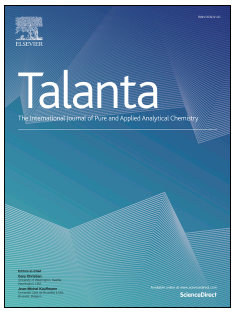
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.



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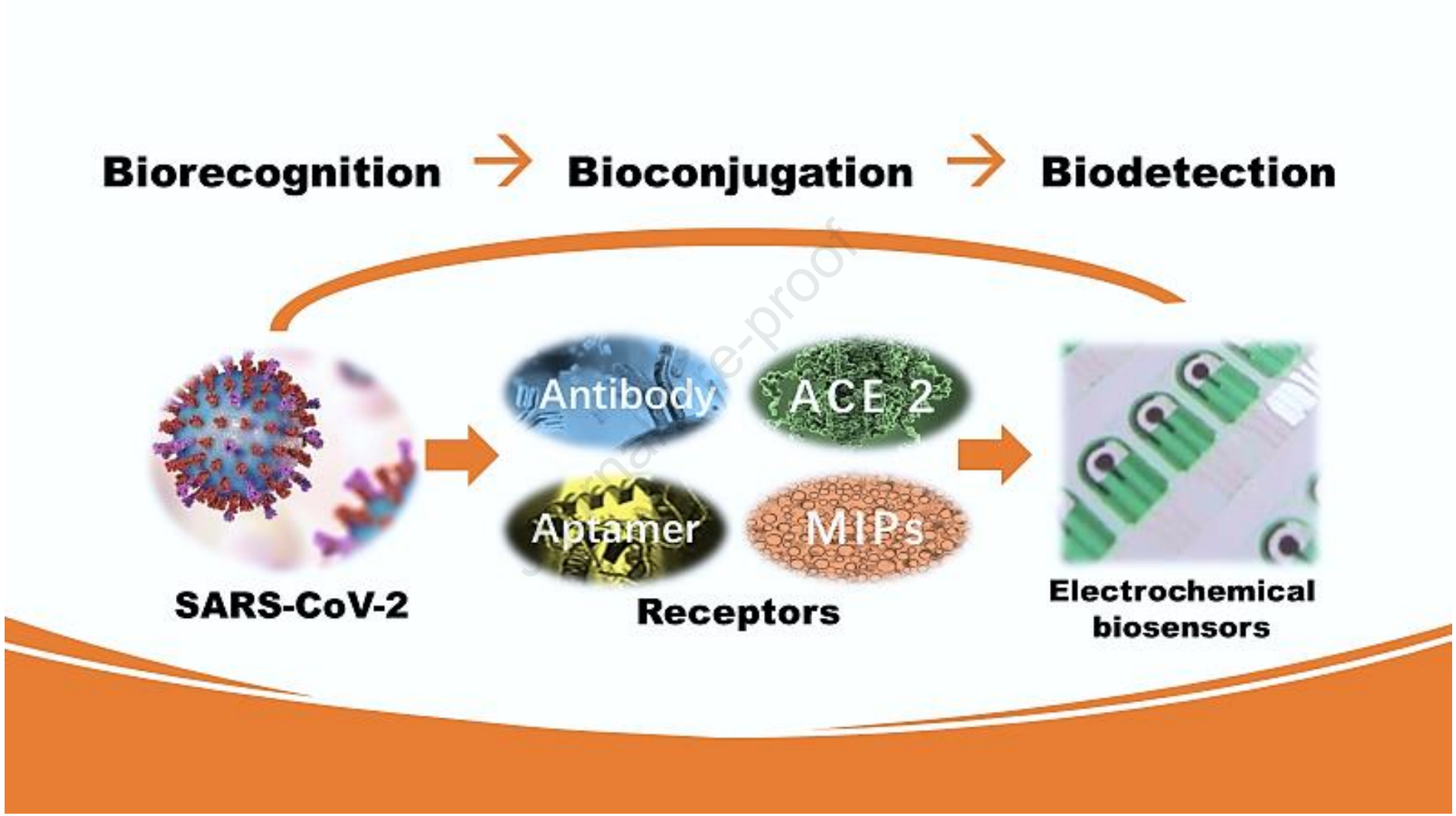
Biorecognition
Bioconjugation
Biodetection
Antibody
ACE 2
Aptamer
MIPs
SARS-CoV-2
Receptors
Electrochemical biosensors

The critical experimental aspects for developing pathogen electrochemical biosensors: a lesson during the COVID-19 pandemic

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Keywords

SARS-CoV-2; Electrochemical biosensor; antibody; aptamer; ACE2; Molecularly imprinted polymers

Abstract

Though the bitter global pandemic posed a severe public health threat, it set an unprecedented stage for different research teams to present various technologies for detecting SARS-CoV-2, providing a rare and hard-won lesson for one to comprehensively survey the core experimental aspects in developing pathogens electrochemical biosensors. Apart from collecting all the published biosensor studies, we focused on the effects and consequences of using different receptors, such as antibodies, aptamers, ACE 2, and MIPs, which are one of the core topics of developing a pathogen biosensor. In addition, we tried to find an appropriate and distinctive application scenario (e.g., wastewater-based epidemiology) to maximize the advantages of using electrochemical biosensors to detect pathogens. Based on the enormous amount of information from those published studies, features that fit and favor wastewater pathogen detection can be picked up and integrated into a specific strategy to perform quantitative measurements in wastewater samples.

1. Introduction

Pathogens, including bacteria, fungi, viruses, and viroid, are the fundamental causes of infectious diseases, resulting in more than 15 million deaths annually [1]. Along with globalization, severe infectious diseases, such as dengue fever [2], Ebola [3], influenza [4], the Middle East respiratory syndrome (MERS) [5], and severe acute respiratory syndrome (SARS) [6] have incessantly posed global public health threats and caused dramatic social and economic disruptions. The recent outbreak of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has caused more than 430,000,000 individual cases and 5,900,000 confirmed deaths until February 2022 worldwide [7]. Meanwhile, scientists worldwide have continuously devoted themselves to developing accurate, rapid, cost-effective, and easy-to-use detection methods to combat several mutated strains of SARS-CoV-2 and tame the resulted outbreaks.

Electrochemical biosensors have demonstrated superior performance among various detection methods. They have been widely accepted as one of the most promising approaches for quantitatively and qualitatively analyzing infection biomarkers in different fluid samples. More importantly, electrochemical biosensors contain sufficient flexibility that is capable of switching between multifarious bioreceptors, such as pathogen cells, antigens, antibodies, epitopes, oligonucleotides, carbohydrates, and phages, while providing fast and accurate point-of-care clinical diagnosis or in-situ environment samples detection [8-10]. During this COVID-19 pandemic, the development and application of electrochemical biosensors for detecting SARS-CoV-2 biomarkers in the swab, saliva, and

wastewater samples have experienced explosive growth. When conducting literature research, it is surprising to see different combinations of rather cutting-edge technologies and relatively conservative approaches that one would hardly imagine could have been published in such a short period. Though the bitter global pandemic triggers this situation, it offers a rare opportunity to compare and conclude what strategy contains high practicality, reassuring familiarity, and straightforward procedure, meanwhile, forecast the trend of developing a novel electrochemical biosensor, particularly for the selection of high specificity/affinity receptors, efficient immobilization methods, and sensitive electrode surface materials.

Given that, due to the accessibility and familiarity, the choice of a specific sensing surface material or electrode type is a usually predetermined aspect in most research teams, while selecting an appropriate and cost-effective receptor (i.e., recognition element) is, therefore, a critical factor with much more room to explore the possibility of a wide range of elements, such as antibodies/antigens, epitopes, enzymes, aptamers, and imprinted polymers. Among many choices, it is evident that antibodies are the most prevalent due to the straightforward design process and proven track records of high selectivity and binding affinity [11]. Apart from the antibody, it should be noted that during the COVID-19 pandemic, some other choices, such as human angiotensin-converting enzyme 2 (ACE2), single-stranded DNA (ssDNA), and molecularly imprinted polymer (MIP), all have received attention due to their low cost, high selectivity, and great flexibility (**Fig.1**). As a result, a review of the criteria for choosing diverse types of receptors is provided in this

work first.

Another essential but sometimes overlooked experimental aspect of developing electrochemical biosensors lies in adopting powerful and easy-to-use immobilization methods. By convention, most research teams tend to choose well-established techniques, such as the classical covalent approach [12], to immobilize a particular receptor (Ricci et al., 2012). This situation is primarily because developing a pathogen detection biosensor would do better to adopt conceptually straightforward and operationally reproducible processes, thereby ensuring a superior level of convenience for method validation and dissemination over different laboratories worldwide. After reviewing most electrochemical biosensors published during the COVID-19 pandemic, only a few techniques have received consistently positive comments and have become widely adopted. In this work, for all the SARS-CoV-2 electrochemical biosensors we found, we sort the immobilization approaches into four main types: classical covalent attachment, non-specific direct adsorption, gold-thiol chemistry binding, and magnet-assisted capturing. Also, many studies combined two or more approaches to avoid situations like random orientation, over-packed density, and denaturation of protein-based receptors.

Unlike the receptors and immobilization methods, the sensing platform materials for electrochemical biosensors are almost always prepared in a unique, signature manner. After reviewing a large number of recently published SARS-CoV-2 electrochemical biosensors, we could hardly find several studies that have used the identical way to prepare their electrodes, and nearly all the working electrode surfaces were undergone more or less

modification to boost some of the fundamental electrochemical features and offer more active sites for the subsequent immobilization step. Nevertheless, it is still possible to find a common point: most studies tend to load their electrode surfaces with gold-based or carbon-based nanoparticles, nanosheets, nanotubes, nanocubes, etc. In this context, we believe it is an excellent opportunity to evaluate different electrode preparation methods in terms of sensing performance, material cost, and ease of use.

Along with the development of analytical biochemistry, different concepts associated with electrochemical biosensors have been constantly proposed and applied, such as immunosensors, aptasensors, label-free type, sandwich-type, screen-printed electrode, multichannel, differential pulse voltammetry, impedance spectroscopy, molecularly/cell imprinted polymer, magnet-assisted, nanoporous materials, and so on. The recent global pandemic sets an unprecedented stage for different research teams to present all the technologies mentioned above in detecting SARS-CoV-2, providing a rare and hard-won lesson for one to comprehensively survey the core experimental aspects of developing pathogens' electrochemical biosensors. We will begin this survey with a section on various receptors, immobilization methods, and sensing surface modification approaches. At the end of this survey, we will provide important clues to help audiences who intend to develop novel yet practical biosensors to equip human society for future pathogen threats.

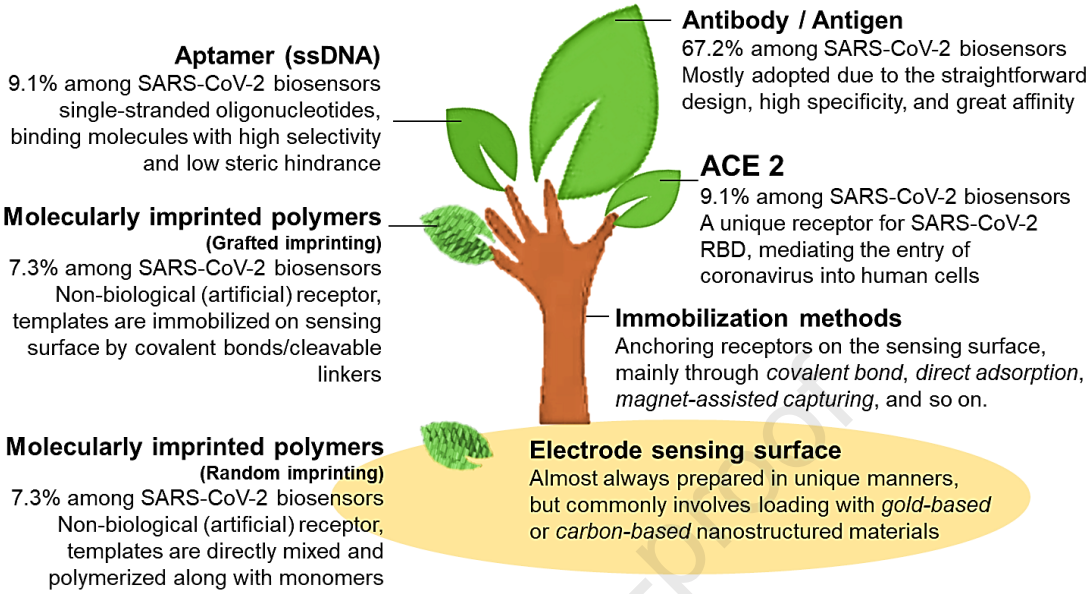

Fig. 1. Schematic diagram of receptors, immobilization methods, and electrode sensing surface associated with the development of the SARS-CoV-2 electrochemical biosensors, the percentile usage rate of each type of receptor were calculated based on the 55 published studies during the COVID-19 pandemic.

2. Antibody receptor

Because antibodies can exhibit remarkable specificity and binding affinity and are competent for almost all pathogens and other infectious agents, they become the "first choice" and "gold standard" when conceiving of developing a new pathogen biosensor. Taken together, it gave the reason for many researchers to call such biosensors the "immunosensors," although many later developed biosensors do not entirely depend on the antibody-antigen conjugation reaction [11]. As shown in **Table 1**, antibodies or antigens are the primary biological receptors adopted in developing electrochemical biosensors to detect biomarkers of SARS-CoV-2.

Table 1. Selected representative studies of SARS-CoV-2 electrochemical biosensors using antibodies/antigens as their receptors.

Receptor	Immobilization strategy	Immobilization mechanism	Sensing surface material	Label-based/ label-free	Output signal [a]	LoD	Reference
Antibody/ antigen (67.2 %) [b]	Classical covalent immobilization Methods (60 %)	Using EDC-NHS [c] as the cross-linker to form strong amide bonds (66.7 %)	Carboxymethyl-chitosan	Label-free	EIS	0.179 fg/mL	[13]
			MUA [d]/AuNPs	Label-free	SWV	1 pg/mL	[14]
			PABE [e]/Carbon nanofiber	Label-free	SWV	0.8 pg/mL	[15]
			EDA/OGCFs [f]	Label-free	DPV	25 pg/mL	[16]
			MAA [g]/AuNPs	Label-free	EIS	3.16 pmol/L	[17]
			PBA [h]/Graphene	HRP-labeled	EIS/DPV	n/a	[18]
		Using other cross linkers to form imine/amine/ amide/thioether bonds (33.3 %)	PBASE [i]/ Graphene oxide	Label-free	SWV	20 µg/mL	[19]
			EpoxyS-Thi-AB [j]	Label-free	EIS	1.2 fg/mL	[20]
			Cysteine-ZnO/ rGO [k]	Label-free	EIS	21 fg/mL	[21]
			Glu-CysAm/ AuNP [l]	Label-free	EIS	0.5 µg/mL	[22]
			PBASE/ Graphene	Label-free	EIS	0.25 fg/mL	[23]

Table 1. (*continued*)

Receptor	Immobilization strategy	Immobilization mechanism	Sensing surface material	Label-based/ label-free	Output signal	LoD	Reference
Antibody/ antigen	Non-covalent adsorption (14 %)	Electrostatic interaction/ van der Waals force	SPCE array [m]	HRP-labeled	CA	0.15 ng/mL	[24]
			AuNPs	Label-free	DPV/CV	0.63 fmol/L	[25]
			PEDOT [n]	Label-free	EIS	n.a.	[26]
			TAPP-DPDD [o]	Label-free	EIS	0.17 fg/mL	[27]
	Magnet-assisted capturing (11 %)	EDC-NHS-Mbs	Pd-Au/ Nanosheet	Label-free	DPV	7.2 pg/mL	[28]
		APBA [p]-Mbs	SPCE	HRP-labeled	SWV	0.20 ng/mL	[29]
		Commercial Anti-mouse IgG-Mbs	SPCE	AP-labeled	DPV	8 ng/mL	[30]
	Other (14 %)	ProtA [q]-mediated immobilization	Cu_2O Nanocube	Label-free	EIS	0.04 fg/mL	[31]
		Commercial His-tagged chelation	$Ni(OH)_2$ NPs	Label-free	DPV	0.3 fg/mL	[32]
		Thiolated antibody binding with Au sensing surface	Ti-Au/P-doped Si-SiO_2	Label-free	EIS	$1*10^5$ gc/mL	[33]

[a] Output signal refers to electrochemical impedance spectroscopy (EIS), Square Wave Voltammetry (SWV), differential pulse voltammetry (DPV), and chronoamperometry (CA); [b] All percentiles are calculated based on the 53 published electrochemical biosensor for the detection of SARS-CoV-2; [c] *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide HCl (EDC) and *N*-hydroxysuccinimide (NHS); [d]11-mercaptoundecanoid acid (MUA); [e] 4-aminobenzoic acid (PABE); [f] Ethylenediamine/oxidized graphitic carbon foil (EDA/OGCFs); [g] Mercaptoacetic acid (MAA); [h] 1-pyrenebutyric acid (PBA); [i] 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PBASE); [j] Epoxy functional group substituted thiophene with acetylene black (EpoxyS-Thi-AB); [k] *L*-cysteine-zinc oxide nanoparticles/reduced graphene oxide (Cysteine-ZnO/rGO); [l] Glutaraldehyde-cysteamine/gold nanoparticles (Glu-CysAm/AuNP); [m] Screen-printed carbon electrode array (SPCE array); [n] Poly 3,4-ethylenedioxythiophene (PEDOT); [o] 5,10,15,20-tetramine (4-aminophenyl) porphyrin -2,2'-bipyridyl-5,5'-dialdehyde (TAPP-DPDD); [p] 3-aminophenyl boronic acid (APBA); [q] Staphylococcal protein A (ProtA).

2.1. Label-free format accounted for the overwhelming majority

At present, one of the primary research focuses associated with the antibody/antigen-based biosensors lies in deciding whether to adopt the label-based (i.e., sandwich-type) or label-free format and use polyclonal or monoclonal antibodies [34-36]. The fundamental difference between the sandwich-type and label-free type is how an antibody-antigen conjugation triggers the transducer to convert the biochemical reaction into an electrochemical signal, such as electrochemical impedance spectroscopy (EIS), Square Wave Voltammetry (SWV), differential pulse voltammetry (DPV), and chronoamperometry (CA).

From the technical perspective (**Fig. 2**), the sandwich-type format typically requires monoclonal antibodies to be first immobilized on the sensing surface and then serve as the receptors to react with target antigens. After the first-run antibody-antigen conjugation, polyclonal antibodies tagged with enzymatic labels (ex. alkaline phosphatase (AP)) are added to the testing solution to conjugate the antigens mentioned above and send out an electrochemical signal produced by the tagged enzymatic reaction (ex. AP converts 1-naphthyl phosphate to 1-naphthol) [37-40]. On the other hand, the label-free format can choose either monoclonal or polyclonal antibodies as the receptors and immobilize them on the sensing surface without additional enzymatic labeling. When testing a potentially infectious sample, the target antigens will conjugate the immobilized antibodies and proportionally cover the sensing surface with the immunological complexes leading to the reduced or perturbed signal intensity [41-43].

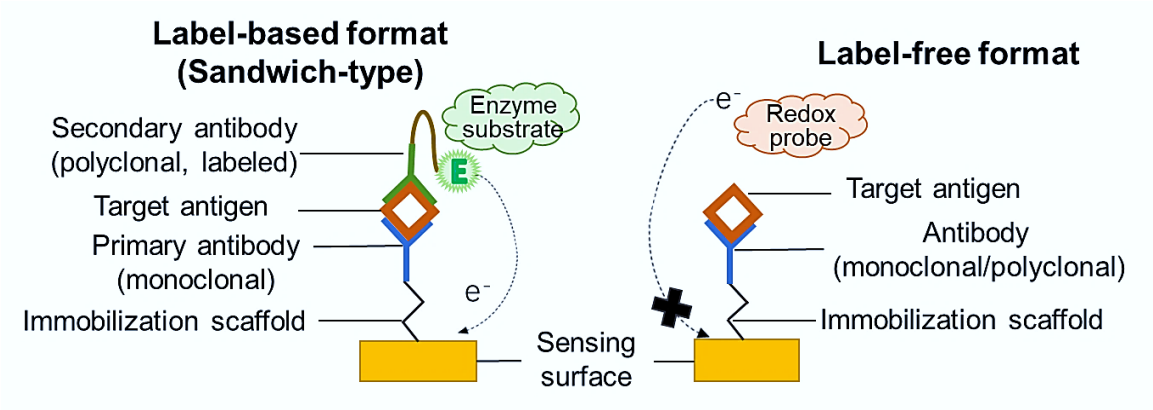

Fig. 2. Schematic diagram of the label-based and label-free immunosensors using antibodies as the biorecognition element.

After going through all the published SARS-CoV-2 electrochemical biosensors, there is no doubt that the label-free format has become the first choice (**Table 1**). This phenomenon can be ascribed to the elimination of preparing the enzyme-tagged secondary antibody in the label-free format, significantly reducing the total workload and cost. Meanwhile, the rapidly growing nanotechnology offers the sensing surface much greater sensitivity to effectively compensate for the heterogeneous diffusion of a redox probe, such as ferri-ferrocyanide, between the solution and electrode interface. Besides, compared with other organic or inorganic analytes, the relatively sizeable conjugated immunological complex can cause a more remarkable signal perturbation, favoring the detection performance of the label-free format [8].

2.2. Antibody immobilization

One way or another, having a robust and efficient immobilization method is an inevitable step in sandwich-type and label-free formats since the sensing performance is directly dictated by the uniform and unhindered presentation of the active protein sites with

equal importance [12]. Among all the adopted immobilization strategies, the classical covalent method accounted for around 60 % of those antibody/antigen-based SARS-CoV-2 biosensors. The powerful covalent bond immobilization is formed typically through reactions between functional groups (ex. amine and carboxyl) present on the protein surface and solid support, triggered by adding a cross-linker agent, such as *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide HCl (EDC) and *N*-hydroxysuccinimide (NHS) [12]. As proteins typically bear many exposed amine groups of lysine residues, using the combination of EDC-NHS became the most used example, which accounted for over 66 % of those covalent bond-based SARS-CoV-2 biosensors (**Table 1**). Other cross-linkers with pyrene and sulfhydryl moieties, such as 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE) and cysteamine (CysAm), were also repeatedly used in developing the SARS-CoV-2 biosensors due to their unique affinity to the graphene and gold functionalized sensing surface through π-π stacking interaction and gold-thiol chemistry, respectively [19, 21-23, 44]. Some representative studies using the above-mentioned covalent methods will be discussed later in this survey.

Besides the covalent bond, direct adsorption is the second most common strategy, which depends on electrostatic interaction and van der Waals force to passively anchor receptors on the sensing surface [26, 27, 45]. The benefit of immobilization using this strategy is neither cross-linker agents nor protein surface modifications are required. However, the direct adsorption method can only offer relatively weak binding, leading to a critical issue that the protein-based receptors could easily leach out from the sensing

surface [12]. Also of note is the involvement of porous organic polymers (POPs) in adsorbing protein-based receptors directly on top of porous solid support. It is believed that POPs can provide high affinity toward different biomarkers through various interactions, including hydrogen bonds, electrostatic interactions, van der Waals force, and π-π stacking interaction [27]. Among all the SARS-CoV-2 electrochemical biosensors, two studies used POPs to achieve the direct adsorption of antibodies on the sensing surface and obtained acceptable detection performance in terms of specificity and sensitivity [26, 27].

It is worth noting that a small minority of research groups adopted remarkably different immobilization strategies, including Staphylococcal protein A-mediated immobilization [31], commercial His-tagged antibody chelation [32], and thiolated antibody binding with the gold-modified sensing surface [33]. From the perspective of developing an electrochemical biosensor, these distinctive methods had a similar advantage: they could offer more oriented immobilization than the classical covalent binding or direct adsorption, ensuring uniform and unhindered presentation of the F_{ab} region on antibody receptors. Nevertheless, the disadvantages are also apparent that they all require additional delicate steps to empower the better-oriented immobilization, which could be slightly tricky to achieve or relatively expensive to obtain.

Interestingly, among all the published SARS-CoV-2 electrochemical biosensors, only one study used two distinctive immobilization strategies (i.e., the classical covalent method and ProtA-mediated immobilization, **Fig. 3**) and compared the difference between the two strategies in terms of the detection sensitivity and dynamic range performance [23]. Based

on the results, the sensitivity in both cases showed a superior low limit of quantification (LoQ) of 0.25 fg/mL, indicating that the orientation of antibody receptors does not significantly affect the detection performance, especially when the target antigens are in trace concentration levels. However, the dynamic range (i.e., 0.25 fg/mL to 1.0 µg/mL) was three-magnitude broader for the ProtA-mediated approach compared to the PBASE-based covalent method (i.e., 0.25 fg/mL to 100 ng/mL) [23], which is an excellent example of how a pathogen biosensor could be affected by the uniform and unhindered presentation of the antibody receptors.

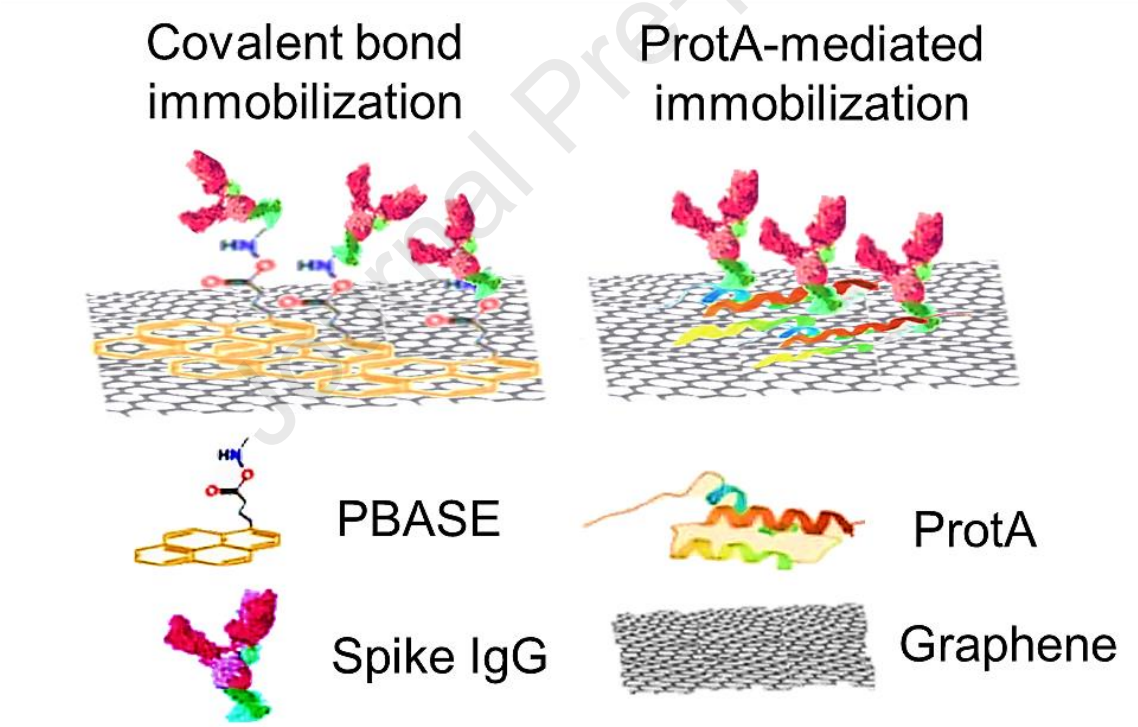

Fig. 3. Schematic illustration of the different immobilization strategies where on the left hand side is the PBASE-based covalent and on the right hand side is the ProtA-mediated (image adapted from Ehsan et al. [23]).

2.3. Why did the magnet-assisted method get a cold shoulder?

Directly immobilizing receptors on the sensing surface may pose several significant

drawbacks. First, the whole immunological chain, assembled layer by layer onto the electrode surface, can undoubtedly lead to the passivation of the sensing capability, which almost always can be found, typically illustrated in a cyclic voltammogram showing the gradually reduced peak heights after each assembling step, in the electrochemical biosensor studies. Additionally, the must-have repeatedly washing steps can cause unpredictable defects on one or more layers, compromising the critical factor of the sensor reproducibility. Another crucial drawback lies in the confined space of a typical electrode platform, which not only hinders the kinetic of immunological reactions but also limits the quantity of total immobilized receptors [46]. Using magnetic beads (MBs) with functionalized surfaces seems able to overcome all the above drawbacks. Plus, MBs allow facile and nonintrusive separation after immunological reactions.

However, after going through all the reported SARS-CoV-2 electrochemical biosensors, it can be found that MBs attracted less attention and only accounted for 11 % of those antibody/antigen-based SARS-CoV-2 biosensors. Presumably, this phenomenon is due to the involvement of the enzyme-labeled secondary antibody, by convention, in the magnet-assisted electrochemical biosensors [29, 30], which is also the main reason for the sandwich-type format becoming significantly less used. To tackle this issue, some research groups have tried to spatially split the immunological chain into "two parts," where the target antigens will first be captured by the MBs surface-assembled antibodies in the test solution, then conjugating the electrode surface-immobilized antibodies with the assistance of a magnet, and eventually generate electrochemical signals in proportion to the level of

sensing surface perturbation caused by the double-antibody conjugated immunological complex. One magnet-assisted SARS-CoV-2 electrochemical biosensors adopted this design, demonstrating an effective way to get rid of the need to prepare the enzyme-labeled secondary antibody while achieving a relatively low LoD and a wide dynamic range [28]. Due to its novel design, a detailed discussion of this "two parts" MPs-assisted method will be provided later in this survey.

2.4. The multifarious electrode surface materials and modification approaches

A typical electrochemical biosensor's working electrode surface is the physical support for receptors on where immunological reactions will occur. Meanwhile, it is also the critical platform that converts immunological activity to electronic signals. From this perspective, it is reasonable that most studies chose carbon-based and gold-based nanostructured materials as their first choice, adding together accounted for around 94 % of all published studies.

A large group of studies used different morphological gold nanoparticles (AuNPs) to modify their sensing surfaces to improve fundamental electrochemical characteristics and form orderly packed self-assembled monolayers (SAMs). Because the fundamental mechanisms of AuNPs and SAMs and their corresponding benefits have been extensively reviewed in other studies, this work will emphasize how to functionalize AuNPs effectively to allow SAMs to anchor receptors in an orderly manner. More specifically, what chemicals have been involved most frequently along with AuNPs to develop the SARS-CoV-2 electrochemical biosensors? As shown in **Fig. 4**, two strategies, namely Glu-CysAm and

MUA/EDC-NHS, were repeatedly adopted in several studies [14, 15, 17, 20, 22, 44, 47]. Both chemical combinations involve using a linkage agent (i.e., cysteamine (CysAm) and 11-mercapoundecanoic acid (MUA)) containing a thiol (-SH) at one end to form gold-thiol bonds (RS-Au) and a functional group (i.e., $-NH_2$ in CysAm and -COOH in MUA) at the other end to either attach to an auxiliary agent (ex., glutaraldehyde) or directly bear the protein receptors through the well-known EDC-NHS-mediated approach. In addition to the standard approach of casting the electrode surface with AuNPs and following the subsequent functionalizing treatments, we found one unusual study that directly assembled the AuNPs-antibody complex in its resuspended solution condition [14]. This study fused several steps, including the AuNPs synthesis, particle surface modification, antibodies immobilization, and target antigens conjugation, into a "one-pot" process, which looked more like it was designed for surface plasmon resonance (SPR) detection. Indeed, this novel biosensor can simultaneously carry out SPR and electrochemical detection, achieving different LoDs of 48 ng/mL and 1 pg/mL, respectively, without conventional electrode surface modifications. Due to its novel design, a detailed discussion of this "one-pot" approach will be provided later in this survey.

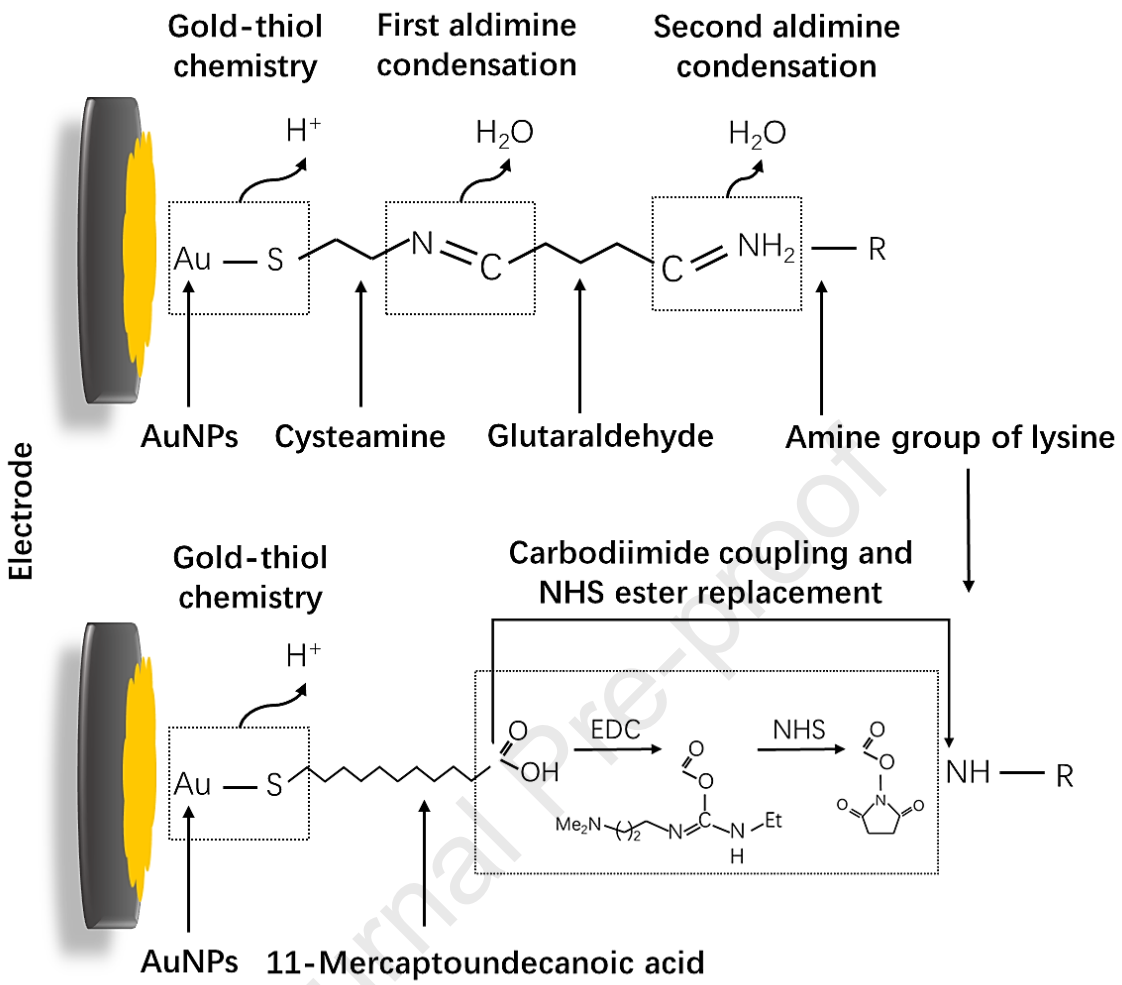

Fig. 4. Illustration of two repeatedly used SAMs combined with covalent bonds methods: (a) using cysteamine and glutaraldehyde through gold-thiol chemistry and double aldimine condensation reactions, (b) using 11-mercapoundecanoic acid (MUA) and EDC-NHS through gold-thiol chemistry, carbodiimide coupling, and NHS ester replacement.

The benefits of using nanostructured carbon in developing electrochemical biosensors have been well studied, including non-toxicity, massive specific surface area, low density, good electrical conductivity, high electronic mobility, and most importantly, ease of production and activation with various active functional groups for anchoring receptors. Thus, nanostructured carbon is another most used electrode material in developing SARS-CoV-2 electrochemical biosensors. It was found that except for the three magnet-assisted

biosensors that directly used unmodified screen-printed carbon electrode (SPCE) as their detection platform [29, 30, 48], other studies involved using carbon-based electrodes all underwent surface modifications with nanostructured carbon, such as graphene, reduced graphene oxide (rGO), and multi-walled carbon nanotubes (MWCNTs). Although different types of nanostructured carbon have been used, two distinctive approaches for effective functionalization of the carbon backbone can be concluded herein, namely the generation of the endogenic active groups through physical-chemical activation and the insertion of the exogenous active groups through linkage agents addition (**Fig. 5**). Of note, the introduction of active groups through the addition of pyrene or quinoline derivatives (via π-π stacking) was adopted in six individual SARS-CoV-2 electrochemical biosensors compared with four that used different physical-chemical oxidation methods, which require generating defects and edges on the sp^2 carbon structure to bear oxygen-based functional groups [49-51]. We understand that each research team usually has a specific preference for handling the carbon backbone activation. To avoid the inevitable adverse impacts on the sensing surface, relatively mild treatments should be considered first as long as the specific chemicals' accessibility stays high.

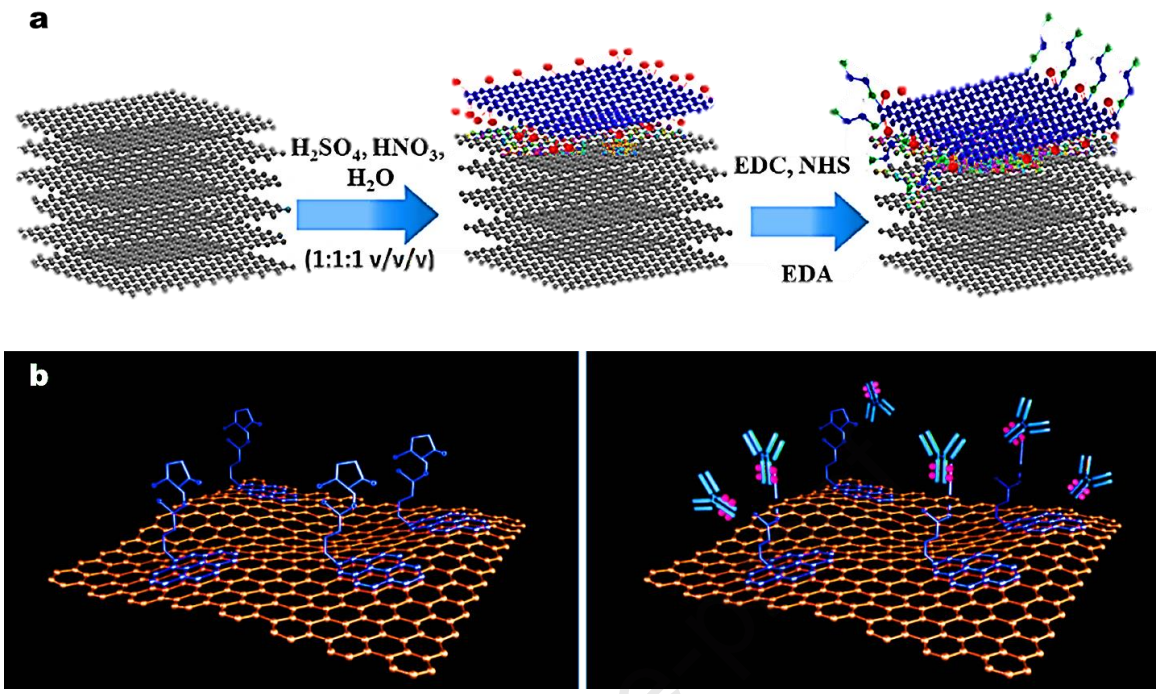

Fig. 5. Illustration of two approaches for effective functionalizing the nanostructured carbon backbone with the receptor-bearing active groups: (a) generation of the active functional groups through strong acids oxidation following with the EDC-NHS treatment (image adapted from Adeel et al. [16]) Copyright [2022] by Elsevier. Reprinted with permission. (b) insertion of 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE) (image adapted from Ehsan et al. [23]).

3. Aptamer and ACE2 receptor

Apart from the conventional antibody/antigen-based receptors, aptamers (i.e., oligonucleotides) and ACE2 (i.e., human angiotensin-converting enzyme 2) have also been utilized to develop the SARS-CoV-2 electrochemical biosensors. Regarding the former, we found five individual studies that used an aptamer receptor to target different SAR-CoV-2-related biomarkers, including the S1 receptor-binding domain (RDB) [52, 53], RNA sequence [54, 55], and nucleocapsid protein (NP) [27] (**Table 2**). On the other hand, we also found five individual studies that adopted ACE2 as their receptors (or used it as an alternative to the secondary antibodies). Of note, due to the nature of ACE2, it can only be utilized to specifically detect the RBD protein within the SARS-CoV-2 spike protein subunit 1 (S1) [56-60].

Given that aptamers and ACE2 still fall into the category of biorecognition elements, they undoubtedly share some common traits with the antibody/antigen-based electrochemical biosensors. As a result, we will mainly focus on the remarkable differences and some unique experimental aspects of the aptamers/ACE2-based SARS-CoV-2 electrochemical biosensors. However, as the total number of both studies is significantly less than the antibody/antigens-based biosensors, this issue more or less hindered us from identifying specific patterns or drawing solid conclusions.

Table 2. Studies of SARS-CoV-2 electrochemical biosensors using an aptamer or ACE2 as their receptors.

Receptor	Target biomarker	Immobilization method	Sensing surface material	Label-based/ label-free	Output signal	LoD	Reference
Aptamer (5/53)	RBD	Thiol-labeled ssDNA binds to AuNPs modified surface (Au-S bond)	CNF [a]-AuNP/ SPCE	Label-free	EIS	0.35 ng/mL (7.0 pM)	[52]
			AuNP/ SPCE	Label-free	EIS	0.06 ng/mL (1.3 pM)	[53]
	Nucleotide sequence		micro-Au/ GNPs [b]	AuNPs-labeled	PO [c]	6.9 cp/μL	[54]
		Streptavidin/ EDC-NHS	CMD [d]/ SPCE	HRP-labeled	CA	0.50 ng/mL (10 pM)	[55]
	NP	Direct adsorption	TAPP-DPDD-POP [e]	Label-free	DPV	0.59 fg/mL	[27]
ACE2 (5/53)	RDB	Glutaraldehyde or EDC-NHS (Covalent method)	SPCE	Label-free	EIS	2.8 fg/mL	[58]
			CysAm-SiO_2@UiO-66 [f]	Label-free	EIS	100 fg/mL	[57]
			CysAm-AuNP	Label-free	EIS	229 fg/mL	[59]
		Magnet-assisted electrochemical assay	SPCE	AuNPs-labeled	DPV	0.35 ag/mL	[60]
			SPAuE [g]	Label-free	CA	22.5 ng/mL	[56]

[a] Carbon nanofiber (CNF); [b] Graphene nanoplatelets (GNPs); [c] Potentiometry (PO); [d] Carboxymethyl-dextran (CMD); [e] 5,10,15,20-tetramine (4-aminophenyl) porphyrin – 2,2'-bipyridyl-5,5'dialdehyde porous organic polymers (TAPP-DPDD-POP); [f] Silicon dioxide nanoparticles modified Universitetet i Oslo-66 metal-organic framework (SiO_2@UiO-66); [g] Screen-printed gold electrode (SPAuE).

3.1. Remarkable virtues of the aptasensors

Compared to the antibody/antigen-based SARS-CoV-2 electrochemical biosensors, the aptamer-based biosensor, also known as aptasensor, demonstrated several attractive features, including its wide range of biomolecule targets, ease of tagging with terminal chemical moieties, and outstanding stability. Although we could only address five electrochemical aptasensors reported during the COVID-19 pandemic, it can still be found that they possess a high versatility, covering a wide range of target biomolecules like SARS-CoV-2 RBD epitope, nucleocapsid phosphoprotein, and the nucleotide sequence. Moreover, it can be found that this versatility is not only exhibited over the different kinds of target biomolecules but also within a single type of analyte. For example, Alafeef et al. [54] simultaneously selected four different antisense single-stranded oligonucleotides (ssDNA) to target two regions within the same SARS-CoV-2 N-gene. The advantage of this design is apparent that amid the fast global spread of COVID-19, it empowers the developed aptasensor with practical implications even if one or more regions of the viral gene mutated.

Secondly, the in vitro combinatorial chemical synthesis offers remarkable convenience to aptamers in designing and tagging terminal functional groups. Indeed, when conceiving an electrochemical aptasensor, the most common strategy is to add thiol groups at the end of aptamer sequences, allowing the formation of the gold-thiol bond to direct attach to either a gold-based sensing surface or signal amplifier [61]. As shown in **Table 2**, three out of five electrochemical aptasensors chose to immobilize their thiolated-aptamers on the

AuNPs modified sensing surface through the facile SAMs process [52-54]. In comparison, only one out of 35 antibody-based biosensors adopted this directly thiolated strategy [33] (see **Table 1**).

Another critical characteristic of aptamers lies in their chemical stability, particularly the resistance to thermal denaturation and harsh treatments [62]. In many real cases, this feature may become more dispositive than other critical technical performances because the distribution of the point-of-care testing kits depends heavily on precisely coordinated cold-chain logistics, and the less temperature-sensitive aptamer-based biosensors can remarkably ease the burden on the logistical cost. After carefully reading all the SARS-CoV-2 electrochemical aptasensors, we found that four (i.e., 80% of total aptamer-based studies) had undergone sensor stability tests over different time spans at 4℃ (or storage in a refrigerator) [27, 52, 53, 55]. Among them, Abrego-Martinez et al. [53] reported an outstanding result in the thermal stability test after 21-day storage at 4℃, in which the impedimetric response of the developed aptasensor only lost 1 % of its sensing capability with respect to the freshly prepared one. In **Table 3**, several representative studies that carried out the storage stability test are listed below. If we take away the particular case of 30-day without significant change achieved under the argon atmosphere [47], the aptasensors generally showed more extended storage stability. It should be mentioned that as one of the most active research teams, Dr. Lokman Liv and his co-workers have consecutively reported the remarkably stable performance of using the argon atmosphere to preserve the sensitivity of biosensors [44, 47, 63, 64], which allows them to be stored

for a long-term at room temperature (i.e., 25℃) or even at higher summer temperature (i.e., 37℃).

Table 3. Stability performance and storage condition of the selected SARS-CoV-2 electrochemical biosensors.

Receptor type	Storage/preserving conditions	Shelf-life/ test period	Signal attenuation/change after storage	Reference
Antibody	Dry using N_2 gas; store at 4℃	14 d	3% reduction of ESI response	[31]
	Store in Ar[1]; at 4℃, 25℃, 37℃	30 d	No significant difference at 4℃ and 25℃, 15.5% reduction at 37℃	[47]
	Store in a dry environment; at 4℃	10 d	No significant reduction but higher signal scattering	[28]
Aptamer	Store in the refrigerator	14 d	No significant reduction in EIS	[52]
	Store at 4℃	15 d	108.7% of the initial EIS signal	[27]
	Store at 4℃ in BB[2]	21 d	Signal loss of 1% to a fresh sensor	[53]
ACE 2	Store dry at 4℃	3 d	50% reduction of the initial	[59]
	Store in PBS at 4℃	6 d	23% reduction of the initial	
	Store at -20℃	5 d	21.6%[3] reduction of the initial	

[1]Store in an argon atmosphere; [2]Binding buffer (50 mM Tris-HCl + 150 mM NaCl + 2 mM $MgCl_2$, pH = 7.5); [3]Calculated value based on the information from .

The schematic designs of the SARS-CoV-2 electrochemical aptasensors are illustrated in **Fig. 6**. Compared with the antibody-based studies, aptasensors showed high diversity in how an electrochemical signal could be triggered due to the conjugation between the target biomarkers and aptamer receptors. Nevertheless, evaluating its sensitivity, specificity, and detection range is crucial when getting back to the basics of a pathogen biosensor. The study reported by Cui et al. [27] provides an excellent example of comparing the core competencies when choosing aptamers or antibodies as receptors to detect the same SARS-CoV-2 biomarker (i.e., nucleocapsid protein, NP). According to the study of Cui et al. [27],

in the same condition, the SARS-CoV-2 immunosensor (antibody-based) showed a better LoD of 0.17 fg/mL and a higher maximum response concentration (MRC) of 200 pg/mL compared to 0.59 fg/mL and 10 pg/mL obtained from the SARS-CoV-2 aptasensor, respectively. Although based on this single study, we cannot simply conclude that antibodies surpass aptamers in pathogen detection performance, the relatively minor interfacial resistance change presenting before and after the aptamer-antigen binding is a general issue associated with the label-free aptasensor system [61, 62]. In other words, the label-free strategy heavily depends on the subtle change in the interfacial electron transfer resulting from the non-electroactive protein covering, which naturally favors the antibody receptor due to its sizeable biomolecular dimension and the subsequently formed insulating layer.

In summary, the recently reported SARS-CoV-2 electrochemical aptasensors exhibited remarkable versatility toward various target biomarkers, a high level of convenience in tagging terminal functional groups, and excellent thermal stability. Though based on the current released studies, the aptasensors seem to have slightly compromised their sensitivity (most at the level of ng/mL or pM) when compared with immunosensors; we believe that along with the more aptasensors being developed, ones with outstanding feasibility and sensitivity could be achieved in the future.

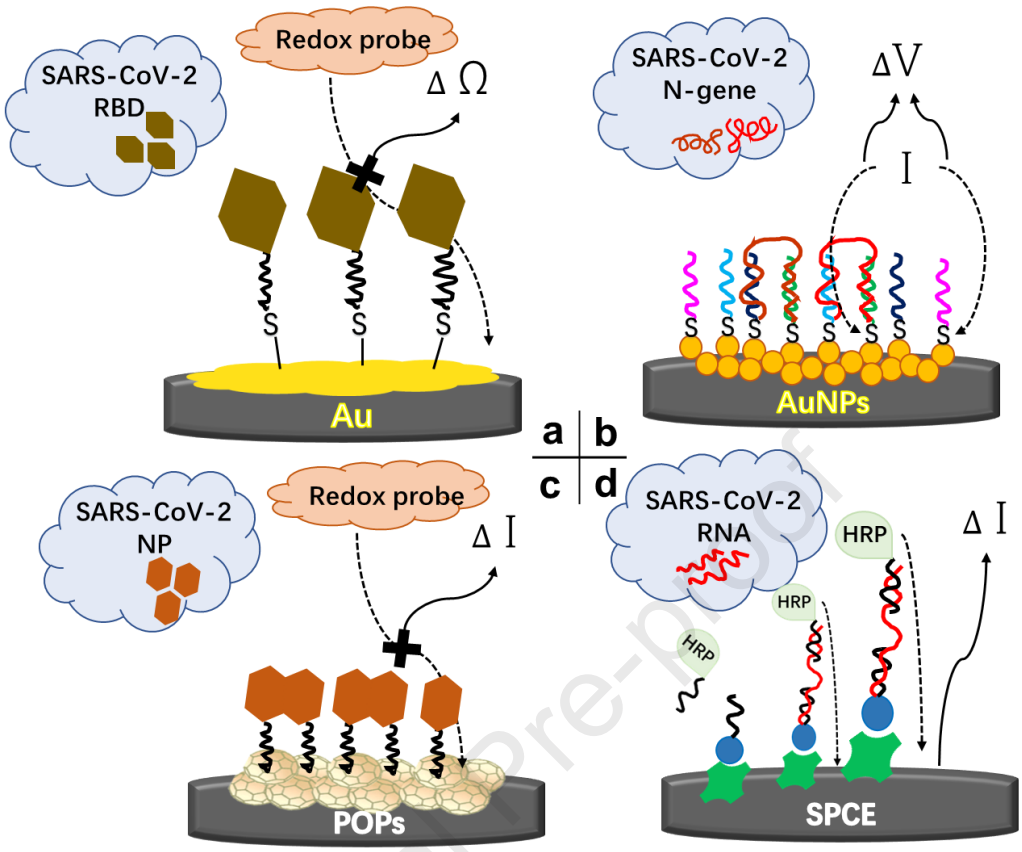

Fig. 6. Schematic diagrams of the four strategies of the aptamer-based SARS-CoV-2 electrochemical biosensors: (a) thiolated aptamer label-free detection [52, 53], (b) thiolated aptamer duplex association detection [54], (c) polymers attached aptamer label-free detection [27], and (d) biotinylated aptamer duplex association with enzyme-labeled detection [55].

3.2. Unique features of the ACE 2-based biosensors

Exploring the strong binding affinity between the RBD within the S1 protein and the human ACE2 receptor offers a unique path to conceiving the SARS-CoV-2 electrochemical biosensor. As mentioned above, we found five individuals harnessed the ACE 2-RBD binding to facilitate the highly selective yet more sensitive detection of SARS-CoV-2. Focusing on those designs purely from a technical perspective, the ACE 2-based biosensors seem to have no significant differences from the antibody/antigen-based biosensor studies.

However, due to the nature of ACE 2, three mentionable features will be discussed below in detail, including its exceptionally high affinity to specific SARS-CoV-2 variants, quantifiable biological activity by testing with its natural substrate (i.e., angiotensin II), and extraordinary performance on the detection sensitivity.

Conducting the selectivity analysis, also known as the cross-reactivity study, is an essential step subsequent to biosensor fabrication. During this step, a fixed concentration of the target analyte undergoes investigations using the established experimental procedure in the presence of potential off-target interfering agents. An interesting observation from the selectivity analysis was reported by de Lima et al. [59], in which the ACE 2-based electrochemical biosensor exhibited a remarkably higher selectivity to the SARS-CoV-2 UK variant B.1.1.7 (Alpha) than the original type. As a result, de Lima et al. [59] interpreted that the more infectious Alpha variant carrying eight mutations in the RBD region (**Fig. 7**) empowers the higher affinity with the ACE 2 receptor, thus significantly enhancing the electrochemical response during the selectivity analysis. Given the fact that the recent variants of SARS-CoV-2, like the SARS-CoV-2 SA variant BA.1 and BA.1.1 (Omicron), seem to evolve continuously toward more infectious, the ACE 2-based detection method can potentially become a more powerful tool in confronting newly mutated strains of SARS-CoV-2 and taming the associated outbreaks.

One common challenge before conducting electrochemical detection is to evaluate the receptor's biological activity in a manner that conveys just how fresh, functional, or well-preserved a biosensor is without actually sacrificing several electrodes to run some

preemptive tests. Given that angiotensin II is ACE 2's natural substrate, analyzing the spontaneous enzymatic activity by exposing the prepared biosensors with angiotensin II offers a viable way to tackle the need for establishing a facile functionality test. This idea was first proposed by Torres et al. [58]. They first applied Nafion as the ACE 2-protection membrane on top of the prepared biosensors. Subsequently, they evaluated the receptor's functionality by one-step measuring the impedimetric response in the angiotensin II solution. In contrast, by convention, to evaluate the freshness and functionality of an electrochemical biosensor, it is necessary to perform actual measurements using the prepared electrode system, resulting in the direct interaction between the receptors and analytes, causing inevitable performance loss.

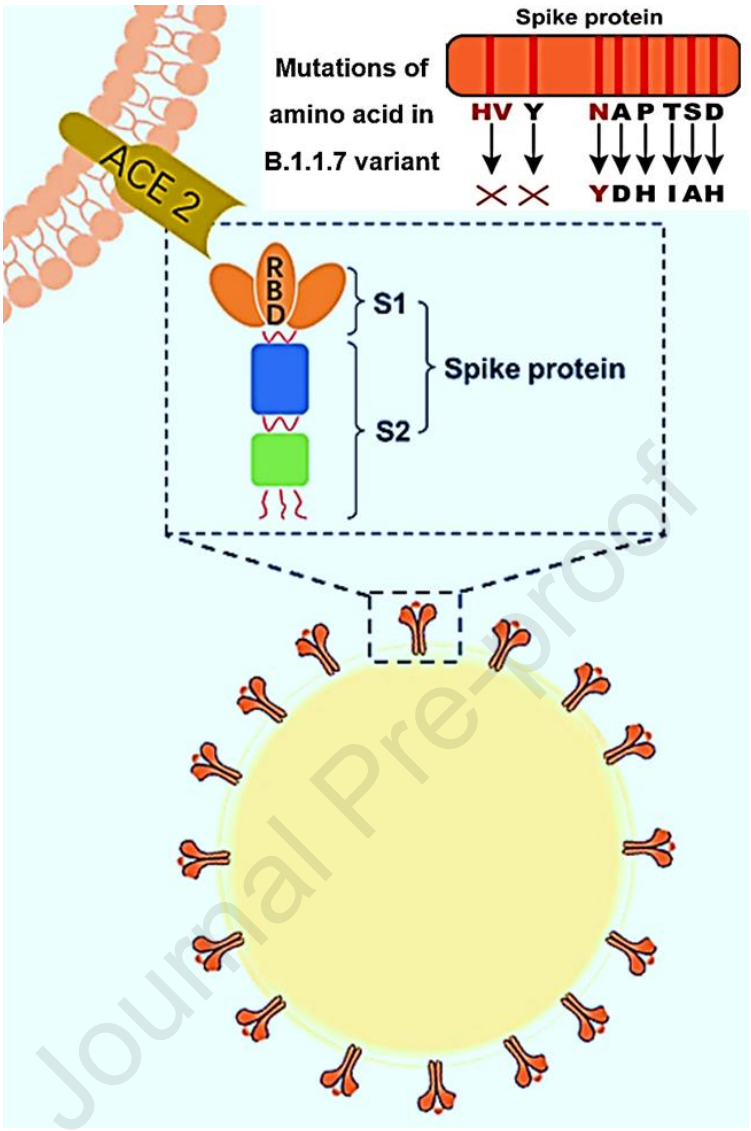

Fig. 7. Illustration of the SARS-CoV-2 spike protein structure and the mutations of spike protein in the more infectious SARS-CoV-2 UK variant B.1.1.7 (Alpha) (image adapted from [65]).

Based on what we have reviewed, achieving a significantly low detection limit (i.e., LoD) is an inescapable theme that has been pursued in all the SARS-CoV-2 biosensor studies. After going through all the LoDs reported recently, it can be found that the ACE 2-based electrochemical biosensors show superior performance in general. As an LoD is usually calculated based on the signal-to-noise ratio, the low LoDs can directly reflect the excellent affinity of ACE 2 to RBD and the minimum non-specific binding risk. It is worth

mentioning that a study with the lowest LoD till now (i.e., 0.35 ag/mL) was also achieved when using ACE2 as its receptor [60]. Considering its remarkable performance regarding the common concern of having a better LoD, a detailed discussion of its sensing mechanism will be provided later in this survey.

4. MIPs receptor

It should be noted the biosensors, as mentioned above, rely on biological receptors recognizing the idiotypic moieties present in SARS-CoV-2. However, fabricating these biosensors using biological receptors is generally believed to be costly and limited in sensor shelf life [66]. Introducing MIPs as artificial biorecognizers for pathogen detection can offer a promising way to enhance the cost-effectiveness and robustness of these biosensors. As discussions of the MIPs' merits have been done in many other works, we direct readers to more specific reviews [67, 68].

During the COVID-19 pandemic, we found eight specific MIPs-based SARS-CoV-2 detection studies (**Table 4**), covering the three mainstream MIPs-related taxonomies, including whole-cell/epitope imprinting, grafted/random imprinting, and bulk/surface imprinting (**Fig. 8**). These three taxonomies assign MIPs to six individual subsets allowing one to quickly pick up appropriate technologies or often combine several to create a feasible technique roadmap. For example, Bognar et al. [69] grafted (i.e., immobilized) the RBD epitope on the gold surface via gold-thiol bonds, then adopted electropolymerization, a typical polymerizing approach within the surface imprinting scope, to fabricate the thin polymer layer using scopoletin as the MIPs monomer. The above example adsorbed and merged the advantages of high selectivity, fast mass transfer rate, and oriented template direction from epitope imprinting, surface imprinting, and grafted imprinting, respectively.

Table 4. Studies of SARS-CoV-2 biosensors using MIPs as artificial biorecognition elements.

Template anchoring strategy	Template anchoring method	Polymerization strategy	Template washing method	Template molecule	Monomer molecule	Sensing surface	LoD	Reference
Grafted imprinting method (4/53)	Cys- peptides bind to Au surface (Au-S bond)	Surface imprinting (electropolymerization)	Electrochemical oxidative desorption	RDB epitope	Scopoletin	Au	100 fM	[69]
	4-ATP w/ DTSSP [a] (Covalent binding of cleavable linker)		Disulfide-bond cleavage via reducing agent	NP	m-PD [b]	Au-TFE [d]	15 fM	[70]
				SP	3-APBA [c]		15 fM	[71]
	EDC-NHS w/APBA (Boronate affinity to glycoprotein)	Bulk imprinting (oxypolymerization)	Organic solvent wash (10 vol% acetic acid and ethanol)	whole-cell	GO-bearing pyrrole [e]	GCE	0.326 fg/mL	[72]
Random imprinting method (4/53)	n. a. [f]	Surface imprinting (electropolymerization)	Alkaline wash	RDB epitope	o-PD [g]	Au-SPE	0.7 pg/mL	[73]
			Acid wash	SP	Pyrrole	Pt-disk	n. a.	[74]
		Bulk imprinting (UV-thermopolymerization)		Whole-cell	AAM-MAA-MMA-NVP-GO [h]	Ag-SPE	0.1 fM	[75]
		Surface imprinting (self-polymerization)	Organic solvent wash (anhydrous ethanol/water)	RDB epitope	DA [i]	MNPs	22.5 ng/mL	[76]

[a] 4-aminothiophenol with 3-3'-dithiobis sulfosuccinimidyl propionate (4-ATP w/ DTSSP); [b] m-phenylenediamine (m-PD); [c] 3-aminophenyl boronic acid (3-APBA); [d] Gold-based thin film metal electrode (Au-TEME); [e] decorated graphene oxide with pyrrole-boronic acid (GO-bearing pyrrole); [f] Not applicable (n. a.); [g] o-phenylenediamine (o-PD); [h] Acrylamide-methacrylic acid-methyl methacrylate-N-vinylpyrrolidone-graphene oxide (AAM-MAA-MMA-NVP-GO); [i] Dopamine (DA)

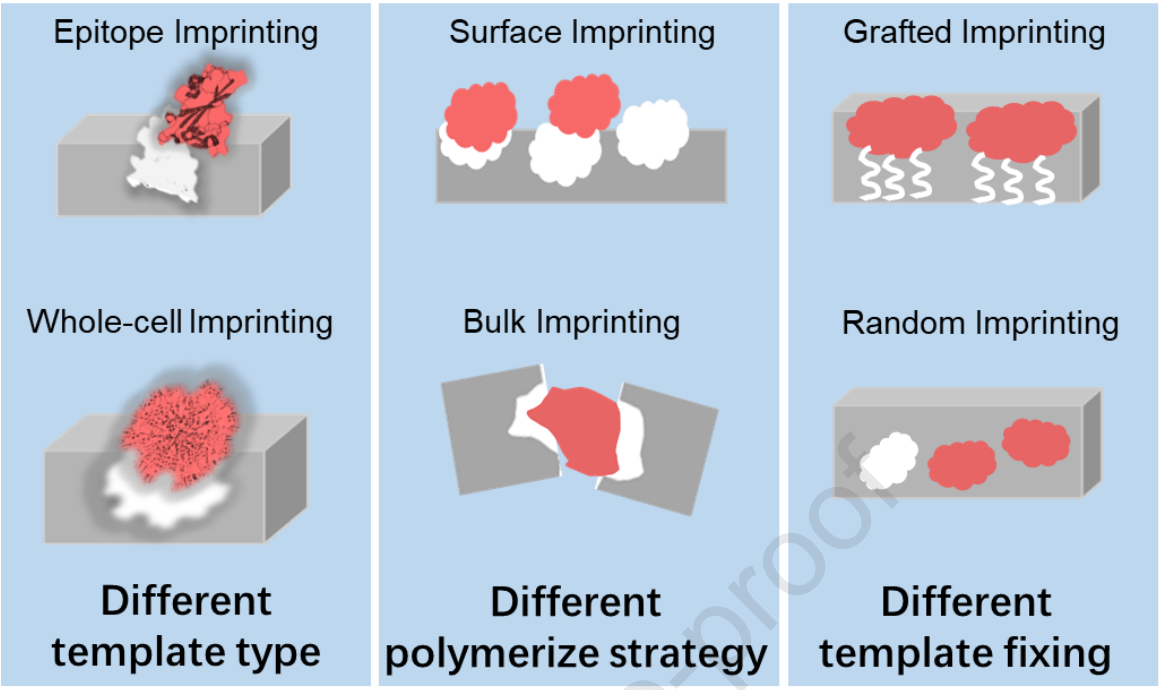

Fig. 8. Illustration of the three mainstream taxonomies of MIPs regarding cell imaging, protein purifying, and pathogen detection applications.

4.1. Grafted imprinting vs. random imprinting

Based on comparing the process complexity within all of the MIPs-related strategies, choosing between the grafted and random imprinting can profoundly impact the ease of the MIPs fabrication due to the additional steps that require attaching/detaching the anchoring chemical linker by introducing suitable cleavable agents or dismountable bonds between templates and a sensing surface. As a result, we categorize all the published MIPs-based biosensors in accordance with the grafted/random imprinting.

As shown in **Table 4**, we addressed equal numbers of studies that have decided whether to adopt the grafted or random imprinting, indicating both strategies have some intrinsic advantages. The grafted imprinting can result in the formation of orderly packed template cavities under the condition of a mild and highly controlled template release

process, such as electrochemical oxidation [69] and the S-S bond cleavage [70, 71]. Other than the gold-thiol and EDC-NHS (covalent) binding methods that have been extensively applied in typical electrochemical biosensor studies, introducing a cleavable linker (i.e., 3-3'-dithiobis sulfosuccinimidyl propionate (DTSSP)) to meet the goal of effective yet more controlled templates anchoring and releasing through the amide bond attachment and S-S bond cleavage is a unique and iconic motion of the grafted MIPs studies. Of note, within the two different grafted studies [70, 71], having the only similarity of both using DTSSP as their template cleavable linker, excellent yet highly comparable detection performances in terms of LoD (i.e., 15 fM and 15 fM), LoQ (i.e., 50 fM and 64 fM), and MRC (i.e., 111 fM and 200 fM) were obtained, indicating the importance of having a reliable cleavable template linker for a grafted MIPs-based biosensor. On the other hand, random imprinting can, to a great extent, simplify the fabrication process and relies on harsh treatments like alkaline/acid wash to thoroughly decompose and release the embedded templates from the polymers layer [73-75]. As shown in **Table 4**, all the random imprinting studies simply washed out their templates using harsh chemical methods. Although the harsh chemical washing may compromise the integrity of the polymers-cavities structure [77], using synthetic materials with high stability can effectively ensure the resistance against most organic or inorganic alkaline/acid solvents. More importantly, the random imprinting strategy can maximize the advantages of adopting the MIPs-based recognition elements, particularly the ease of operation and excellent robustness.

4.2. Surface imprinting vs. bulk imprinting

If we judge purely by counting the number of related studies, the surface imprinting strategy had an enormous superiority over the bulk imprinting, showing that the spotlight has been preferentially cast on the surface imprinting in recent years. After further narrowing the surface imprinting strategy down to a specific polymerization technology, we found that the electropolymerization method accounted for over 80 % of those surface imprinting studies. The significant trend in the prevalence of electropolymerization is partly due to the attractive features of the subsequent electrochemical detection system, such as its excellent readout sensitivity and the apparent portability [68]. Not to be overlooked is that electropolymerization can precisely control the polymer thickness by adjusting the electrochemical parameters (e.g., scanning cycles in the cyclic voltammetry). This feature can avoid the "embedding" phenomenon commonly associated with MIPs fabrication and offers more functioning cavities during the detection process. For instance, Bognar et al. [69] reported that, due to the electro-inactive feature of the poly-scopoletin layer, the obtained MIPs is strictly self-limiting and can result in a highly conformal film with thickness up to 10 nm, which is perfect for accommodating the SARS-CoV-2 RBD with the size of *ca.* 20 kDa.

On the other hand, although only two studies were found, a trend can still be found that the whole-cell imprinting and bulk imprinting seem to merge and become interchangeable when considering developing a MIPs-based pathogen biosensor. Of note, bulk imprinting has been frequently mentioned as a suboptimal method, having some

bottleneck problems like slow binding kinetics, severe template residues problem, and heterogeneity in the resulted polymer structure [77, 78], which could be used to explain why the whole-cell imprinting method was not received as much attention as the epitope imprinting method. Presumably, the study report by Sukjee et al. [75] can overturn the stereotype of bulk imprinting. Their study used four monomers mixed with graphene oxide to successfully fabricate the MIPs layer to detect the SARS-CoV-2 whole-cell in wastewater, achieving an excellent detection sensitivity of 0.1 fM and a high maximum response level at 100 fM, respectively. Due to its straightforward design and great practical significance, a detailed discussion of this whole-cell imprinting study will be provided later in this survey.

5. Selected studies of the electrochemical biosensors

Although the criteria for which characteristics should be considered with high priority remains a subject of discussion, some key features, such as sensitivity, specificity, dynamic ranges, feasibility, material costs, and resistance against fouling and passivation, have been mentioned repeatedly in the studies of electrochemical detection of the SARS-CoV-2 biomarker. Accordingly, four SARS-CoV-2 electrochemical biosensors, each representing a unique design, are selected to undergo detailed discussions below.

5.1.MBs-assisted label-free "two parts" method

As the whole immunological chains typically take place on the surface of MBs, the magnet-assisted electrochemical assay requires using enzyme-labeled secondary antibodies to react with the corresponding enzymatic substrate and then transfer the electronic signal to the sensing surface. The consequence of adopting the conventional MBs-assisted method, aside from the raised material cost and tedious preparation process, is that the enzyme-labeled secondary antibodies do not directly contact the sensing surface. In other words, the enzymatic reaction will be taken place far away from the sensing surface, adding another diffusion-controlled step (i.e., the enzymatic reaction products diffuse to the sensing surface) to accomplish the sensing and thereby limiting the sensitivity and dynamic range of the method [46].

As shown in **Fig 9a**, Zhao et al. [28] reported a novel MBs-assisted label-free electrochemical immunosensor to detect the SARS-CoV-2 S1 antigen with a low LoD of 7.2 pg/mL and a wide dynamic range from 0.01 to 1,000 ng/mL. In their work, the

antibody-functionalized MBs no longer fully supported the whole immunological chain but only served as half of the immunological reaction support and sample separators. On the other hand, the electrode modified with Pd-Au nanosheets was used as the other half of the immunological reaction support allowing the same antibodies to be immobilized on the top. After immunological conjugating, the electrochemical signal would be proportionally changed due to the surface perturbation caused by the double-antibody conjugated immunological complex. This design has great practical significance since it inherits virtues from the conventional labeled-based MBs-assisted method, including effectively extending the space for immunological reactions from the electrode surface to the bulk solution and magnetically enriching the viral concentration in advance of the assay. Nevertheless, most importantly, it can avoid the conventional involvement of the enzyme-labeled antibody in the MBs-assisted assay. In comparison with the other two MBs-assisted antibody-based electrochemical biosensors [29, 30], the study reported by Zhao et al. [28] brings down the LoD from the level of ng/mL to pg/mL, which of the MBs-assisted electrochemical immunosensor is a big step forward.

5.2.AuNPs-antibodies suspension method

As we discussed earlier, the AuNPs modified electrode surface is not merely the perfect physical support for receptor immobilization due to the formation of uniform SAMs but also plays a critical role in enhancing the sensing performance by improving many essential characteristics of the electrode surface. Thus, using AuNPs-modified electrodes to develop an electrochemical biosensor has received much attention. However, AuNPs-

modified electrodes are not yet practical for mass-product, as the surface modification protocols are often too delicate to be implemented by industry [46]. As an alternative, Karakus et al. [14] immobilized the SARS-CoV-2 SP antibodies directly on the AuNPs in their resuspended condition and came up with an interesting colorimetric-electrochemical-hybridized detection method.

As shown in Fig.9b, the merits of this method lie in the following aspects: 1. the MUA-caped AuNPs can form a uniform SAMs layer around nanoparticles through the thiol-terminated moiety and use the carboxyl-terminal to prevent collision between nanoparticles while enabling the MUA-AuNPs for the subsequent antibody immobilization. 2. when testing samples, the presence of SARS-CoV-2 will lead to the aggregation of AuNPs, enabling rapid colorimetric detection as a preemptive move to decide the necessity of the following electrochemical detection. 3. the electrochemical detection process can be performed using unmodified disposable screening-printed electrodes, avoiding the delicate electrode surface modification process and significantly improving the practicability. 4. the authors took advantage of the cathodic response from heteroatoms (i.e., reducing reaction) like carbonyls from the antibodies themselves, thereby achieving the label-free detection even without adding redox couples. It should be noted that due to the combination of the colorimetric and electrochemical detection, the prepared AuNPs-antibodies complexes have to be kept in a well-suspended condition, which prevents from using a separator like MBs to enrich the immunological reaction products in the vicinity of the electrode surface and limit the electrochemical assay to obtain a comparable sensitivity to other studies.

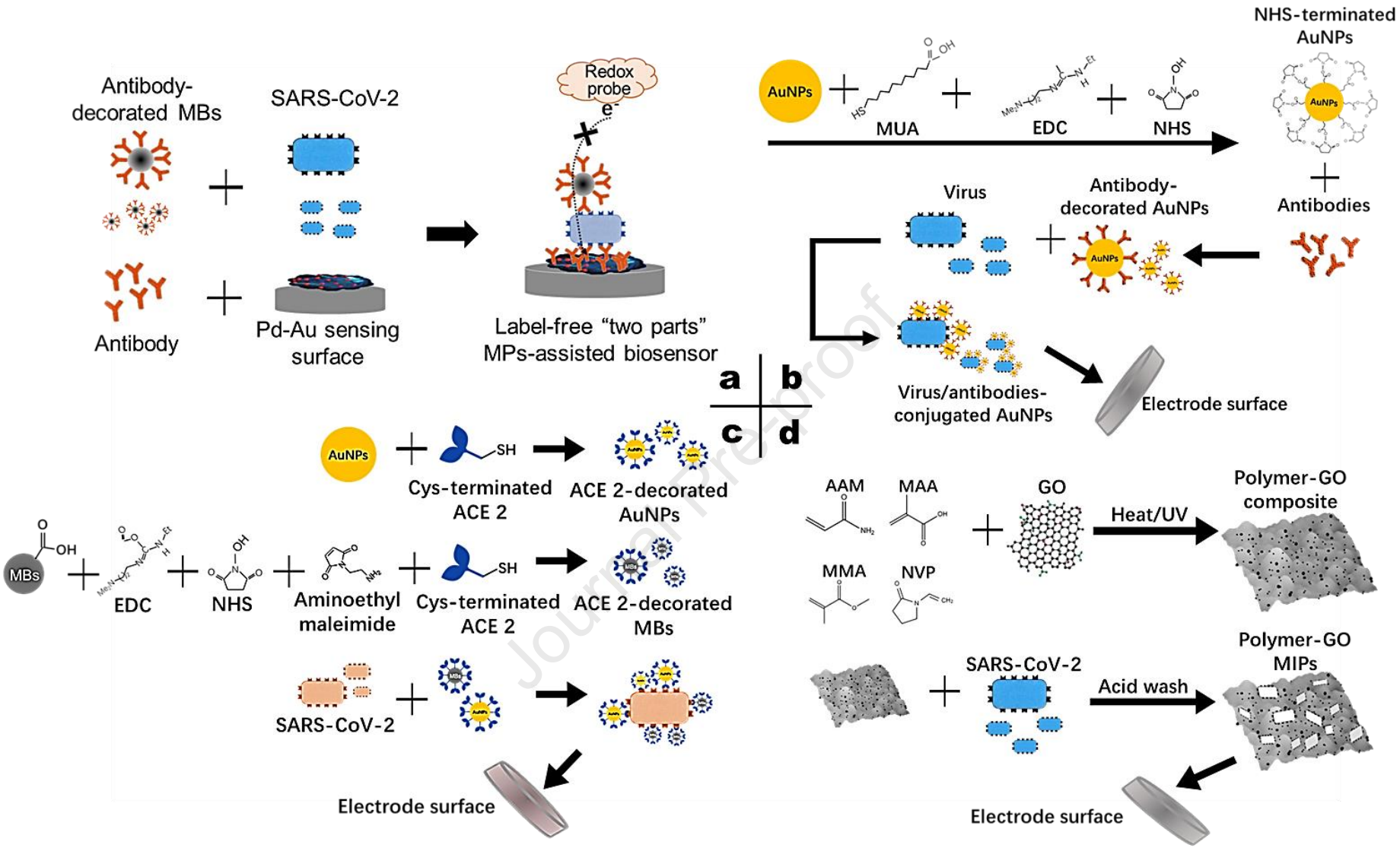

Fig. 9. Schematic diagrams of the selected studies: (**a**) MBs-assisted label-free "two parts" method [28], (**b**) AuNPs-antibodies suspension method [14], (**c**) MBs-ACE 2/AuNPs-ACE 2 suspension method [60], and (**d**) Polymer-GO composite whole-cell MIPs method [75].

5.3.MBs-ACE 2/AuNPs-ACE 2 suspension method

Considering that ACE 2-RBD binding plays a critical role in the fast-spreading of the SARS-CoV-2 pandemic, one does not need to overemphasize the great potential of using ACE 2 as the receptor to develop highly sensitive detection methods. As we mentioned, one of the ACE 2-based electrochemical biosensors obtained the lowest LoD of 0.35 ag/mL among all the SARS-CoV-2 detection studies [60], once again proving ACE 2 is a powerful candidate when considering to select a receptor for the SARS-CoV-2 detection.

Apart from the natural affinity of ACE 2 toward RBD peptides, its novel design also accounted for a large part of the excellent detection performance. In comparison with the above-mentioned AuNPs-antibodies suspension method [14], Nascimento et al. [60] 's study applied a similar approach to utilize AuNPs as the signal amplifier under their suspension condition and thus avoiding the delicate electrode surface modification process (**Fig. 9c**). In addition, they used nanoscale MBs functionalized with ACE 2 as the separators to enrich the concentration of viruses on the electrode surface. By convention, the value of LoD is calculated based on the signal-to-noise ratio. Thus, repressing non-specific adsorption is another critical factor when designing a high-sensitivity biosensor. As shown in **Fig. 9c**, the electrochemical response can only be triggered after the SARS-CoV-2 viral cells synchronously attached ACE 2-AuNPs and ACE 2-MBs, forming a sandwiched structure AuNPs-ACE 2-RBD/Virus/RBD-ACE 2-MBs. This design can ensure a minimum noise signal level produced by non-specific bindings, which share a similar design philosophy with the typical sandwich-type immunosensors. In summary, three

specific reasons worked together to empower the excellent detection performance: using ACE 2 as the receptors, adding MBs as the separators, and adopting the sandwich-type-like design philosophy.

5.4.Polymer-GO composite whole-cell MIPs method

Although the research interest has been primarily shifted toward targeting small molecules like epitopes through surface imprinting, the Holy Grail of MIPs technology is to imprint complex templates such as the whole-cell of SARS-CoV-2 to exhibit multiple recognition mechanisms based on cell shape, size, and the entire surface biochemical interaction with MIPs [67]. The study reported by Sukjee et al. [75] demonstrated that whole-cell MIPs could be used as artificial receptors to harness an electrochemical biosensor with a surprisingly high sensitivity of 0.1 fM. The experimental procedures of this imprinting study, including the ratio optimization of different monomers, the addition of GO suspension, the polymerization process, and the acid wash of whole-cell templates, were all straightforward to be understood and easy to be replicated in other virus detection experiments. Thanks to the outstanding resistance to harsh environments, this whole-cell-imprinted MIPs-based electrochemical biosensor is the only one we found that can be used directly to detect wastewater samples without tedious pretreatment and preconditioning steps.

However, one step within the fabrication process (i.e., the electrochemical reduction of the polymer-GO composite MIPs) may need to seek a different measure to improve the selectivity further. Undoubtedly, GO is an insulator nanoflake, which requires an additional

reduction process to eliminate the surface functional groups and promote electron transfer. It is presumably because of such considerations that Sukjee et al. [75] performed the electrochemical reduction of the polymer-GO composite MIPs right before the electrochemical detection. However, it is well-known that abundant hydrophilic polar moieties and oxygen-based functional groups on GO surfaces can lead to the homogeneous dispersion of the GO-embedded composite [79], more importantly, having many active functional groups within the MIPs cavities can facilitate more specific recognition of the exposed antigens on the cell surface [72]. In other words, the electrochemical reduction process was likely to cause a downgrade of the interaction between the MIPs and the entire cell surface, compromising the whole-cell MIPs recognition only via a few basic features like viral shape and size. To balance the needs between the high electron transfer rate and surface interactions, alternatives such as adding graphene with pyrene derivatives (e.g., 1-pyrenebutyric acid) to the polymer composite can avoid facing a similar antithesis by using GO directly.

6. Prospects for the future

According to the early statistics obtained using the widely adopted RT-PCR method, the SARS-CoV-2 viral loading in respiratory samples varies from 641 copies/mL to 1.34×10^{11} copies/mL, with a median of 7.99×10^{4} in throat samples and 7.52×10^{5} in sputum samples [80]. Considering *ca.* 100 copies of the spike protein per virion and *ca.* 180-200 kDa of molecular weight [81], it can be expected that an electrochemical biosensor if having a dynamic range approximately from 2.0 pg/mL to 20 μg/mL, will be capable of detecting or diagnosing the SARS-CoV-2 biomarker (e.g., spike protein) with comparative performance to the conventional RT-PCR method. As a result, most electrochemical biosensors mentioned in this work have had good enough performance to become a powerful tool for point-of-care (POC) tests.

After looking to the website of the U.S. Centers for Disease Control and Prevention (CDC) [82], many commercial products have been developed and authorized for rapid COVID-19 POC tests. However, those POC products almost all belong to the lateral flow immunochromatographic assay (e.g., RapCov™ Rapid COVID-19 Test by ADVAITE, BinaxNOW™ COVID-19 Ag Card by Abbott, and QuickVue® SARS Antigen Test by Quidel). One can hardly find any electrochemical POC products that became commercialized after the recently explosive growth in the number of published relevant studies. It should be noted that the electrochemical biosensors cannot compete with the lateral flow immunochromatographic assay regarding the material cost and ease of operation. Furthermore, antigen tests are generally considered inferior to RT-PCR, making

the users, to some extent, acquiesce to its relatively compromised detection performance. In summary, it seems that the electrochemical biosensor is not a perfect candidate for POC, not because it cannot offer enough sensitivity or become further miniaturization but because the lateral flow immunochromatographic assay contains a higher level of reassuring familiarity as a mature POC product, which renders essential information by taking as little as possible effort to learn the specimen collection and handling with the minimum cost of mass production, distribution, and storage.

Herein, we believe it is essential to find appropriate and distinctive application scenarios to maximize the advantages of electrochemical pathogen biosensors. For example, using wastewater-based epidemiology (WBE) to track the magnitude and distribution of an infectious disease like SARS-CoV-2 may be one of the suitable applications for electrochemical pathogen biosensors. Recently, WBE has been proposed in many epidemic areas over the world. However, all the WBE studies chose RT-PCR as the reference method to measure the viral concentration from wastewater samples [8]. It should be noted that when testing a wastewater sample, RT-PCR is highly susceptible to the presence of inhibitors and contaminants, which can lead to false-negative results [83]. In addition, running RT-PCR tests typically requires 4-6 hours of sophisticated technician labor in a clean, centralized laboratory environment. The above limits in the PCR-based method may give electrochemical biosensors a chance to demonstrate their excellent characteristics in quantitative measurement, ease of operation, and in-situ detection potential.

Based on the enormous amount of information from those published electrochemical biosensors, features that fit and favor wastewater pathogen detection can be picked up and integrated into a specific strategy to perform measurements in wastewater samples. As shown in **Fig. 10**, we proposed an electrochemical biosensor for wastewater pathogen detection by fusing three featured technologies, including the MBs-assisted primary concentration of virion particles from a relatively large sample size of wastewater, the AuNPs-mixed polymer composite for the enhancement of detection sensitivity, and the MIPs-based whole-cell imprinted receptor to confer the excellent resistance toward the harsh wastewater environment, and all of which were mentioned in this review. The MBs-assisted concentration method is already a mature technology widely applied in DNA/RNA purification during qPCR tests. Unlike traditional concentration methods (i.e., PEG-based separation, membrane filtration, and ultrafiltration [84]), adopting MBs can quickly separate the virion particle from the wastewater matrix in a non-destructive fashion, providing the first line of defense against the harsh wastewater environment. However, due to a wastewater sample's complex matrix, some small substances may inevitably contaminate the concentrated viral pellet. Thanks to the relatively large and morphological featured MIP cavities; the whole-cell imprinted MIPs layer can recognize viral cells based on the size and shape identification, ignoring the interference from those small substances like anions, cations, dissolved organics, or surfactants, and setting the second line of defense against the impurities from wastewater.

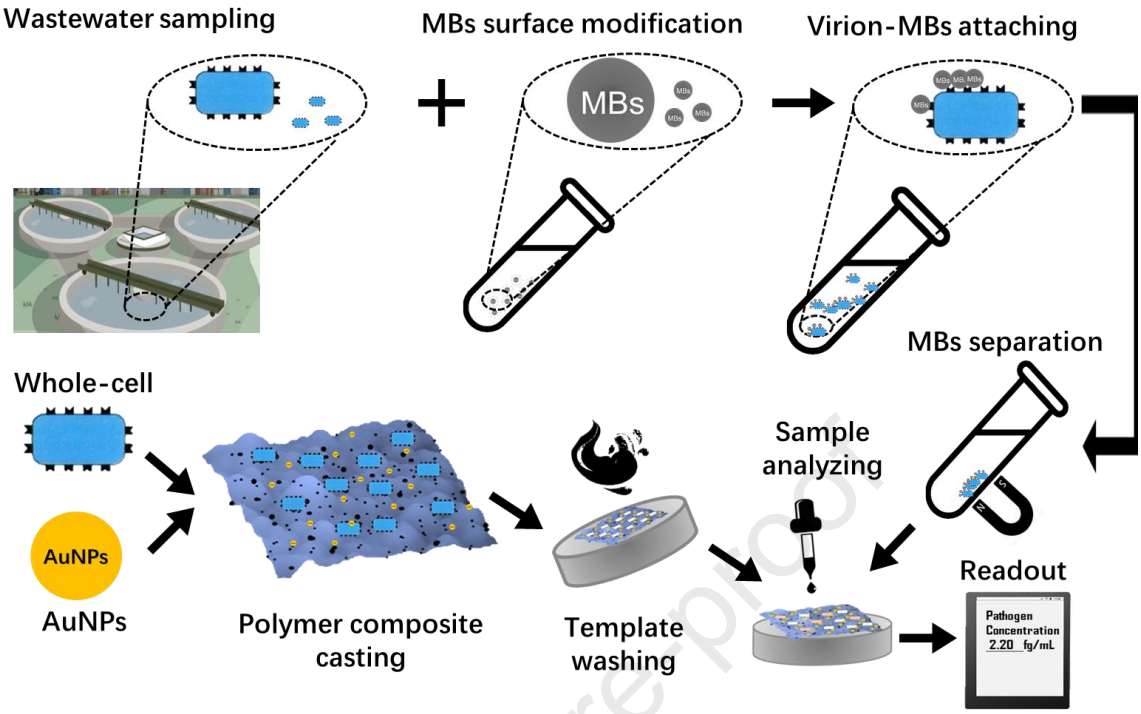

Fig. 10. Illustration of a proposed method for the in-situ quantitative detection of pathogens in the wastewater sample using the MBs/AuNPs-assisted whole-cell MIPs electrochemical biosensor.

Author contributions

CM and DL contributed to conception and design; CM contributed to collecting and assembling relevant information; CM and DL contributed to drafting the article; HG, ZY, DZ, JL, QF, and PK contributed to reviewing. DL contributed corresponding. All authors had final approval of the article.

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Competing interests

The authors declare no competing interests.

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- Evaluate the effects of different receptors in SARS-CoV-2 electrochemical biosensors
- Dig deep into the rationale why different studies chose specific detection strategies
- Point out the importance of finding appropriate and distinctive application scenarios
- Propose the WBE to maximize the advantages of electrochemical pathogen biosensors

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: