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Electrochemical Methods in Clinical Immunoassays and Nucleic Acid Analyses

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Abstract: The applications of electrochemical methods in immunoassays and nucleic acid analyses are discussed. Instead of a comprehensive literature review, this paper focuses on the techniques that have already been commercialized and used in clinical diagnoses. Learning from the success of these methods will help us to further understand the strengths and limitations of electroanalytical technology. A personal perspective of the research and development in these fields has been presented.

Key words: electrochemical method; immunoassay; nucleic acid analysis; clinical diagnostics
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Electrochemical methods have been widely applied to the studies of chemical problems. Their important practical applications in chemical analyses include, among others, ionic selective electrodes, electrochemical gas sensors, and electrochemical glucose sensors. Various strategies have also been proposed to employ electrochemical methods in immunoassays and nucleic acid analysis. Quite a few literature reviews have appeared and provided up-to-date overview of these studies[1-2]. Here, the discussion mainly focuses on the electrochemical techniques that have already been commercialized and used in clinical immunoassays and molecular diagnostics. The accuracy and reproducibility of these methods have generally been evaluated strictly to justify commercialization, and thus, the discussion on their strengths and limitations should be more meaningful.

Immunoassays and nucleic acid analyses play important roles in in-vitro diagnostics (IVD). The immunoassay is a technique employed to detect or quantify a target substance, usually a specific protein, in a blood or body fluid sample using an immunological reaction. Clinical immunoassays include the tests for pregnancy, infectious diseases, cardiovascular diseases, cancer biomarkers, etc. Different from the protein-based immunoassays, molecular diagnostic tests are based on the analysis of DNA or RNA by detecting their specific sequences, variations, and quantities. The clinical implementation of nucleic acid analysis is growing rapidly for diagnosis, disease screening and monitoring, as well as pharmacogenomics/precision medicine.

A variety of analytical methods have been used in immunoassays and nucleic acid analyses, including fluorescent, chemiluminescent, colorimetric, chromatographic, mass spectroscopic, electrochemical methods, etc. The performance of an analytical technique is typically evaluated in terms of sensitivity and specificity. "Analytical sensitivity" represents the smallest amount of target analyte detectable in a sample, while "analytical specificity" refers to the ability to detect the particular target in the presence of other substances. There are two ways to enhance the assay sensitivity, i.e., signal amplification and target amplification. Assay specificity mainly relies on the properties of probes and target amplification primers.

In the immunoassay, the quality of the probe (i.e., antibody) is crucial for a highly specific detection. As protein targets are difficult to amplify, signal amplification is necessary to achieve high sensitivity. The

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studies of analytical chemistry in this field concentrate on the development of various labeling techniques to amplify the detection signals, and thus, improve the assay sensitivity.

The targets in nucleic acid analysis are DNA or RNA molecules that can be efficiently amplified by using polymerase chain reaction (PCR) or reverse-transcription (RT) PCR. These target amplification methods can be extremely sensitive, and even a single copy of DNA or RNA could be detected. The design and optimization of amplification primers and probes determine the assay specificity. The introduction of some special secondary structures (such as hairpin shaped molecular beacon) or the modification of the oligonucleotide primers and probes (such as locked nucleic acid) can be used to improve the detection specificity.

1 Electrochemical Methods in Immunoassays

Commonly used methods in clinical immunoassays include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, immunochromatography, etc. Since the 1990s, an electrochemical method, i.e., the electrogenerated chemiluminescence (ECL), has been widely adopted in clinical diagnostics. The excellent performance of the ECL technology has made it a mainstream immunoassay method and set the standard in immunochemistry[3-8].

1.1 History of ECL Technology

ECL is a process to generate luminescence during electrochemical reactions in a solution. Detailed ECL researches date back to the mid-1960s. By alternate pulsing of an electrode potential, the electroactive species A and D (they could be the same molecule) in the solution will accept or loss electrons to form their free radical ions. The electron-transfer reaction between the radical ions near the electrode surface is very energetic (typically 2 ~ 4 eV) and leads to the formation of excited states. The energy is released as light when the excited states return to the ground states. This process is typically called annihilation ECL route. Some polycyclic aromatic hydrocarbons (such as rubrene and 9,10-Diphenylanthracene) and metal complexes (such as $Ru(bpy)_3^{2+}$) can efficiently produce ECL via the annihilation route.

$$A + e \rightarrow A^{\bullet -} \text{ (reduction)} \quad (1)$$

$$D - e \rightarrow D^{\bullet +} \text{ (oxidation)} \quad (2)$$

$$A^{\bullet -} + D^{\bullet +} \rightarrow A^* + D \text{ or } A + D^* \text{ (excited-state formation)} \quad (3)$$

$$A^* \rightarrow A + h\gamma \text{ or } D^* \rightarrow D + h\gamma \text{ (light emission)} \quad (4)$$

The ECL involves very exergonic reactions and serves as a test for theories of electron transfer. Basically, in the homogeneous electron transfer reaction (eq 3), the intersection of the potential energy surface of the reactants with that of the electronic ground-state products could generate a larger energetic barrier than the intersection with respect to one producing an excited state; therefore, the formation of the ground-state products becomes less favorable as compared with the formation of the excited states. In other words, the reaction rate decreases as the driving force (free energy change) increases. The existence of efficient ECL processes is a good indication of inverted region behaviour suggested by the Marcus theory of electron transfer[9]. The study on ECL at the liquid/liquid interface further provided experimental evidences to demonstrate the heterogeneous inverted region electron transfer[10].

The ECL technology can be used not only to study the properties of radical species and electron-transfer reactions, but also to work for quantitative chemical analyses. In the early studies, most ECL experiments were conducted in aprotic solutions, such as acetonitrile, DMF and THF, in order to obtain relatively stable radical ions. Sometimes, purification and deaeration of the solutions were also necessary. These stringent requirements have greatly limited the application of ECL technology in chemical analysis.

In the early eighties of last century, a new type of highly efficient ECL reaction, coreactant ECL, was discovered in aqueous solutions, allowing the wide applications of the ECL method in analysis. The most commonly used coreactant ECL system is $Ru(bpy)_3^{2+}$/tripropylamine. The reaction mechanism is complicated and can be simplified as follows:

$$Ru(bpy)_3^{2+} - e \rightarrow Ru(bpy)_3^{3+} \quad (5)$$

$$Pr_3N - e \rightarrow [Pr_3N^{\bullet+}] \rightarrow Pr_2NC^{\bullet}HEt + H^+ \quad (6)$$

$$Ru(bpy)_3^{3+} + Pr_2NC^{\bullet}HEt \rightarrow Ru(bpy)_3^{2+*} + \text{products} \quad (7)$$

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\gamma \quad (8)$$

where Pr = $CH_3CH_2CH_2^-$, Et = $CH_3CH_2^-$

The coreactant ECL method can be used to detect the coreactant species, such as amines and organic acids. More importantly, by labeling antibodies with the ECL tag, highly sensitive immunoassays can be achieved. The ECL immunoassay technology was initially developed by Igen International which was acquired by Roche Holding for $1.4 billion in 2003. After more than 20 years' continuous development and marketing, Roche ECL-based Elecsys® assays has become a new generation mainstream immunoassay technology, and has been widely adopted in clinical laboratories all over the world.

1.2 Features and Advantages of the ECL Immunoassay Technology

Figure 1 outlines the principle of a typical sandwich assay of an antigen. First, the antibody modified with a biotin and the $Ru(bpy)_3^{2+}$-labeled antibody are mixed with the sample of interest. If antigen is present, a sandwich-structured immunocomplex is formed. Streptavidin-modified magnetic beads are subsequently added to bind the immunocomplexes via the biotin/streptavidin interaction. The magnetic beads are then flushed in to an electrochemical cell and captured on the working electrode surface by applying a magnetic field. After washing, a solution containing tripropylamine is pumped into the cell. Upon application of a sweep or step to positive potentials, the coreactant and bead-bound $Ru(bpy)_3^{2+}$ are oxidized, and emission of light is detected with the photomultiplier. Light intensity is used to quantify the antigen in the sample. After the ECL measurement, the magnetic field is switched off and the beads are washed out of the cell. The electrode surface is regenerated by in situ electrochemical cleaning.

The unique features of the ECL immunoassay include at least the following:

1) The small molecule label, $Ru(bpy)_3^{2+}$, has little influence on the antigen/antibody interaction, and is highly stable with long shelf-life. 2) The immune reactions take place in a homogenous solution, en-

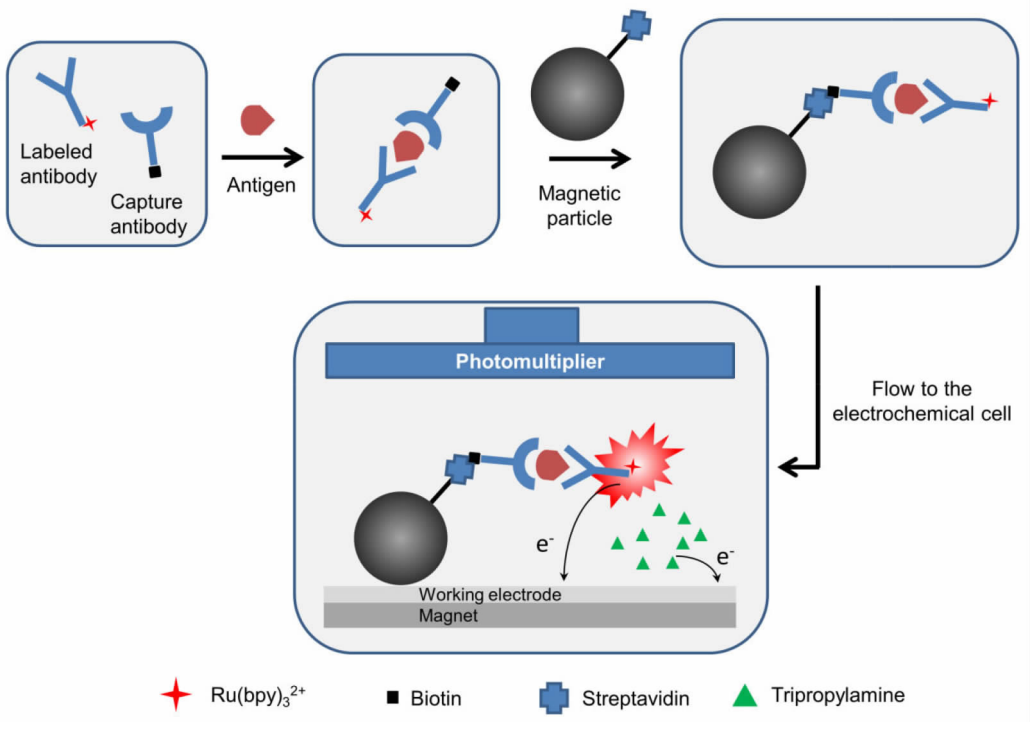

Fig. 1 Representation of ECL-based immunoassay (sandwich assay)

abling fast response time. For the majority of assays, the incubation time is only around 18 minutes. 3) The immunocomplexes are brought to the electrode by the magnetic beads, and washed away after the ECL measurement. No modification of the electrode surface is required, which makes the regeneration of the electrode surface simple and the results highly reproducible. 4) No light source is needed, avoiding background signals from light scattering and luminescent impurities. 5) The reaction between ECL label and coreactant is specific, avoiding the interference from possible side reactions. 6) The ECL reactions are spatially and temporally controllable, making it feasible to optimize the measurement and automate the workflow. This is the advantage of ECL over other chemiluminescent assays. 7) During the ECL reactions, the labels are regenerated, allowing each label molecule to participate in multiple ECL reaction cycles.

Beside the sandwich assay described in Figure 1, ECL immunoassay can also be run in a number of other formats, such as direct, competitive, and bridge assays.

The major advantages of ECL immunoassays are the rapid response time for faster reporting of patient results, the wide measuring range (linear response over six orders of magnitude) for fewer dilutions and repetitions, the superior precision and sensitivity for accurate and precise patient results, low sample volume (patient-friendly 6 ~ 50 μL per test), the long shelf-life, high on-board and lot-to-lot stability due to highly stable constituents.

1.3 Clinical Applications of ECL Immunoassays

The fully automated Roche Elecsys® platform offers a wide range of immunoassays for clinical diagnostics in the fields of infectious diseases, oncology, cardiovascular diseases, women's health, etc. Infectious disease assays include, among others, Elecsys® HBsAg II (for diagnosis or screening of hepatitis B), Elecsys® HIV combi PT (for the early detection of HIV infection), and Elecsys® Syphilis (for diagnosis or screening of Syphilis); examples of tests for oncology diagnostics are Elecsys® Tg II (for monitoring and early detection of tumor recurrence after thyroidectomy in patients with differentiated thyroid carcinoma), Elecsys® total PSA (for early detection and monitoring of prostate cancer), and Elecsys® ProGRP (tumor marker test for use with blood samples to support treatment of lung cancer patients). With these broad assay menus, the ECL technology has rapidly become a mainstream method and a replacement for other methods in clinical immunoassays.

2 Electrochemical Methods in Nucleic Acid Analysis

The extraordinary success story of the ECL immunoassay technology demonstrates that electrochemical methods could be very effective in amplifying the detection signals, and thus, achieve high analytical sensitivity. However, the clinical applications of electrochemical methods in nucleic acid-based molecular diagnostics are not as successful. Despite of the large number of literature reports on the nucleic acid analysis by using different electrochemical approaches[2], the commercialized platforms are rare and out of the mainstream.

Currently, the fluorescence-based real-time PCR is the dominant technology adopted for molecular diagnostic in clinical settings. As mentioned above, PCR is a very powerful method in molecular biology, enabling the production of millions of copies of specific nucleic acid sequences in a short duration. This extremely sensitive method allows the detection of a single copy of a DNA sequence. A major problem in the application of PCR is the carry-over contamination caused by the aerosolized PCR products, which may lead to false positive results. The contamination that was common and difficult to deal with became a hurdle for the clinical adoption of PCR-based diagnostics. However, nowadays there are a variety of ways to prevent the carryover contaminations and the reliability of PCR techniques has been significantly improved. In addition, the emergence of fully integrated and automated PCR detection instruments, such as Roche cobas® and Cepheid GeneXpert® systems, has greatly facilitated the implementation of PCR-based methods. For other strategies used to en-

hance the sensitivity of nucleic acid detection by signal amplification, including the ECL, it is extremely hard to compete with the highly sensitive and reliable PCR technology.

Actually, although the PCR technology can efficiently amplify the target molecule, the detection of the amplicons still needs to use a chemical analysis method. Fluorescence is most commonly used for real-time or end-point detection. Electrochemical technology can also be employed as a flexible and simple method for the analysis of the PCR products. In the electrochemical detection, expensive and fragile optical sensors are not required, which may make the instrument more robust and less costly, and avoid complex optical calibration procedures. There are a few successful examples that integrate PCR amplification and electrochemical detection, including Genmark DX eSensor XT-8 and ePlex platforms, Atlas Genetics io™ System, and Elice DetScan™ technology.

The eSensor XT-8 and ePlex platforms use oligonucleotide probes labeled with ferrocene molecules to generate electrochemical current signals. The target gene is first amplified by PCR and the PCR products are then treated with enzymes to obtain single-stranded DNA which allows the binding of the signal probe. After hybridization of the target and the signal probe, the solution is pumped through a microfluidic chamber to the electrode surface modified with the capture probe and the target/signal probe hybrids are immobilized and analyzed by electrochemical detection. Based on the eSensor XT-8 platform, Genmark DX has been marketing a number of clinically valuable assay kits, including the respiratory viral panel, cystic fibrosis genotyping test, and warfarin sensitivity test, which are FDA cleared panel for IVD use. The new ePlex platform fully integrates nucleic acid extraction, amplification, and detection, offering comprehensive panels on a scalable, sample-to-answer system.

The Atlas Genetics io™ system is also a fully automated platform, comprising of a small, low cost, easy-to-use instrument and a test-specific disposable cartridge. Different electrochemical labels, with discrete oxidation properties, are used to modify the oligonucleotide probes for the detection of different targets, enabling multiplex analyses. This approach rapidly amplifies the number of targets that can be detected from a single patient specimen. Atlas Genetics' primary focus is to provide solutions for sexually transmitted infections and hospital acquired infections. Companion diagnostic assays for personalized medicine are under development.

The above two electrochemical platforms are mainly used for the end-point analysis of PCR products, but are difficult to conduct real-time PCR monitoring and quantitative nucleic acid detection. Elice DetScan™ technology was described firstly in 2006 for a non-optical real-time PCR experiment. The detection is based on a measurement of the interaction between electrochemical binding probes and nucleic acids, as illustrated in Figure 2A[11]. A type of DNA intercalating redox probe molecule, $Os[(bpy)_2DPPZ]^{2+}$, is used to generate the electrochemical signal. Upon binding to the amplified double-stranded DNA, the probe becomes less electrochemically detectable due to steric and/or diffusional constraints compared with its free counterpart. It has been found that the redox intercalator is chemically stable under PCR cycling and does not significantly inhibit PCR. The electrochemical signal can be obtained by square wave voltammetry during PCR cycling, which is exponentially decreased as the amount of amplicon is raised with the number of PCR cycles (Figure 2B). The principle is analogous to that of real-time PCR using ds-DNA intercalating fluorescent dyes (such as SYBR green). The DetScan™ method has also been used to monitor isothermal helicase-dependent amplification of nucleic acids. Because no target-specific probe is involved in the detection, the assay specificity could not be as good as that of the two electrochemical methods mentioned above. Compared with fluorescent real-time PCR, both of the sensitivity and the stability of DetScan™ technology has yet to be further improved.

3 Conclusions and Outlook

The advances in science and technology have

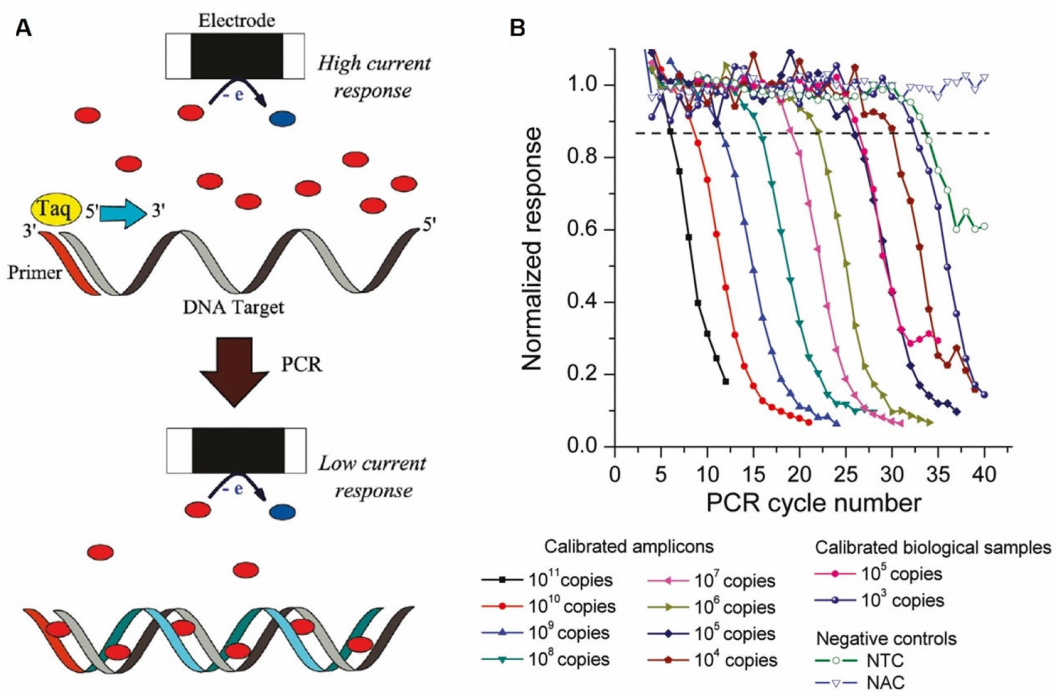

Fig. 2 A. Scheme's principle of DetScan technology; B. Real-time electrochemical-based PCR curves (Reprinted with permission from Ref 11. Copyright 2011 American Chemical Society.)

been profound and brought us material comfort and convenience. Today, people pay more attention to health care issues and medical progresses. Analytical chemistry has, therefore, become more focused on clinical analysis and diagnostics. A large number of electrochemical approaches have been proposed for immunoassay and nucleic acid analysis.

Thanks to the excellent sensitivity and reliability, the ECL technology has been developed as the mainstream immunoassay technology. However, further study is required to clarify the complicated light emission mechanism which involves the chemical and electrochemical reactions of short-lived radical species. The deep understanding of the coreactant ECL systems would help to optimize the reaction conditions and search for more efficient and less toxic coreactants. In recent years, many new types of luminescent metal complexes with high emission efficiency have been synthesized, and some of them may serve as efficient ECL labels. In addition, the labels with different emission wavelengths could be used for multiplex ECL detection.

A lot of highly sensitive electrochemical strategies other than the ECL technology have been reported in the literature for use in immunoassays[1]. Whether these methods can be commercialized and adopted clinically depends largely on their reliability. It should be noted that some of the sensitive approaches involve complicated modification of the electrode surface and tedious experimental protocols, which makes their reproducibility a serious concern.

In the field of molecular diagnostics, the fluorescence-base PCR technology is very likely to remain dominant for a long time. The "PCR amplification/electrochemical detection" strategies may find applications for point-of-care testing in decentralized laboratories and near-patient environments, if the detection systems could demonstrate their unique advantages in automation, integration, and miniaturization.

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免疫检测及核酸分析中的电化学方法

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摘要：本文介绍电化学方法在免疫检测及核酸分析中的应用. 鉴于有关方面的文献综述已有多篇，这里着重于已经商品化和临床应用的电化学分析技术. 希望从这些成功地应用于体外诊断的方法中得到一些启示，并提出作者个人对于该领域研究前景的看法.

关键词：电化学方法；免疫检测；核酸分析；临床诊断