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An overview of recent analysis and detection of acetylcholine

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ABSTRACT

Acetylcholine (ACh), the major neurotransmitter secreted by cholinergic neurons, is widely found in the peripheral and central nervous systems, and its main function is to complete the transmission of neural signals. When cholinergic neurons are impaired, the synthesis and decomposition of ACh are abnormal and the neural signalling transition is blocked. To some extent, the concentration changes of ACh reflects the occurrence and development of many kinds of nervous system diseases, such as Alzheimer's disease, Parkinson's disease, Myasthenia gravis and so on. Thus, researches of the physiological and pathological roles and the tracking of the concentration changes of ACh in vivo are significant to the prevention and treatment of these diseases. In the paper, the pathophysiological functions and the comprehensive research progress on detection methods of ACh are summarized. Specifically, the latest research and related applications of the optical and electrochemical biosensors are described, and the future development directions and challenges are prospected, which provides a reference for the detection and applications of ACh.

1. Introduction

Acetylcholine (ACh) is a neurotransmitter that is present in both the peripheral nervous system (PNS) and central nervous system (CNS) of mammals [1]. Research has shown that ACh is associated with various diseases, including Alzheimer's disease (AD) [2], Parkinson's disease (PD) [3], Myasthenia gravis (MG) [4], and acute organophosphorus pesticide poisoning (AOPP) [5]. In particular, the concentration of ACh in AD patients is far below the normal value. The detection of ACh is important for the diagnosis, treatment, and follow-up of these diseases [6].

In recent years, many advances have been made in ACh detection method (Fig. 1). The conventional analytical methods include capillary electrophoresis (CE), chromatography/mass spectrometry (CG/MS), and neuroimaging (NI). These types of methods can realize the quantitative and non-invasive analysis of ACh. However, these methods still have disadvantages, for instance, complex operation, high technical requirements, and insufficient accuracy and sensitivity. Therefore, it is desirable to improve these conventional methods and develop new detection technologies. Photoelectrochemical biosensing is a new detection technology based on the photoelectric conversion characteristics of substances. Because this method is simple, fast, and has high sensitivity, it has been widely used for ACh detection. ACh photoelectrochemical biosensors can be divided into optical biosensors and electrochemical biosensors, depending on the detection method. The detection methods can also be classified into direct detection based on enzymes, indirect detection based on enzymes and nano-enzymes, and direct detection based on the catalytic oxidation of nanomaterials.

To date, there have been many reviews about ACh at different perspectives. In most reviews, various neurotransmitters, including ACh, were focused. For example, Arumugasamy et al. [7] summarized the role of nanomaterials in diagnostic tools and the different techniques used in the accurate detection of neurotransmitters. Hasanzadeh et al. [8] more focused on the corresponding sensitivity and sample matrix of neurotransmitters electrochemical sensors. Sangubotla et al. [9] preferred to discuss the various analytical techniques in the detection of neurotransmitters involved in Alzheimer's disease. All of the above reviews are summarized from different aspect of multiple neurotransmitters such as detection nanomaterials, sample matrix and related diseases, which give us a rapid understanding of the research status of different neurotransmitters. However, these reviews are relatively general and not detailed enough for a single neurotransmitter. To date, the introduction of the pathophysiology and systematic detection about a specific neurotransmitter has not been reported. Thus, ACh was selected in the paper, the distribution, pathological and physiological effect, and related diseases of ACh were overviewed. Then, the conventional instrumental ACh analysis methods are summarized and compared. Especially, the latest research and related applications of the optical and

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electrochemical biosensors are described, and the future development directions and challenges are prospected.

2. ACh

2.1. Source and distribution

ACh was discovered in 1921, Loewi proved that ACh was a chemical transmitter of cardiac vagal nerve endings. This neurotransmitter was officially named ACh in 1926 [10]. Studies have shown that ACh is present in both the PNS and CNS of mammals1. In the PNS, ACh is mainly released by parasympathetic preganglionic and postganglionic fibers and sympathetic preganglionic fibers. In addition, ACh can also be released by the postganglionic fibers in the sympathetic nerves that innervate the sweat glands, and the sympathetic vasomotor fibers and somatic motor fibers in skeletal muscle. In the CNS, ACh is secreted by cholinergic neurons, which can be divided, according to their distribution, into neurons in the basal forebrain central cholinergic system and brainstem cholinergic system. The former includes thalamic-specific sensory-cortical projection nuclei (such as the posterior ventral nucleus) and the piriform region, amygdala, and hippocampus of the limbic system, with the most abundant cholinergic neurons [11,12]. The latter mainly includes the motor neurons of the anterior horn of the spinal cord and some nuclei of the ascending reticular excitatory system of the brain stem, such as the cuneus nucleus and the striatum.

2.2. Physiological action

The main physiological function of ACh is to transmit nerve signal impulses as shown in Fig. 2 [12]. ACh is synthesized from choline (Ch) and acetyl-CoA catalyzed by choline acetyl transferase (ChAT). ACh is transported to the synaptic cleft via the vesicular acetylcholine

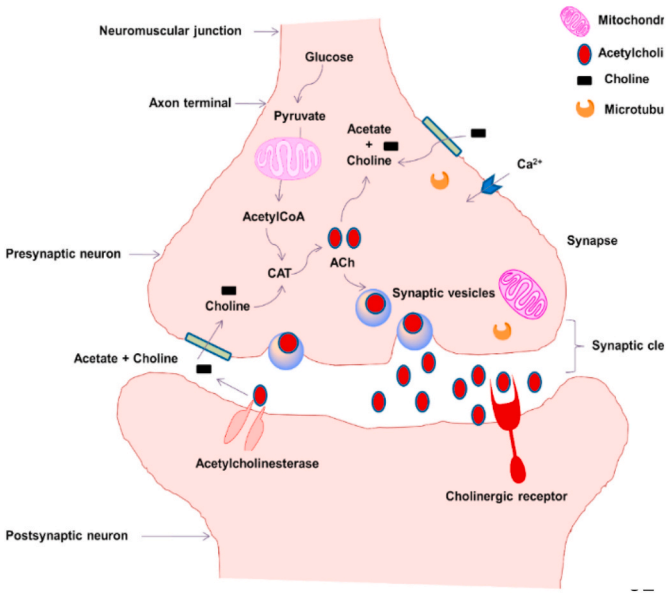

Fig. 2. The process of ACh transmits nerve impulses through synaptic gaps [12].

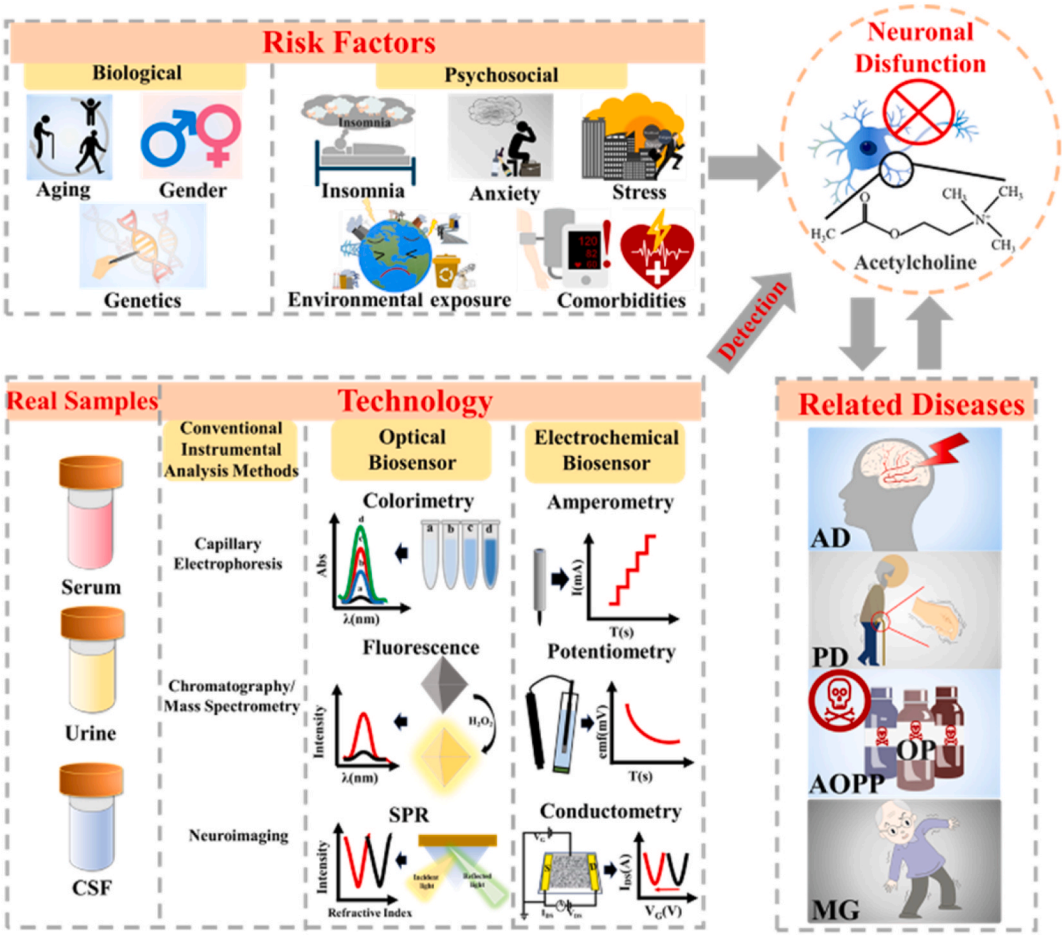

Fig. 1. Etiology of diseases related to ACh and summery of ACh detection methods.

transporter (VAChT) and synaptic vesicles. ACh quickly binds to and activates the acetylcholine receptor (AChR) on the postsynaptic membrane, completing signal transmission. After activation, ACh rapidly disconnects from the receptor and is hydrolyzed into acetate and Ch by free acetylcholinesterase (AChE) in the synaptic cleft. Ch is then transported to the synapse by the choline transporter (ChT) to participate in the synthesis of ACh.

The AChRs, which are responsible for receiving the signal from ACh, include the muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) [13]. The physiological effects of AChR are different at different sites. The mAChR is widely present in the effector cells innervated by parasympathetic postganglionic fibers, and can be divided into five subtypes M1–M5. In the CNS, M1, M2, and M3 are mainly related to learning and memory. In the PNS, M1, M3, M4, and M5 are mainly related to muscle contraction and hormone level regulation. The nAChR is composed of five subunits (α, β, γ, and δ), and the α4β2 and α7 subtypes are the most abundant in the brain. The α4β2 subtype is involved in the regulation of neurotransmitter release and plays a direct role in nicotine addiction [14]. The α7 subtype is highly expressed in areas of the brain associated with cognitive and memory function and is involved in sensory and information processing [15].

2.3. Associated diseases

Pathological changes in ACh levels can reflect the occurrence and development of various nervous system diseases, such as AD, PD, MG, and AOPP. AD is a neurodegenerative disease and the cholinergic hypothesis is one of the current hypotheses for its development. According to this hypothesis, the main etiology of AD is cholinergic synaptic disorder, and the gradual decline of cognitive ability in AD patients may be related to damage of the cholinergic system [16]. Positron emission tomography (PET) [17] has shown that levels of AChE in the amygdala, hippocampus, and cerebral cortex were significantly reduced, and the AChR was largely absent, in AD patients. When the cholinergic system is damaged, the synthesis and decomposition of ACh will be abnormal, leading to impaired nerve signal transmission. Research has shown that the normal body ACh concentration is approximately 8.66 ± 1.02 nM, and in AD patients the ACh concentration is < 6 nM [6]. PD is a common movement disorder in the elderly. The typical characteristics of PD are the degeneration and necrosis of dopaminergic neurons in the substantia nigra of the midbrain, which results in an imbalance between the dopaminergic neurons and cholinergic interneurons in the striatum [3]. This imbalance may result in PD patients having an imbalance in the hypercholinergic system, and the cholinergic interneurons become more and more active, which leads to an increase in ACh levels. MG [4] is caused by a combination of the AChR antibody and AChR produced by the patient, which impede AChR signal transmission and eventually leads to muscle dysfunction. In AOPP [5], organophosphorus pesticides enter the body and quickly combine with AChE to form phosphorylated cholinesterase, which results in ACh being unable to be hydrolyzed and accumulating in large quantities in the body, finally causing symptoms of cholinergic hyperexcitability.

In summary, the cholinergic system plays an important role in various nervous system-related diseases. Changes in the ACh concentration can reflect the occurrence and development of various nervous system diseases. Therefore, it is important to develop an accurate, rapid, and reliable method for the detection of ACh in biological body fluids.

3. Conventional instrumental analysis methods

Currently, the conventional instrumental methods for the analysis of ACh include CE, CG/MS, and NI. CE is a liquid phase separation technology with a capillary as the separation channel and a high voltage direct current field as the driving force. CE has the advantages of a high separation efficiency, requiring only a small sample size, and a relatively short analysis time. Combining the separation ability of CE and the principle of enzyme catalysis, AChE and cholinesterase (ChO) modified electrodes were used as detectors for the column catalytic separation of ACh in CE [18]. Mukherjee [19] et al., using butyryl choline as an internal standard, used two electrodes as detectors for capillary electrophoresis to detect Ch and ACh. The linear ranges of Ch and ACh were 2–2000 and 10–2000 μM, the limit of detection (LOD) was 0.1 and 38 μM, respectively. The sensor improved the detection ability of the electrode and effectively separated the target substance, providing a new selective strategy for the detection of cholinergic biomarkers.

CG/MS includes single instrument analysis, such as MS and high-performance liquid chromatography (HPLC), and mixed methods. Chromatography-mass spectrometry (CG-MS) and ultra-performance liquid chromatography-MS/MS (UPLC-MS/MS) are the most common types of mixed method analysis [20,21]. These methods can directly and quantitatively detect ACh in the cerebrospinal fluid and serum of patients with CNS diseases, and are useful in the treatment and observation of central nervous system diseases. However, MS, the earliest method used for detecting ACh, lacks sensitivity [22]. HPLC is often used in combination with electrochemical sensors, but there is a rapid reduction of sensitivity in the later stages of this analysis because of the instability of the electrodes [23]. The specificity of analysis of ACh is insufficient using GC-MS, and a long analysis time was performed. Therefore, in recent years, UHPLC-MS/MS has been developed for the detection of ACh. Zhang et al. [24] simultaneously determined the content of ACh and iso-ACh in human peripheral blood by UPLC-MS/MS, which indicated the importance of ACh in the PNS for cell proliferation, gland secretion, and immune regulation, and promoted research on ACh-related non-nervous system diseases. However, these methods also have disadvantages such as high maintenance cost and complex operation.

NI is a technology that can directly or indirectly image the function and structure of the nervous system and is an important means of medical diagnosis [25]. PET, magnetic resonance imaging (MRI), and magnetic resonance spectroscopy (MRS) are NI methods used to detect ACh. PET is often used with radioactive tracers to identify CN damage in the brain. MRS is a non-invasive research method, which can directly and quantitatively detect ACh, reflecting the concentration of ACh by detecting the proton nuclei (1H-MRS) in Ch [26]. However, 1H-MRS lacks sufficient specificity for the ACh analysis due to the overlap of the resonances of the trimethylamine groups of Ch, glycerophosphocholine, phosphocholine and betaine. In recent years, magnetic resonance imaging (MRI) has been used to detect ACh, mainly based on the development of new nuclear magnetic resonance molecular contrast agents for brain imaging. For example, Luo [27] has developed a new type of nanoscale MRI contrast agent. AChE- and pH-sensitive contrast agents were fixed on the surface of polymer nanoparticles and used for live monitoring of ACh imaging in mice. At present, NI is primarily used in the auxiliary diagnosis of clinical neurological disorders and generally qualitative studies. There are relatively few studies on quantitative ACh detection using NI, which requires further development and research.

4. Optical biosensors

The methods using optical biosensors that have been used to analyze ACh mainly include colorimetry (CM), fluorescence analysis (FLA), and surface plasmon resonance (SPR). FLA can be further divided into derivation, quenching, fluorescence resonance energy transfer (FRET), and surface plasmon-enhanced energy transfer (SPEET) methods (Table 1).

4.1. Colorimetry

Colorimetric methods detect the concentration of a target object by detecting the color change of UV–visible light absorption, which has the advantages of easy visualization and simple and fast operation. There

Table 1
Comparison of ACh optical biosensors.

Target	Method	Signal element	Detection range(μM)	LOD(μM)	Samples	Ref.
Glu/ACh	CM	ABTS	0.5-10^2, 10-15	0.10, 1.6	Serum, Milk	[30]
ACh		Fe_3O_4NSs/rGO	0.1–10^4	3.9×10^{-2}	Milk	[31]
ACh		AgNPL	10–10^3	0.50	Serum	[33]
ACh		TMB	0.50-6 × 10^3	0.35	Serum	[43]
ACh		TMB	10-8 × 10^3	1.24	–	[44]
ACh		TMB	0.025–0.9	7.50×10^{-3}	Urine	[45]
ACh/Ch		OPD	0.2-160,0.08-120	0.1563,7.26×10^{-2}	Serum	[46]
ACh/Ch	FLA	CDs	0.5-60,0.10-40	0.50,0.10	–	[10]
ACh/Ch		[a]UCNP	0.2-160,0.08-120	7.65×10^{-2},2.97×10^{-2}	Serum	[46]
ACh/Ch		[b]AUR	0.1-10,0.5-10	3.6×10^{-2},2.7×10^{-2}	Serum	[34]
ACh/Ch		MIL-101(Fe)	10^{-2}-10^2,0.1–10	8.9×10^{-3},0.02	Milk, Plasma samples	[35]
ACh		rGO/GO-NA	0.0-5.0 × 10^2	33	–	[36]
ACh		BSA-AuNCs	1.0×10^{-4}-2.0×10^{-2}	5.0×10^{-6}	Blood	[42]
ACh/AChEIs		[c]PCHL	10^{-3}-10	–	–	[47]
ACh/Ch		[d]CTVs	–	–	–	[48]
ACh		[e]HCs	–	–	–	[49]
ACh		Fluorescein	0.10-5.0 × 10^4	2.0 × 10^2	Cerebrospinal fluid in mice	[50]
ACh		BN	0.001–80, 80-2.0 × 10^2	2.7×10^{-3}	Serum	[51]
ACh		[f]LCs	0.01-1 × 10^3	1.45×10^{-2}	–	[52]
ACh/Ch	FRET	AuNCs@ChO	1–10	–	–	[38]
P1/Aβ/ACh		ThT@Er-MOF	0–0.033, 0–0.04, 0–0.01	5.2×10^{-11}, 1.4×10^{-5}, 4.9×10^{-5}	Cerebrospinal fluid	[39]
ACh/Ch		Cds/ZnS/[g]FB	-,0-100	–	Living cells	[53]
ACh	SPEET	Tb^{3+}	-	-	Neuromuscular junctions	[40]
ACh	SRP	Ta_2O_5	3.8×10-2	0.0–10	Blood	[42]

[a] UCNP: Upconversion nanoparticle,
[b] AUR: Amplex ultrared,
[c] PCHL: A photonic crystal hydrogel layer,
[d] CTVs: Cyclotriveratrylenes,
[e] HCs: Hemicryptophanes,
[f] LCs: Liquid crystal,
[g] FB: cationic boronate-protected fluorescein.

are two main detection methods for ACh colorimetry. One is based on the fact that produced by the hydrolysis of ACh can be catalyzed by horse radish peroxidase (HRP). This catalysis results in color changes of substrates such as o-phenylenediamine (OPD), tetramethyl benzidine (TMB), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) [28]. HRP is prone to inactivation and expensive, so nano-enzymes that simulate HRP activity were developed. They provide a method for simple preparation, convenient storage, good stability and less affected by temperature and pH [29]. Su et al. [30] have constructed a colorimetric sensor for the detection of glucose (Glu) and ACh using Ni–Co layered double hydroxides (Ni–Co LDHs) as a nanomaterial enzyme to catalyze the color-change reaction of ABTS. The linear range was 0.5–100 and 10–15 μM, and the LOD was 0.1 and 1.62 μM, respectively, for the detection of Glu in human serum and ACh in milk. Ferric oxide nanoparticles (Fe_3O_4NPs) also have HRP catalytic activity. Qian et al. [31] have prepared Fe_3O_4NPs/reduced graphene oxide (rGO) as a nano-enzyme by a solvothermal method for the detection of ACh. The magnetic Fe_3O_4NPs and rGO can improve the stability and increase the surface area of biosenor. A LOD of 39 nM was used for the determination of ACh in milk, and the recovery was 87.0%–115.2%. Although the crucial progress has been made in nano-enzyme, poor catalytic selectivity and limited catalytic activity have hindered its continued development. To solve these problems, single-atom catalysts (SACs) was reported by Zhang and co-workers [32]. Downsizing catalyst nanoparticles to single-atom scale can lead to the maximized atom utilization and the enhanced catalytic activity. They have exploited the splendid oxidase-like Fe–N–C SAC for the detection of OP and AChE activity. The second detection method is based on the fact that H_2O_2 generated by an enzymatic reaction can directly change the color of nanomaterials. Oh et al. [33] have shown that H_2O_2 generated by the hydrolysis of ACh could etch silver nanoplates (AgNPL) and eventually cause a color change of the AgNPL to achieve the quantitative detection of ACh, with a linear range of 10–1000 μM and a LOD of 0.5 μM. However, CM has the disadvantages of colour interference, high background signals and insufficient sensitivity.

4.2. Fluorescence analysis

4.2.1. Derivative method

The fluorescence derivation method uses a chemical reaction to convert weak or non-fluorescent substances into highly fluorescent products that reflect the concentration of the substance to be measured [34]. However, most fluorescent compounds are aromatic amines that have carcinogenic risks and need to be used with stabilizers, which increases the complexity of the sensing system. Guo et al. [35] have constructed a nano-enzymes, MIL-101(Fe), which has the dual functions of HRP activity and fluorescence emission(Fiugre 3 A). MIL-101(Fe) can catalyze the hydrolysis of ACh to produce H_2O_2, which decomposes to OH groups. At the same time, MIL-101(Fe) can be oxidized by OH to produce 2-hydroxyterphthalic acid that has high fluorescence. This method does not need to introduce a fluorescent agent and is more environmentally friendly than other methods using fluorescent compounds. The linear ranges were 0.01–80 and 80–200 μM, and the LOD was 2.7 nM. Mangalath et al. [36] have developed a pH-responsive fluorescent biosensor using the H^+ generated from the hydrolysis of ACh, which could cause the enhancement of weak graphene oxide-naphthalimide (GO-NA) fluorescence. The linear range was 0–0.5 mM and the LOD was 3.3 mM. This method simplified the sensing system and reduced the detection cost.

4.2.2. Quenching method

As the fluorescence properties of some materials, such as carbon quantum dots (C-dots, CDs) and gold nanoclusters (AuNCs), can be quenched by H_2O_2, ACh can be detected by the quenching of signals [10]. The tyrosine residues containing phenol groups in bovine serum albumin (BSA) can reduce Au^{3+} to luminescent AuNCs, and H_2O_2 can cause the fluorescence quenching of AuNCs. Li et al. [37] have constructed a BSA-AuNCs-modified two-enzyme ACh biosensor (Fig. 3B),

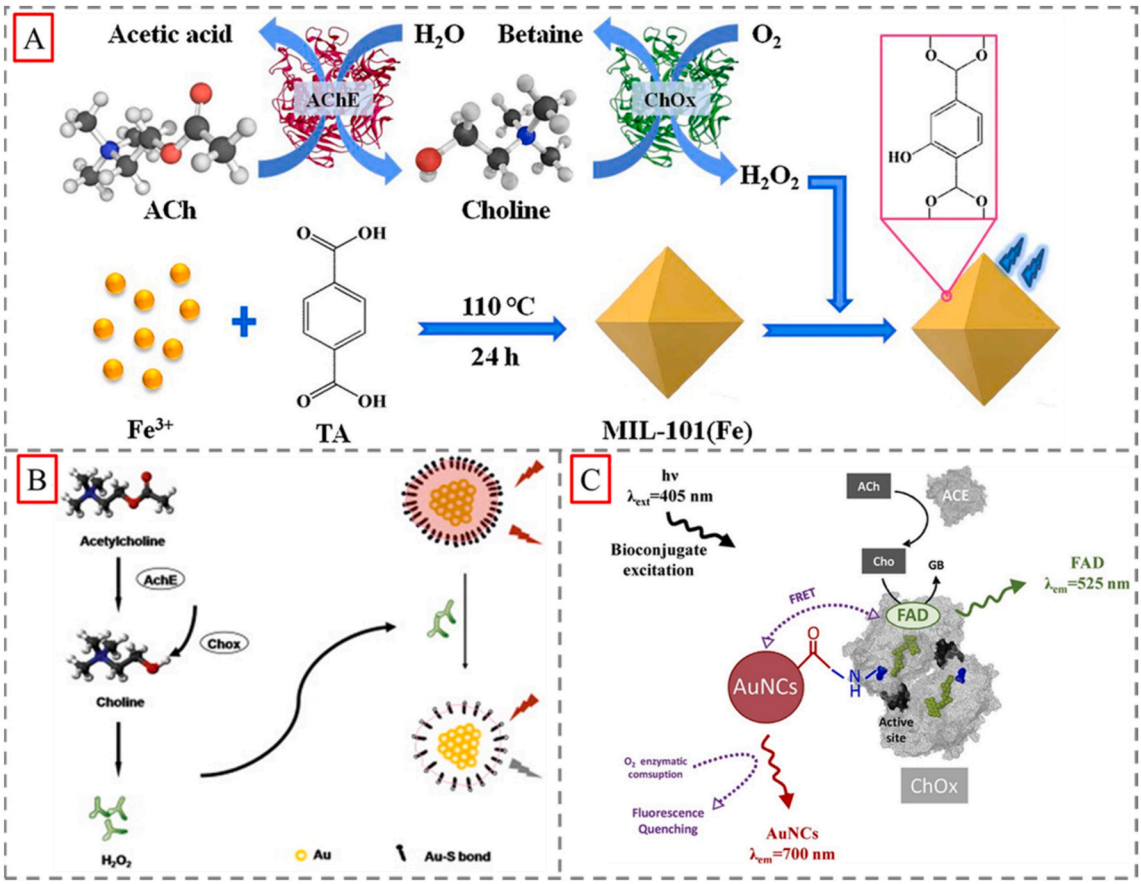

Fig. 3. Optical biosensor of ACh (A–D): A [35] Derivation of Synthesis process and signal principle of nanometer enzymes with HRP activity and fluorescence emission, B [37] Indirectly fluorescence-quenching, the principle is as follow: The tyrosine residues containing phenol groups in BSA can reduce Au^{3+} to luminescent AuNCs, and H_2O_2 produced by hydrolysis of ACh can cause the fluorescence quenching of AuNCs. C [38] The FRET between AuNCs and FDA and the quenching of AuNCs by O_2 were used to the coordinate detection of ACh.

with a linear range of 0.1–20 nM and a LOD of 5 pM. The recovery of ACh in human blood was 93%–102%, and the relative standard deviation (RSD) was 1.06%–2.12%.

4.2.3. *Fluorescence resonance energy transfer*

FRET is the transfer of energy from the donor to the acceptor when two fluorogroups, which act as the energy donor and energy acceptor, are in close proximity (<10 nm). AuNCs are often used as energy receptors in FRET systems because of their high extinction coefficients. Martin et al. [38] (Fig. 3C) connected AuNCs containing ChO with flavin adenine dinucleotide (FAD) by a covalent bond and used the synergistic effect of the FRET phenomenon between AuNCs and FAD, as well as O_2, on the fluorescence quenching of AuNCs to detect ACh, with a linear range of 1–10 μM and RSD of 4%. Wang et al. [39] synthesized a metal-organic framework material (Er-MOF) as the "donor" and used the fluorescent dye thiofuran t(ThT) as the "receptor". Using FRET, the hydrogen bonding and electrostatic interactions between ThT and Er-MOF enabled a double-emission ThT@Er-MOF ratio fluorescence sensor to be constructed. The linear ranges of presenilin 1, amyloid beta, and ACh in the CSF of AD patients were 0–33, 0–40, 0–10 nM, and the LOD values were 0.517 pM, 0.142 nM, and 0.492 nM, respectively.

4.2.4. *Surface plasmon-enhanced energy transfer*

SPEET is based on the fact that the surface plasmon resonance of noble metal nanoparticles can induce a fluorescence quenching effect through dipole-surface interactions between specific ions [40]. For example, H^+ can quench the fluorescence centered on terbium(III) (Tb^{3+}). Therefore, based on the above principle, Mukhametshina et al. [41] developed a method using the quenching of Tb^{3+}-centered luminescence because of the H^+-induced degradation of luminescent Tb^{3+} complexes doped into silica nanoparticles when acetic acid was produced from the enzymatic hydrolysis of ACh. At the same time, α-bungarotoxin was used to stain neuromuscular junctions, and the endogenous ACh released by the neuromuscular junctions could be detected qualitatively.

4.3. *Surface plasmon resonance*

SPR is a phenomenon of resonance that occurs after the free electrons on the surface of a metal medium absorb the energy of the incident light. After the target molecule is combined with the surface of the metal medium designed to adsorb biomolecules, the refractive index changes and the resonance spectrum shifts allow quantitative detection. Kant et al. [42] have deposited multilayers of silver metal (AgNPs) and embedded AChE modified with antalum(v) oxide (Ta_2O_5) nanoflakes on the fibers. The principle of SRP was used to detect ACh with a linear range of 0–10 μM and a LOD of 38 nM.

Now, with the optimization and improvement of optical sensors, the linear range of ACh is increased and LOD is greatly reduced. The ACh optical sensors enable visualization and optical changes mainly by applying nano-enzyme that can simulate HRP. However, most of them require both AChE, ChO and nano-enzymes. Specifically, the techniques such as SPEET, FRET and SRP take advantage of the optical properties of specific metal materials, MOF, and other novel materials to detect ACh. These techniques can greatly reduce the use of AChE and ChO, and have a good application prospect in the fluorescence detection of ACh.

5. Electrochemical biosensors

5.1. Amperometric biosensors

Currently, there are two kinds of amperometric biosensors used for the detection of ACh: enzymatic biosensors, which use the reaction of an enzyme and substrate to detect the current generated by H_2O_2, and non-enzyme biosensors, in which metals and metal oxides are used to build catalytic nanomaterials to detect ACh. The reaction current is detected through a redox reaction.

5.1.1. Enzymatic biosensors

AChE and ChO are commonly used enzymes for the detection of ACh. To improve the shortcomings of natural enzymes, technologies using immobilized enzymes have been developed where free enzymes are fixed on a carrier [54], such as adsorption, entrapment, covalent attachment and cross-linking (Fig. 4). Carrier materials with good thermodynamic and chemical stability and high biocompatibility, such as metals, metal oxides, polymers, and metal-organic framework (MOF), should be selected to improve the capacity for supporting enzymes (Table 2).

Adsorption is a simple immobilization method, which immobilizes enzymes through weak interaction forces, such as hydrogen bonding, van der Waals forces, and electrostatic interactions. Ionic liquids, which have good electrical conductivity, thermal stability, and solubility, can provide a good environment for immobilized enzymes. For example, Albishri [6] was the first to unite GO and the ionic liquid 1-allyl-3-methylimidazolium -bis (trifluoromethylsulfonyl) imide (AMIM TFSI) on a modified glassy carbon electrode. Through static adsorption AChE and ChO were immobilized, and this method was used in the detection of serum samples of Ch and ACh, the LOD values were 0.885 and 1.352 nM, respectively, and the linear range was 5–1000 nM. The adsorption method has little effect on the conformational changes of the enzyme and is simple. There are many types of carriers available, but this method also has disadvantages, such as the weak binding force between the enzyme and carrier, easy detachment of the enzyme, and limited size.

The embedding method involves the encapsulation of enzymes using carrier materials while maintaining the enzyme activity [55]. Common carrier materials include proteins and coagulants. BSA is often used as a binding and transport carrier for bioactive molecules and drugs, and is useful as a substrate for enzyme embedding [56]. Kaur et al. [57] embedded AChE within BSA and this material was fixed on a glassy carbon electrode modified with a polyaniline-zeolite hybrid material (PANI-nano-ZSM-5) and used to detect ACh and organophosphorus pesticides. The linear range of ACh was 1–1000 μM, and the LOD was 0.1 μM. This method is simple to operate and does not involve a conformational change of the enzyme, but the diffusion of the enzyme is limited and this method is not suitable for the catalysis of macromolecular substrates.

The covalent attachment method uses the functional groups of amino acid residues on the surface of an enzyme, such as amino (-NH_2), carboxyl (-COOH), and sulfhydryl (-SH) groups, to react with specific functional groups on the carrier material and immobilize the enzyme through a covalent bond [54]. The cross-linking method uses the bifunctional group structure of crosslinking agents to link the enzyme on the carrier with covalent bonds. Glutaraldehyde (GA), (3-aminopropyl) triethoxysilane (APTES), isocyanate and N, N′-ethylene bis maleimide are the commonly used bifunctional reagents, in which GA is most commonly used. Both methods immobilize the enzyme stably, with a strong interaction between the enzyme and the carrier, and detachment of the enzyme rarely occurs. Akhtar et al. [58] have constructed a microfluidic structured-dual electrodes sensor comprising of a pair of screen printed carbon electrodes (SPCE) (Fig. 5A). One of them was coated with gold nanoparticles and the other with a porous gold layer, followed by electropolymerization of 2, 2:5,2-terthiophene-3-(p-benzoic acid) (pTTBA) on both the electrodes. Then, AChE and ChO were covalently attached onto the different electrode respectively to reduce interference in the actual samples. The LOD was 0.6 ± 0.1 nM with a linear range of 0.7–1500 μM. Chuanhan et al. [59] used platinum nanoparticles (PtNPs) and a Zn-MOF modified gold electrode to co-immobilize AChE and ChO to detect ACh (Fig. 5B), and applied this method to the detection of ACh in clinical serum samples, finding a linear range of 0.01–500 μM and a LOD of 0.01 μM. Kucherenko et al. [60] have developed a microarray electrode for the simultaneous determination of Glu, lactic acid, pyruvate, glutamic acid, Ch, and ACh. Through GA cross-linking, different enzymes were fixed on different platinum-plate electrode surfaces with detection lines ranging from 0.001 to 0.01 and 0.2–2.5 mM. Although the stability of an immobilized enzyme using a chemical method is good, the spatial conformation of the enzyme can easily be affected leading to a decline in the activity. Therefore, crosslinking near the active site of the enzyme should be avoided as far as possible when using this method.

Although natural enzyme is easily inactivated, its excellent sensitivity and specificity still play a role in the amperometric biosensors of ACh. At present, chemical methods are more used in natural enzyme immobilization. The carrier materials have gradually transitioned from single nanoparticles to polymer materials, MOF materials and advanced composite materials to obtain better biocompatibility.

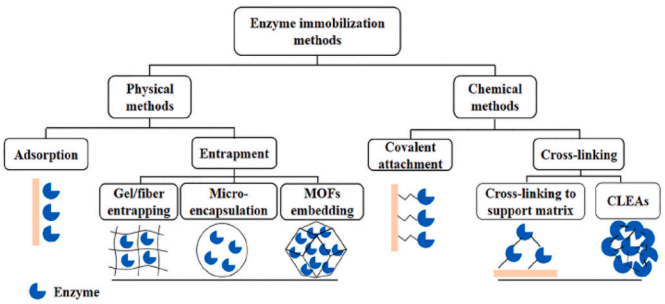

Fig. 4. Enzyme immobilization methods [54].

5.1.2. Non-enzymatic biosensors

Compared with natural enzymes, non-enzymatic biosensors have the advantages of simple preparation, lack of denaturation, and good stability and can provide effective enzyme-like catalysis over a wide range of pH values and temperature [76]. Transition metals, such as Ni, Cu, W, and alloys, and their oxides are widely used in non-enzymatic biosensors.

Research has shown that Ni matrix composites have excellent electrocatalytic activity and these were the first materials to be used in an ACh non-enzyme biosensor [77]. Sattarahmady et al. [78] synthesized a porous NiO-modified carbon paste electrode with a large specific surface area, which had a linear range of 0.25–5.88 mM and a LOD of 6.7 μM for ACh. However, the toxicity of Ni is high, and the electrode wears out quickly with long-term use, and the service life is limited. Cu, as a cheap and easy to obtain material, also has a good catalytic effect on ACh. Balasubramanian et al. [79] constructed a B- and N- doped mesoporous carbon nanocatalyst embedded in Cu@Cu_2ONPs for the determination of dopamine and ACh (Fig. 6A). The catalytic capacity could be improved by B and N doping, with a linear range of 0.004–542 and 0.3–2602 μM and LOD of 0.5 and 17 nM, for dopamine and ACh, respectively, with a recovery of 97.8%–100.2% and a RSD of 1.29%–1.98%, respectively. WO_3 is also used as an enzyme catalytic material for ACh. Anithaa et al. [80] deposited WO_3 films on indium tin oxide electrodes (ITO) by a spin coating method (Fig. 6B). The crystal structure, surface morphology, and electron mobility of WO_3 were changed by injecting a low-energy nitrogen ion beam (100 keV). The electrical conductivity and catalytic activity of WO_3 were also improved. The linear range was 0.1–8000 μM and the LOD was 28 nM for ACh detection

Table 2
Comparison of ACh enzymatic biosensors.

Target	Immobilization	Electrode	Sensor	Detection method	Detection range(μM)	LOD(μM)	Samples	Ref.
Ch/ ACh	Adsorption	GCE	AChE-ChO/GO-IL/AMIM TFSI/ GCE	ADPSV	– 5×10^{-3}-1×10^{-6}	8.85×10^{-4}, 1.352×10^{-3}	Serum	[6]
Ch/ ACh		Pt	AChE-ChO/[a]PDDA/ZnO/ MWCNTs	CV	1.0-0.8 $\times 10^{3}$, 1.0–10^{3}	0.3	Serum	[61]
ACh		GCE	AChE/ZnS/ZnO/Ta_2O_5 –SiO_2	DPV	0.1-1.2 $\times 10^{3}$	1.16×10^{-2}	Serum	[62]
ACh	Entrapment	GCE	AChE/PANI-Nano-ZSM-5	SWV	1.0–10^{3}	0.1	–	[57]
ACh		Pt	AuNPs/AChE/sol–gel/ MWCNT/ChO	CV	5–400	0.1	–	[63]
Ch/ ACh	Cross-linking	GCE	AChE-ChO/QDs/rGO	CV、ECL	10-210, 10-250	8.8, 4.7	–	[64]
ACh		ITO	AChE-ChO/rGO/PtNP	CA	5.0×10^{-3}-700	5×10^{-3}	Serum	[65]
ACh		GCE	AChE-ChO/GO-AuNPs-CS/ Fe_3O_4–TiO_2@NH_2	ECL	6.7×10^{-3}-0.92 $\times 10^{3}$	2.2×10^{-3}	Serum	[66]
ACh		Au	AChE-ChO/CS/Fe@AuNPs	SWV	5.0×10^{-3}-400	5×10^{-2}	Serum	[67]
ACh		FTO	AChE-ChO/Fe_2O_3/rGO/ [b]PEDOT	EIS	4.0×10^{-3}-800	4.0×10^{-3}	Serum	[68]
ACh		Pt	AChE-ChO/[c]PPy-PVS	CV	10^{-5}-10^{-3}	5.0×10^{-3}	Artificial blood	[69]
ACh		GCE	AChE/[d]$PNR_{Ethaline}$/Fe_2O_3NP	CA	2.5–60	1.04	Urine	[70]
ACh		Au	AChE-ChO/MWCNT-MnO_2/ rGO	CV	0.1–100	0.1	Serum	[71]
ACh		CPE	AChE-ChO/CDs-APTES	CV	10^{-5}-10^{-2}	5.0×10^{-3}	Artificial blood	[72]
		CPE	AChE-ChO/[e]PAMAM-Sal	CV	10^{-2}-100	5.0×10^{-3}	Artificial blood	[73]
ACh	Covalent attachment	SPCE	AChE-ChO/AuNPs/pTTBA	CA	0.7×10^{-3}-1.5×10^{3}	2.62×10^{-3}	Serum	[58]
ACh		Au	AChE-ChO/MOF/PtNPs	DPV	0.01–500	0.01	Serum	[59]
ACh		GCE	AChE-ChO/CS-MWCNTs-Fe_3O_4NPs	CV	0.02–0.111, 0.111–1.87	6.1×10^{-4}	Serum	[60]
ACh		ITO	AChE-ChO/AuNPs-GO	CV	10^{-4}-1.0	1×10^{-4}	Serum	[74]
Ch/ ACh	Covalent attachment and cross-linking	CFE	[f]Naf-FCNTs/GA/AChE-ChO/ [g]PoPD	CV	5.2 ± 0.03 $\times 10^{3}$; 5.9 ± 0.07 $\times 10^{3}$	4.5×10^{-2}	–	[75]

[a] PDDA: poly (diallyldimethylammonium chloride),
[b] PEDOT: poly(3,4-ethylenedioxythiophene),
[c] PPy-PVS: polypyrrole-polyvinylsulpfonate,
[d] PNREthaline: poly(neutral red)-deep eutectic solvent,
[e] PAMAM-Sal: Synthesis of denritic macromolecule.
[f] Naf-FCNTs: nafion-functionalized multiwall carbon nano tubes,
[g] PoPD: poly(o-phenylenediamine).

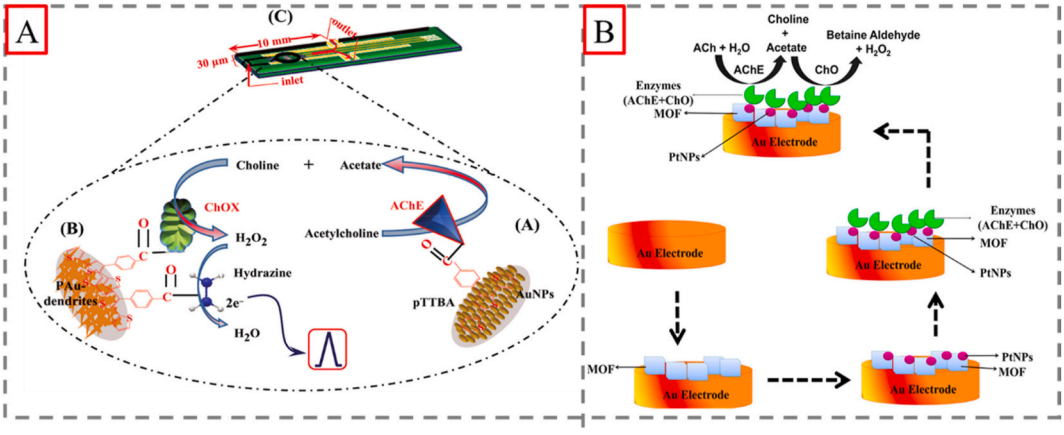

Fig. 5. Enzymatic biosensor of ACh: A [58]. A microfluidic structured-dual electrodes sensor have been constructed by a pair of SPCE. One of them was coated with gold nanoparticles and the other with a porous gold layer, followed pTTBA on both electrodes. Then, AChE and ChO were covalently attached onto the different electrode respectively., B [59]. PtNPs and Zn-MOF were modified on gold electrode to co-immobilize AChE and ChO. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in human serum samples with a recovery of 97.3%–99.6% and RSD of 4.1%.

In addition, using alloy materials can result in a synergistic effect of the two metals, which has attracted extensive attention in the field of non-enzymatic electrocatalysis. Because of the good adsorption capacity and anion exchange performance of LDH [81], Wang et al. [82] have developed a three-dimensional flower-like NiAl-LDH/CDs nanomaterial. Compared with the traditional two-dimensional sheet LDH, the specific surface area of the NiAl-LDH/CDs nanomaterial was larger, and the linear range was 5–6885 μM, the LOD was 1.7 μM, and the RSD was

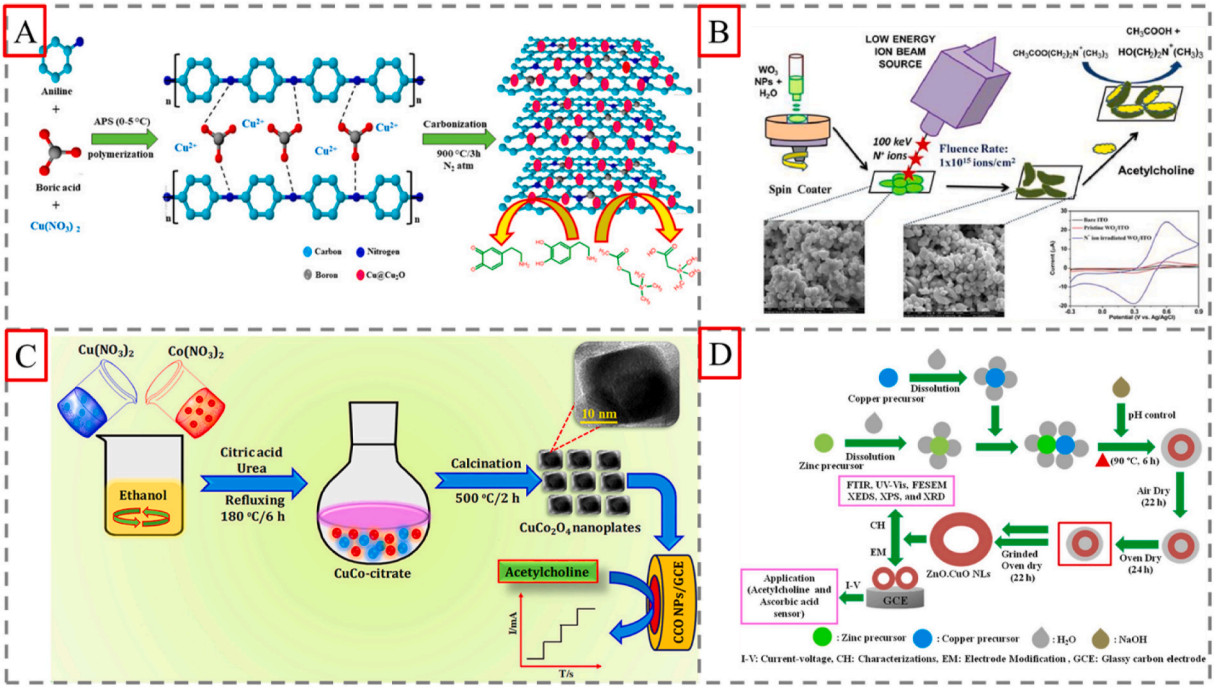

Fig. 6. Non-enzymatic biosensors of ACh: A [79]. A mesoporous carbon nano-enzyme doped with B- and N- for catalyzing ACh and DA., B [80]. WO_3 films were deposited on ITO by a spin coating method, furthermore, a low-energy nitrogen ion beam (100 keV) was injected to improve the crystal structure, surface morphology, electron mobility, electrical conductivity and catalytic activity of WO_3., C [84]. A spinel nanoparticles ($CuCo_2O_4$NPs) was synthesized by hydrothermal reaction as a nano-enzyme to catalyze ACh., D [85]. The ZnO·CuO NLs was prepared in alkaline phase by improving a facile wet-chemical technique and the non-enzymatic simultaneous detection of ACh and ascorbic acid was achieved.

4.7%. Spinel nanoparticles (AB_2O_4NPs) have also been preliminarily used in a non-enzymatic sensor for ACh [83]. A $CuCo_2O_4$NPsmodified glassy carbon electrode (Fig. 6C) has been constructed by Balasubramanian et al. [84] The synergistic effect of Cu and Co was used to improve the catalytic activity. The linear range was 0.2–3500 μM, and the LOD was 30 nM. Nanoleaves from doped metal oxide might be considered for higher research in sensor development having their distinctive criteria with massive exterior region, good porosity, permeability, and stability. Hussain et al. [85] have improved a facile wet-chemical technique, which prepares the zinc oxide doped copper oxide nanoleaves (ZnO·CuO NLs) in alkaline phase and achieved the non-enzymatic simultaneous detection of ACh and ascorbic acid (Fig. 6D). The linear range was 100.0 pM–100.0 mM, the LOD was 14.7 and 12.0 pM respectively.

At present, few materials can directly catalyze of ACh, and mainly are some transition metals. The catalytic performance and specificity are also limited. The catalytic performance can be improved through the modification of materials by polymetallic composite and doping of non-metallic elements.

5.2. Potentiometric biosensors

Potentiometric biosensors are based on ion-selective electrodes (ISEs), and ion-sensitive field-effect transistors (ISFET) are used to detect objects by converting changes in ion concentrations into measurable potentials.

ISEs are composed of a sensitive membrane, internal reference solution, internal reference electrode, and inert cavity. The sensitive membrane forms the core, and the ion carrier on it converts the ion concentration to a measurable potential value by forming complexes with the target ion. The sensor constructed by this method which has the advantages of requiring only a small volume, convenient carrying, and low cost [86]. The current ionic carriers for the detection of ACh include β-cyclodextrin (β-CD), molecularly imprinted polymers (MIPs), and calixarene. β-CD has an annular cavity, which shows molecular recognition functions via the hydrophobic effect, hydrogen bonding, and van der Waals forces [87]. He et al. [88] have constructed a non-enzymatic ISE (Fig. 7A) by coating a conductor with poly(3, 4-ethyldioxythiophene)-doped poly(styrene sulfonate) (PEDOT/PSS). ACh was recognized and captured by the β-CD, and a linear range of 10^{-5}–10^{-1} M and a LOD of 5.69 ± 1.06 μM were observed for the detection of ACh in rat brain homogenate. MIPs are materials obtained by mixing template molecules, functional monomers, cross-linking agents, and other substances in a solution for polymerization, before the elution of template molecules. The MIP has cavities with the same spatial structure and binding sites as the template molecules and has a specific spatial recognition ability for the target molecules [89]. Sacramento et al. [90] have prepared a MIP using ACh as the template through a bulk polymerization method as a modified ISE (Fig. 7B) to detect ACh in human serum. The LOD was 3.45×10^{-5} M, the recovery was 89.4%–90.8%, and the average RSD was 5.0%. Calixarene is an aromatic electron-rich macrocyclic compound with a basket-like structure, which can form host-guest complexes with target molecules [91]. The size of the calixarene cavity determines the selectivity and binding strength of target ions. The ACh can be recognized through the host-guest recognition between the aminomethyl group of ACh and the macrocyclic cavity of calixarene. Mousavi et al. [92] have combined AuNPs and *para*-Sulfonated calix[n]arene (pSCn), a class of water-soluble calixarenes with open and rigid cavities, to fabricate novel sensor (Fig. 7C), with a linear range of 1 nM–1 mM. This biosensor could directly detect ACh in artificial cerebrospinal fluid and brain homogenate from a mouse model of AD without prior separation. However, deprotonation of the phenol groups of calixarenes at basic pH conditions increases the water solubility of the calixarenes, leaving the leakage of the ionophore a potential concern. Chen et al. [93] have proposed a family of hydrophobic macrocycle Oxatub [4]arenes as ACh selective ionophores. Oxatub [4]arene is a flexible macrocycle and thus possesses numerous conformations due to the naphthalene flipping. The

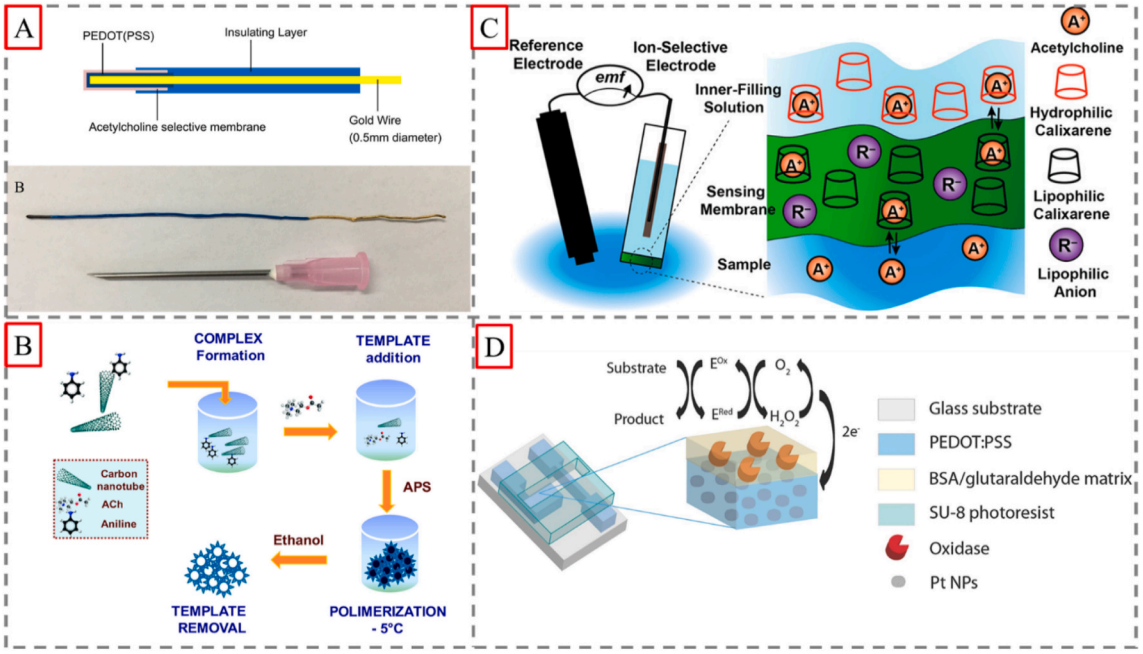

Fig. 7. Potentiometric biosensor of ACh: A [88]. A non-enzymatic ISE by coating a conductor with poly(3,4-ethyldioxythiophene)-doped poly(styrene sulfonate)., B [90]. A MIP using ACh as the template through a bulk polymerization method was modified on ISE to detect ACh., C [92] A novel sensor combin AuNPs and para-Sulfonated calix[n]arene (pSCn), a class of water-soluble calixarenes with open and rigid cavities, for detection of ACh., D [95] AChE, ChO, and glutamate oxidase were immobilized on PtNPs and the PEDOT/PSS-modified the biosensor was used to detect AChE and glutamate.

conformational plasticity and adaptability enables oxatub [4]arene to host a wide range of organic cations. The detection limit of this ISEs is 100 ± 20 nM, and the ACh concentration in mouse brain homogenate has measured.

An ISFET consists of an ISE sensitive film, a metal oxide, and a semiconductor field effect transistor (FET). The FET uses the field effect to control the output current with high sensitivity and has a small size and is easy to carry [94]. For general ISE electrodes, the ISFET combines the advantages of an ISE and a FET. Kergoat et al. [95] have immobilized AChE, ChO, and glutamate oxidase on PtNPs and a PEDOT/PSS-modified the biosensor to detect AChE and glutamate. The linear range of AChE and glutamate was 0.9–14 μM and the LOD was 5 μM (Fig. 7D).

5.3. Conductometric biosensors

Graphene field-effect transistors (GFETs) are a class of conductance biosensors that detect targets by measuring the conductivity offset. GFETs consist of source and drain electrodes, a gate, and conductive graphene material between the source and drain electrodes, and often have silver/silver chloride as the gate [96]. GFETs use enzyme catalyzed hydrolysis to cause local pH changes, which lead to graphene charge density changes that affect the conductivity of the graphene devices to shift the Dirac point (Dirac, the lowest electrical conductivity) [97]. Myung et al. [98] have prepared a GFET (Fig. 8A) to detect ACh, which had a linear range of 1–10 mM. Fenoy et al. [99] have modified poly (3-amino-benzylamine-co-aniline) (PABA) on GO to achieve a method with good conductivity, stability, and sensitivity (Fig. 8B), with a linear range of 5–1000 μM, a LOD of 2.3 μM, and a RSD of 2.6%, for the detection of ACh in urine. GFETs are more suitable for enzyme modification than most other currently available biosensors. However, this type of sensor has been less studied and still needs further development.

In conclusion, the potentiometric and conductometric biosensors have good sensitivity and accuracy to directly detect the amount of ACh. Among the above biosensors, besides the major enzymatic biosensors, there is a new idea to directly capture ACh by constructing specific recognition elements such as β-CD, MIPs and calixarene. Nevertheless, these recognition elements are often difficult to distinguish ACh and the substance of similar structure, especially in actual samples. Although these kinds of biosensors have excellent performance and good stability, they specificity still needs to be improved, so it is difficult to be applied to the detection of complex actual samples at present stage.

6. Conclusion and outlook

In this paper, the physiological effects of ACh and the existing methods for the analysis and detection of ACh are introduced, and the latest research and applications of ACh photoelectric biosensors are described. According to the detection principle, ACh analysis methods can be divided into three categories: direct detection based on natural enzyme hydrolysis, indirect detection based on a combination of a natural enzyme and nano-enzyme (simulating HRP activity), and direct detection based on the catalytic nanomaterials. The following development trends can be identified.

(1) Natural enzyme catalysis has the advantages of good specificity and high sensitivity, but it has the following disadvantages: a high price, enzyme activity that is easily influenced by the environment, and enzymes that can easily detach after immobilization. The stability of the sensors also needs to be improved. To provide a better microenvironment, reduce the detachment of the enzyme, maintain enzyme activity, and effectively increase the enzyme load, the development of enzyme-carrier materials with good biocompatibility and the optimization of the enzyme fixation mode, are current research topics of interest.
(2) Nano-enzymes and nanomaterials that directly catalyze ACh have the advantages of convenience, economy, and good stability, they are more suitable for the application in actual samples. The inner core of them are artificial materials, and the future research direction of their both to construct new nanomaterials for detection. But their applications are slightly different. Nano-enzymes are more likely to be used in optical biosensors, while the

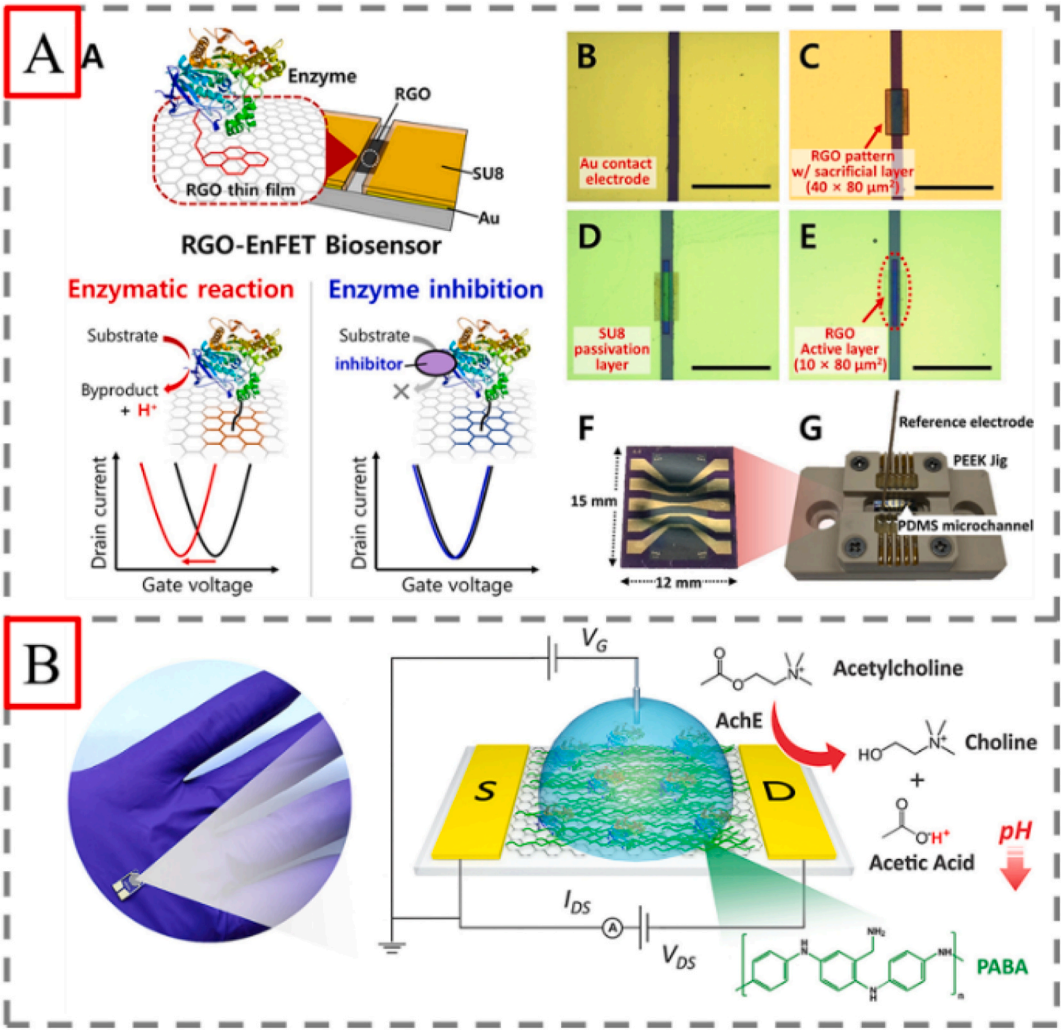

Fig. 8. Conductometric biosensor of ACh: A [100]. A reduced graphene oxide-based enzyme-modified field-effect transistor was fabricated to study the enzymatic kinetics between acetylcholinesterase AChE and acetylcholine ACh., B [101]. A new strategy of AChE immobilization on graphene field-effect transistors. The film of the copolymer PABA provide the suitable electrostatic charge and non-denaturing environment for enzyme immobilization.

catalytic nanomaterials are more common in electrochemical biosensor. Currently, the major challenges are the specificity and catalytic capacity of these materials needs to be improved.

SAC is a kind of nano-enzyme with excellent specificity in recently. It greatly improves the catalytic activity of nano-enzymes by controlling material structure and simulating the active site of nature enzyme. At present, SAC that can simulate HRP activity has been widely used, but how to analyze and simulate the active site of AChE and ChO is a great challenge. Meanwhile, the preparation methods and technologies of SAC can also provide a new reference direction for exploitation of the direct catalytic nanomaterials. Therefore, further development is necessary to improve the catalytic activity of materials, such as optimization of the particle size and porosity of various special structures. Finally, the specific detection of ACh in body fluids in the presence of dopamine, adrenaline, and other interfering substances will be the focus of future practical applications.

Declaration of competing interest

There are no conflicts to declare.

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