensors for the detection of single AD biomarkers.

AD Biomarker	Biorecognition Molecules	Techniques	Linear Range of Detection	Detection Limit	Refs.
A β oligomers	Protein-binding peptide	SWV	0.5 – 20 nM	0.048 nM	[168]
	Cellular prion protein (residues 95-110)	EIS	0.5 – 100 pM	0.5 pM	[169]
	Cellular prion protein	EIS	0.1 pM – 10 nM	0.1 pM	[170]
A β ₁₋₄₀	mA β ab	EIS	1–104 pg/ml	4.81 pg/ml	[55]
A β ₁₋₄₂	Specific RNA aptamer	DPV	0.002–1.28 ng/ml	0.4 pg/ml	[166]
	A β ₁₋₄₂ -binding peptide	DPV	3–7000 pg /ml	0.2 pg/ml	[167]
	Anti-A β ₁₋₄₂	CV	0.5 – 500 ng/ml	0.1 ng/ml	[171]
Total A β	Anti mA β antibody	EIS	2.65 nM–2.04 μ M	0.57 nM	[172]
S100B protein	His ₆ -RAGE domain	SWV	1 – 20 pM	0.52 pM	[128]
	Monoclonal anti-S100B	DPV	0.1 – 100 pg/ml	0.1 pg/ml	[173]
	His ₆ -RAGE domain	SWV	1 – 20 pM	0.9 pM	[123]
Glycated albumin	His ₆ -RAGE domain	SWV	1– 20 pM	2.3 pM	[162]
APOE4	HRP-labelled APOE4 antibody	CV	1 – 10 ng/ml	0.3 ng/ml	[161]
Tau-441	Oriented antibodies 39E10	CV, EIS	0.01 pM-10nM	0.03 pM	[137]
	Tau protein	CV, EIS	0.2 – 1.0 μ M	0.2 μ M	[138]
	Anti-tau antibody	EIS, DPV	0.5 – 15.1 nM	0.15 nM	[61]

(A β) was reported recently. This approach revealed immobilization of a specific RNA aptamer on the gold nanostructure (synthesized by electrodeposition using polyethylene glycol) (Fig. 7). Binding of A β peptide to specific RNA aptamer was detected by ferro/ferricyanide redox marker. The applicability of the devel-

oped aptasensor was tested in the real samples (human blood serum and artificial cerebrospinal fluid) for the demonstration of its viability [166].

Another platform designated for peptide-based biosensor employs irregular shaped microporous gold nanostructure electrodeposited on a polycrystalline

gold surface using sodium alendronate. A β_{1-42} -binding peptide was immobilized on the gold nanostructure by using a specific peptide sequence that creates a strong bond with the surface of a microporous gold nanostructure through the thiol group of its cysteine residue. In selecting this peptide sequence, a high specificity to capture A β_{1-42} was considered. Ferro/ferricyanide redox probe was employed as a redox marker to electrochemically follow the binding of A β_{1-42} by the peptide. The applicability of biosensors for the quantitation of A β_{1-42} was tested in spiked serum samples and artificial cerebrospinal fluid. The presented electrochemical biosensor is free of interferences and able to detect A β_{1-42} with a detection limit of 0.2 pg mL⁻¹ [167]. It is critically important to diagnose AD at the early stages of its progression, which allows successful treatment and recovery of patients. Therefore, it is essential to develop simple and sensitive diagnostic methods that can detect AD biomarkers at very low concentrations in biological fluids. Some of the recently reported biosensors are able to determine AD biomarkers with remarkable sensitivity (the values of detection limit at pM) (Table 2).

5.2. Multiplex Assays

It is imperative to deviate from the detection of single AD biomarker and develop a diagnosis methodology of multiplex biomarker detection to reflect the complexity of AD pathogenesis. Simultaneous detection of two or more markers achieves higher sensitivity than one marker to distinguish Alzheimer's disease patients and others, indicating that each marker may be complementary and not redundant with the other for an accurate diagnosis. Measurement of different analytes in a single sample from individual patients in parallel appears to considerably improve the accuracy of AD diagnosis [174].

Simultaneous detection of a series of clinically relevant protein biomarkers is indispensable for clinical applications. However, the levels of different biomarkers may cover an expansive range; a biosensor for such markers requires not only high sensitivity but also a broad detection range [175]. For instance, the cut-off levels of A β_{1-42} , t-tau and p-tau181 are 530 pg/mL (117.4 pM), 350 pg/mL (7.6 pM) and 80 pg/mL (1.7 pM) [176]. Hence, although many biosensors for specific biomarkers have been reported, the development of multiplex detection of AD biomarkers remains strongly limited.

Simultaneous sensing of multiple specific biomarkers can be achieved through either multi-label or multi-

-electrode approaches. In multi-label systems, a single electrode is used for the detection of various biomarkers. On the other hand, multiple sensing areas are used to detect multiple biomarkers in multi-electrode systems [177]. The designing of a biosensor that is capable of simultaneous determination of two or more analytes in a single measurement is a great challenge. The following examples of electrochemical biosensor for multiple AD biomarker detection will be presented herein.

In the first paper, the novel ratiometric electrochemical biosensor for the dual determination of copper ions (Cu²⁺) and A β_{1-42} based on a 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and poly(diallyldimethylammonium chloride) (PDDA)-bi functionalized single-walled carbon nanotubes (CNTs) composite with the detection limits of 0.04 μ M for Cu²⁺ and 0.5 ng mL⁻¹ for A β_{1-42} , respectively, was presented by Yu *et al.* [178]. Neurokinin B (NKB) immobilized on the ABTS-PDDA/CNTs modified electrode surface was used as specific recognition of Cu²⁺ element by forming a [Cu₁₁(NKB)₂] complex with Cu²⁺. The modified electrode also generated the electrochemical signal toward the A β_{1-42} monomer, when a certain amount of the monomer was added to Cu²⁺-contained PBS buffer, due to the release of copper ions from the complex through A β binding to Cu²⁺. Consequently, the designed electrochemical approach was capable of monitoring two important biological species: copper ions, which are directly involved in A β aggregation and A β_{1-42} ; by one single biosensor in plasma and hippocampus of normal and AD rats [178].

Recently, the simultaneous determination of four AD biomarkers on one microchip has been presented by Song *et al.* [179]. Sensitive and selective detection of multiple biomarkers including Tau, ApoE4, Amyloid- β and miRNA-101 on mini-pillar-based biosensor that confines the reagent in open-channel microreactors for simultaneously sensing multiple biomarkers is achieved. Such a mini-pillar sensor mainly consists of a mini-pillar array with an electrode array for anchoring the droplets to electrochemical signal acquisition and circuit integration unit for aggregation of multiple signals (Fig. 8).

The mechanism of proteinaceous biomarkers, including Tau, ApoE 4 and Amyloid- β sensing, is based on electrode resistance change with the electrochemical signals fluctuations (Fig. 9a). After immobilization of antibody and block protein on gold nanodendrites working electrode, the initial signal is recorded. The antibody-antigen specific recognition causes the increases of surface resistance and signal decrease. The

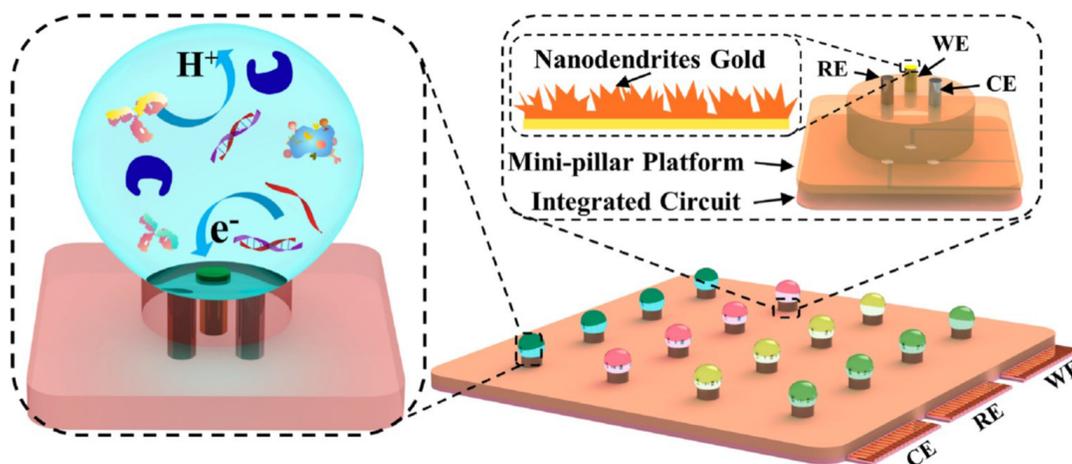


Fig. (8). The open-channel mini-pillar sensor for multiple electrochemical detection. Reprinted with permission from Ref [179]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

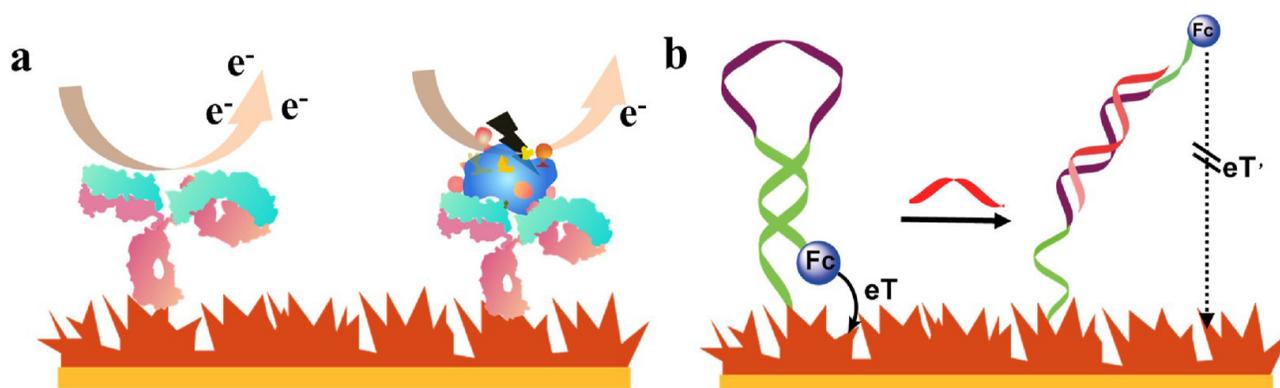


Fig. (9). Electrochemical array toward multiple and simultaneous AD biomarkers detection. **a)** The mechanism of electrochemical detection of proteinaceous biomarkers. **b)** The mechanism of electrochemical detection of miRNA-101. Reprinted with permission from Ref [179]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

gold nanodendrites are obtained by electrodeposition onto the working electrode and caused enhanced sensitivity *via* improving probe-binding capacity and response signal.

The detection of miRNA-101 is based on altering the positioning of the reporter (Fc) relative to the electrode surface (thereby producing a target-dependent change in current) during the combining of miRNA-101 to redox-reporter-modified hairpin DNA probe immobilized on working electrode by Au-S bond. All target biomarkers concentrations are determined by using the signal fluctuations (Fig. 9b) [179].

CONCLUSION AND FUTURE DIRECTIONS

With the increasing aging population, the number of people with AD is set to rise. The need is now

greater than ever to develop technologies for the rapid, sensitive, reliable and cost-effective methods for AD biomarkers detection based on new analytical technologies. In this review, we have presented a snapshot of the recent developments in this field and an overview of the pace at which electrochemical biosensors have sought to address this gap. Moving forward, there are definitely challenges that must be addressed, for the field to continue its growth momentum.

The important strategy of development is the capability for integration of analytical technologies on a single platform. The development of electrochemical biosensors that allow for simultaneous detection of multiple biomarkers of an AD can achieve higher detection sensitivity while reducing false positives. This can be achieved by the simultaneous use of biorecogni-

tion molecules with different specificities equipped with appropriate redox active labels, so that many AD biomarkers could be detected in parallel. However, there are still problems arising from mutual interference or overlapping of the electrochemical signal. This challenge can be achieved by finding appropriate redox active labels which differ in electrochemical behavior when attached to biorecognition molecules on the same electrode platform.

Another challenge is to improve the sensitivity and specificity of biosensors and make them affordable and accessible at the point of care. In recent years, nanomaterials have been widely used in biosensing as one of the candidates for further sensitivity actions in the development of highly sensitive devices for early diagnosis and point-of-care applications. New advances in nanomaterials, nanofabrication technologies and biomimetic surfaces can all be further explored for developing biosensors that continue to push the boundaries of AD biomarkers detection.

There is a need for transition from development stages towards commercialization of biosensors for point-of-care diagnostics of AD. This is especially important for biosensors where detection limits and the sensitivity achieved are several orders of magnitude better than physiologically and diagnostically relevant levels. By driving the development of fabrication techniques of biosensors, through product development, other practical aspects, including costs and portability, could also be examined.

LIST OF ABBREVIATIONS

mAb	= Monoclonal Antibody
mA β b	= Monoclonal amyloid-beta antibodies
HRP	= Horse Reddish Peroxidase
AuNPs	= Gold Nanoparticles
SAM	= Self Assembled Monolayer
DPTA	= Thiol Derivative of Pentetic Acid
His ₆ -RAGE	= His-tagged domains of Receptor for Advanced Glycation End products
A β	= Amyloid beta
MCH	= 6-mercaptohexanol
Fc	= Ferrocene
MS	= Mass Spectrometry
MRI	= Magnetic Resonance Imaging

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

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