**Electrogenerated chemiluminescence peptide-based bioassay**

**Abstract:** Electrogenerated chemiluminescence (ECL) involves the generation of species at electrode surfaces that then undergo electron-transfer reactions to form excited states that emit light. The ECL method is becoming an increasingly promising method in the life sciences, environmental analysis, and clinical analysis, owing to its good selectivity, high sensitivity, wide dynamic concentration response range, and potential and spatial controllability. Here, we give a mini-review on the advances of the ECL peptide-based bioassay using peptide as the molecular recognition element. First, a general history of ECL development is presented. Then, the general strategies of the ECL peptide-based bioassay are reviewed. Particular attention is paid to the related progress in the last 5 years. Finally, we conclude with the future challenges and prospects in the development of the ECL peptide-based bioassay.

**Keywords:** bioassay; electrogenerated chemiluminescence; peptide.

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**Introduction**

Electrogenerated chemiluminescence (electrochemiluminescence, ECL) involves the generation of light through electrochemical processes. ECL analysis has been widely used in the life sciences, environmental analysis, and clinical analysis due to its advantages, such as good selectivity, high sensitivity, wide dynamic concentration response range, and potential and spatial controllability. A bioassay involves the use of a biological molecular recognition element to determine the biological activity of a substance. A biological molecular recognition element (e.g., antibody, peptide, aptamer, enzyme, nucleic acid, and receptor) is the key element because its affinity and specificity strongly affect the analytical performance of a bioassay (Iqbal et al. 2000). An ideal molecular recognition element should possess the characteristics of high selectivity, fast response, robust performance, and versatility for various targets. Antibodies have been the most commonly used component because of their specificity, affinity, and versatility. However, antibodies also exhibit certain limitations. For instance, most antibodies are isolated via hybridoma technology that requires B cells in antigen-immunized animals, which suffers batch-to-batch variation. Antibodies are sensitive to temperature and prone to irreversible denaturation. In addition, antibodies have high molecular weight, and it is difficult to achieve site-specific labeling. Therefore, it is highly desirable to seek an alternative ligand as a new molecular recognition element for bioassay.

Recently, peptides obtained using phage display show molecular recognition properties, in a manner similar to antibodies, but with improved characteristics, such as stability and resistance to harsh environments, production synthetically in a reliable and cost-effective manner, and amenability compared to antibodies to engineering at the molecular level. Additionally, due to their small size, peptides may achieve higher density for immobilization and bind to epitopes that are not easily accessed by antibodies (Petrenko and Vodyanoy 2003, Tsuji et al. 2013). With these advantages, significant advances have been made on the peptide-based bioassay. Hence, we give a mini-review on the advances of the ECL peptide-based bioassay using peptide as the molecular recognition element. First, a general history of ECL development is presented. Then, the general strategies of the ECL peptide-based bioassay are reviewed, including the two types of the ECL peptide-based bioassay: one uses peptide as the alternative to antibody and the other uses peptide as the substrate for specific enzyme. Particular attention is paid to the related progress in the last 5 years. Finally, we conclude with the future challenges and prospects in the development of the ECL peptide-based bioassay.
History of ECL

The phenomenon of luminescence upon electrolysis was first observed in 1927 when Grignard compounds were oxidized at several hundred volts (Dufford et al. 1927). The subsequent publications concentrated on the investigation of the mechanism and nature of ECL, especially polyaromatic hydrocarbons and metal complexes [Tris(2,2′-bipyridyl)ruthenium(II), Ru(II), TBR, Ru(bpy)] in the 1960s to 1970s (Hercules 1964, Visco and Chandross 1964, Santhanam and Bard 1965, Tokel and Bard 1972, Tokel-Takvoryan et al. 1973). Although ECL studies were performed in organic solvent in the absence of oxygen and water in this early stage, the analytical application of ECL was not much concerned (Cruser and Bard 1967, 1969). The major difficulties with the ECL systems in aqueous solutions are (a) the very low solubility of the organic compounds, (b) the reactivity of the radical ions with water, and (c) the limited potential range of water. The ECL emission in an aqueous system was first observed based on the reaction of electrogenerated Ru(bpy)32+ in the oxidation of oxalate ion (Rubinstein and Bard 1981). Leland and Powell (1990) reported, for the first time, the coreactant ECL system of Ru(bpy)32+-tripropylamine (TPA) in aqueous solution. Their pioneering research opened the door for the application of the ECL in analytical science (Lee 1997).

There are usually two types of ECL processes: radical ion annihilation and a coreactant participation. ECL emissions in organic solvent are produced by electrochemically oxidizing and reducing the ECL emitters (R) to get stable radical cations (R·+) and anions (R·−), respectively. The produced radical ions are annihilated by the oppositely charged radical ions to generate the excited state species (A*; the S-route, an “energy-sufficient system”) or 1A* (the T-route, an “energy-deficient system”), which was named as ion annihilation (Park and Bard 1976, Yeh and Bard 1976). In a coreactant participation process, ECL is frequently generated at an electrode in a solution containing luminophore species (“emitter”) and coreactant. A coreactant ECL system was first reported by Bard, in which Ru(bpy)32+ and oxalate were oxidized at the same time at a platinum electrode in acetonitrile, where the oxidation of oxalate produced a strong reductant, CO2−, which ultimately caused the reduction of Ru(bpy)32+ and the production of the excited state (Chang et al. 1977). Yuan et al. (2012) reviewed the typical coreactants for the Ru(bpy)32+ system, such as oxalate, persulfate, TPA, NADH (nicotinamide adenine dinucleotide, reduced disodium salt), 2-(dibutylamino)ethanol, vitamin C derivatives, alcohols, ketones, nucleic acid, formic acid, formaldehyde, organic acids, amines, hydrogen peroxide (H2O2), oxygen, hydrazine, and relative derivatives. Since the coreactant ECL system of Ru(bpy)32+ was widely studied, ECL proved to be useful for analytical applications (Li et al. 2009).

Now, ECL has received increasing attention in bioassay, and the increasing interest is reflected in the growing number of reviews. Miao (2008) gave an excellent review on the fundamentals of ECL, the mechanisms of different ECL systems, and the developments in the ECL field, such as ECL instrumentation and ECL luminophores and ECL applications with emphasis on the detection of biorelated species between 2003 and early 2007; 526 references were cited. Qi et al. (2009) reviewed the applications of nanomaterials in ECL biosensors from 2004 to 2008; 106 references were cited. Rampazzo et al. (2012) reviewed nanoparticles in metal complex-based ECL for highly sensitive applications; 194 references were cited. Deng and Ju (2013) reviewed the application of nanomaterials for ECL bioassay in enzymatic sensing, immunoassay, DNA analysis, and cytosensing; 218 references were cited. Mirasoli et al. (2014) reviewed the applications of miniaturized analytical devices based on chemical luminescence detection since 2009; 105 references are cited. From the above-mentioned reviews, it can be seen that the ECL bioassay mainly focused on the following aspects: ECL emitters, molecular recognition elements or target, immobilization methods, and the exploration of ECL signal amplification strategies.

ECL peptide-based bioassay

Peptide is combinatorial protein molecule in which a variable peptide sequence with affinity for a given target protein is displayed on an inert, constant scaffold protein (Colas et al. 1996, Mascini et al. 2012). Peptide is selected from combinatorial libraries based on its affinity to the target protein or small molecule and is expressed in bacterial cells, such as Escherichia coli. Excellent reviews dealing with the detailed technical aspects of combinatorial artificial protein selection technology are available (Colas 2000, Geyer and Brent 2000, Mascini et al. 2012). Here, we outline two general design strategies for the ECL peptide-based bioassay: one uses peptide as the alternative to antibody and the other uses peptide as the substrate for specific enzyme.

Peptide as the alternative to antibody

Because peptides can specifically bind with a target, they can be used as the alternatives to antibodies to detect proteins and small molecules with high sensitivity and
selectivity, comparable to and sometimes even better than antibodies. Two configurations of binding model are usually used in the ECL peptide-based bioassay. One is a system where the peptide ligand is fixed on the sensor chip and target is flowed or incubated with the peptide ligand. The other is the homogeneous model where the peptide is conjugated with target in homogeneous solution. The binding between peptide and target converts to physically detectable ECL signals. We summarized a schematization of possible configurations of the ECL peptide-based bioassay as shown in Figure 1.

### Peptide fixed on the electrode surface

A specific peptide (FYSHSFHENWPS) can selectively bind to cardiac troponin I (cTnI) with a dissociation constant of the complex in a nanomolar level (Park et al. 2010). Using the specific peptide as the molecular recognition element, we developed an ECL peptide-based biosensing method for the determination of cTnI with gold nanoparticle amplification (Shan et al. 2014). Capture peptide was self-assembled on the surface of gold nanoparticle-modified gold electrode through a thiol-containing cysteine at the end of the capture peptide to obtain ECL biosensor. When the biosensor reacted with target cTnI and then incubated with ECL probe (bis(2,2′-bipyridine)-4,4′-dicarboxybipyridine ruthenium-di(N-succinimidyl ester) [Ru(bpy)2(dcbpy)NHS, Ru1] labeled signal peptide), a strong ECL response was electrochemically generated. The good affinity of peptide and the amplification of gold nanoparticles resulted in a sensitive detection of cTnI from $1.0 \times 10^{-12}$ to $3.0 \times 10^{-10}$ g/ml with a detection limit of 0.4 pg/ml. The peptide fixed on the electrode surface provides a simple and selective heterogeneous method for the assay of biomedical samples, especially when it contains a high concentration of interference proteins. The process is complicated because it includes immobilization and washing steps.

### Homogeneous model

Another configuration is the homogeneous model. The binding of target to peptide in solution results in the change of ECL signal. One homogeneous ECL method for the determination of cTnI was reported in our group (Wang et al. 2012). In this work, the peptide (FYSHSFHENWPSK) as the molecular recognition element was labeled with ruthenium complex through NH$_2$-containing lysine on the peptide via acylation reaction and used as an ECL probe. In the presence of cTnI, a decrease in ECL signal was observed upon the binding event between the ECL probe and target cTnI. The larger difference between small peptide and protein in homogeneous ECL peptide-based bioassay results in a sensitive and selective determination of cTnI with a detection limit of $1.2 \times 10^{-10}$ g/ml. However, the binding interaction between peptide and target is limited due to the limited binding epitopes, and the ECL signal is limited because only one ECL molecule is attached directly to each peptide.

Binding interactions can be increased by the binding of multiple ligands on one entity to multiple receptors (Terskikh et al. 1997, Vance et al. 2008). Thus, the employment of polyvalent binding motif of the probe on the appropriate platform for the target protein is promising in the development of a sensitive peptide-based bioassay. We developed a homogeneous ECL peptide-based method by incorporating liposome and magnetic bead as amplification platform for the determination of cTnI (Qi et al. 2013). The principle scheme is shown in Figure 2. Liposomes were

![Figure 1](image.png)  
**Figure 1** Scheme of possible configurations of the ECL peptide-based bioassay in the binding model. (A) sandwich assay; (B) competitive assay; (C) magnetic bead-based homogeneous model.
synthesized using a standard sonication procedure and used as the carrier of ECL signal reagents for signal amplification. The magnetic capture peptides were synthesized by covalently attaching the peptides to the surface of magnetic beads via acylation reaction, and the liposome peptides were synthesized by covalently attaching the peptides to the signal reagent-encapsulated liposomes. In the presence of cTnI, sandwich-type conjugates were generated in the incubation of the magnetic capture peptides and the liposome peptides. After a magnetic separation, the sandwich-type conjugates were treated with ethanol; thus, a great number of the ECL reagents were released and measured by the ECL method. The large amounts of ECL reporter molecules \(1.9 \times 10^7 \text{Ru}1\) molecules/liposome and both large active surface areas of the liposome and magnetic beads providing polyvalent binding motifs for the target protein result in an extremely low detection limit of 4.5 pg/ml for cTnI, which opens a new door to the ultrasensitive detection of proteins in clinical analysis.

**Figure 2** Schematic diagram of the liposome peptide-based ECL method for the determination of cTnI. Reprinted with permission from Qi et al. (2013). © 2013 American Chemical Society.

**Peptide as the substrate for enzyme**

Peptide can be used as the substrate for specific enzyme to measure the enzymatic activity with high degree of specificity and good stability (Denmeade et al. 1997). One is based on the cleavage of peptide by target enzyme and another is based on the phosphorylation of peptide by protein kinase in the cosubstrate of ATP molecule.

**Cleavage of peptide**

A peptide with the amino acid sequence HSSKLQ can be used as a substrate to measure prostate-specific antigen (PSA) enzymatic activity in extracellular fluids with high degree of specificity and good stability (Denmeade et al. 1997). Several peptide-based methods were developed for the detection of PSA based on the target-induced cleavage of peptide, such as fluorescent peptide-based assay (Choi et al. 2013, Lee et al. 2013) and electrochemical peptide-based assay (Roberts and Kelley 2007, Zhao et al. 2010). We developed an ECL peptide-based method for the determination of PSA based on the target-induced cleavage of peptide (Qi et al. 2012). A specific peptide with a sequence of CHSSKLQK was labeled with Ru(bpy)\(_2\)(dcbpy)NHS and immobilized onto a gold electrode surface via a self-assembling technique to obtain the ECL-PB biosensor. The peptide substrate was recognized and cleaved by PSA, resulting in the decrease of amount of ECL emitter and then the decrease of ECL signal. The direct transcription of peptide cleavage events into an ECL signal provides a simple, sensitive method for the detection of PSA. The detection limit of 0.038 ng/ml was obtained. However, in this protocol, the ECL intensity of the biosensor decreased with the increase of PSA concentration, which is known as a “signal-off” mode. The signal-off property is undesirable because it is more susceptible to background variation and the room for signal change is also small.
Therefore, another “signal-on” ECL peptide-based biosensor for the determination of PSA by combining both signal enhancement from gold nanoparticle and signal quenching from ferrocene was fabricated recently in our laboratory (Qi et al. 2014). The specific peptide (CHSSKLQK) served as a substrate, and Ru(bpy)$_3^{2+}$ was used as an ECL signal. The schematic diagram of the fabrication process of ECL-PB biosensor for the determination of PSA is shown in Figure 3. Gold nanoparticles were first modified onto Nafion-modified glassy carbon electrode by casting the mixture of Nafion and gold nanoparticles, and then Ru(bpy)$_3^{2+}$ was effectively incorporated into the composite film of Nafion based on the electrostatic interaction. Then, ferrocene-labeled specific peptide was self-assembled on modified electrode. In the absence of PSA, a lower ECL signal was observed due to the quenching of the attached Fc on the ECL of Ru(bpy)$_3^{2+}$. In the presence of PSA, PSA recognized and cleaved the peptide on the electrode surface, resulting in the decrease of the amount of Fc on the electrode surface and thus an increase of ECL signal. A signal-on ECL peptide-based method was developed for the determination of PSA via target-induced cleavage of peptide. Due to the enhancement of the gold nanoparticles on the ECL signal and the low background of ECL-PB biosensor from Fc quenching, a low detection limit of 8×10$^{-13}$ g/ml was obtained for PSA.

Phosphorylation of peptide

The serine, threonine, or tyrosine residues in peptide or protein can be phosphorylated by protein kinase in the presence of ATP molecule. The phosphorylation of proteins catalyzed by kinases is known as a control mechanism with most aspects of physiology functions. The measurement of kinase activities and the identification of their potential inhibitors are not only necessary for understanding many fundamental biological metabolism processes but also important for the research of cancer development, diagnosis, and treatment. Therefore, peptide can be used as the substrate to assay the activity of protein kinase. The ECL method was also employed as the detection technique to assay protein kinase activity. The generally used methods for the measurement of protein kinase activities mostly relied on phosphor-specific antibodies bound to the phosphorylated proteins specifically (Figure 4A). For example, Xiao et al. (2007) developed a sensitive ECL method for the detection of protein tyrosine kinases with 240 fmol/well of the kinase reaction product using ruthenium derivative-labeled monoclonal antibody against phosphotyrosine. Other ECL strategies were also proposed to measure protein kinase activities, including employing adenosine 5’-[c-thio] triphosphate (ATP-s) as cosubstrate and gold nanoprobe as ECL signal (Figure 4B).

Figure 3  Schematic diagram of the fabrication process of ECL-PB biosensor for the determination of PSA. Reprinted with permission from Qi et al. (2014). © 2014 American Chemical Society.
or employing zirconium cations (Zr\(^{4+}\)) as linking agent to recognize phosphates and rapidly link two biomolecules containing phosphate groups (Figure 4C).

Xu et al. (2010) reported an ECL biosensor using gold nanoparticles as signal transduction probes for the kinase activity and inhibition analysis. The schematic representation of ECL strategy for kinase activity detection is shown in Figure 5. Kemptides (CLRRASLG) were self-assembled on the gold electrode to obtain peptide-modified electrode. After the phosphorylation reaction in the presence of protein kinase A (PKA) using ATP-s as the cosubstrate, thiolated substrate peptides were introduced on the electrode surface. Then, gold nanoparticles were specifically conjugated to the thiophosphate group. Based on the catalysis of gold nanoparticles to the luminol ECL reaction, PKA was measured in the range of 0.07–32 U/ml with a low detection limit of 0.07 U/ml. The method was also used to quantitatively evaluate the inhibition of kinase in the presence of small molecule.

Since then, various nanoparticles, including gold nanoparticle and silica nanoparticles, with different strategies have been used in the ECL peptide-based bioassay for the determination of protein kinase. For example, Zhao et al. (2012) used DNA-modified gold nanoparticles...
that can absorb a great deal of Ru(II) by electrostatic interaction to develop the ECL method for the determination of protein kinase and inhibition. First, an ECL nanoprobe was prepared by conjugating gold nanoparticles with phosphorylated DNA capture probes and TBR-cysteamine. In the presence of PKA and ATP, substrate biotinylated peptides were phosphorylated and then conjugated to streptavidin-coated magnetic beads via the biotin-streptavidin interaction. Zr⁺⁺ cations are used for linking ECL nanoparticles with peptides and result in the capture of ECL nanoparticles on the surface of magnetic beads. The complexes were then captured and enriched on the electrode surface for ECL detection. The linear range was from 0.01 to 50 U/ml and the detection limit of PKA is 0.005 U/ml.

Chen et al. (2013) developed an ECL method for the determination of PKA and inhibition by using linking agent of Zr⁺⁺ cations and signal amplification of Ru(II) encapsulated phosphorylate-terminated silica nanoparticles (R-PSiNPs). R-PSiNPs were prepared by the controlled synchronous hydrolysis of tetraethoxysilane and 3-(trihydroxysilyl)-propylmethyl-phosphonate in water-in-oil microemulsion. Peptide (CLRRASIG) is self-assembled on the gold electrode surface via Au-S bonding and then phosphorylated by PKA in the presence of ATP. R-PSiNPs were subsequently grafted to electrode surface by linking with phosphorylated peptide in the presence of Zr⁺⁺. The method can be employed to assay PKA activity with a detection limit of 0.005 U/ml.

Liang et al. (2014) reported an ECL biosensor for the determination of protein kinases and inhibition using enzyme-functionalized gold nanoparticles as amplification platform. Biotin-DNA and glucose oxidase (GOx) were first conjugated to gold nanoparticles to form GOx/AuNPs/DNA-biotin nanoprobe. Peptides were first assembled onto gold electrode surface and then phosphorylated by PKA in the presence of TPA. The assembled phosphorylated peptides are subsequently recognized by biotinylated anti-phosphoserine antibody. Then, the nanoprobe was bound to the biotinylated anti-phosphoserine antibody on electrode surface through a biotin-avidin interaction. Gox assembled on the nanoprobe can catalyze glucose to generate H₂O₂ in the presence of O₂, whereas the ECL reaction occurred in the luminol system. This biosensor can also be used for the quantitative analysis of ellagic acid.

Challenges and prospects

We summarized the ECL peptide-based bioassay using peptide as the recognition molecule element. This is not a comprehensive review and only summarizes a fraction of research activities that exploit peptide as the recognition element in bioanalytical chemistry. Peptide has been shown to be a versatile and effective molecular recognition element in bioassay. Combined with nanoparticles such as gold nanoparticles, silica nanoparticles, or liposomes, the ECL peptide-based bioassay is expected to pay increasing attention in biomedical fields, such as diagnostics, bioseparation, and therapeutics.

Although the ECL peptide-based bioassay opens new opportunities in analytical science, we must emphasize that the strategies proposed to date have not been exploited fully for their analytical potential. First, the type of peptide is limited for further application. Additionally, a fundamental understanding of peptide-target binding or cleavage interactions in terms of binding affinity and peptide secondary structure is also needed, which will lead to improved utilization of peptide for bioassay. Moreover, emission in ECL biosensors based on the amount of ECL agent and coreactant, the mass transport through the immobilization matrix, and the charge-transfer kinetics in the heterogeneous reaction system is not understood. Therefore, the refinement of the ECL peptide-based bioassay is needed for the following challenging issues: (a) more specific peptides selected from combinatorial libraries for diagnostics; (b) more research work focused on conjugation or nanomaterial labeling with emphasis on labeling degree, bioactivity, stability, and storage; (c) good identification of electron transfer and ECL mechanism between peptide and electrode; (d) better understanding of peptide-target binding or cleavage interactions in terms of binding affinity and peptide secondary structure; and (e) development of high-throughput, miniaturized ECL peptide-based bioassay or biosensor array for multiplexing target detection.

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