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Electrochemiluminescence of tris(2,2′-bipyridyl)ruthenium and its applications in bioanalysis: a review

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ABSTRACT: Electrochemiluminescence (ECL) of tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] is an active research area and includes the synthesis of ECL-active materials, mechanistic studies and broad applications. Extensive research has been focused on this area, due to its scientific and practical importance. In this mini-review we focus on the bio-related applications of ECL. After a brief introduction to $Ru(bpy)_3^{2+}$ ECL and its mechanisms, its application in constructing an effective bioassay is discussed in detail. Three types of ECL assay are covered: DNA, immunoassay and functional nucleic acid sensors. Finally, future directions for these assays are discussed. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: electrochemiluminescence; tris(2,2′-bipyridyl)ruthenium (II); applications

Introduction

Electrochemiluminescence (or electrogenerated chemiluminescence, ECL) is a means to emit measurable luminescent signals by converting electrochemical energy into radiative energy via an electrochemical reaction (1). Luminescent signals can be obtained when the excited state of ECL-active species generated at electrode surfaces decays to the ground state. ECL has become a very active area of research in the past few decades. Many materials have been explored to investigate their ECL behaviour and they can be classified into two types, based on their chemical nature: (a) transition metal complexes; and (b) organic molecules and nanomaterials. Among these systems, the ECL of tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] (including its analogues) and its co-reactants as a highly sensitive detection method has received considerable attention, due to its advantages over existing systems in clinical testing and biomolecule detection. To date, a comprehensive monograph and several excellent reviews have been published in this area (1–16).

In this minireview, we focus on the state of the art in ECL based on $Ru(bpy)_3^{2+}$ for bioanalytical applications. We discuss $Ru(bpy)_3^{2+}$ based ECL mechanisms, its applications in DNA detection, immunoassay and functional nucleic acids sensors and provide a summary and outlook for this technology. Readers are referred to the monographs and reviews for more detailed information (1–16).

Mechanisms of $Ru(bpy)_3^{2+}$-based ECL

The mechanisms of $Ru(bpy)_3^{2+}$ based ECL have been extensively studied by many researchers and are well summarized in several reviews (1,7,13,15,16). Three different mechanisms have been proposed: the 'oxidative–reductive' co-reactant pathway; the 'reductive–oxidative' co-reactant pathway; and the hot electron-induced pathway. Most of the bioapplications of $Ru(bpy)_3^{2+}$-based ECL are based on 'oxidative–reductive' ECL, where $Ru(bpy)_3^{2+}$ (and its analogues) is used as the ECL luminophore and tri-*n*-propylamine (TPrA, and its alternatives) is widely used as the co-reactant. Here we focus on the $Ru(bpy)_3^{2+}$–TPrA system in order to elucidate the 'oxidative–reductive' reaction mechanism.

As shown in equations 1– 4, when a potential is applied to the system, $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$ at the electrode surface. Then $Ru(bpy)_3^{3+}$ can be further reduced with $TPrA^{\bullet}$ (which is from the oxidation of TPrA at the electrode surface) to produce the excited state $Ru(bpy)_3^{2+*}$. When $Ru(bpy)_3^{2+*}$ decays to the ground state, $Ru(bpy)_3^{2+}$, a red light will be emitted around 620 nm (17):

$$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+} \quad (1)$$

$$TPrA - e^- \rightarrow [TPrA^{+\bullet}] \rightarrow TPrA^{\bullet} + H^+ \quad (2)$$

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

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\nu \quad (4)$$

As shown in the scheme above, the ECL luminophore $Ru(bpy)_3^{2+}$ is regenerated during the reaction, which results in enhanced sensitivity compared with chemiluminescent systems. Also, the ECL signal intensity correlates with the concentrations of $Ru(bpy)_3^{2+}$

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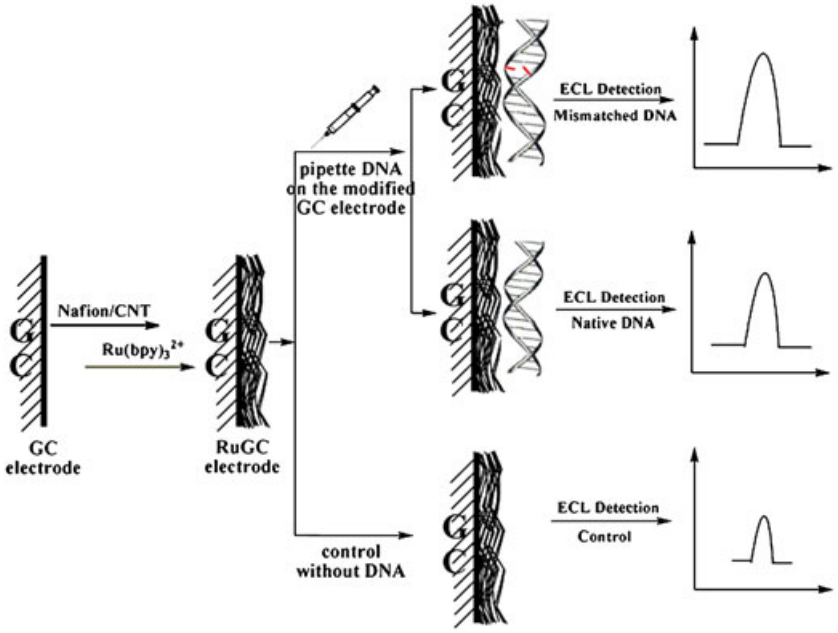

Figure 1. Schematic diagram of glassy carbon (GC) electrode modification and DNA ECL detection procedures (26). Reproduced with copyright permission from Elsevier.

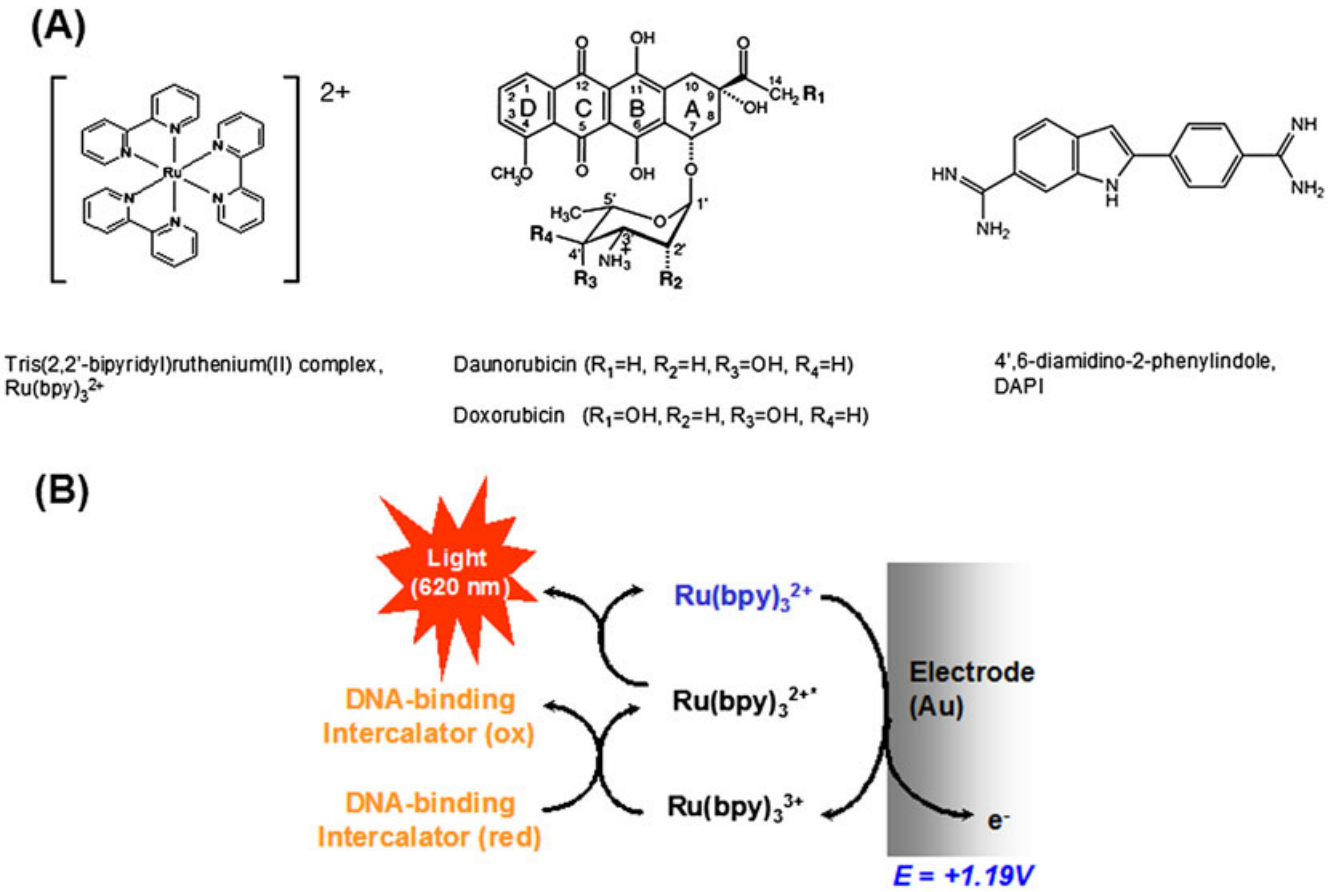

Figure 2. DNA detection based on $Ru(bpy)_3^{2+}$ with DNA-binding intercalators as the co-reactant (28). Reproduced with copyright permission from Elsevier.

and the co-reactant, so ECL can be used to detect both. If the concentration of $Ru(bpy)_3^{2+}$ is constant, the ECL intensity is positively related to the concentration of the co-reactant; thus, it could be used for determination of the reactant. Both the solid-state ECL and separation techniques-coupled ECL detection fall into this category. If the co-reactant is in excess, the ECL

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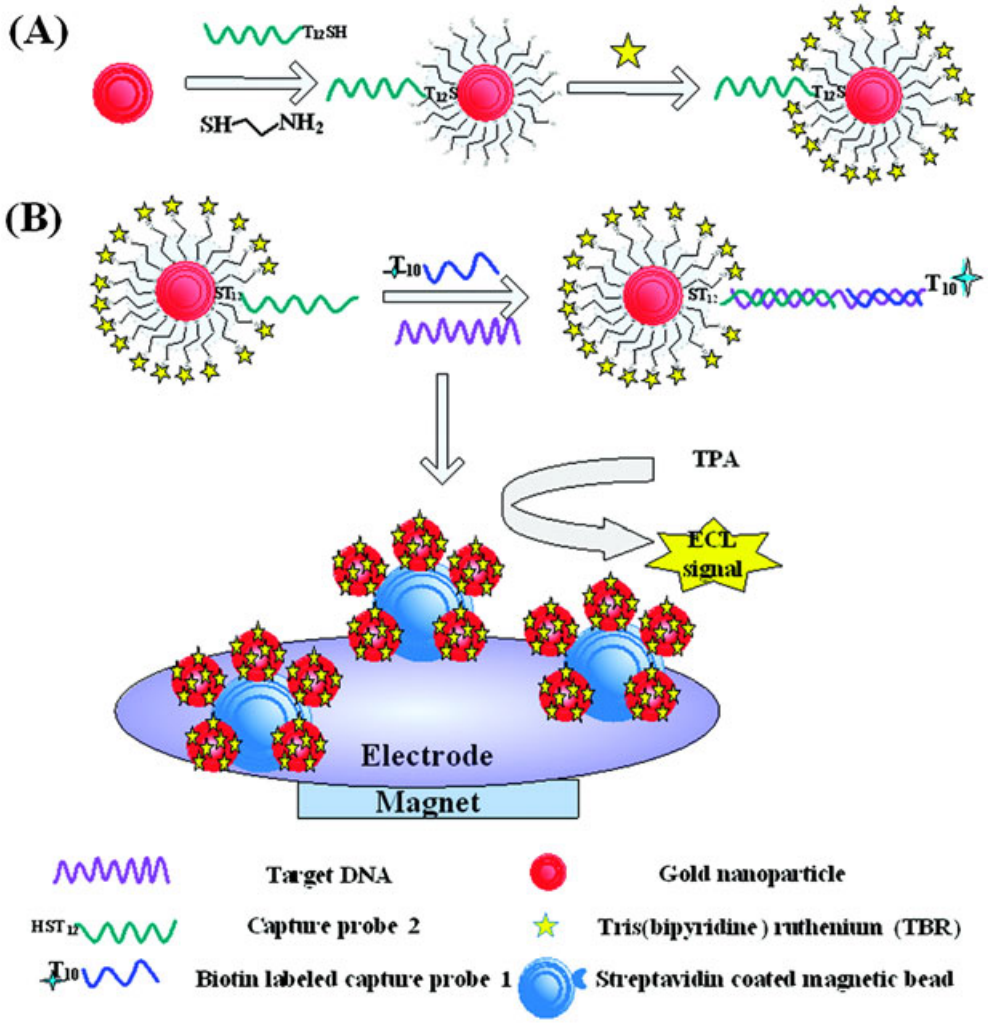

Figure 3. DNA detection based on a bar code and magnetic microparticle method (33). Reproduced with copyright permission from ACS.

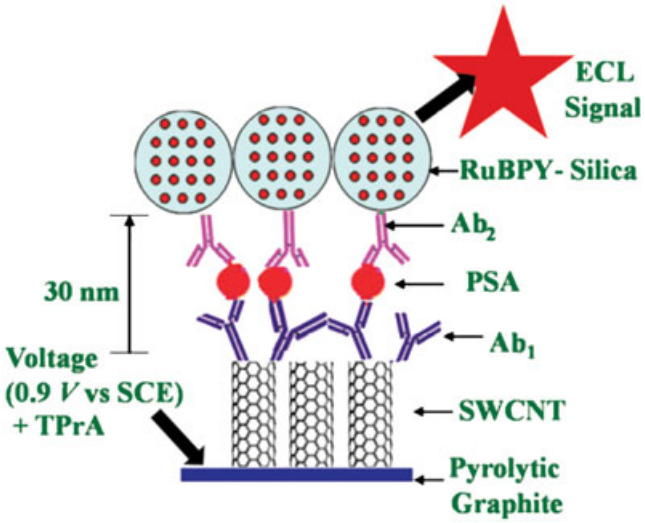

Figure 4. Sandwich type of ECL sensor with $Ru(bpy)_3^{2+}$-doped silica nanoparticles as enhanced ECL tags for detection of cancer biomarker PSA (43). Reproduced with copyright permission from RSC.

intensity is positively related to the concentration of $Ru(bpy)_3^{2+}$. In this case, however, instead of detection of $Ru(bpy)_3^{2+}$, the analyte of interest is detected via conjugation with $Ru(bpy)_3^{2+}$ analogue as an ECL label. Most of the examples discussed below fall into this category.

$Ru(bpy)_3^{2+}$-based ECL for bioapplications

$Ru(bpy)_3^{2+}$-based ECL has been widely used in analytical science due to its inherent advantages, such as extreme stability, good water solubility, high sensitivity, spatial and temporal controllability, wide dynamic range, wide range of analytes and compatibility with separation techniques. Many analytes of interest, including amines, amino acids, drugs, alcohol, oxalate, glucose and certain biomolecules, have been successfully determined with low detection limits (7,14,15,18–22). These analytes usually act as the alternative co-reactant to TPrA. On the other hand, non-co-reactant analytes, such as most biomolecules, can be readily detected combined with $Ru(bpy)_3^{2+}$ labelling. Besides, other types of label-free ECL sensors (such as those based on interactions between DNA and its intercalators) have also been fabricated. Both labelled and label-free $Ru(bpy)_3^{2+}$-based ECL sensors for bioapplications are discussed in this section.

DNA detection

DNA detection is of great importance in clinical testing, pathogen detection, forensic chemistry and mutated gene

diagnosis associated with human diseases. Owing to its excellent analytical performance, $Ru(bpy)_3^{2+}$-based ECL has been widely used in DNA detection (23–36).

Rusling's group reported the direct ECL detection of DNA, using a $[Ru(bpy)_2(PVP)_{10}]^{2+}$-modified electrode (23). Their method also differentiated chemically damaged DNA and its native counterpart. Wang *et al*. developed a label-free ECL approach for sensitive DNA detection with a $Ru(bpy)_3^{2+}$-modified electrode, based on the catalytic oxidation of guanine and adenine bases in nucleic acids (Figure 1) (26). Their approach distinctly discriminated double-stranded DNA and its thermally denatured counterparts, with a low concentration of 30.4 nmol/L. Further, a single-base mismatch detection of a *p53* gene sequence segment was successfully realized at 0.393 nmol/L.

An interesting label-free ECL sensor for DNA detection was developed by using DNA-binding intercalators as the co-reactant (Figure 2) (28). Certain double-stranded DNA intercalators were screened; among them, doxorubicin, daunorubicin and 4′,6-diamidino-2-phenylindole (DAPI) were found to be good

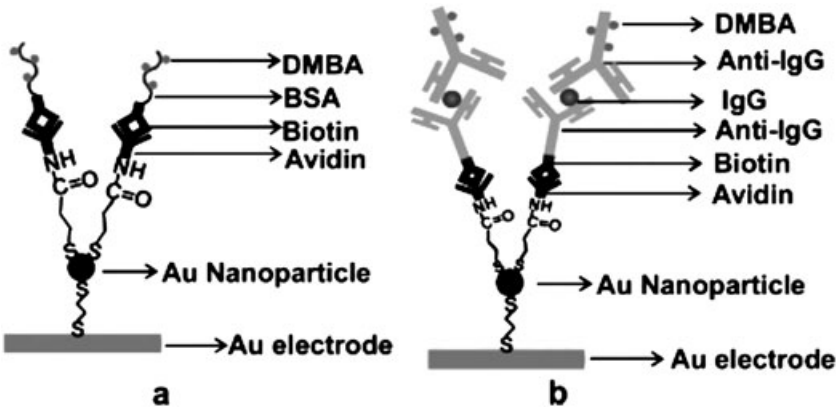

Figure 5. BSA and IgG detection with DMBA as ECL tags and gold nanoparticles as amplifying elements (46). Reproduced with copyright permission from ACS.

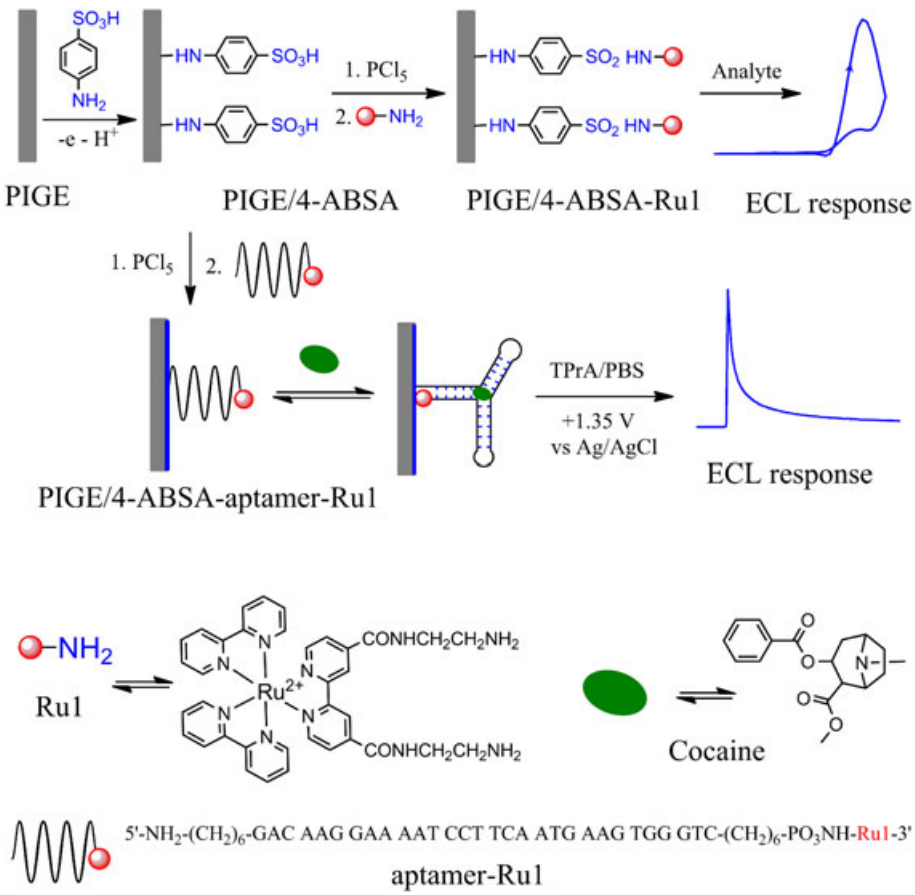

Figure 6. Scheme for fabrication of a ECL aptasensor for cocaine detection (49). Reproduced with copyright permission from ACS.

co-reactants for $Ru(bpy)_3^{2+}$ ECL. The double-stranded DNA interacted with these intercalators, producing strong ECL signals. However, single-stranded DNA could not interact with these intercalators; thus, limited or zero ECL signals were obtained. Based on this mechanism, the detection of a single nucleotide polymorphism for hepatitis C virus was accomplished with DAPI-intercalated $Ru(bpy)_3^{2+}$ ECL.

For most DNA sensors, the probe strand was labelled with $Ru(bpy)_3^{2+}$ or its analogue. Further amplification protocols were developed to improve the sensitivity. A PCR-free quantitative detection of nucleic acid targets was reported using $Ru(bpy)_3^{2+}$-labelled DNA as a bar code (31). Recently, cysteamine rather than DNA was used as a bar code for single-mismatched DNA detection in human serum (33). As shown in Figure 3, combined with bar code and magnetic microparticles, the sensing system had a detection limit as low as 100 fmol/L. By quenching of $Ru(bpy)_3^{2+}$ ECL with ferrocene, a quantitative DNA detection method was reported by Cao and co-workers (37). Also, by employing a 'molecular beacon'-like design, a turn-on DNA sensor was fabricated (38). In this design, $Ru(bpy)_3^{2+}$ was immobilized on an electrode surface, and ferrocene was labelled on one end of the hairpin DNA. Without target DNA, the $Ru(bpy)_3^{2+}$ ECL was quenched by ferrocene. However, the presence of target DNA would open the hairpin structure and thus restore the ECL intensity.

Immunoassay

For immunoassay, most of the biological targets are not ECL-active, so ECL tags are required to label the biomolecules with $Ru(bpy)_3^{2+}$ derivatives (15,35,39–45). To improve both the sensitivity and biocompatibility, a $Ru(bpy)_3^{2+}$ derivative of dendritic structure was used for multi-labelling a biomolecule at a single site (39). Other interesting amplification methods were also developed (35,40–45). By encapsulating thousands of $Ru(bpy)_3^{2+}$ molecules into micro- or nano-structures, ECL tags with enhanced sensitivity could be obtained and further used for immunoassay. For example, a sandwich type of ECL sensor for prostate-specific antigen (PSA) detection was fabricated (Figure 4). Using $Ru(bpy)_3^{2+}$-doped silica nanoparticles as enhanced ECL tags and carbon nanotube forests as efficient electrical communication pathways, PSA in cell lysates and human serum samples was successfully detected (43).

Alternatively, ECL co-reactants could also be used as tags for labelling (46,47). For example, 4-(dimethylamino)butyric acid (DMBA), an analogue of TPrA, was explored as an ECL tag for

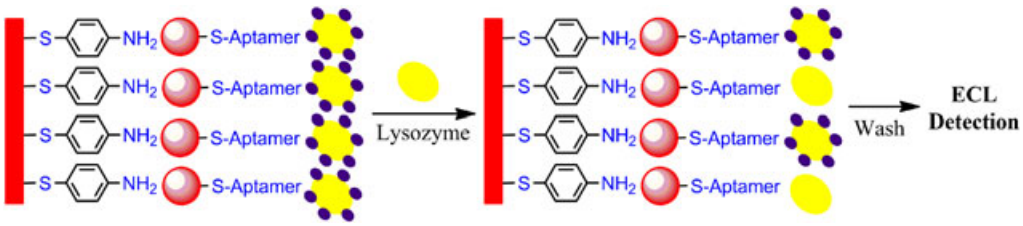

Figure 7. Aptamer-based biosensor for the detection of lysozyme by increasing sensitivity with gold nanoparticle amplification (54).

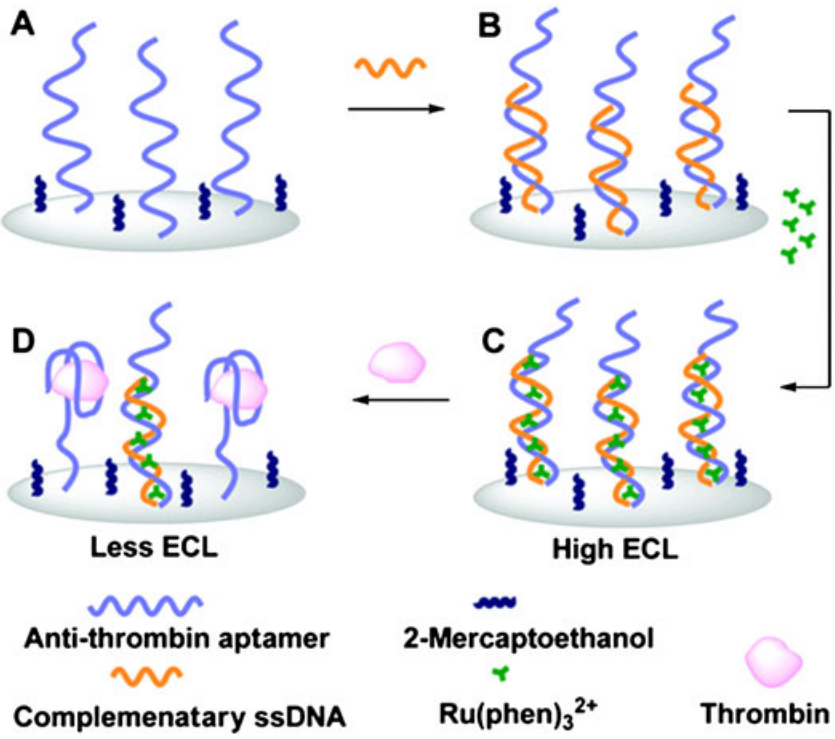

Figure 8. Schematic for the principle of the ECL aptasensor for thrombin detection (64). Reproduced with copyright permission from ACS.

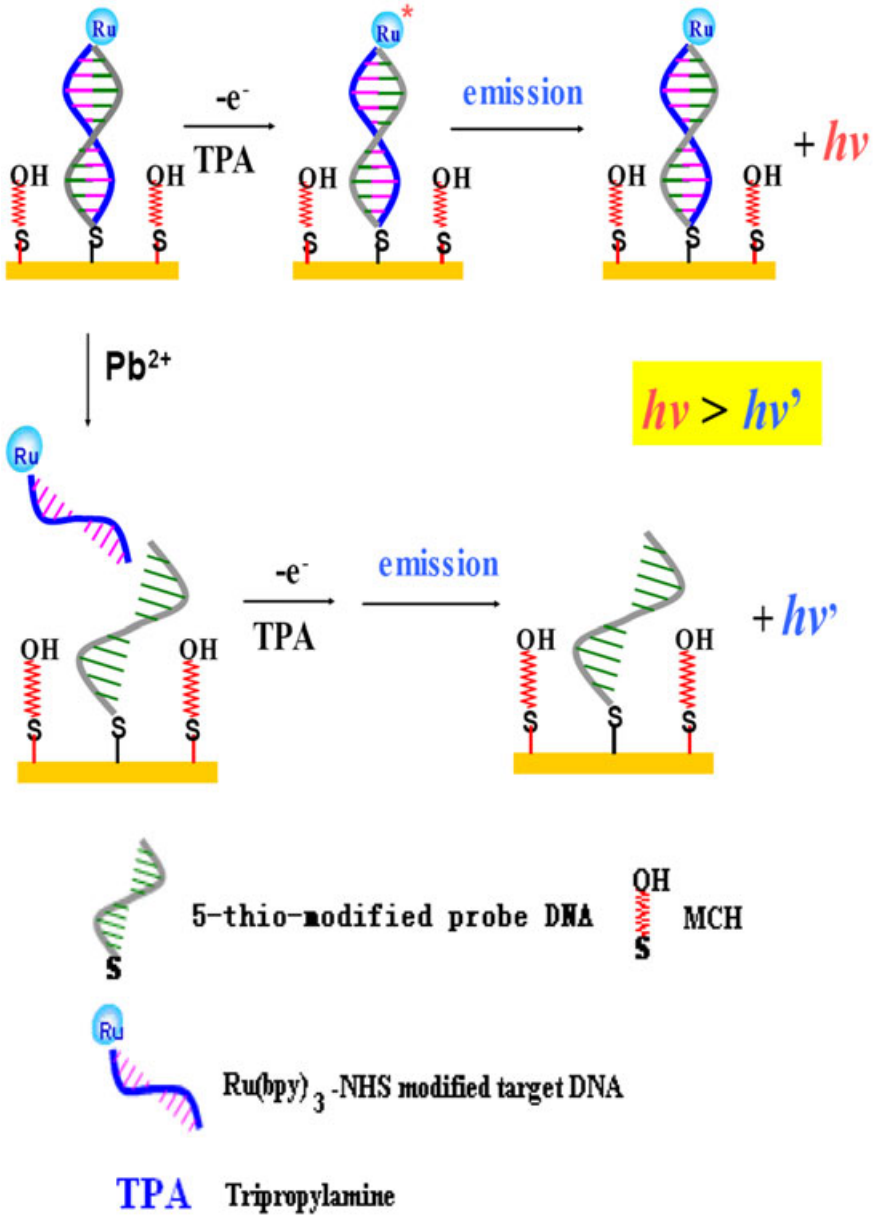

Figure 9. ECL detection of Pb^{2+} based on the Pb^{2+}-dependent DNAzyme (65). Reproduced with copyright permission from RSC.

biological substance labelling and ECL detection (Figure 5) (46). Specifically, bovine serum albumin (BSA) and anti-immunoglobulin G (IgG), tagged with DMBA, were used as models for ECL detection of BSA and IgG in the presence of $Ru(bpy)_3^{2+}$ solution. By further employing gold nanoparticles as amplifying elements, a 10- and six-fold sensitivity enhancement was obtained for BSA and IgG over their direct immobilization on an electrode using DMBA labelling.

Functional nucleic acid sensors

As functional nucleic acids, aptamers and DNAzymes are gaining more and more attention from researchers (48). For a given target, the highly-selective aptamer (or DNAzyme) can be obtained from test tubes via an approach called 'systematic evolution of ligands by exponential enrichment' (SELEX). Since their targets could range from metal ions, small molecules, proteins to even cells and bacteria, functional nucleic acids have been adopted as recognition elements to construct novel sensors.

Aptamers are functional nucleic acids that can bind to specific targets. Combined with ECL detection, many sensors based on aptamers (aptasensors) have been developed (16,49–66). More than 10 years ago Bruno *et al.* employed ECL for selection of an aptamer to anthrax spores (50). ECL-based aptasensors have already been used to detect small molecules and proteins. A highly sensitive and reusable ECL aptasensor for cocaine detection has been fabricated by immobilizing the $Ru(bpy)_3^{2+}$-attached cocaine aptamer on a modified paraffin impregnated electrode surface (Figure 6) (49). The 'as-prepared' aptasensor showed a very low detection limit of 10 pmol/L for cocaine and a good selectivity toward cocaine, heroin and caffeine. A structure-switching aptasensor with a solid-state ECL sensing platform was

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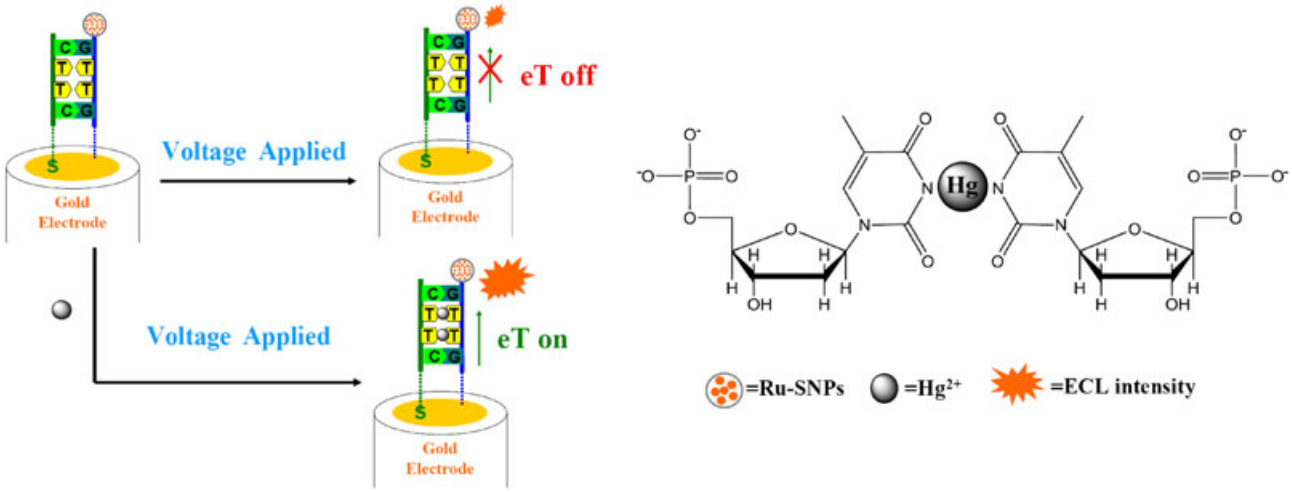

Figure 10. ECL detection of Hg^{2+} based on the T-Hg^{2+}–T pair (67). Reproduced with copyright permission from RCS.

developed for the detection of adenosine (66). Although it was a signal-off sensor, it showed good selectivity and sensitivity toward adenosine detection. An interesting label-free ATP aptasensor was reported by Xu *et al.* (61). Their design was based on the fact that the ECL of $[Ru(bpy)_2dppz]^{2+}$ was negligible in aqueous solution, and increased ca. 1000 times when $[Ru(bpy)_2dppz]^{2+}$ was intercalated into the nucleic acid structure. When ATP apatamer was used as the probe, ATP could bind to the aptamer more strongly than $[Ru(bpy)_2dppz]^{2+}$, so it could specifically affect the ECL of $[Ru(bpy)_2dppz]^{2+}$ and thus provide a method for ATP detection. The sensor had good selectivity (against CTP, GTP and UTP) and a low detection limit of 100 nmol/L ATP.

Several aptasensors for protein detection have also been reported. Most of these studies used either thrombin or lysozyme as the model proteins. For example, a lysozyme aptasensor was reported based on competition assay, where native lysozyme had higher affinity towards its aptamer than the $[Ru(bpy)_2(dcbpy)NHS]$-labelled lysozyme (Figure 7) (54). With gold nanoparticle amplification, a 100 fmol/L detection limit was obtained. The sensor was successfully used in the detection of lysozyme in egg. Yin and co-workers reported a simple, selective and sensitive label-free ECL aptasensor for thrombin detection, using the intercalation of $Ru(phen)_3^{2+}$ into double-stranded DNA (Figure 8) (64). Using their method, a mass detection limit of 0.2 amol/L for thrombin was obtained. Since the design was independent of conformational changes, it could be readily extended to targets whose aptamers have no specific conformational changes.

DNAzymes are catalytic nucleic acids capable of catalysing a broad range of reactions, such as cleaving nucleic acid substrates. Most of the DNAzymes sensors developed so far have been based on metal ion-dependent DNAzymes. Recently, Chen and co-workers extended DNAzymes sensors from fluorescent, colorimetric, electrochemical types, etc. to an ECL type (Figure 9) (65). The enzyme strand was first immobilized on an electrode surface, then the $Ru(bpy)_3^{2+}$-tagged substrate strand was hybridized with the enzyme strand to form double-stranded DNA. The presence of lead (Pb^{2+}) would release the cleaved substrate fragment and decrease the ECL signal. The limit of detection for Pb^{2+} was 0.1 nmol/L, which was lower than most fluorescent, colorimetric and electrical biosensors.

Besides metal ion-dependent DNAzymes, there are other types of specific interaction between metal ions and DNA. For example, thymine–thymine mismatches in double-stranded DNA could be selectively stabilized by forming thymine–Hg^{2+}–thymine (T-Hg^{2+}–T) base pairs. This phenomenon has been widely used for Hg^{2+} sensing. A highly sensitive and selective signal-on ECL sensor was also constructed for Hg^{2+} detection (67). As shown in Figure 10, without Hg^{2+}, the ECL intensity of the sensor was low because of the mismatch of T–T; however, with Hg^{2+}, the mismatch of T–T switched to stable T-Hg^{2+}–T base pairs, which gave enhanced ECL signals.

Conclusions and outlook

Among the most ultrasensitive detection methods, $Ru(bpy)_3^{2+}$ ECL has been widely used for both biological and non-biological assays, due to its high sensitivity, small sample volume and wide dynamic range. The related techniques have already been commercialized for immunoassays. Although impressive analytical performance has been achieved, multiplex detection is still an emerging area that will engage future research. Multiplex detection might be accomplished with highly spatial and temporal sensitive devices, such as CCD and fibre. Second, single-molecule, single-cell and single-bacterium-based assay will be another interesting and promising research field, which will advance our fundamental understanding of these systems. Third, the exploration of ECL for imaging, especially for *in vivo* imaging, will provide more bioanalytical information (both spatial and temporal) than simple biosensors. This will supply important insights into biological systems. Finally, more attention should be focused on the miniaturization of the current instrumentation, which will not only reduce the cost of clinical assay but also provide a chance to convert the bench technique to a bed-side one.

Acknowledgements

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LUMINESCENCE
The Journal of Biological and Chemical Luminescence

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