

Amine-Rich Nitrogen-Doped Carbon Nanodots as a Platform for Self-Enhancing Electrochemiluminescence

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Abstract: Amine-rich nitrogen-doped carbon nanodots (NCNDs) have been successfully used as co-reactant in electrochemiluminescence (ECL) processes. Primary or tertiary amino groups on NCNDs have been studied as co-reactant sites for Ru(bpy)₃²⁺ ECL, showing their eligibility as powerful alternatives to tripropylamine (TPrA). We also report the synthesis and ECL behavior of a new covalently linked hybrid of NCNDs and Ru(bpy)₃²⁺. Notably, the NCNDs in the hybrid act both as carrier for ECL labels and as co-reactant for ECL generation. As a result, the hybrid shows a higher ECL emission as compared to the combination of the individual components, suggesting the self-enhancing ECL of the ruthenium complex due to an intramolecular electron transfer process.

Carbon nanodots (CNDs), quasi-spherical nanoparticles with size below 10 nm,^[1-3] are expected to have a huge impact in biotechnological and environmental applications, based on their high potential as a nontoxic, fluorescent alternative to the popular semiconductor-based quantum dots (QDs).^[4,5] In addition, properties such as water solubility, chemical inertness, facile modification and high resistance to photobleaching^[3,4,6] are important for their analytical and bioanalytical applications.^[1,4,5] In this latter fields, electrochemiluminescence (ECL) is becoming an increasingly popular biosensing technique. ECL is a redox-induced light emission in which high-energy species, generated at the electrodes, undergo a high-energy electron transfer reaction forming an excited state that emits light.^[7] The excited state can be produced through the reaction of radicals generated from the same chemical species (emitter), in the so-called


annihilation mechanism, or from two different precursors (emitter and co-reactant), via co-reactant ECL.^[7,8] In the annihilation mechanism, the application of oxidative conditions to a luminophore followed by reductive conditions (or viceversa), generates high-energy species that react with one another producing ECL emission. In contrast, in co-reactant ECL, both luminophore and co-reactant are first oxidized or reduced at the electrodic surface forming radicals and intermediate states. The co-reactant radical oxidizes or reduces the luminophore producing its excited state. Thus, depending on the nature of the co-reactant, both “oxidative–reduction” or “reductive–oxidation” mechanisms are possible. The main advantage of the co-reactant pathway is that the formation of radicals in aqueous solutions, and the consequent generation of ECL, is attainable without potential cycling and at less extreme potentials compared to common organic solvents, opening up a wide range of bioanalytical applications.^[7]

The most employed ECL luminophore is ruthenium(II) tris(2,2'-bipyridyl) (Ru(bpy)₃²⁺) because of its luminescence efficiency, reversible electrochemical behavior, chemical stability and versatility.^[9-11] Alkyl amines are the most representative class of “oxidative–reduction” co-reactants and tripropylamine (TPrA) provides the optimum ECL signal when used in combination with Ru(bpy)₃²⁺, resulting in their wide use in commercial ECL immunoassays.^[8,12,13] However, TPrA has several disadvantages: not only it is toxic, corrosive, and volatile, but also needs to be used in high concentrations (usually up to 100 mM) to obtain good sensitivity.^[14] Alternative co-reactants have to obey to some stringent requirements, such as matching redox potentials, fast charge transfer kinetics and rapid degradation routes to produce a high-energy radical capable to initiate the ECL process.^[8] Moreover, good solubility and high chemical stability are two crucial conditions. Recently, suitably designed fluorophores carrying groups acting as co-reactant have attracted a lot of attention.^[15-20] They would not only avoid the addition of TPrA, but also provide a self-enhanced ECL emission due to an intramolecular, and thus more efficient, electron transfer. In the ECL context, the use of CNDs as carrier for fluorophores has so far not been reported, while their use as co-reactant is still at the initial stages.^[21-23] However, apart from a recent study in which benzylic alcohol moieties were used as co-reactant sites,^[21] the identification of the functional groups responsible of their co-reactant behavior is often difficult, though fundamental. Recently, we reported a straightforward approach to the synthesis of nitrogen-doped CNDs (NCNDs), using a microwave reactor under controlled conditions.^[24] The NCNDs thus produced showed a rather homogeneous size distribution with an average size of

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2.5 ± 0.8 nm, multiple oxygen and nitrogen functional groups on their surface and excellent solubility in water (up to 80 mg mL⁻¹). The presence of abundant amino groups on their surface (1350 μmol g⁻¹ based on Kaiser test), would allow the easy insertion of interesting functional groups and/or molecular moieties through standard organic chemistry procedures.^[24]

Herein, we report a NCNDs/Ru(bpy)₃²⁺ system in which primary or tertiary amino groups on the NCND surface act as powerful “oxidative–reduction” co-reactant for Ru(bpy)₃²⁺ ECL generation in aqueous solution, suitable for bioanalytical purposes (Figure 1). Moreover, we show that NCNDs, in covalently linked systems with Ru(bpy)₃²⁺ (**Ru-NCNDs**, Figure 1), are not innocent fluorophore carriers, but also act as co-reactant in the ECL process. Notably, the **Ru-NCND** hybrid shows an increased ECL signal as compared to the system of the individual components NCNDs/Ru(bpy)₃²⁺. Thus, the latter acts as an ECL self-enhanced platform due to an intramolecular electron transfer reaction.

To be active as ECL co-reactant, the amino functional groups should have a hydrogen atom attached to the α-carbon, in order to produce a strongly reducing intermediate, which supplies chemical energy to generate the luminophore emissive excited state.^[25] In NCNDs, the presence of primary amino groups was already confirmed by a strongly positive Kaiser test.^[24] In addition, our previously reported 2D-NMR studies of NCNDs demonstrated the presence of -CH₂NH₂ groups.^[24] Also, the high current and steep slope of the mono-electron oxidation peak (+1.14 V vs. SCE) in the cyclic voltammogram (CV) of NCNDs (Figure S1, black trace and Figure S2 in the Supporting Information) suggest that a high number of amino groups on the NCND surface can be easily oxidized. Moreover, among the variety of amine derivatives investigated (aromatic, aliphatic, primary, secondary and tertiary; Figures S1 and S3), in the same solvent and at the same concentration, the strong similarity with the CV of phenethylamine and benzylamine (Figure S1) further corroborates that the NCND amino groups may have a hydrogen atom attached to the α-carbon. Therefore, we envisage that these amino groups should be capable of forming reductive or oxidative species during an unidirectional voltammetric scan.^[12,26]

Since Ru(bpy)₃²⁺ and NCNDs have almost the same HOMO values (-6.1 and -5.7 eV respectively),^[27,28] both species can be oxidized at the electrode surface upon applying a positive voltage. Thus, the ECL signal of a 0.1 mM Ru(bpy)₃²⁺ solution in PBS (pH 7.4) was recorded upon addition of NCNDs (0.1 mg mL⁻¹) at a glassy carbon electrode (during a cyclic voltammetry between +0.5 V and +1.4 V) and compared to the one recorded without NCNDs (Figure 2a, bottom part). Ru(bpy)₃²⁺ background ECL emission (Figure 2a, bottom part, black trace) in aqueous solutions can be attributed to a light-emitting reduction of Ru(bpy)₃³⁺ by OH⁻ ions (chemiluminescence) or other reducing agents, rather than the annihilation reaction.^[29–32]

The presence of NCNDs (Figure 2a, bottom part, red trace) results in a four-time intensity enhancement of the Ru(bpy)₃²⁺ ECL signal rising at +1.0 V and peaking at +1.1 V. This result confirms the NCNDs co-reactant behavior during the oxidation process, which can be further corroborated by the higher ECL signal of Ru(bpy)₃²⁺ upon increasing the concentration of NCNDs from 0.002 to 0.1 mg mL⁻¹ (Figure 2b). In order to prove the efficiency of our system over time, several cycles were performed, scanning between +1.1 V and -0.9 V, at a rate of 0.05 V s⁻¹. In contrast to TPrA, the ECL response with NCNDs is relatively constant (within 10%) for more than 15 cycles (Figure S4), revealing their great electrochemical stability, the abundant presence of available amino groups for further ECL cycles and their consequent eligibility for biosensing platforms.

CV measurements were performed to shed light on the co-reactant mechanism. The CV of a 0.1 mM Ru(bpy)₃²⁺ solution in PBS (pH 7.4) shows the typical reversible oxidation peak at +1.1 V (Figure 2a, upper part, black trace).^[28] However, upon addition of 0.1 mg mL⁻¹ NCNDs (Figure 2a, upper part, red trace) the intensity of the oxidation peak of Ru(bpy)₃²⁺ increases, while the reduction peak becomes lower. This behavior is commonly observed in the system Ru(bpy)₃²⁺/TPrA and suggests a NCNDs catalytic effect on the Ru(bpy)₃²⁺ oxidation.^[27,33]

From the observed behavior we suggest a possible mechanism, sketched in Figure 3. Upon oxidation, the amino groups of NCNDs become a reductive unstable intermediate able to form the excited-state Ru(bpy)₃^{2+*} through highly energetic electron transfer. The latter emits light and

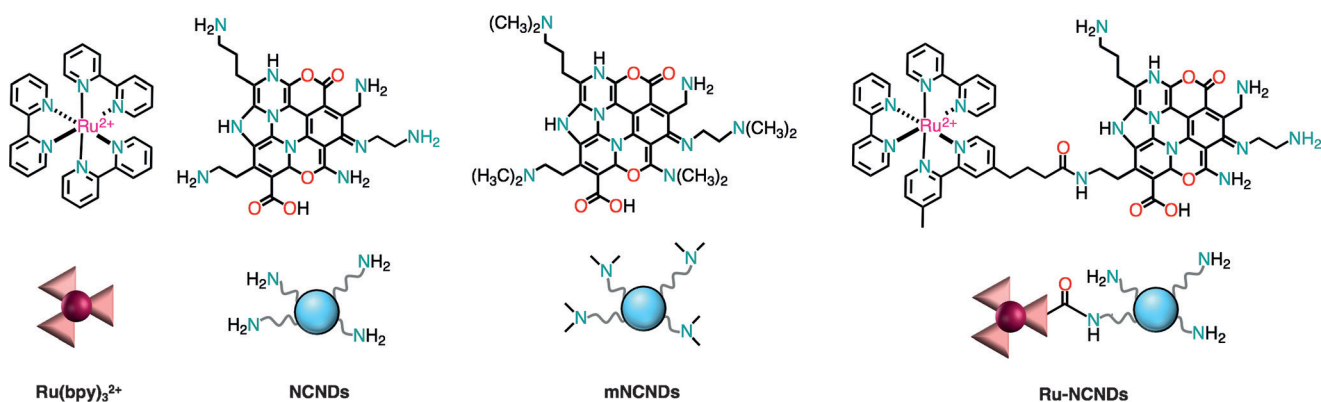


Figure 1. Tris(2,2'-bipyridine)ruthenium(II) (Ru(bpy)₃²⁺), nitrogen-doped carbon nanodots (NCNDs) and methylated-NCNDs (mNCNDs) on the left, covalently linked Ru(bpy)₃²⁺-NCNDs (Ru-NCNDs) hybrid on the right.

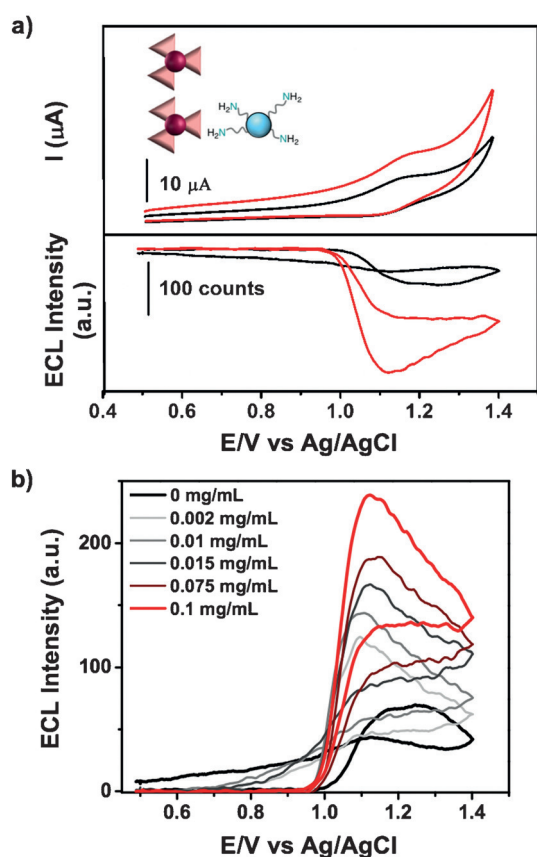


Figure 2. a) CV (upper part) and ECL (bottom part) potential curves of $0.1 \text{ mM Ru}(\text{bpy})_3^{2+}$ (black traces) and $\text{Ru}(\text{bpy})_3^{2+}/\text{NCND}$ system (red traces) in PBS solution (pH 7.4) on GC electrode ($d=3 \text{ mm}$). Scan rate 0.05 Vs^{-1} , potential referred to Ag/AgCl (KCl sat.) at room temperature. Platinum wire as counter electrode and the peak height scaled with the scan rate in CV. b) ECL intensity during a potential scan of 0.1 mM (pH 7.4) of $\text{Ru}(\text{bpy})_3^{2+}$ in PBS solution (pH 7.4) upon addition of different concentrations of NCNDs ($0\text{--}0.1 \text{ mg mL}^{-1}$). GC electrode ($d=3 \text{ mm}$), potential referred to Ag/AgCl (KCl sat.) at room temperature. Platinum wire as counter electrode.

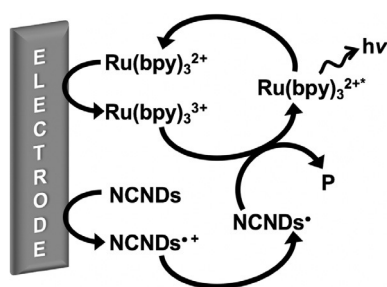


Figure 3. ECL proposed mechanism for $\text{Ru}(\text{bpy})_3^{2+}/\text{NCND}$ system.

produces $\text{Ru}(\text{bpy})_3^{2+}$, which in turn can be re-oxidized for a new cycle.

Since generally the ECL intensity increases using amines as co-reactant in the order primary < secondary < tertiary,^[34] we converted the primary groups of NCNDs to tertiary amines through an Escheweiler-Clarke methylation reaction (detailed experimental conditions in the Supporting Information). The methylated NCNDs (mNCNDs) were fully char-

acterized and the changes observed in their photophysical properties are consistent with the NCND surface modification (Figure S6). The new peak at $+0.8 \text{ V}$ vs. SCE in the CV matches with the one of TPrA (Figure S7) and can be attributed to the formation of tertiary amines on the surface of mNCNDs. ECL experiments for solutions at the same optical density in the metal to ligand charge transfer band ($^1\text{MLCT}$) of $\text{Ru}(\text{bpy})_3^{2+}$, reveal that the ECL activity is ten times higher for mNCNDs compared to NCNDs (Figure 4a,

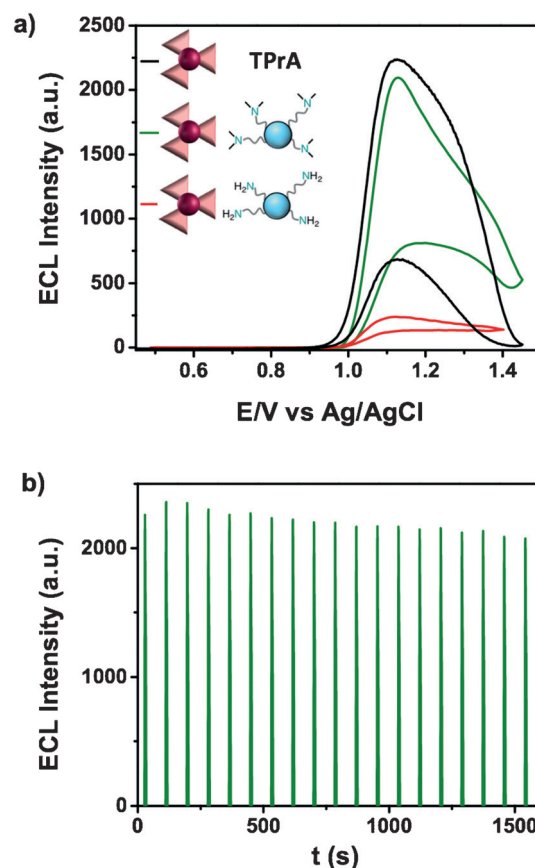


Figure 4. a) ECL emission of $\text{Ru}(\text{bpy})_3^{2+}$ enhanced by 0.1 mg mL^{-1} NCNDs (red trace), 0.1 mg mL^{-1} mNCNDs (green trace) and 20 mM TPrA (black trace) in PBS solution (pH 7.4). GC electrode ($d=3 \text{ mm}$), potential referred to Ag/AgCl (KCl sat.) at room temperature. Platinum wire as counter electrode. b) ECL responses of $\text{Ru}(\text{bpy})_3^{2+}/\text{mNCND}$ system in PBS solution (pH 7.4) obtained during a continuous potential scan between $-+1.1 \text{ V}$ and -0.9 V (20 cycles under scan rate 0.05 Vs^{-1}). GC electrode ($d=3 \text{ mm}$), potential referred to Ag/AgCl (KCl sat.) at room temperature. Platinum wire as counter electrode.

green and red traces). In addition, as in the case of NCNDs, higher ECL signals for $\text{Ru}(\text{bpy})_3^{2+}$ were observed upon increasing the concentration of mNCNDs (Figure S8). Interestingly, constant ECL response (within 10%) were obtained for over 20 cycles (Figure 4b). Thus, tertiary amines on the mNCND surface provide an enhanced ECL activity with respect to primary amines, as usually observed with all the alkyl amines commonly used as “oxidative-reductive” co-reactants.^[35,36] Moreover, a comparison with the widely used TPrA clearly demonstrated the potential of amine-rich

NCNDs as alternative co-reactant species. Interestingly, a small amount of mNCNDs (0.1 mg mL^{-1} , corresponding to a concentration around 0.13 mM in amines) gave the same $\text{Ru}(\text{bpy})_3^{2+}$ ECL intensity as the one recorded using TPrA 20 mM (2.86 mg mL^{-1}), which corresponds to an improvement of ca. 150 times (Figure 4a, green and black traces) despite of the slower diffusion coefficient of the mNCNDs vs. TPrA (Figure S9 and Table S1).^[37]

We finally investigate the efficiency of NCNDs as co-reactant when covalently linked with $\text{Ru}(\text{bpy})_3^{2+}$ (**Ru-NCNDs**). The **Ru-NCND** hybrid was prepared through a condensation reaction of the NHS-ester activated ruthenium bipyridine derivative and the amines of NCNDs (detailed experimental condition in Supporting Information). Transmission electron microscopy (TEM) was employed to probe the morphological features of the nanoconjugate. TEM images showed that the quasi-spherical shape of NCNDs is retained, while the average size ($4.5 \pm 0.4 \text{ nm}$, FWHM: 0.798; Figure S10) increases in the hybrid system. ICP-MS measurements revealed the presence of about three ruthenium complexes for each dot, in accordance with the increased size of the hybrid (details in the Supporting Information). Fluorescence anisotropy measurements were used to further demonstrate the increased size of the hybrid compared to the bare NCNDs (Figure S11). This technique is commonly used for rotational time measurements associated to the emission transition moment that lies along the fluorophore structure.^[38] The rotational time depends on the viscosity of the solvent and on the size of the fluorophore.^[39] The bigger the fluorophore is, the longer is the rotational time necessary to get a depolarized emission. As expected, the rotational time of the anisotropic emission of NCNDs increases from 1.45 ns to 2.3 ns , for the free and the **Ru-NCND** hybrid, respectively.

Additionally, the FT-IR spectrum of **Ru-NCNDs** indicates that the ruthenium bipyridine complexes have been covalently attached to NCNDs through the amide linkage (Figure S12). The three peaks corresponding to the vibrations of the NHS-ester bonds at 1814 , 1783 , and 1736 cm^{-1} observed for the Ru-COOSu , disappear in the hybrid spectrum for the benefit of two characteristic vibrations of amide I (at 1653 cm^{-1}) and amide II (1547 cm^{-1}).

Photophysical measurements of the **Ru-NCNDs** hybrid were compared with those of $\text{Ru}(\text{bpy})_3^{2+}$ and NCNDs used as references. The absorption spectrum of the hybrid system revealed the main features of the two references with a slight shift of the metal to ligand charge transfer, ¹MLCT, band of $\text{Ru}(\text{bpy})_3^{2+}$ at lower energy (Figure S13). Even the luminescence spectrum of **Ru-NCNDs** ($\lambda_{\text{exc}} = 340 \text{ nm}$) exhibits two distinct contributions at 400 nm and at 630 nm corresponding to the ³MLCT transitions of the ruthenium bipyridine complex and to the NCND-centered emission (Figure S15). The excited state lifetime of **Ru-NCNDs** is monoexponential when detected in the ruthenium emission band ($\tau = 363 \text{ ns}$, $\lambda_{\text{exc}} = 440 \text{ nm}$), while a complex decay (tri-exponential) is observed for the NCND emission ($\tau_1 = 12 \text{ ns} - 12\%$; $\tau_2 = 4 \text{ ns} - 26\%$; $\tau_3 = 1 \text{ ns} - 62\%$; $\lambda_{\text{exc}} = 375 \text{ nm}$), without significant variations respect to the individual components in the same solvent (Table S2). The absolute photoluminescence quantum yield (PLOQY) of $\text{Ru}(\text{bpy})_3^{2+}$ moiety in the hybrid was found

to be 4%, similar to the complex alone, indicating that no quenching processes occur due to the attachment on NCNDs. The CV of the **Ru-NCND** system shows the same profile of $\text{Ru}(\text{bpy})_3^{2+}$ both in oxidation and reduction plus an additional peak ($+1.09 \text{ V}$ vs. SCE) attributable to the oxidation of the free amino groups on the NCND surface (Figure 5a). In addition, we estimated the diffusion coefficients for both hybrid and references (Figure S14 and Table S1), observing a decreased value in the case of the hybrid, as expected. Finally, to evaluate the NCND co-reactant behavior in **Ru-NCNDs**, we recorded the ECL curve of the nanosystem in PBS solution, comparing those obtained for $\text{Ru}(\text{bpy})_3^{2+}$ or for the uncoupled system $\text{Ru}(\text{bpy})_3^{2+}/\text{NCNDs}$ in the same conditions and adjusting to the same optical density of $\text{Ru}(\text{bpy})_3^{2+}$ (Figure S16). It was observed an ECL emission intensity two times higher for the **Ru-NCND** hybrid with respect to the uncoupled components (Figure 5b, blue and red traces). According to ECL self-enhanced systems already reported,^[15-19] we hypothesize that in **Ru-NCNDs** the intra-

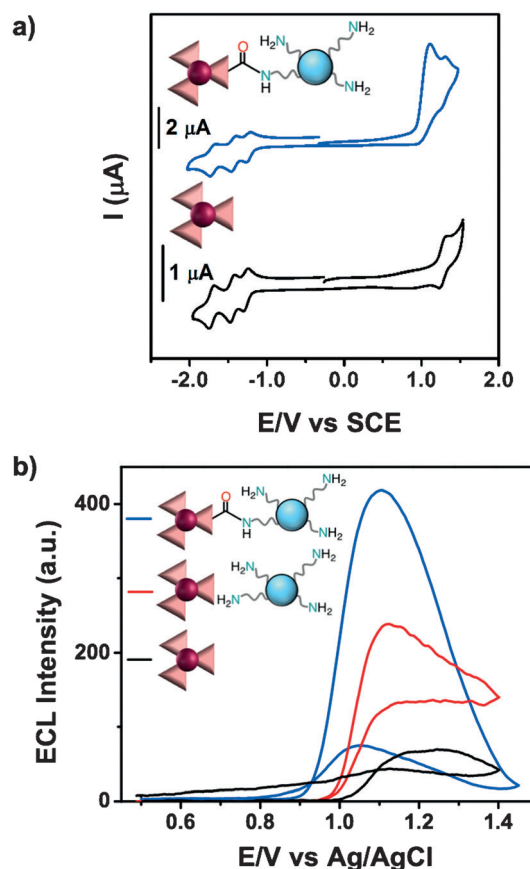


Figure 5. a) CVs of **Ru-NCNDs** (blue trace) and $\text{Ru}(\text{bpy})_3^{2+}$ (black trace) at the same concentration of chromophores (0.26 mM) in 0.1 M TBAPF_6 DMF solution. GC electrode ($d = 3 \text{ mm}$). Scan rate 0.1 V s^{-1} , potential referred to SCE at room temperature using platinum wire as counter electrode. The peak height scales with the scan rate. b) ECL intensity during a potential scan of 0.1 mM PBS solution (pH 7.4) of $\text{Ru}(\text{bpy})_3^{2+}$ (black trace), upon addition of 0.1 mg mL^{-1} of NCNDs (red trace) and of **Ru-NCNDs** (blue trace) with a concentration of ruthenium complex of 0.1 mM . Scan rate 0.05 V s^{-1} . GC electrode ($d = 3 \text{ mm}$), potential referred to Ag/AgCl (KCl sat.) at room temperature. Platinum wire as counter electrode.

molecular electron transfer is more efficient compared to the intermolecular reaction due to the shorter electron-transfer path and minor energy loss.

In summary, we demonstrate that NCNDs carrying amino groups on the surface act as powerful co-reactant species to promote ECL. Their simple and cost effective production, high aqueous solubility and low toxicity make NCNDs a promising alternative to the conventional co-reactant species.

Moreover, we probed, for the first time, their use in covalently-linked system with ECL labels as interesting new platforms for self-enhancing ECL.

This work will open new possibilities towards self-enhanced hybrids for immunoassay applications, where the use of nanodots, each bearing more ECL fluorophores to the electrode surface, will result in a further signal enhancement due to the increased density of ECL probes at the electrode surface.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: carbon dots · co-reactant · electrochemiluminescence · hybrids · self-enhancing

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