Journal of Pharmaceutical and Biomedical Analysis 224 (2023) 115195

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Electrochemical devices for cholesterol detection

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A B S T R A C T

Cholesterol can be considered as a biomarker of illnesses such as heart and coronary artery diseases or arteriosclerosis. Therefore, the fast determination of its concentration in blood is interesting as a means of achieving an early diagnosis of these unhealthy conditions. Electrochemical sensors and biosensors have become a potential tool for selective and sensitive detection of this biomolecule, combining the analytical advantages of electrochemical techniques with the selective recognition features of modified electrodes. This review covers the different approaches carried out in the development of electrochemical sensors for cholesterol, differentiating between enzymatic biosensors and non-enzymatic systems, highlighting lab-on-a-chip devices. A description of the different modification procedures of the working electrode has been included and the role of the different functional materials used has been discussed.

1. Introduction

Speaking about sensors and lab-on-a-chip devices for pharmaceutical and biomedical analysis, it is inevitable to think of the electrochemical analysis of cholesterol. The particular chemical characteristics of cholesterol allows it to organize cell membranes, determine the lipid phase balances, form lipid domains and myelin, act as a precursor to steroid hormones and vitamin D, regulate protein receptor function and traffic and affect both the pathophysiology and biological responses to drugs used to treat human diseases, in some cases by directly controlling the passage of the drug across membranes and living cells [1,2]. Considering the huge importance of this biomolecule, the exhaustive control of its levels through easily accessible analyses is an urgent public health issue since it would be useful in the diagnosis and prevention of a number of clinical disorders, including heart diseases. Moreover, the determination of cholesterol content in food is also vital to select low intake cholesterol diets and lifestyle recommendations adjusted to each individual in particular [1,3,4]. Methodologies of high analytical resolution are continuously explored to evaluate the presence of small amounts of cholesterol in biological entities [1]. In this way, the electrochemical detection of this compound could be really interesting to produce point-of-care devices, considering the versatility of size, geometry and nature of electrodes that can be integrated and the minimum instrumentation required [2,5–8]. Thus, highly selective enzymatic electrochemical sensors have been developed in last decades, mainly based on the high specificity of cholesterol oxidase (ChOx) and its combination with peroxidases [2,9,10]. These enzymatic assays allowed not only the determination of the free cholesterol form, but also of its esterified form, both frequently present in biosamples by means of bienzymatic systems based on the joint modification with ChOx and cholesterol esterase (ChE) [11–14]. Despite the high selectivity and exceptional sensitivity of enzyme-based sensors they present some drawbacks related to the decrease of enzyme activity with the use of the sensor, being also affected by temperature and pH among other parameters [15,16]. In order to overcome these drawbacks, nonenzymatic cholesterol sensors have been used recently [11]. Thus, the main focus of this review is to provide an updated critical overview of different reported electrochemical sensing and biosensing strategies for cholesterol detection, ranging from classical devices architectures to intelligent sensor systems.

2. Electrochemical biosensors

Research on the electrochemical analysis of cholesterol initially focused on enzyme-based biosensor platforms, with related publications dating back to the early 1990s and even earlier [17]. Chemical reactions between cholesterol and immobilized biomolecules on a working electrode lead to a measurable current, or potential [18], related to the concentration of this target analyte. ChOx, which catalyses the oxidation and isomerization of cholesterol to cholest-4-en-3-one with H_2O_2 production, has mainly been used with this aim. The simplest biosensors are based on recording the amperometric current due to the H_2O_2 oxidation, which is proportional to the amount of cholesterol originally present in the sample, according to Fig. 1A [19–22]. In order to decrease the high anodic potential usually required on monitoring this process, + 0.7 V approx., an alternative strategy has been to replace the natural acceptor

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https://doi.org/10.1016/j.jpba.2022.115195
Received 24 October 2022; Received in revised form 28 November 2022; Accepted 30 November 2022
Available online 5 December 2022

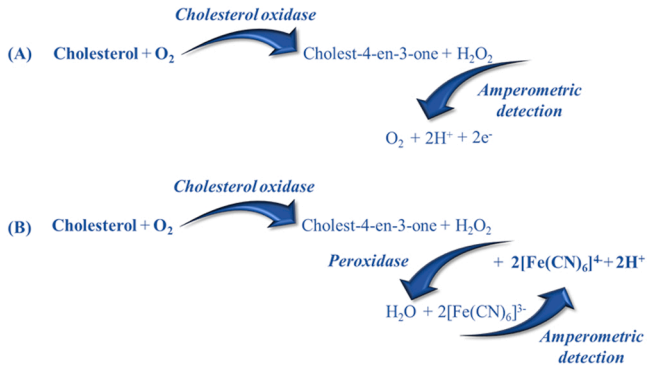

Fig. 1. Cholesterol detection mechanisms for (A) first-generation cholesterol oxidase-based biosensors and (B) second-generation by enzymatic biosensors based on the use of potassium ferrocyanide as mediator.

of electrons (O_2) by mediators, such as Prussian Blue [23] or hydroxymethylferrocene [24,25].

To minimize potential interferences from electroactive substances commonly present in samples containing cholesterol, low-potential amperometric measurements of the enzymatically generated H_2O_2 has also been attempted modifying metals and carbon surfaces of solid electrodes by carbon nanotubes (CNTs) [26], graphene [27,28], metallic nanoparticles (NPs) [29–31], transition metals nanomaterials [32–34], rare earth metal oxides nanomaterials [35], conductive polymers [36–38], quantum dots [39–41], hybrid combinations of them [42,43,52–55,44–51], and even redox mediators [4,56–60] (Table 1). The incorporation of these materials leads to improve the catalyst activity [47], the electrical conductivity [52,61], the enzyme loading, and provides even a platform for biocompatibility and immobilization chemistry suitable for the enzyme attachment [40,55]. In fact, the enzyme immobilization is a critical step in assembling electrochemical biosensors and plays an important role in their performance. Different approaches have been followed for enzyme immobilization on the above-mentioned electrodes surfaces, including from the simple adsorption to permselective membranes, which encloses the enzyme avoiding leakage and providing protection to interferences, controlled molecular architectures or covalent procedures that lead to oriented and highly reproducible systems (Table 1). The validity of most of these devices has mainly been checked by analysing cholesterol content in serum samples. Since the detection of total cholesterol (free and esterified) is desirable, ChE has often been used for this analysis, both immobilized [4,29,34–36,56] or in solution [28,42,62]. This enzyme hydrolyses cholesteryl esters to produce free cholesterol, which is subsequently oxidized by cholesterol oxidase with H_2O_2 production.

Moreover, bienzymatic sensors have been developed with this aim as well (Table 1). ChOx has been combined with a peroxidase enzyme and a redox mediator, such as potassium ferrocyanide, being in this case the resulting potassium ferricyanide reduced at the electrode surface related to cholesterol concentration (Fig. 1B) [63,64]. Further modification of the electrode surface with CNTs prior to enzyme immobilization has been shown to promote electron transfer, improving sensitivity and linearity [65], as well as with AuNPs/chitin-ionic liquid/poly(3,4-ethylenedioxypyrrole)/graphene-multiwalled carbon nanotubes-1,1′-ferrocenedicarboxylic acid-ionic liquid [66] or AuNPs/Poly-(diallyldimethyl-ammonium chloride)/CNTs with hydroquinone as redox mediator [48]. The development of redox mediator-free bienzymatic devices has been achieved as well, usually combining horseradish peroxidase (HRP), which can facilitate direct electron transfer to the electrode, and suitable matrices such as nanoporous Au networks [67], AuNPs/CNTs/poly(allylamine hydrochloride) [68], polyaniline [69] or poly(N[3-(trimethoxysilyl)propyl]aniline) [70] that allow immobilizing high loading of enzymes.

Cytochrome P450scc [71,72] and apolipoprotein B-100 antibody [73] have also been used, to a lesser extent, for the sensitive detection of cholesterol. Cytochrome P450scc catalyses the cholesterol side chain cleavage reaction, the initial and key step in the regulation of steroid hormone biosynthesis. Their specific interaction allows recording a current time response as a function of the cholesterol concentration. Therefore, Cytochrome P450scc has been immobilized, by crosslinking with glutaraldehyde and bovine serum albumin or in an agarose matrix [71], onto screen-printed rhodium graphite electrodes or by adsorption onto CNTs/AuNPs/screen-printed rhodium graphite electrodes [72]. The interaction of lipoproteins and apolipoprotein B-100 antibody is also well known. A NiO thin film/ITO has been utilized as efficient matrix for the covalent immobilization of this antibody using carbodiimide/hydroxysuccinimide chemistry [73].

3. Electrochemical non-enzymatic sensors

As it has been described above, enzymatic biosensors present high sensitivity, specificity and rapidness in the determination of cholesterol. Most of these methods are based on the use of cholesterol selective enzymes which are expensive and require a friendly environment to retain their catalytic properties, being affected by pH and temperature [16]. As a result, non-enzymatic cholesterol sensors have been developed trying to overcome these disadvantages.

3.1. Non-enzymatic cholesterol detection on bare electrodes

Bare electrodes have been used for the direct electrooxidation of cholesterol in organic medium, being first reported by Kowalski et al. in 2005 [74]. Thus, cholesterol can be detected at far positive potentials on a Pt electrode using glacial acetic acid, containing sodium perchlorate and sodium acetate, as supporting electrolyte. The direct oxidation of cholesterol in this medium gave rise to two acetoxycholesterol species as main products (Fig. 2). However, when the direct oxidation process takes place in dichloromethane with tetrabutylammonium tetrafluoroborate (TEABF 4) using separated cathodic and anodic compartments, a dicholesteryl ether is obtained (Fig. 2) [75]. Thus, the electrochemical oxidation of cholesterol may lead to different products, depending on the solvent and supporting electrolyte used. In this way, the oxidation mechanism showed in Fig. 3 was proposed to occur in acetonitrile containing lithium perchlorate using a carbon fiber column electrode [76]. This direct oxidation can also be followed in acetonitrile containing perchloric acid medium when using a GCE and a boron-doped diamond (BDD) electrode, being properly applied to the determination of cholesterol in dairy products [77]. Pencil lead electrodes have been used as well in acetonitrile medium containing, in this case, acetone and lithium perchlorate. This electrode exhibited a high sensitivity and a wide linear range for voltammetric and amperometric measurements of serum cholesterol and skin cholesterol [78].

The registered current due to the direct electrooxidation of cholesterol can be enhanced when using nanoporous Pt [79], Au/Pt [80], and Cu_2S [81] working electrodes. These porous electrodes present high surface/volume ratio, thereby being favourable for electrochemical analysis at lower positive potentials. This decrease in the potential may also be achieved using electrochemical methods based on the indirect electrochemical oxidation of cholesterol. These methods often involve the presence of redox mediators (M) which oxidized state (M_{ox}) forms adducts with cholesterol, giving oxidized products (P) according to the scheme shown in Fig. 4 [82,83].

Several works describe the indirect oxidation of cholesterol using ionic species as mediators such as Co^{2+} cations, which have been used for the electrocatalytic oxidation of cholesterol through electrochemically generated Co^{3+} cations in acetonitrile containing lithium perchlorate [84]. The same electrochemical medium has been used by Maki et al. [85] for the indirect oxidation of cholesterol, due to the formation of a Tl(II)-hematoporphyrin-O_2 adduct. Bromine species have also been selected as redox mediators using a Pt electrode and different

Table 1
ChOx amperometric biosensors based on the use of nano-sized materials or polymers for cholesterol detection.

Working electrode	Nano-sized materials/polymers modification	Immobilized biomolecules	Immobilization procedure	Redox mediator	Real sample	Reference
Au	CNTs	ChOx	Layer-by-layer deposition of poly (diallyldimethylammonium chloride)	–	–	[26]
Au	1.6-Hexanedithiol/Au NPs/11-mercaptoundecanoic acid	ChOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	–	Serum	[42]
Au	AuNPs/acetone-extracted propolis/CNTs	ChOx	Adsorption	–	–	[52]
Au	AuNPs/CNTs/poly(allylamine hydrochloride)	ChOx/HRP	Layer by layer assembly technique	–	Serum	[68]
Pt	Polypyrrole-polyaniline film	ChOx	Glutaraldehyde crosslinking	–	–	[37]
Si/Ag	ZnO nanorods	ChOx	Nafion membrane	–	–	[32]
Si/Ag	ZnO nanotubes	ChOx	Nafion membrane	–	Serum	[33]
Ti	Nanoporous Au networks	ChE/ChOx/ HRP	Entrapment in chitosan	–	Margarine, butter and fish oil	[67]
Waxed graphite	Pt decorated CNTs	ChOx	Tetraethoxysilane sol-gel	–	Serum	[46]
Graphite	–	ChOx/HRP	Graphite composite	$[Fe(CN)_6]^{4-}$	Butter, lard, egg yoke	[64]
GCE	Reduced graphene oxide	ChOx	Polypyrrole entraptmen	–	Serum	[27]
GCE	Reduce graphene oxide	ChOx	Layer-by-layer deposition of polyethylene imine	–	Serum	[28]
GCE	AgNPs	ChOx	–		Serum	[31]
GCE	Cu-Pt-Bi nanocomposite	ChOx	Nafion membrane	–	Serum	[30]
GCE	Dithienolpyrrole(ariyl)aniline	ChOx	Glutaraldehyde crosslinking	–	Serum	[38]
GCE	Poly (propylene imine) dendrimer and core-multishell CdTe/CdSe/ZnSe quantum dots	ChOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	–	–	[40]
GCE	Cerasome /graphene quantum dots	ChOx	Layer-by-layer deposition of polyethylene imine	–	Serum	[41]
GCE	AuPtNPs/chitosan/1-butyl-3-methylimidazolium chloride	ChOx	Glutaraldehyde crosslinking	–	Serum	[62]
GCE	AuNPs/Graphene nanoplatelets	ChOx	Nafion membrane	–	–	[47]
GCE	AuNPs/Poly-(diallyldimethyl-ammonium chloride)/ CNTs	ChOx	Nafion membrane	–	Serum	[48]
GCE	Au/Pt functionalized ZnO nanorods/CNTs	ChOx	Adsorption	–	Serum	[49]
GCE	Polypyrrole/AuNPs/CNTs	ChOx	Adsorption	–	Serum	[51]
GCE	Polyaniline/CNTs/PtNPs	ChOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	–	Serum	[54]
GCE	AuNPs/Graphene nanosheets	ChE/ChOx	Entrapment in chitosan/polyvinyl alcohol	Ferri/ ferrocyanide	–	[56]
GCE	–	ChOx/HRP	–	$[Fe(CN)_6]^{4-}$	–	[63]
GCE	AuNPs/Poly-(diallyldimethyl-ammonium chloride)/ CNTs	ChOx/HRP	Nafion membrane	Hydroquinone	Serum	[48]
GCE	AuNPs/chitin-ionic liquid/poly(3,4-ethylenedioxypyrrole)/graphene-multiwalled carbon nanotubes-1,1′-ferrocenedicarboxylic acid-ionic liquid	ChE/ChOx/ HRP	Glutaraldehyde crosslinking	–	Serum	[66]
GCE and SPCE	Polyethylene imine/reduced graphene oxide	ChOx/GOx	Nafion membrane	Ferrocene	Serum	[58]
SPCE	Polyaniline/crystalline nanocellulose/ 1–butyl-3–methylimidazolium chloride	ChOx	Glutaraldehyde crosslinking	–	–	[53]
SPCE	PtNPs/Reduced graphene oxide/Poly(3-aminobenzoic acid)	ChOx/GOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	–	Serum	[55]
SPE	CNTs	ChOx	Entrapment in polydopamine	Prussian Blue	Serum	[59]
SPCE	AuNPs/Graphene oxide	ChE/ChOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	$AgNO_3$	–	[4]
SPCE	PtNPs/ferrocene carboxylic acid/chitosan/reduced graphene oxide	ChE/ChOx	Adsorption	Ferrocene carboxylic acid	Serum	[60]
SPCE	CNTs	ChE/ChOx/ POD	Carboxymethyl cellulose layers	$[Fe(CN)_6]^{4-}$	Serum	[65]
AuSPE	Nanostructured Pt thin films/Polydopamine	ChOx	Covalent graft by Schiff base or Michael-type additions	–	–	[43]
ITO	Au nanostructures	ChE/ChOx	Glutaraldehyde crosslinking	–	Serum	[29]
ITO	Anatase titania nanofibers	ChE/ChOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	–	–	[34]
ITO	*n*-Tm_2O_3 nanorods	ChE/ChOx	Electrostatic interactions	–	Serum	[35]
ITO	Polyaniline film	ChE/ChOx	Glutaraldehyde crosslinking	–	Serum	[36]
ITO	Thioglycolic acid capped CdS quantum dots	ChOx	Covalent interactions via carbodiimide/hydroxysuccinimide ligand chemistry	–	–	[39]

(*continued on next page*)

Table 1 (*continued*)

Working electrode	Nano-sized materials/polymers modification	Immobilized biomolecules	Immobilization procedure	Redox mediator	Real sample	Reference
ITO	ZnO NPs/Chitosan	ChOx	Physiosorption	–	–	[44]
ITO	SnO_2 NPs/Chitosan	ChOx	Physiosorption	–	–	[45]
ITO	Polyaniline	ChE/ChOx/POD	Glutaraldehyde crosslinking	–	–	[69]
ITO	–	ChOx/HRP	Entrapment in poly(N[3-(trimethoxysilyl)propyl]aniline)		Serum	[70]
FTO	AuNPs/ZnONPs/CNTs	ChOx	Adsorption	–	Serum	[50]

AuSPE: gold screen-printed electrode; FTO: Fluorine doped tin oxide; GCE: Glassy carbon electrode; GOx: Glucose oxidase; IDEs: nano-sized carbon interdigitated electrode; ITO: Indium Tin Oxide; POD: peroxidase; SPCE: screen-printed carbon electrode.

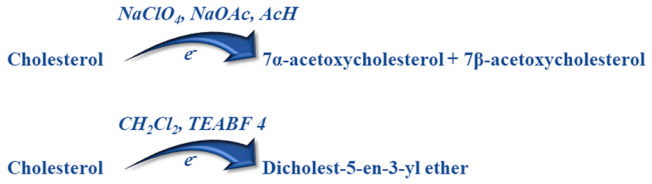

Fig. 2. Electrochemical oxidation of cholesterol at Pt electrodes.

Fig. 3. Electrochemical oxidation of cholesterol in acetonitrile using bare electrodes.

Fig. 4. Scheme of the indirect oxidation of cholesterol in presence of redox mediators.

media, including dimethylformamide [16] and acetonitrile [86], following a peak attributed to Br/Br^+ redox pair. When a mixed medium of dimethylformamide and water [87] is used, the proposed redox mechanism is slightly different, since the species acting as mediator were the BrO^- ions electrogenerated under galvanostatic conditions from Br^-.

The indirect determination of cholesterol can also be performed using electrochemical methods based on indicator displacement assay. These methods rely on a competition between an electroactive indicator and the analyte. The presence of the analyte provokes the displacement of the indicator from a complex, which causes a change in the registered electrochemical response using different electroanalytical techniques [82]. In this way, a complex formed with methylene blue and graphene functionalized with β-cyclodextrin was added to the electrochemical cell for the indirect determination of cholesterol. Cholesterol replaces the methylene blue molecule, which then moves to the solution, being electrochemically detected using a Pt wire electrode [88]. A similar procedure has been developed by Canganboina and Doong [89] using a GCE. In this case, functionalized nitrogen-doped graphene quantum dots covalently linked to β-cyclodextrin were added to the electrochemical cell for the analysis of cholesterol in serum samples through the described host-guest interaction.

Most of the methods implied a high redox potential for the detection of cholesterol, which is naturally related to a lack of selectivity due to the presence of other compounds likely to be oxidized at lower potentials. Thus, different modified electrodes have been developed in order to reduce the overvoltage.

3.2. Non-enzymatic cholesterol detection on electrodes modified with nanomaterials

Among the different modified electrodes used in the determination of cholesterol, those based on the modification of the working electrode with nanomaterials stand out. The special electrochemical properties of these materials, already described above, have made their use frequent in the development of different types of enzymeless-based sensors. Thus, a carbon nanotube electrode fabricated on an insulating Teflon material has been used for the sensitive and selective analysis of cholesterol in sulfuric acid medium, following the electrochemical signal obtained at a low potential (−0.27 V), according the mechanism described in Fig. 5 [90].

The analysis of cholesterol in dairy products has also been performed using a carbon paste electrode modified with CNTs by mixing graphite powder with a composite of polyaniline, starch and CNTs. In this case, the effective interaction of the composite material with cholesterol generated an oxidation signal at a lower potential value [91]. Carbon nanofibers-based electrodes have also been described for the analytical determination of cholesterol, using a working electrode constructed by the dispersion of the carbon nanofibers with electro-conductive Cu/Ni bimetallic NPs, which helped to grow a poly methyl orange film on the electrode surface. This polymeric film acted as the recognition element for cholesterol determination in serum samples [92]. Other carbon containing nanomaterials often used in the determination of cholesterol have been graphene [93,94] and reduced graphene oxide [95]. Thus, Rengaraj et al. [93] developed a sensitive sensor for the analysis of cholesterol in milk samples based on a NiO/graphene composite. The sensor showed a high sensitivity related to the exceptional catalytic activity of NiO. In the same way, the modification of a carbon paste electrode with reduced graphene and CuO led to the development of a very sensitive sensor for the simultaneous analysis of cholesterol, ascorbic acid and uric acid by square wave voltammetry [95]. Graphene has also been used in combination with other materials such as polyaniline nanofibers for the generation of a nanocomposite used in the modification of an ITO electrode, being the obtained sensor highly selective towards cholesterol [94].

Other nanomaterials widely used in the development of sensitive sensors for the analysis of cholesterol have been metallic NPs. In this way, planar carbon electrodes modified with CoNPs and NiNPs have been employed for the simultaneous amperometric determination of insulin, glucose, cholesterol, and uric acid in serum samples using a

Fig. 5. Scheme of the oxidation of cholesterol in H_2SO_4.

sequential-injection system [96]. In the same way, different types of AuNPs and AgNPs (individual NPs, core-shell NPs, nanoalloys and NPs synthesized electrochemically) have been immobilized on a GCE, showing a great capability for electrochemical oxidation of cholesterol [97]. Another sensitive sensor based on modified GCEs involved the deposition of porous tubular AgNPs on the electrode surface. The developed sensor allowed the analysis of cholesterol in serum samples by means of amperometric measurements in a NaOH solution, showing these porous NPs have higher electrocatalytic activity to the electrochemical oxidation of cholesterol than solid nanorods [98]. Likewise, nanoporous Pt modified electrodes demonstrated high sensitivity for the continuous free-cholesterol monitoring using a micro-needle array system [99].

Nanomaterials based on transition metals have also demonstrated electrocatalytic activity in the analysis of cholesterol. Thus, an amperometric nonenzymatic sensor, based on the simple synthesis of Cu_2S nanoroses on a Cu rod to generate an integrated electrode, has demonstrated a good sensitivity and a great selectivveity in the analysis of cholesterol in the presence of common interferents [81]. Oxides of these transition metals have also been employed in the development of sensitive sensors. In this way, an amperometric sensor based on the use of a hybrid materiaglinsull as working electrode, consisted of TiO_2 nanotubes decorated with Cu_2O NPs, offered good results in the analysis of cholesterol in serum samples. The obtained electrochemical response was related with the high O_2 adsorption capacity of Cu_2O NPs and can be explained through the mechanism described in Fig. 6 [100]. The indirect oxidation of cholesterol to oxysterol was also achieved by means of a disposable sensor based on the modification of a SPCE with hybrid nanocomposites of NiO, MoS_2 and poly(methyl orange) which acts as a cholesterol-identifying agent [101]. In the same way, the formation of hydroxyl radicals also explains the oxidation of cholesterol by means of carbon paste electrodes modified with Fe_2O_3 NPs [102]. Other hybrid material selected as well for the analysis of cholesterol has been a mixture of CuO NPs with polyaniline nanofibers and murexide [103] and oxidized Zn-In nanostructures [104], which follow an oxidation process based on the oxidation of H_2O_2 on the electrode surface (Fig. 7).

Nanoclays are also among the nanomaterials used in the improvement of the electrochemical properties of common working electrodes in the determination of cholesterol. In this way, a nanocomposite gel, form by the synthetic discotic nanoclay Laponite and the soft phyllosilicate Montmorillonite, was deposited on an ITO electrode for the construction of a sensitive sensor based on the direct oxidation of cholesterol by cyclic voltammetry [105,106].

Mixtures of different nanomaterials, including metallic NPs in combination with carbon nanomaterials such as CNTs [107] or nanomaterials based on transition metals [108,109] have also been used in the construction of improved non-enzymatic sensors for cholesterol. Thus, an ITO electrode modified with PtNPs and CNTs has been used for the analysis of cholesterol in phosphate buffer by amperometric measurements with a wide linear range and sensitivity in the presence of common interfering species [107]. Accordingly, AgNPs and ZnO nanorods have been combined for the modification of a Cr-Pt microelectrode for the development of sensitive amperometric sensors [108] and AuNPs, CdS quantum dots and TiO_2 nanotubes were deposited on a titanium substrate [109] generating a biosensing platform for the amperometric determination of cholesterol in serum samples through the mechanism described in Fig. 6. Finally, hybrid nanostructures of carbon nanomaterials and oxides of transition metals, such as MnO_2 nanoclusters, were electrodeposited over a graphene modified pencil

Fig. 6. Schematic of oxidation of cholesterol through the formation of hydroxyl radicals.

Fig. 7. Schematic of oxidation of cholesterol through the oxidation of H_2O_2 at the electrode surface.

graphite electrode for the direct electrooxidation of cholesterol to a mixture of 5-cholesten-3-one and 4-cholesten-3-one, which allowed the analysis of cholesterol in serum samples by differential pulse voltammetry [110].

3.3. Non-enzymatic cholesterol detection on electrodes modified with conducting polymers

The modification of the working electrode with conducting polymers also offers important advantages in the development of electrochemical sensors, including the enhancement of the electrical conductivity and the electroactive surface area. Thus, a conductive matrix based on a poly (ionic liquid) poly(vinylbutylimidazolium)–cobalt olyoxometalate supported on carbonaceous materials has been tested for the development of a sensitive sensor for the analysis of cholesterol in serum samples, based on the increase of the electrochemical signal obtained for ferricyanide in the presence of cholesterol [111]. Serum samples have also been successfully analysed using carbon fiber paper electrodes modified with polypyrrole and dispersed amorphous Ru-Pi nanoclusters, which favour the electrochemical oxidation of cholesterol with a negative potential shift [112]. The analysis of cholesterol in food samples has been carried out as well using conducting polymers modified electrodes based, in this case, on a biocomposite of poly (3,4-ethylenedioxythiophene) with taurine, which demonstrated an exceptional electrocatalytic activity towards cholesterol detection [113].

3.4. Non-enzymatic cholesterol detection on electrodes modified with molecularly imprinted polymers

Molecularly imprinted polymers (MIPs), based on the formation of selective cavities in a polymeric matrix, have also been employed as the recognition element in the development of cholesterol sensors. Most of these sensors were based on the decrease of the electrochemical signal of the $[Fe(CN)_6]^{3-/4-}$ probe in presence of cholesterol, due to occupation of binding sites in the polymeric film by cholesterol molecules [114]. The formation of the MIP film has been carried out using different procedures, including electropolymerization of the monomer on the electrode surface in the presence of cholesterol as a template [114–117] Thus, 2-mercaptobenzimidazole [114–116] and aminothiophenol [117] have been electropolymerized on Au electrodes and AuNP/polydopamine/-graphene modified GCEs, respectively for the analysis of cholesterol in serum samples [117]. Procedures based on imprinted self-assembled films on Au electrodes have also been carried out using different self-assembled monolayers generated in presence of the cholesterol molecules. Thus, hexadecyl mercaptan [118–120], p-aminothiophenol [121], and stearylmercaptan [122] have been selected for the generation of sensitive sensors based on the formation of Au–S bonds on the electrode surface. Moreover, photopolymerization procedures have been employed for the generation of MIPs based on the copolymerization of acrylic acid, metacrylic acid and N-vinylpyrrolidone [123] and methacrylic acid, ethylene glycolmethacrylate and 2,2-dimetthoxy-2--phenyliacetonephenon [124]. In the last case, the MIPs generated were integrated with CNTs and casted onto a AuNPs modified SPCE. A carbon nanotube@molecularly imprinted polymer has also been used as the recognition element of cholesterol of sensors based on ceramic carbon electrodes. In this case, the MIP was synthetized by means of a crosslinking method using chitin and toluene-2,6-diisocyanate as cross-linker [125]. Finally, a novel graft-co-polymerization technique was implemented for the generation of a MIP based on silylated graphene oxide–grafted-chemically modified nanocellulose used in the

modification of a GCE [126].

3.5. Non-enzymatic cholesterol detection on electrodes modified with cholesterol recognition elements

As it has been above-described, electrochemical methods based on indicator displacement assay have successful been applied in the indirect electrochemical determination of cholesterol. Therefore, the incorporation of recognition elements based on host-quest type interactions to different electrode surfaces has also been carried out for the development of selective cholesterol sensors. Different molecules have been used with this aim, β-cyclodextrin being the most frequently selected. Thus, inclusion complex of β-cyclodextrin and methylene blue has been used in the modification of Au electrodes [127] and GCEs, previously modified with poly(N-acetylaniline) and graphene [128]. These electrochemical sensors produced a measurable electrochemical signal, which proportionally decreased after the addition of cholesterol. Calixarenes molecules, such as calix[6]arene, have also been used in the development of host-guest sensors based on a graphene modified GCEs [129]. More recently, Jahani et al. [15] have developed a sensor based on the modification of GCE with a polymer of intrinsic microporosity, using methylene blue as the probe molecule as well.

Finally, it can be mentioned another modification of the working electrode, based on a recognition element, which includes the electrochemical adsorption of diphosphonic acid of 1,4-diacetylglycoluril which its structure is similar to flavin adenine dinucleotide, an active center of ChOx, allowing the sensitive and selective determination of cholesterol in dairy products by differential pulse voltammetry [130].

4. Lab-on-a-chip electrochemical devices for cholesterol determination

Lab-on-a-chip devices offer a long list of attractive advantages for research in numerous fields requiring a low consumption of sample, reagents and energy, rapid response and portability, which is especially interesting in the case of cholesterol analysis even at patient's home, for routine personal healthcare monitoring [5,6131]. In this way, electrochemical detection offers simple and inexpensive platforms for the development of miniaturized cholesterol sensors as point-of care testing devices [6].

Non-enzymatic lab-on-a chip systems have scarcely been developed for the determination of cholesterol. A miniaturized non-enzymatic cholesterol sensor based on host-guest interactions was obtained using a SPCE modified with β-cyclodextrin and CNTs, being successfully applied to the analysis of cholesterol in serum samples [132]. The same detection system was applied by Willyam et al. [133] using a β-cyclodextrin and methylene blue complex which was immobilized on the surface of Fe_3O_4 magnetic NPs. The sensor allowed the indirect determination of cholesterol after dropping the complex solution on the SPCE surface. Au electrodes have also been used for the development of a sensor chip mounted in a battery-operated handheld instrument, to monitor cholesterol contents in food samples. The selectivity of the sensor was achieved by means of the modification of the electrode surface with an alkanethiol self-assembled monolayer [134].

The development of enzymatic lab-on-a chip devices for cholesterol determination has attracted more attention in recent years, using platforms such as polydimethylsiloxane, silicon and paper substrates for microfluidic chips, screen-printed strips or more novel concepts based on contact lens.

Polydimethylsiloxane is an attractive material for microchip fabrication due to its high chemical resistance properties, low cost, easy fabrication, non-toxicity and biocompatibility [135–138]. Microchannels were usually fabricated on it by standard procedures of photolithography and coupled directly to the electrodes system inserted into the chip [135–139]. Different schemes have been developed for the electrochemical detection, incorporating CNTs [135] and nanostructured metal oxides [136,137,139] onto the microelectrode surface to increase the loading of ChOx. For instance, Wisitsoraat et al. immobilized ChOx and potassium hexacyanoferrate, prepared in polyvinyl alcohol solution, on a CNTs working electrode, using a Ag reference and a Pt counter electrode as well [135]. Similarly, ChOx has been immobilized onto anatase-titanium dioxide/ITO [136], ChOx and ChE onto NiO nanorods/ITO [137], ChOx and ChE onto chitosan/anatase titanium dioxide/ITO [139], and ChOx onto NiO thin film/Pt using [$Fe(CN)_6^{3-}$] species as mediator in solution [138].

Moreover, silicon wafers have also been used for microfluidic devices, being electrodes and passivation layers constructed by photolithographic techniques. Cholesterol microbiosensors were fabricated by modifying the Au working electrode by ChOx co-immobilized thioglycolic acid self-assemble monolayers, using $K_3Fe(CN)_6$ as mediator [140]. Sensitive cholesterol microsensors, consisted of ChOx, HRP, and polyaniline on Au electrodes on Si_3N_4/Si substrates, were developed based on the enzyme-induced conductivity change of polyaniline with fast response (Fig. 8) [131].

Filter paper microfluidic electrochemical biosensors, which have the ability to wick liquids in very small volumes by capillary action, have been reported as well for simple and low-cost point-of-care cholesterol diagnostics. Microfluidic channels and electrodes were fabricated by photolithographic methods, respectively, being then ChOx immobilized onto a NiONPs/graphite electrode [141].

In relation to enzymatic screen-printed lab-on-a-chip devices, Foster et al. described a patented diagnostic device using screen-printed graphite electrodes, intended for use in a doctor's office, based on a feeder strip in which the sample is transferred by capillary action from one end to the amperometric detection zone containing ChOx and ferrocyanide as mediator [3]. Screen-printed strips were also designed using ChOx / ChE / Fe_3O_4 / SPCE [142], ChOx/carboxymethyl cellulose/tetracyanoquinodimethane/SPCE [143], ChOx/polyaniline/polyvinylpyrrolindone/graphene/paper based SPCE [144] and ChOx/nanoporous Au/paper based SPCE [145]. Likewise, screen printed electrodes fabricated onto polyethylene terephthalate substrate have been used as electrochemical test strips in smartphone-based microdevices with 1 μL finger pricked whole blood drop. ChOx and [$Fe(CN)_6]^{3-}$ were immobilized onto SPCEs to build the amperometric analyzer [146].

Most of these devices, photolithographic and screen-printed electrodes, have been fabricated as planar electrodes. On the contrary, Gao et al. described a multi-channel portable electrochemical detector based on the use of microneedle electrodes, with a relative specific surface for enzyme immobilization that enhances sensitivity and facilitates direct and continuous monitoring of the analytes in the skin fluids [147]. Summarizing, the design for the simultaneous detection of glucose, uric acid, and cholesterol consisted of the magnetorheological drawing lithography of microneedles on a flexible substrate, sputter coating of Au/Ti film on the surface of microneedles, electro-deposition of polyaniline nanofibers and PtNPs and modification with GOx, uricase and ChOx. Recently, a 3D printed biochip has also been applied as a proof-of-concept to the simultaneous amperometric determination of two cardiac biomarkers (cholesterol and choline) in the same blood droplet, through enzymatic assays developed on the two miniature integrated working electrodes of a 4-electrode biochip. ChOx and choline oxidase were immobilized by adsorption and protected by a Nafion

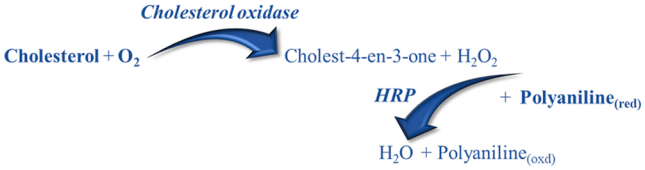

Fig. 8. - Cholesterol detection mechanism based on the enzyme-induced conductivity change of polyaniline.

membrane [148]. Even more recently, wireless and soft smart contact lens that enables real-time quantitative recording of cholesterol in tear fluids using a smartphone have been reported. The electrochemical cholesterol biosensor utilizes a two electrode system that consists of a Nafion/ChOx/Prusian Blue based working electrode and a reference electrode, which allow recording a chronoamperometric current at − 0.1 V [149].

5. Conclusions

This review focuses on the description of selected achievements in cholesterol electrochemical determination. A great development in sensitive and selective electrochemical determination of cholesterol sensors, including enzymatic and non-enzymatic sensors, has been observed considering the large number of references reported. Despite the high price of enzymes and their handling problems related to their denaturation and easy loss of activity, most of the sensors developed for the determination of cholesterol are enzymatic sensors due to their high selectivity of the recognition processes related to enzymatic reactions. ChOx has been the most employed enzyme in the development of enzymatic biosensors using different modified electrodes, incorporated to both mediated and non-mediated devices. Non-enzymatic sensors have been based on the modification of electrodes of different nature with different materials in order to find the desired selectivity and sensitivity. It should be highlighted that nanoscale materials have been among the most widely used in improving the properties of both enzymatic and non-enzymatic systems, mainly due to their catalytic properties. Bienzymatic systems based on the combination of ChOx with HRP have also been developed to record low-potential amperometric measurements of the enzymatically generated H_2O_2. Some of these electrochemical platforms have been used for the development of lab-on-a-chip devices for easy and low-cost point-of-care cholesterol analysis. Most of these miniaturized enzymatic systems have been based on the use of microfluidic devices or screen-printed electrodes. Enzymeless miniaturized systems have been less developed, finding few references related to the use of screen-printed electrodes coupled to a battery-operated handheld instrument. Therefore, there is still an open field of research aimed at the development of small-size sensors that allow rapid and in situ analysis of cholesterol, avoiding the inherent problems of working with enzymes while maintaining their selectivity.

CRediT authorship contribution statement

Olga Domínguez Renedo: Writing – original draft, Writing – review & editing, **Ana Marta Navarro Cuñado:** Writing – original draft. **M. Asunción Alonso Lomillo:** Writing – original draft, Writing – review & editing, Supervision.

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

No data was used for the research described in the article.

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