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Electrochemical Immunosensors, Electrochemical Detection Techniques, Methods and Application of Microfluidics in Immunoassay

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Abstract:
The use of electrochemical methods for purifying and separating wastewater has recently attracted a lot of interest. Since electrons are the primary reagent and they do not generate solid residue, they are considered cleaner than numerous other physiochemical and membrane-based technologies. In addition, the rapid response time, great selectivity, dependability, and sensitivity of electrochemical techniques have made them attractive as tools for water treatment. These methods have progressed to the point where they are not only efficient and small, but also inexpensive. The environmental contamination caused by perchlorate, nitrate, and other salts is a big problem because these substances are widely utilised as rocket and missile propellants and in other industrial applications. Since the problem of environmental contaminations is becoming worse by the day, effective methods of removing these species are urgently required. After reviewing the literature, it is clear that in order to effectively remove species like perchlorate and nitrate on an industrial scale, a new electrochemical reduction method utilising selective electrocatalysts following a stable mechanistic path is necessary. Among the many difficult problems with electrochemical reduction techniques is the requirement to develop economically viable or cost-effective processes. In addition, photo-electrochemical techniques are still in the early stages of study, thus further in-depth investigations are needed to fully understand their potential. It is also difficult to detect these ions in water. Therefore, additional study is needed to develop effective methods for detecting nitrate and perchlorate. Molecular diagnostics, immunoassays, and biochemical analyses have all benefited greatly from point-of-care testing in recent years, particularly in areas with limited access to laboratory facilities. Microfluidic chips offer numerous notable benefits over other point-of-care testing technologies. All steps of a biological reaction—from loading reagents to detecting their presence—can be carried out on a single microprocessor thanks to technology that miniaturises traditional laboratory equipment. This has led to the microfluidic platform being extensively studied in recent decades for applications in environmental monitoring, healthcare, and food safety. In this article, we will take a look back at the most recent developments in the use of microfluidics in immunoassay. There are two components to a microfluidic platform, passive manipulation and active manipulation, that are based on the various fluid driving forces. Active manipulation encompasses the introduction of pressure, centrifugal, electric, optofluidic, magnetic, and digital microfluidics; passive manipulation centres on capillary-driven microfluidics.

Keywords: Microfluidics in Immunoassay, Electrochemical Immunosensors, Detection, Techniques, Application.

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Introduction

Numerous analytes in the fields of medicine, biochemistry, and environmental science necessitate the development of rapid and easy analytical procedures. Because of their specificity and sensitivity, immunoassays and immunosensors that depend on interactions between antibodies and antigens offer a potential method of investigation in this regard. The formation of a persistent compound on the surface of an immunoassay device or an immunosensor allows for high specificity by molecular recognition of target analytes, typically antigens, by antibodies. Conversely, sensitivity is conditional on a number of variables, such as the choice of detecting device for gauging the analytical signal [1-3], the orientation of the immobilised antibodies once they have been attached to the surface of the immunoassay or immunosensor, and the employment of high affinity analyte-specific antibodies. The limitations of previous methods of immunoassay and immunosensor detection are circumvented by electrochemical detection. For instance, immunoassays combined with optical detection achieve limited sensitivity in the analysis of coloured or turbid samples, while radioimmunoassays often raise concerns about the short half-life of radioactive substances, potential health hazards, and disposal issues. On the other hand, these issues are not present in electrochemical immunoassays and immunosensors, which allow for quick [4, 5], easy, and cost-effective detection. Also, unlike in bulk solutions, the important reactions in electrochemistry happen at the interface between the electrode and the solution. Together with advancements in micro- and nano-electrochemical sensors, electrochemistry provides the additional benefit of being able to detect analytes in extremely small amounts. An electrochemical immunoassay is defined in this research as a solid-phase system where an antibody-antigen reaction occurs but the associated [6, 7] electrochemical detection is performed at a different location. In contrast, the immunoreaction and electrochemical detection take place inside an electrochemical immunosensor, making it a standalone device [8-11]. The aforementioned assessments have previously reported on several elements of older immunoassays. To lay the groundwork for developing immunoassays and immunosensors, we must first understand the fundamentals of immunoassay formats, such as antibody structure and the antibody-antigen interaction. So that those who aren't familiar with the field can easily grasp its relevance, we will next provide detailed explanations of the most recent advances in electrochemical immunoassays and immunosensors.

IMMUNOASSAYS AND IMMUNOSENSORS

Quantitative analysis is performed in immunoassays by using antibodies as the principal binding agents for the target antigen, which is frequently the analyte. Analysing the antigen-antibody binding process and distinguishing between bound and unbound antigen are the end goals of an immunoassay. Put simply, the fractional occupancy of the recognition sites is the key determinant of all immunoassays. The evaluation of occupied sites or, more indirectly, the measurement of unoccupied sites can both form the basis of such a measurement.

Antibody Immunotherapy Techniques

Immunoassay systems, whether competitive or non-competitive, must take the method of capture antibody immobilisation on a solid phase into careful consideration during design. The selected approach has the advantage of producing an immobilised capture antibody that is orientated to interact favourably with its target antigen with minimum steric hindrance, which is a desirable quality. It is also ideal to be able to immobilise the antibody while preserving its antigen-binding capabilities [12-15]. The sensitivity and dynamic range that an immunosystem is capable of clearly depend on all of these characteristics. Capture antibodies can be immobilised on solid phases using a variety of techniques, such as physical adsorption, electrostatic/physical trapping in a polymer matrix, or covalent attachment.

The effect of biotin on streptavidin binding

Immunoassay systems have made extensive use of specific affinity interactions for antibody immobilisation in the past few years. One such example is the interaction between streptavidin and biotin. You can utilise this method to immobilise the capture antibody in immunoassay/immunosensor systems, as well as nucleic acids, polysaccharides, and proteins [24]. Biotinylating the capture antibody and coating a solid phase with avidin or streptavidin are the

common steps in the process. Among the greatest non-covalent association free energies measured thus far, the dissociation constants of biotin-avidin and biotin-streptavidin interactions are on the order of 10_15 mol L_1. Additionally, the complexes are resistant to dissociation when exposed to chemicals like detergents and protein denaturants, and they can endure high temperatures and pH fluctuations. Importantly, this immobilisation method keeps the immobilised antibody's biological activity [16, 17]. When it's necessary to keep the binding affinity for biotin high while minimising non-specific binding by charged species, neutravidin, an almost neutrally charged (pI of 6.3) variant of avidin, is utilised. Recent reports have detailed the development of an electrochemical immunosensor that can detect Mycobacterium tuberculosis by immobilising a capture antibody through the biotin-streptavidin interaction. A streptavidin-modified SPCE had a biotinylated anti-M. tuberculosis antibody attached to its surface in this system. At first, the electrode was subjected to an anodic current of 25 μA for 2 minutes in a solution of 0.1 mol L_1 H2SO4. Because of its enhanced hydrophilicity, the adsorptive capabilities of the SPCE were intended to be improved. The electrode surface was treated with streptavidin solution after pretreatment and left on overnight [18-23]. A bovine serum albumin (BSA) solution was used to inhibit any remaining free sites of the SPCE. After that, the electrode was treated with biotinylated anti-M. tuberculosis, and the biotin-streptavidin interaction was left to run for 90 minutes. The sensor surface was prepared for capture by the immobilised capture antibody after a distant incubation of antigen M. tuberculosis and monoclonal mouse anti-M. tuberculosis was performed. Finally, AP-labeled rabbit anti-mouse antibody was introduced to complete the immunosensor construction. Subsequently, AP was used to transform the substrate 3-indoxyl phosphate into its Indigo product. Cyclic or square-wave voltammetry was used to generate the analytical signal after indigo was transformed into hydrosoluble indigo carmine.

Methods for Electrocatalysis

An electrocatalytic antibody was created by loading Pd NPs onto an anti-TNF-α (tumour necrosis factor-α) antibody. This innovative method for electrochemical immunoassay sensing was developed by the group of Polsky et al. A palladium shell is produced from gold particles that have been covalently attached to an antibody. There is no need for any other reagents other than the oxygen that is already present in the solution because the Pd NPs are very reactive during the oxygen reduction reaction. The electrochemical methods, such as linear sweep voltammetry, demonstrated that the Au/Pd-modified antibody exhibited remarkable catalytic activity in the reduction of oxygen. To detect TNF-α, a full sandwich immunoassay was utilised, which included the immobilisation of a diazonium-antibody probe, the capture of the target analyte, and the immobilisation of an NP-antibody label [24-27]. A cathodic current is caused by a decrease in oxygen in the buffer, which happens when the concentration of TNF-α increases. From 1 ppt to 100 ppb TNF-α, the assay reveals a detection range with extremely high signals at −400 mV. Using an electrocatalytic silver-enhanced AuNP method, Lee et al. previously demonstrated sensitive and selective electrochemical hybridisation detection. An indium tin oxide (ITO) electrode-deposited array of gold nanoparticles (AuNPs) was employed to amplify the signals of a DNA hybridisation assay with potential applications in clinical diagnostics. Coating the ITO with an electro-conducting polymer, poly(2-aminobenzoic acid) (PABA), and avidin molecules, which are promising immobilisation platforms for DNA biosensors, demonstrated that the NP electrocatalytic characteristics were sensitive to surface modifications of the electrode surfaces. Various types of AuNPs exhibited variations in electrocatalytic efficiency [28, 29] when silver was catalytically electrodedeposited on PABA-coated ITO surfaces with avidin covalently linked to the PABA [30, 31]. The hybridisation indication was streptavidin-5-nm AuNP, and the immobilisation platform was an avidin-modified (by PABA) ITO electrode, which allowed the signal to be amplified through the silver electrodeposition procedure. Using linear sweep voltammetry, this setup produced a signal-to-noise ratio of 20.

Electrocatalytic approach caused by AuNPs to improve the sensitivity of magneto-immunosensing technology was disclosed, combining Lee's idea with magnetic collecting. 140 After successfully integrating NPs for diagnostics, this system was built to identify cancer cells. For in situ identification and quantification of tumour cells, the system utilised AuNPs in conjunction with an electro-transducing platform/sensor composed of screen-printed carbon electrode. The detection limit was set at 4000 cells per 700 μL of solution. Cell surface proteins were the targets of particular antibodies attached to AuNPs, allowing for the selective detection of cells. Taking advantage of the catalytic characteristics of AuNPs on hydrogen evolution, detection was accomplished in just a couple of minutes without the need for chemical agents. The technology has the potential to be used in clinical diagnostics as either an

immunosensor or a DNA sensor, depending on its configuration [32-39]. In an electrochemical cell, chemical processes utilising electrical charges provide the basis of electrochemical analytical techniques. A non-conductive membrane separates two electrodes that are connected by an applied current in this apparatus. Amperometry, which is based on the current produced by reduction-oxidation (redox) reactions, is the basis for the measurement. There is a connection between electrochemical-based sensors and electrochemical methods used in hydrogen analytics. At a reasonable price, these sensors are an established technology. Their linear output, low power consumption, good resolution, outstanding repeatability, and precision after calibration make them gas-specific in the ppm range. Nevertheless, temperature has a significant impact on electrochemical sensors. Their lifespan is 6-12 months, and it might be reduced in the presence of moisture and other gases. In order to measure hydrogen concentrations in real time, Grimmer et al. suggested an electrochemical technique that uses the electrochemical oxidation of hydrogen in a combination of dry and humid gases generated under dynamic conditions. A reference electrode, supplied with a known flow of hydrogen, served as the reference for his electrochemical cell, while a test electrode received the gaseous mixture including hydrogen. Both the steam iron process and the catalytic hydrolysis of borohydride for hydrogen storage were effective tests of this approach. For a maximum hydrogen flow of 100 ml (H2).min−1 [40-43], the test cell's limit of quantification was 0.30 ml (H2).min−1. For the purpose of detecting and quantifying hydrogen on the surface of solid materials, electrochemical, spectrometric methods like Rutherford backscattering spectrometry [91], and potential distribution techniques are more commonly employed.

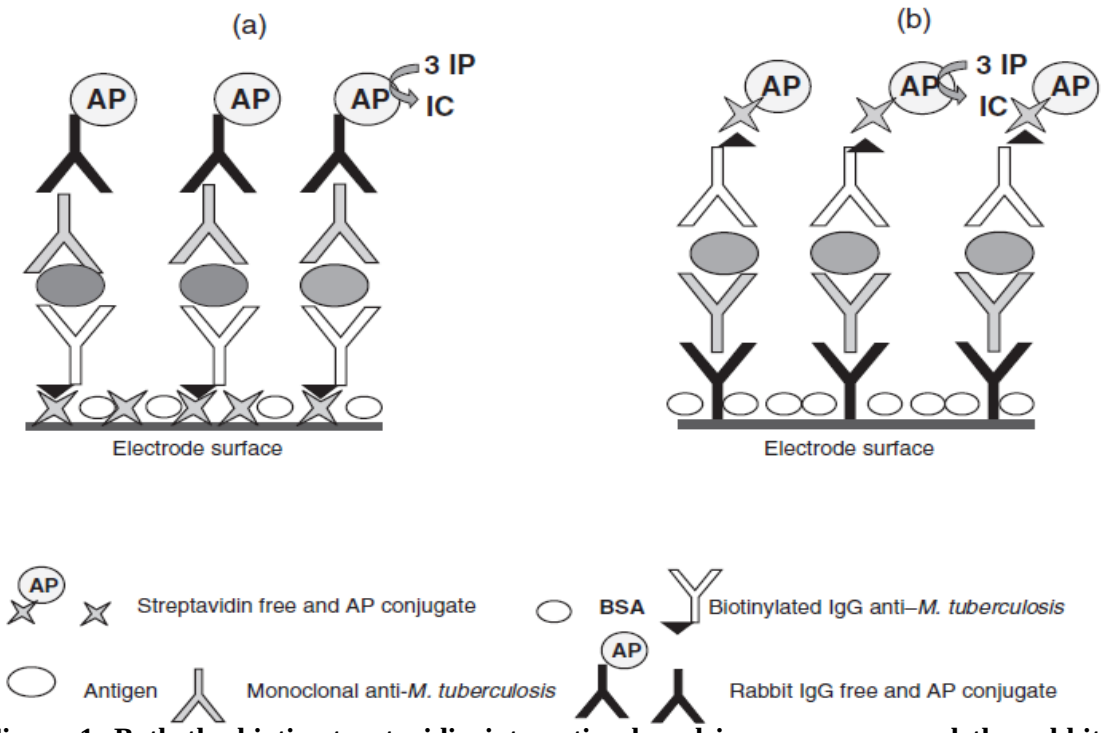

Figure 1. Both the biotin-streptavidin interaction-based immunosensor and the rabbit IgG-modified SPCE-based immunosensor are shown schematically.

Antibody-binding proteins

A bacterial antibody-binding protein is another popular method for capturing antibodies in immunoassay systems using affinity-based immobilisation. Among them, Protein A and Protein G are both very prevalent. In order for the immobilised antibody's antigen binding sites to be orientated away from the solid phase and able to bind the target analyte, these proteins bind to antibodies through their non-antigenic (Fc) regions. It is unnecessary to biotinylate antibodies since these proteins connect directly with their Fc regions. Protein A, first isolated from Staphylococcus aureus cell walls, has a molecular weight of about 42 kDa. Its ßNH2 terminal is where you'll find five Fc binding domains. Protein A can only bind to three subclasses of human IgG, however: IgG1, 2, and 4. Protein A binds slightly to mouse IgG but not to goat or rat IgG. Protein G is the second type of bacterial antibody binding protein. It is found on the cell surface of group C and G streptococci [44, 45]. Protein G shows selectivity for subclasses of antibodies from numerous species and has three Fc binding domains near its C-terminal. Protein A-GEB is a protein-

based biocomposite that was described by Zacco et al. in 2004 as a stiff material for electrochemical immune-sensing scaffolding. This biocomposite serves as a transducer for the electrochemical signal and also offers a way to safely immobilise the capture antibody. To create the biocomposite layer, a 1:4 (w/w) ratio of graphite powder to epoxy resin was mixed, and then 2% (w/w) of protein A was added. Protein A-GEB was cured for one week after the produced paste was transferred to an electrically-contact cylindrical sleeve [46, 47]. A model competitive immunoassay was used to examine the layer's suitability as a scaffold for electrochemical immunosensing. Protein A was initially interacted with by introducing rabbit antibody (RIgG) to the layer and enabling it to do so via its Fc regions. The next step was to attach biotinylated anti-RIgG to the RIgG that had been immobilised. To begin the immunoassay, we added the substrate H2O2 and streptavidin-labeled HRP to bind to the bound anti-RIgG. A concentration of 2 pmol or 10 pmol of anti-RIgG was detected by the test. Alumina and abrasive paper polishing can regenerate the Protein A-GEB layer, as demonstrated by these researchers. This process creates a mirror finish with newly exposed Protein A, which can be reused in future experiments.

Polymers with conductivity

It is common practice to immobilise capture antibodies in immunoassay systems using conducting polymers like polyaniline, polypyrrole, or polythiophene. The polymers can be incorporated into immunoassay systems that utilise amperometric, potentiometric, or impedimetric techniques. When necessary, conducting polymers can eliminate the requirement for a mediator by providing a direct path for electron transfer from an enzyme to the surface of an electrode. It is possible that "reagentless" or "label-free" immunosensing might be made possible by conducting polymers. Entrapping antibodies within the chains of conducting polymers is a typical method for immobilising these molecules. Typically, the antibody is introduced into a monomer solution and then co-immobilized onto the surface of the sensor along with the polymer. But the antibody could get denaturated and lose its action if it gets trapped. Additionally, a significant amount of the immobilised antibody will be stuck inside the polymer matrix and cannot bind to its antigen. Instead of entrapment, the antibody can be covalently attached to active groups on a conducting polymer film that has already been immobilized [48, 49]. An amperometric immunosensor for the precursor protein vitellogenin (Vtg) was recently published by Darain et al., which is based on the conducting polymer poly-terthiophene carboxylic acid (TTCA) placed on a screen-printed carbon array (SPCA). A monomer solution of TTCA in a 1:1 ratio of di(propylene glycol) methyl ether and tri(propylene glycol) methyl ether was used to electropolymerize the SPCA [36]. Three times, the potential was cycled between 0.0 and 1.6 V (versus Ag|AgCl). After submerging the array in a solution of N-(3-dimethylaminopropyl)-ethylcarbodiimide (10 mmol L_1) for 4 hours at room temperature, the TTCA-coated SPCA was functionalised with EDC. This was followed by coupling the EDC-functionalized polymer to HRP and a monoclonal anti-Vtg antibody. The sensor was incubated in a solution containing Vtg and Vtg that was labelled with glucose oxidase (GOx) to generate a competitive immunoassay. The addition of glucose caused GOx to produce H2O2, which was then decreased by the immobilised HRP. The enzyme channelling process was carried out solely by Vtg-GOx bound by the capture antibody due to the presence of the TTCA layer [50-54]. Unbound Vtg-GOx in the bulk solution did not provide any notable electrocatalytic effect. A separation-free immunosensor is a common name for this. At _0.3 V (versus Ag|AgCl), the amperometric signal was recorded, which is catalysed by the enzyme channelling. This immunosensor was able to detect Vtg at a limit of 0.09 ng mL_1, which is based on four times the standard deviation of the blank.

Self-assembled monolayers

An additional appealing option for immobilising the capture antibody in immunoassay systems is self-assembled monolayers (SAMs). Using the property of alkanethiols to spontaneously chemisorb to metals like gold or silver allows for the assembly of highly ordered monolayers. Due to the lack of a stable oxide in ambient circumstances, gold electrodes are commonly utilised. To begin making an alkanethiol SAM, a gold-sulfur bond is formed via chemisorption of the alkanethiol's sulfhydryl group to the surface of the gold. A widely-accepted hypothesis for the creation of this bond proposes that the S-H bond is added to the surface of gold by oxidation, and then hydrogen is reduced via reduction.

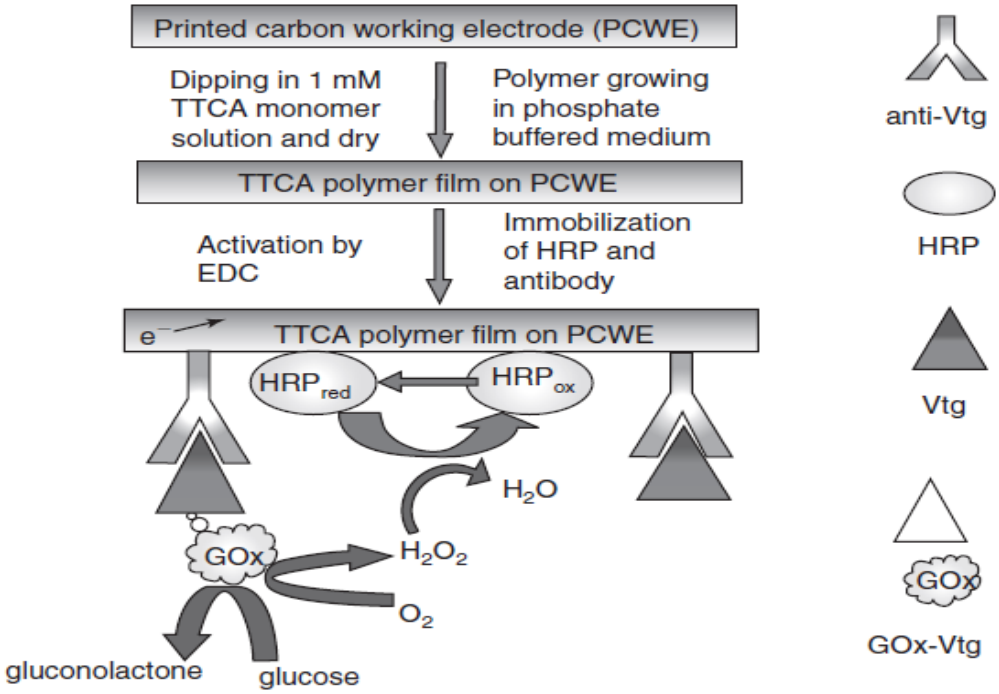

Figure 2. The immunoassay's schematic depicts the steps for preparing the electrodes and producing the signal using a TTCA film.

The transformation of carboxylic acid groups into active esters has a net effect. After that, the activated SAM was let to sit in a goat anti-rabbit IgG solution for two hours. Scientists looked at the film's resistance to non-specific protein interactions and the immobilised capture antibody's capacity to participate in specific immunoreactions. A fibrinogen protein solution was introduced to the SAM. Fourier transform-infrared reflection absorption spectroscopy (FT-IRRA) was used to characterise the SAMs after they were rinsed thoroughly. Even though the SAM had terminal ßCOOH groups, it was able to withstand non-specific protein interactions since the spectra did not show an amide I or amide II band. Also, once the antibody was immobilised, it was shown that the SAM was not reactive to non-specific contacts. To test the immobilised capture antibody's immunoreactivity, rabbit anti-sheep IgG was used as an antigen. As a consequence, the amide bands in the FT-IRRA spectra grew significantly, suggesting the existence of a particular interaction. Electrostatic interactions allow antibodies to be adsorbed on charged SAMs, in addition to covalently adhering to them [55-59]. Antibody adsorption orientation has been studied by Chen et al. in relation to surface and solution parameters. The physisorption of anti-human chorionic gonadotrophin (hCG) IgG to COOH- and NH2-terminated SAMs was investigated in this study. hCG is a hormone that is utilised in pregnancy testing and has dual capabilities as a tumour marker. The isoelectric focussing technique was employed to ascertain that the isoelectric points (IEPs) of IgG, its Fc fragment, and F(ab)2 were 6.8, 8.3, and 6.0, equivalently. To find out how much of the surface area of COOH- and NH2-terminated SAMs were covered by adsorbed antibodies, surface plasmon resonance was employed. To enhance the electrostatic interaction between the IgG and the SAMs, a slightly acidic solution (2.1 mM PBS with 0.3 mM NaCl) was utilised. The COOH-terminated SAMs were seen to adsorb a significantly higher amount of IgG. We hypothesised that IgG would adsorb more effectively on COOH-terminated SAMs at pH values below its IEP and on NH2-terminated SAMs at pH values above its IEP. At pH 6.25, the COOH-terminated SAMs exhibited a maximum adsorption of about 5 mg m_2, while the NH2-terminated SAMs exhibited a maximum adsorption of around 2.3 mg m_2 at the same pH. This unexpected outcome was thought to be caused by the uneven structure of the IgG (Fc and F(ab)2) regions. The estimated orientation of the adsorbed antibody can be derived from a highly idealised packing of antibodies [60-64]. Surface coverage is anticipated to fall within the range of 2.6 mg m_2 to 5.5 mg m_2 when antibodies are orientated "end on" (Fc area closer to the SAM) or "head on" (F(ab)2 region closer to the SAM). Conversely, it is anticipated that a surface coverage of around 2.0 mg m_2 occurs when antibodies are adsorbed "side on" (with one Fab region attached to the SAM and the other to the Fc area). It is likely

that the IgG linked to the COOH-terminated SAMs is orientated head or end on, according to these values. At different pH levels, the ratio of hCG to immobilised anti-hCG IgG was examined in order to delve deeper into the direction of adsorbed antibodies. The studies showed that the ratio for IgG adsorbed to the positively charged NH2 surface near the IEP of IgG was 0.48, which is higher than the other concentrations. This points to an increase in the number of accessible F(ab)2 regions for analyte binding. Consequently, a positively charged NH2-terminated SAM is the surface of choice for immunosensing systems that use capture antibody adsorption to a SAM as their immobilisation mechanism. The ideal conditions for adsorption include a low ionic strength and a pH close to the antibody's IEP. Proteins A and G, which are covalently attached to SAMs, provide an additional means of controlling the orientation of capture antibodies on their surfaces. Our lab has recently shown that Protein A can be covalently coupled to a thioctic acid SAM that has been placed on a gold disc electrode [65, 66]. This study used EDC to activate the thioctic acid SAM to its o-acylurea intermediate. Next, 30 μg of Protein A was added to the electrode surface in a solution. Protein A's lysine residues were able to bind more effectively after an overnight incubation at 4oC of the electrode. Once the surface of the sensor was prepared, it could be incubated with the capture antibody and an immunoassay could be run..

Antibody fragments

Immunoassay methods can also make use of antibody fragments (Fab) instead of complete capture antibody molecules. Antibodies are typically fragmented by proteolytic enzymes such papain, chymotrypsin, and trypsin. To remove the disulphide bonds that hold the two chains of the F(ab)2 fragment together, chemicals like dithiothreitol or 2-mercaptoethalamine are usually used after enzymatic digestion. Two Fab fragments, one with a thiol group at the end, are the outcome of this process. The pieces can thus self-assemble on a gold surface with little to no further reagent because of their strong affinity for the metal. The resulting stacks are organised in a way that makes the antibody-binding areas easier to reach for binding antigen. Zhang and Meyerhoff recently published an immuno-assay for C-reactive protein (CRP) detection using Fab fragments attached to magnetic particles coated with gold. Placing the gold-plated particles in a solution containing anti-CRP Fab fragments allowed them to be immobilised on their surface. This process was carried out overnight at 4oC. Next, varying concentrations of CRP were added to the Fab-coated beads and left to incubate. The particles were resuspended and rinsed extensively before being exposed to an excess of goat anti-human CRP labelled with HRP. The HRP substrate (3,3 ,5,5 -tetramethylbenzidine) was added to the two-site sandwich immunoassay after an additional washing and resuspension procedure [67, 71]. A part of the supernatant's absorbance was measured at 490 nm after the enzymatic reaction was stopped with H2SO4. The absorbance was plotted against the concentration of CRP to create a calibration plot. This immobilisation method was successful in achieving a detection limit of 0.14 ng mL_1. The outcomes were contrasted with a comparable setup where Fab was covalently attached to uncoated magnetic particles through a tosyl reaction with the amine groups of the fragments, rather than being coated. The minimum detectable concentration using this method was 1.9 ng mL_1. Based on the findings, immobilisation using a self-assembled layer of Fab fragments on top of gold-plated magnetic particles is an appealing option since it improves antigen binding orientation. There are many other types of solid-phase immunoassays that could benefit from this technology, even if it is not an electrochemical immunoassay system.

Advanced Electrochemical Methods for Detection

Several techniques for immobilising capture antibodies onto solid phases of systems have been covered in the preceding sections. The next step is to build an immunocomplex using the established protocol for an immunoassay. In order to quantify the amount of analyte present, a detection method is therefore required. Detection in biosensor technology has traditionally relied on electrochemical methods. Reasons for this include electrochemistry's many advantageous properties, such as the sensitivity of its transducers, their compatibility with current miniaturisation and microfabrication technologies, their low power consumption, their affordable cost, and their independence from sample colour and turbidity. The majority of antibodies and antigens cannot function as redox partners on their own, so in order to facilitate an electrochemical response in immunoassays and immunosensors, it is common practice to conjugate a suitable label to a specific component of the immunocomplex [72-75]. The amount of the analyte in a sample solution can be quantitatively determined from the electrochemical signal that is generated. Electrochemical detection techniques are commonly employed with immunoassay systems and immunosensors; these methods are classified according to the type of signal measured and include potentiometry, amperometry, voltammetry, and, more

recently, electrochemical impedance spectroscopic measurements. What follows is an explanation of the four main approaches, with comments based on more current research that has tackled issues related to these methods.

Detectors of immunoconcentration

When an antibody binds to its antigen, a potentiometric immunosensor detects the resulting change in voltage between an indicator and a reference electrode. So far, potentiometric detection-based immunosensors have been mostly unheard of. The minimal change in potential that results from the interaction of an antibody with its antigen is one of the key drawbacks of this detection method. It is also possible that the sample matrix interferences will make it impossible to identify this tiny signal. Because of this, the sensitivity and dependability of these sensors are frequently poor. One modern potentiometric immunosensor uses a screen-printed electrode coated with polypyrrole to detect enzyme-labeled immunocomplexes. At least four cycles of cyclic voltammetry with aqueous pyrrole and sodium dodecyl sulphate were used in the electro-polymerization technique for the construction of this electrode. The final state of the polypyrrole film was achieved by applying a constant voltage. These electrodes, coated with polypyrrole, not only demonstrated enhanced sensitivity but also remained stable at 37oC for a minimum of four months. The antibody was bound to streptavidin-coated polypyrrole or adsorbed directly onto the polypyrrole layer, depending on the method used to immobilise it. Hepatitis B surface antigen or the cardiac marker troponin I were added to the sample solution before the immunoelectrode was incubated. Adding a signal antibody coupled to HRP finished the sandwich immunoassay. In a pH 7.4 solution of 0.01 M PBS, potentiometric experiments were carried out. The enzyme turnover was initiated by adding the active substrate, o-phenylenediamine dihydrochloride, and the potential was monitored 60 s later. To effectively minimise matrix interferences, it was necessary to deliberately separate the immunoreaction from the detecting phase. There was a direct correlation between the magnitude of the antibody-analyte reaction and the sample concentration of the analyte [76, 77], as measured by the change in potential. The process of the potential change, which was described as a "charge-step procedure," is not entirely obvious, although it is caused by the HRP conversion of o-phenylenediamine dihydrochloride to 2,3-diaminophenazine. Potential changes were thought to have occurred because the enzymatic process changed the solution's pH and ionic strength, which in turn changed the polypyrrole layer's physical (porosity, density, thickness) and electrochemical (conductivity, charge) properties. Troponin I has a reported sensitivity of 0.4 pmol L_1 and hepatitis B surface antigen of 50 fmol L_1.

Intravenous immunosensors

Amperometry involves measuring, under controlled potential conditions, the current generated by the oxidation or reduction of an electroactive analyte species at the surface of an electrode. The amount of analyte present is then proportional to the current magnitude. Because antibodies and antigens do not have inherent electroactivity, an appropriate label is required to induce an electrochemical reaction at the immunosensors in an immunocomplex. Enzyme labels, such as oxidoreductases, hydrolytic enzymes, and AP, are commonly employed in this regard due to their capacity to produce an electroactive product after catalysing the conversion of a substrate. The amount of analyte present can be quantitatively attributed to the amplitude of the current resulting from the product's redox reaction. As an additional example of this signal-generation strategy, consider the enzymatic conversion of 4-aminophenyl phosphate (4-APP), the substrate of AP, to 4-aminophenol (4-AP). Afterwards, a Faradaic current proportionate to the analyte concentration is generated by oxidising 4-AP to 4-quinone imine (4-QI). An important benefit of enzyme labels is that they can amplify the signal that can be detected with very small amounts of enzyme, thanks to the catalytic impact.

Immunoassays that measure voltage

Typically, while developing electrochemical immunosensors, one electrode is used to immobilise an immunocomplex, and then the label on the immunocomplex is used for detection at the same electrode. A newer and more popular transducer option for electrochemical immunoassays is interdigitated array (IDA) microelectrodes. An IDA typically has two interdigitated microelectrode "fingers" for its basic architecture. To achieve "redox" cycling of the electroactive species to be detected, the two interdigitated electrodes of an IDA employed as a sensing electrode in a voltammetric experiment are typically kept at different potentials. As shown in Scheme 1, a two-electron oxidation of 4-AP to 4-QI is promoted by applying an oxidising potential to one of the two IDA electrodes. After that, 4-QI finds

its way to an electrode that is held at a lower potential, where it is reduced to 4-AP. From there, it can go through another oxidation at the electrode that is next to it. By increasing the ratio of the Faradaic current to the background current, this redox cycling enhances the signal-to-noise ratio, which in turn lowers the detection limits and increases sensitivity. Analytical chemistry and biosensor systems found numerous uses for IDA electrodes as electrochemical detectors due to these characteristics [60]. As a detector in immunoassays for mouse IgG, Thomas et al. utilised an IDA that comprised 25 sets of platinum microelectrodes with 1.6 μm gaps and 2.4 μm widths in a recent application. With an 87% redox cycling and a detection limit of 3.5 fmol mouse IgG, the signal was four times stronger than with single-electrode detection.

Nanoprobes functionalised with noble metal nanoparticles

Functionalising Ag NP with nanocarriers such CNT [48], graphene, or carbon nanosphere allows for the construction of various nanoprobes in electrochemical immunoassays, thanks to the low oxidation potential of the NP and its ease of use in electrochemical stripping analysis. The carboxylated CNT's inherent reduction property allowed for the in situ deposition of Ag NPs on this support from solution-based silver ions, eliminating the need for an additional reduction agent. These NPs were subsequently utilised to link the antibody through the streptavidin assembly. The immunosensor surface was coated with high-content Ag NPs by a sandwich-type immunoreaction, which further induced silver deposition and significantly amplified the detection signal. For the simultaneous electrochemical measurement of CEA and AFP, this approach demonstrated extremely high sensitivity with wide detection ranges and detection limits as low as 0.093 and 0.061 pg mL−1, respectively, based on the electrochemical stripping detection of the Ag NPs at the immunosensor.

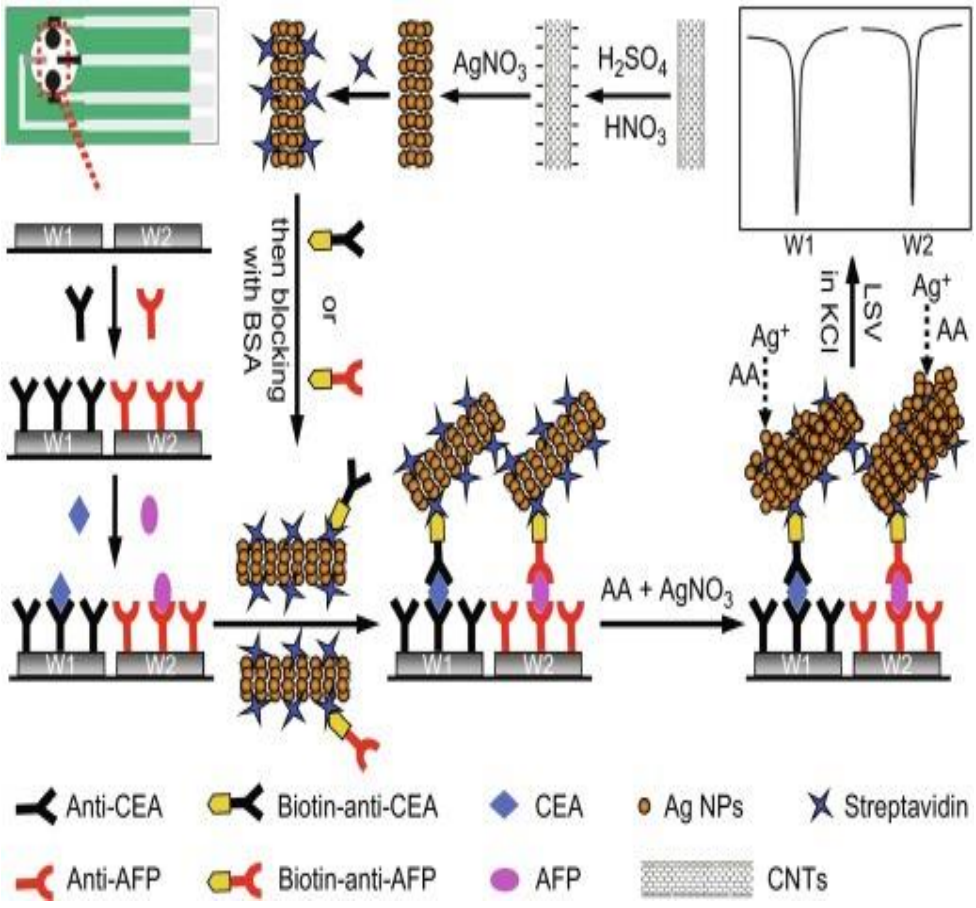

Figure 3. The schematic depicts the steps involved in preparing the immunosensor array and trace tag, as well as the detection approach, which involves analysing the immunosensor surface for Ag NPs using linear-sweep stripping voltammetry.

Immunosensors and impedimetric assays

The immunosensor surface's interfacial charge, capacitance, resistance, mass, and thickness will change as a result of the immobilised antibody's particular molecular recognition of the antigen. Since these interfacial changes can potentially lead to the quantitative detection of the analyte of interest, electrochemical approaches that track them are gaining popularity. An electrochemical system is subjected to a constant DC potential in an EIS experiment, on top of which is applied a sine wave potential signal with a modest amplitude, typically 5 to 10 mV peak-to-peak. Applying a sinusoidal potential and measuring a sinusoidal current yields the impedance, according to Ohm's law. In this circuit, RS is the ohmic resistance of the electrolyte solution, Cdl is the double layer capacitance, Rct is the charge transfer resistance that exists in the electrolyte solution when a redox probe is present, and ZW is the Warburg impedance that results from the diffusion of redox probe ions from the bulk electrolyte to the electrode interface. Keep in mind that an immunocomplex structure on an electrode surface is not anticipated to impact either RS or ZW, which are bulk properties. Conversely, the insulating and dielectric characteristics of the contact between the electrode and electrolyte solution determine Rct and Cdl.

Systems for Microfluidic Electrochemical Immunoassays

Using direct electrowetting-based valves, an integrated microfluidic device was realised with injection, flushing, and sensing functions. Two substrates made up the device: one made of poly(dimethylsiloxane, or PDMS), and the other of glass, which had driving and sensing electrodes attached to it. Hydrophilic flow channels created with a dry-film photoresist layer allowed liquids to move spontaneously, enabling microfluidic transport. Gold working electrodes acted as valves to regulate the injection and flushing of liquids. The glass substrate may have had a through-hole or the channels used to build the valves. We partially simulated a sandwich immunoassay by detecting immobilised antigens to show that the technique might be used in an immunoassay. An anti-human AFP antibody or human α-fetoprotein (AFP) was attached to a platinum working electrode in the chamber through the use of a plasma-polymerized film (PPF). A potential was applied to the injection valves [78, 79], and the solutions were injected one by one into a reaction chamber located in the middle of the chip. The chamber was then incubated for appropriate durations. A filter paper was positioned beneath the apparatus, and the solutions were then absorbed after being flushed through the flushing valve. We used electrochemical detection after incubation with the appropriate antibodies that had been labelled with glucose oxidase (GOD). The amount of immobilised antigen determined the obtained current in both cases. The detection limit was set at 0.1 ng, and the calibration curves were sigmoidal. Many times, biochemical tests include adding different solutions to a reaction chamber and then exchanging them. The immunoassay is a good illustration of this. Because molecule diffusion affects analysis time, doing immunoassays in a microfluidic system can drastically cut down on analysis time. The expectations surrounding the micro-scale assay are increasingly growing, even if many ongoing investigations are still limited to conducting immunoassays in a micro-flow channel. Using electroosmotic flows in a network of flow channels is one of the representative alternatives for achieving on-chip analysis. Nevertheless, the system's miniaturisation is out of sync with its need for a big power supply. Contrarily, systems with integrated pumps and valves have only just become a reality; this is likely attributable to the technical challenges associated with integrating microfluidic components. However, there have been reports of a few gadgets that make it possible to handle and combine solutions. The use of integrated magnetically operated microvalves has allowed a research group from the University of Cincinnati to develop an on-chip immunoassay. Incorporating sensing capabilities for a variety of target analytes, our research group has manufactured microfluidic devices with active transport capabilities. To broaden the device's potential uses, we continued working on an active microfluidic device that could sequentially inject and flush solutions, allowing it to handle many solutions at once. Direct electrowetting was employed to achieve this goal. When the device's size is decreased, the effect of interfacial tension becomes more significant. Hence, microfluidic transport studies are increasingly focussing on interfacial tension as a driving mechanism. The elimination of moving components like a diaphragm and the consequent reduction in the need for peripheral equipment like a power source and detection devices constitute a compelling argument in favour of this microfluidic system. Microfabrication allows for the production of electrodes for use in microfluidic transport and sensing. Previously, we mobilised liquids using pumps made of strips of extended gold working electrodes.

Modern Developments in the Use of Microfluidics for Immunoassays

Enabling the full biochemical process, including reagent loading, reaction, separation, and detection, on the microchip, microfluidic chips use technology that miniaturises typical laboratories by fabricating microchannel networks. Fluid metering, fluid valving, mixing, transport, and incubation of reagents are common elements of microfluidic platforms. The following benefits are associated with these microfluidic chips. First, by reducing the assay volume, the number of reagents needed can be significantly cut down. As the characteristic scale falls, the surface force (capillary force, etc.) becomes more important in the motion of the liquid, allowing for passive liquid propulsion systems like capillary test strips to function. With a low Reynolds number, Third laminar flow can provide a stable liquid-liquid contact, which allows for increased sensitivity. The concentration of an analyte (often a protein) in a solution can be determined with the use of an antibody or antigen in an immunoassay, a type of biochemical test. ELISA, radioimmunoassays, fluorescence immunoassays, chemiluminescence, the list goes on. Despite immunoassay's widespread use, its promotion in POCT is hindered by the old method's enormous experimental equipment and complicated operation stages. Traditional immunoassay has its limitations, but when combined with microfluidic technology, it can significantly improve upon them. Improving the performance of immunoassays for the detection of analytes in secretory fluids like blood has been the subject of much research in recent years. Meanwhile, hundreds of thousands of researchers have documented the use of microfluidics in immunoassay in their published publications. Some of their attempts at improvement centre on streamlining processes, some on bringing disparate systems together, and yet others on making things more sensitive. In order to achieve perfect integration, superior packaging, and sensitivity, researchers are working hard to design a comprehensive microfluidic platform. Although numerous reviews have covered various microfluidic applications and microfluidic technologies in general, none have focused on immunoassay platforms. One example of a niche area that needs attention is biomedical analysis using centrifugal microfluidics. On top of that, microfluidic immunoassay has been undergoing fast invention and development. While Tan et al. created a reusable optofluidic point-of-care testing platform for the sensitive detection of biomarkers with easy procedures, an ultra-low-cost paper centrifugal operational system that can be operated manually was invented in 2017. Researchers studying POCT in resource-constrained settings should, therefore, immediately conduct a review regarding the immunoassay's implementation on different microfluidics.

Many advances in microfluidic labs on a chip that are passively driven have been made throughout the years. Typically, microfluidics may be driven and advanced without the need for an external actuator. The numerous distinct benefits of passive microfluidic systems are categorised according to the forces employed, which can be surface tension, capillarity, or gravity-driven flow. To begin with, it can be easily faked. Second, there's no need for qualified operators, therefore less expertise is required. thirdly, because it doesn't need any external power, it's cheaper than active microfluidic systems. Improvements in valve control, an unparalleled degree of integration, and high sensitivity are just a few of the recent successes that have resulted from researchers combining immunoassays with a passive microfluidic system. - A Automated passive microfluidic immunoassay devices like this are just as good as, if not better than, those exact laboratory tests. In areas where resources are scarce [80, 81], the system shows great promise as a tool. Directed Microfluidics by Capillaries. The Laplace capillary pressure formula tells us that as the capillary radius gets smaller, the capillary pressure will go up, because $r \sim 2 \lambda/R$ represents the relationship between the surface tension of the liquid and the capillary radius. The flow can be driven by capillary pressure alone, without the need for any other energy source. It can significantly decrease the size of the driving device when used to microfluidic chips. Improved Capillary Microfluidic Immunoassay Through System Integration, Portability, and Step-Simplified Procedures. The capillary microfluidic immunoassay has been the subject of intensive study with the goal of improving its system integration, making it more portable, and streamlining its operating steps. This would allow for unrestricted control of the fluid regardless of the setting of time. Within 60 minutes of using this equipment 82, 83], the carcinoembryonic antigen (CEA) could be detected with a LOD of 0.3 ng/mL. To get the most out of microfluidic devices, you should steer clear of complex systems, like those that require multiple steps to operate. A straightforward microfluidic platform driven by capillaries and omitting the cleaning phase was announced in 2018. Using integrated microbeads, Epifania et al. achieved a single-step mode of operation for mycotoxin detection.

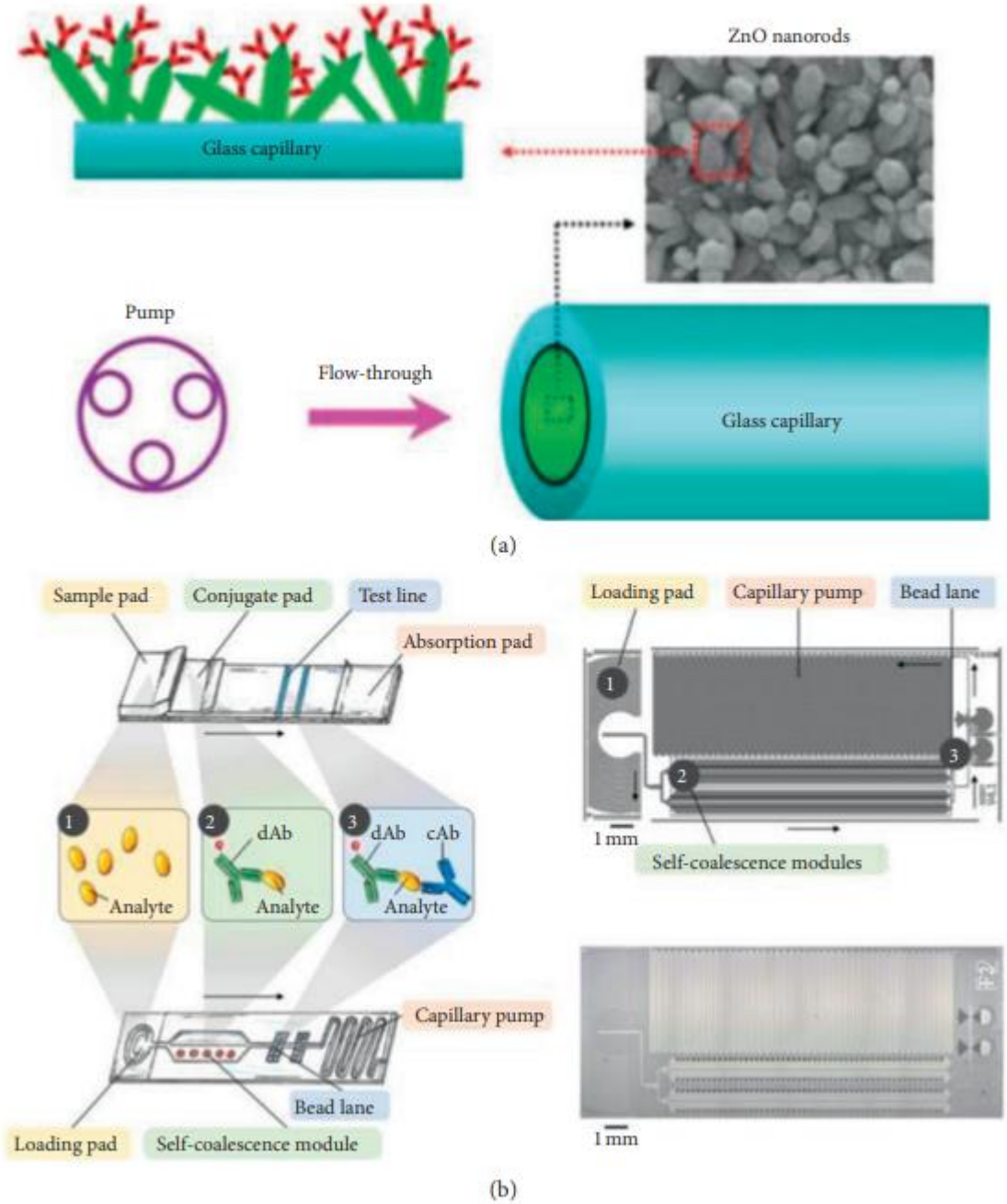

Figure 4. (a) the illustration of the ZnO nanorod-decorated glass capillaries immunoassay. (b) flowchart of the transposition from a lateral flow assay to a microfluidic chip.

CONCLUDING

The capillary action in hydrophilic flow channels or a through-hole can be controlled with valves that operate on the principle of direct electrowetting, allowing for the timely injection and flushing of solutions. The straightforward design and operation make it easy to incorporate numerous flow channels and manage numerous solutions. Biochemical analyses involving reagent exchange may be possible with this equipment. In this regard, the immunoassay serves as an exemplar. Electrochemical immunoassays and immunosensors continue to be the subject of intense research, despite the fact that immunoassay techniques have been around for more than 20 years. The selectivity of these immune systems is remarkable. Studies are ongoing to determine the best methods for minimising steric hindrance and optimally aligning antibodies on solid phases. The sensitivity of studies using immunoassays and immunosensors will surely be improved by future advancements in this field. With the use of electrochemical detection, these systems will provide quick, easy, and cost-effective analyses that are both sensitive and selective. In order to employ a more direct signal-generation technique, there is an ongoing interest in creating and using

appropriate labels for electrochemical immunoassays. An interesting and simpler alternative to schemes utilising labels, a label-free strategy has begun to be facilitated by the application of electrochemical impedance spectroscopy. However, in order to attain the necessary sensitivity and dynamic range in amperometric detection, a great deal of research must be conducted in this field. Further advancements in the production of miniaturised immunoassay devices are also anticipated as a result of promising developments in microfluidic electrochemical immunoassay systems. In light of the growing trend of home-based diagnostics, this will pave the way for the creation of portable instruments for rapid on-site pharmaceutical and clinical diagnosis. Wanting to combine immunosensors in an array shape to analyse numerous analytes at once is another obstacle in this field. In light of these promising prospects, new immunosensor technologies are expected to be developed soon.

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