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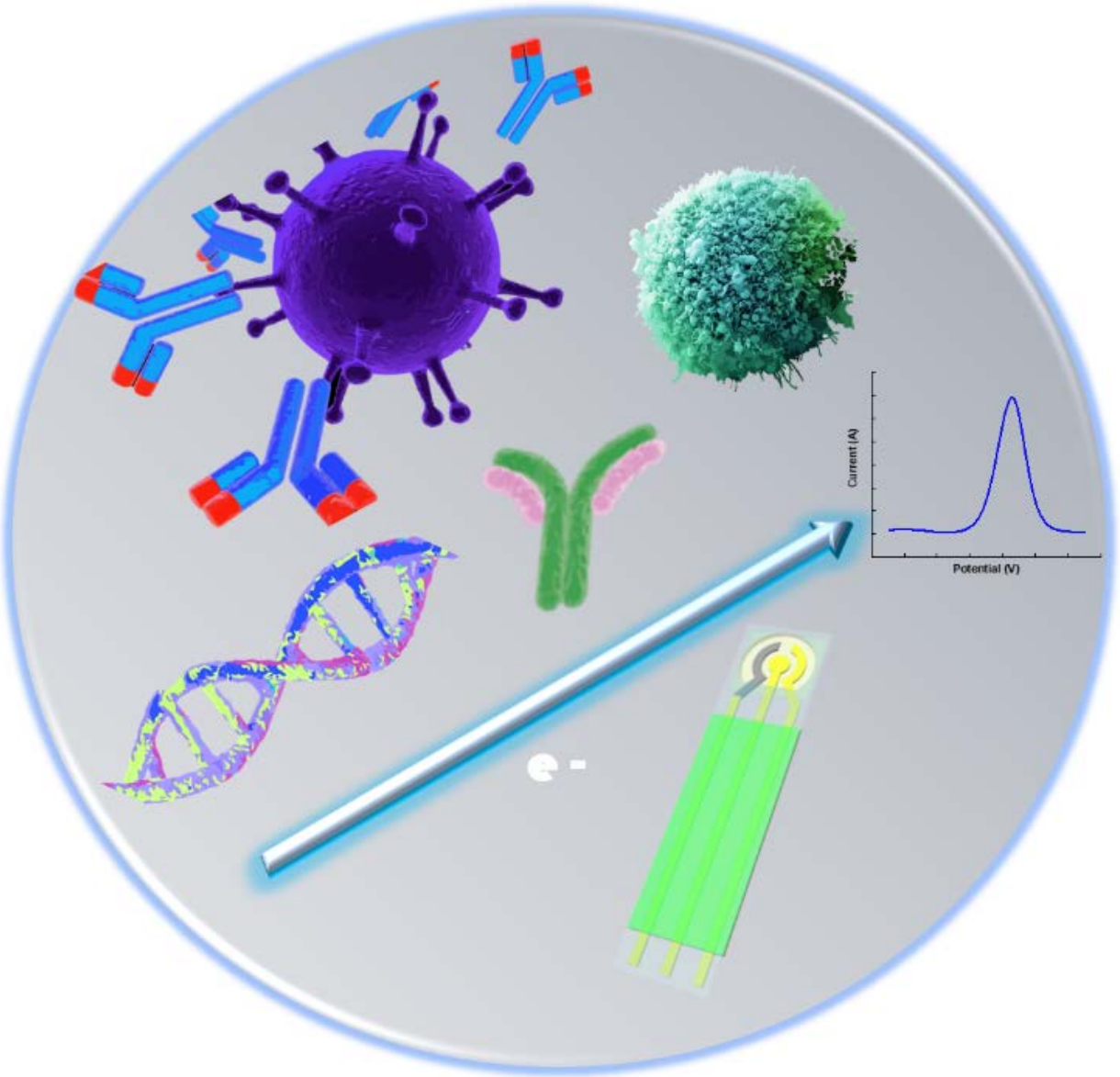
MINIREVIEW

Recent Advances on Electrochemical Biosensing Strategies toward Universal Point of Care Systems

Yifan Dai, [a b] and Chung Chiun Liu*[a b]

Key words: analytical electrochemistry · universal biosensor · point of care· biosensing strategy



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Abstract: A number of very recently developed electrochemical biosensing strategies are promoting electrochemical biosensing systems into practical point-of-care applications. Focus of research endeavors in biosensing community has transferred from detection of a specific analyte to the development of general biosensing strategies that can be applied for a single category of analytes, such as nucleic acid, protein and cell. In this review, we describe most recent cutting-edge researches on electrochemical biosensing strategies. These described developments resolved critical challenges on application of electrochemical biosensors to the practical point-of-care systems, such as rapid readout, simple biosensor fabrication method, ultra-high detection sensitivity, direct analysis in complex biological matrix and multiplexed targets analysis. This review provides general guidelines both for scientists in the biosensing research community and for the biosensor industry on development of point-of-care system, benefiting global healthcare.

1. Introduction

The advances in understanding of molecular pathways on the regulation of diseases promote the development of point-of-care diagnosis.[1] The information from broad assessments of biomolecular signatures from nucleic acid, protein and cell during disease progression is utilized as a general standard to quantitatively evaluate the severity of the disease.[2] The existence of certain biomolecules in human fluids has been recognized as the confirmation of a corresponding disease, such as cancers,[2a, 3] neurodegenerative disorders.[4] Therefore, techniques on early detection of biomolecules are extremely critical for medical industry, especially meaningful for disease diagnosis and drug treatment.[5]

Current techniques for biomolecular quantification involve the use of mass spectrometry,[6] ELISA,[7] Western blot,[8] and Northern blot.[9] However, these techniques are expensive, complex and time-consuming, therefore not suitable for the contemporary needs of point-of-care disease evaluation. With the unresolved medical demands on an accurate, simple, cost-effective, time-efficient and sensitive biomarker detection system, recent scientific endeavors lead to many novel biosensing systems, such as field-effect transistor,[10] surface plasmon resonance sensor,[11] and electrochemical biosensor.[1g, 1i, 1j, 12] Among these emerging diagnostic platforms, electrochemical biosensor stands out for wide developments toward point-of-care diagnostics owing to the advantages discussed below.[1i, 13] Every sensing system must consist of three essential elements: a sensing element, a bio-recognition element, and a transduction element. As for electrochemical biosensing system, it possesses a simple and cost-effective sensing element (cost less than $1 for each sensor), such as single-use sensor,[14] sensor array.[15] The processing method of bio-recognition element in an electrochemical biosensing system is simple and efficient, delivering an effective bio-interface without complex operations. Furthermore, the cost-effectiveness of the electric (signal) transduction system, such as linear sweep voltammetry, differential pulse voltammetry, making the whole sensing system applicable in undeveloped area. Generally, the development of electrochemical biosensing system has created a positive and robust impact on global health.

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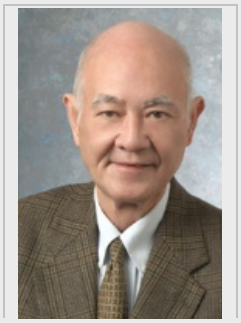

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2. Current limitations on electrochemical biosensing system

However, many technical challenges still linger, limiting the utilizations of electrochemical biosensing system. First, for the detection of early-stage disease, the abundance of biomarkers in human fluid is low comparing with that of irrelevant biomolecules, hence, requiring high-sensitivity and high-differentiation ability for accurate analysis. Second, the matrix effect caused by the biomolecules (other than the target) from human fluid interferes the target recognition process (during mass transportation), delivering a high-possibility of false positive result. In addition, for practical applications, fast turnaround time is required. Therefore, the fabrication method of biosensor needs to be simple and

Table 1. Summary of electrochemical biosensing strategies covered in this review.

Ref.	Target	Sensing Element	Recognition element	Transduction element	Detection limit
[24]	MicroRNA	Au@MNPs network + gold electrode	DNA probe	Methylene blue	fM
[28]	Methylated DNA	IMB+ carbon electrode	DNA antibody	HRP/H_2O_2/HQ	pM
[33]	Circulating tumorNA	High-throughput Nanostructured gold electrode	Combinatory DNA probe + clamp probe	$[Ru(NH_3)_6]^{2+}$ +$[Fe(CN)_6]^{3-}$	nM
[38]	DNA	Gold electrode	DNA probe	Methylene blue	pM
[41]	DNA	Gold electrode	Target induced DNA strand displacement	Ferrocene	fM
[48]	Antibody	Gold electrode	DNA probe + complementary DNA strand	Methylene blue	nM
[49]	Small molecule	Gold electrode	Antibody+ DNA probe + complementary DNA strand	Methylene blue	nM
[53]	Thrombin	Nanostructured gold electrode	Aptamer+ charge neutralizer displacement	$[Ru(NH_3)_6]^{2+}$ +$[Fe(CN)_6]^{3-}$	fM
[54]	HE4/Tau	Gold electrode	Antibody+ charge neutralizer	$[Fe(CN)_6]^{2+/3+}$ /$[Ru(NH_3)_6]^{2+/3+}$	pM
[59]	Methyltransferase	Two electrode system (up & down)	Hemimethyla-ted DNA +restriction enzyme	Methylene blue +$[Fe(CN)_6]^{3-}$	fM
[67]	E. coli/ B. subtilis	MB +carbon nanotubes electrode	Antibody	Metabolic activity indicator	5 cell/mL
[69]	VCaP	High-throughput Nanostructured gold electrode + Metal nanoparticles	Anti-EPCAM + cell surface aptamer/antibody	Metal nanoparticles	2 cell/mL

efficient. Recent studies have demonstrated novel electrochemical biosensing strategies that are able to overcome the issues mentioned. In this review, we focus on the very recent advances of electrochemical biosensing strategies that deliver simple, high-sensitivity, non-fouling biosensing platforms (Table 1). The selected works demonstrated robust applicability of the developed sensing methods as general detection platforms targeting a specific class of the analyte, including nucleic acid, protein and cell. Other progressive advances on electrochemical detection of small molecules and electrochemical applications in diagnosis, prognosis and therapeutic actions in different sample matrixes were comprehensively reviewed, therefore not included in this work.[16] In this Minireview, each strategy review starts with the description of the three fundamental elements of the biosensing system, following by the discussions on the design of the biosensing strategies. The selected cutting-edge electrochemical biosensing strategies provide solid foundations for further research endeavors for the development of the universal electrochemical detection platform for point-of-care systems.

3. Detection of nucleic acid

Nucleic acid is the fundamental composition of human genome.[17] The advancing understanding of transcription and translation process of genomic information promoted the development of disease diagnostic and treatment from nucleic acid level.[18] However, the low abundance of relevant nucleic acids and the complexity of the human fluid significantly impeded the accuracy and sensitivity of nucleic acid detection. Herein, we discussed the most recent studies on nucleic acid detection, which significantly enhanced the detection sensitivity and reduced the matrix effect on the detection performance.

3.1 Gold coated magnetic nanoparticles network

It is crucial to detect ultralow concentrations of microRNA, which is a blood-borne biomarker for various disease. [19] Minute variations of microRNA concentration in human blood is critical for disease progression, long before disease can be probed on imaging scans.[20] However, the complex composition of human blood provides significant interference for detection. Studies demonstrated multiple ways to reduce the matrix effect, such as the application of magnetic beads for target separation,[21] and construction of non-fouling surface through surface chemistry.[22] Magnetic beads based biosensors have been widely developed owing to their simplicity of application.[21b, 23]

Very recently, an electrically reconfigurable DNA-gold-coated magnetic nanoparticles (DNA-Au@MNPs) network was designed to distinguish small variations of microRNA concentrations from blood sample directly (Figure 1A).[24] The sensing element in this design was gold-coated magnetic nanoparticles, which were used for immobilization of recognition element, and more importantly,

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the magnetic property of the nanoparticles was able to assist the separation of captured microRNA target from complex sample matrix. The recognition element for the capture of microRNA was a probe DNA sequence complementary to the microRNA target designed with a thiol linker (to tether onto gold surface) and a methylene blue label (as electrochemical transduction element). Once the recognition element linked with the Au@MNPs, the DNA-Au@MNPs were then mixed with sample containing microRNA target for hybridization of 30 min. The target captured DNA-Au@MNPs were separated from sample matrix through external magnet and collected onto a gold electrode for electrical reconfiguration through square-wave voltammetry. The electrochemical quantification was achieved through the methylene blue redox signal difference between non-hybridized DNA-Au@MNPs (baseline) and target captured DNA-Au@MNPs, because the formation of double helix on Au@MNPs surface increased the space between Au@MNPs inside the network therefore leading to an increase of electron transfer length during the redox reaction and an impedance of electron tunneling through the formed Au@MNPs network. In addition, the electrical polarization of the Au@MNPs network reconfigured the network formation, leading to a suppression of electrochemical current. The construction of Au@MNPs network provided an ultra-amplification of change of electrochemical signal once trace amount of target captured, resulting in a femto-to-attomolar sensitivity level with capability on detection microRNA directly from complex biological matrix.

3.2 Immunomagnetic beads with selected hybridization

Another important type of nucleic acid drew significant attentions to biosensing development is DNA methylation, which is an early prognostic sign of cancer progression.[25] DNA methylation is a covalent attachment process of a methyl group onto the carbon-5-position at cytosine within the CpG dinucleotides, leading to a repression on gene transcription, which is considered to be an early event of cancer cell progression.[26] The clinical adoption of methylation assay, such as bisulfite assay, enzyme based assay, is generally poor due to the complexed detection process and expensive instrument.[27] However, the advancement in electrochemical biosensing strategy provides great potentials on rapid and cost-effective analysis of specific DNA-methylation in human serum and cells.

In order to achieve the accuracy and the sensitivity for DNA-methylation detection, the differentiation of methylated cytosine and adenine from regular DNA strand is required. Recently, a strategy involved a precise capture of target by multiple recognition elements and an amplification of detection signal strategy was applied for biosensing development. An immunomagnetic beads (IMB) with selected hybridization based electrochemical strategy was applied to quantitatively access the tumor suppressor gene methylations (Figure 1B).[28] The sensing element in this design contained two parts, a specific 5-methyl-cytosine antibody linked magnetic beads and a screen-printed carbon electrode. The recognition element was also consisted of two parts, the specific methylated DNA antibody on the magnetic beads and a synthetic DNA probe (containing horseradish peroxidase (HRP) linker) complementary to the specific methylation sequence on the target. The transduction element was the electrochemical signal obtained by the HRP reduction of hydrogen peroxide. The construction of immunomagnetic beads was through activation of carboxylic group linked magnetic beads by EDC/NHS chemistry for the immobilization of specific antibody. The IMBs were then directly incubated into the sample for target capture along with the complementary DNA sequence for methylation sequence hybridization for 30 min. After target capture, the target-IMBs were incubated with HRP solution in order to further generation of electrochemical signal for 15 min. The amount of HRP linked onto the IMBs was proportional to the amount of methylated DNA capture. Conventional HRP/H_2O_2/hydroquinone electrochemical method was applied for signal generation. H_2O_2 was reduced by IMB linked HRP and the reduced form of HRP was regenerated by hydroquinone (acted as mediator for enhancing electron transfer). The mediator was oxidized during the HRP enzymatic reaction, therefore further electrochemical reduction of the mediator provided signal for the quantification of methylated-DNA captured. This electrochemical strategy was directly applied for analysis of 6-metyhlguanine-DNA methyl transferase (MGMT) gene in human serum, human cells and tumor tissues with a detection limit at 1.2 pM.

This robust electrochemical biosensing strategy was also demonstrated through high-sensitivity, rapid simultaneous detection of miRNAs directly from metastatic cancer cells and human tumor tissues with a pico-molar sensitivity.[29] Similar sandwich setup based on magnetic separation and enzyme labelling was demonstrated by the detection of exosomes, indicating the versatility of this strategy.[30] In addition, building on the same design logic involving the nucleic acid hybridization and amplification system, a comparison study demonstrated 11 different methodologies for nucleic acid detection, confirming the diversity and generality of this electrochemical sensing strategy for high-sensitivity nucleic acid detection.[31]

3.3 Combinatorial probes based clamping strategy

A major challenge for electrochemical detection of circulating nucleic acids is to differentiate small quantity of mutated sequence from highly expressed normal sequence in human blood in order to prevent the matrix effect.[32] A combinatorial probes based strategy for high-throughput electrochemical biosensing strategy for circulating tumor nucleic acids is proved to be an effective mean by directly quantifying mutation-bearing nucleic acid sequences from human serum within 30 min (Figure 1C).[33] The sensing element was a nanostructured gold based micro-electrode.[34] The recognition element was consisted by two steps. First step aimed at removing the irrelevant wild-type nuclei c acids through complementary peptide nucleic acid probes directly in the sample.[35] The second recognition step was the application of immobilized complementary peptide nucleic acid sequence specifically to the target of interest. The transduction element was based on the electrochemical reduction reaction of $[Ru(NH_3)_6]^{3+}$, which was electrostatically interact with the phosphate backbone of nucleic acid.[36] This electrochemical biosensing strategy was demonstrated by the detection of epidermal growth factor receptor (EPFR). Various combinatorial probes were designed according to the coding region of the EPFR gene for target capture. To mediate the interference from wild-type EGFR, clamp probes, which were used to treat the sample first, were applied to associate with the wild-type EFGR before introduction the sample onto the electrode, preventing the false-positive possibility upon combinatorial probe capture.[37] By application a high-throughput sensor strategy, combinatorial probes, targeting different mutations point, were applied onto the sensing system, delivering a multiplexed detection and evaluation of a broad range of sequence alterations of EPFR from human sample.

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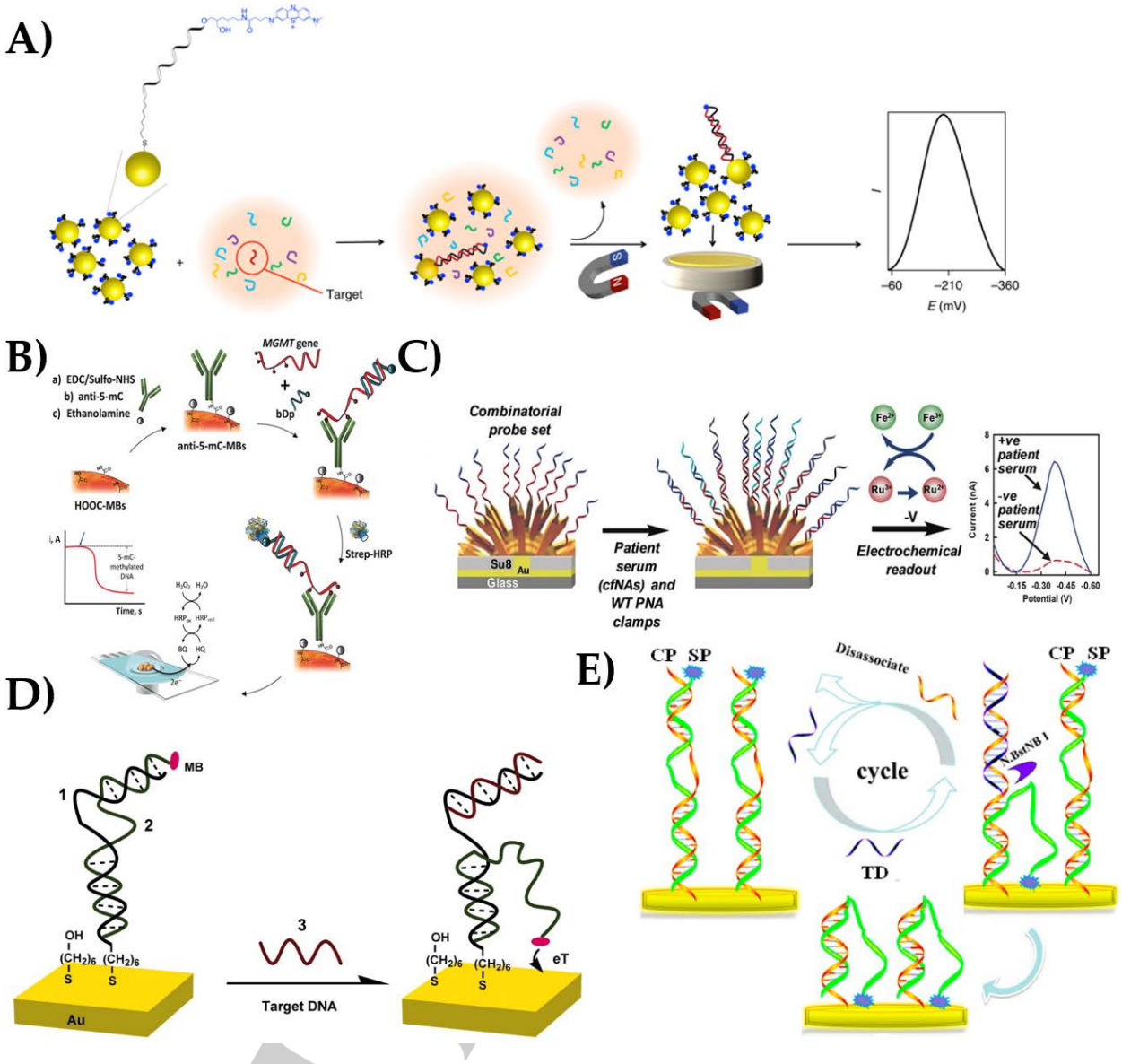

Figure 1. A) DNA probe (-methylene blue)-Au@MNPs were mixed with sample for target capture; Au@MNPs were separated by magnetic onto the gold electrode, going through electrical reconfiguration; square-wave voltammetry was applied to transduce the electrical signal for quantification. (Adapted from [24]. Copyright 2018 Nature Publishing Group.) **B)** IMBs were functioned with target specific antibody; secondary target complementary probe (with signal generator) was applied for further capture; electrochemical signal was acquired through H_2O_2/HRP/HQ system (Adapted from [28]. Copyright 2018 John Wiley and Sons.) **C)** Combinatorial probe set was applied on nanostructured gold-electrode for target capture after the treatment of samples with nucleic acid clamps for non-target genes; electrochemical signal was generated by the reaction of $[Ru(NH_3)_6]^{2+/3+}$ through corresponding interaction with nucleic acid backbone (Adapted from [33]. Copyright 2018 John Wiley and Sons). **D)** Target hybridization induced conformational change of methylene blue linked signaling DNA probe, leading to a shorter electron transfer distance (higher electrochemical reaction kinetics) (Adapted from [38]. Copyright 2006 National Academy of Sciences). **E)** Target hybridization induced strand displacement assay with the application of nicking endonuclease for non-enzymatic cycling signal amplification (Adapted from [41]. Copyright 2014 American Chemical Society).

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3.4 Binding induced biomolecular switches

Another critical challenge limited electrochemical biosensors from practical application is that the binding event of target biomolecule cannot directly generate a specific measurable transduction signal. In order to generate a specific measurable signal from target binding achieving rapid detection, binding induced biomolecular switches were developed for electrochemical biosensing (Figure 1D).[38] The sensing element was a gold electrode based system. The recognition element was target complementary DNA probe (1), which would be displaced the presence of target. The transduction element was based on the methylene blue tag on a signaling DNA probe (2), which was complementary to the probe (1). With the presence of target, DNA probe (2) was displaced from the double helix formation, partially liberating the methylene blue tag collide to the electrode. Therefore, with a shorter electron transfer distance, the electrochemical reaction signal of methylene blue upon target hybridization was higher than the non-target condition. This electrochemical biosensing strategy achieved a single-step detection of DNA target at a subpico molar concentration level. The utilization of target induced conformational change of probe biomolecules (aptamers) was also demonstrated recently for detection of protein and small molecules from complex matrix.[39]

Built on the concept of binding induced biomolecular switches, nucleic acid strand displacement was widely investigated as molecular motor because the strand displacement process is a non-enzymatic and kinetically controlled amplification reaction cascade.[40] The application of strand displacement on biosensor design not only delivered the direct induction of measurable signal upon target binding but also provided multiple non-enzymatic signal amplification cycles with the presence of target, enhancing the detection sensitivity. An electrochemical detection strategy combined target induced strand displacement with endonuclease nicking was developed and demonstrated for the detection of Bacillus subtilis related nucleic acid strand (Figure 1E).[41] The sensing element was a gold electrode system. The recognition element was based on target induced strand displacement reaction and further endonuclease nicking resulted cyclic amplification. The transduction element was based on the electrochemical reaction of ferrocene linked on SP. CP and SP (partial complementary sequences) were first constructed on the electrode surface. With the presence of target, the non-complementary part of CP/SP was initiated the displacement, leading to the formation of CP/target double helix and the release of ferrocene linked SP closer to the electrode. After the displacement, in order to further amplify the detection signal, the nicking endonuclease was applied to recognize and cleave the specific restriction site, resulting the release of target from the CP. The released target would hybridize with other available non-displaced CP/SP, delivering the cyclic amplification through releasing more SP strands onto the electrode surface (higher electrochemical reaction signal of ferrocene). A sub-femto molar detection of target nucleic acid directly in whole blood was achieved through this strategy.

The presented nucleic acid detection strategies demonstrated the recently developed robust biosensing methods, establishing some critical aspects for the construction of point-of-care systems. To achieve non-fouling detection based on the in-situ buildup of affinity biosensor, the application of magnetic beads for target separation was a general strategy to prevent non-specific detection signal. Also, to prevent the non-specific capturing of non-relevant nucleic acid, nucleic acid clamp was applied to decrease the interference from those nucleic acids with single-mutation difference with the target sequences. For the enhancement of detection sensitivity, secondary recognition agent with enzymatic tag was applied for signal generation. In addition, target induced strand displacement created a signal-on platform, which provided non-enzymatic amplification of detection signal through strand displacement cycles. These biosensing strategies for nucleic acid detection can be diversely applied for the detection of any other clinical relevant nucleic acid. Moreover, some innovative combinations of the described sensing, recognition, and transduction elements may bring in new aspects for future nucleic acid detection systems.

4. Detection of protein

Protein biomarker, such as autoantibody,[42] circulating antigen,[43] is an effective evaluation standard for various cancer severity and drug treatment.[2a, 44] Comparing with conventional methods on protein detection such as ELISA, Western Blot, electrochemical strategies stand out for extensive research endeavors, owing to its ease-of-use and cost-effectiveness.[13d, 45] However, suffering from complicated biosensor fabrication process, limited-sensitivity, and low performance in complex matrix, limited types of electrochemical biosensor were applied for practical clinical usage. Recent advances in electrochemical protein biosensing strategies delivered simple, high-sensitivity and non-fouling platforms, moving the industry toward clinical applications.

4.1 Steric hindrance effect strategy

Matrix effect for on-chip protein detection is significant, due to the nature metal affinity with some of the plasma compositions, such as albumin,[46] , fibrinogen.[47] Therefore, one aspect of an effective biosensing strategy must be preventing the plasma protein from transporting onto the electrode surface. Recently, a one-step DNA-based electrochemical sensor utilized the steric hindrance effect provided by the protein target, delivering a multiplexed protein detection strategy in human blood with a sensitivity at nano-molar sensitivity (Figure 2A).[48] The sensing element in this design was a three electrode system with gold as working electrode, on which the recognition element was immobilized through the gold-thiol chemistry. The recognition element contained two parts. The first part was a target recognition element linked with a DNA strand on which a signaling redox label was attached on the other end. The second part was a surface immobilized capturing DNA strand complementary to the sequence of the signaling DNA strand. The transduction element was the electrochemical redox signal from methylene blue (the signaling redox label). The first part of the recognition element was first mixed with sample (containing target). The target was then captured by the small recognition element on the signaling DNA and the signaling DNA strand was bonded with the surface immobilized capturing DNA. Therefore, with target protein linked on the recognition element, the diffusion of other copies of the signaling strand would be impeded by the steric hindrance effect created by the target protein, leading to fewer redox label attached on the gold surface and thus generating lower electrochemical redox current. This detection system was applied into a multiplex task for the detection of anti-Dig and anti-DNP targets in whole blood samples. Owing to the steric hindrance effect on preventing biomolecules diffusion onto the electrode surface, the complex matrix presented a very minor signal loss comparing with the detection performance in buffer solution.

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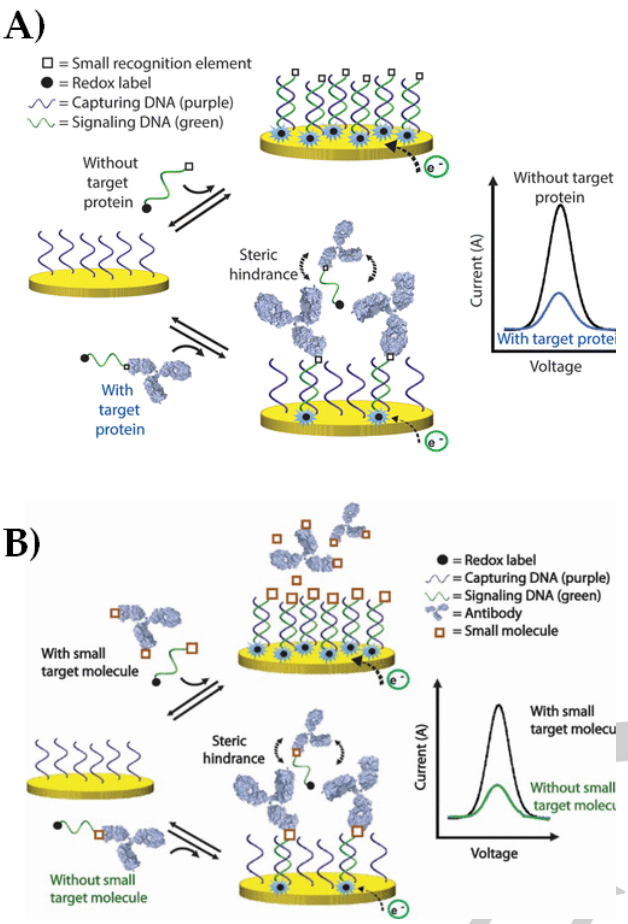

Figure 2. A) Capturing DNA was immobilized on the sensor surface to interact with either target containing signaling probe (methylene blue linked) or non-target signaling probe; electrochemical signal was acquired from different concentrations of methylene blue on the surface, which was influenced by the steric hindrance effect caused by the target captured on the surface (Adapted from [48]. Copyright 2015 American Chemical Society). B) After the immobilization of captured DNA, signaling DNA was mixed with the target-specific antibody for small molecule capture; once the target was captured by antibody, the antibody would no longer able to bond onto the surface (no steric hindrance effect leading to an increase in electrochemical signal from methylene blue) (Adapted from [49]. Copyright 2017 American Chemical Society).

Built on the steric hindrance effect, another electrochemical biosensing strategy further incorporated competitive binding into the design for the detection of small molecules based on antibody captured (Figure 2B).[49] Same sensing element and transduction element were applied as the previous design.[48] Comparing with the previous design, the recognition element in this design added one more compound, the target corresponded antibody. With the presence of the target small molecule, the antibody recognized the target and formed a complex. Therefore, less antibody would form conjugate with signaling DNA due to competitive binding, delivering the hybridization of signaling DNA with its complementary strand on the gold surface, further leading to a high electrochemical current outputs. With the absence of the target small molecule, the antibody binding sites were occupied by the signaling DNA and the further hybridization of antibody-signaling DNA with surface complementary DNA strand created steric hindrance, impeding further signaling DNA from hybridization onto the gold surface, therefore creating a low electrochemical signal. This strategy achieved a pico-molar detection of small molecule in human blood within 20 min. Owing to that the recognition strategy of this design was antibody-based, it can also be applied as a general platform for the detection of protein.

4.2 Electrostatic charge based strategy

A conventional method for achieve biomolecular detection from complex matrix is sample dilution, therefore leading to less interference substances.[50] However, upon dilution, the concentration of target of interests is also reduced. Therefore, the development of a high-sensitivity electrochemical biosensing strategy is desirable. Biosensors that aim at quantification of biomolecules through the change of electrostatic charge of the sensor surface have been demonstrated for many high-sensitivity applications.[51] However, these charge based characterization methods typically required external charged nanoparticles to enhance the electrostatic interaction and the electrochemical signal, increasing the complexity of the design of the sensing system.[52] Recently, direct utilization of analyte charge character was adopted for development of electrochemical detection strategies. A neutralizer displacement assay was demonstrated for detection of protein analyte, thrombin at sub-pico molar level (Figure 3A).[53] The sensing element was a developed nano-structured gold electrode.[34] The recognition element was a thiolated thrombin aptamer, known to fold into a G-quadrtet structure upon binding. The transduction element was the conventional $[Ru(NH_3)_6]^{2+/3+}$ based redox system. The special property of this design was the addition of positively charged neutralizer (lysine residues contained peptide), which was applied for reducing the charge effect of the aptamer on electrochemical detection of target (eliminating the background signal). When the target protein was introduced into the system, the structural change of the aptamer was induced upon the target binding activity. Consequently, the release of neutralizer (caused by the displacement) changed the detection surface charge to negative, leading to a high electrochemical reduction current of $[Ru(NH_3)_6]^{3+}$ based on the electrostatic interaction of the positively charged probe with the negatively charged surface biomolecules.

A recent universal electrochemical biosensing strategy divided protein analytes (based on their amino acids sequence) into two categories, positively and negatively charged targets. A neutral charged biomolecules surface was constructed for electrochemical detection of Tau protein and HE4 protein with a sub-pico molar sensitivity (Figure 3B).[54] The sensing element was a single-use gold sensor prototype.[55] The recognition element was target specific antibody. The transduction element was $[Ru(NH_3)_6]^{2+/3+}$ or $[Fe(CN)_6]^{3-/4-}$ based redox system. Unlike conventional use of redox system as surface impedance indicator,[56] this biosensing strategy applied redox system for electrostatically interacting with correspondingly charged protein and further quantification by the electrochemical redox reaction based on the kinetic difference. After incubation of thiol modified antibody onto the gold surface,[55] in order to provide an electrostatic interaction

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environment only for the target, two strategies were applied in sequence to maximize the electrostatic interaction. First, based on the charge of the target, the charge of the immunoglobulin G antibody was neutralized by immobilization of thiol modified poly-arginine or poly-glutamic acid onto the gold surface, providing a neutral charged environment for target sensing. Second, the pH condition of the testing environment was altered to trigger the charged condition of the target. Therefore, experimentally, after incubation of target, the corresponding redox system was electrostatically attracted onto the target, delivering a short electron transfer distance and high redox activity. Therefore, the concentration of the target was positively correlated with the electrochemical current outputs based on the redox system.

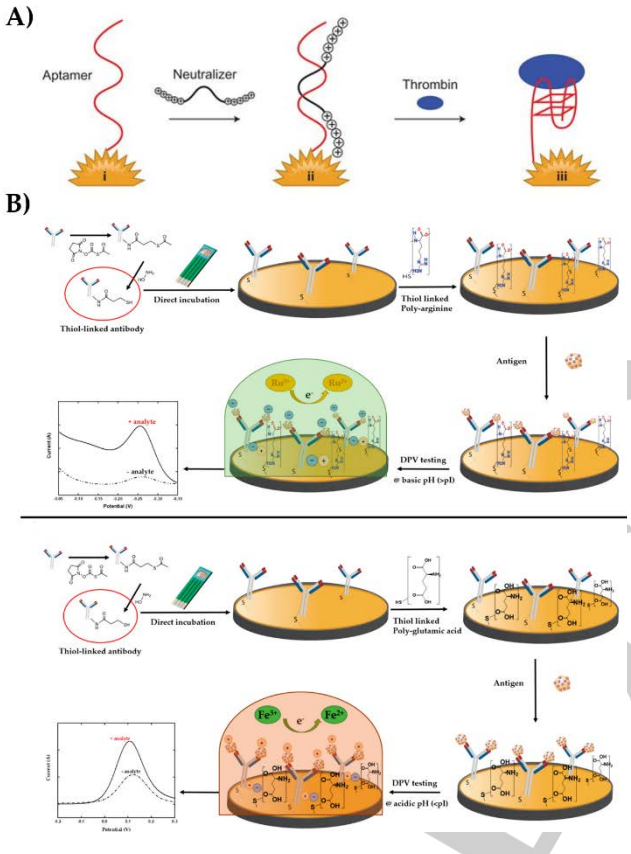

Figure 3. A) Aptamer was immobilized onto a nanostructured gold electrode along with a charge neutralizer; upon target binding, displacement happened and led to the change of electrostatic interaction (Adapted from [53]. Copyright 2012 Nature Publishing Group). **B)** After antibody was immobilized onto the electrode**,** the thiol-linked charged amino acids were tethered onto the electrode to form neutral charged surface; upon introduction of the target, correspondingly charged redox probe was applied to the surface to interact with analyte and caused the increase of electrochemical signal owing to the decrease of electron diffusion distance (Adapted from [54]. Copyright 2019 American Chemical Society).

4.3 Electrochemistry directed formation of bio-surface for controlled biosensing and the application of DNA charge transport

The formation of biomolecular layer is an essential step for the development of electrochemical biosensing.[45, 55, 57] Controlled and regulated formation of biomolecules monolayer is critical and beneficial for electrochemistry guided electron transfer and understanding of surface biomolecular reaction, therefore leading to an enhanced, reproducible highly-sensitive performance for electrochemical biosensing. An electrochemical approach for guided patterning DNA array on a gold-sensor surface was developed (Figure 4A).[58] This controlled formation of the DNA monolayer bio-electrode was then further used to detect human methyltransferase,[59] an enzyme causing DNA-methylation (further leading to cancer progression).[25a, 26, 60] To achieve controlled grafting of DNA monolayer on gold electrode, a two-electrode system, a patterning electrode and a substrate electrode (up and down formation), was applied (Figure x). First, a mixed self-assembled monolayer (containing 12-azidododecane-1-thiol and 11-mercapto-undecylphosphoric acid) were first formed on the bottom gold electrode. A mixed solution of [Cu-(phendione)$_2$] [SO_4] and an alkyne-labeled DNA sequence were placed inside the two electrode system. The electrochemical activation reaction of Cu(II) to Cu(I) facilitated the azide−alkyne cycloaddition between alkyne-modified DNA dulexes and an azide-terminated thiol, achieving controlled formation of DNA monolayer. Through controlling the position of the patterning electrode and repeating the same process with different DNA sequences, a multiplexed DNA monolayer surface with controlled distance was formed on the substrate electrode.

The electrochemically guided DNA monolayer formation strategy was applied to produce a low-density DNA monolayer, which was used to detect methyltransferase from tumors through electrochemical evaluation of DNA charge transport (Figure 4B).[59] The sensing element in this design was a two electrode cell constructed by 15 substrate electrodes and 15 patterning/detection electrodes as a high-throughput detection array. The recognition element was hemimethylated DNA, which can be specifically methylated by the target. The transduction element was the electrochemical signal from methylene blue along with ferricyanide. As shown in Figure x2, with the target presented in the sample (blue line), the surface immobilized hemimethylated DNA was transformed to a methylated duplex. Methylation-specific restriction enzyme, BssHII (purple), which specifically cuts the hemimethylated DNA,[61] was then added onto the electrode. Therefore, with the target treated DNA, the restriction enzyme could not perform the function, therefore leaving a full duplex DNA strand for the electron transfer upon the generation of methylene blue signal.[62] When the target was not presented (red line), the surface immobilized hemimethylated DNA was cut into fragments by the addition of restriction enzyme, leading to a disconnection of electron transfer between methylene blue and the electrode surface. This strategy demonstrated a detection level at nano-molar level directly in human biological sample.

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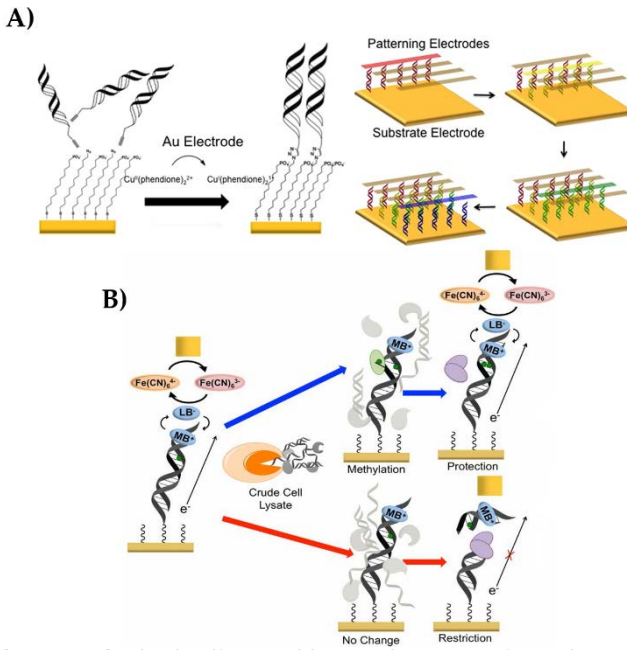

Figure 4. A) Mixed self-assemble monolayers was formed and alkyne labeled DNA was then introduced for immobilization; electrochemical activation reaction of Cu(II) to Cu(I) facilitated the azide−alkyne cycloaddition, achieving controlled formation of DNA monolayer based on the two electrode system (Adapted from [58]. Copyright 2013 American Chemical Society). **B)** With a constructed hemimethylated DNA surface, methyltransferase was incubated to interact with the captured DNA; BssHII was then applied for DNA cutting; based on the existence of methyltransferase, the amount of electrochemical signal generated by methylene blue would be different caused by the change of electron transfer ability of the DNA (Adapted from [59]. Copyright 2014 National Academy of Sciences).

The application of protein detection for point-of-care system is very promising because of the following reasons. First, protein type biomarkers are the most discovered and well-understood clinical relevant markers. Second, nature provides high-affinity antibody-antigen pair that can be directly utilized as a highly-specific recognition element for protein detection assay design. Therefore, a general applicable antibody based protein detection strategy can be utilized for different biomarkers, saving the amount of research endeavors for specific design in order to detect some other protein markers. Also, the size and abundance of protein in biological fluids are relatively larger and higher than those of nucleic acid markers, therefore relatively easier to reach the required detection sensitivity. As demonstrated above, surface chemistry is critical for increasing detection sensitivity and reducing matrix effect. The construction of target triggered steric hindrance biomolecular interaction interface led to non-fouling surface through limiting the access of other biomolecules onto the electrode surface. Also, the electrochemistry directed immobilization of biomolecules onto electrode surface was applied to control the surface density of the tethered biomolecular layer. The ideal surface density condition can prevent the penetration of non-specific biomolecules into the electrode surface, also delivering the non-fouling detection interface. For high-sensitivity detection, the use of selected transduction probe was demonstrated to take the electrostatic charge of the analyte directly into account for quantification. All the presented electrochemical strategies embrace a simple biosensor construction property. Through the selection and combination of these sound techniques, the design of the point-of-care system has the versatility and freedom to achieve the detection of different types of protein biomarkers.

5. Detection of cell

Cell detection is vital for the development of liquid biopsy for the analysis of human blood on monitoring tumor progression and bacterial infection.[44b, 63] The standard cell detection involves the use of cell viability assay, which is time-consuming and limited on detection sensitivity, therefore not ideal for point-of-care development.[64] Recent progresses of electrochemical strategies on cell detection demonstrated efficient and accurate biosensing systems on detection of cells directly from human blood.

5.1 Multiplexed recognitions of cell based on sensing array and individual metal nanoparticle

Recent progress on cancer pathogenesis pointed out the importance of cancer cell transportation during metastasis.[2d] Therefore, quantification of circulating cancer cells can be pivotal for metastatic process control.[63c, 65] One of the challenges for cell detection is the accuracy on cell recognition, because the diversity of protruded protein composition on the cell membrane augments the recognition difficulty. Moreover, the presence of non-target cells in the sample impedes the interaction of recognition element with the target cell. Therefore, electrochemical detection of circulating cancer cells in human blood with high-specificity and high-sensitivity is a critical challenge for non-invasive point-of-care cell detection development.[66]

Recently, a highly-specific electrochemical analysis of cancer cells based on multiplexed marker analysis was developed for circulating cancer cell analysis in human blood with a profiling limit of two cells/sensor (Figure 5).[67] The sensing element was a gold-nanoflakes based sensor array with eleven sensing cells (Figure 5A). The recognition element contains anti-epithelia cell adhesion marker (EPCM) immobilized on the gold electrode for cell capture from bloodstream and metal nanoparticles (MNPs) linked specific antibody or aptamer for recognition of cell surface marker, enhancing the detection specificity. The transduction element was based on electrochemical oxidation signal of MNPs (Figure 5B). The various types of MNPs provided different electrochemical signal at different potentials, delivering a multiplexed identification of cell surface marker (Figure 5C). Cell suspension was directly incubated onto the gold electrode to allow the target cell recognition and binding activity by Anti-EPCAM for 1 hr. The MNPs linked with different target surface markers' recognition elements were then incubated for the recognition of surface marker and further electrochemical signal transduction. This strategy was showcased for the detection of VCaP cells for prostate cancer with the presence of blood cells. By utilizing different recognition elements, this universal strategy can be used to identify other specific cells. Most importantly, the sensitivity level of this approach was clinical relevant with a detection limit of 2 cells/mL.[68]

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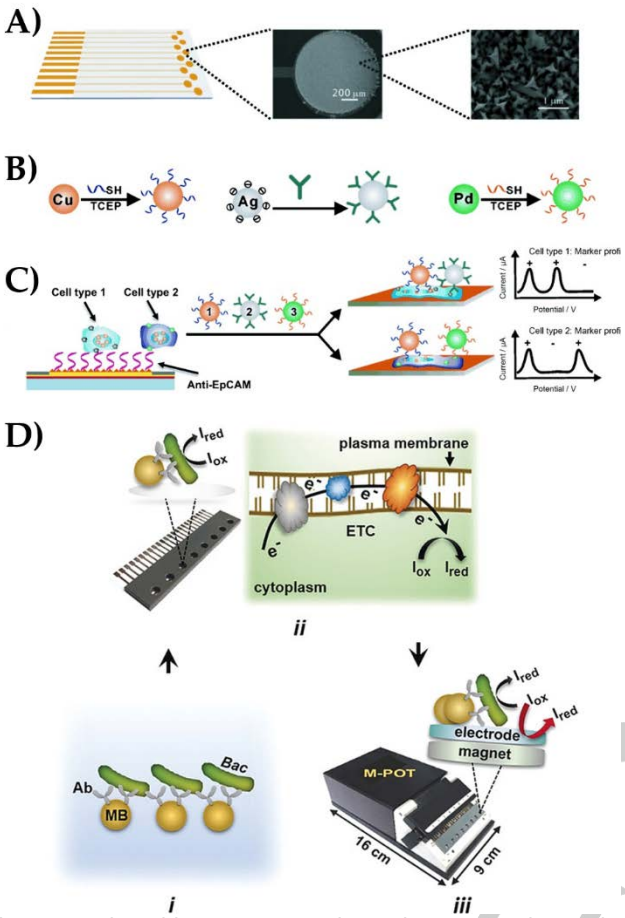

Figure 5. A) Gold nanostructured sensing array. B) Metal nanoparticles linked recognition elements functioned as signal generator. C) Target cell captured by Anti-EpCAM formed surface and the metal nanoparticles were then introduced; the signal generation was based on whether the metal nanoparticles would recognize its corresponding target (Adapted from [67]. Copyright 2014 John Wiley and Sons). D) Magnetic beads with linked antibody were used to capture bacterial cell; the magnetic beads were separated and moved onto the carbon nanotube sensor array; metabolic activity indicator was used to interact with cells and also functioned as electrochemical signal generator (Adapted from [69]. Copyright 2018 John Wiley and Sons).

5.2 Superparamagnetic bead and carbon-nanotube based micro-chip array

Rapid infection diagnosis is beneficial for global health from reduction of risks on infection recurrence and patient mortality. Thus, it is crucial to monitor bacterial cell concentration in bloodstream directly with a rapid and simple biosensing system. Very recently, an immune-affinity enrichment strategy using magnetic beads with carbon-nanotube based electrochemical sensor array was implemented as a point-of-care system for bacterial cell detection (Figure 5D).[69] The sensing element consisted of a superparamagnetic bead (MB) (for the immobilization of recognition element) and a carbon-nanotube based microchip array (for the collection of electrochemical signal). The recognition element was cell-specific antibody built on the MB through recombinant protein A/G coating on the bead surface. The transduction mechanism was based on the reaction between the target cell and the specific metabolic activity indicator, which was further used to generate electrochemical reduction signal. The antibody coated MB was first immersed in biological sample for apprehension of target cell for 30 min. The target-MB complexes were then separated by magnet and placed onto the electrochemical sensor array for reaction with specific metabolic activity indicator, which was chemically reduced by the metabolically active bacteria. Amperometric read-out was then directly acquired from the reaction mixture through a multiplexed potentiostat. The decrease of reduction current based on the metabolic activity indicator was utilized for quantification of cell. This electrochemical strategy could sense E. coli and B. subtilis at a concentration of 5 cell/mL within 30 min, four times more efficient than standard cell viability assay.

The development of electrochemical biosensing strategy for cell detection can be revolutionized for clinical cancer evaluations. However, one of the greatest challenge for cell detection is the specificity of detection. The recognition method for cell detection mainly relied on specific affinity element (such as antibody) to capture the surface protein on a specific type of cell. The interference from other surface protein can cause steric hindered low-efficient hybridization and non-specific capturing. To increase the detection sensitivity, nano-structured electrode was demonstrated to increase the capture efficiency. The use of multiplexed profiling of cell surface protein increased the target specificity. Also, the electrochemical evaluation of specific metabolic activity indicators upon cell capture was applied to increase the specificity of cell detection. Generally, for future cell detection, the design of high-specificity electrochemical strategies will be beneficial to the ultimate goal for point-of-care system.

Besides those electrochemical strategies for the detection of above described three classes of biomolecules, the electrochemical detections of small molecules and ions are also critical to point-of-care diagnosis, such as non-invasive, non-fouling continuous monitoring of glucose, lactates, hormones and metal ions from biological fluids (such as sweat, tears and saliva).[16a, 70] Extensive researches for the development of these electrochemical biosensors based on wearable devices for healthcare monitoring can be found in previous reviews.[16a, 16c, 71] As for the affinity detection of small molecules, such as hormones, same strategy design logic as antibody based protein detection can be applied directly, because many protein type receptors are commercially available for small molecule capture.

6. Summary and outlook

In conclusion, electrochemical biosensors have been widely developed for the detection of biomolecules ranging from small nucleic acid fragments to large human cells. With progressing understandings by the research community on the outcomes of electrochemical biosensing system, instead of focusing on the detection of a specific analyte, the majority of the cutting-edge researches concentrates on the advancement of general detection strategies aiming at detection of a single category of the analytes. These progresses in biosensing strategies have robustly promoted the capability of electrochemical biosensor

on quantitative analysis of target in real sample, leading to more emerging healthcare industries toward clinical point-of-care systems. In this review, we present very recent reports on the developments of electrochemical biosensing strategies based on the category of the analyte, including nucleic acid, protein and cell. Configurations of sensing element, recognition element, and transduction element were described for each biosensing strategy, providing in detail descriptions of the experimental application and reasoning of the specific design. These demonstrated strategies resolved critical challenges in electrochemical biosensing research, such as rapid readout, simple biosensor fabrication method, ultra-high detection sensitivity, direct analysis in complex biological matrix and multiplexed targets analysis.

For future biosensing development, one of the ultimate goals for point-of-care system is to conduct multiplexed and simultaneous sensing of various biomarkers on a single sensor, delivering a time-efficient, cost-effective and comprehensive analysis of biomarkers. Based on the significant progress of biosensing strategy design in recent years, multiple targets can be captured at once through different recognition elements functionalized sensing elements. To achieve multiplexed electrochemical signaling simultaneously, multiple electrochemical signaling probes with different activation potentials are necessary to indicate different targets concentrations. However, for now, there are limited types of electrochemical probe systems with distinguishable activation potential. In future, electrochemists can focus on identifying more electrochemical redox species with separating potentials in order to achieve the ultimate multiplexed point-of-care system. Also, in order to integrate the electrochemical biosensor into real world medical market like the success of glucose sensor, researches in simple methods for maintaining the activity of recognition element are needed. As we noticed from the reviewed electrochemical biosensing strategies, most of the designs aim at utilizing the properties of the biomolecule to generate a mean of detection. Building upon the fundamental aspect of biomolecular interactions, biochemical reaction and electrochemical reaction, the design of electrochemical biosensing strategies becomes more and more precise and effective on biomolecular detection. The combinations of these strategies can lead to a universal point-of-care system that is able to detect various categories of analytes simultaneously from one human sample. Significant amount of efforts had been absorbed to the development of sensing and transduction elements in the last decade. In near future, with improving systematic comprehending of human immunology from biochemistry aspect, scientists will be able to engineer and imitate specific immunologic reactions inside human and apply those onto the development of electrochemical biosensing strategy. We believe that significant research developments based on mimic and manipulation of human immune system will lead the biosensor industry to another huge transformation, benefiting the world-wide healthcare industry with an ultimate universal point-of-care system.

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Conflicts of interest

The authors declare no conflict of interest.

Keywords: analytical electrochemistry • biosensing strategy • universal biosensor • point-of-care

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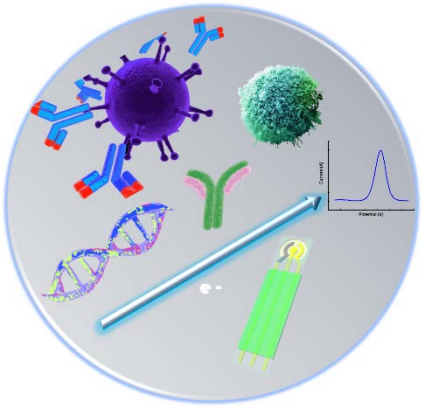

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