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Review Article

Advanced carbon nanomaterials for electrochemiluminescent biosensor applications

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Abstract

Electrochemiluminescent biosensors are nowadays an established technology in the field of immunosensors and diagnostics. Along with the advent of nanotechnology, the marriage between electrochemiluminescence and nanomaterials results in promising enhancing strategies in many biosensor applications. Among nanomaterials, carbon-based ones are the most used, as (i) scaffolds, (ii) luminophores and (iii) electrode materials of the sensor. In this review, we describe the importance of a rational modification and functionalization of carbon nanomaterials to optimize electrochemiluminescence signal, and we also resume the latest and most relevant applications of electrochemiluminescent biosensors based on carbon nanomaterials.

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Introduction

Nanotechnology is nowadays permeating our society with applications in many different fields, such as electronics, catalysis, optics, biology and medicine [1]. With their peculiar mechanical, optoelectronic and physicochemical properties, nanomaterials can improve current technologies or pave the way for new future applications in various fields, such as biosensing [2], in particular, electrochemical biosensing [3–5].

Electrogenerated chemiluminescence or electrochemiluminescence (ECL) is currently a leading transduction technique in immunosensors, with many important applications also from the industrial point of view [6,7]. ECL is chemiluminescence where light emission is triggered by an electrochemical reaction [8]. Thanks to the combination between electrochemical and spectroscopic methods, ECL owns several advantages over chemiluminescence and photoluminescence, such as (i) superior temporal and spatial control on light emission, (ii) intrinsically very low background (i.e. high sensitivity), because of the absence of excitation light and (iii) broad dynamic range and fast measurement in low volume of sample. In particular, ECL has been a great success, as a transduction method, in the biosensor fields, thanks to the unique signal-to-noise ratio also in real and very complex matrix such as cell lysates, urines and blood. For the biosensor application, the electrochemical reactions are upon the luminophore and a sacrificial molecule (i.e. the coreactant), in the so-called 'coreactant ECL', by applying only one single potential step. Two pathways, of coreactant ECL mechanism, are available: 'oxidative reduction' and 'reductive oxidation'. The former concerns the oxidation of the coreactant, which undergoes a homogeneous follow-up reaction, yielding a strongly reducing radical intermediate. Then, the radical is able to reduce the oxidized luminophore to its excited state, which emits light. The latter mechanism is transposed to reduction straightforwardly [9].

As a matter of fact, the number of scientific publications on ECL has been exponentially increased in the last 20 years, reaching almost 3000 publications in 2018.

In the quest for ever-increasing sensitivity, ECL has been coupled to nanotechnology to optimize the signal

generation. The combination between nanomaterials and ECL led to many applications, in particular, concerning luminescent nanoparticle (NP) probes [9,10].

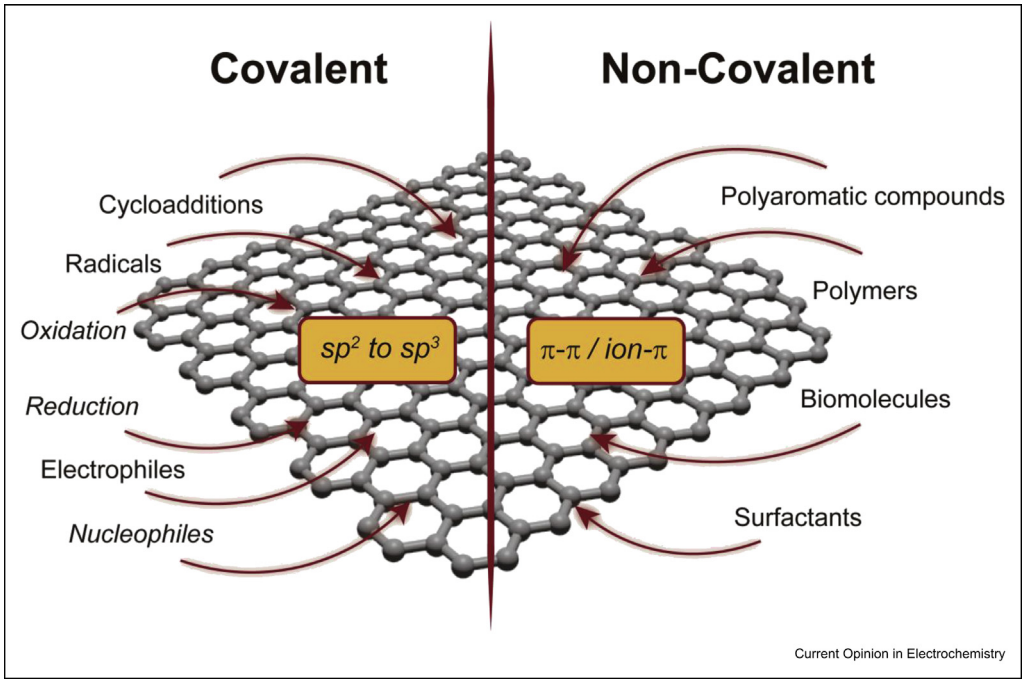
Herein, we highlight application of carbon nanomaterials in these fields and the latest developments concerning the fabrication of electrochemiluminescent biosensors with carbon nanotubes (CNTs) [11], graphene (G), graphene oxide (GO) and reduced GO (rGO) [12], carbon dots (CDs) [13] and graphitic carbon nitride (g-C_3N_4) [14]. In particular, we focused our attention on the crucial role played by the rational modification and functionalization of carbon nanomaterials to enhance the (i) electrical conductivity, (ii) surface area and (iii) stability and (iv) to optimize the biorecognition. Thanks to their unique propriety and versatility, carbon nanomaterials represent a fundamental building block that enables the detection of a wide range of analytes, such as metal ions, cancer biomarkers, toxins and cells. Future challenges and research trends will be also pointed out along with the discussion.

Functionalization strategies of carbon nanomaterials

Nanomaterials can be successfully functionalized *via* noncovalent interactions, such as pi—pi, pi—ion and ion—ion interactions, using amphiphilic units, polymers or metals, or *via* covalent functionalization. In the latter case, typical reactions include oxidation, reduction, cycloaddition, radical and nucleophilic/electrophilic additions (Figure 1) [11,15]. For oxidized nanomaterials, such as oxidized CNTs, GO, rGO and CDs, the chemical strategies also include the chemistry of the oxygenated functions that were created during their corresponding synthesis. The properties of the final material strongly depend on the functionalization strategy adopted as the chemical functionalization can change the intrinsic properties of the nanomaterial. For instance, CNTs and G display properties dependent on their aromatic structure. The use of covalent modification can disrupt the aromaticity with the introduction of defects, such as sp^3 carbon atoms, which deteriorate the associated particular properties. Therefore, the choice of the functionalization strategy is crucial. Ideally, it needs to find a compromise between new features while maintaining the intrinsic properties of interest for the envisioned application. For example, GO and rGO, which are obtained from the oxidation of graphite, are widely used as transducing components. The introduction of oxygenated groups makes them easy to handle in water/polar solvents and useful for *post*-functionalization steps.

The foremost step in the construction of a biosensor is the immobilization of a receptor onto a transducer [16]. The two most common methods of immobilization are physical adsorption and anchoring through chemical means. The direct adsorption method of a receptor is

Figure 1



General representation of functionalization possibilities for carbon nanomaterials. Adapted from the reference: https://link.springer.com/article/10.1007/s12274-014-0622-9 [64].

highly used in sensing because it is a simple and fast approach that does not require any previous chemical modification [17]. However, it is not a controllable process, it is nonspecific and it does not yield stable interfaces. Immobilization *via* chemical bonding demonstrates a good stability and flexibility and high binding strength, preventing the desorption of the receptor. To bind the receptor by chemical immobilization, several covalent and noncovalent approaches have been developed, which include amide bonds with carboxylic groups, thiol—ene reactions, the use of NPs, pi—pi interactions and the immobilization in biomatrices. For this purpose, the chemical modification of carbon-based nanomaterials in a previous step to immobilization is mandatory. However, it is worth mentioning that many biomolecules readily adsorb on aromatic structures, which sometime makes difficult to distinguish among adsorbed, noncovalently and covalently attached carbon nanomaterials.

In addition, the proper orientation of the receptor biomolecules, such as nucleic acids or antibodies, anchored to the electrode surface is critical because it must retain its bioactivity, while providing accessibility to the target analyte and a direct interaction with the transducer surface [18]. Immobilization with specific chemical reactions (i.e. via chemical linkers) can avoid the unwanted heterogeneity in molecule orientation, improving the recognition efficiency. For example, covalent bonding is frequently used in the immobilization of DNA receptors [19]. The DNA probe is typically linked to the thiol or amine groups at the end of 3' or 5' which binds covalently to the carbon nanostructure with specific functional groups, yielding a good oriented receptor.

The covalent immobilization is also useful to yield stable interfaces compared with physical adsorption. In many ECL sensors, where moderately high potential is applied, covalent bonding between the carbon nanostructure and the electrode surface is necessary to create a stable interface. One of the most important steps for building an ECL biosensor is the immobilization on the transducer. In fact, for an ECL transduction, it is necessary for a system to preserves its stability when a potential, typically between 0.8 and 1.4 V, is applied. For instance, noncovalent immobilization is usually not stable upon the application of this working potential.

Generally, the chemical modification is also able to overcome the limited low solubility/dispersibility of large and nonoxidized carbon nanomaterials as CNTs and G. The solubility/dispersibility is a crucial point, particularly, for a scale-up production of sensing devices. Thus, the chemical modifications are used to improve sensing devices by oxidizing nanomaterials, the introduction of polar groups *via* small moieties or polymers that can yield solutions, stable dispersions or inks.

Carbon nanotubes

CNTs have high electron conductivity and a large specific surface area, combined with fast kinetics for the coreactant oxidation (i.e. tri-*n*-propylamine) compared with usual electrode materials, such as indium tin oxide (ITO) [20].

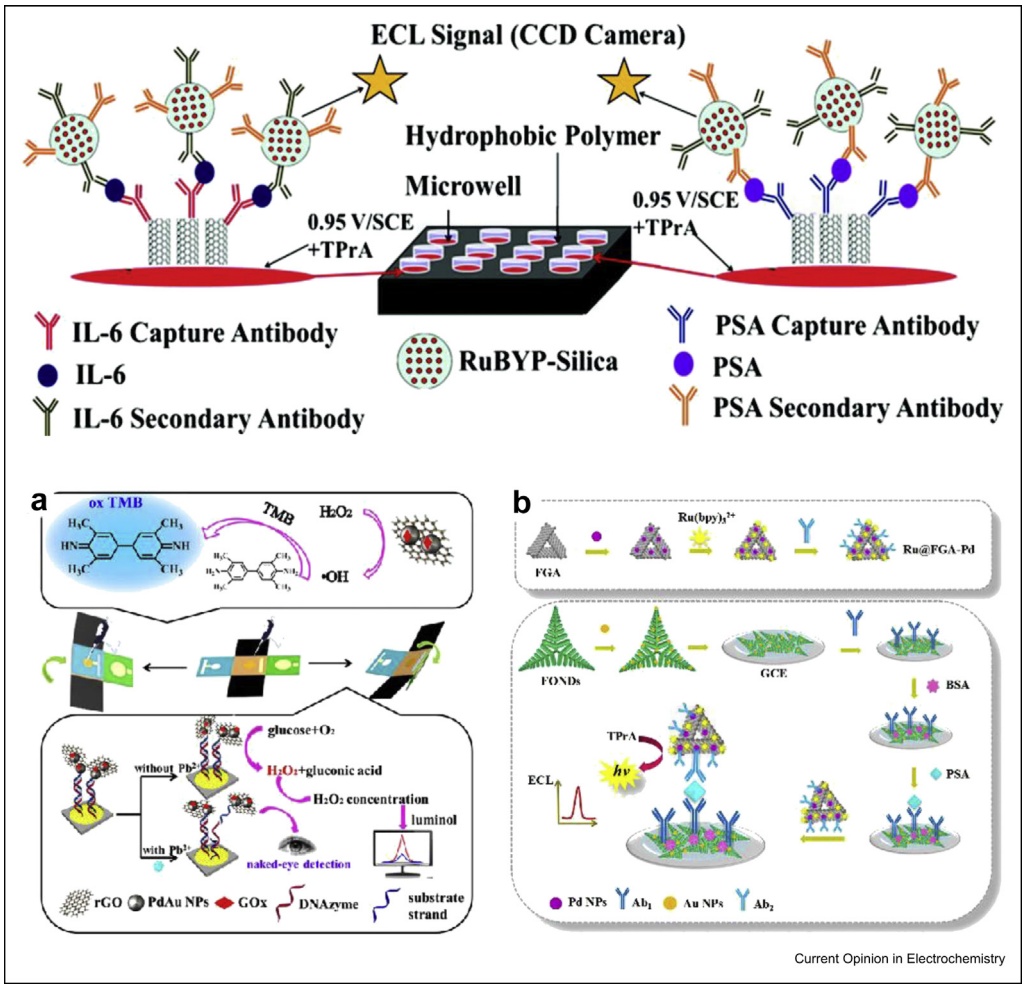
In specific sensors, the chemical modification is also useful for anchoring the nanomaterial as a transducing component on the electrode. In the aforementioned 'on-electrode' strategy, a covalent modification was used to yield a very stable interface under working conditions between modified CNTs and the working electrode. Besides, chemical modification can be useful to control the orientation of nanostructures on the electrode surfaces. Sardesai et al. [21] built an ultrasensitive ECL immunosensor array to detect two prostate cancer biomarkers. This CNT-based array was able to detect simultaneously the two corresponding biomarkers with a detection limit at picomolar for millilitre concentrations (Figure 2). The high sensitivity is due to a forest of perpendicularly oriented CNTs to the electrode surface that provides a large conductive and functionalized surface area for the attachment of receptors. This particular orientation of the CNT surface was possible because the oxidation of CNTs is preferential towards CNT tips. Thus, the terminal CNT stands in upright bundles on the electrode surface because of electrostatic interactions between the terminal carboxylic groups and the electrode surface.

These intrinsic characteristics make CNTs highly suitable to carry the recognition unit (e.g. the antibody) [22•], the luminophore or a catalyst to amplify the light signal. The wiring enables electric contact even at a long distance from the electrode surface, leading to signal enhancement compared with flat electrodes ([22•], [23]).

CNTs have been applied to the recognition of many cancer biomarkers, such as prostate-specific membrane antigen [22•], carcinoembryonic antigen (CEA) [24] and carbohydrate antigen 19-9 for pancreatic cancer [25], or small organic molecules such as organophosphorous pesticides [26] or diclofenac [27].

Furthermore, CNTs can be decorated with metal NPs that act as a catalyst for the electrochemical oxidation of the coreactant or luminophore itself, for example, using luminol or a luminol derivate [25,26]. Analogues of CNTs, single-walled carbon nanohorns, have been applied forATP detection. Single-walled carbon

Figure 2



Top: schematic representation of a CNT-based ECL biosensor for detection of prostate cancer biomarkers (adapted from Sardesai et al [21]). Bottom: **(a)** schematic representation of the lab-on-paper device for lead ion sensing (adapted from Xu et al [31•]) and **(b)** scheme of the sensor assembly based on 3D nanostructured graphene aerogel for prostate-specific antigen (adapted from Yang et al [34•]). CNT, carbon nanotube; ECL, electrochemiluminescence; rGO, reduced graphene oxide; CCD, charge coupled device; PSA, prostate specific antigen; IL, interleukin; FOND, functionalized Fe2O3 nano dendrites; FGA, functionalized graphene aerogel supported; TMB, 3,3′,5,5′-tetramethylbenzidine; BSA, bovine serum albumin; GCE, glassy carbon electrode; NP, nanoparticles.

nanohorns act both as a scaffold to carry the ATP aptamer and quencher of the coreactant ECL $Ru(bpy)_3^{2+}$/tri-*n*-propylamine system to reach a detection limit of 0.5 ng mL^{-1} [28].

Graphene

Graphene, GO and rGO find similar application as CNTs, mainly as a platform for the antibody, DNA or aptamers or metal NPs, or a scaffold for the metallopolymer [29]. With the high content of oxygen moieties, GO is highly preferred compared with G because of its water solubility and ease of bioconjugation.

An attractive feature of GO is the possible application, directly in contact with the working electrode or bound in a sandwich-type immunoassay (Figure 2). We may refer at these two strategies as 'on-electrode' and 'in-electrode', respectively [30].

Both on-electrode and in-electrode strategies can be applied to completive immunoassay resulting in a decrease in ECL with the increase of the analyte, because of quenching effects, or by blocking the electrode surface ([31•] [32•], [33]) or usual signal increase with the concentration of the analyte [34•-37].

GO finds many applications for a wide variety of analytes, such as heavy metal ions, such as Hg^{2+} [37] and Pb^{2+} [31•], cancer biomarkers (prostate-specific antigen, p53, mucin 1) ([32•] [34•], [35]), organic molecules, such as toxins (aflatoxin M1) [33] and nervous stimulant (clenbuterol) [36], and biomarkers, for

monitoring the damage of DNA by oxidative stress (8-oxo-7,8-dihydro-2-deoxyguanosine) [29].

Carbon dots

Carbon dots (CDs) show, thanks to their duality in their chemical composition, two important applications in ECL sensors: as coreactants or as luminophores. The ECL properties of CDs depend on the carbon core nature, functional groups and doping heteroatoms. However, most CDs used in ECL studies possess plenty of oxygen-containing functional groups such as carbonyl, carboxyl, hydroxyl and epoxide/ether. The oxygen-rich CDs are synthesized by oxidizing large carbon materials in a top-down approach or by pyrolysis process from molecular precursors in a bottom-up approach [13]. These functional groups furnish water solubility, labelling and ECL efficiency because the oxygenated groups facilitate the electrogeneration of the corresponding radicals [38]. For instance, CDs with different oxidation degrees were synthesized for comparing their ECL efficiency [38]. The indispensable contribution of oxygen-containing functional groups to the ECL property of CDs was verified by the stronger ECL response of the highest oxidized nanomaterial. In addition, their functionalization allows the combination with other materials such as metals and metal oxide nanostructures.

On the other hand, CDs in combination with metal-based quantum dots generate new ECL systems for biosensing because of a synergic ECL signal enhancement [39,40]. In 2017, Zhou et al. [39] have fabricated a novel ECL-sensing platform for the detection of two biological markers for tuberculosis infection in human serum. The reported biosensor exhibited a parallel detection for both biomarkers with a detection limit of 10 fg mL^{-1}. The key issue of the highly sensitive detection is the ECL system based on nanocomposites of gold NPs (AuNPs) and CDs (AuNPs@CDs). The nanocomposite of AuNPs@CDs was synthesized by the ionic interaction between aminated AuNPs and CDs.

Nitrogen-doped carbon dots can successfully replace tri-*n*-propylamine as the coreactant for ruthenium(II) tris(2,2′-bipyridyl) [41••-43] because they are rich in amines (Figure 3). However, nitrogen-doped carbon dots can be used as luminophores, as well [44•-46], for a range of wavelength emission from 400 nm to 550 nm. On the contrary, CDs are used only as luminophores ($\lambda_{Em} \approx 400-700$ nm), mainly with the coreactant peroxydisulfate or hydrogen peroxide, in 'reductive oxidation' ECL [47-51•].

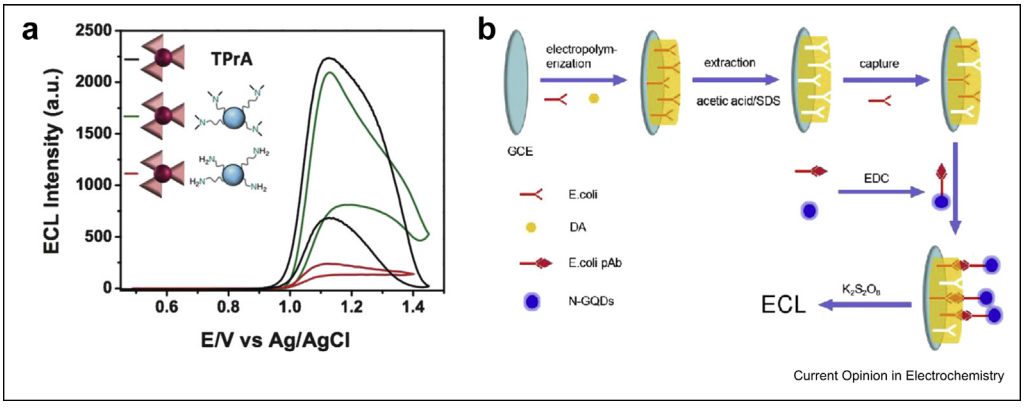
CD-based biosensors have been developed for several analytes, such as *Escherichia coli* [44•], human IgG protein [47], cancer biomarkers (liver cancer noncoding RNA and CEA) ([49], [51•]), microRNAs [45] and organic molecules, such as pentachlorophenol [42], dopamine [43], glucose [48] and lincomycin [50].

Graphitic carbon nitride

Graphitic carbon nitride finds applications as an alternative luminophore to the conventional ruthenium(II) tris(2,2′-bipyridyl). The ECL of g-C_3N_4 is commonly generated by the 'reductive oxidation' coreactant mechanism with peroxydisulfate, for a range of wavelength emission from 350 to 650 nm (Figure 4a) [52]; furthermore, triethanolamine has been proposed for the 'oxidative reduction' coreactant mechanism [52,53].

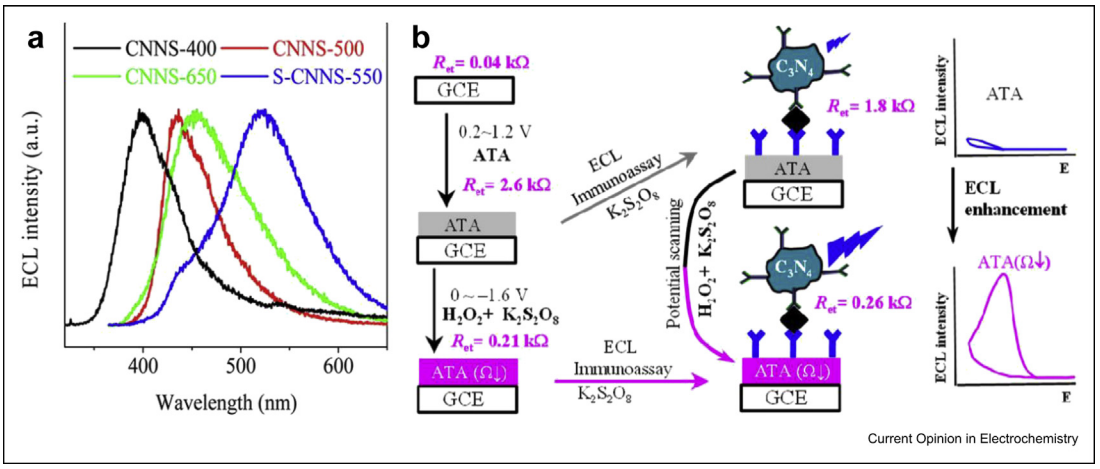
Graphitic carbon nitride is usually deposited on the surface of the electrode, or it can be also bound to the electrode after target analyte recognition, by sandwich immunoassay (Fig. 4b) ([54•] [27]). The bioconjugation of g-C_3N_4 with a secondary antibody is

Figure 3



Schematic rapresentation of electrochemiluminescence for carbon nanodots. **(a)** ECL emission of $Ru(bpy)_3^{2+}$ with 0.1 mg mL^{-1} NCDs (red trace), 0.1 mg mL^{-1} methylated NCDs (green trace) and 20 mM TPrA (black trace) in phosphate buffer solution (pH 7.4) at the glassy carbon electrode (adapted from Carrara et al [41••]). **(b)** Fabrication process of an ECL immunosensor with CDs as luminophores (adapted from Chen et al [44•]). ECL, electrochemiluminescence; NCD, nitrogen-doped carbon nanodot; N-GQDS, nitrogen-doped graphene quantum dots; GCE, glassy carbon electrode; DA, dopamine; SDS, sodium dodecyl sulfate; TPrA, tripropyl amine; EDC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide.

Figure 4



Schematic rapresentation of electrochemiluminescence for graphitic carbon nitrite. **(a)** Tuning of the ECL emission for g-C_3N_4 by modulating the temperature of synthesis (adapted from Zhou et al [52]). **(b)** Example of fabrication of the ECL immunosensor using g-C_3N_4 as the luminophore (adapted from Jin et al [54•]). ECL, electrochemiluminescence; g-C_3N_4, graphitic carbon nitride; ATA, 2-aminoterephthalic acid; GCE, glassy carbon electrode; CNNS, carbon nitride nanosheets.

carried out by reaction at carboxylic moieties of oxidized g-C_3N_4, in a similar fashion as for GO.

Biosensors with g-C_3N_4 respond with ECL emission proportional to increasing analyte concentration. However, quenching mechanisms have been proposed, as for cyanide [55] or metal ion detection [52].

The target analytes of the biosensor detected using g-C_3N_4 are CEA [54•]; proteins and enzymes (acetylcholinesterase and concanavalin A) [53,56]; metal ions (Ni^{2+}, Cd^{2+} and Cu^{2+}) [52]; small organic molecules, such as diclofenac [27], 17β-oestradiol [57], dopamine [58] and 8-hydroxy-2′-deoxyguanosine [59]; and *E. coli* [60].

Carbon nanomaterials as electrode materials

The previous examples showed how carbon nanomaterials can be integrated in ECL biosensors to carry out many different functions, however, by using a conventional electrode material as platforms, mainly glassy carbon or ITO. Here, we show how carbon nanomaterials can replace conventional electrode materials to make robust electrodes for ECL.

CNTs are highly promising for the development of electrodes, especially in ECL imaging. Deposition of the CNT layer from solution [20] or inkjet printing ([61],[62]) has been used to fabricate the CNT electrode on polymer films or glass.

Paramagnetic polystyrene beads labelled with ruthenium(II)tris(2,2′-bipyridyl), similar to commercial immunoassay, were deposited on a CNT electrode and imaged using a microscope. When compared with ITO, the CNT electrode showed higher ECL emission from the beads [20]. This enhanced ECL is ascribed to the effect of the catalytic surface of CNTs towards the oxidation of the coreactant, tri-*n*-propylamine [63]. Furthermore, a biosensing application of this ECL imaging technique was developed for the investigation of single cells [61]. By labelling specific proteins of the cellular membrane, it was demonstrated that ECL microscopy allows observation of membrane details, which are difficult to resolve by classic photoluminescence microscopy, paving the way for promising applications in ultrasensitive cell imaging assays [62].

Conclusion

Here, we showed how the main groups of carbon nanomaterials, such CNTs, G, CDs and g-C_3N_4, allow ECL to benefit from higher sensitivity, and new mechanisms as well, towards many different analytes of interests. The ECL sensors, which integrate carbon nanomaterials, can be used to detect a wide range of analytes, from simple metal ions, proteins and cancer biomarkers, to cells, which makes ECL one of the most relevant techniques in electrochemical sensors. To offer a general guide for the manufacture of ECL biosensors, we have also emphasized how the chemical modification of carbon nanomaterials can improve the sensing performance.

We may prospect a wide application of carbon nanomaterials in point-of-care testing, as it is nowadays for inkjet-printed paper fluidic substrates and screen-printed electrodes, to fabricate low-cost and disposable ECL sensors. Carbon nanomaterials could find application as they meet several parameters, such as accessible production, reproducible preparation, easy to process,

and available bioconjugation strategies. As CNTs are nowadays regularly applied in screen-printed electrodes, we may infer other carbon nanomaterials, such as those described in this review, will be used profitably as long as fundamental and applied research will keep challenging the current limits, in particular, accessible production and cost, and reproducible preparation procedures.

We believe that there is still a huge room for improvement and that the research in this field will continue to grow exponentially. In particular, regardless of the role of the carbon material, either as a transducing component, coreactant or luminophore, its chemical modification is usually a prerequisite for manufacturing the device. The modification of the carbon material allows the implementation of lacking features, yet required, or the fine-tuning of key properties to achieve an optimal functionality. Therefore, the modification can implement recognition specificity by attachment of sensing molecules on the carbon material, by tuning of emission and charge transfer efficacy and by improving the material processability.

Conflict of interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.coelec.2019.04.018.

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- • of special interest
- •• of outstanding interest

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