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Enzyme-based biosensors: emerging tools for advanced biomedical applications

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ABSTRACT

Proteins and enzymes are fundamental elements for the correct regulation of cell metabolism and their dysregulation or malfunction can lead to the onset of several pathologies. Altered enzymes can participate in tumour formation and progression, but they can also take part in neurodegenerative and inflammatory diseases. Enzyme-based biosensor can become important tools for disease monitoring and drug screening in a faster and cost-effective manner. This work shows the importance of these molecules, giving particular attention to their role in cancer development and neurodegenerative diseases. After the introduction many examples of enzyme immobilization techniques and detection methods are presented, showing most recent advances and innovations. After these general aspects of enzyme-based biosensor, several applicative systems for the detection of specific molecules and drug screening are presented with the intention to show the versatility of these devices. In conclusion, possible future developments of enzymatic biosensors are described, including the synergy with artificial intelligence system and the first studies of self-powered devices, describing the cutting-edge technologies of this field and new possible applications.

1. Introduction

Enzymes and proteins in general are essential elements of our body, because they have fundamental roles in cell metabolism and proliferation. Several new pathways and factors involved in the development of diseases have been recently discovered and particular attention is placed on them. This is due to the fact that their alteration or dysregulation can lead to the development of the pathologies. Enzymes are particularly involved in diseases which the cellular metabolism is accelerated, like cancer.

Another class of diseases in which they are involved is inflammatory diseases, in which enzymes and proteins take part in the activation of the immune system leading to an overreaction. As can be seen from [Table 1](#) there are different inflammatory responses due to specific enzymes or receptor in various body districts. Usually, these diseases are chronic and targeting these specific proteins could be effective.

The most relevant examples of enzyme dysregulation in cancer (HKII, PKF, PKM2, LDH-A), neurodegeneration (BACE1, GLUT1, KGDHC) and inflammation (NLRP3, NF- κ B, COX-2) are here further describes and summarized in [Table 1](#).

Cancerous cells are characterized by high consumption of glucose via the anaerobic glycolysis process [1–3]. This leads to an increase of the enzymes required for that type of metabolism, like hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK).

HK catalyses the first step of the glycolytic pathway where glucose is phosphorylated to glucose-6-phosphate (G-6-P), essential to allow the cell to produce energy [3]. There are four isoforms of HK, from HK I to HK IV, that have a different affinity to glucose [3]. In normal tissues isoform II is very little expressed, instead in tumours it is highly present [1,3]. Therefore, inhibiting this enzyme may reduce cancer progression [1,3].

PFK catalyses the phosphorylation of fructose-6-phosphate (F6P) into fructose-1,6-bisphosphate (F1,6BP) [2]. There are three different isoforms of this enzyme and the overexpressed ones depend on the type of tumour [2,4]. For example, PFK-L is present in human mammary tumour [2], instead PFK-P is associated to breast carcinoma, hepatocellular carcinoma, prostate cancer, glioblastoma, and leukaemia [4]. The inhibition of PFK activity can slow down tumour progression [2,4].

PK catalyses the direct transfer of phosphate from phosphoenolpyruvate (PEP) to ADP to produce ATP and pyruvate [5]. PK presents four

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Table 1
Diseases related to enzymes and proteins alteration.

Disease	Type	Enzyme	Dysfunction	Mechanism of action	Ref.
Cancer	General	Hexokinase isoform II (HK II)	Overexpression	Metabolic enzyme essential for the glycolytic process. The isoform II is present in high quantities in cancer tissue.	[1,3]
	General	Phosphofructokinase isoforms L and P (PFK-L/PFK-P)	Overexpression	Enzyme of the glycolytic process. The isoform majorly expressed depend on the tumour type.	[2,4]
	General	Pyruvate kinase isoform M2 (PKM2)	Overexpression	Enzyme of the glycolytic pathway. The isoform M2 is overexpressed in multiple cancer types.	[5,6]
	General	Lactate dehydrogenase isoform A (LDH-A)	Overexpression	LDH oxidate NADH to NAD ⁺ with the conversion of pyruvate into lactate.	[7–9]
	General	Phosphoinositide 3-kinases class I (PI3K)	Overactivation	In multiple cancers is more present the isoform A. PI3K/AKT/mTOR is a pathway that promotes cell proliferation. Its dysregulation can lead to cancer.	[10]
	Colorectal and gastric	Phosphoglycerate mutase 5 (PGAM5)	Overexpression	PGAM5 is involved in mitochondrial homeostasis. In high levels it promotes gastric and colorectal cancer proliferation.	[11,12]
	Colorectal and pancreatic	Protein arginine N-methyltransferase 1 (PRMT1)	Overexpression	PRMT1 increases chromatin accessibility and promotes gene transcription.	[40,41]
	Prostate	Protease-activated receptor (PAR)	Overexpression	PAR are transmembrane proteins involved in multiple cellular processes involved in cancer progression. They are activated by proteases, highly present in the prostate.	[42,43]
	Prostate	Partition defective 3 (Par3)	Overexpression	Pars are proteins essential for cell polarity. Their dislocation is related to tumorigenesis.	[44]
	Prostate	Protein arginine N-methyltransferase 5 (PRMT5)	Overexpression	PRMT5 control histone methylation and gene transcription. High levels of PRMT5 repress miRNAs that target tumour promoting genes.	[45,46]
	Oral squamous cell carcinoma	Tumour necrosis factor alpha-induced protein 2 (TNFAIP2)	Overexpression	TNFAIP2 promotes inflammation and cell proliferation promoting the NF- κ B pathway. The NF- κ B promotes tumour invasion and migration.	[47,48]
Neurodegenerative diseases	Oral	Matrix metalloproteinases 2 and 9 (MMP-2, MMP-9)	Overexpression	Class of enzymes involved in the extracellular matrix turnover. High levels of MMPs promote cancer diffusion.	[13,14]
	Alzheimer disease	β -amyloid (A β)	Accumulation	A β is a protein fragment. A β accumulation could be related to changes in its precursor metabolism.	[16–18]
	Alzheimer disease	Glucose transporter 1 (GLUT1)	Activity decrease	GLUT1 is a membrane channel that allow the glucose to enter in the neurons. Its reduction could increase neurons death.	[19,20]
Inflammatory	General	α -ketoglutarate dehydrogenase complex (KGDHC)	Activity decrease	KGDHC is a rate-limiting enzyme of the tricarboxylic acid cycle. Reactive oxygen species can influence the activity of KGDHC and have an impact in neurodegenerative diseases.	[21,22]
	Chronic obstructive pulmonary disease	Bromodomain-containing protein 4 (BRD4)	Overexpression	BRD4 regulates transcription of genes. It promotes macrophage polarization and increases inflammatory response.	[49]
	Blau syndrome	Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)	Activity increase	NF- κ B is a transcription factor. Its activation increases inflammatory response.	[50–52]
Coronary remodelling	Neovascular retinal disease	C-chemokine receptor type 2 (CCR2)	Upregulation	CCR2 is a receptor responsible of macrophages activation and inflammatory response.	[53–55]
	Inflammatory bowel disease	NOD-like receptor family pyrin domain containing protein 3 (NLRP3)	Excessive expression, aberrant activation or polymorphism	NLRP3 is a pattern recognition receptor activated by a variety of stimuli and its overactivation lead to inflammatory and chronic diseases.	[23,56,57]
Cystic fibrosis		Cyclooxygenase isoform 2 (COX-2)	Overexpression	COX-2 in an enzyme involved in the metabolic pathway of arachidonic acid (AA). High levels of AA can lead to heart diseases.	[28]
		Cystic fibrosis transmembrane conductance regulator (CFTR)	Channel dysfunction	CFTR is channel expressed on the apical side of airway epithelial cells. Its dysfunction causes airway surface dehydration and compromises the airway microenvironment.	[26,27,58]
Small intestine disease		Sodium-glucose cotransporter 1 (SGLT1)	Channel upregulation	SGLT1 is a glucose transporter. Its inhibition can enhance mitophagy and improve cell homeostasis.	[24,25]
Cells ferroptosis		Glutathione peroxidase 4 (GPX4)	Under expression	GPX4 is an enzyme involved in redox reactions.	[29,30]

(continued on next page)

isoforms and in cancerous tissues is more present the isoform M2 [5,6]. The presence of PKM2 promotes tumour growth and suppresses cell apoptosis [6]. Targeting PKM2 with some small molecules has been used in preclinical studies to interfere with tumour growth [6].

In tumours tissues, there is also high presence of lactate dehydrogenase (LDH). LDH catalyses the transformation of pyruvate to lactate with the oxidation of NADH to NAD⁺ [7]. In tumours it is more expressed the isoform A of LDH [7,8]. The high levels of LDHA allow the cells to sustain high consumption of glucose even in hypoxic conditions [7]. However, even in normal conditions tumour cells transform the pyruvate into lactate with the Warburg effect, allowing a faster production of ATP [7,9]. Targeting and inhibiting LDHA could be a strategy to treat cancer [7,8]. It can also be used as a biomarker for enzyme-based biosensors to monitor the development of the disease in real-time.

Cell over proliferation is not only due to a rapid metabolism but can also depend on other pathways. Phosphoinositide 3-kinases class I (PI3K), involved in the PI3K/AKT/mTOR (PAM) pathway, promotes cell survival, growth and proliferation [10]. In most human cancers a precursor of the PI3K is mutated and this accelerates carcinogenesis and cancer progression via PAM pathway hyperactivation [10]. Inhibiting PI3K seems to have a beneficial effect on treating cancer [10]. Mutated PI3K can become a target to develop biosensor capable of monitoring oncogenic pathways.

Enzymes are involved in most common tumours, interesting the gastrointestinal tract, the prostate and the mouth. In all this cases there is a cellular over proliferation due to enzymes upregulation, like in the case of phosphoglycerate mutase 5 (PGAM5). In cellular stress situations PGAM5 exits the mitochondria, binds to other proteins in the cytosol and promotes epithelial cells growth [11]. This can lead to the development of certain tumours, like the colorectal cancer [11]. In gastric cancer was seen that PGAM5 also promoted the PI3K/AKT pathway [12]. Therefore, inhibiting this enzyme could become a strategy to control colorectal and gastric cancer pathogenesis [11,12].

To note the role of matrix metalloproteinases (MMPs) in oral cancer. MMPs are a class of enzymes involved in the extracellular matrix and basement membrane degradation [13]. They are essential for normal tissue turnover, but in high levels they can facilitate cancer diffusion and formation of metastasis [14]. Inhibiting this type of enzyme reduces the invasion of oral cancer cells [14].

MMPs are not only involved in the diffusion of oral cancer, but also in other oral diseases, like periodontitis [13]. Indeed, high levels of MMPs, in particular MMP-1, MMP-8 and MMP-9, bring to periodontal tissue destruction [13]. The most active one is MMP-1 that breaks collagen fibers reducing the integrity of the periodontal tissue [15]. Inhibiting MMP-1 could be a possible therapy for this disease [15].

In neurodegenerative diseases are involved various proteins. In Alzheimer's disease there is an accumulation of β -amyloid peptide ($A\beta$) [16,17]. $A\beta$ comes from the amyloid precursor proteins (APP), proteins that are cleaved by different enzymes [16]. If the reaction is performed by a β -secretase, it will produce $A\beta$ [16,17]. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is a β -secretase enzyme and it's essential to produce $A\beta$ [17,18]. The inhibition of BACE1 could represent a therapeutic approach for Alzheimer's disease [18]. BACE1 can also be used as biomarker for enzymatic biosensors in early diagnosis of neurodegenerative diseases related to $A\beta$ accumulation.

Alzheimer's disease is not only related to $A\beta$ accumulation, but also

to other proteins dysregulation, like the glucose transporter 1 (GLUT1) [19]. GLUT1 is a transmembrane transporter that facilitates the diffusion of glucose and other saccharides into neurons and it is less expressed in this disease [20]. GLUT1 is also related to the accumulation of $A\beta$ in the brain [19]. Therefore, GLUT1 could be an important target for the treatment of Alzheimer's disease [19].

In various neurodegenerative diseases there is cellular activity reduction that could be related to α -ketoglutarate dehydrogenase complex (KGDHC) [21,22]. KGDHC is a mitochondrial enzyme that regulates the cellular metabolism in the brain [22]. Reduction of KGDHC reduces the ability of the cells to diminish reactive oxygen species [21]. Therapies that increase its amount may have positive effects on these diseases [21].

In inflammatory bowel diseases (IBD) the development of the pathology is usually related to NOD-like receptor family pyrin domain containing protein 3 (NLRP3) activation [23]. When NLRP3 is in the active form, it promotes inflammatory pathways that can lead to IBD [23]. Targeting the NLRP3 inflammasome could have promising effects on treating IBD [23]. NLRP3 has also specific activation for IBD, therefore can be used as a biomarker in biosensors for an early detection of the disease.

The intestine could also be affected by glucose absorption related pathologies. Sodium-glucose cotransporter 1 (SGLT1), among other transporters, regulates the absorption of glucose in the intestine [24]. SGLT1 inhibition can have a positive effect in recovery from radiation-induced intestinal injury (RIII), that usually involves patients who sustain radiotherapy [25]. The inhibition of SGLT1 promotes mitophagy and mitochondrial homeostasis via inhibiting the PI3K/AKT/mTOR pathway [25]. Therefore, SGLT1 is a promising therapeutic target for RIII [25].

Another disease that involves the dysregulation of a channel is the cystic fibrosis, in which the cystic fibrosis transmembrane conductance regulator (CFTR) has an aberrant behaviour [26]. CFTR is located in the apical part of airway epithelial cells. Its activity defects result in altered ions transport that causes dehydration and hypersecretion of mucus leading to airway obstruction [27]. CFTR could be a promising target for cystic fibrosis treatments [26,27].

The dysregulated activity of some enzymes could also lead to heart diseases. For example, the overexpression of cyclooxygenase-2 (COX-2), due to high levels of arachidonic acid, could bring to coronary remodelling [28]. The pathway that determines the onset of the disease is complex and several proteins could be targeted [28]. Measuring the levels of COX-2 with an enzyme-based biosensor could be an effective strategy to have a continuous monitoring of the risk to develop heart diseases.

Sometimes the disease is due to an under activation of an enzyme, like for ferroptosis. Ferroptosis is a type of cellular death due to the rupture of the cytoplasmic membrane [29]. Glutathione peroxidase 4 (GPX4) seems to influence ferroptosis reducing the lipid oxidation and increasing membranes stability [29,30]. Therefore, its activation reduces cells death, but its selective inhibition could promote cellular death and GPX4 could become a target for cancer therapies [31,32].

Not only the management of these diseases is complex, but also their diagnosis reveals to be quite difficult to perform in a sustainable way.

Many types of cancer are detected only at an advanced stage, when treatment options are limited and prognosis is poor [33]. Only less than

Table 1 (continued)

Disease	Type	Enzyme	Dysfunction	Mechanism of action	Ref.
Periodontitis		Matrix metalloproteinases 1, 8 and 9 (MMP-1, MMP-8, MMP-9)	Overexpression	It stabilizes cells membranes and its inhibition can cause cells death. Class of enzymes involved in the extracellular matrix turnover. High levels of MMPs causes periodontal tissue destruction.	[13,15]

50 % of cancers are detected in an early stage and established screening approaches are present only for few cancers type, like cervical, breast and colorectal cancer [33]. This screening usually involves the acquisition of images with different techniques. The most used ones are x-rays, magnetic resonance imaging (MRI) or positron emission tomography (PET), that are not invasive techniques but are time consuming, expensive and will miss many of the smaller tumours [33]. A promising alternative is to identify small molecules associated with the development of cancer, like circulating tumour DNA (ctDNA) [33]. But the detection of these biomarkers in blood is complicated, especially in the early stages of the pathology, by the fact that they are present at extremely low concentrations [33].

Also for neurodegenerative diseases, the diagnosis is done by imaging techniques, that are not suitable for conducting screening tests because the acquisition is too expensive [34]. For these diseases, especially Alzheimer's one, the diagnosis is usually done only after the appearance of the first symptoms and first lesions in the brain [35]. Usually at this stage the available treatments are useless to patients [34]. An alternative possibility could be the analysis of the cerebrospinal fluid for the detection of A β [34]. Several techniques have been developed, like enzyme-linked immunosorbent assay (ELISA), mass spectroscopy, electron microscopy and others, but they are costly, time consuming or lack in sensitivity [34].

Several widely used analytical techniques also present well-documented limitations. For example, ELISA and other immunoassays can suffer from cross-reactivity, variability between batches, reduced sensitivity for low-abundance analytes and long assay times due to multiple incubation and washing steps [37,38]. Imaging-based diagnostics such as MRI, PET or CT, while essential in clinical practice, often fail to detect small or early-stage lesions and require high-cost infrastructure and specialized personnel [36]. Compared to these approaches, enzyme-based biosensors provide rapid analysis, require minimal sample preparation and can achieve high sensitivity and specificity through the intrinsic selectivity of the enzyme.

The ELISA test is also used in inflammatory disease to evaluate the presence of specific molecules [39]. It is widely used for the high quantitative and easily reproducible results [39]. But it also has some drawbacks, indeed it requires high levels of biological material, usually not available, and the dynamic range is narrow compared to other technologies [39].

To partially solve the diagnosis problems, a biosensor could be used. A biosensor is normally composed by three parts: a biological recognition element, a transducer and a signal processor [59]. The biological recognition element interacts with the target molecules specifically and the transducer converts the biochemical information into a measurable signal. The signal is read by the signal processor [59]. For the bio-recognition element, different types of molecules could be used, such as antibodies, nucleic acids and enzymes [59]. Enzymes are the most used due to their selectivity and specificity [59,60]. In addition, enzyme biosensors could find applications in a variety of fields [60]. The signal produced by the enzyme can then be transduced in different ways. The most diffused are electrochemical and optical transduction [59,61]. In electrochemical detection an electrical quantity, like current (amperometric detection), charge (potentiometric detection) or impedance (impedentiometric detection), is measured [59,61]. For the optical detection, absorbance, reflectance or fluorescence are the main quantities detected [59,61]. The best technique to use depends on the application and on the enzyme selected.

Biosensors are spreading relatively fast due to the advantages they bring. They have high sensitivity and specificity, but in a miniature device easy to use [62]. Enzyme based biosensors offer a rapid, extra-laboratory analysis with substantial decrease of cost per sample and can be used for real-time diagnosis [63,64]. They also bring advantages in the analysis of complex biological fluids, like blood [62]. Moreover, they play an essential role in early detection of diseases [61]. For these reasons, these devices are widely used in health-care

applications and could find relevant usage in point of care diagnostic.

In the context of biosensing, enzymes can play two complementary roles. They may function as biomarkers to be detected, when their altered expression or catalytic activity reflects a pathological condition, or they may serve as functional biorecognition elements, where their catalytic specificity enables the detection of other analytes such as metabolites, nucleic acids or proteins. The specific role of the enzyme depends on the sensing strategy and on the biomedical question addressed.

The aim of this study is to give a general view of the state of the art, recent development, application and future prospective of enzyme-based biosensor. It underlines their use in the diagnostic field and in disease monitoring, but also their role in the development of new drugs and therapies for human diseases, showing their potential and critical issues. First the principles of enzyme immobilization and the main transduction mechanisms are presented, followed by representative applications grouped by pathological area. In each case the specific role played by the enzyme within the sensing system is highlighted. In conclusion it describes new frontiers of enzymatic biosensors and in which direction they are evolving, including possible future applications. In all sections, particularly attention is placed on the innovations, trying to put on display the cutting-edge technologies of this field.

2. Enzymatic biosensors: base principles and technological innovations

In the construction of an enzymatic biosensors there are two main aspects that need to be considered: the enzyme immobilization and the detection method. They are fundamental for the proper functioning of the system and are not independent from each other: based on the detection method some immobilization techniques work better and vice versa. These two aspects will be described in the following paragraphs.

2.1. Enzyme immobilization

To ensure stability, durability and consistency of the system is fundamental to confine the enzyme in a specific region of the biosensor and avoid its dispersion [59,61]. To do so there are different techniques that can be adopted. The most appropriate one depends on a lot of factors, like the structure of the enzyme, the substrate that supports the enzyme, the operating environment and the detection method used [61]. The different techniques can be divided in two main categories, depending on the nature of the interactions that confine the enzyme. The first possibility is to use physical forces to confine the enzymes on the substrate of interest. In this category there are mainly three possible techniques that are adsorption, confinement and entrapment. The other possibility is to immobilize the enzyme with covalent bounds and there are two principal methodologies: cross-linking and covalent bonding. These techniques are illustrated in a schematic way in Fig. 1 and are described in more details below.

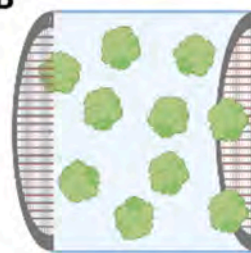
- Adsorption (Fig. 1-A): the enzyme molecules are adsorbed directly onto the support. This technique is the simplest one, the enzyme doesn't resent of the immobilization and its activity is high [65]. But the interaction is only based on weak bonds, like hydrogen bond and Van der Waals forces, and therefore the molecules can desorb easily. This implies that the stability of the system is quite low [65].
- Confinement (Fig. 1-B): the enzyme is confined between semi-permeable membranes. The membranes have a porosity that allow the passage of the substrate while avoiding the exit of the enzyme [61]. This technique is one of the oldest, but it is still promising. There are different commercially available membranes in different materials and with different pores dimensions [65]. They can be made of both natural and synthetic polymers. The confinement could also be done using liposomes (Fig. 2-B), that are made by biological membranes [65–67]. The advantage of this technique is that the enzyme is not bounded to anything, therefore its activity is

Physical

A



B

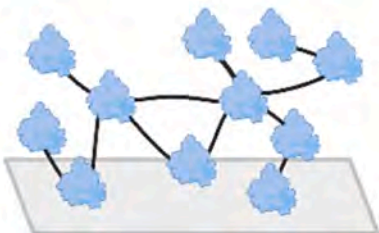


C



Chemical

D



E

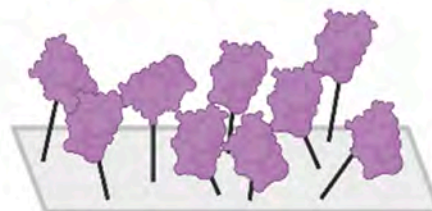


Fig. 1. Physical immobilization by adsorption (A), confinement (B), entrapment (C); chemical immobilization by cross-linking (D), covalent bonding (E).

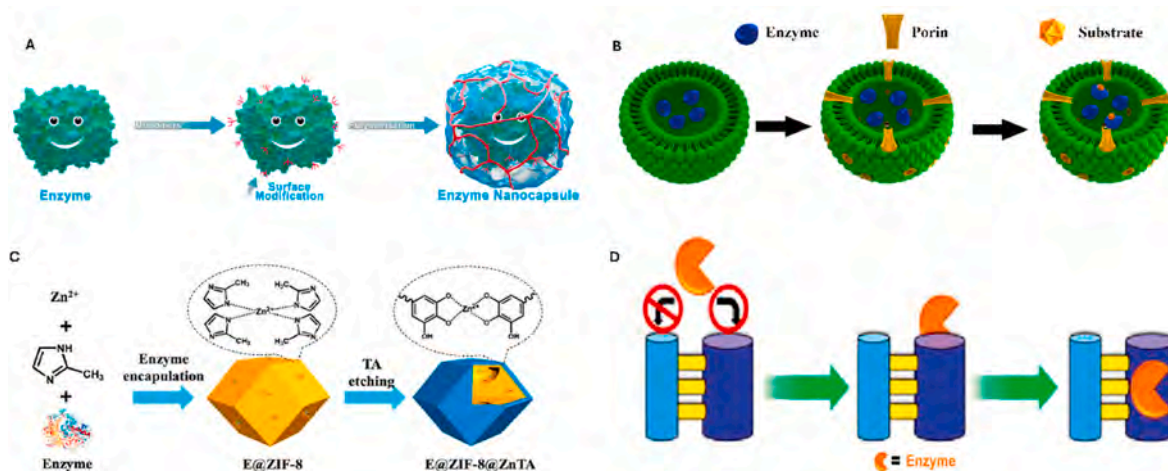


Fig. 2. Different methods of enzymatic confinement: creation of a polymeric nano capsule around the enzyme (A) [66], confinement of the enzyme using liposomes (B) [64], MOFs synthesis with enzyme to entrap it into the porosity of the material (C) [67], diffusion of enzymes into already synthesised MOFs (D) [68].

unaffected [65]. The enzyme is also in a favourable environment, thanks to the high presence of water, that protect it from denaturation and deactivation [68]. It has also some drawbacks, indeed encapsulating more catalytic proteins in the same membrane could lead to aggregation and reduction of activity. This problem can be solved with more complex procedures in which is possible to do single enzyme entrapment [68,69]. It could be done with polymers or with metal-organic frameworks (MOFs). Using polymers, the membrane is created by polymerization of the monomer around the enzyme itself (Fig. 2-A) [69]. MOFs, instead, are more ordered porous structures and allow a higher control over the enzyme distribution after the immobilization. For the confinement, their synthesis is done in a solution containing the enzyme (Fig. 2-C) [68]. This strategy has some limitations, because the procedure must be done in mild conditions to avoid the denaturation of the enzyme. Usually, MOFs are synthesised in organic solvents and therefore the available ones for this type of immobilization are quite low [68].

With MOFs is also possible to immobilize the enzymes by diffusion into the structure with a hierarchical porosity (Fig. 2-D) [68].

- In this case there are no restraints on the synthesis, because it is done separately from the immobilization. During the immobilization the enzyme diffuses only in the bigger pores and channels, while the substrate can also diffuse in the smaller ones (Fig. 3-A, B). Moreover, the larger size of channels and bridge windows (Fig. 3-C, D) facilitated the diffusion of substrate and coenzyme and left enough space for the mutual recognition of the immobilized enzyme and coenzyme [68]. The main disadvantage of this technique is the mass transfer through the device, because it is limited by the presence of the porous structure. It is also possible that on the surface of the cage could grow some other microorganism [65].
- Entrapment (Fig. 1-C): the enzyme is locked between polymer chains of substrate or membrane, without covalently bonding to them [65]. One problem of entrapment is the loss of enzyme activity due conditions used in the process [61,65]. To avoid the issue is possible to do the entrapment in hydrogel matrices. This offers a

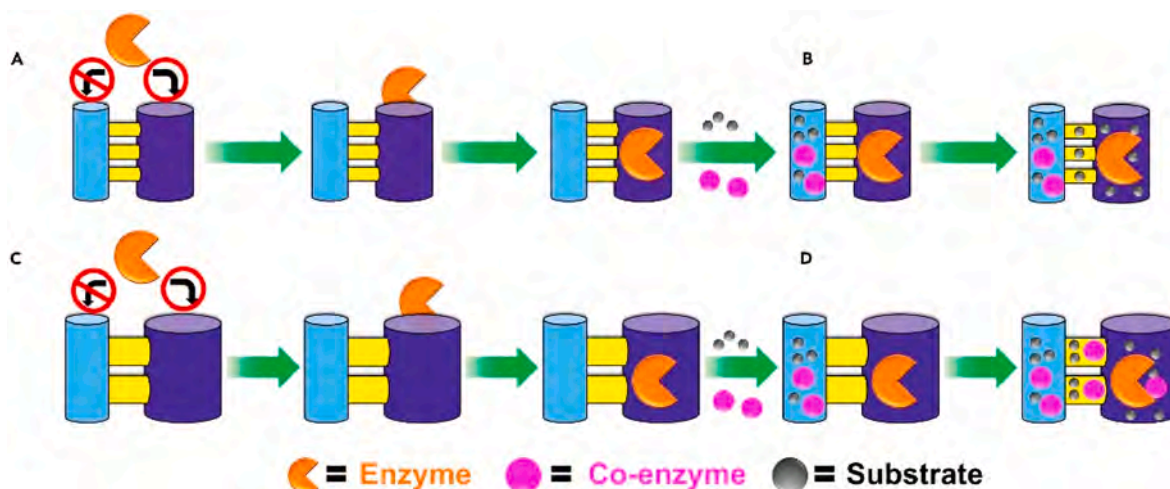


Fig. 3. Enzyme diffusion into porous structure. Narrow channels and windows (A) show slow diffusion rates of substrate and coenzymes and limited space for enzyme-coenzyme recognition (B); wide channels and windows (C) show fast diffusion rates of substrate and coenzymes and sufficient space for enzyme-coenzyme recognition (D) [67].

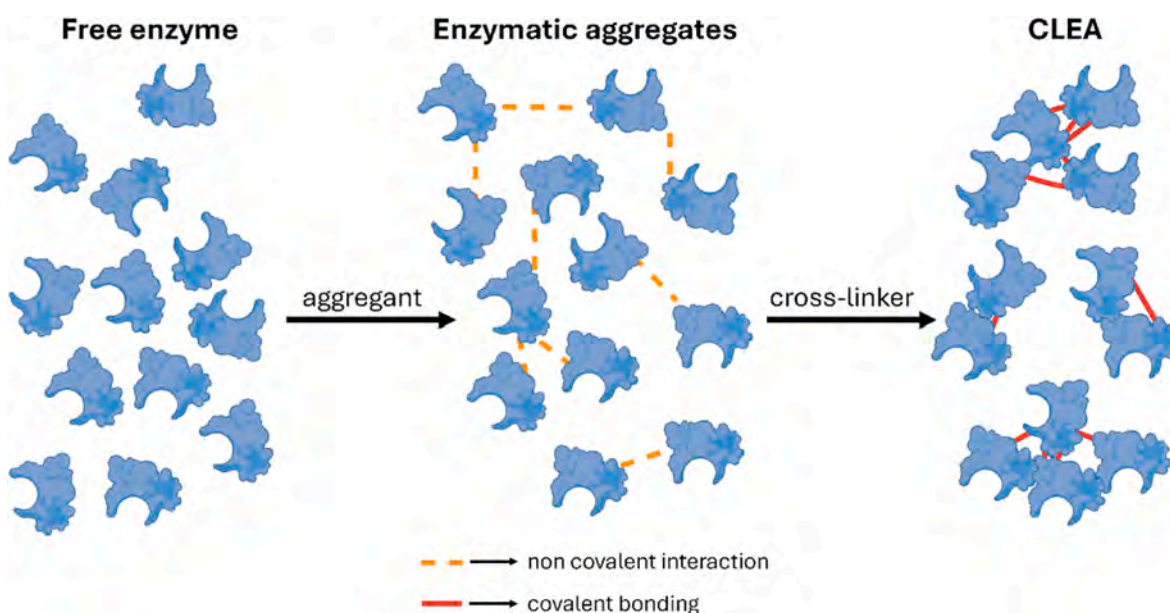


Fig. 4. Enzyme aggregation to form CLEAs.

suitable environment for the enzyme, that can retain its activity [70, 71]. The obtained system is also stable and could be used for high number of measurements [70,71]. The main issue of using hydrogels is that in aqueous environment they undergo swelling, the spaces between the chains increases and the possibility of the enzyme to escape is higher, leading to a reduction of the stability of the system.

- Cross-linking (Fig. 1-D): the enzyme creates covalent bonds with other enzymes or the matrix [61,65]. The most common used cross-linker is glutaraldehyde, that is cheap and a great cross-linker for commercial scale operations [65]. This method stabilizes adsorbed molecules and increases attachment and limiting their desorption [61]. Some drawbacks of crosslinking are the reduction of enzyme's activity, low reproducibility and low mechanical stability [65]. The reduction of activity is due to the covalent bonding that can limit the capacity of the enzyme to catalyse the reaction. The low reproducibility is due to the random interactions between different molecules, resulting in a different network each time. These problems could be overcome by more complex techniques like

cross-linked enzyme aggregates (CLEAs, Fig. 4). In CLEAs the enzymes aggregates are created by addition of salts, organic solvents or non-ionic polymers at the enzyme solution [65,72,73]. In the aggregates the enzymes are held together by non-covalent bonding without perturbation of their tertiary structure [73]. The aggregate precipitation technique is also used to purify proteins and CLEA methodology combine purification and immobilization in a single operation [73]. After the precipitation the aggregates are made permanently water insoluble by covalent crosslinking [73]. Then the aggregates are fixed to the substrate. This methodology has higher costs, but it guarantees a high catalytic activity compared to other techniques [65,73].

- Covalent bonding (Fig. 1-E): the enzyme is covalently bonded to the solid carrier and there is no leakage of the enzyme even in harsh conditions [61,65]. The bonding method depends on the enzyme and on the substrate used and could involve different functional groups. It is also necessary to do the reactions in mild conditions to avoid enzyme denaturation. This technique allows high stability, but there

is a loss of activity of the enzyme [61,65]. This is probably due to the binding of groups of the active site or the increase of the rigidity of the enzyme.

From the above description is clear that the immobilization method can influence the activity of the enzyme and the stability of the system. The systems that use a covalent immobilization, i.e. cross-linking and covalent bonding, generally present a higher stability and the system can be used for a longer period of time. Indeed, the ones that are based on physical interactions give more freedom to the enzyme allowing for a higher activity, but there is an increase in the probability to lose the enzyme itself. In general, at the increasing of the stability there is a reduction of the enzymatic activity. Therefore, it's fundamental to find an equilibrium between these two aspects.

2.2. Detection methods

Enzyme-based biosensors take advantage from the specificity of the reaction that involves the enzymes to detect specific molecules. The biological information produced by the reaction is converted by the transducer into a visualizable signal, usually an electric or an optic quantity.

2.2.1. Electrical detection

There are several techniques that uses electric quantities for the detections. In the following paragraphs, the most common one will be briefly described.

2.2.1.1. Amperometric. Amperometric biosensors work on the principle of change in current according to the change in chemical concentration of the molecule of interest [61]. There are a lot of different structures for amperometric enzyme-based biosensor, but in general they can be divided in three generations (Fig. 5) according to the pathway of electron transfer during the biochemical reaction [59].

In the first generation (Fig. 5-A) the electric signal is caused by the generation of H_2O_2 in the reaction catalysed by the enzyme [59]. At the electrode H_2O_2 is converted into water and oxygen. This generation has some drawbacks, such as electroactive interference and high redox potential for the redox indicator [59].

In the second-generation of biosensors (Fig. 5-B), H_2O_2 is replaced by electron transfer mediator. It serves as electron carriers, which promote electrons transfer from the redox active centres of the enzyme to the electrode [59]. This can reduce the redox potential at the electrode and the influence of interfering substances [59]. Usually, the electron transfer is a cofactor involved in the reaction. One of the most used for dehydrogenase-based biosensors is nicotinamide adenine dinucleotide (NADH) [59]. NADH is reduced or oxidated at the electrode site.

The biosensors of the third generation (Fig. 5-C) rely on the direct electron communication of the enzymatic redox centre and the electrode [59]. In this case the electrons involved in the reaction are directly transferred to the working electrode and detected. The contact of the redox enzyme with the conductive electrode is essential to facilitate electron exchange [74]. The detected current depends also on the

accessibility of the active site. To facilitate fast electron transfer, a monolayer of the enzyme is preferred due to the proximal distance to the electrode [74]. To facilitate even more the electron transfer, usually the enzyme is covalently bonded to the electrode and the electrode itself is nanostructured with conductive nanoparticles, like carbon nanotubes and metallic nanoparticles [74]. The most used metallic nanoparticles are based on gold or silver. The electrode modification is fundamental to increase the electroactive surface and improve electron exchange, increasing the measured current. The materials for the electrode nano-structuring are not subject of this review, but more information can be found in Refs. [75–77].

In amperometric detection is usually used a three electrodes setup with working, reference and counter electrode. The working electrode is the one responsible of the detection and is modified with the nanoparticles previously mentioned to improve the response. The reference electrode is usually an Ag/AgCl electrode and the counter is a Pt wire. In this setup the voltage is applied between working and reference electrode [61]. With this configuration there are no limitations in the control of the potential generated at the working electrode, but miniaturization is more complicated [61].

To have a good response is fundamental to have a good contact between the enzyme and the electrode and usually the enzyme is covalently bonded to the modified working electrode. This gives high stability to the system, but the retained activity could decrease. An alternative is to use the confinement method, but in this case is necessary an intermedicator to transport the electrons from the active site to the electrode. This type of systems falls in the second-generation of biosensors. Usually, in biosensors applied for batch measurements is used a covalent immobilization of the enzyme onto the electrode. For example, in the study presented by Narwal et al., lactate dehydrogenase is covalently bonded to a gold electrode [78]. The system has a stable response over 80 days with a linear range between 0.01 and 55 mM. The great characteristics of this system came from the covalent bonding of the enzyme to the surface of the electrode that gives high stability and an intimate contact between the enzyme and the surface.

In other solutions the enzyme is first entrapped in a membrane and then the membrane is bonded to the electrode surface. For example, in the study conducted by Guan et al. glucose oxidase is enclosed in liposome (Fig. 2-B) that then are linked to a glassy carbon electrode (GCE) using electrostatic interactions [67]. To ensure a good stability the GCE is modified with chitosan and to facilitate substrate transport protein channels were included in the liposomes. This system has a good stability and linear response, but it also guarantees a good environment for the enzyme that retains its activity. A similar approach was used in a study conducted by Dhanjai et al., but the enzyme is entrapment as a single molecule in a polymeric membrane [69]. The membrane then is bonded to GCE modified with carbon nanotubes using again electrostatic interactions with chitosan. This system has high stability also in organic solvents and at high temperatures and a good linear range. The advantage of single molecule entrapment is that the molecules can't aggregate.

In contrast, for flux analysis, the enzyme is typically confined to a specific region, and a cofactor carries the electrons to the working

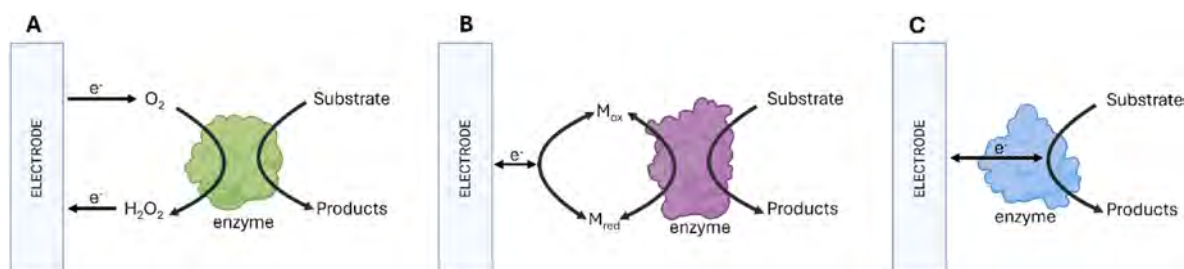


Fig. 5. Different generations of amperometric enzyme biosensors: first generation (A), second generation (B) and third generation (C).

electrode as the solution flows through the biosensor. This approach was used in the study carried out by Josypčuk et al. in which the selected enzyme was confined by linking it to a silver amalgam [79]. The system was developed in subsequent studies with different enzymes and geometries [80,81]. The confinement could also be done in other ways, like using magnets, as has been done by Sheng et al. [82]. In the study glucose oxidase was bonded to magnetic nanoparticles, that were held in place by an external magnetic field. In these studies, the biorecognition and detection regions are separated. This increases the system versatility and it is possible to use different enzymes without changing the electrode [79]. The study carried out by Tvorynska et al. shows that the system response doesn't depend on the distance between the reaction site and the electrode [80]. There are also some complications due to the presence of the flowing liquid and it is necessary to find the best flux to have a good detection. Indeed, the measured current increases as the mass transport increases [80,81], but then it reaches a plateau. If the flow is too high, it is not beneficial for the detection and there is a waste of reagents, therefore it is fundamental to optimize the mass flow entering the system.

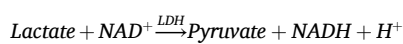
Amperometric biosensors are spreading thanks to their fast, accurate and specific response compared to other detection techniques [59,61]. They also have some drawbacks, like the signal reduction from fouling agents and interference from the chemicals present in the sample [61]. The three-electrode configuration allows for good measurements but reduces the possibility to miniaturize the system and in many cases, it may not be practical to design such complex construct [61].

2.2.1.2. Potentiometric. In potentiometric biosensors, the measured parameter is oxidation and reduction potential of the electrochemical reaction in which change in potential corresponds to the change in charge and hence change in current [61]. In this technique is measured the potential between a working electrode and a reference electrode across some interface and there is a negligible bias current [83]. This technique is simple, compact and has low power consumption [83]. It is also miniaturizable without loss of sensitivity [83]. The generated potential is specific for a particular reaction and type of species examined [61]. There are different methodologies that can be used for the detection. The most common one is ion-selective electrode (ISE) [83]. ISEs are characterized by the presence of a selective membrane and an ion transport or exchange causes a variation in the membrane potential [83]. The enzymes catalyse the reaction and the produced ions are detected [83]. A more sensitive option to ISE is to use field-effect transistors (FET). A schematic illustration of a FET is present in Fig. 6. The recognition element, like enzyme or DNA probe, is linked to the gate

electrode and the binding of the analyte to the biorecognition element leads to a change in the charge distribution of the semiconductor layer underneath, and therefore a change to the overall device conductance that can be measured [83]. With FET biosensor is possible to detect really low concentrations, in the orders of nM [83,84]. They can be used for selective detection in complex fluids, like blood, or for tracking the level of specific molecules, like neurotransmitters [83,84]. The problem with enzymes is their lack of stability, high-cost and time-consuming procedures and difficulty of quality control [84].

Recently potentiometric enzymatic biosensors are developing thanks to the possibility of miniaturization as the potential is independent from the electrode size [85]. Another limitation is the time resolution, indeed usually the acquired potential is an equilibrium one [85]. This is fine for measurements in static conditions, but if the phenomenon is dynamic, like the change of neurotransmitters concentration in the brain, is needed a faster detection [85]. Therefore, efforts in non-equilibrium, and transient potentiometry are presently emerging, opening for both high temporal resolution and continuous monitoring [85].

2.2.1.3. Impedimetric. With electrochemical impedance spectroscopy (EIS) is possible to measure a variation of impedance or capacitance between two electrodes. It is a steady-state technique that utilizes small signal analysis, allowing it to probe signal relaxations across a wide range of applied frequencies. Furthermore, EIS exhibits high sensitivity even with minimal changes in analyte concentration [86]. The main limitation of this technique is the dimensions required for the equipment necessary for the measurement [86]. In the study conducted by Kumar et al. an impedimetric biosensor was developed for the detection of lactate in human sweat [86]. LDH was immobilized onto a screen-printed carbon electrode and was used to catalyse the following reaction:



Then NADH is subjected to the following equilibrium:



The described reaction produces charged ions in the enzymatic layers that can be detected with EIS. The developed system shows a linear range between 0.1 and 100 mM with high stability and repeatability. It also shows high specificity and could detect lactic acid also in the presence of interfering compounds.

Therefore, EIS has high sensitivity and selectivity and with the miniaturization of the instrumentation could be used for portable

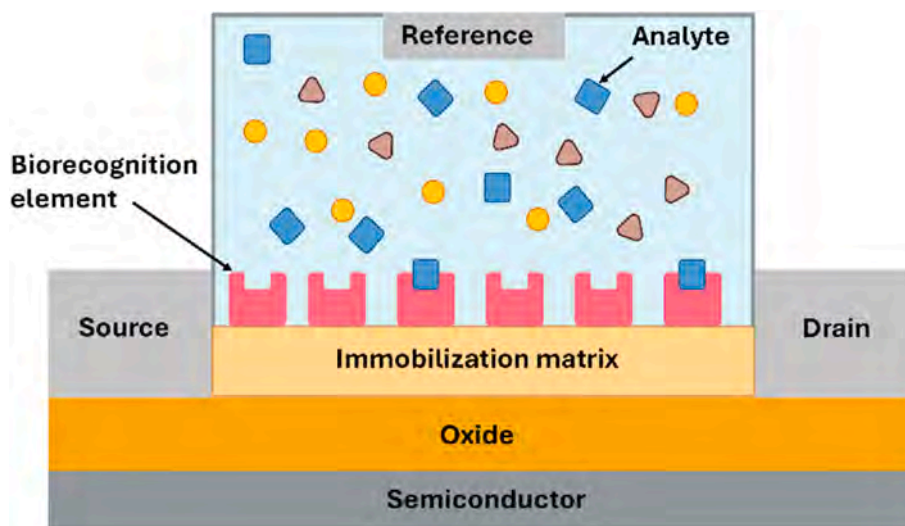


Fig. 6. Schematic illustration of bio-FET electrode for potentiometric detections.

devices in point-of-care diagnostic.

2.2.1.4. Voltammetric. Voltammetric biosensors are based on electro-analytical chemistry techniques in which quantitative analyte sensing is made by varying the potential and measuring the resulting current as an analyte reacts electrochemically with the working electrodes surface [87]. There are different techniques, but the most applied one is linear sweep cyclic voltammetry (CV) [59,87]. CV has different advantages, such as high sensitivities and low detection limits, quantitative analysis of processes and fast and clear characterization of the process [87]. With CV the reduction potential of the species present in the solution is detected. If different species have closer or overlapped reduction or oxidation potential, it will result in poor selectivity and reproducibility [87]. Another drawback is the fouling of oxidation products at the electrodes. This problem can be reduced using carbon-based electrodes, thanks to the enhanced electron transfer rate that is achievable with them [87].

2.2.2. Optical detection

Optical biosensors base the detection on photons in the UV, visible or near-infrared spectra [61]. In this type of sensors, the optical transduction is fundamental, because it allows the measurement of the target analytes through converting the analyte-biomolecule reciprocity into a measurable optical signal [59]. For the measurement, different techniques can be used depending on the type of transducer used. The most used ones are absorption, luminescence, chemiluminescence [59].

2.2.2.1. Absorption. This technique evaluates the change in absorption of the solution in which the reaction is happening. This methodology of detection works only if the products of the reaction have significantly different absorbance at a certain wavelength compared to the reagents [59]. The absorbance is evaluated with the Lambert-Beer law, described by equation (1).

$$A = \epsilon bc \quad (1)$$

Where A is the absorbance, ϵ is the molar absorption coefficient of the analyte at a particular wavelength, b is the free path of the light and c is the concentration of the analyte [59].

To increase the response is possible to use chromogenic substrate that can change colour reacting with some products of the reaction catalysed by the enzyme [88,89]. One of the biggest problems of this technique is the interference with other compounds. Indeed, in complex fluids, like biological fluids, is probable that different molecules have the absorbance peak at the same wavelength and with this method they are not distinguished. To solve this issue is necessary a pretreatment to remove all the interfering substances using complex and time-consuming procedures [90]. In the study presented by Bi et al., a microfluidic device was created to evaluate the amount of ascorbic acid (AA) using UV/Vis spectroscopy [90]. The system was able to detect the interested molecule even in the presence of the most common interferants. Therefore, the pretreatment for eliminating interfering substances can be skipped or greatly simplified. The microfluidic device can detect AA when its concentration was ten time lower than interfering substances, like caffeine. If the difference in concentration is higher, they recommend doing pretreatments to clean the solution.

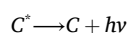
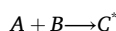
2.2.2.2. Luminescence. In this technique is measured the intensity of the light emitted by the solution. It is linked to the concentration of the analyte by the Parker's law, shown in equation (2).

$$c = \frac{F}{2.31 I_0 \phi \epsilon b k} \quad (2)$$

Where c is the concentration, F is the luminescence intensity, I_0 is the intensity of excitation light, ϕ is the luminescence quantum yield, ϵ is the molar absorbance of the analyte at the excitation wavelength, b is the

path length of light through solution and k is a constant [59]. The light emission is associated with a reaction with the analyte [59]. For example, in the study of Jenie et al. the fluorescent emission is obtained with resazurin, that is an oxidation indicator [91]. The system used lactate dehydrogenase and the reaction catalysed by the enzyme is associated with the oxidation of resazurin. When resazurin is oxidated, it emits light that can be detected. It is also possible to use a quenching effect, in which the reaction reduces the intensity of the emitted light (Fig. 7) [92]. In the study conducted by Kim et al., the enzyme was entrapped in a hydrogel with quantum dots (QD). The QD are used to emit the light and the products of the reaction act as quenching agents reducing the intensity of the light emission. The system has a good response and is also possible to entrap different enzymes.

2.2.2.3. Chemiluminescence. In chemiluminescence the light is emitted because of a chemical reaction [59]. The general reaction is the following:



This method is normally used in presence of oxidases that produces H_2O_2 that can react with luminol to determine the chemiluminescence effect. Chemiluminescence can also be used to have quenching effects. In this case the radiation emitted is absorbed by a fluorescent acceptor present near the light emitter [59]. It is also possible to do flow-through chemiluminescence biosensors, like has been done by Lan et al. [93]. Glucose oxidase and horseradish peroxidase are immobilized with sol-gel methods with gold nanoparticles. Glucose oxidase produces the H_2O_2 that then is used by the horseradish peroxidase to react with luminol to produce the chemiluminescence effect. Gold nanoparticles are used because they facilitate the chemiluminescence reaction enhancing its effects. Chemiluminescence detection is becoming increasingly important in various fields because of the very low detection limit, rapidity and wide linear working range that can be achieved using relatively simple instrumentation [93].

If the reaction is an electrochemical reaction, it is defined electrochemiluminescence. It includes electro generation of reactive intermediates to form electronically excited luminous force [61]. Usually, the emission is in the visible or near-infrared spectra [61]. In the study carried out by Ferraraccio et al. glucose and lactate dehydrogenase have been encapsulated in alginate hydrogel and the interaction between NADH and tris(2,2'-bipyridyl) ruthenium (II) is used to have the electrochemiluminescence effect [94]. The system presented good stability and reproducibility. It also showed a good sensitivity in the presence of common interfering species.

3. Specific applications of enzymatic biosensors

The main advantage of biosensors is their high specificity and excellent sensitivity, allowing the detection of molecules that are present in low concentrations. For this reason, the main applications of biosensors are in the diagnostic field, where sensitive, rapid and minimally invasive measurements are required. In recent years they also emerged as an opportunity to address research gaps for in-vivo measurement, broadening their utility beyond traditional laboratory settings [95]. They are emerging also as a promising tool for drug screening evaluating the inhibitory effect of the drug on the target enzymes. In the following sections, the most representative applications of enzyme-based biosensors are discussed and organized according to the major pathological areas described in the Introduction. Each set of examples is contextualized in relation to the underlying disease mechanisms and to the transduction strategies presented earlier in the manuscript. Additionally, one example involving a DNA-based catalytic system is included. Although DNAs are not proteins, they exhibit

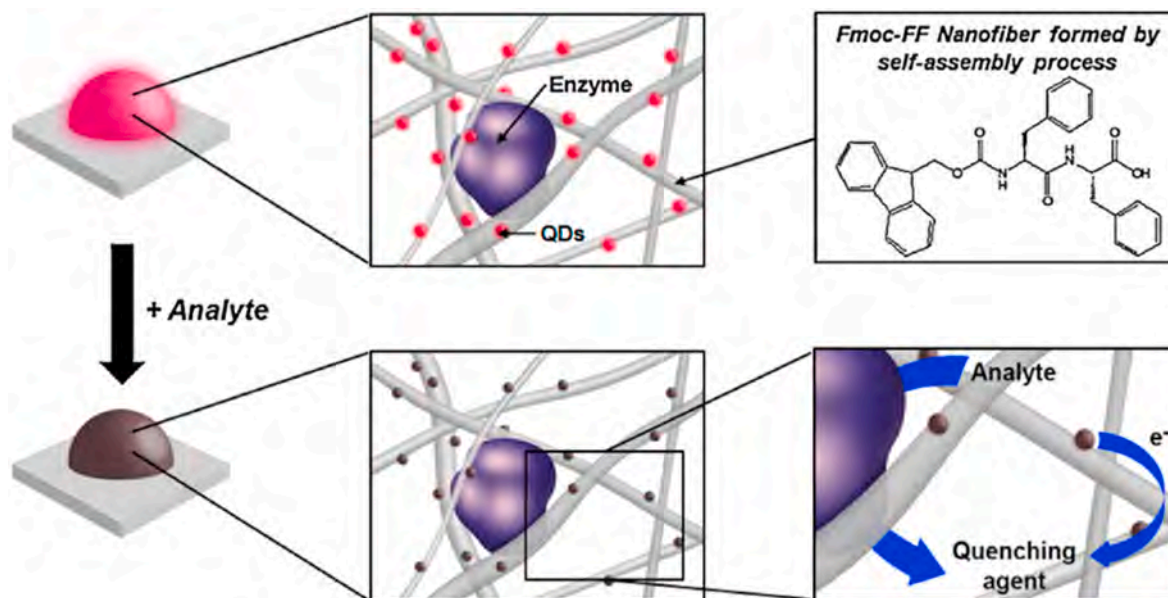


Fig. 7. Luminescence biosensors with quenching effect on quantum dots [89].

catalytic behavior analogous to natural enzymes and therefore fall within the conceptual scope of this review, especially in the context of nucleic-acid-related diagnostics. Finally, selected applications in drug screening are also reported, highlighting how enzymatic biosensing platforms can be leveraged for inhibitor evaluation and therapeutic development.

3.1. Cancer detection

Cancer-related enzymatic biosensors rely on highly sensitive electrochemical and optical transduction methods, as cancer biomarkers often appear at extremely low concentrations during the early stages of the disease. In the following examples, different detection principles are compared in terms of suitability for metabolic biomarkers (e.g., lactate), redox-active species (e.g., ROS), nucleic acids (cfDNA) and proteolytic enzymes (MMPs), highlighting the rationale behind the selection of each sensing strategy. For the detection of cancer, the first step is the identification of the biomarkers that can be used for the diagnosis. Then they need to be quantified in patient samples, and this reveals difficult to perform because, as already said, they are present in extremely low concentration, particularly in early stages of the pathology [33]. Therefore, using really high specific biosensors, such as enzymatic ones, is fundamental.

As mentioned in the Introduction, one of the most diffuse molecules in different types of cancers is lactic acid or lactate, therefore its detection is used for cancer diagnosis. Different enzymatic biosensors for the detection of this molecule have been developed. Azzouzi et al. presented an amperometric biosensor for the detection of L-lactate [96].

They used a screen-printed carbon electrode modified with graphene oxide (RGO) and gold nanoparticles (AuNPs). A gel matrix was prepared with tetramethoxysilane and methyltrimethoxysilane, in which the RGO-AuNPs compound and L-lactate dehydrogenase (LDH) were incorporated. A schematic representation of the working principle is shown in Fig. 8. In brief words, LDH catalyse the reaction from lactate to pyruvate with the consequent reduction of the NAD^+ to NADH. Then at the electrode, NADH is oxidized back to NAD^+ and the electron transfer is measured as a current. Therefore, the system measures the concentration of NADH that is directly related to the concentration of L-lactate. The obtained device presents a detection limit of $0.13 \mu\text{M}$ and has good reproducibility and stability. It can also detect L-lactate without interferences from commonly interfering compounds such as urate, paracetamol and L-ascorbate. Therefore, the developed system is a good platform for L-lactate detection.

This example was one of the first study for the detection of L-lactate. Recently other systems have been presented. Tvorynska et al. have developed an amperometric flow biosensor for this aim [97]. In this case the enzyme used is lactate oxidase (LOx) that is covalently bonded to mesoporous silica powder (SBA-15). LOx is involved in the metabolism of lactate and catalyse its transformation in pyruvate with the production of H_2O_2 in presence of oxygen. The system monitors the electrochemical reaction by evaluating the oxygen consumption via its reduction at negative potential. The mesoporous silica powder with the enzyme is pressed in a Plexiglass tube to form a column-like reactor in which the flow can pass through. After inlet liquid has passed the bio-recognition area, the analyte concentration is measured through a three-electrode system. The working electrode is a silver amalgam

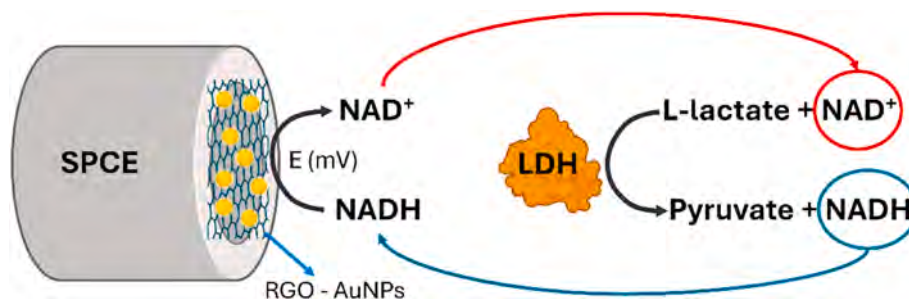
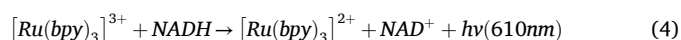


Fig. 8. Schematic representation of the NADH detection.

screen-printed electrode. The silver amalgam was chosen because it facilitates the reduction of oxygen and therefore it is a good transducer for the detection of oxygen consumption. The system has a good response with a working range compatible with the detection of lactic acid in biological fluids. Using a negative potential for the detection system, the effect of the most common interfering substances was reduced and this allows a good detection in complex fluids, like *saliva*.

Ferraraccio et al. developed a hydrogel to entrap LDH and glucose dehydrogenase (GDH) for the detection respectively of L-lactate and glucose [94]. The entrapment was done by a self-assembly of the hydrogel that was deposited onto a glassy carbon electrode. For the detection, electrochemiluminescence method was used, in particular the emission of light came from the reaction between NADH and tris(2, 2'-bipyridyl) ruthenium (II) ($[Ru(bpy)_3]^{2+}$). NADH was a product of the reactions catalysed by both enzymes, and it's oxidized to NAD^+ by $[Ru(bpy)_3]^{3+}$ that is produced by electrochemical oxidation at the electrode surface. When $[Ru(bpy)_3]^{3+}$ is reduced back to $[Ru(bpy)_3]^{2+}$, there is the emission of a photon. Details of the reactions are presented in equation (3) and equation (4). With this method was possible to use the same luminophore for the detection of both L-lactate and glucose, but the simultaneous detection was not possible because the two peaks of emission were at the same potential. The system was tested with interfering substances (ascorbic acid, dopamine, citric acid and urea) and a significantly higher response is achieved for the two analytes. The system also showed a good linear range, with reproducible and repeatable measurements.



The amount quantification of the enzymes in biological fluids is an alternative to the determination of the reaction products concentration, previously described, to evaluate the overexpression of LDH. In this case, the enzyme is no more the functional unit for the detection of a specific substrate, but it becomes the biomarker itself. This methodology was followed by Jenie *et al* [91]. They prepared a fluorescence biosensors with a substrate of porous silicon as enhancer of the fluorescence. The fluorophore used is resazurin that is covalently bonded to the silicon substrate. The system is exposed to a solution containing the enzyme and all the cofactor needed for the reaction, in this case NAD^+ and L-lactate. The oxidation of the resazurin is associated to the reduction of NAD^+ to NADH catalysed by the enzyme. This causes an increase in the fluorescence emission. A schematic representation of the working mechanism is shown in Fig. 7. The obtained system has a working range compatible with the enzyme concentration in human biological fluids, good selectivity for LDH and faster detection compared to other techniques.

In addition to these examples of enzymatic biosensors for the detection of lactate, other biomarkers can be used for cancer detection.

Indeed, tumour environments are characterized by high presence of reactive oxygen species (ROS), that can be used as analyte for the diagnosis. Zouleh et al. developed a system for the detection of superoxide anion ($O_2^{\cdot-}$) by electrochemical analysis [98]. They used a gold electrode modified with reduced graphite oxide and yttrium hexacyanoferrate. Superoxide dismutase (SOD) was immobilized over the electrode with chitosan and glutaraldehyde. SOD converts $O_2^{\cdot-}$ into H_2O_2 that can be used for the measurement. The obtained system shows high selectivity, not having a response for interfering substances such as dopamine, glucose, acetaminophen, ascorbic acid, uric acid and NaCl. The system was also tested for the detection of $O_2^{\cdot-}$ produced by prostate cancer cells and it demonstrated to effectively monitor in real-time the superoxide anion produced by the cells.

Tumours can also be detected using biomarkers that are specific to each type. Dempsey et al. developed a system for the detection of lung cancer by measuring the activity of proteases [99]. In this study the

enzyme is used as the biomarker and the aim of the biosensor is to detect its concentration. Protease enzymes are a large family of enzymes that regulate tumour cell invasion, angiogenesis, epithelial to mesenchymal transition and malignancy [99]. The researchers linked tetrakis-carboxyphenyl-porphyrin (TCPP) to graphene nanoparticles using specific peptides degraded by proteases. When these peptides are cleaved, the quenching effect of the graphene nanocrystal is lost, allowing TCPP to emit detectable fluorescence. By measuring this fluorescence, it is possible to determine the amount of enzyme present in the sample analysed. The system was tested with human plasma and showed promising results. To determine the presence or the absence of the tumour from the fluorescence, they developed a machine learning algorithm. They tested the system over 750 samples and it showed high specificity (82 %) and sensitivity (90 %). More details of the usage of artificial intelligence systems are presented in chapter 4.

For pancreatic cancer, carnitine is used as a biomarker, in particular, it is evaluated its presence in saliva [100]. Zhang et al. developed an electrochemical biosensor for the detection of carnitine by cathodic stripping voltammetry [100]. Carnitine acetyltransferase was mixed with bovine serum albumin and cross-linked with glutaraldehyde to obtain a solution that was casted onto gold electrode (Fig. 9). This enzyme transfers an acetyl group from the acetyl-coenzyme A (acetyl-CoA) to the carnitine with the formation of acetyl-carnitine and CoA. CoA is characterized by the presence of a thiol group that makes it easily adsorbed on the gold electrode surface. For the detection they used cathodic stripping voltammetry (CSV).

The system was developed for the detection of carnitine in the saliva and was tested with common interfering substances. The electric signal from the carnitine is at least three times higher than for other compounds, therefore the system has good selectivity. It also has high sensitivity and in future it could be used for clinical applications.

Also circulating free DNA (cfDNA) can be used as a biomarker for cancer diagnosis. Ying et al. developed a biosensor for the detection of cfDNA in human serum [101]. For the detection they used DNA G-quadruplex/hemin enzyme, that is a synthetic enzyme. It's an enzyme based on nucleic acid; therefore, it's different from usual enzymes, but it has an analogous behaviour. It has been used for a chemiluminescence biosensors, as it can catalyse the reaction between luminol and H_2O_2 and emits light [101]. For the detection are prepared complementary probe to the target DNA with pending extremities. If the extremities are placed near to each other, they will form a G-quadruplex that combines with the hemin group to form the DNA G-quadruplex/hemin enzyme. This enzyme can catalyse the reaction involving the luminol and emit light. A schematic representation of the mechanism can be found in Fig. 10. The amount of light emitted is correlated to the amount of cfDNA in the sample. The system has a detection limit compatible with the detection of cfDNA in patients. The device was tested with serum samples from healthy, cholecystitis or with gall bladder cancer (GBC) patients. It was able to identify all the patients with GBC but not distinguish between healthy and cholecystitis patients. Anyway, the novel method using DNA G-quadruplex/hemin enzyme could be effectively used for the detection of cfDNA in patients with GBC.

For gastric cancer diagnosis, D-amino acids are important biomarkers [102]. Li et al. developed an electrochemiluminescence biosensor for the detection of D-alanine [102]. They used H_2O_2 as an etching agent to activate the light emission of gold nanostructures modified with MnO_2 . The nanostructure is deposited onto a glassy carbon electrode. D-amino acid oxidase (DAAO) is used to produce H_2O_2 , since it catalyses a reaction involving D-amino acids that has H_2O_2 as a product. The modified electrode is inserted in a solution containing D-amino acids and DAAO and the intensity of the light emission depends on the concentration of the amino acids. MnO_2 has a quenching effect on the emission of the gold nanostructure and in presence of H_2O_2 it is removed, turning on the emission. The system has a really good response and is able to detect D-amino acids in biological fluids, like human saliva.

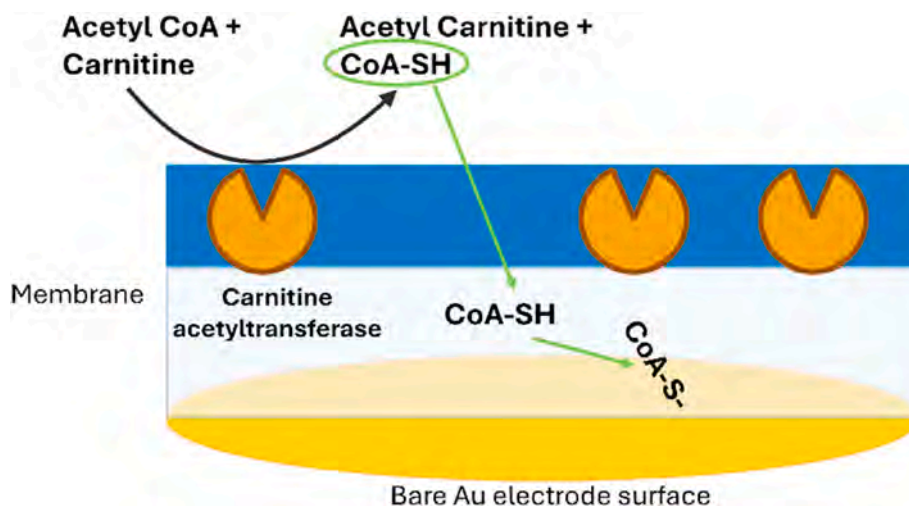


Fig. 9. Schematic representation of the working mechanism for carnitine detection.

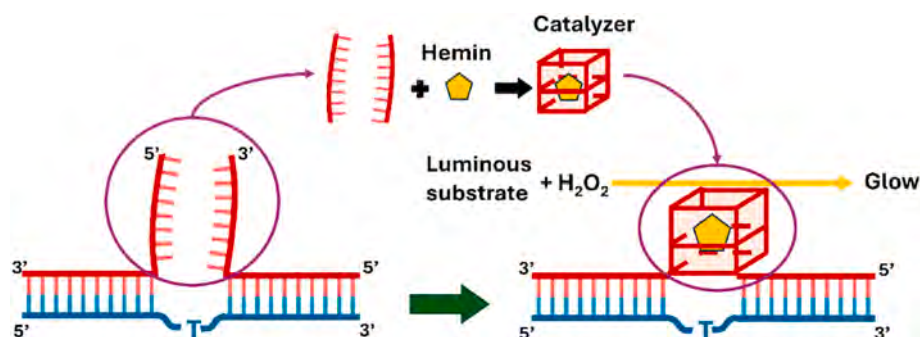


Fig. 10. Schematic representation of the detection of cfDNA: the DNA sequence labelled with the "T" represent the target DNA, the other two are the probes; the red box is the G-quadruplex formed by the pending extremities of the probes.

In lung cancer there is an overexpression of matrix metalloproteinases (MMPs), in particular MMP-1, MMP-2 and MMP-9 [103]. As has been said in the Introduction, MMPs are involved in tumour angiogenesis and formation of metastasis. Moreover, they are present in various cancers and diseases in addition to lungs [13–15]. Kowalczyk et al. developed a system for the simultaneous monitoring of MMP-1, MMP-2 and MMP-9 activity with voltammetric detection [103]. The aim of the device was to evaluate the concentration of the three enzymes in analysed samples, making the enzymes the biomarker itself. On a gold electrode were bind peptide sequences with specific cleavage sites for the three enzymes. At the extremity of each peptide a specific molecule was bound: methylene blue for MMP-1, anthraquinone for MMP-2 and ferrocene for MMP-3. Each enzyme cuts its peptide and with a voltammetric measurement can be quantified the concentration of the specific molecule bonded to the peptide, that is equal to the concentration of the enzyme itself. Therefore, the enzyme concentration can be evaluated by this indirect methodology. A schematic representation of electrode preparation and working mechanism can be seen in Fig. 11. The system was tested with plasma and tumour or tissue extracts from patients. The amount of active MMPs detected by the sensor is equal to what find with ELISA test, showing that the developed system is a valuable alternative to traditional tests, with the advantage of simultaneous multiple enzyme detection.

3.2. Neurodegenerative diseases detection

Neurodegenerative disorders involve heterogeneous classes of biomarkers, ranging from misfolded proteins (p53, amyloid- β , tau) to

neurotransmitters (acetylcholine, glutamate). Because these targets require diverse recognition principles, the biosensors developed for neurodegeneration employ several detection mechanisms, including immuno-electrochemical, optical and SPR-based approaches. The following examples illustrate how each method aligns with the biochemical properties of the target.

The protein p53, particularly in its unfolded configuration is one of the most promising biomarkers for neurodegenerative diseases. This protein regulates gene expression but in oxidative environments can translocate into mitochondria, leading to apoptosis or necrosis. This phenomenon seems to play an important role in neurodegenerative diseases, like Parkinson's, Huntington's and Alzheimer's diseases [104]. Amor-Gutiérrez et al. designed and produced an electrochemical biosensor for the detection of p53 [105]. The system is based onto screen-printed carbon electrodes modified with gold nanoparticles deposited onto the electrode by electrochemical reduction of salt precursor. The surface is further modified with antibodies for the unfolded p53. The detection mechanism is based on two steps. Initially the system is placed in contact with the solution to analyse and the p53 present binds to the antibodies. Then an unfolded p53 modified with biotin is added in a known concentration and let react with the residual antibodies. At the end alkaline phosphatase modified with streptavidin (S-AP) in a fixed concentration, 3-indoxyl phosphate (3-IP) and silver nitrate are added, let react and measured the response with sweep voltammetry. S-AP is an enzyme that, thanks to the streptavidin, specifically interacts with the avidin present on the modified p53. AP hydrolyses 3-IP with the formation of indoxyl intermediates that reduces the silver ions to metallic silver. It will adsorb on the surface of the

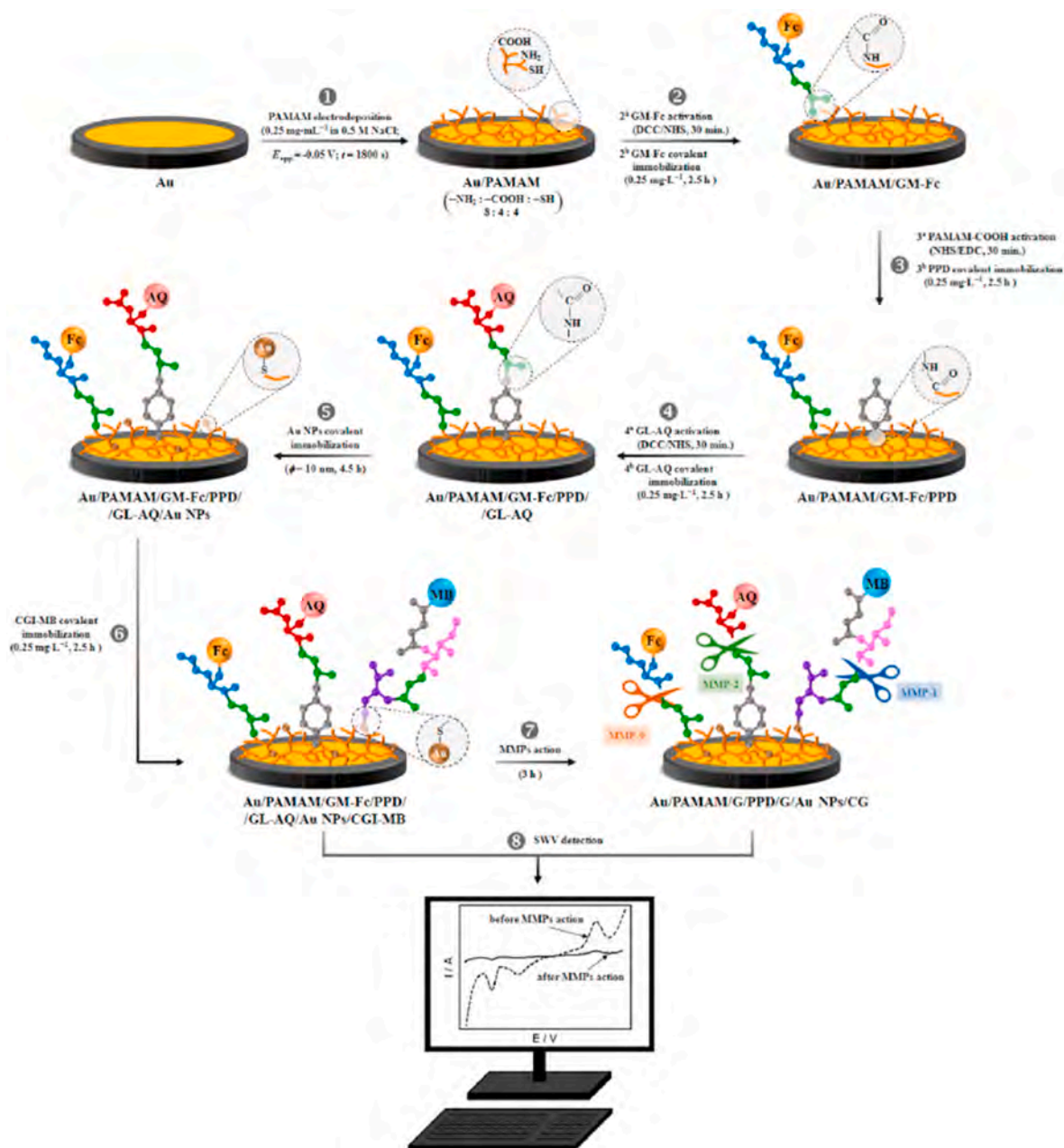


Fig. 11. Schematic representation of electrode modification and working mechanism for the detection of MMPs [100].

electrode and its amount is measured with sweep voltammetry. The intensity of the peak current is proportional to the quantity of silver deposited on the electrode and therefore on the amount of modified p53 added. The quantity of this p53 is inversely proportional to the concentration of unfolded p53 in the solution. The system was tested with plasma of patients with Alzheimer's disease or Mild Cognitive Impairment (MCI) and compared with ELISA conventional test. The obtained results with the two methods are comparable and the developed system can find the unfolded p53 in patient plasma with high sensitivity.

For Alzheimer's disease specifically, one of the most important biomarkers is the β -amyloid ($A\beta$). Diba et al. presented a biosensor to identify amyloid- β 1–42 [106]. The system is based on electrochemical measurements with screen printed carbon electrode modified with gold nanoparticle. On the electrode is immobilized an antigen for $A\beta$ and antifouling molecules to avoid the deposition of other molecules on the electrode surface during the measurements. Another antigen modified with alkaline phosphatase (ALP), which presents different recognition

sequences, is added for the recognition. Therefore, there is the formation of antigen sandwich on the electrode surface (Fig. 12). With the second antigen is also added 4-aminophenol phosphate (APP) that is dephosphorylated by the interaction with ALP. Then the product is further oxidized on the electrode surface with the formation of a peak current measured with cyclic voltammetry. The intensity of the peak depends on the quantity of ALP present in the system and therefore on the amount of $A\beta$. Also in this case, the system was tested with patient serum and plasma and it was able to quantify correctly the amount of amyloid- β 1–42 present in different samples.

In Alzheimer's disease another important biomarker for early diagnosis is the phosphorylation of tau proteins on specific amino acids. In particular, one of the two most relevant phosphorylation for the formation of the pathology are the one at serine 396 and serine 404. Zhang et al. developed a system for the ultrasensitive detection of phosphorylated tau in these two specific points [107]. The system is based on optic detection and uses superparamagnetic iron oxide nanoparticles

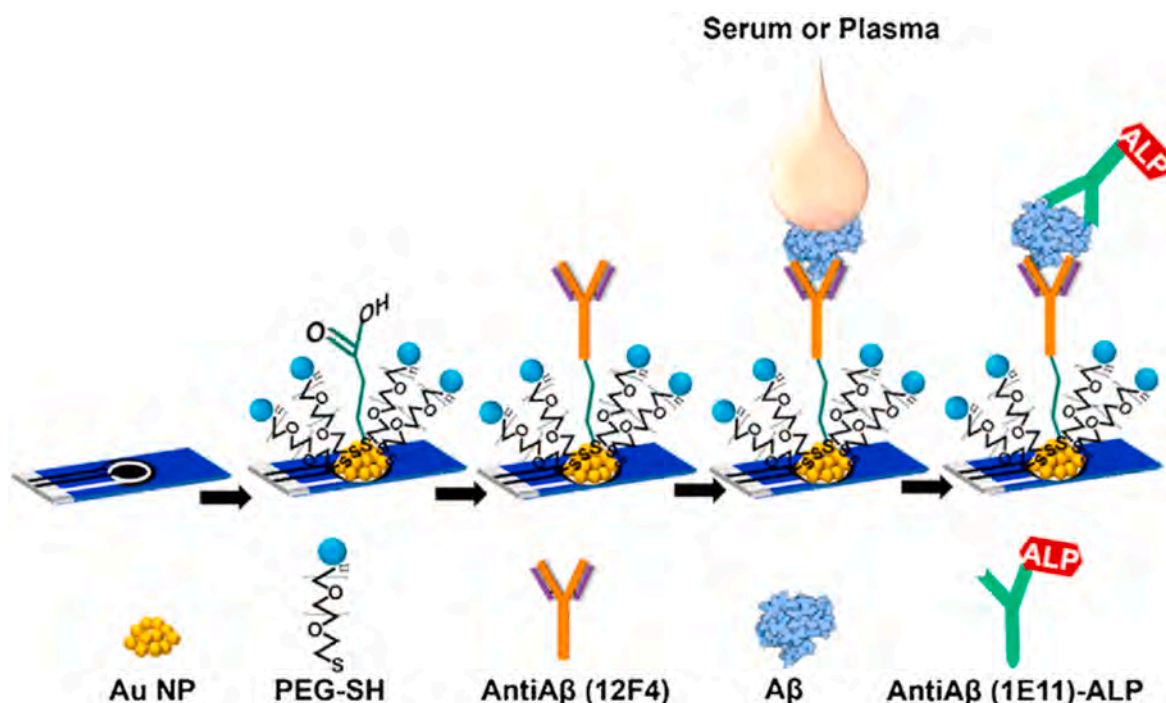


Fig. 12. Schematic representation for antigen sandwich formation for $A\beta$ detection [103].

modified with specific antigens for the phosphorylated tau. These magnetic nanoparticles are put in contact with the blood sample and the recognition element is added. It is composed by gold nanoparticles modified with other antigens for the tau protein. The antigen is modified with horseradish peroxidase (HRP) to achieve a colorimetric detection. Indeed, 3,3',5,5'-tetramethylbenzidine (TMB) is added in the sampling solution, that in presence of HRP react and forms an intense blue colour. The measurements were carried out in a qualitative way by the direct observation of the colour or quantitatively by measuring the absorbance of the solution. The gold nanoparticles were also modified with a Raman reporter, therefore was also possible to do the detection with Raman spectroscopy. The system showed a low limit of detection (LOD) (1.5 pg of phosphorylated tau per ml) and with the Raman spectroscopy was possible to achieve high sensitivity and anti-interference abilities.

Acetylcholine is another important biomarker for the early diagnosis of Alzheimer's disease. Kant et al. proposed a fiber-optic based biosensor for the detection of acetylcholine [108]. Over the core of an optic fiber was made to grow tantalum oxide (Ta_2O_5) nanocrystals and over them was immobilized acetylcholinesterase. The sensing mechanism is based on resonance frequency of the optic fiber probe, in particular a frequency shift is measured. The shift depends on the concentration of acetylcholine in the solution. Indeed, acetylcholinesterase transform acetylcholine in choline and acetate in presence of water. This causes a change in the refractive index of the sensing surface that result in a shift of the resonance frequency. This is because the refractive indices of Ta_2O_5 , choline and acetate are different. The developed system has a working range compatible with the clinical applications and, thanks to the highly specificity of the enzyme used, has no interference from the common substances present in the sample.

Acetylcholine is not only present in the central nervous system but also plays important roles in the gastrointestinal tract. Several studies have reported alterations in the gut of Alzheimer's disease models, even during the early stages of the pathology [109]. Henderson et al. developed an enzymatic biosensor to evaluate the level of acetylcholine in the gut of mice [110]. They prepared a microelectrode made of platinum covered with Pt nanoparticles and poly(*m*-phenylenediamine) (pmPD). The nanoparticles have the aim to increase the electrochemically active

area. The active surface was modified with two enzymes, acetylcholinesterase (AChE) and choline oxidase (ChOx), that are immobilized by crosslinking with glutaraldehyde. The aggregates diffuse into the pores of pmPD and are stable on the electrode surface. The system is based on an indirect detection of acetylcholine; indeed, it is not an electroactive specie, and its concentration is measured from the products of its oxidation. AChE hydrolyses acetylcholine into acetate and choline. Choline is subsequently oxidized by ChOx with the production of H_2O_2 and betaine aldehyde. Then H_2O_2 diffuses into the pores of pmPD and is oxidized on the electrode surface, generating a current response proportional to the concentration of acetylcholine. The system allows a good evaluation of acetylcholine concentration with a working range in the physiological limit. It also has a fast response (around 2 s), that is significantly lower for common interfering substances. Though, it gives a response in equal amount for both acetylcholine and choline due to realization choices, therefore it cannot be used for the measurement of acetylcholine concentration in samples from patients. However, it can be used to monitor the cholinergic neuronal function.

Another neurotransmitter important for the development of different neurodegenerative diseases is glutamate. Glutamate is the most important excitatory neurotransmitter of the mammalian central nervous system (CNS). However, glutamate overstimulation is also implicated in neuronal cell death [111]. Alsharabi et al. prepared an ultrasensitive electrochemical biosensor for the detection of glutamate [112]. The electrode was based on nickel foam covered with reduced graphite oxide (rGO). Over the rGO was immobilized glutamate dehydrogenase (GLDH) with covalent bonding using glutaraldehyde. GLDH transforms glutamate into 2-oxoglutarate and ammonium and uses NAD^+ as a cofactor for the reaction. NAD^+ is reduced to NADH and the electron transfer is measured as a current at the electrode. The intensity of the current depends on the concentration of glutamate in the analysed solution. The system is blind to interfering substances and has high reproducibility (over 20 cycles) and stability (86 % of retained activity after 10 weeks of storage). The system has also a working range in the physiological range of glutamate, therefore, it can be used for the detection of the neurotransmitter in biological fluids.

3.3. Diabetes detection and monitoring

One of the most diffused metabolic diseases is diabetes. The following examples illustrate how amperometric, optical and chemiluminescent strategies are adapted to achieve high sensitivity and rapid response for continuous metabolic monitoring. Glucose biosensors are the first biosensors to be fabricated [59], therefore nowadays the requirement is to increase the detection limit and sensitivity. Bi et al. developed a biosensor based on dendritic gold nanostructure with glucose oxidase immobilized on it [113]. The system was obtained by modifying a carbon fiber cloth electrode with electrodeposition of gold to obtain the dendritic structure. Over this structure was adsorbed 4-mercapto-benzoic acid that was used to covalently bond the glucose oxidase (GOx) by EDC/NHS methodology. After some enzymes are linked to the surface, enzymatic aggregates are created by crosslinking different molecules with glutaraldehyde. For the detection is used an electrochemical method and is used ferrocenemethanol (FcOH) as an electron transporter onto the electrode surface. GOx interact with glucose and passes from the oxidized form to the reduced one using the cofactor FAD. Then the reduce form of GOx oxidises again by reducing FcOH, that is oxidated at the electrode surface. A schematic representation of the working mechanism can be seen in Fig. 13. The measured current is proportional to the concentration of glucose in the sample and the system shows a higher sensitivity compared to other glucose biosensor, with a wider working range and a lower LOD. It has also a significantly higher (20 times) response for glucose compared to the most common interfering substances.

Huang et al. developed an ultrasensitive glucose biosensor using electrochemiluminescence detection [114]. They used as a substrate a vertically ordered mesoporous silica film (VMSF) modified with a monolayer of glucose dehydrogenase (GDH). The VSMF was deposited onto indium tin oxide (ITO) glass with an electrochemical procedure and over it was covalently bonded the GDH using the (3-glycidyoxypropyl) trimethoxy silane (GPTMS). $[\text{Ru}(\text{bpy})_3]^{2+}$ was used for the electrochemiluminescence detection because it undergoes oxidation and reduction with the emission of UV light. In presence of glucose, GDH oxidise it by reducing the NAD^+ into NADH that is subsequently oxidized back to NAD^+ reducing the $[\text{Ru}(\text{bpy})_3]^{2+}$. The reduced form of $[\text{Ru}(\text{bpy})_3]^{2+}$ is then oxidized to the original form at the electrode surface with the emission of a UV photon. Therefore, the intensity of the emission is proportional to the concentration of glucose in the solution. The NAD^+ present in the solution act also as a co-reactant with $[\text{Ru}(\text{bpy})_3]^{2+}$ enhancing the electrochemiluminescence emission. The system has high sensitivity for glucose with a working range between 0.01 and 1000 μM and a LOD of 1.5 nM. It also shows high selectivity for glucose with no response for interfering substances, and it retains over 75 % of activity after 14 days of storage. It was also tested with human

plasma and was able to precisely measure the concentration of glucose even in complex fluids.

For the monitoring this disease is not only necessary to track blood glucose levels but also lactate levels, especially in the case of type II diabetes [115]. Daboss et al. developed a biosensor for the simultaneous detection of glucose and lactate [116]. The system is based on the measurement of H_2O_2 produced by the oxidation of glucose or lactate respectively by glucose oxidase or lactate oxidase. The working electrode was modified with Prussian Blue nanoparticles covered with a nickel hexacyanoferrate shell to improve the stability. These particles are then reduced to Prussian White on the electrode surface. Over the modified working electrode was casted a solution containing the enzymes and perfluorosulfonated ionomer to obtain a membrane after solvent evaporation. For the simultaneous detection are prepared two different electrodes, one with glucose oxidase and one with lactate oxidase. When the two enzymes catalyse the reaction, one of the products is H_2O_2 , that is reduced to hydroxyl ions by the oxidation of Prussian White to Prussian Blue. Prussian Blue then immediately reduces back to Prussian White at the electrode surface and a current proportional to the amount of H_2O_2 can be measured. For the analysis, the two electrodes are placed in a microfluidic device. The system was tested both with human serum and sweat. With serum was tested only the glucose biosensor and before the measurement is needed a dilution of the sample to bring the glucose concentration in the working range of the device. Instead, for sweat analysis, both the electrodes were tested. Also in this case, was needed a dilution but was possible to monitor in real time the concentration of glucose and lactate. The results of the biosensor were compared with conventional measurements techniques and between them there was almost a perfect correlation. Thereby the device can be used for precise monitoring of the patient disease.

Another important biomarker for diabetes diagnosis is glycated hemoglobin (HbA1c), that can be used to monitor the levels of glucose in the blood [117]. Zhou et al. presented an enzymatic biosensor for electrochemiluminescence detection of HbA1c in a simple manner [118]. The system is made of three pads: a base pad for support, an electrode pad for the electric measurements and a detection pad to evaluate the amount of HbA1c. The base pad is a thin polyethylene terephthalate (PET) plate. The electrode pad is done by carbon screen printing onto a hydrophobic cloth. The detection pad is still done by screen printing onto a hydrophobic cloth but using PET ink to create a hydrophilic zone for the reaction. Over this hydrophilic zone are placed the luminol for the chemiluminescence and fructosyl amino-caid oxidase (FAOD) used for the detection. The three parts are then assembled and placed in a protection cover to form the device. The production of H_2O_2 from the FAOD is used for the detection. Indeed, FAOD react with fructosyl valine (FV) with the formation of H_2O_2 . To release the FV from the red blood cells is needed a pretreatment of the sample. In the

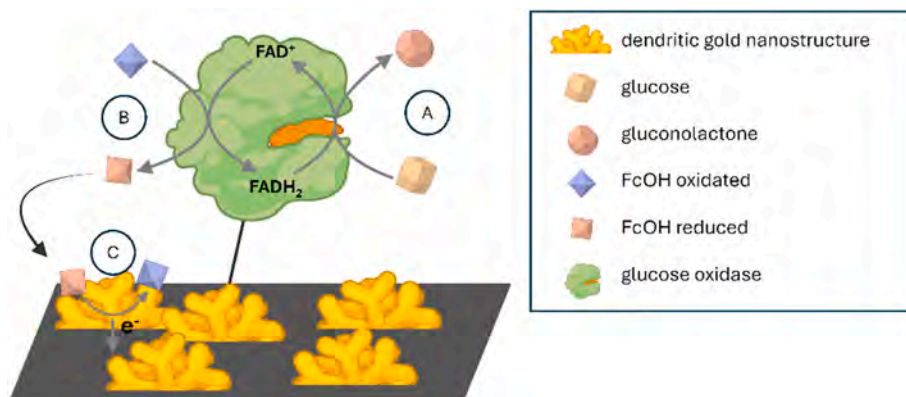


Fig. 13. Schematic representation for the detection of glucose: glucose oxidation (A) and simultaneous FcOH reduction (B), FcOH oxidation at surface electrode (C). It is represented only one enzyme molecule for clarity purposes only.

pretreatment the first step is the hydrolysis of red blood cells, followed by a digestion of the proteins with a specific protease solution. After the pretreatment a drop of the sample can be placed onto the detection pad and it will go only in the hydrophilic zone, where can interact with FAOD. The H_2O_2 produced react with the luminol to emit a fluorescence in the UV spectra. The system was able to evaluate the concentration of HbA1c in human blood and the response was independent from the presence of interfering substances.

For the monitoring of diabetes, it's also important to control the concentration of insulin in blood. For this purpose, Syed et al. developed a colorimetric biosensor [119]. The system is based on antibody for the insulin immobilized on magnetic nanoparticles. With these particles is possible to separate the insulin from the rest of the sample using an external magnetic field. Then another antibody for the insulin is added, modified with horseradish peroxidase. This second antibody is needed for the colorimetric detection. 3,3',5,5'-tetramethylbenzidine (TMB) is used for the detection: it is oxidated by the horseradish peroxidase producing a blue compound. Then is measured the absorbance of the sample with UV/Vis spectroscopy and the measured signal is proportional to the concentration of insulin. The system was able to measure the concentration of insulin both in human plasma and saliva. The results were validated with surface plasmon resonance imaging and there is high correlation between the two. The system also has a linear range compatible with the physiological values of insulin.

A similar approach was followed by Nandhakumar et al. [120]. For the recognition element they used the membrane of human hepatocellular carcinoma cells (HepG2). These cells have highly presence of insulin and glucagon receptor on the membrane and the specific recognition is used for the detection. The membrane of the cells is deposited onto screen printed gold electrode and for the detection are added specific antibodies for insulin and glucagon modified with horseradish peroxidase (HPR). It is also added a solution with H_2O_2 and TMB. In presence of the two analytes, the antigens bind to the membrane and the HPR oxidise the TMB. The oxidized form of TMB is then reduced on the electrode surface and a current is measured. The intensity of the current is proportional to the concentration of insulin or glucagon. Using a cell membrane the adsorption on the electrode is reduced and the background noise is lower compared to other recognition techniques. The system is able to quantify the concentration of insulin and glucagon simultaneously and there is no crosstalk between the two systems. The simultaneous detection is a great advantage for the monitoring of diabetes.

3.4. Inflammatory diseases detection

Inflammatory diseases involve complex enzymatic cascades and dysregulated signalling pathways. Biosensors targeting inflammation-related enzymes and receptors must therefore be highly selective and capable of operating in complex biological fluids. The following examples illustrate how enzymatic biosensing principles can support early detection and monitoring of inflammatory responses. For inflammatory diseases, an important biomarker is NF- κ B which is associated to inflammation but also to tumour development, as can be seen from the Introduction. Lu et al. presented a biosensor for the detection of NF- κ B p50 [121]. NF- κ B is a transcription factor and acts binding to the DNA; therefore, they used a double strand DNA (dsDNA) to isolate NF- κ B from the analysed solution. Differential pulse voltammetry (DPV) technique is used for the detection adopting a modified gold electrode with single strand DNA (ssDNA) covalently bonded to the surface. The detection is based on the specificity of exonuclease III (Exo III) in degrading dsDNA. A solution containing dsDNA is mixed with the solution containing NF- κ B p50, then is added the Exo III and the Cas12a/crRNA duplex to create a Cas12a/crRNA-target triplex. At the end the modified electrode is added and the impedance is measured. If in the analysed solution there is the NF- κ B factor it will bind to the dsDNA, inhibiting the activity of the Exo III resulting in a high signal measured

with the DPV. The working mechanism is shown in Fig. 14. To increase the response is used a Catalytic Hairpin Assembly (CHA) to increase the number of dsDNA in the absence of NF- κ B and increase the reduction of the signal. The system has a high selectivity with a LOD of 0.24 pM and there is a direct correlation between the concentration of NF- κ B and the signal measured with the DPV. The system has also a high selectivity and the response is significantly lower even for other types of NF- κ B, like the p65. In addition, the stability is high with no significant loss over 14 days. The system was tested 10-fold diluted nuclear extract with a recovery rate higher than 99.4 %, showing the ability to detect NF- κ B in real samples.

3.5. Cystic fibrosis detection

In patient with cystic fibrosis, a correlation with high levels of fatty acids and the disease has been found [122], therefore measuring their amount can be used for the diagnosis. Giaretta et al. developed a biosensor for the detection of linoleic acid [123], an acid that belongs to the fatty acids category. For the detection they prepared cotton threads modified with a conductive polymer, PEDOT:PSS, and two enzymes, horseradish peroxidase (HRP) and lipoxygenase (LOX) (Fig. 15). The threads were modified by immersion of the fibers in the solution containing the PEDOT:PSS, the horseradish peroxidase and the lipoxygenase. For the detection the impedance of the modified threads is measured. Lipoxygenase catalyses the formation of H_2O_2 starting from fatty acids. Then horseradish peroxidase interacts with H_2O_2 , reducing it to water causing a reduction in the resistance of the cotton fiber by transferring the electrons to the PEDOT. The response of the system is achieved only in the presence of both enzymes. The system shows a wide linear range with a detection limit of 161 nM, an order of magnitude lower compared to other linoleic acid sensors. The sensor was able to detect linoleic acid in solutions containing different interfering substances and there was not a significantly difference between the solution with linoleic acid only. The system was also able to detect linoleic acid in the presence of others fatty acids, sowing a good selectivity. Therefore, it is possible to use this biosensor for the detection of linoleic acid in complex solutions, like saliva or other biological fluids.

One main limitation of the development of new biosensors for the detection of cystic fibrosis is the lack of specific biomarkers. Therefore, it is fundamental to find them and then develop a proper enzymatic biosensor for their detection.

3.6. Drug screening

Enzymatic biosensors for drug screening usually evaluate the inhibition of the enzymatic activity after the administration of the drug. Leote et al. developed an electrochemical bienzymatic biosensor to evaluate the activity of pyruvate kinase and its inhibition [124]. Pyruvate kinase has an important role in cancer metabolism and there are different therapies to inhibits its activity. The proposed system based its detection on co-immobilization of pyruvate kinase and pyruvate oxidase. The working electrode is made of a SiO_2/Si wafer covered with one layer of titanium and one of gold. Then the enzymes are immobilized by crosslinking with glutaraldehyde. The detection mechanism is based on the reactions catalysed by the two enzymes. Pyruvate kinase transform phosphoenolpyruvate into enol pyruvate with the production of ATP from ADP. The enol pyruvate in acid environment is converted into pyruvate that is used by the pyruvate oxidase to produce acylphosphate, H_2O_2 and CO_2 . Then the H_2O_2 is reduced at the electrode surface to H_2O and an electrochemical signal can be measured. For the measurement was used cyclic voltammetry and fixed potential amperometry. The intensity of the signal depends on the amount of H_2O_2 produced that is stoichiometrically equal to pyruvate, therefore can be used to monitor the activity of the pyruvate kinase. To evaluate the level of inhibition, first the response in the absence of the inhibitor was measured and then the response after the incubation with a solution containing a known

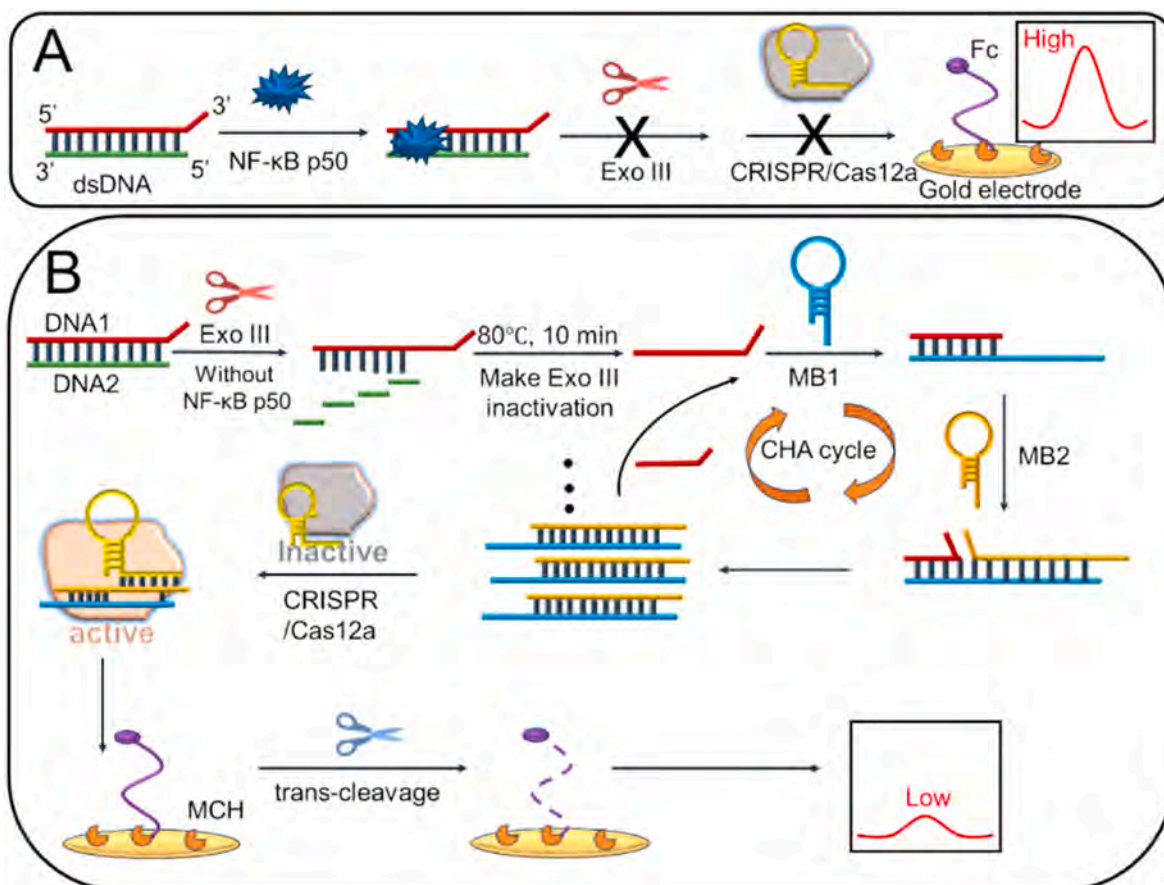


Fig. 14. Schematic representation of the mechanism to detect NF-kB p50: process in the presence of NF-kB p50 (A) and in its absence (B) [118].

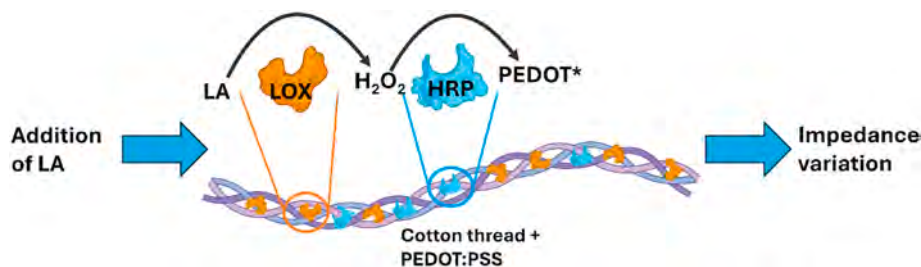


Fig. 15. Schematic representation of cotton threads modification and working mechanism for the detection of linoleic acid.

amount of the inhibitor. The system was tested only with shikonin, that is one of the most used inhibitors of pyruvate kinase and understanding the inhibition mechanism and kinetic was possible. To do so, they measured the inhibition and activity of the enzyme with different concentration of phosphoenolpyruvate and ADP, keeping constant the concentration of the inhibitor. The developed system has high affinity for pyruvate and can simultaneously elucidate the drug inhibition mechanism, with great significance in pharmacology for discovering new drugs and increase drug therapy efficiency.

Li et al. presented a biosensor for the evaluation of the activity of acetylcholinesterase by multicolour visual assay [125]. The system was based on an electrospun membrane modified with gold nanopyrramids to achieve the colorimetric detection. The system is based on the reaction catalysed by the acetylcholinesterase: this enzyme transforms acetylcholine in thiocholine, that subsequently cause the release of Fe^{2+} from $\alpha\text{-FeOOH}$ nanorods. The iron ions catalyse H_2O_2 to produce $\bullet\text{OH}$, that interact with the gold nanopyrramids causing the variation of the colour.

A schematic representation of the working mechanism is shown in Fig. 16. The change of colour can be easily evaluated with an RGB analysis using a smartphone, allowing an easy monitoring. To evaluate the inhibition a calibration curve was computed with a linear range between 0.01 and 500 U/l, using solution with well-known enzyme activity. At the increase of the inhibition there is a shift towards the red colours, while if the inhibition is lower, the colour shift towards the blue. With this sensor, the evaluation of the inhibition efficiency was also possible using equation (5) measuring the wavelength of the peak in different conditions with UV/Vis spectroscopy.

$$\eta = \frac{\lambda_i - \lambda}{\lambda_{\text{control}} - \lambda_i} \quad (5)$$

Where η is the inhibition efficiency, λ_{control} is the maximum absorbance wavelength of blank control (system with only gold pyramids), λ_i is the maximum absorbance wavelength of the system in presence of the enzyme and the inhibitor and λ is the maximum absorbance wavelength

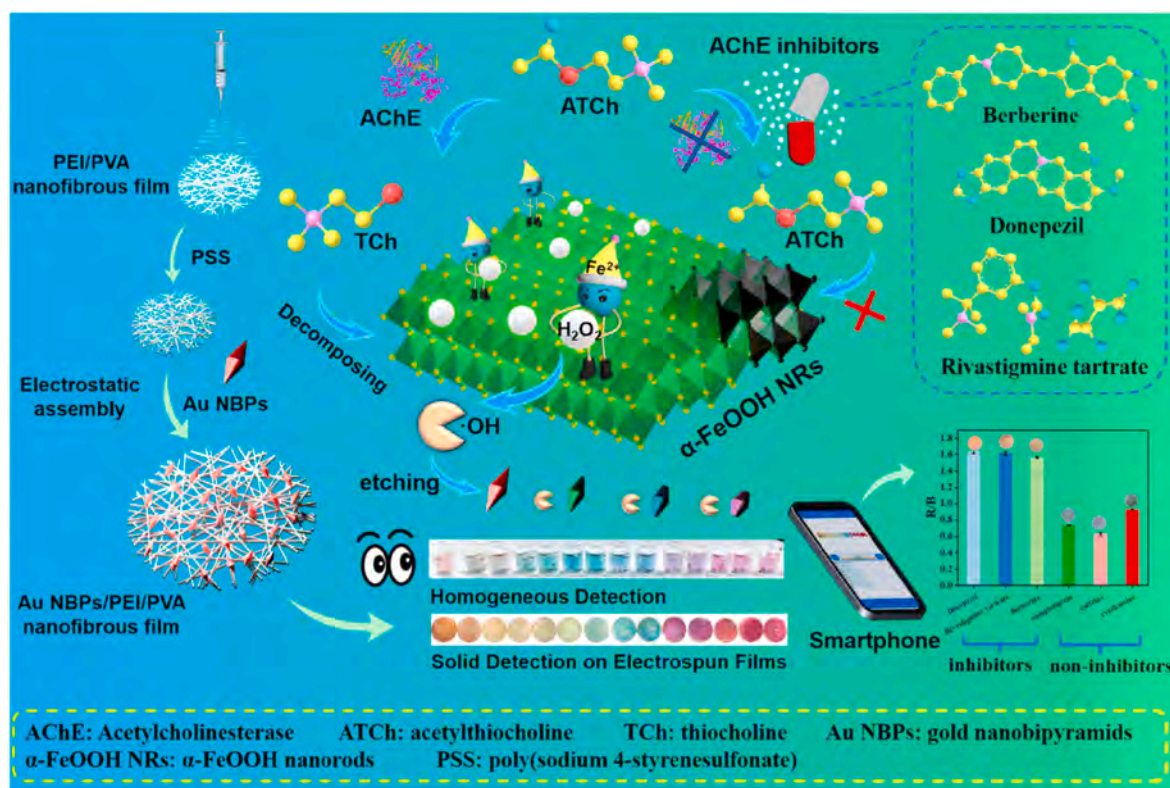


Fig. 16. Schematic representation for the evaluation of the inhibition level of acetylcholinesterase (AChE) [122].

of the system with only the enzyme. Therefore, the developed system can be used to evaluate the inhibition level of different drugs or molecules against acetylcholinesterase.

Wei et al. developed a biosensor for the evaluation of the activity of α -glucosidase using a colorimetric detection [126]. The system is based onto a cellulose membrane modified by covalently bonding the enzyme. The detection is based on 4-Nitrophenyl α -D-glucopyranoside (PNPG), that can be catalysed by the α -glucosidase 4-Nitrophenyl (PNP), producing a yellow compound. The PNPG is adsorbed to the surface and therefore if the enzyme is active the cellulose membrane turns from white to yellow. The inhibition level was evaluated with the colour intensity reduction compared to the initial relative intensity. With the increase of the inhibition the intensity of the colour gradually reduces, making the cellulose membrane to turn back to white. The system was tested with both known inhibitor and non-inhibitor molecules, and the reduction of the colour is present only with the inhibitors, therefore the system has a selective response. The system also had a high stability, with no changes of colour intensity over 120 days. Also, the detection time was fast and require only 80 s. Therefore, the presented device can be used for fast and reliable recognition of inhibiting molecules for α -glucosidase and for new drug discovery.

The biosensors used for drug screening are used for preliminary evaluation of the efficacy of the drug. These systems can be used to test all the candidate compounds and see if they have an effect on the desired enzyme. If the result is negative, then the drug is directly discarded and will not be used in the successive preclinical trials. This could have a huge impact on reducing development time and costs to obtain new drugs. These models have no capability of predicting the behaviour in vivo, due to their simplicity compared to a living organism, therefore cannot be used as a substitute to any of the other conventional tests. However, they can give useful information about the working mechanism of the drug and its effect on the enzyme.

4. New frontiers of enzymatic biosensors

4.1. Artificial intelligence

As described in the previous chapter, with enzyme-based biosensors it is possible to detect specific molecules in really low concentrations. This is useful for diagnostic, but for a more accurate diagnosis, a multiomic approach is taking place. The multiomic approach is based on the analysis of a variety of data and on the extraction of information from their aggregation. It has been developed only recently, thanks to the diffusion of artificial intelligence and machine learning methods to extract the information from the collected data. Artificial intelligence method can become really powerful tools in the healthcare industry in different sectors, such as the decision-making process, knowledge extraction, diagnosis, new drug discovery, patient assistance, personalised medicine and many others [127]. The main difficulty to create a reliable artificial intelligence (AI) system is the possibility to collect data of good quality. Indeed, usually the datasets used for the training are not consistent and have missing values.

In the following paragraphs are presented some examples of enzymatic biosensors that are associated to AI, underlining the implications of its usage.

4.1.1. Diagnosis

Enzymatic biosensors can be useful for both data collection and drug screening associated to AI. Indeed, with this type of biosensors it is possible to construct systems with reliable data acquisition, as shown in the previous chapter, and use this data to train AI models for the diagnosis of diseases. In the diagnostic field AI system can be useful in finding patterns that are really difficult to detect with other methodologies. The knowledge extracted from the data acquired with the biosensor can then be used to perform reliable diagnosis.

This approach was followed by Dempsey et al. and, in their article, they described a biosensor to evaluate the enzymatic activity of

proteases to detect lung cancer [99]. As already described in chapter 3, this system uses a machine learning algorithm to detect the presence or absence of the tumour. They used Emerge, a fitting system that tries to find the algorithm that better represent the relation between the biosensor activity, i.e. the emission of light, and the presence or absence of the tumour. From Fig. 17 we can see that the difference between the activity of the sensor in patient with cancer or without cancer is minimal. Using the artificial intelligence tool, it was possible to distinguish the two categories of patients with a specificity of 82 % and a selectivity of 90 %. This example shows that with AI it is possible to extract information from data that in other ways would be really complicated. This information can then be used for the diagnosis of the pathology with a high accuracy.

4.1.2. Biosensor preparation

AI can also be used to facilitate the process of developing an enzyme-based biosensor. Jesus et al. used an artificial neural network to evaluate the activity of a biosensor composed of a conjugated polymer and enzyme [128]. They perform the test using urease and bonded it to different copolymers films. The detection of the system was based on absorbance: urease, in presence of urea, promotes the formation of ammonia basifying the environment. The change in pH leads to the interconversion between acid and basic forms of the bromocresol purple indicator, causing an increase in absorbance at 588 nm. Absorbance, time and urea concentration were the variables given as input to the artificial neural network (ANN). The ANN uses this data to evaluate the reaction progress and give information about the catalytic activity. Therefore, with this tool is possible to test different substrate for the enzyme immobilization and find the best one. The system can also be used as a predictor model to find out how the reaction will evolve in subsequent time steps. This device is not only helpful in the construction of the system but can give valuable information on how the reaction changes and will evolve at the variation of boundary conditions.

From this example emerge how machine learning algorithm can become really useful in the creation of models to predict the response of the system at the variation of the environmental conditions in which the system operate. These models allow the identification of the optimal working conditions in a faster way. Several parameters can impact the efficacy of a biosensor, include the type of working electrode used, the concentration of enzymes and stabilizing agents, the stabilization procedure, temperature and pH. In the study presented by Farahbakhsh et al., they evaluated the performance of different ML system to find out the best model to predict the biosensor behaviour and find the best parameters [129]. They developed an amperometric biosensor using alcohol oxidase. For the biosensor preparation, they modified a glassy carbon electrode with gold nanoparticles and chitosan. The chitosan was used as stabilizer agent for the enzyme immobilization. The enzyme was

added with the chitosan solution and it's entrapped between the polymeric chains. To facilitate the electron transport, prussian blue was added as an intermediary for electron transfer. After the preparation of the electrode, they started to develop different ML algorithm to find the best working conditions of the system. To evaluate the performance of the biosensors, they used the maximum current measured and the Michelis-Menten constant. They tried four different algorithms, including multilayer perceptron (MLP), deep learning networks (DNN), decision tree (DT) and random forest (RF). All these models can be used for predictions. The best predictive model was the MLP and it was used to evaluate how temperature, pH and ethanol concentration changed the measured current. The increase of the temperature brings an initial increase of the current, but then there is a decrease, and this is true for all values of pH. The pH doesn't have effect on the maximum current measured, but it changes the temperature at which the peak is measure. Therefore, for each pH value there is an optimal temperature. At the increase of the ethanol concentration there is an increase in current, but the slope depends on temperature and pH. The presented biosensor was not thought to be used for the detection of ethanol, but for current production, but this approach can also be followed to find the best operating condition for amperometric biosensors, since the working mechanism is the same. This article shows how ML can facilitate the optimization of biosensors, reducing the number of tests that are needed for the characterization. A drawback of this system is that a model that works well for a biosensor probably won't be the best for another one and further studies are needed on this topic.

4.1.3. Enzyme modification and drug screening

AI can also be used to find the best mutation of an enzyme leading to an increase of the biosensor response.

D'Oelsnitz et al. used a convolutional neural network (CNN) to enhance the activity on an enzyme and increase the production of amaryllidaceae alkaloids [130]. This are complex plant secondary metabolites with recognized therapeutic value that are used in several disease, such Alzheimer's disease. The problem of these compounds is that they are really difficult to extract. For this reason, in this study d'Oelsnitz et al. modified norbelladine 4'-O-methyltransferase (Nb4OMT) to have a higher production of 4'-O-methylnorbelladine (4NB). The Nb4OMT enzyme is responsible for the production of 4NB. With a CNN they generated mutations in the original structure of the enzyme to increase it bioactivity. A schematic representation of the development process is shown in Fig. 18. To test the performance of the new developed enzymes, they built a biosensor using a transcription factor that is regulated by the concentration of 4NB. Also the transcription factor was modified using the same CNN to improve the affinity with 4NB. The modified enzyme showed 60 % improvement in production compared to conventional methods, two times higher

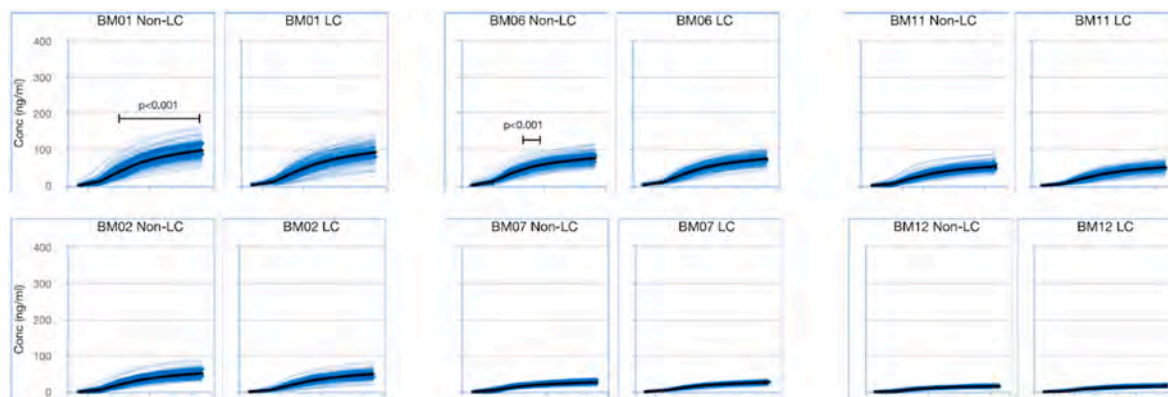


Fig. 17. Biosensor signal recorded at different timepoints (blue lines) and average (black line) for patients with lung cancer (LC) or without lung cancer (Non-LC). Only few biomarkers are reported (for all the data and more information see Ref. [96]).

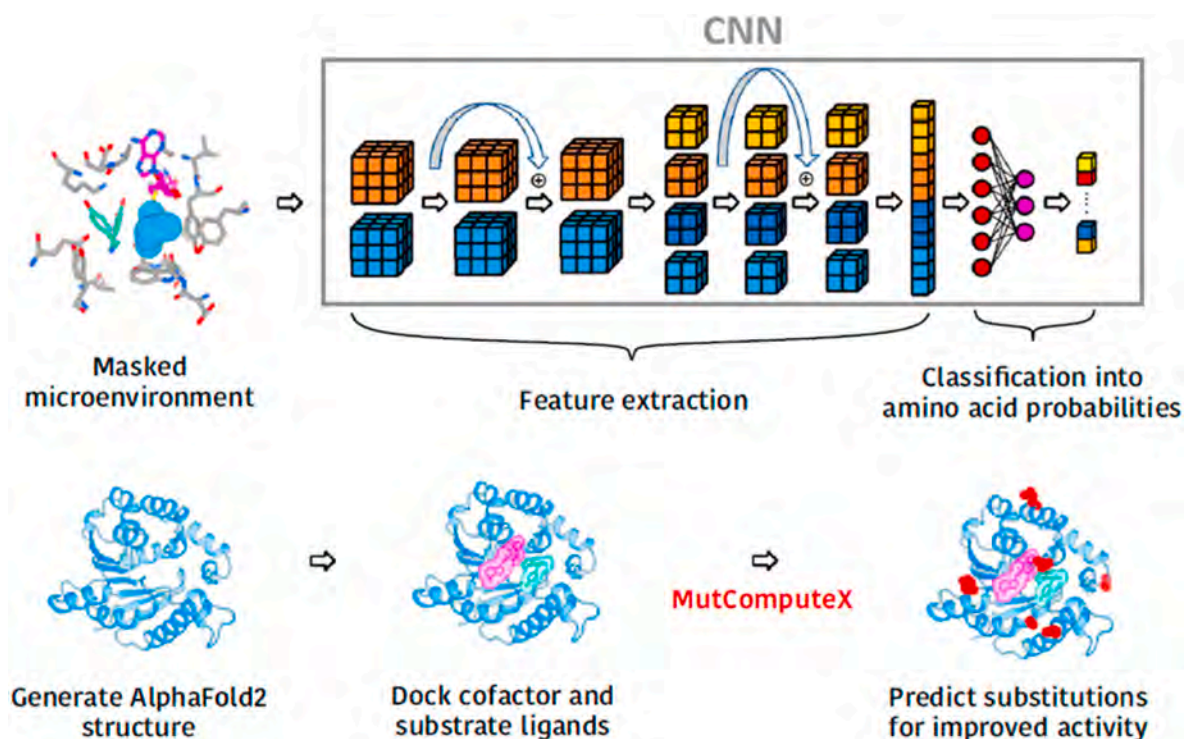


Fig. 18. Schematic representation of the workflow to modify of an enzyme using CNN to improve its bioactivity [127].

catalytic activity and three times lower formation of regioisomers. This works shows the potential of custom biosensor, such as rapid collection of phenotype data under a wide variety of experimental conditions, including determining the kinetics of product formation among strain and enzyme variants. It also opens the way to the formation of ad-hoc built enzymes for the ultra-highly sensitive detections of molecules, really useful for drug screening and early diagnosis.

This system can also have repercussions in new drugs discovery, thanks to AI ability in the optimization of the drug structure design and providing a quicker validation [131]. The AI can generate new molecules or modify existing compounds to increase its activity and enzymatic biosensors can be used as high throughput system to test drugs in a faster, efficient and low costs manner. The AI is also becoming more used to identify possible biomarkers relevant for the diagnosis. This can be helpful for the development of biosensors, because one of the limitations of the development of this system is to find the molecule to detect, and AI can help in this. Therefore, a synergy between artificial intelligence and biosensors can bring high advantages in the healthcare industry.

From these examples is evident how the integration of artificial intelligence methods with biosensors can be beneficial for the development of the biosensors itself. It can also help in the interpretation of the measurements taken with the biosensor and extract new knowledge from them.

4.2. Self-powered biosensors

One big limitation of wearable devices is the power needed to the proper functioning of the system. Usually these devices rely on batteries, that require recharging, battery swap or even substitution of the device. With moder technologies wearable devices are becoming more popular, especially for continuous monitoring of diseases. For this reason, the problem of the power source needs to be solved and enzymatic biosensors may be helpful. Veenuttranon et al. developed a self-powered biosensor for the detection of glucose [132]. The wearable device can convert chemical energy into electric energy and detect glucose in

sweat. The data are then transmitted wirelessly with a specific system. The device is formed by two different electrodes, a cathode and an anode. On both electrode is immobilized glucose oxidase (GOx) that carries out both the functions of detection and energy harvesting. At the bioanode there is the oxidation of glucose by GOx and the subsequent electron transfer toward the printed nanocomposite-based electrode through 1,4-naphthoquinone (NQ). NQ acts as a redox mediator and facilitate the electron transfer towards the anode. The reaction catalysed by GOx happens also at the biocathode and this time is of interest the H_2O_2 produced as a product of the reaction. At the cathode H_2O_2 is reduced with the help of prussian blue. Therefore, the electrons that are produced at the anode can flow through the external load, powering it, and finish at the cathode to allow the reduction of H_2O_2 . A schematic representation of the geometry of the system and of the working mechanism is shown in Fig. 19. To prepare the two electrodes, a new screen-printable ink was developed, allowing compositional changes and supporting the fabrication of highly flexible devices. To improve the electros exchange, the ink was modified with nanostructures, such as carbon nanotubes. The enzyme was immobilized onto the surface of the two electrodes using polyurethane polymers. The detection of glucose is based on measuring the current that the GOx produces to power the device. Indeed, this current is proportional to the concentration of glucose present in the sweat. Therefore, this system is able to simultaneously self-power itself and detect glucose. The system was able to produce enough power for micro-electronics circuits such as DC-DC converter or wireless transmitter. It has a working range between 0 and 20 mM of glucose solution and can monitor the concentration of glucose in that range. Therefore, this biosensor can be embedded in wearable systems for the continuous monitoring of glucose levels without the need of an external power source. This device is really innovative and opens the doors to the fabrications of other devices for the continuous monitoring of diseases in a non-invasive way.

Xia et al. have developed a self-powered enzymatic biosensor for the detection of lactate in human sweat. The system is based onto a textile matrix made with cellulose fibers (TMC) [133]. To improve the electrons transfer, the surface was modified with carbonous nanostructures, like

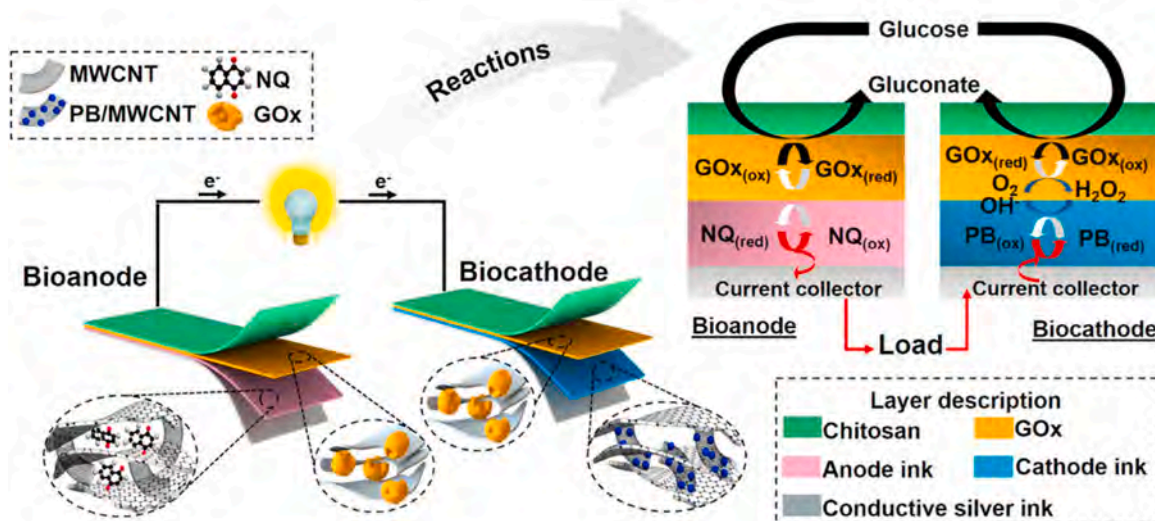


Fig. 19. Schematic representation of the geometry of the system and of the working mechanism [129].

carbon nanotubes and reduced graphene oxide. This modified membrane was used to prepare both the anode and the cathode of the system. The anode was further modified with lactate dehydrogenase (LDH) immobilized with a polymeric hydrogel. The cathode was modified with laccase, still immobilized with the same polymeric hydrogel. Using the hydrogel was advantageous because it allows the enzyme to retain a high catalytic activity. Then the two electrodes were assembled to form the system. To produce energy are used two opposite reactions at anode and cathode. At the anode, LDH converted lactate to pyruvate, while NAD^+ is reduced to NADH. NADH is then oxidized on the modified electrode surface with the regeneration NAD^+ and the accumulation of electrons on the surface. On the cathode surface, laccase is reduced by the electrons produced at the anode by the LDH. Therefore, if the two electrodes are connected to an external load, they can power it. The usage of TMC allow the system to be flexible and elastic, but resistant and able to withstand loads, therefore it can be used for wearable applications. The system was able to produce enough power to self-sustain itself and to have a good detection of lactate. The detection limit was quantified at $9.49 \mu\text{M}$ and the response with interfering substances (NaCl, KCl, urea and glucose) was lower than 8 %. Therefore, the developed biosensor can be used for continuous monitoring of lactate for patients with certain diseases, but also for healthy people while they are practicing sport activities. This device shows that is possible to create power by combining two redox reactions also from waste products like lactate and simultaneously monitor its concentration.

One limitation of wearable biosensors is that the performances are highly influenced by the contact between the device and the skin. For a good detection, the biosensor needs to be in close contact with the patient skin, and therefore a system to push it close to the epidermis is needed. Thanks to the flexibility of the biosensors they can be integrated into elastic induments, keeping a good contact without discomfort for the patient. A future option may be the implantation of the device directly under the skin, but this requires future developments, especially with regards to materials and their biocompatibility.

Self-powered biosensors can also be applied for multiple biomarkers detection for in vitro applications. Wang et al. prepared a biosensor based on glucose oxidase (GOD) for the detection of two different typer of miRNAs [134]. These small molecules can play an important role in different pathologies development, including cancer, but their detection is complicated by the fact that are present in extremely low concentrations [135]. The proposed system is based on carbon paper (CP) electrodes, modified for both the detection and the power generation. At the biocathode, the CP is modified with gold nanoparticles and bilirubin

oxidase (BOD), while at the bioanode, the CP is modified with gold nanoparticles and a single strand DNA (DNA1) for the miRNAs capture. The role of the gold nanoparticles is to increase the electroactive area and improve the electron transfer, while the BOD acts as the electron acceptor. The system evaluates the presence of miRNA-21 and miRNA-155, turning in an "on" or "off" state. When miRNA-21 is present, it hybridises to the DNA1, allowing the binding of GOD modified with DNA2 to the anode surface, by hybridizing with another sequence of miRNA-21. The GOD will oxidise the glucose with the formation of electrons at the anode, while the BOD is reduced at the cathode. This allows the formation of a high open circuit tension indicating the "on" state of the sensor. In the presence of miRNA-155, it will replace the miRNA-21 and will detach the GOD from the surface. In this case a DNA3 modified with SiO_2 nanospheres and gold nanoparticles will hybridize to the miRNA-155, inhibiting the interaction of the GOD with the anode. In this case the open circuit tension will be lower, indicating the "off" state of the biosensor. In Fig. 20 is shown a schematic illustration of the construction and working mechanism of the biosensor. For the detection is used the variation of open circuit tension, both for a qualitative and quantitative measurements. From the increase or decrease of the open circuit tension, was possible to evaluate if miRNA-21 or miRNA-155 were respectively present and from the magnitude of the variation was possible to quantify their concentration in serum. For the miRNA-21 the detection limit was quantified at 0.17 fM , while for the miRNA-155 it was 0.37 fM , allowing for an ultrasensitive detection of miRNAs. They also measured the response of different interfering substances, like other miRNAs or nucleotide sequences with only one base mismatch and the response was at least four times lower in both cases. This self-powered biosensor allows for ultrasensitive detection of more compounds simultaneously, combined with the production of energy to power the device.

Self-powered biosensors are rapidly spreading because they do not require an external energy source and probably in the next years they will increase in number and will be able to detect a greater variety of biomarkers. The next step of this typology of biosensors is the integration of drug administration in an autonomous way. They will be able to detect the concentration of a certain molecules and release the right amount of drug needed for the therapy. Therefore, they will be able to simultaneously monitor the pathology evolution and give the therapy to the patient. There are already several examples of patches for smart drug delivery, like the work presented by Ye et al. [136], but they are usually for the treatment of diabetes. In future years probably there will be the production of enzymatic biosensor also for the treatment of more

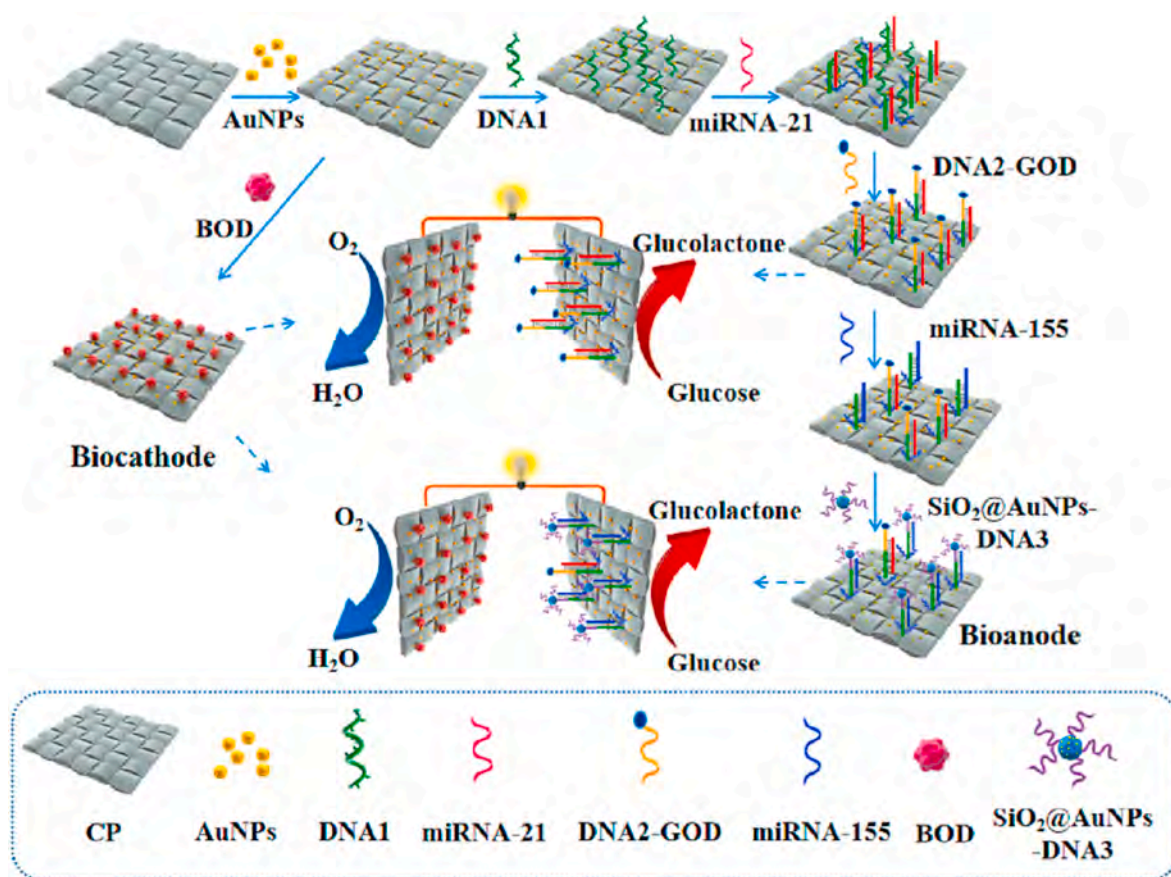


Fig. 20. Schematic illustration of “on-off” self-powered biosensors for ultrasensitive detection of miRNA-21 and miRNA-155 [131].

complex pathologies.

5. Conclusions

From the Introduction, it is clear how important are proteins and enzymes in the development of a disease. They carry out fundamental roles in our body and their dysregulation or alteration may lead to the onset of different pathologies (Table 1). Therefore, their monitoring is essential to trace out the formation and the evolution of the pathology. To perform these tasks, are needed devices with extremely high sensibility and with detection limits in the micromolar concentrations. Devices like so may be enzymatic biosensors, thanks to the high affinity of the enzymes for the substrates involved in the reactions they catalyse. The development of an enzymatic biosensor is complex and there are several factors that need to be considered, like the immobilization technique and detection method. Each decision taken during the fabrication of the device leads to different performance of the final product and the best solution depend on several factors, first of all the enzyme used and the final goal of the sensor. There are already several biosensors for the monitoring and the detection of different biomarkers, each one with its own characteristics. One common element to all is the usage of nanostructured materials, that are essential to increase the active area of the device and facilitate the reactions involving the enzymes. The first enzymatic biosensor was for glucose monitoring and nowadays they have reached an ultra-sensitivity and are usually associated to the simultaneous detection of other metabolic compounds. Recently biosensors for other diseases are developing, especially regarding molecules involved in tumour development and progression. These enzymatic biosensors may have an important role in the diagnosis, especially in early stage of the pathology thanks to their high sensitivity and selectivity. Particular attention has also been placed onto

neurodegenerative disease, due to the recent knowledge acquired on the topic. Several enzymatic biosensors have been developed for an ultra-sensitive detection of biomarkers involved in this typology of diseases, leading to the possibility of an early diagnosis. Enzymatic biosensors can also be useful for drug screening applications. Indeed, several enzymes can be targeted by different drugs to have therapeutic results and evaluate the effect of the drug on the enzyme is fundamental. For the development of new drugs, several different compounds must be tested and high throughput system, like enzymatic biosensors, can make the difference and change the production of drugs, reducing costs and development time.

Enzymatic biosensors allow for low concentration detection of biomarkers, but from these values is difficult to perform a diagnosis. A help can be given by artificial intelligence (AI) system, and in the next years more applications of biosensors associated to artificial intelligence algorithms will be developed. The first devices have already been fabricated with good performances especially in the diagnostic field and they will develop towards a multiomic approach. Artificial intelligence can also help in the development of the device itself under different aspects. With AI it is possible to create predictive models to know how well the biosensor will perform, without the need to execute of all the characterization test. It can help to choose the best substrate for the enzyme immobilization and identify the best working conditions for the system, reducing the time needed for the development of the device. It can also help for the construction of the enzyme itself, suggesting modification to the original structure to enhance the affinity for the molecule to detect. This will increase the sensitivity of the device leading to a higher accuracy. There are already some models to perform this task and many others will be developed. The main advantage brought by AI is the possibility to carry out tasks that with other systems seems impossible to do, and that's why they are spreading in all fields. The evolution of

biosensors is not only with AI, but also towards self-powered biosensors. They allow a continuous monitoring of the biomarkers, without the need of external power sources, since the biosensor will produce the energy it needs. These devices can find vast applications in the wearable devices, not only for patients and for the monitoring of their disease, but also for sport medicine, monitoring substances contained in sweat.

Enzymatic biosensors bring some unique characteristics and advantages, can find applications in several fields and with the help of AI can become really powerful tools to perform tasks in a way that with already present technologies will be almost impossible to do in a sustainable way.

CRedit authorship contribution statement

Edoardo Bocina: Writing – original draft. **Clarissa Cocuzza:** Methodology, Investigation. **Chiara Vincenzi:** Methodology, Investigation. **Debora Fino:** Supervision. **Valentina Cauda:** Supervision. **Marco Piumetti:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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