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Nanomaterials for biosensing with electrochemiluminescence (ECL) detection

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1. ABSTRACT

Analytical applications of nanomaterials used in electrochemiluminescence (ECL)-based detection methods are reviewed. Among nanomaterials, carbon-based nanomaterials (carbon nanotubes, graphene), metal nanoparticles, quantum dots, inorganic metal complexes and conducting polymers are considered. The most common mechanisms of ECL detections are also described in this review. Finally, challenges and perspectives of the use of such materials in chemical analysis are discussed.

2. INTRODUCTION

Electrogenerated chemiluminescence, also known as electrochemiluminescence (ECL), has become an important detection method in analytical chemistry. ECL can be defined as the emission of light generated by relaxation of excited state molecules that are produced during an electrochemical initiated reaction. Generation of light from electrochemically induced reactions is a well known process for over hundred years. In the early 1900's, pioneer works from Duffort and Harvey reported the

emission of light from Grignard (1) and luciferin derivative compounds (2-5), respectively. Then, in the early 60's, ECL emission generated from aromatic compounds were observed by Visco (6,7), Hercules (8) and Bard (9). ECL is increasingly becoming very popular in chemical analysis and this is reflected by the increase number of reviews published in this area over the last decade (10-20). ECL allows the detection of analytes at low concentrations over a wide linear range, since the absence of an excitation light source produces very low background signals and virtually no scattering. Therefore, high sensitivity detection is achieved without the use of expensive instrumentation. Another significant advantage of the ECL over conventional spectroscopic techniques is that the ECL signal occurs only in the presence of a concomitant electrochemical initiation reaction, which is dependent of the applied potential: without the application of a suitable potential, the electrochemical reaction does not occur and the system will not emit light. This fact, coupled with the possibility to place the optical fiber very close to the electrode surface, allows accurate spatial and temporal control over the reaction with concomitant high selectivity and improvement of the signal-to-noise ratio. Another advantage of the ECL over electrochemical techniques is the absence of electrical interferences. Indeed, the detection of light enables multi-parameter detection and different species can simultaneously be detected using lifetime or wavelength discrimination. The combination of ECL with chromatographic and electrophoretic systems allowed the separation and detection of complex mixtures, since the reactants can be generated and activated *in situ*. Another important advantage of ECL is that the reactive species can be immobilized and preconcentrated on electrode surfaces. This allows a considerable increase of the light intensity and at the same time the regeneration of the reactants at the electrode surface. Even though several reagents can produce ECL, a large part of the analytical applications is covered only by a few reactions and amongst them, inorganic complexes such as ruthenium bipyridyl complexes, $[Ru(bpy)_3]^{2+}$, and organic molecules such as luminol are the most popular. Only recently, the use of ECL in chemical analysis has received an unexpected boost by the discovery that quantum dots (QDs), such as CdSe and CdTe, can emit light under the application of a suitable potential. Some of the most studied ECL reactions have involved the use of polyaromatic hydrocarbons, but with very limited applications in chemical analysis due to their insolubility in aqueous solutions. The aim of this review is to give a general overview of the basis of the ECL process, along with the latest developments and applications of this analytical technique in chemical analysis. Particular emphasis will be given to the co-reactant-based ECL applications (luminol, ruthenium complex derivatives) using nanomaterials such carbon-based materials (carbon nanotubes, graphene), polymers, metal nanoparticles and quantum dots.

3. PRINCIPLES OF ECL

3.1. Ion annihilation ECL

There are two dominant pathways to produce ECL, *i.e.* the annihilation and the co-reactant pathways; however, most of the analytical applications reported in the literature are based on the latter one. The ion annihilation process involves the formation of an electrochemically generated intermediate species at the electrode that interacts and undergoes the formation of both a ground and electronically excited states (13, 15) . Let consider a species R, where the radical ions are produced by anodic oxidation and cathodic reduction reactions such as:

$R^1 - e^- \rightarrow R_1^{\bullet +}$ (oxidation at the electrode)

$R^1 + e^- \rightarrow R_1^{\bullet -}$ (reduction at the electrode)

$R_1^{\bullet -} + R_1^{\bullet +} \rightarrow R_1 + {}^1R_1^*$ (annihilation process)

${}^1R_1^* \rightarrow R_1 + h\nu$

In energy sufficient systems, the formation of the excited state is energetically accessible to the redox process and the ion annihilation occurs via the singlet route "S-route". In this case, the light will be emitted by relaxation. A typical example of ion annihilation process via the S-route is the ECL of 9,10-diphenylanthracene (DPA)(6-9). It is also known that annihilation processes may also occur even though the radical cations and radical anions are from different molecules.

In contrast, in energy deficient systems where the excited singlet state ${}^1R_1^*$ is inaccessible to the redox process, the emitting species is formed via a triplet-triplet annihilation route "T-route". This pathway involves the formation of triplet intermediates. Assuming the radical cations ($R_1^{\bullet +}$) and radical anions ($R_2^{\bullet -}$) are from different molecules, the emission of light may occur through the S-route, *i.e.*:

$R_1^{\bullet +} + R_2^{\bullet -} \rightarrow {}^3R_1^* + R_2$

or through the T-route, *i.e.*:
${}^3R_1^* + {}^3R_2^* \rightarrow {}^1R_1^* + {}^1R_2^*$

In the latter case, the excited species can be either ${}^1R_1^*$ or ${}^1R_2^*$, depending on their relative energies. Usually, in energy sufficient system, the S-route is the dominating pathway. Examples of ECL processes using the T-route are the ECL of rubrene (21) and of ruthenium tris-bipyridyl derivatives. In this case the proposed mechanism is the following:

$[Ru(bpy)_3]^{2+} - e^- \rightarrow [Ru(bpy)_3]^{3+}$ (oxidation at the electrode)

$[Ru(bpy)_3]^{2+} + e^- \rightarrow [Ru(bpy)_3]^{+}$ (reduction at the electrode)

$[Ru(bpy)_3]^{3+} + [Ru(bpy)_3]^{+} \rightarrow [Ru(bpy)_3]^{2+*} + [Ru(bpy)_3]^{2+}$

$[Ru(bpy)_3]^{2+*} \rightarrow [Ru(bpy)_3]^{2+} + h\nu$ (λ = 610 nm)

The excited state can also be produced via an alternative hot electron mechanism, i.e.:

$[Ru(bpy)_3]^{3+} + e^- \rightarrow [Ru(bpy)_3]^{2+*}$

The reduction of $[Ru(bpy)_3]^{2+}$ occurs at quite negative potentials, therefore Pt electrodes cannot be used in aqueous solutions in this specific case, since there would be evolution of hydrogen at the electrode surfaces. This situation can be avoided using metal oxide such as the tantalum (Ta/Ta_2O_5) oxide electrodes. Ion annihilation can lead to the formation of excimers (excited dimers) and exciplexes (excited complexes), and in this case there is an additional pathway, known as the "E-route". The main advantage of the ion annihilation process is that it requires only the ECL species, solvent and supporting electrolyte in order to generate light. Other metal complexes are suitable for ECL and among them Ir, Os, Mo, Re, Tb have received some attention even though the Ru, Os , and Ir derivatives are the most used (12).

3.2. Co-reactant ECL

3.2.1 $Ru(bpy)_3^{2+}$-based ECL

Another pathway to produce ECL is defined as the "co-reactant ECL". The major advantage on using the co-reactant pathway is that this facilitates the generation of ECL in aqueous solution, opening up a wide range of opportunities for the use of ECL in chemical analysis. Co-reactant ECL is often generated by applying a suitable potential to the electrode with the luminophore species either immobilized on the electrode or in solution, and in the presence of a species that acts as a co-reactant. When a potential (positive or negative) is applied to the electrode, both the luminophore species and the co-reactant undergo oxidation or reduction with concomitant formation of radicals and intermediate states. Then, the decomposition of the intermediate states leads to the formation of highly-reactive oxidizing or reducing species, which will then interact with the oxidized or reduced luminophore to produce the excited stated and subsequent ECL emission. Typical examples are the ECL of oxalate ($C_2O_4^{2-}$), and tripropylamine, TPA, as co-reactants. When $C_2O_4^{2-}$ is the co-reactant, the proposed pathway is the following (22):

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

$[Ru(bpy)_3]^{3+} + C_2O_4^{2-} \rightarrow [Ru(bpy)_3]^{2+} + C_2O_4^{-\bullet}$
$C_2O_4^{-\bullet} \rightarrow CO_2^{-\bullet} + CO_2$

$[Ru(bpy)_3]^{3+} + CO_2^{-\bullet} \rightarrow [Ru(bpy)_3]^{2+*} + CO_2^-$
$[Ru(bpy)_3]^{2+*} \rightarrow [Ru(bpy)_3]^{2+} + h\nu$

In this reaction, the excited state is produced via direct reduction of $[Ru(bpy)_3]^{3+}$ by the radical $CO_2^{-\bullet}$, however, a second mechanism via annihilation may occur, *i.e.*,

$[Ru(bpy)_3]^{2+} + CO_2^{-\bullet} \rightarrow [Ru(bpy)_3]^{+} + CO_2^-$
and $[Ru(bpy)_3]^{3+} + [Ru(bpy)_3]^{+} \rightarrow [Ru(bpy)_3]^{2+*} + [Ru(bpy)_3]^{2+}$

One of the most common ECL reactions involves the use of TPA as co-reactant. The occurrence of ECL using amines enables the detection of a large variety of analytes. The proposed mechanism is the following:

$[Ru(bpy)_3]^{2+} - e^- \rightarrow [Ru(bpy)_3]^{3+}$
$[Ru(bpy)_3]^{3+} + TPA \rightarrow [Ru(bpy)_3]^{3+} + TPA^{+\bullet}$

$TPA^{+\bullet} \rightarrow TPA(H_{-1})^{\bullet} + H^+$

$[Ru(bpy)_3]^{3+} + TPA(H_{-1})^{\bullet} \rightarrow [Ru(bpy)_3]^{2+*} + TPA(H_{-1})^{\bullet+}$
$[Ru(bpy)_3]^{2+*} \rightarrow [Ru(bpy)_3]^{2+} + h\nu$

Similar to the oxalate reaction, the excited state can be generated via annihilation through the reduction of $[Ru(bpy)_3]^{2+}$ by the radical species:
$TPA - e^- \rightarrow TPA^{+\bullet}$

$TPA^{+\bullet} \rightarrow TPA(H_{-1})^{\bullet} + H^+$
$TPA(H_{-1})^{\bullet} - e^- \rightarrow TPA(H_{-1})^{\bullet+}$

The TPA in the solution is either oxidized or reduced in the same potential step as the luminophore species. Through electron transfers or chemical reactions, the co-reactant generates a product that reacts with the ECL luminophore to generate an excited state. In the case of $[Ru(bpy)_3]^{2+}$-based ECL, tertiary amines such as tripropylamine, TPA, are the dominant co-reactants. However, others species include the use of peroxydisulfate ($S_2O_8^{2}$), hydrazine and hydrogen peroxide ions, which operate in the reductive-oxidation mode, while oxalate and pyruvate ions operate in the oxidative-reduction mode. Co-reactants are often used in the following cases: (i) when the radical cations ($R^{+\bullet}$) and anions ($R^{-\bullet}$) are unstable, (ii) when they cannot be formed due to the narrow potential window of the solvent, or (iii) when the annihilation process is not very efficient. The co-reactant-based approach is particularly useful in chemical analysis in aqueous solution when it is necessary to avoid the quenching of oxygen (this is often the case in the ion annihilation process). This allows the analysis to be performed without degassing the samples. It is worth to mention that only the luminophore species can be regenerated at the electrode, while the co-reactant is consumed during the electrochemical reactions. This pathway has been extensively utilized in chemical analysis for the ECL detection of amino acids, proteins, cyclic and aliphatic amines, and pharmaceuticals (12). It has been shown that the tertiary amines produce higher ECL signals than secondary and primary amines. Most of the ECL reactions between the co-reactant and $[Ru(bpy)_3]^{2+}$ involve oxidative-reduction steps and the related ECL mechanism depends on the analyte. Therefore different co-reactants correspond to different ECL pathway. Most of the ECL studies reported the use of TPA as a co-reactant, however, because of its toxicity and volatility, a recent work reported a detailed investigation of the ECL of $[Ru(bpy)_3]^{2+}$ using different amines (23): 2-(dibutylamino)ethanol was shown to be a suitable co-reactant for ECL detection.

3.2.2 luminol-based ECL

The ECL of luminol, in the presence of hydrogen peroxide in alkaline media, is very similar to the chemiluminescence (CL) process induced by chemical

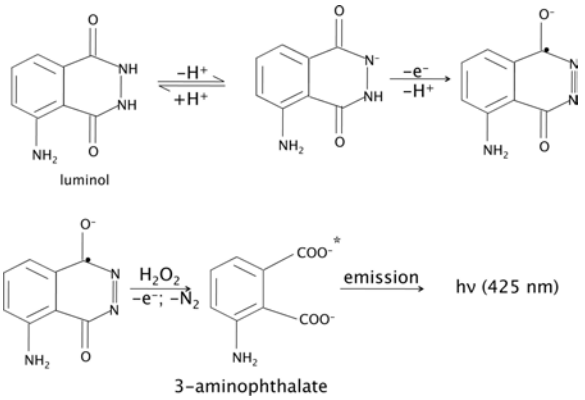

Figure 1. The mechanism of ECL reaction of luminol in the presence of H_2O_2 as the co-reactant.

oxidation (24, 25). The CL from luminol is well known and extensively used in forensic investigations. The first report of ECL from luminol was reported in the early 1950's (26), followed by studies of ECL at Pt electrodes (27). ECL from luminol was observed in alkaline media after electrochemical oxidation with hydrogen peroxide as a co-reactant (16). However, a recent work questioned the use of the co-reactants and showed that stable ECL from luminol without co-reactants is possible (28). Luminol can produce light under different experimental conditions, including different solvents, electrode materials, presence and absence of oxygen. The ECL is also dependent from the direction of the applied potential (anodic and cathodic) (15). The mechanism for the luminol ECL reaction in the presence of H_2O_2 is the following (Figure 1):

In alkaline media, luminol forms an anion, which is electrochemically oxidized. The oxidation of the azo-compound produces a 3-aminophtalate in excited state. Different pathways are involved during the oxidation processes, which depend on the applied potential. Hydrogen peroxide can participate as a HO_2^- or $O_2^{-\bullet}$. Then, deprotonation occurs only in alkaline media (29, 30). Many enzymes produce hydrogen peroxide during their catalytic reactions and hence, the combination with luminol could create a powerful tool for the detection of biological analytes at very low concentrations. So far, ECL from luminol is able to detect hydrogen peroxide at pM concentrations, however, when used in combination with enzymes, there are some severe limitations, since the enzymes suffer the strong basic environment of the solution. Therefore, for practical applications, a compromise has to be achieved. Luminol derivatives have been used extensively in labeling biomolecules. Recently, it has been demonstrated that ECL can be coupled with scanning electrochemical microscopy (SECM) to create a novel SECM/SECL (scanning electrochemiluminescence) technique, allowing the imaging of the biocatalytic activity of enzyme-polymer spots. In that work, Lei *et al.* developed a novel SECM/SECL apparatus (see Figure 2) for imaging the catalytic activity of GOx-resydrol derivative. Combination of SECM and ECL was made possible by integrating a photomultiplier tube (PMT) within a SECM setup which is mounted on the top of an inverted microscope. The H_2O_2 locally generated by the glucose oxidase (GOx)-catalyzed reaction, reacted with oxidized luminol that was simultaneously electrochemically generated at the positioned SECM electrode tip. By using a phase-sensitive lock-in amplifier, the potential applied to the SECM tip was swept to invoke an associated oscillation of the ECL. Images of the local immobilized enzyme activity obtained both by ECL and SECM were compared to elucidate the pathway in which the SECM and SECL signals are generated (see Figure 3).

4. ECL FROM CARBON-BASED MATERIALS

Carbon nanotubes (CNTs) have received considerable interest over the last decades due to their mechanical and exceptional electrical properties, which make them an attractive material for analytical applications (35-38). However, these applications require the solubilization of CNTs with polymers in order to form stable CNTs/polymer composites, which could then be cast onto electrodes (39, 40), as well as the concomitant immobilization of the luminophore. For instance, ruthenium complex derivatives are widely used in combination with CNTs for ECL detection of DNA. It is well known that sensitive and selective detection of DNA is central for clinical tests, pathogen detection, and for the detection of genetic disease based on oligonucleotide hybridization. The formation of CNTs/polymers/$[Ru(bpy)_3]^{2+}$ composites have been reported by Tao *et al.* (41). They reported the use of the Ormosil/MWNTs/$[Ru(bpy)_3]^{2+}$ composites for detection of herring sperm (HS) double-stranded (*ds*) DNA. They proposed the following pathway for ECL emission using $[Ru(bpy)_3]^{2+}$:

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

$$[Ru(bpy)_3]^{3+} + DNA(guanine) \rightarrow [Ru(bpy)_3]^{2+} + DNA^{+\bullet}(guanine_{ox})$$

$$[Ru(bpy)_3]^{3+} + DNA^{+\bullet}(guanine_{ox}) \rightarrow DNA^{2+}(guanine_{2ox}) + [Ru(bpy)_3]^{2+*}$$

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

DNA hybridization using SWNTs/$[Ru(bpy)_3]^{2+}$ has recently been reported by Li *et al.* (42). A thiol-modified ss-DNA self-assembled monolayer (SAM) was deposited on a gold electrode that was previously hybridized with target ss-DNA and probe DNA, respectively. The probe DNA was labeled with $[Ru(bpy)_3]^{2+}$-functionalized CNTs. This approach allowed the detection of perfectly-matched target ss-DNA at a picomolar level (30). Another interesting approach for detection of DNA has been recently reported by Zhang *et al.* (43). In this case, a thiolated hairpin DNA SAM was assembled on gold electrodes as the recognition element and a ruthenium complex (N-hydroxysuccinimide derivative) used as the luminophore. In the absence of target ss-DNA, the ECL probe immobilized on the gold electrode generates a strong ECL signal when it is in a folded configuration due to the vicinity of the termini to the electrode surface. Upon addition of target ss-DNA, the

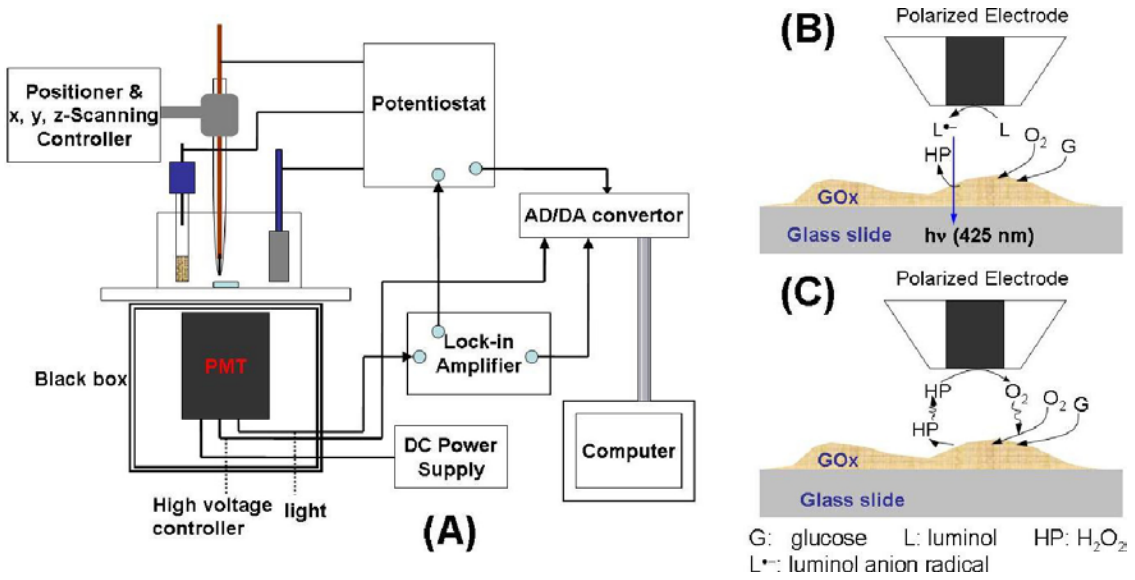

Figure 2. Schematic diagram of the SECM/SECL setup with integrated lock-in amplifier; (b) scheme of SECL and SECM (c) for studying local biocatalytic activity of enzyme−polymer spots. Reproduced with permission from Reproduced with permission from (34).

ECL probe on the electrode is converted into a double helix due to the hybridization, resulting in the tag moving far from the electrode surface. This caused a significant quenching of the ECL signal, which is associated with the increase of the target DNA. A detection limit up to 90 pM of complementary target ss-DNA was measured. Recently, Wei *et al.* reported a study regarding DNA detection via ECL, using a Nafion/MWNTs/$[Ru(bpy)_3]^{2+}$ composite (44). This investigation allowed the "label free" detection of DNA, and was based on the electrocatalytic detection of guanine and adenine bases. ECL signals of double-stranded DNA were obtained, allowing salmon-testes DNA detection at nanomolar concentration. In a similar way, other approaches involved the use of different polymers like sulfonated polystyrene/CNTs/$[Ru(bpy)_3]^{2+}$ composites, as recently reported by Li *et al.* (45). On the other hand, Fang *et al.* have described a novel ECL sensor by combining within the ECL an avidin/biotin-based systems with $[Ru(bpy)_3]^{2+}$ (46). This novel combination allowed the quantification of bovine serum albumin (BSA) up to nanomolar concentrations. Other analytes of clinical relevance such as glucose (47, 48), carbamate derivatives (49), and famotidine (50) have been quantified via ECL. For instance, an interesting approach for the detection of glucose has been designed by encapsulating glucose oxidase within a CNTs/Nafion/GCE (47). ECL was also employed for the detection of important neurotransmitters such as dopamine and epinephrine by quantitatively measuring the inhibition effect of the ECL signal using a Nafion/CNTs/$[Ru(bpy)_3]^{2+}$ electrode (51). This electrode configuration has been demonstrated to be useful for achieving the desired sensitivity (up to fractions of millimoles) and permselectivity, even in the presence of a 200-fold excess of ascorbic acid, which is the major interferent species in the determination of neurotransmitters. It was possible to achieve such result due to the ion exchange capabilities of Nafion. This polymer is negatively charged due to the presence of the sulfonic groups, which makes this approach effective in incorporating positively charged species, and at the same time, in repelling the negative charged species. ECL detection of glucose using a hybrid MWNTs/Pd NPs/Nafion/luminol composite has also been reported by Chen *et al.* (52). They were able to detect glucose in the range 0.5-40 μmol L^{-1} with a detection limit of 0.09 μmol L^{-1}. The mechanism of the ECL reaction is shown in Figure 4. An interesting immunoassay sensor for the detection of alpha-fetoprotein (AFP) has been reported by Wohlstadter *et al.* (53). They developed a novel sensor using a SWNTs/polyethylene vinylacetate (EVA)/$[Ru(bpy)_3]^{2+}$ composite. They designed a novel AFP immunoassay by functionalizing a streptavidin-coated nanotubes-EVA composite with biotinylated anti-AFP. This SWNTs/polyethylene vinylacetate (EVA)/$[Ru(bpy)_3]^{2+}$ electrode allowed the detection of AFP with a detection limit in the range 0.1-100 nM.

Similarly, Cai *et al.* developed an ECL-based lactate (LA) biosensor using luminol as signalling species (54). Lactic dehydrogenase (LDH) and pyruvate oxidase (PYOD) with nicotinamide adenine dinucleotide (NAD) as coenzyme were immobilized on CNTs. They were able to achieve a detection limit for lactate of 10 pM.

The recent discovery of graphene (55-57) seems to represent a further expansion of the use of carbon-based materials for biosensing (58, 59). Recently, Fan *et al.* reported the use of graphene oxide nanoparticles (GO NP) suspension for ECL detection of TPA (60). Interestingly, they were able to observe single ECL events from individual graphene oxide nanosheets (see Figure 5). In a subsequent work, Li *et al.* were able to produce a graphene/Nafion/$[Ru(bpy)_3]^{2+}$ composite and they tested this material for detection of TPA (61). The introduction of

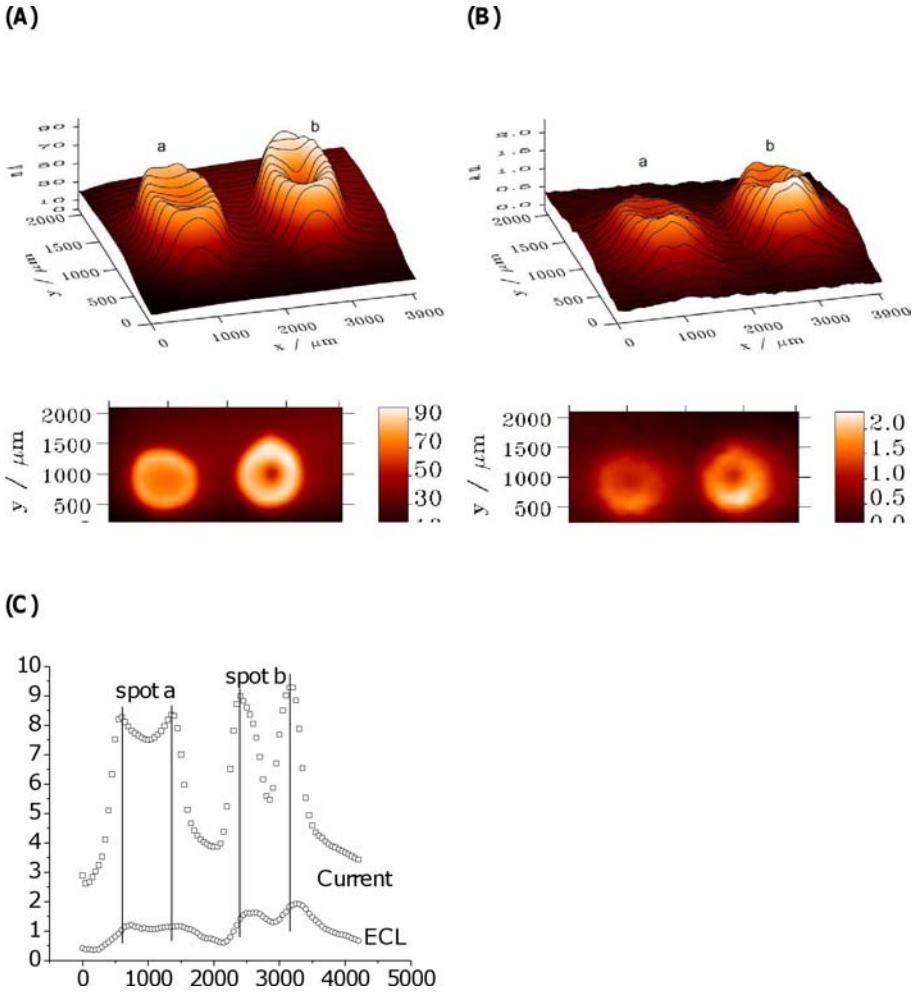

Figure 3. (A) Generation-Collection (GC)-SECM image of GOx−polymer spots in 100 mM glucose and 0.20 M phosphate buffer (pH 6.8); (B) SECL image of the same spots as SECM in 100 mM glucose, 1 mM luminol, and 0.20 M Tris−HCl buffer (pH 8.5) using a 250 μm Pt disk as tip electrode (RG 2.2). Amplifier parameters: OSC frequency, 3 Hz; amplitude, 0.3 V_{pp}; sensitivity, 5 mV; ac gain, 10 dB; (C) *x*-line scans through the spot centers from the SECM and SECL images in panels A and B. Reproduced with permission from (34).

graphene into Nafion facilitated the electron transfer of $[Ru(bpy)_3]^{2+}$ and at the same time improved the long-term stability and sensitivity. Concentrations of TPA up to 50 nM were easily achieved. Other examples involved the use of ruthenium bipyridyl derivatives covalently attached to CNTs for detection of TPA up to pM concentrations (62). Another approach involved the functionalization of carboxy-terminated MWNTs with a (2,2≠-bipyridyl)phenanthroline ruthenium(II) derivative. In this case, the carboxy-terminated groups of MWNTs were activated with succinimide and carbodiimide derivates and then bound, by mean of an amidic bond, with bis(2,2≠-bipyridine)-5-amino-1,10 phenanthroline ruthenium(II). The system showed excellent sensitivity toward the detection of micromolar concentrations of TPA (63). An interesting ECL study has recently been reported by Zheng *et al.* (64). In this work, the authors reported for the first time the electrochemical formation of water soluble carbon nanocrystals (C NCs) from graphite rods. The peculiarity of the C NCs is that during their electrochemical synthesis, an ECL signal at 535 nm was observed without the addition of co-reactants (see Figure 6). At the same time, ECL signal from C NCs was detected in the presence of 0.1 M $S_2O_8^{2-}$, as a co-reactant. This experimental evidence is relevant in chemical analysis because it suggests that C NCs might be suitable for use in ECL without the addition of co-reactant, opening novel applications in biosensing and imaging.

Lin *et al.* reported the fabrication of a novel ECL biosensor for the detection of hypoxanthine (65) (see Figure 7). Hypoxanthine (HX) is an essential metabolite for adenine nucleotide degradation, which is mainly accumulated in biological tissues. The determination of HX is important for the quality control of food. They used an electrically heated carbon paste electrode (HCPE) modified with xanthine oxidase (XOD). The use of heated electrodes is of great interest from both theoretical and practical points of view (66). This method allows the direct electrical

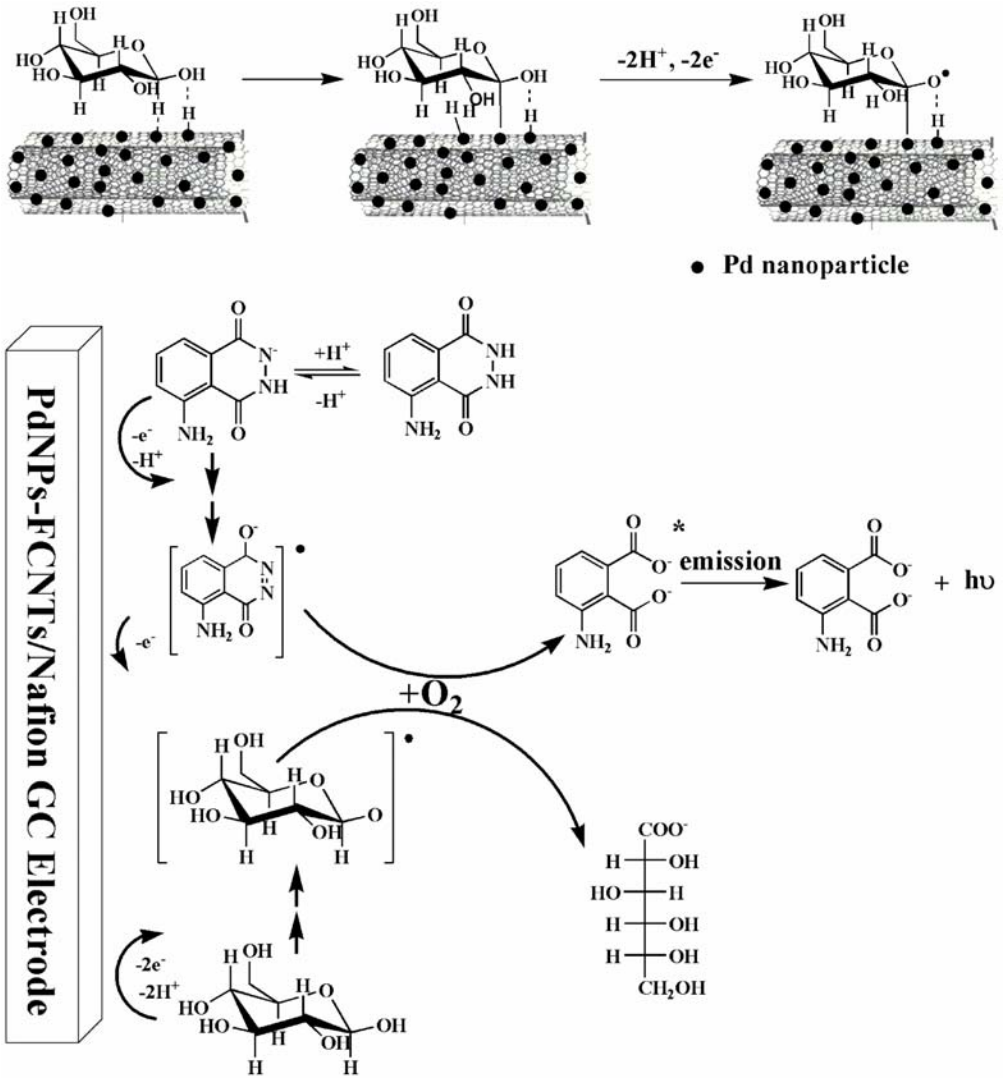

Figure 4. Mechanism of the glucose-luminol system at Pd NPs/CNTs/Nafion composite. Reproduced with permission from (52).

heating of the electrode, but leaving the bulk solution's temperature unchanged. The results showed an enhancement of the ECL intensity of luminol after addition of hypoxanthine (HX) to the solution, and a linear relationship between the ECL intensity and the concentration of HX. However, since the activity of XOD is highly dependent on temperature, the biosensor is very sensitive to the temperature of the electrode. Also, because the temperature of the electrode affects the diffusion and convection of the luminescent compounds near the electrode surface, as well as the catalytic activity of the enzyme, this has to be controlled and optimized carefully. The key feature of the designed biosensor is that the temperature of the electrode should be controllable, so the most suitable temperature for the enzyme reaction can be reached. The results showed that the ECL enzyme biosensor exhibited the best sensitivity at an electrode temperature of 35 °C for the detection of HX. The detection limit was 30-fold lower than that at room temperature (25 °C).

5. ECL FROM INORGANIC NANOTUBES

Beyond carbon-based materials, other inorganic-based nanomaterials have been employed in ECL detection. An interesting class of materials that is promising in ECL is constituted by TiO_2-derivative nanotubes (TNTs). This biocompatible material produced using a hydrothermal method (67) has recently been employed for the ECL detection of choline (68, 69) and clonazepam (70). Dai *et al.* developed a method to immobilize TNTs and choline oxidase on a chitosan modified GCE via electrostatic adsorption and covalent interaction, respectively (69). The enzymatic oxidation reaction of choline oxidase produced hydrogen peroxide, which was detected using a luminol-based ECL reaction. A scheme of the process is shown in Figure 8. The use of TNTs not only provided a biocompatible microenvironment for the immobilized enzyme, which resulted in an excellent stability and long lifetime of the biosensor, but also exhibited great

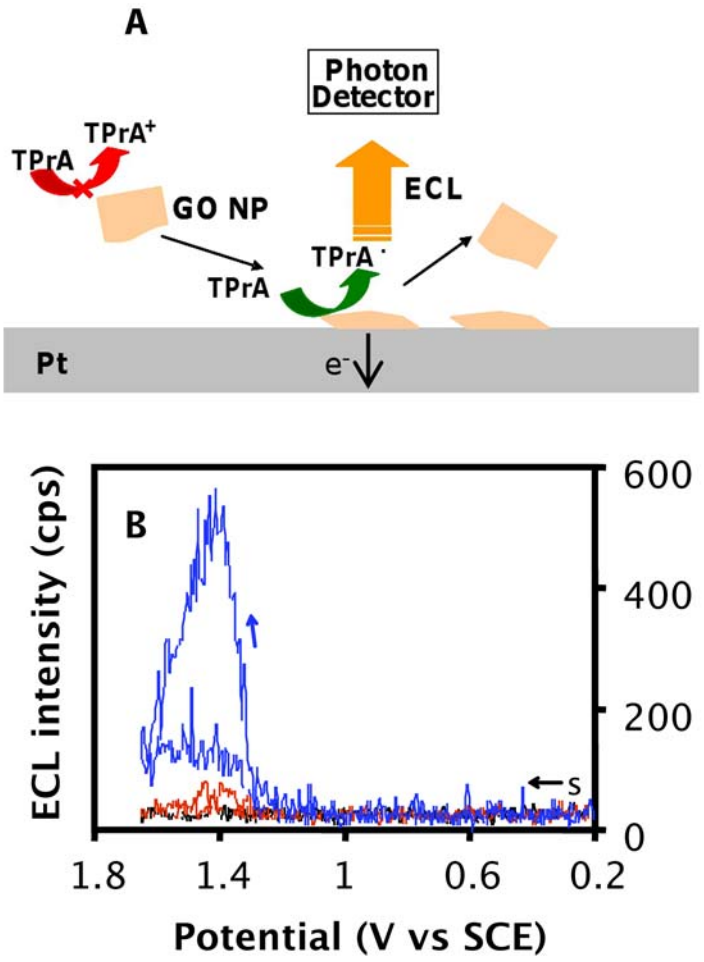

Figure 5. (A) Schematic diagram of the ECL mechanism of TPA at GO NP; (B) typical ECL spectrum of 13 mM TPA at graphene oxide. The solution contained 0.1 M $NaClO_4$ as the supporting electrolyte and phosphate buffer (pH 7.0). Reproduced with permission from (60).

enhancement of the ECL luminol signal with concomitant improvement in the ECL signal.

Other nanomaterials have been explored for potential use as ECL sensors: some of the examples include polyvinylbutiral/MWNTs/$[Ru(bpy)_3]^{2+}$ (71), halloysite-CNTs/poly-dopamine/]$Ru(bpy)_3]^{2+}$ (72), ZnO/CNTs (73), CNTs/glucose oxidase/Nafion (47, 48), and Co-porphyrin/MWNTs/$[Ru(bpy)_3]^{2+}$ composites (74). An interesting class of nanomaterial that has recently been reported is constituted by CdS nanotubes (75). The ECL from CdS nanotubes has been studied by Jie *et al.* (76): CdS nanotubes were entrapped in carbon paste electrodes and used for ECL detection of H_2O_2 in the presence of $S_2O_8^{2-}$ as a co-reactant. Interestingly, two ECL peaks were observed, and they were attributed to two different ECL mechanisms: the first one was attributed to the annihilation process between the reduced and the oxidized specie of CdS NTs, while the second one was attributed to the electron-transfer reaction between the reduced species of CdS NTs and the co-reactants ($S_2O_8^{2-}$ or H_2O_2). An interesting method for the production of Au nanoring for the detection of NADH has also been reported by Chovin *et al.* (77). They measured the concentration of NADH via ECL using $[Ru(bpy)_3]^{2+}$ as a luminophore. In this work, $[Ru(bpy)_3]^{2+}$ mediated the NADH oxidation and NADH acted as a co-reactant. The proposed method has also the advantage that allowed the imaging of the NADH oxidation at the Au nanorings.

6. ECL DETECTION FROM NANOPARTICLES

Nanoparticles (*i.e.* inorganic and organic nanoparticles) are receiving considerable attention over the last few years due to their optical, electrical, electrochemical and luminescent properties which make them attractive materials for applications in biosensing (78-81). The biocompatibility and low cost of silica nanoparticles (Si NPs) make them an attractive material for these applications. The use of Si NPs in ECL detection has been reported by Bae *et al.*(82): octadecyl-functionalized Si NPs deposited on ITO showed ECL in either the anodic and

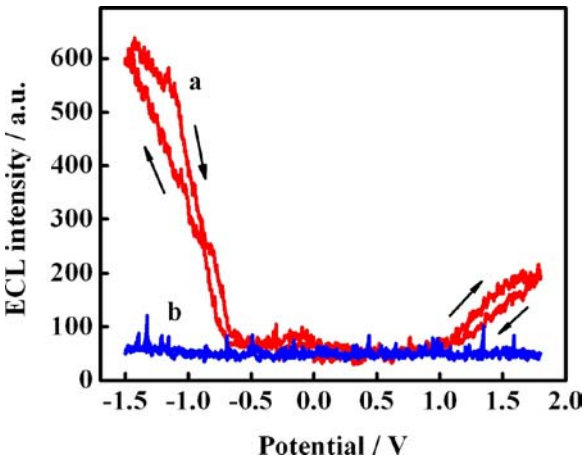

Figure 6. ECL responses with (red line, a) and without (blue line, b) C NCs at a Pt electrode in 0.1 M PBS (pH 7.0). The scan rate is 0.1 V s^{-1}. Reproduced with permission from (64).

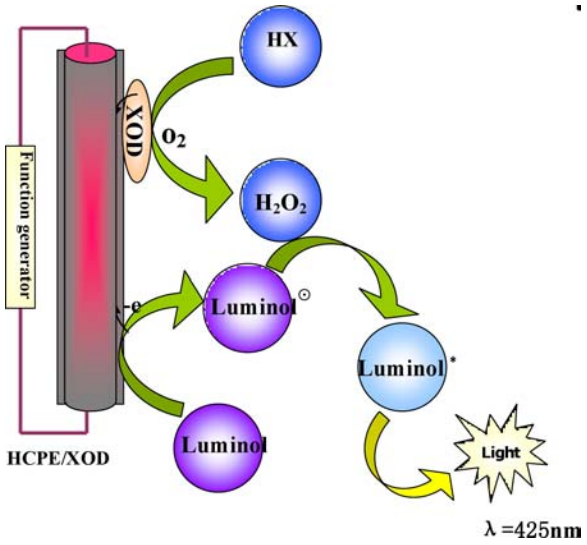

Figure 7. Schematic diagram of ECL biosensor for the detection of hypoxanthine. Reproduced with permission from (65).

cathodic sweep potentials using $S_2O_8^{2-}$ as a co-reactant. The elucidation of the mechanism of ECL emission remains to be clarified even though the cathodic ECL has been attributed to the holes injection from the sulfate radicals ($SO_4^{\bullet}$) to the valence band of negatively charged Si NPs. In another work, Qian *et al.* reported the one-step synthesis of $[Ru(bpy)_3]^{2+}$-functionalized Si NPs: they were able to detect TPA with a detection limit of 50 nM (83). An interesting work from Zhang *et al.* reported an investigation on the use of $[Ru(bpy)_3]^{2+}$-functionalized nanoparticles (Ru NPs) conjugated with chitosan for detection of TPA (84). Ru NPs exhibited remarkable sensitivity with detection limits for TPA up to nanomolar concentrations. Zhang *et al.* reported an interesting method to assembly Si NPs with $[Ru(bpy)_3]^{2+}$: they co-immobilized $Ru(bpy)_3^{2+}$-silica NPs (RuDS) with MWNTs using hydrophobic interactions (85). MWNTs were used to either immobilize RuDS, and facilitate the electron transfer between $[Ru(bpy)_3]^{2+}$ and the electrode. Detection limits of TPA up to nM concentrations were obtained. Another efficient platform for ECL detection has been reported by Zhou *et al.* (86). They fabricated $[Ru(bpy)_3]^{2+}$-Nafion nanofibers using a one-step electrospinning technique. These nanofibers formed a porous 3-D nanostructure and possessed a high surface-area-to volume-ratio and therefore a larger amount of $[Ru(bpy)_3]^{2+}$ was deposited. This method was tested for the ECL detection of phenolic compound with a detection limit of 1 nM in the case of phenol. Yang *et al.* reported the fabrication of a novel sandwich-type ECL immunosensor constituted by $[Ru(bpy)_3]^{2+}$-doped Si NPs to label a secondary antibody (87).

Carboxylated-terminated MWCNTs were deposited on the electrode surface and bonded with avidin. Then, biotinylated antibodies were immobilized on the surface of the electrode by employing biotin/avidin interactions. Later, the electrode was incubated with IgG antigen and then with the secondary antibody which was labeled to $[Ru(bpy)_3]^{2+}$-doped Si NPs. A strong ECL signal was obtained and the amplification analysis of protein interaction was achieved with linear range between 0.05-200 ng mL^{-1} and detection limit of the order of pg mL^{-1}.

Bae *et al.* developed a novel ECL sensor for the detection of DNA using a dendritic signal amplification strategy (88). They used two different $[Ru(bpy)_3]^{2+}$-doped Si NPs probes coated with complementary DNA, which was self-assembled as a sandwich-like type dendritic architectures on an Au grid. This dendritic amplification route was tested in conjunction with the ECL detection of the target DNA. This method allowed a 5-fold enhancement of the ECL signals compared to other amplification methods. The higher sensitivity allowed by the dendritic amplification route was attributed to the hybridization between the DNA and the complementary DNA on the additional probe. Detection limits as low as 1 fM of target DNA were achieved.

Liu *et al.* reported the development of an Au NPs/GOx composite film for ECL detection of glucose. In this study, Au NPs were deposited on a 3-D network of silica network and glucose concentrations in μM range were detected using luminol as a co-reactant (89).

Core-shell Si NPs constituted by magnetic iron oxide particles coated with silica have been used as a novel platform for ECL detection using TPA as co-reactant (90). These nanoparticles showed good stability and biocompatibility associated to high selectivity. Magnetic nanoparticles are widely used in chemical analysis. For instance, paramagnetic beads are promising materials for the detection of *Clostridium Botulinum* toxins in food (91): streptavidin-coated paramagnetic beads were bound to biotinylated serotype-specific antibodies. A ruthenium chelate labeled anti-serotype antibody acted as a luminophore species for ECL emission. This setup allowed the detection of this toxin up to pg mL^{-1} concentrations.

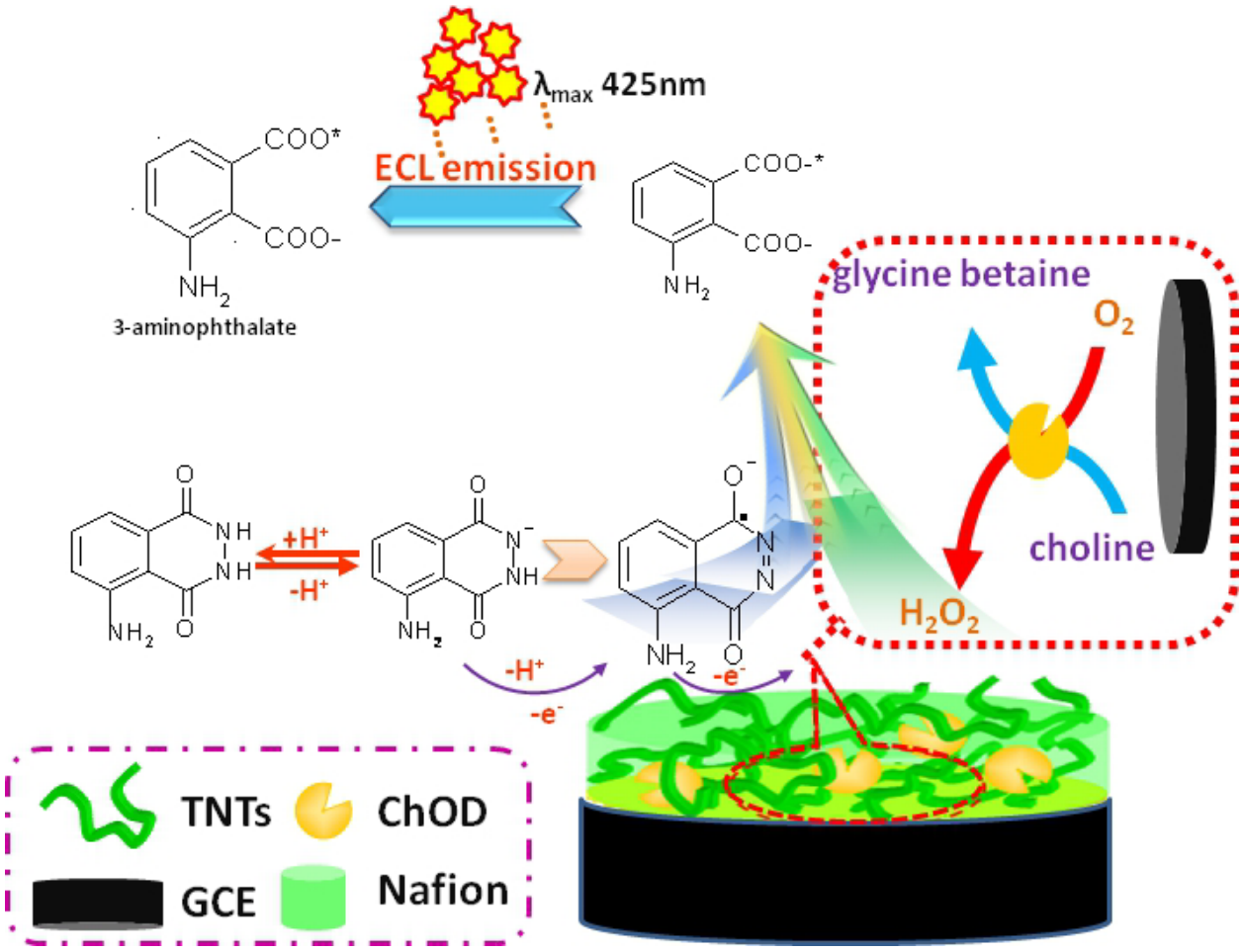

Figure 8. Schematic diagram of the TNTs-based ECL biosensor. Reproduced with permission from (69).

Magnetic Fe NPs were also used as an immunosensor for DNA detection (92).

Du *et al.* reported the synthesis of Pt NPs within a polymer membrane such as Eastman AQ55D for ECL detection of TPA (93). They claimed that the electroactivity of Pt NPs and the permselectivity properties of the Eastman AQ55D membrane allowed the detection of TPA up to fM concentrations. Zhang *et al.* reported the fabrication of thin films of Si and Au NPs for ECL detection of TPA (94). Detection limits up to 10^{-8} M were achieved. Ding *et al.* reported the fabrication of a $[Ru(bpy)_3]^{2+}$-zirconia-Nafion-based composite film for detection of pharmaceuticals (lidocaine, tramadol, ofloxacin) coupled with an electrophoretic microchip for applications in clinical diagnostics (95, 96). Bringing adequate selectivity is one of the major issues for clinical applications. This can be achieved by immobilizing biomolecules and/or enzymes for specific reactions. An interesting approach has been reported by Zhang *et al.* They reported the incorporation of alcohol dehydrogenase (ADH) within $[Ru(bpy)_3]^{2+}$-Au NPs using the layer-by-layer method (97). They fabricated an ECL biosensor for the detection of alcohol. A positively charged $[Ru(bpy)_3]^{2+}$ species was immobilized on ITO electrodes via electrostatic interaction with negatively charged Au NPs. Then, Au NPs were bounded with the amine and cysteine residues of the ADH enzyme, facilitating the electron transfer and allowing the ECL detection of ethanol. This concept is very interesting, since it could be extended to other enzymes as appropriate. ECL has been used recently as a bio-bar code assay for the detection of genetically modified organisms (GMO): Zhu *et al.* described a polymerase chain reaction (PCR)-free ECL bio bar code based on oligonucleotide-modified Au NPs (98). The systems consisted of $[Ru(bpy)_3]^{2+}$-labeled bar code DNA (TBR), nucleic acid hybridization using Au-NPs and biotin-labeled probes, and a selective capture of the hybridization complex using streptavidin-coated paramagnetic beads. The detection of target DNA was achieved by measuring the ECL emission from TBR. This configuration allowed the detection of target nucleic acids with high speed and sensitivity as well as the detection of GMO fragments from real GMO products. Compared with the PCR bio bar code method, the ECL-based bio bar code method is faster and simpler, since it avoids the use of expensive PCR instrumentations as well as the multiple hybridizations and all the washing steps used in the other PCR-based assays (99). This method can directly detect target DNA fragments from raw materials and, importantly, it does not require tedious purification procedures.

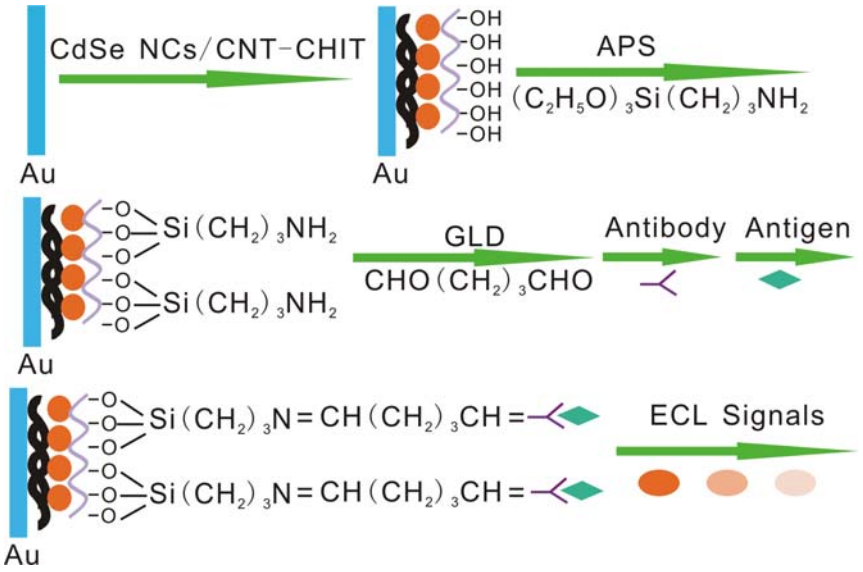

Figure 9. Schematic diagram of the CdSe QDs/MWNTs-based ECL immunosensor for the detection of IgG. Reproduced with permission from (119).

Nanoparticles composed by organic materials such as conducting polymers have recently been reported. Chang *et al.* reported on the development of a novel setup based on the ECL detection of a single polyfluorene derivative (F8BT) nanoparticle (100). They used a novel single-molecule spectroelectrochemistry (SMS-EC) technique described by the same authors in their previous works (101, 102). Because in the ECL process the emission is triggered electrochemically, and the signal-to-noise ratio is high with low background currents, this approach could provide the basis of a new and highly sensitive detection method in chemical analysis. Following this work, these authors recently reported on the ECL emission induced by single-particle collisions (103). Fan and Bard investigated the ECL emission from a single-particle (Pt NPs) collision event. The collision of a single Pt NP deposited on an ITO electrode generated an enhancement of the ECL signal. This amplification is possible because the system allows the discrimination of events occurring at the substrate from those at the Pt NP. A large ECL amplification during the oxidation of $[Ru(bpy)_3]^{2+}$ in the presence of TPA was observed. In this case, each Pt NP collision is characterized by a unique photon spike, which is dependent on the frequency and amplitude, and therefore, on the size and concentration of Pt NP. The high sensitivity provided by the SMS-EC can be translated in future developments of new and promising applications in chemical analysis.

7. ECL FROM QUANTUM DOTS

Quantum dots (QDs) or semiconductor nanocrystals are a very attractive class of materials because of their excellent luminescent properties and applications in many areas of nanotechnology, such as medical diagnostics, imaging, and chemical sensing (104-111). Recent works from the Bard's group showed that QDs are electrically excitable in both nonaqueous (82, 112, 113) and aqueous media (114, 115). For instance, CdSe QDs can be oxidized and reduced during the potential scan based on the following mechanism (82):

$$R^{\bullet -} + R^{\bullet +} \rightarrow R^* + R$$

$$R^* \rightarrow R + h\nu$$

The ECL emission occurs when the electrogenerated reduced species, $R^{\bullet -}$, collides with the oxidized form, $R^{\bullet +}$, producing R* by the annihilation process as described previously. Since the emission spectra of QDs are size dependent, it is expected that the ECL behavior will be size dependent too. This was in fact demonstrated by Liu *et al.* (116). The dependence of the ECL intensity with the size of the particles is related to the band gap of QDs, which increases with the decrease of the QDs size (117).

Recently, interesting reports on ECL from QDs applied to chemical analysis have been published in the literature. For instance, Jang *et al.* reported on the ECL detection coupled with enzymatic reactions (118). A thioglycolic acid (TGA)-protected CdSe QDs/glucose oxidase (GOx) composite was formed for the detection of glucose via ECL. Glucose was detected by measuring the quenching of the ECL response from the composite after addition of glucose. Significantly, this method could potentially be extended to other classes of enzymes for specific applications.

An ECL immunosensor for detection of human immunoglobulins (IgG) has been reported. Jie *et al.* developed this immunosensor using a CdSe QDs/MWNTs-chitosan/3-aminopropyl-triethoxysilane (APS) composite (see Figure 9) (119). Due to the presence of reactive amine groups, this system could be used as an efficient cross-linker for the conjugation of biomolecules, such as

antibody immobilization. To increase the ECL signal, $S_2O_8^{2-}$ was used as a co-reactant. The experimental data showed that APS catalyzes the reaction of CdSe QDs with $S_2O_8^{2-}$ based on the following mechanism:

$CdSe + ne^- \rightarrow nCdSe^{-\bullet}$

$S_2O_8^{2-} + RC_3H_7NH_2 \rightarrow SO_4^{2-} + SO_4^{-\bullet} + RC_3H_7NH_2^{+\bullet}$

$CdSe^{-\bullet} + SO_4^{-\bullet} \rightarrow CdSe^* + SO_4^{2-}$

$CdSe^* \rightarrow CdSe + h\nu$

The results also showed comparable values with those obtained via the ELISA method as an indication of the suitability of such method (99).

A recent investigation reported the use of CdSe QDs/Au NPs/anti-PAB composite as a non-labeled ECL immunosensor for detection of human prealbumin (PAB, antigen) (120). In this work, the immunosensor measured the inhibition of the ECL reaction on the CdSe QDs (with $S_2O_8^{2-}$ as a co-reactant) operated by the immunocomplex. This method allowed the detection of PAB at ng mL^{-1} concentrations.

TGA-protected CdSe QDs were also used for the ECL detection of thiols (121). It is believed that the intermediate $OH^{\bullet}$ radical is the key specie for producing holes-injected QDs. Thiol compounds have been used as model molecules for $OH^{\bullet}$ radical annihilation and to investigate the quenching effects on the ECL emission. In the case of QDs it has been demonstrated that both O_2 and H_2O_2 can act as a co-reactants in the ECL process. The related ECL mechanisms are the following (122):

$O_2 + H_2O + 2e^- \rightarrow HO_2^- + OH^-$
$2CdS^{-\bullet} + HO_2^- + H_2O \rightarrow 3OH^- + 2CdS^*$
$2CdS^{-\bullet} + H_2O_2 \rightarrow 2OH^- + 2CdS^*$
$CdS^* \rightarrow CdS + h\nu$

The thiols derivatives investigated were γ-*L*-glutamyl-*L*-cysteine-glycine, GSH and *L*-cysteine that are biomolecules involved in many biological processes such as Parkinson's and Alzheimer's diseases (123). The results showed high sensitivity, suggesting the suitability of such method for the simultaneous detection of both scavengers and generators of hydroxyl radicals in clinical samples.

In another work, Jie *et al.* reported on the use of a thioacetic acid-(RSH)-protected CdS QDs/cysteamine/Au NPs composite for the detection of low-density lipoproteins (LDL) using $S_2O_8^{2-}$ as a co-reactant (122). This system was used for the detection of LDL proteins by measuring the quenching of the ECL signal resulting from the specific interaction of the LDL with a LDL receptor ligand (apoB100). This approach allowed the detection of LDL proteins at extremely low concentrations (0.025-0.16 ng mL^{-1}) with the possibility to reach detection limit of 0.006 ng mL^{-1}.

Recently, a novel method for preparing polyamidoamine (PAMAM, generation 4 containing 64 amino groups, G4)-protected CdS QDs has been reported. Lu *et al.* developed an *in-situ* induction precipitation method followed by electrochemical reduction (124). This method showed a significant increase of the ECL signal in the presence of $S_2O_8^{2-}$, compared to bare CdS QDs. Interestingly, the proposed method showed that the ECL signal can be modulated and stabilized by changing the duration time of the potential pulse and using high scan rates.

Other approaches reported the combination of QDs with CNTs. For instance, CdS QDs/CNTs composites have shown to be a useful platform for ECL detection. Chen *et al.* reported the synthesis of CdS QDs/CNTs composite for ECL detection in the presence of H_2O_2 (125). Similarly, ECL of ZnS NPs in alkaline aqueous solution in the presence of $S_2O_8^{2-}$ as co-reactant has been reported by Shen *et al.* (126).

For the use of QDs in medical diagnostics, it is of crucial importance to produce QDs stable in aqueous solutions. ECL from water soluble CdTe NCs has been recently reported (107). Liu *et al.* produced mercaptopropionic acid-capped CdTe QDs for ECL detection of neurotransmitter (cathecol) derivatives (107). The ECL emission involved the generation of superoxide ions at the ITO electrode surface, which then injected an electron into CdTe NPs to form a CdTe anion species. The collisions produced between the CdTe anion species and the oxidation products led to the formation of species in an excited state that emitted light at around 580 nm.

The detection of cathecols was achieved by measuring the quenching of the ECL using the electro-oxidized products of cathecols (dopamine and *l*-adrenalin). Interestingly, common interferences such as the uric and ascorbic acids did not quench significantly the ECL emission from CdTe QDs (107). It has also been demonstrated that the quenching mechanism is operated via an ECL energy-transfer rather than a charge transfer process.

TGA-protected CdTe NCs were also produced for the ECL determination of H_2O_2 in aqueous solutions (127). The ECL emission intensity was linear with H_2O_2 concentration in the range between 0.2-10 μM with a detection limit of 0.06 μM. Noticeably, the ECL spectrum of TGA-capped CdTe NCs exhibited a peak at around 620 nm, which was red-shifted of about 50 nm with respect to the photoluminescence peak, suggesting that surface states were playing an important role in the ECL emission.

8. ECL FROM POLYMER AND METAL COMPLEXES THIN FILMS

One of the major disadvantages on using solution phase reactants is the loss of signal due to diffusion of the ECL reagent out of the detection zone, as well as its consumption. To overcome these problems, several strategies have been developed in order to immobilize the

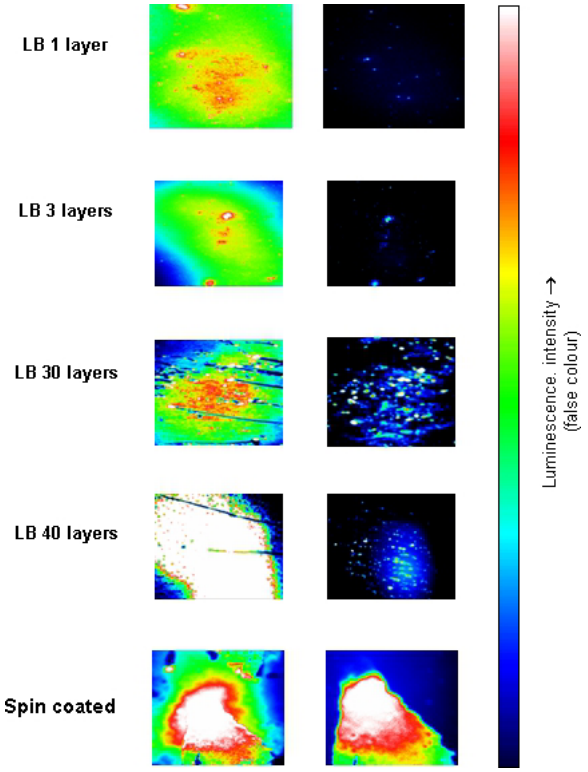

Figure 10. Epifluorescence images of LB-Nafion films with increasing number of layers (1, 3, 30, and 40) and spin-coated Nafion film before (left side) and after (right side) application of 1.2 V. The right side images were acquired 1 s after the oxidizing potential step for the LB films and 20 s for the spin-coated Nafion film. The films were loaded in 10^{-3} M $[Ru(bpy)_3]^{2+}$. The size of the imaged area is 300 × 300 μm^2. Reproduced with permission from (132).

luminophores at the electrode surface. Among the immobilization methods to anchor the luminophore to the electrode surface, the self-assembly (SAM) and the Langmuir-Blodgett (LB) methods have received particular attention.

Dennany *et al.* reported an investigation on $[Ru(bpy)_2dcb]^{2+}$ SAMs deposited on optically transparent fluorine doped tin oxide (FTO) electrodes, where dcb is 4,4-dicarboxy-2,2-bipyridine and bpy is 2,2´-bipyridine (128). The surface coverages were in the range of 10^{-10} mol cm^{-2}, which are consistent with those expected for a close packed monolayer. A clear ECL response was observed in the presence of oxalate and amino acid derivatives as the co-reactants. Interestingly, proline derivatives were the most efficient species for generating ECL. The ECL linear range was between 0.2 to 10 nM.

Other ruthenium complex derivatives were assembled as SAM on electrode surfaces: for instance, Bertoncello *et al.* reported a study of SAMs of $[Ru(bpy)_2(bpySH)](PF_6)_2$, deposited on Pt micro and macroelectrodes (129). These SAMs displayed luminescence properties similar to those corresponding to powder samples. Significantly, efficient ECL was generated using tripropylamine as the co-reactant.

The ability to fabricate nanostructured films is an important goal for the production of devices and sensors. Bertoncello *et al.* described a novel procedure to incorporate $Ru(bpy)_3^{2+}$ into Nafion Langmuir-Schaefer (LS) (130, 131). In this case, Nafion LS films with incorporated $Ru(bpy)_3^{2+}$ were fabricated using a novel "one-step" procedure that allowed $Ru(bpy)_3^{2+}$ to be directly incorporated during the formation of the film at the air-water interface. ECL was then demonstrated for a 6 nm thick Nafion LS films and interestingly, it was shown that thinner the films, higher the ECL signal. This behavior was explained in terms of electron hopping between the oxidized and the reduced ruthenium species, which is significant in thicker films. Whereas, for thinner films the generation of the ECL reagent occurs rapidly and the limitation switches to become diffusion in solution. Moretto *et al.* investigated LB films of Nafion using epifluorescence imaging (132). They showed that lowering the film thickness down to nanometric level is effective in shortening the switching time and the apparent diffusion coefficient in the Nafion LB film. Typical epifluorescence imaging of Nafion LB films with incorporated $[Ru(bpy)_3]^{2+}$ are shown in Figure 10.

The possibility to immobilize DNA with incorporated a luminophore directly on the electrode surface has been investigated (133). For instance, Miao *et al.* immobilized single-stranded DNA (ssDNA) on an Au electrode, followed by hybridization with the complementary target strand of ssDNA tagged with the luminophore $[Ru(bpy)_3]^{2+}$. The electrode was placed in a solution or flow cell containing a co-reactant. ECL generation was triggered by applying a potential pulse. This method was used for the detection of DNA derived from the *Bacillus Anthracis*, and TPA was used as a co-reactant. Also, this method has been used for the detection of biomolecules of clinical relevance. C-reactive protein (CRP) is demonstrated to be as one of the key markers of inflammations as well as of myocardial infarction. Using a biotynilated anti-CRP onto an Au electrode modified with avidin, Miao *et al.* quantified CRP in human plasma and serum. CRP and anti-CRP tagged with $[Ru(bpy)_3]^{2+}$ labels were then conjugated to the surface, followed by immersion of the modified electrode in a TPA solution for ECL generation (133). Similarly, the same authors detected DNA hybridization using polystyrene microbeads and an unsoluble $[Ru(bpy)_3]^{2+}$ derivative (134). The scheme of the procedure utilized for the ECL detection is shown in Figure 11, while a typical SEM image of the polystyrene microbeads after DNA hybridization is shown in Figure 12, respectively.

Recently, Zhang *et al.* reported an investigation on the DNA sensor for the sequence-specific DNA detection using GOx-based biocatalyzed ECL and non-fouling surface (135). In this approach, a glucose oxidase labeled sandwich-type DNA sensor was built on a non-fouling surfaces made of a mixed SAMs incorporating

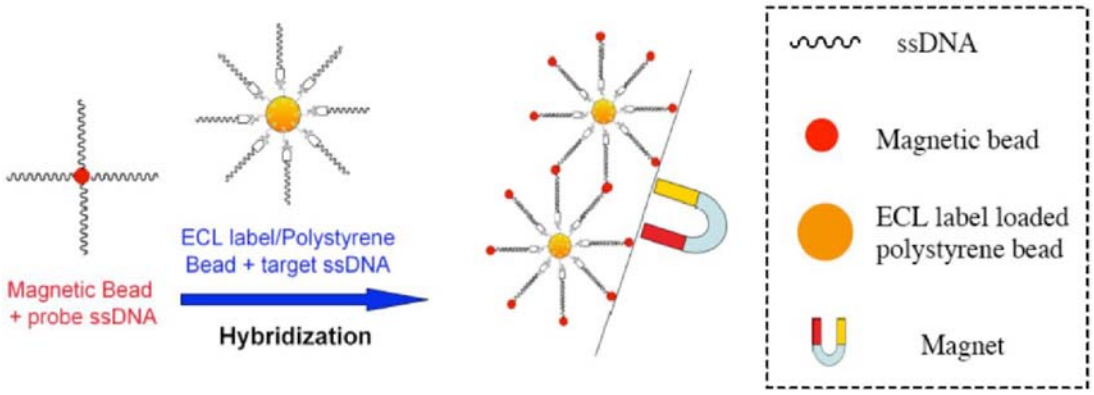

Figure 11. Schematic diagram of DNA hybridization on a polystyrene bead as the ECL label carrier and a magnetic bead for the separation of analyte-contained ECL label/polystyrene beads. Reproduced with permission from (134).

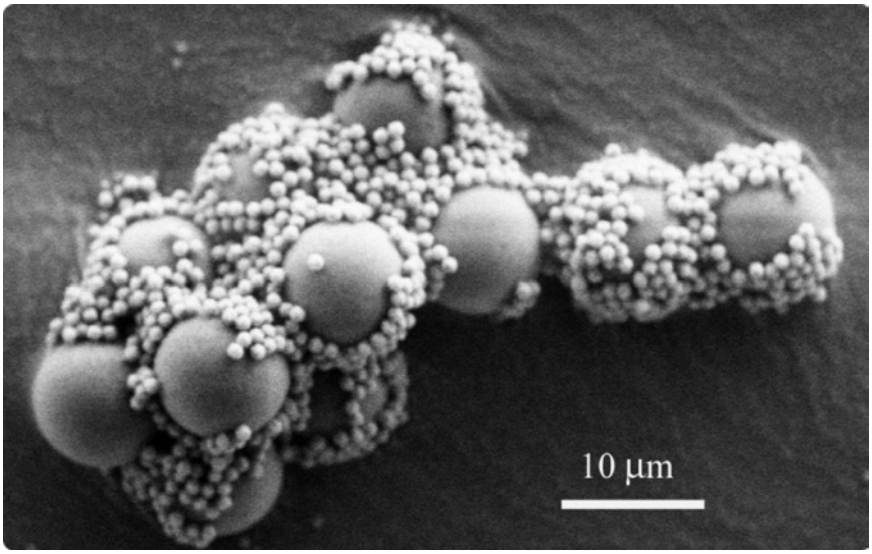

Figure 12. SEM image of polystyrene microbeads after DNA hybridization between probe DNA-MB and complementary DNA-Ru(concentrations of both DNA were 5 μM, and the size of the PSB and the MB was 10 μm and 1.0 μm, respectively. The ratio of the MB to PSB was 29. Conductive carbon tape was used as the substrate, and the specimen was precoated with a 300-nm Cr thin film and imaged with a LEO 1530 scanning electron microscope at 4 kV. Reproduced with permission from (134).

thiolated oligonucleotides and oligo(ethylene glycol) (OEG) thiols (SH-DNA/OEG). The sequence-specific DNA sensing was accomplished by the ECL signal of luminol with H_2O_2 generated *in situ*. The protein-resistant non-fouling surfaces significantly suppressed the non-specific adsorption of the enzyme label on electrode and reduced the background noise of the sensor. The sensor showed the ability to detect up to 1 pM of target DNA. In complicated biological fluids such as human serum, this non-fouling platform-based sensor also revealed superior performance over conventional sandwich-type DNA sensors. An interesting approach to immobilize luminophores on the electrode surface has been developed by Dennany *et al.* (136). They synthesized a novel metallopolymer, $[Ru(bpy)_2(PVP)_{10}]^{2+}$, where bpy is 2,2´-bipyridyl and PVP is poly(4-vinylpyridine). This metallopolymer has been studied with oxalate, TPA and other small molecules as the co-reactants. A key aspect of the metallopolymer thin films is that the concentration of the luminophore (Ru) is higher (typically at molar concentration) than in solution, (micromolar concentration). A disadvantage is that the high concentration of the luminophore in the film may be a limiting factor since the self-absorbance can be significant and therefore affecting the overall luminescence efficiency. Also, self-quenching phenomena may introduce some significant limitation. For instance, the emission intensity decreased by about 80% when just a fraction, 20%, of a $[Ru(bpy)_2(PVP)_{10}]^{2+}$ film is oxidized, *i.e.*, the loss of emission intensity greatly exceeds the expected one on the basis of the reduced luminophore concentration due to the quenching effect of Ru^{3+}. This effect can be overcome

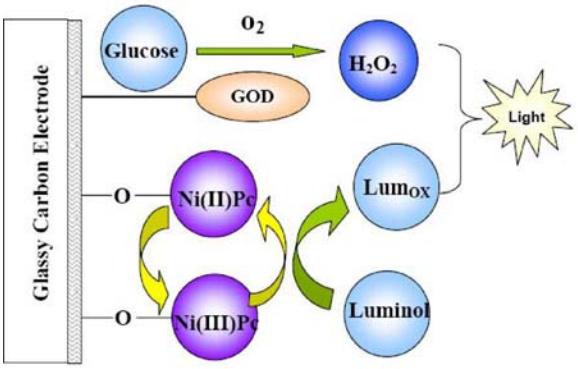

Figure 13. The principle of the Ni(II)Pc/GOx/luminol ECL biosensor. The marked layer on the GCE corresponds to MWNTs. LumOX is the product of the oxidation of luminol. Reproduced with permission from (153).

decreasing the loading of the ECL generating reagents in order to minimize the quenching. The immobilization on electrode surfaces brought significant positive benefits, for example, the overall efficiency of the ECL reaction for the metallopolymer film is almost four times higher than that for $[Ru(bpy)_2(PVP)_{10}]^{2+}$ dissolved in solution (128). A complementary method to improve the ECL efficiency has recently been reported by Devadoss *et al.* (137). They fabricated DMAP-functionalized Au NPs/$[Ru(bpy)_2PVP_{10}](ClO_4)_2$ composite and studied the ECL behavior in the presence of TPA. They found that, despite Au NPs quenched the metallopolymer emission, the ECL signal was enhanced of about three times compared to the pure metallopolymer.

Metallopolymers have found some interesting applications in the ECL detection of DNA. Dennany *et al.* investigated the use of the metallopolymer $[Ru(bpy)_2PVP_{10}](ClO_4)_2$ as the luminophore, using guanine as a co-reactant (138). Ultrathin films (~10 nm) of the metallopolymer were assembled with DNA using the layer by layer (LBL) method. Significant ECL was observed when the guanine bases were present in the oligonucleotide films. It has been suggested that the ECL mechanism involves the interaction of guanine radicals with Ru^{3+} to generate the Ru^{2+*} excited state, or the reduction of Ru^{2+} to Ru^{+} by the guanine radicals, followed by annihilation of Ru^{3+} and Ru^{+} as seen previously. In a similar way, a study related to the DNA damage has been reported by changing the metal centre from ruthenium to osmium (139). The redox potential of the osmium polypyridine centers is approximately 500 mV less positive than for ruthenium. Therefore, the osmium polymer does not generate an ECL signal in the presence of guanine. However, due to its lower oxidation potential, the oxo-guanine specie is more easily oxidized than guanine. Thus, oxo-guanine can reduce the electrogenerated Os^{3+} sites to produce electronically excited Os^{2+}* centers that emit approximately at 700 nm. This approach for detecting oxidative and chemical damage within DNA has also been extended to oxo-adenine (138, 139). Thin films of DNA, $[Ru(bpy)_2(PVP)_{10}]^{2+}$ and $[Os(bpy)_2(PVP)_{10}]^{2+}$ reveal that Os^{2+} centres can selectively catalyze oxo-guanine, while the higher oxidizing potential of Ru^{2+} sites with a formal potential of approximately +1.1 V are capable of oxidizing both oxo-adenine and guanine. Other studies reported the use of thin films of different polymers and composites. Iridium complex derivatives have shown to be a promising class of material for ECL detection. For instance, $[Ir(ppy)]_3$ and$[Ir(btp)_2(acac)]$ incorporated in polymers display ECL upon application of a suitable positive potentials in the presence of TPA as a co-reactant (140). Other relevant systems include orthometallated iridium (III) complexes. It has been demonstrated it is possible to tune the excited states and generate efficient ECL, by changing the ligands attached to the metal center, (140, 142). However, the studies related to the osmium complexes for ECL production are somehow limited due to the intrinsic limitations, such as a larger spin–orbit coupling, which results in shorter excited state lifetimes and weaker emission (143). However, osmium compounds possess some advantages over ruthenium compounds: they are more photostable and they usually oxidize at less anodic potentials (144), and the longer emission wavelength may be more suitable for other specific analytical applications. Poly(luminol) films including its derivatives have been shown as interesting materials for ECL (145-149). An interesting approach has been developed by Li *et al.* (150). The authors prepared a composite film of poly(luminol-benzidine) (PLB) on the graphite electrode by electropolymerization of luminol and benzidine in acidic medium. They found that PLB composite film presented better ECL performances for H_2O_2 than the bare poly(luminol) film. Interestingly, the polymeric 3-aminophthalate presented higher fluorescence quantum yield than the pure poly(luminol) film. They were able to detect concentrations of hydrogen peroxide in the μM range. Sassolas *et al.* successfully incorporated an enzymatic matrix such as choline oxidase (ChOD) within poly(luminol) film for the detection of choline (151). They detected choline in the linear range 80 nm-130μM (152). In another work, Qiu *et al.* immobilized GOx within films of Ni(II)tetrasulfonated phthalocyanine/MWNTs (see Figure 13), reaching detection limit of 80 nM for glucose (153). Dai *et al.* reported a graphite/poly(methylmethacrylate) (graphite/PMMA) composite for detection of vitamin C based on luminol ECL (154). They observed the inhibition of the ECL when vitamin C was added. Their method allowed the detection of vitamine C up to 10 nM.

9. MISCELLANEOUS

Detection of DNA using fluorescence-based DNA chips are probably one of the most used tool for gene analysis. However, this approach requires expensive equipment and often tedious procedures for labelling. In the late 90's, interesting works from Kulmala *et al.* on ECL production from the injection of tunnel-emitted hot electrons and from a pulse-polarized oxide-covered aluminium electrode into aqueous Tb(III) ion solution were reported (155). The same authors reported cathodic ECL generated from oxide-covered aluminium electrodes in the presence of lanthanide(III) (156) and rare earth(III) chelates (157), luminol (158) and $[Ru(bpy)_3]^{2+}$ (159). The cathodic ECL induced by hot electrons is a similar process to the

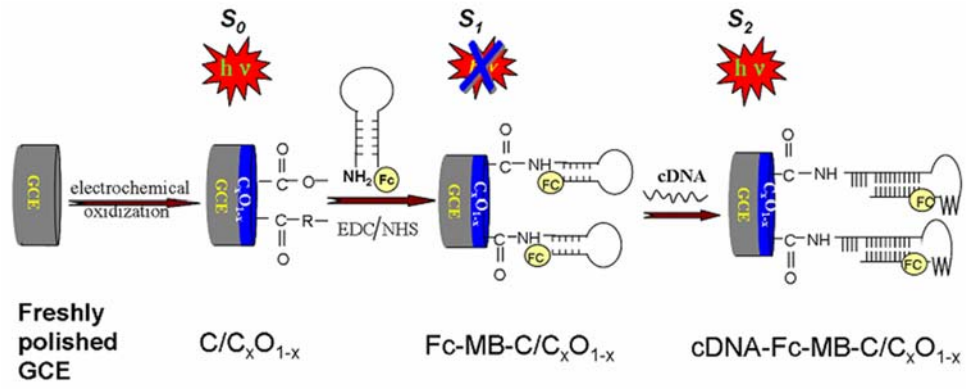

Figure 14. Schematic diagram of the reagentless Fc-MB DNA biosensor. Reproduced with permission from (162).

conventional ECL with the exception that the light emission is initiated using hot energetic electrons generated from an oxide thin film-covered electrode under cathodic pulse polarization (160). Typically, Al/Al_2O_3 and Si/SiO_2–based electrode substrates are used to generate energetic electrons by direct tunneling during the cathodic pulse polarization. A nanometer scale thick oxide film (*ca.* 5 nm) is required (158, 159, 161).

The most significant advantages of the cathodic ECL consist in the low detection limits and wide linear ranges achieved at the same time (158, 159). Later, this method was used by Spehar-Deleze for biorecognition detection in DNA hybridization (161). Nonetheless, complicated labeling processes are still required. In a recent attempt to circumvent laborious labeling procedures, Wu *et al.* described a reagentless DNA biosensor based on cathodic ECL using an oxide-covered glassy carbon electrode (162). A schematic diagram illustrating the procedure for the fabrication of the biosensor is shown in Figure 14.

Firstly, a thin oxide film of *ca.* 3 nm was formed on the electrode surface. This allowed the generation of energetic electron during a cathodic pulse polarization cycle. Cathodic ECL at a C/C_xO_{1-x} electrode was observed. A label-free immunosensor was then fabricated using the carbonyl groups, which can be linked covalently with antibodies. This work was based on the quenching of cathodic ECL operated by ferrocene.

By applying a cathodic pulse polarization cycle, an ECL signal (S_0) is produced at the C/C_xO_{1-x} electrode. The ECL signal (S_1) is quenched when a probe constituted by a molecular beacon (MB) is covalently attached to an amino functionalized ferrocene (Fc) group. When the probe interacts with the complementary target DNA (*c*DNA), the hybridization forced the stem to stay apart. Then, Fc is moved away from the electrode surface and the ECL signal (S_2) is restored. The quenching of the ECL signal at different Fc concentrations is shown in Figure 15. With this method, *c*DNA concentrations between 10^{-11} M – 10^{-8}M were achieved using cathodic ECL with detection limits up to 5 pM (162).

10. ECL FROM METAL IONS

The detection and quantification of metal ions are of considerable importance because of their relevance in many physiological (163, 164), environmental (165, 166), and industrial processes (167). Several analytical methods are currently available for monitoring metal ions and these include atomic absorption spectroscopy (AAS) (168), inductively coupled plasma mass spectrometry (ICP-MS) (169), and voltammetric methods (170-172). A gas-phase chemiluminescence (CL) method was first described by Fujiwara (173) and Fraser (174, 175) in the early 80's. They quantified trace of As, Sb, Sn, and Se with ozone oxidation. Then, years later this method was further improved by Galban (176), and more recently, Dasgupta *et al.* reported arsenic speciation using a liquid chromatographic system with gas-phase CL (177), as well as a novel system for the quantification of arsenic in drinking water (178, 179). In one of their work (178), arsenate was reduced electrochemically to arsine that reacts with ozone with concomitant light emission. The simplicity and sensitivity of ECL suggested that this technique can be fruitfully employed for the detection of dissolved aqueous metal ions (180). The ECL efficiency can be maximized using suitable organic ligands that, in virtue of their metal-to-ligand charge transfer interactions (MLCT), form excited state species with consequent light emission. Many organic ligands form stable complexes with metal ions. For instance, 1,10-phenanthroline has been shown to form a complex with Cd(II) allowing the quantification of cadmium via ECL at ppb level concentrations (178, 181). Richter *et al.* used the chelating agent 2,9-dimethyl-1,10-phenanthroline (dmp) for the quantification of Cu(II) ions (182). ECL was generated by reducing Cu^{2+} ions to Cu^{+} using hydroxylamine hydrochloride. The complex $Cu(dmp)_2^{+}$ was oxidized using TPA as a co-reactant. This configuration allowed the detection of Cu ions at ppb concentrations (182, 183). Several metal ions and organic compounds such as phthalazine, quinazoline, quinoxaline,

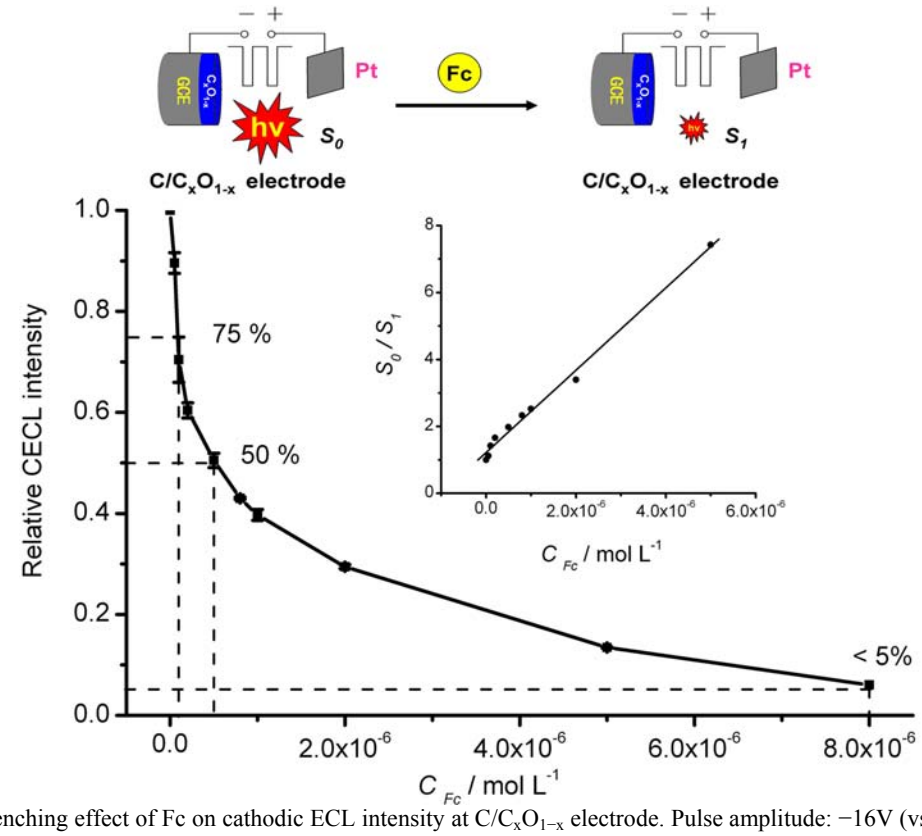

Figure 15. Quenching effect of Fc on cathodic ECL intensity at C/C_xO_{1-x} electrode. Pulse amplitude: −16V (vs. Ag/AgCl), pulse width: 0.4 ms, duration time: 9.6ms and total time: 20 s. Insert: intensity Stern–Volmer quenching plot for cathodic ECL at C/C_xO_{1-x} electrode by Fc. Solution: 0.2M BBS (pH 8.0) containing 0.1M Na_2SO_4 and 1mM $K_2S_2O_8$. Reproduced with permission from (162).

1,10-phenanthroline, 4,7-phenanthroline, 2,2:6,6≠≠-terpyridine, benzo[c]cinnolinephenazine, 2,3-diphenyl-1,4-diazospiro[4,5]deca-1,3-diene, 2,2-bis(2-pyridyl)pyrazine, 2,3-diaminonaphthalene, and 2,2'-bipyridine were investigated (180). The ECL emission has been shown to be strongly dependent on the ligand modification, type of surfactant used, and interactions between surfactant and TPA (184). As an example, Richter *et al.* reported the ECL detection of metal cations (Pb, Hg, Cu, Ag and K) using a ruthenium complex containing a crown ether moiety (185, 186), while Lin *et al.* reported the use of a novel azacrown ether appended Ir(III) complexes for ECL detection of Ba(II) and Ag(I) in acetonitrile (187). The iridium(III) complexes showed superior ECL recognition properties for cations than the analogous ruthenium(II) complexes. Instead, Zhang *et al.* utilized luminol in weakly acidic solutions to detect Co(II) (188). They found that Co(II) greatly enhances the ECL of luminol with the possibility to detect Co(II) up to ppt concentrations.

11. FINAL REMARKS

ECL is being widely used in different analytical applications ranging from medical diagnostics to biodefense. To date, there are more than one hundred assays for biomarkers, including those for thyroid diseases, tumor and cardiac markers, cancer-research, cell signaling pathways, diabetes, nucleic acids, and other analytes of significant clinical relevance. Recently, ECL and the combination of electrochemical and chromatographic techniques with CL have found relevant applications in the environmental field (detection and speciation of heavy metals), opening new perspectives for CL and ECL-based detection methods. ECL is set to play an increasing role in these areas and specifically in the development of novel portable and high sensitivity devices, e.g., biomedical point of care devices and nanosensors. Many of these applications will rely on the friendly uses of such instrumentations, as well as its high sensitivity and selectivity. These advances will mainly be driven by novel advanced materials, especially metal nanoparticles and quantum dots, as well as thin films of inorganic metal complexes (Os, Ru, Ir), organic molecules (conducting polymers). These materials can be assembled using a variety of methods as nanocomposites on different conducting surfaces, such as carbon-based materials (graphene, carbon nanotubes, diamond and carbon paste electrodes). These nanomaterials in combination with novel microfluidic platforms will lead to the development of ultra-sensitive and economically affordable lab-on-a-chip

systems for a variety of biomedical and environmental applications.

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Abbreviations: ADH = alcohol dehydrogenase, AFP = alpha-fetoprotein, APS = 3-aminopropyl-triethoxysilane, bpy = 2,2'-bipyridine, BSA = bovine serum albumin, ChOD = choline oxidase, CL = chemiluminescence, CNTs = carbon nanotubes, CRP = C-reactive protein, dcb = 4,4-dicarboxy-2,2-bipyridine, DMAP = 4-(dimethylamino)pyridine, DPA = 9,10-diphenylanthracene, ECL = electrochemiluminescence, EVA = polyethylene vinylacetate, FTO = fluorine doped tin oxide, F8BT = poly(9,9-dioctylfluorene-co-benzothiadiazole), GCE = glassy carbon electrodes, GMO = genetically modified organism, GO NP = graphene oxide nanoparticle, GOx = glucose oxidase, GSH = gamma-L-glutamyl-L-cysteine-glycine, HCPE = electrically heated carbon paste electrode, HX = hypoxanthine, ITO = indium tin oxide, LB = Langmuir-Blodgett, LBL = layer-by-layer, LDH = lactic dehydrogenase, LDL = low-density lipoproteins, LS = Langmuir-Schaefer, MWNTs = multi walled carbon nanotubes, NAD = nicotinamide adenine nucleotide , NCs = nanocrystals, NPs = nanoparticles, OEG = oligo(ethylene glycol), Oxalate = C2O42-, PAB = human prealbumin antigen, PAMAM = polyamidoamine, PCR = polymerase chain reaction, Peroxidisulphate = S2O82-, PLB = poly(luminol-benzidine), PMMA = poly(methylmethacrylate), PMT = photomultiplier, ppy = 2-phenylpyridine, PYOD = pyruvate oxidase, PVP = poly(4-vinylpyridine), Ru DS = $[Ru(bpy)_3]^{2+}$ functionalized Si nanoparticles, Ru NPs = $[Ru(bpy)_3]^{2+}$ functionalized nanoparticles, $[Ru(bpy)_3]^{2+}$ = tris(2,2'-bipyridyl)ruthenium(II), RSH = thioacetic acid, SAM = self assembly monolayer, SECL = scanning electrochemiluminescence, SECM = scanning electrochemical microscopy, SMS-EC = single molecule spectroelectrochemistry, SWNTs = single walled carbon nanotubes, TBR = $[Ru(bpy)_3]^{2+}$ labelled bar code DNA, TGA = thioglycolic acid, TPA = tripropylamine, TNT = TiO2 nanotubes, XOD = xanthine oxidase

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