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Review Article

Recent advances in surface modification and antifouling strategies for electrochemical sensing in complex biofluids

Daniel P. Carroll and Paula M. Mendes

Abstract

The ability of biosensing systems to act selectively and sensitively in complex biological fluids will play a significant role in future healthcare developments. In this short review, we discuss recent advancements in surface modification strategies, which have seen electrochemical biosensors perform with high accuracy in real patient samples (plasma, urine, whole blood, sweat). We discuss novel substrate and interfacial modifications for imparting surfaces with antifouling properties. This has allowed analytical devices to detect cancer biomarkers with a sensitivity of 2 pg/mL in whole blood. We also examine nanobodies (Nbs) for use as robust receptor components, which have recently been shown to have single molecule detection limits of the SARS- CoV-2 S1 spike protein in unprocessed saliva. Although such progress has been made, the review also highlights that current platforms are still limited in their capacity to control biointeractions at the sensing interface and long-term stability continues to be a barrier to many biosensors achieving commercialisation. Finally, future prospects are discussed including the use of stimuli-responsive surfaces for increased control over specific and non-specific biointeractions and on-demand biosensing.

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Keywords

Electrochemical Biosensors, Complex Biofluid Analysis, Surface Modification, Antifouling Surfaces, Nanobodies.

Introduction

After Clark and Lyons developed the first electrochemical glucose sensor in the early 1960's the biosensing field saw an initial surge in commercial success [1]. This was in the form of home glucose meters and pregnancy tests, which was driven by the large market size for such products. As the 80s and 90s rolled by, many people assumed it was just a matter of time before biosensors would be adapted for the rapid detection of all clinically relevant analytes. However, as of today blood glucose monitoring remains the dominant medical application for electrochemical biosensors, making up around 73% of the global market [2]. Other applications for biosensors such as cell therapy bioprocessing and environmental monitoring have been significantly hindered by the lack of commercially available analytical devices [3,4]. One reason for this is the large number of biomolecules present in clinically relevant samples. This can cause a build-up of unwanted material at the sensing interface which can drastically impact the resistive and capacitive properties of the receptor component thus, hampering electrochemical signal transduction [5]. Furthermore, while affinity-based biosensors have demonstrated remarkable sensitivity (fM-pM detection limits), they have suffered from surface instability. Commonly used receptor elements such as enzymes, antibodies, and aptamers rely on physiological conditions to retain their activity. This has hindered their commercial viability as the surface is often denatured during storage, shipping, or while the device is sitting idle [6]. These two issues of controlling surface interactions and long-term stability remain major challenges currently facing the biosensing field. However, the story does not end there. Recently, nanostructured electrodes and specific interfacial chemical modifications have enhanced the ability of electrochemical biosensors to function effectively in complex biological fluids. The following section highlights some of these strategies which should be considered when designing biosensing surfaces moving forward.

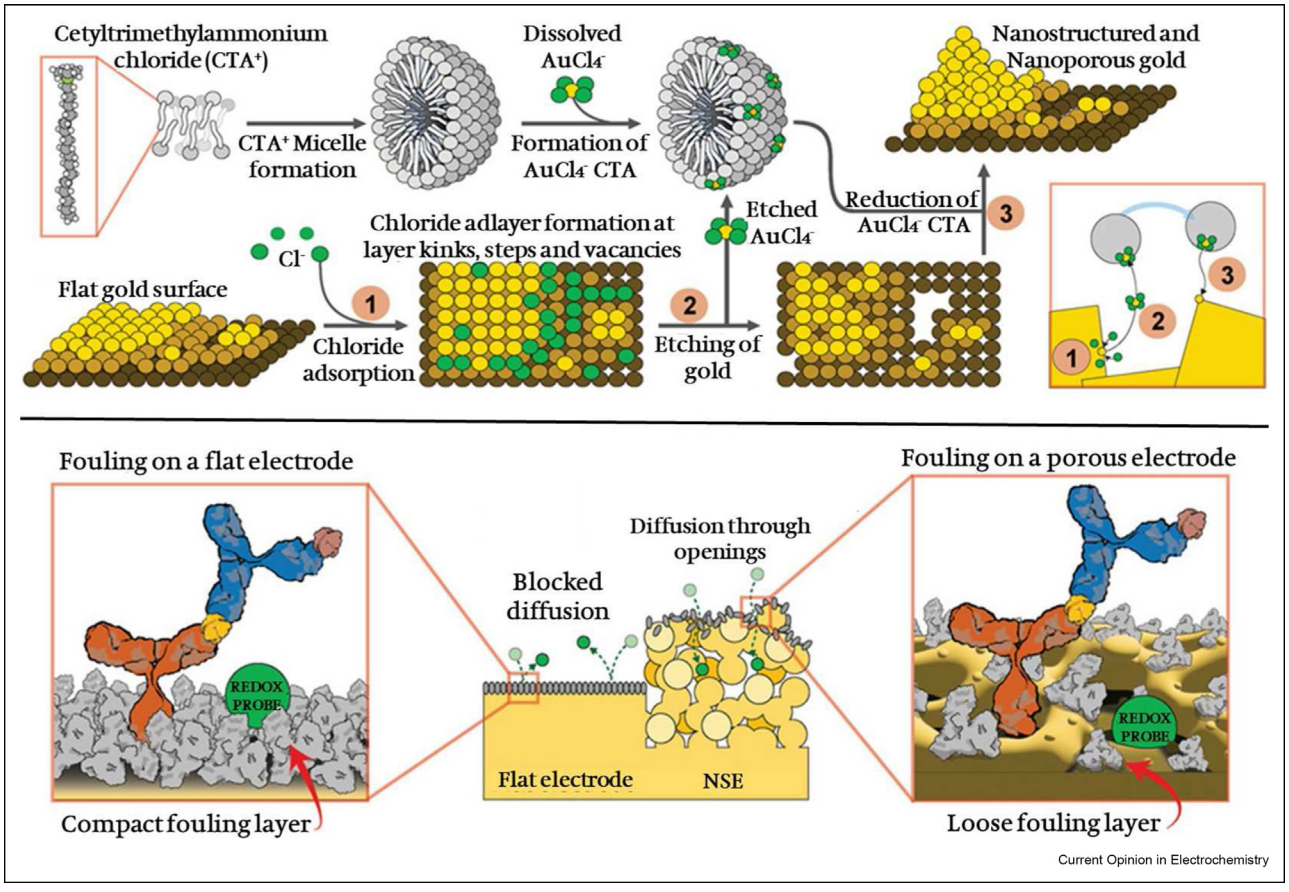
Antifouling strategies

Nanostructured Electrodes (NSE)

Recent strategies for minimising the impact of surface fouling and increasing sensitivity have taken the form of NSEs. These can be defined as electrodes (typically gold) which contain surface features (e.g., pores) with dimensions in the range of 1−100 nm [5,7−10]. They can be fabricated through different methods but usually involve some combination of etching, annealing, electro-oxidation, and electro-reduction. The porous surface prevents unwanted material forming a compact planar film on the electrode's surface which would otherwise significantly limit the movement of charged species across the interface (Figure 1). This prevents the loss of sensitivity and allows biorecognition events at the surface to be detected by the transducer. When compared to the equivalent planar electrodes exposed to fouling conditions, NSEs have demonstrated a 4000% decrease in the charge transfer resistance (Rct), when measured using electrochemical impedance spectroscopy (EIS) [5*]. This facilitated the discrimination between healthy and prostate cancer patients through the detection of cancer biomarkers known as tumour-derived extracellular vesicles (tEVs). A limit of detection (LOD) of 60 vesicles μL^{-1} was achieved for $CD9^{+}$EVs in unprocessed plasma. In comparison, current enzyme-linked immunosorbent assays (ELISAs) could only achieve an LOD of 700 veicles μL^{-1} in phosphate-buffered saline (PBS). Furthermore, flat gold electrodes were electrically passivated and could not be tested in plasma.

However, it is important to highlight that simply fabricating a nanoporous surface will not automatically improve a biosensors sensitivity. The pore's dimensions play a key role in the system's analytical performance and need to be optimised before sensitive antigen detection can be carried out. Daggumati and co-workers used thermal annealing to increase the size of the pores on NSEs, which were fabricated through a dealloying process for the development of an aptamer-based biosensor. The different surfaces were exposed to bovine serum albumin (BSA) and the NSE with the smaller pore size (14 nm) showed the lowest reduction

Figure 1



Top: Schematic of the NSE fabrication process, cetyltrimethylammonium chloride (CTA^{+}) ions form micelles that coordinate with chloroauric acid. 1) Electrochemically driven adsorption of chloride adlayers on gold. 2) Etching of the gold generating chloroauric acid. 3) Electrochemically driven reduction of gold-laden CTA^{+} micelles. Bottom: Comparison of a fouling layer on the flat gold surface vs the NSE. Adapted with permission from Sabaté del Rõo et al. [5].

in signal suppression (Figure 2), [10]. This was a result of size exclusion mechanisms as the BSA was too large to fit into the pores on the unannealed surface [11]. This pore size dependence on the surface's antifouling properties indicates that the size of the pores would need to be optimised for different applications, depending on the size of the background entities which could potentially foul the interface.

More recently, tighter control over pore size was achieved by combining thermal annealing and electrochemical coarsening [8**]. When used for the development of an aptamer-based biosensor, a 200% increase in signal gain was achieved compared to a planar electrode with the same footprint (Figure 3, (a)). This behavior can be explained by considering the decrease in Debye volume inside the nanopore, which extends the electric double layer (EDL) farther into the solution. This in turn results in a reduction to the charge screening effect at the interface. Accordingly, the stronger electric field inside the nanopore increases the chances of electron transfer occurring between the redox reporter and the surface for any given conformation of the aptamer probe (Figure 3, (b)).

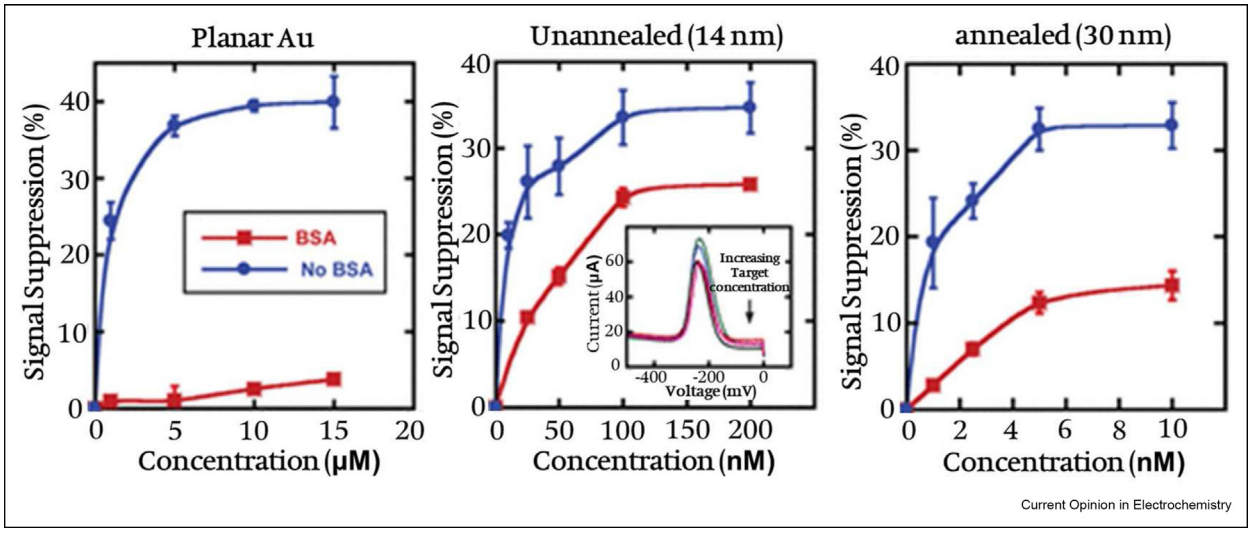
The optimised nanoporous surface displayed a 24-fold increase in signal level and an almost fourfold improvement to the LOD for the detection of doxorubicin compared to an equivalent planar electrode. The small size of the nanopores may also prevent larger molecules from interacting with the binding domain on the aptamer, making such platforms attractive for the detection of small molecules in highly complex backgrounds.

NSE's also have a larger electroactive area compared to an equivalent planar electrode with the same footprint. This has been shown to increase receptor immobilisation yields which can improve the LOD and dynamic range of electrochemical biosensors [12]. Furthermore, the formation of such nanopores have been shown to be compatible with microfabricated thin-film electrodes [5]. This property would allow NSEs to be integrated with more sophisticated microfluidic/microarray platforms. However, such fabrication still requires specialist facilities and equipment therefore, it is also worth considering interfacial modifications as a more convenient approach for reducing the effect of surface fouling.

Interfacial chemistry

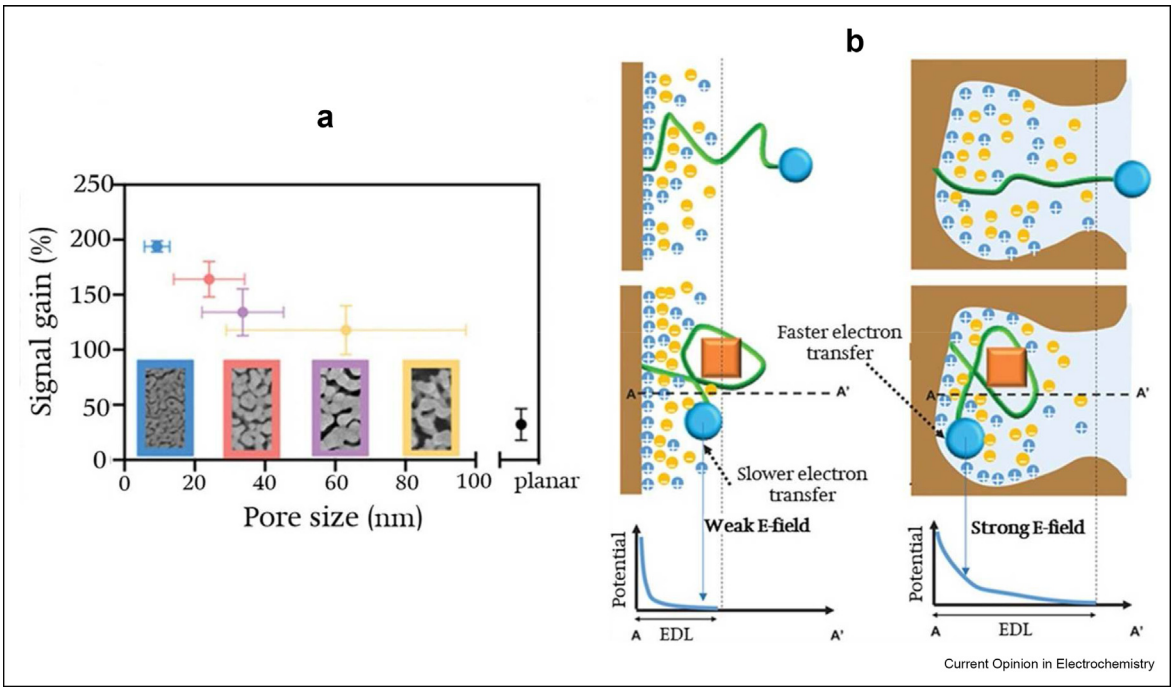
Like NSEs, chemical modifications can be made to electrode surfaces to both increase sensitivity and reduce biofouling. Typically referred to as functionalisation layers they can serve as anchor points for receptor components keeping them in spatial contact with the transducer (electrode). This allows electrochemical changes to the interface following biointeractions to be detected [13–15]. Traditionally, such layers consisted of alkane thiol, self-assembled monolayers (SAMs) which spontaneously organise themselves onto gold surfaces [16–18]. Antifouling was achieved with end-groups or bioconjugating other molecules which strongly hydrated the surface, this prevents biomolecules non-specifically adsorbing onto the interface. However, such surfaces

Figure 2



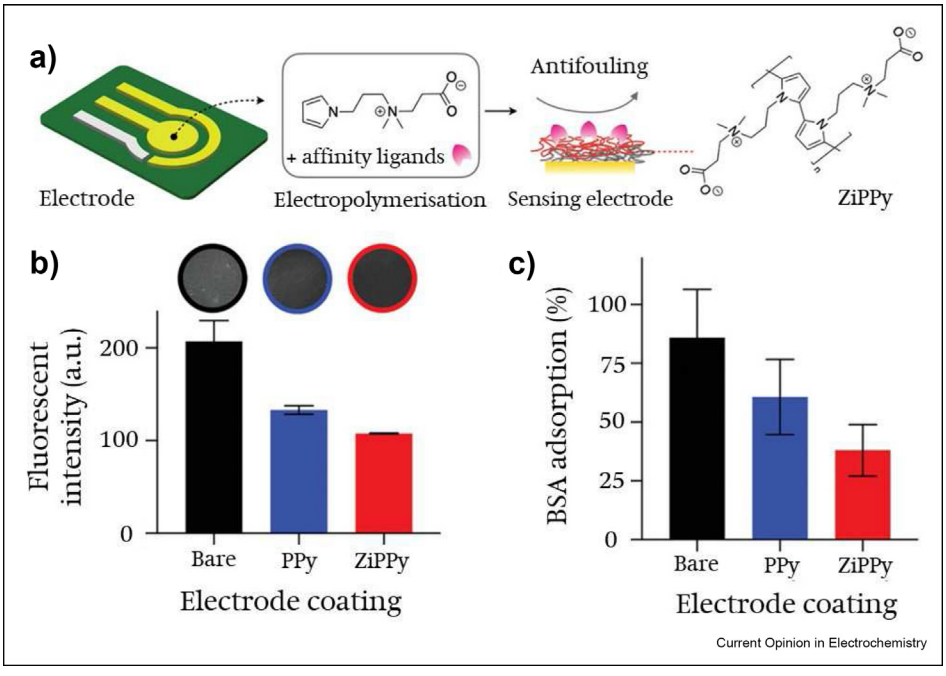
Aptamer sensor response upon target hybridisation with (red) and without (blue) BSA on planar, unannealed, and annealed NSE surfaces. Adapted with permission from Daggumati et al. [10]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Figure 3



a) Impact of pore size on signal gain **b**) scheme of how the EDL from a planar (left) and NSE (right) affects electron transfer from a redox reporter (blue circle) tethered to the aptamer (green). During electrochemical measurement, the redox reporter interacts with the EDL (shaded blue region), where a closer distance between the reporter and the electrode surface leads to faster electron transfer. In the nanoporous electrode, the reporter experiences stronger electric fields. A and A′ represent the electrode surface and the maximum distance of the reporter from the electrode, respectively. Adapted with permission from Fu et al. [8]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Figure 4



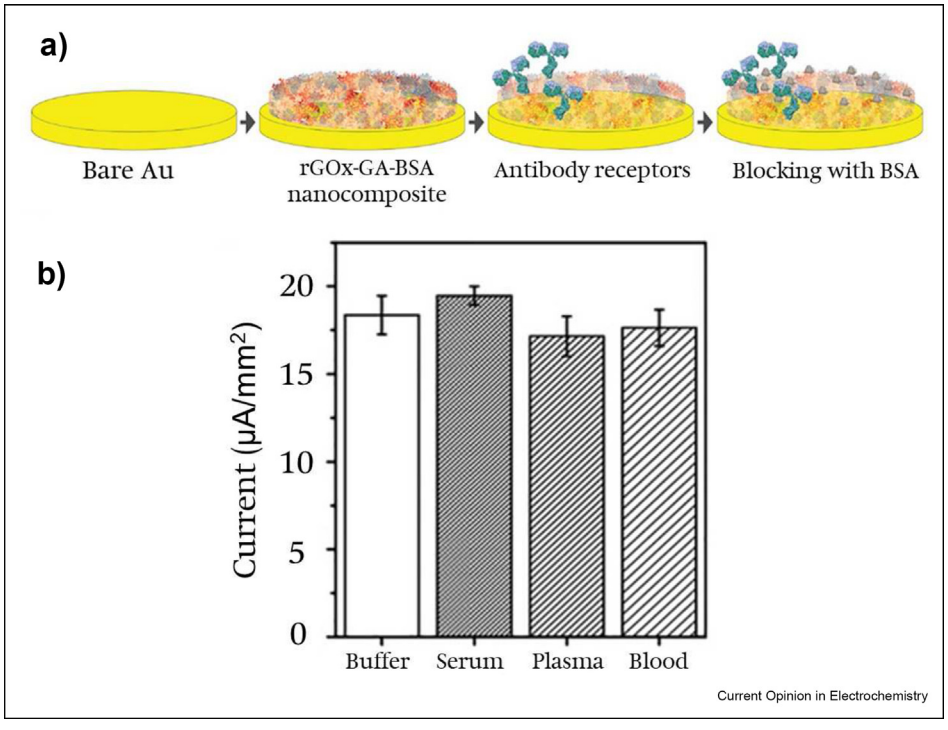
a) Zwitterionic pyrrole (ZiPy) monomers in the presence of affinity ligands are electropolymerising into zwitterionic polypyrrole (ZiPPy) through cyclic voltammetry (CV). **b**) Bare, polypyrrole (PPy)-coated, and ZiPPy-coated electrodes were incubated with fluorescently labelled streptavidin solution, washed, and imaged. **c**) Electrodes were incubated with 10% BSA, and the remaining BSA in the supernatant was quantified. Adapted with permission from Kilic et al. [20*].

suffered from limited stability as SAMs are prone to oxidation in air or solution [19]. Over the past number of years, the use of zwitterionic molecules has become increasingly common, not only to prevent undesired biointeractions but also to improve surface conductivity [20].

Figure 4 shows the fabrication of an antifouling surface with embedded affinity ligands using zwitterionic polypyrrole (ZiPPy). This allowed for high conductivity while preventing proteins from interacting with the interface (Figure 4: B) and C)). Also, both antifouling and receptor elements are functionalised to the surface in a single step thus simplifying the fabrication process. Furthermore, utilising electrochemical grafting for film fabrication also greatly improves the stability of the platform compared to SAM based systems, due to the formation of stronger surface bonds [21,22]. These properties allowed the system to be adapted for the detection of SARs-CoV-2 antibodies in saliva samples down to 50 ng/mL, without the need for sample purification, dilution, or secondary labelling.

While impressive, such systems have struggled to retain relevant analytical performance in more complex biofluids, such as whole blood. This has remained the holy grail in clinical diagnostics for life threatening diseases for decades. However, over the past two years Zupaňcic and co-workers have shown multiplexed detection of endogenous sepsis markers from real patient samples (whole blood diluted by 50%) by utilising nanocomposites for electrochemical biosensors [23**]. The nanocomposite consisted of BSA containing a network of reduced graphene oxide (rGOx) nanoparticles cross-linked using glutaraldehyde (GA). This surface showed no change in current density when investigated with CV after exposure to serum, plasma, and whole blood for 1 h (Figure 5). The antifouling properties of the interface arise from the enhanced porosity of the cross-linked BSA matrix, which size excludes particles from diffusing through the pores, thus blocking their access to the electrode surface [24**]. The applicability of the nanocomposite was demonstrated by incorporating three different receptor types (antibodies, phosphorylcholine, and Fc-mannose binding lectin) for the detection of four different sepsis biomarkers (procalcitonin (PCT), C-reactive protein (CRP), syndecan-1, and pathogen-associated molecular patterns (PAMPs)). All target analytes were detected within clinically significant ranges without any cross-reactivity, demonstrating for the first time a multiplexed electrochemical sensor for different sepsis biomarkers in whole blood.

Figure 5



a) Preparation of the electrochemical biosensor. **b**) Oxidation currents of ferri-/ferrocyanide couple obtained after exposure of nanocomposite coated electrodes to buffer (PBS), human serum, plasma, and whole blood. Adapted with permission from Zupaňcïïc et al. [23].

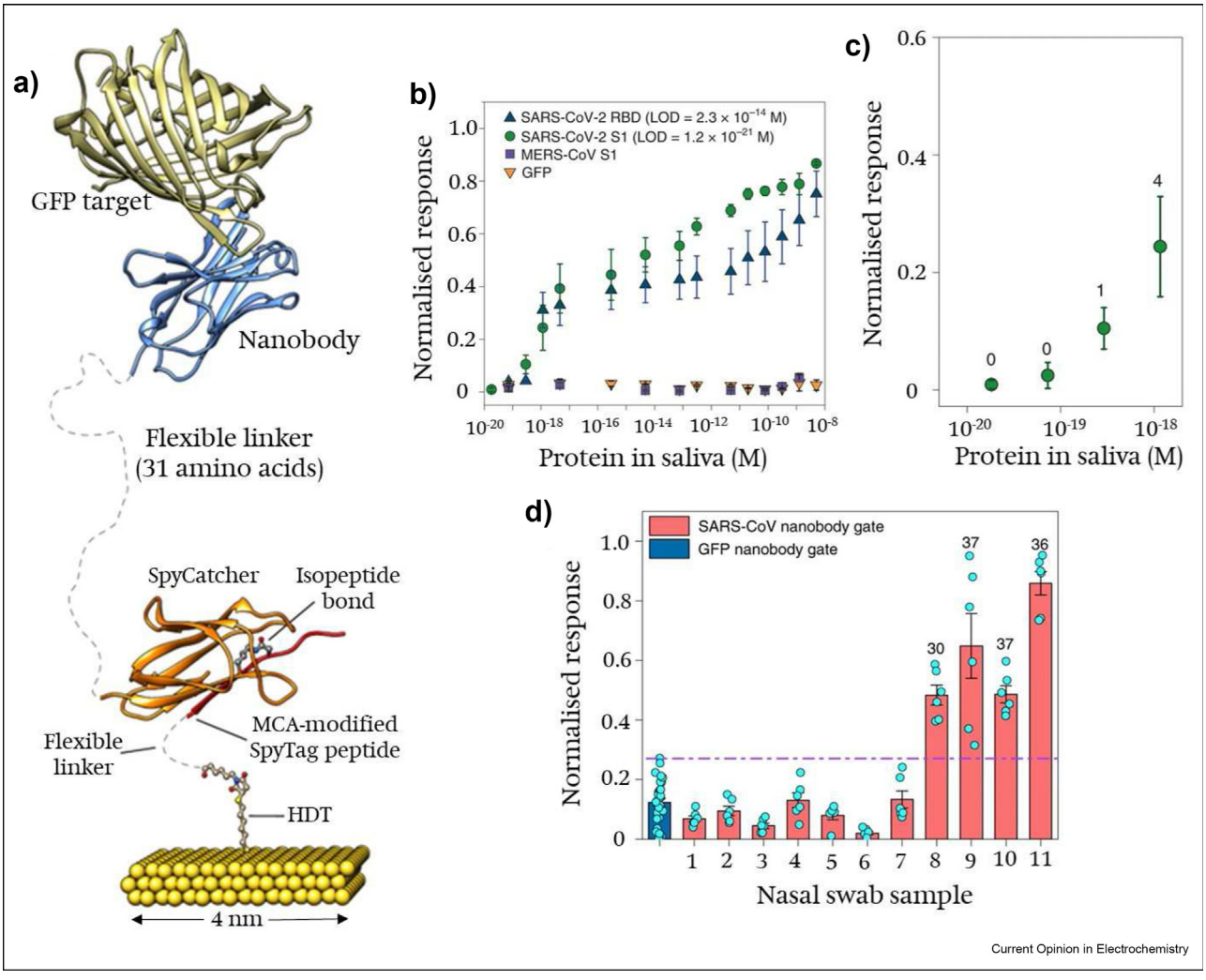
This is a major step forward in the detection of disease biomarkers in complex biofluids using electrochemical biosensors.

While the above reports are highly promising, the continued use of biological components as receptor elements will always be accompanied by stability issues. Despite Zupančič and co-worker's platform displaying sensitive detection in whole blood, it would likely be unsuitable for storage, shipping, or long-term monitoring of analytes due to the receptor components being reliant on strict physiological conditions to retain their activity [25]. Therefore, more robust receptors are sorely needed to bridge this gap.

Nanobodies (Nbs)

Nbs are the single variable domain on the heavy chain of Camelid anti-bodies and have a wider operational window in terms of temperature and pH compared to other biological receptor units [26]. They are small (15 kDa) allowing for increased surface densities while retaining the specificity of their larger antibody counterparts. They are robust which can facilitate the development of biosensing platforms with suitable stability for long term use, storage, and shipping [27]. Reports published over the past two years have demonstrated attomolar sensitivities for SARS-CoV-2 and MERS spike proteins in saliva [28,29]. Guo and co-workers functionalised Nbs to organic

Figure 6

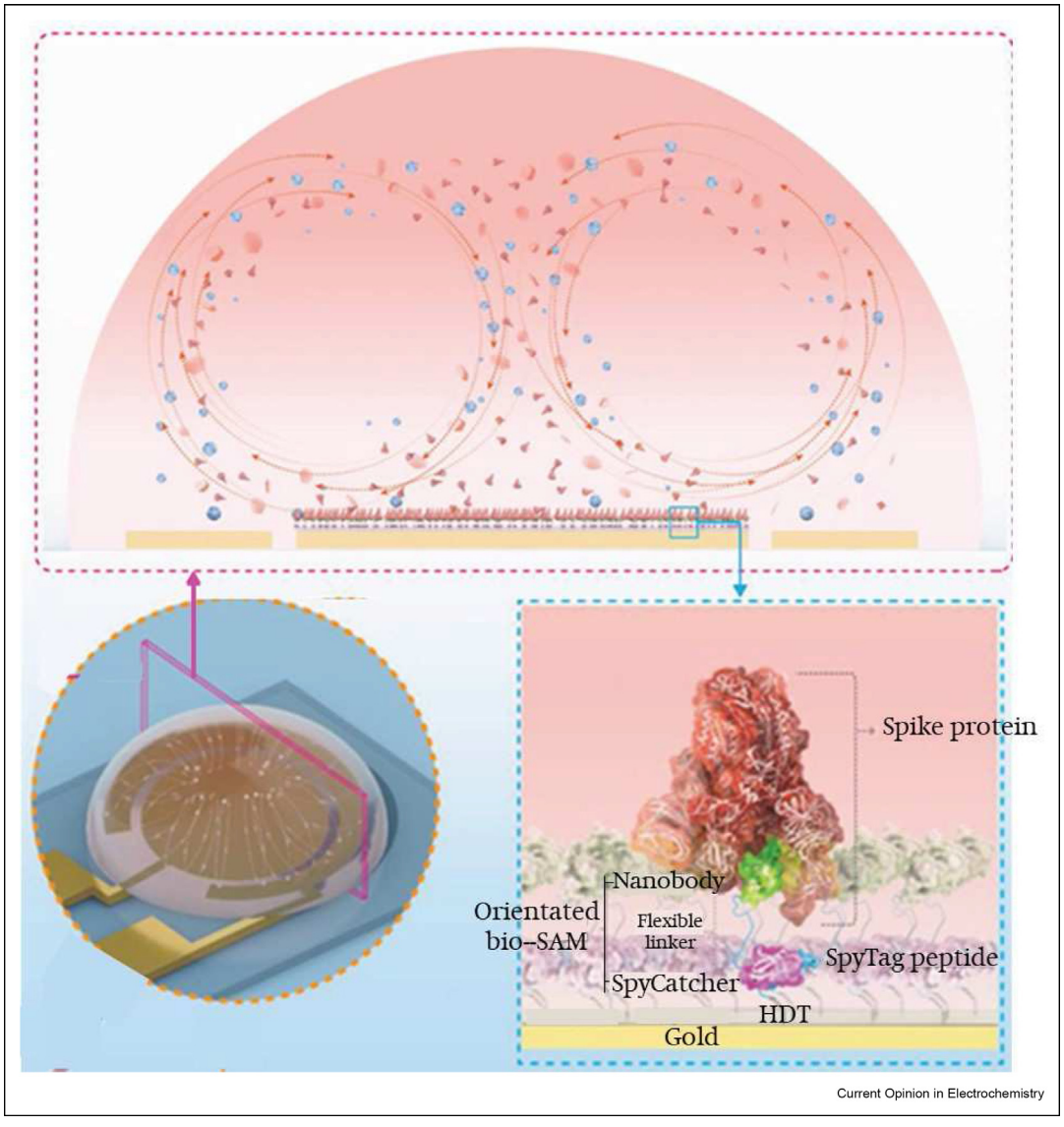


a) Structural model of the complete biorecognition layer assembled from 1,6-hexanedithiol (HDT), SpyTag, and nanobody–SpyCatcher. **b**) Response to SARS-CoV-2 proteins, GFP and MERS-CoV S1 spiked into human saliva. **c**) Normalised response of SARS-CoV-2 sensors at single-molecule concentrations. **d**) Normalised sensor response for nasopharyngeal swab samples (n = 11) from healthy volunteers (1–7) and COVID-19-positive samples from walk-in patients (8–11). Cycle threshold values from RT-PCR are indicated above the positive samples. Nature©, adapted from Guo et al. [28**].

electrochemical transistors (OECT) with controlled orientation via nanobody–SpyCatcher fusion proteins on disposable gate electrodes (Figure 6). The platform demonstrated accuracy and sensitivity on par with reverse transcription-polymerase chain reaction (RT-PCR) tests, in the detection of endogenous spike proteins from unmodified nasopharyngeal samples.

Controlling the Nbs orientation increases the likely hood that the binding pocket will be facing out into the surrounding environment. This is highly desirable for all biosensors/assays as it enhances the binding capacity towards target antigens. Furthermore, the flexibility introduced to the system by the amino acid linkages give the Nb and SpyCatcher domains the ability to rearrange themselves on the surface. The flexibility imparted to the receptor on the surface more closely resembles their behaviour in solution. Biointeractions at an artificial interface are often diffusion limited due to the depletion of analytes close to the surface. When not limited by diffusion, it has also been found that the forward and reverse reaction rates are lower for surface reactions compared to reactions in solution [30]. Therefore, further investigations should be carried out to better

Figure 7



Schematic of the ACET enhanced nanobody-OECT biosensor. 10 μL of the sample solution is introduced on top of the concentric gate electrode. A function generator supplies an AC potential and induces ACET to concentrate the target molecules (e.g., spike protein) on the surface with immobilised nanobodies. This process takes 2 min. The electrode is then rinsed with phosphate-buffered saline (PBS), flipped, and mounted on top of the channel for signal acquisition. Adapted with permission from Koklu et al. [29].

understand the effect receptor flexibility has on the binding kinetics at the surface. This could lead to a significant improvement in the analytical performance of electrochemical biosensors.

The sensitivity and robustness of this Nb receptor system has seen it adapted into other biosensing platforms which utilise alternating current electrothermal flow (ACET) [29**]. This induces directional convection currents which can drive the analyte towards the surface regardless of its charge or hydrophobic properties (Figure 7). This produced a 5-fold reduction in immunocomplex formation time compared to periodic manual mixing. The ACET also helped reduce fouling in complex media by washing away non-specifically bound species from the sensor surface. This produced a LOD for the SARS-CoV-2 spike protein of 100×10^{-18} M and a large dynamic range of 10^{-18} to 10^{-9} M in diluted saliva.

The platforms discussed above have the potential to be adapted for the detection of any analyte providing that an associated Nb already exists or could be produced. However, the use of SAMs in these platforms limits the benefit of the Nbs increased stability. While the receptors themselves are stable to a wide range of temperatures, and pH, the 1,6-hexanedithiol layer would not remain stable for more than a few days [31,32].

Summary and outlook

The electrochemical biosensing field is for the first time in a position where almost all the necessary components for the development of sensitive and robust analytical devices have been demonstrated. NSEs have shown excellent antifouling capacity by preventing the formation of compact fouling layers on the sensing surface. This has led to the successful discrimination between healthy and prostate cancer patients through the detection of tEVs in urine and plasma samples. The use of nanocomposites (BSA crosslinked with rGOx) has preserved oxidation current responses (100%) after incubation in highly complex media (plasma, whole blood). This was achieved by preventing molecules from reaching the surface through size exclusion mechanisms. This approach allowed cancer biomarkers to be detected with pg/mL sensitivity in whole blood. Furthermore, replacing traditionally used biological receptor components with Nbs has the ability to provide long-term stability and increased receptor immobilisation yields, resulting is enhanced LODs and wider dynamic ranges. Nbs have already proven their effectiveness in the detection of the SARs-CoV-2 spike protein, with single molecule detection limits achieved. In theory they can be adapted for the detection of any biomolecule, providing the associated Nb exists or could be produced. Likewise, the limited stability of SAMs can be overcome by substituting them for electrochemically grafted films. With abundant examples now in the literature, the stronger surface bonds make these films more stable to temperature, pH, and electrical potentials [33–38].

The key to unlocking widespread use of electrochemical biosensors in real patient samples will involve bringing together surface components, which have individually proven to be extremely effective for the detection of analytes in complex biofluids. An electrochemically grafted film coupled with Nbs as the receptor components could have similar sensitivities to some of the platforms listed above, while remaining stable for 30 days or more. The last piece of the puzzle, which seems to be under reported in the literature is the ability to control the surface biointeractions in an on-demand fashion. By preventing pre-mature binding events (specific and non-specific) this would allow biosensing surfaces to be in constant contact with complex media and detection carried out only when it is required. One exciting area known as stimuli-responsive surfaces holds great potential to meet the demand of on-demand biosensing [39–41].

Declaration of competing interest

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

Data availability

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

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The nanostructured electrode surface prevented the formation of a compact fouling layer and compared to an equivalent planar electrode a 4000% decrease in the charge transfer resistance was observed in fouling conditions. Tumour-derived extracellular vesicles were detected with a LOD of 60 vesicles μL^{-1} in unprocessed plasma. In comparison, current ELISAs could only achieve an LOD of 700 vesicles μL^{-1} in PBS. Furthermore, flat gold electrodes were electrically passivated and could not be tested in plasma. This demonstrates how nanostructured electrodes can significantly enhance the analytical performance of electrochemical biosensors in highly complex media.

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Showed when aptamer probes were located inside the nanopores on the surface a 200% increase in signal gain could be achieved. The authors showed this was due to a decrease in the Debye volume inside the nanopore which extends the electric double layer farther into the solution. This in turn results in a reduction in the charge screening effect at the interface. Accordingly, the stronger electric field inside the nanopore increases the chances of electron transfer occurring between the redox reporter and the surface for any given conformation of the aptamer probe. This resulted in a 24-fold increase in signal level and an almost fourfold improvement to the LOD for the detection of Doxorubicin compared to an equivalent planar electrode.

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Electrochemical grafting allowed the surface to be modified with zwitterionic polypyrrole and affinity ligands in a single step. The zwitterionic functionality not only improves the antifouling properties by strongly hydrating the surface but also improves surface conductivity and stability. SARs-CoV-2 antibodies were detected with this platform in saliva samples down to 50 ng/mL, without the need for sample purification, dilution, or secondary labelling.

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Using the nanocomposite discussed below (**3) multiplexed detection of sepsis biomarkers was demonstrated in whole blood for the first time. Procalcitonin (PCT), C-reactive protein (CRP), syndecan-1, and pathogen-associated molecular patterns (PAMPs)) were detected within clinically significant ranges without any cross-reactivity. This is a major step forward in the detection of disease biomarkers in complex biofluids using electrochemical biosensors.

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The authors developed a nanocomposite consisting of bovine serum albumin containing a network of reduced graphene oxide nanoparticles crosslinked using glutaraldehyde. This was functionalised to a gold electrode which then showed no change in current density when investigated with CV after exposure to serum, plasma, and whole blood for 1 h. This was due to the nanocomposite preventing the diffusion of particles to the surface through size exclusion mechanisms.

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