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miRNAs and their Applications in Neurogenerative Diseases Detection: An Electrochemical Approach

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ABSTRACT

MicroRNAs (miRNAs) are extensively studied noncoding and conserved post-transcriptional gene regulators in the genome. Since their discovery in 1993, miRNAs have gained significant interest due to their involvement in numerous biological processes. In addition, their altered expression levels in various diseases have led to the study of their potential use as biological biomarkers in several pathologies, including neurodegenerative pathologies. Neurodegenerative diseases (NDs) encompass a diverse group of pathologies of diverse etiology, but they share common features and mechanisms leading to neuron destruction and death. The conventional diagnosis of ND involves a comprehensive clinical examination, consideration of the patient's medical history, neuroimaging and MRI techniques, and clinical laboratory tests. However, most ND symptoms become evident up to 10-15 years after disease onset. This highlights the need for early biomarkers and rapid diagnostic platforms that are sensitive and enable periodic preventive controls in people with risk factors. This mini review addresses the challenges in the clinical diagnosis of neurodegenerative diseases and explores the potential of miRNAs as early biomarkers for detecting ND. In addition, the development of electrochemical biosensors and bioassays for specific targets are presented in this revision.

Keywords: Neurodegenerative Diseases; Biomarker; Point-of-Care; Micrornas; Biosensor

Abbreviations: NDs: Neurodegenerative Diseases; WHO: World Health Organization; AD: Alzheimer Diseases; CSF: Cerebrospinal Fluid; RNA: Ribonucleic Acid; PD: Parkinson's Disease; PCR: Polymerase Chain Reaction; PoC: Point-of-Care; SPE: Screen-Printed Electrode; CSF: Cerebrospinal Fluid; GoX Graphene Oxide; SWCNTs: Single-Walled Carbon Nanotubes

Neurodegenerative Diseases

Neurodegenerative diseases (NDs) are characterized by the gradual degeneration and destruction of motoneurons. Neurodegenerative diseases involve processes such as protein aggregation and/or degradation, oxidative stress, mitochondrial dysfunction, and bioenergetic damage [1]. Conformational changes in protein sequences lead to the accumulation of misfolded proteins both inside and outside of the cells. The most relevant NDs include Alzheimer's disease (characterized by abnormal tau protein phosphorylation), Parkinson's disease (marked by intraneuronal aggregates of α-synuclein), Lewy body dementia and Huntington´s disease [2]. Gradual neuronal degeneration leads to a loss of functionality and personal independence, making caregivers and medical attention necessary. The impact of NDs is substantial, with global healthcare costs exceeding hundreds of billions of dollars annually, according to the World Health Organization (WHO) [3]. Expenses include social and healthcare costs, as well as the loss income for affected individuals and their caregivers [4,5]. Due to their high social and economic impact, the WHO has declared dementia, predominantly caused by Alzheimer's, [6] as a priority disease in the Mental Health Action Programme, and the European Parliament has

also emphasized the fight against Alzheimer diseases (AD) through the Declaration on priorities in the fight against Alzheimer's disease (2010/C 76 E/17) [7].

The prognosis for NDs is complex due to factors such as age, family history, and genetic or environmental influences. Diagnosis typically involves a comprehensive clinical examination, medical history assessment, neuroimaging (MRI, PET scans), genetic analysis for ND-related mutations, and cerebrospinal fluid (CSF) tests [7] which require lumbar puncture. However, early diagnosis is challenging as initial clinical symptoms often manifest 10-15 years after the onset of anatomophysiological changes [8,9]. Currently no single test can accurately identify most of these neurodegenerative diseases. Moreover, postmortem examination by immunohistochemistry is often necessary for their correct diagnosis [10]. Early diagnosis is critical to slow their progression. It was demonstrated that several pathologies, such as cardiovascular diseases, cancer or NDs, exhibit altered levels of miRNAs, which can remain stable in blood, CSF, urine, or saliva. Thus, these molecules hold promise as potential early biomarkers. Identifying ND-related miRNAs could guide the development of new detection strategies for these small nucleic acids in biological fluids, enabling less invasive and more specific diagnostic methods for NDs. Therefore, the level of specific miRNAs is of great clinical relevance, encouraging the development of new detection strategies for small nucleic acids in biological fluids and favoring their use as early biomarkers by using less invasive and more specific methods for ND diagnosis (see Figure 1).

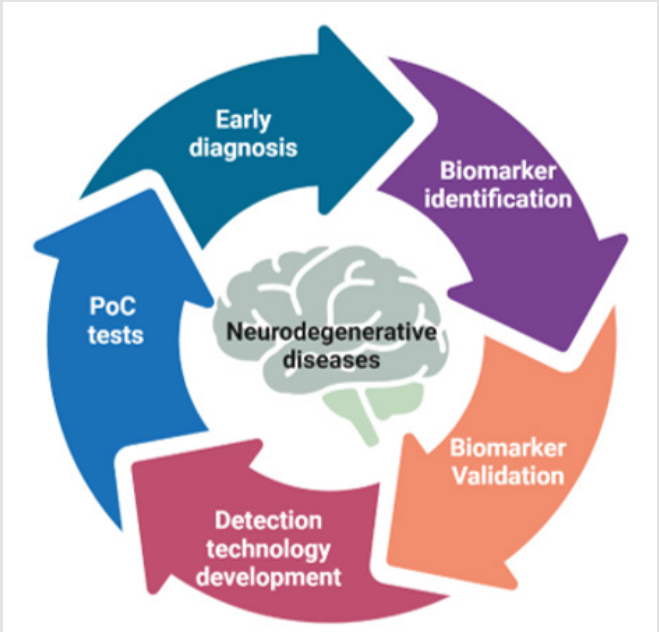

Figure 1: Scheme of the main elements necessary for early neurodegenerative disease diagnosis.

The miRNAs

MicroRNAs (miRNAs) are short fragments of noncoding ribonucleic acid (RNA), approximately 18-25 nucleotides in length that play a role in gene expression regulation at the post-transcriptional level. They were first described in the late 20th century during studies on developmental regulation in the nematode Caenorhabditis elegans. [11] These small RNA molecules are found in multicellular organisms and are involved in regulating physiological events such as cell proliferation, differentiation or apoptosis by inhibiting the translation of target messenger RNA (mRNA) molecules. This regulatory mechanism primarily involves binding to mRNA sequences, hindering the translation mechanism and preventing protein formation. [12] miRNAs are highly conserved across species and are typically located in intragenic regions of the genome, mostly within introns and less frequently in exons. The main characteristic of miRNAs is that they are derived from endogenous precursors, exhibit phylogenetic conservation and do not encode protein products. miRNAs play crucial roles in embryonic development and tissue differentiation, exerting control at the post-transcriptional level in almost all cell types [13]. This characteristic, along with the discovery of altered miRNA expression profiles in various diseases, has led to their investigation as potential biomarkers. In 2008, it was shown by Mitchell et al. that miRNAs are not limited to the intracellular environment, since they are present in the bloodstream [14]. Later,

their presence in other body fluids, such as saliva, tears, urine, serum and cerebrospinal fluid (CSF), was also reported. These circulating miRNAs are protected from degradation by ribonucleases in the extracellular environment through their binding to lipoprotein or their incorporation into vesicles, rendering them highly stable under adverse conditions [14]. However, isolation procedures are usually necessary prior to their identification and study.

Circulating miRNAs as Disease Biomarkers

In recent years, alterations in miRNA patterns at the cellular/tissue level, specifically associated with pathological processes such as inflammation or neurological disorders, have been reported [15]. These unique expression profiles do not depend on race, age or sex, making miRNAs excellent candidates as diagnostic biomarkers [15,16]. Due to their stability in body fluids and the specific expression profiles that can reflect the status of specific organs or conditions, circulating miRNAs have been studied as ND biomarkers. For example, a study by Khoo SK et al. in 2009 demonstrated the efficacy of circulating miRNAs as biomarkers for Parkinson's disease (PD) [15]. The study recruited patients diagnosed with idiopathic PD from a Health Care Parkinson's Center, as well as healthy individuals. Peripheral blood samples were collected to isolate total RNA and microRNA. This work identified nine predictive Parkinson classifier pairs and 13 miRNAs with altered expression levels in patients [15] compared to healthy controls. For the predictive Parkinson classifier pair, the interpretation was as follows: pair (miR-1307/miR-632) "If miR-1307 expression is higher than miR-632 expression, the patient is predicted to have PD; otherwise, the patient is predicted to be a healthy control".

MicroRNA Detection Procedure

The detection of miRNAs as biomarkers in biological fluids requires accurate, sensitive, reproducible and multiplexed methods [17]. In general, the choice of method will depend on the quantity and quality of the biological sample. The detection process comprises three stages: isolation, amplification, and detection. The isolation of miRNA from the biological matrix is crucial. This involves pretreatment of the sample, using chaotropic agents, detergents and/or organic solvents to promote the lysis of lipidic vesicles and lipoproteins [17]. In addition, centrifugation or solid-phase extraction through affinity columns is also necessary to remove particulate components and debris from the sample. Due to their low abundance, miRNA purification could necessitate the use of amplification methods to facilitate their detection. Polymerase chain reaction (PCR) is the most widely used amplification method to detect and amplify low-abundance nucleic acids. Conventional PCR amplifies a specific segment by repeated thermal cycles of denaturation, annealing and extension. In the case of RNA templates, a reverse transcription step is added to convert RNA to DNA (RT–PCR protocol). In the last decade, several powerful isothermal amplification strategies have been developed for the sensitive detection of miRNAs. The isothermal amplification technique replicates nucleic acids at a constant temperature, eliminating the need for thermal cyclers and making them more cost-effective and simpler to implement in point-of-care (PoC) tests. These techniques include a variety of methods in which different combinations of enzymes or primer designs are involved in their amplification protocol [18].

However, amplification techniques need to be integrated into detection platforms to enable the correct identification of specific miRNA sequences. Standard methods for miRNA detection are based on gel electrophoresis and molecular hybridization with fluorescence probes [19]. However, from a medical standpoint, the urgent need for current diagnostic devices is not only focused on high sensitivity but also on portable and simple-use devices that can be performed at PoC. Biosensor technology has the potential to speed up detection and to increase specificity and sensitivity. Moreover, they may enable high-throughput analysis and may be used for early diagnosis. Today, they are replacing other more sophisticated techniques, and they will be an important tool in healthcare applications.

Electrochemical Biosensors for ND Diagnosis

A biosensor is an electronic device used to transform a biological interaction into an electrical signal. This device is based on the direct spatial coupling of the immobilized biologically active element, the so-called "bioreceptor", with a transducer that acts as a detector and electronic amplifier [20]. Electrochemical transduction offers the advantages of high sensitivity, which can be enhanced by attaching biocatalytic labels to bioreceptor-target complexes to amplify the detection signal, is readily miniaturized, and has a low cost of production since it does not require expensive instrumentation for read-out (Figure 2) [21]. Although several alternatives have been proposed and different miRNA detection strategies have been reported, the development of sensor platforms for the diagnosis of neurodegenerative diseases is limited. The initial electrochemical approaches were based on enzyme-linked magneto-immunoassays, as proposed by Erdem et al. in 2013 [22]. They used a screen-printed electrode (SPE) array combined with streptavidin-magnetic particles as a solid support to immobilize biotinylated specific capture probes to miR-15a. For detection, an alkaline phosphatase-streptavidin conjugate was used. Other examples include the multiplexed detection of three different miRNAs (miRNA-15a, miRNA-16, and miRNA-660), all AD related, using voltammetry readout [23]. The same research group has also developed voltammetric and impedimetric sensors for the detection of miRNA-34a, another AD biomarker [24,25]. They modified pencil graphite electrodes with graphene oxide (GOx). While the sensor showed good selectivity for miRNA-34a, the detection limits were moderate. Other electrochemical strategies,

such as electrochemical impedance spectroscopy using molybdenum disulfide nanosheet-modified electrodes, have been developed with better performance. However, this methodology has been developed for cancer biomarkers [26].

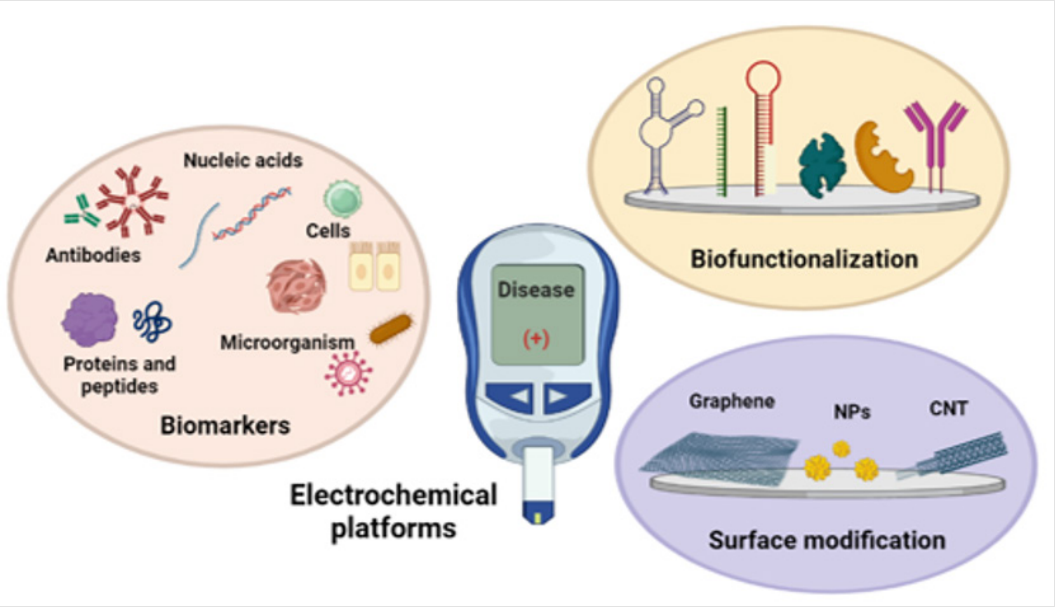

Figure 2: Schematic representation of biosensor components and its applicability.

The incorporation of nanomaterials such as graphene, graphene oxides, and gold nanostructures in the development of diagnostic platforms, especially in the fabrication of electrochemical biosensors, has been shown to be highly attractive for improving the sensitivity of the methods. Liu et al. fabricated triangular electrodes by combining nanomaterials such as GOx and gold nanostars conjugated with capture probes for the detection of miRNA-137 as an Alzheimer's biomarker [27]. The obtained results have demonstrated detection limits on the order of 10 fM. Another approach for the same target was reported by Naderi-Manesh's group. They developed an electrochemical nanobiosensor for ultrasensitive detection of miR-137 using electrodes modified with gold nanowires, electrochemically reduced GOx, and doxorubicin to enhance the sensitivity to the fM range. [28] This system, tested in human serum, has revealed its potential as a diagnostic tool for Alzheimer's disease. Another example is SPE modification with gold nanoparticles and methylene blue-labeled anti-miRNA-29a probes, reported by Miglione et al. [29]. These electrodes were used for square wave voltammetry measurement for miRNA-29a detection, a class of miRNAs known to regulate the pathogenesis of AD. This sensor was evaluated in serum and buffer, showing limited detection limits.

Other sensing designs, in this case for miR-137 and miR-142 detection (AD biomarkers), have reported a fluorescence nanobiosensor coupled with isothermal amplification of miRNAs by hybridization chain reaction [30]. The levels of these miRNAs were quantified using SYBR green as a fluorescent marker and graphene oxide (GoX) as a fluorescence quencher. Fluorescence intensity was used to quantify the miRNA levels based on the creation of hybridization events when the target miRNA was present in a serum sample. In the same way, a label-free electrochemical nanobiosensor was used for the detection of miR-155, a biomarker for multiple sclerosis. The high sensitivity of the nanobiosensor was also based on electrode modification techniques. In this case, single-walled carbon nanotubes (SWCNTs) and polypyrrole nanocomposites were used on a graphite sheet substrate to enhance bioreceptor immobilization performance [31]. To our knowledge, although microfluidic electrochemical platforms (μPADs) or colorimetric paper-based sensors have been developed for miRNA detection, [26-33] none of them have been designed as biomarkers of neurodegenerative diseases.

All the platforms mentioned before could be capable of giving quick answers to patients, reducing the time gap between diagnosis/treatment and disease onset. Currently, the main limitation is the lack of validation with real samples since most of them were tested in buffer of artificial spiked samples.

Conclusion

Currently, there is an urgent demand for new diagnostic tools for point-of-care applications. Moreover, the early detection of neurodegenerative diseases such as Alzheimer's and Parkinson's, among others, may reduce the devastating effects on families and patients, including increasing their quality of life and extending their life expectancy. Such diseases are often detected in the last stages, when visual and evident cognitive effects are observed. However, some biochemical changes and protein expression in the patients may be detected approximately 10-15 years before the first symptoms of the disease appear. The development of novel point-of-care tests for early diagnosis needs a multidisciplinary approach where several elements are necessary: biomarkers, sample matrix, readout systems, isolation and amplification strategies, clinical studies, etc. Here, we showed the importance of microRNAs and their potential application in the early diagnosis of certain neurodegenerative diseases. Moreover, the use of electrochemical biosensors and biosensors may be a useful tool due to their advantages (low cost, miniaturization, user-friendly instrumentation). Finally, more relevant studies combining different electrochemical strategies, such as those used on magnetic beads and amplification and labeling techniques, were presented.

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