RuSiNPs@N,S-GQDs as self-enhanced anodic elect

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A competitive ECL immunosensor using novel self-reinforced solid-state ECL marker RuSiNPs@N,S-GQDs for accurate and rapid assessment of okadaic acid levels in shellfish.

Abstract

A competitive electrochemiluminescence (ECL) immunosensor is proposed to accurately and rapidly assess okadaic acid (OA) levels in shellfish using a novel self-reinforced solid-state ECL marker, which is essential for ensuring seafood safety. Graphene quantum dots doped with nitrogen and sulfur (N,S-GQDs) were synthesized, for the first time, through the electrolysis of graphite in 3-(N-morpholine) propane sulfonic acid solution. Intriguingly, these N,S-GQDs exhibited exceptional co-reactant properties, significantly enhancing the anodic ECL performance of Ru(bpy)3 2+ in a phosphate-buffered saline solution. Following the functionalization of Ru(bpy)3 2+-doped silica nanoparticles (RuSiNPs) with poly(diallyldimethylammonium) chloride, we achieved a well-dispersed assembly of N,S-GQDs on the exterior of the RuSiNPs through electrostatic interactions. Importantly, the core–shell structure of RuSiNPs@N,S-GQDs efficiently encapsulated both the luminophore and co-reactant, thus improving the transfer rates of electrons, shorting interaction distances, and reducing energy loss during light emission. Leveraging this "bright" ECL beacon, the ECL immunosensor demonstrated remarkable analytical performance, yielding a low half maximal inhibitory concentration (IC50) of 0.14 ng mL−1, an extensive linear range spanning 0.003–40 ng mL−1, and impressively low limit of detection of 0.001 ng mL−1 for OA determination.

Keywords: N,S-GQDs · Ru(bpy)☒²☒-doped silica nanoparticles · Okadaic acid · Electrochemiluminescence · Immunosensor

Introduction

Electrochemiluminescence immunoassay (ECLIA) represents a sophisticated fusion of electrochemiluminescence (ECL) and immunochemistry and has gained prominence as an invaluable tool within the biosensing field due to its attributes, including miniaturization, precise controllability, heightened selectivity, and remarkable sensitivity. Key to the establishment of high-efficiency ECLIA protocols is the acquisition of ECL markers having excellent biocompatibility and high ECL activity. In ECLIA research, Ru(bpy)3 2+ dominates as the preferred ECL label because of its high ECL efficiency, favorable operating pH (i.e., neutral conditions), robust stability, and in situ regenerative capabilities.

Typically, the ECL emission of Ru(bpy)3 2+ is predicated on the interaction between a luminophore and intermediate product generated from co-reactants such as tripropylamine, met

hamphetamine, or codeine. Although these co-reactants significantly raise the ECL respons e of Ru(bpy)3 2+, they exhibit inherent limitations. First, organic amines have undesirable v olatility and toxicity, which reduces the accuracy of Ru(bpy)3 2+-based ECL at low concentra tions. Second, the existence of organic compounds in ECLIA solutions can disrupt antibody-antigen interactions. Finally, in most Ru(bpy)3 2+-based ECLIAs, Ru(bpy)3 2+ is fixed on bio molecules, whereas the co-reactants remain in solution. Thus, the large electron transfer di stance impedes efficient interaction between Ru(bpy)3 2+ and co-reactants in the solid/solu tion phase, leading to energy dissipation and diminished sensitivity.

To circumvent these challenges, substantial efforts have been dedicated to discovering alte rnative co-reactants and developing innovative solid-state ECL markers tailored for ECLIA. C onsidering the rapid advancements in nanotechnology, carbon-based materials, specificall y carbon nanodots (C-dots) and graphene quantum dots (GQDs), have emerged as viable co -reactants for Ru(bpy)3 2+-based ECL. Notably, Long et al. were pioneers in elucidating the c o-reactant behavior of C-dots in the anodic ECL of Ru(bpy)3 2+, and Qi et al. reported the sig nificant boosting of Ru(bpy)3 2+ ECL through the use of GQDs as co-reactants.

However, there has been limited research into the use of carbon-based materials as ECL co-reactants; further, there is a need for a comprehensive understanding of the specific mecha nisms underpinning the enhancement of Ru(bpy)3 2+ ECL. Recent investigations have indic ated that doping GQDs with heteroatoms allows for the fine-tuning of their bandgaps and lo cal electron densities, which allows for activity enhancement for specific applications. Thes e advancements have encouraged our research into heteroatom co-doped GQDs, exemplifie d by N,S-GQDs, and our quest to unravel their co-reactant mechanisms governing the ECL o f Ru(bpy)3 2+.

Subsequently, a challenge arose of devising solid-state composites containing both ECL lu minophores and co-reactants. Recent work by Zhong et al. has offered intriguing solutions t o this problem. In addition, Zhuo et al. conceived an ECL luminophore combining a Ru com plex with poly(ethylenimine) within a single composite, thereby enhancing the luminous eff iciency of Ru(bpy)3 2+. Later, Wang et al. reported that Pd nanowires could immobilize poly amidoamine (PAMAM) dendrimers and Ru(bpy)3 2+ together, yielding a novel ECL derivative boasting remarkable ECL emission characteristics.

Inspired by these endeavors, our research has centered on designing a composite that incor porates both Ru(bpy)3 2+ (as the luminophore) and N,S-GQDs (as the co-reactant), and we h ave shown the potential of this approach for the development of high-performance ECL bea cons. Crucially, by immobilizing Ru(bpy)3 2+ within silica nanoparticles to generate RuSiNP s, we avoid the wastage of precious, stable, and electrochemically reversible Ru(bpy)3 2+. T o date, there have been no prior reports detailing the utilization of N,S-GQDs in conjunction with RuSiNPs for the construction of self-enhanced ECL sensors.

In previous studies, we focused on the detection of okadaic acid (OA), the principal constitu ent accountable for diarrheal shellfish poisoning (DSP), which makes it a key safety indicato

r for shellfish consumption. In accordance with the guidelines set by the European Food Saf ety Authority (EFSA), the permissible maximum level for OA in shellfish is 45 µg kg−1. In a pr evious study, we harnessed P,S-GQDs as the ECL probe, successfully achieving the sensitive detection of OA with a minimal detection limit of 0.005 ng mL−1.

Building on this foundation, the present investigation focuses on the synthesis of N,S-GQDs via an electrochemical approach, employing 3-(N-morpholine) propane sulfonic acid (MOP S) as the precursor. These N,S-GQDs, in turn, exhibit a pronounced sensitization impact on t he ECL of Ru(bpy)3 2+. Exploiting this synergy, N,S-GQDs and RuSiNPs were combined to cr eate self-enhanced ECL nanocomplexes having a core–shell architecture. Leveraging their e xpansive specific surface areas and augmented ECL performance, RuSiNPs@N,S-GQDs are e mployed as immune markers, allowing for the formation of an ECL immunoprobe (RuSiNPs @N,S-GQDs-Ab, where Ab indicates an antibody).

Additionally, in this study, the carboxylated multiwall carbon nanotubes-poly(diallyldimeth ylammonium) chloride-Au nanocluster (CMCNT-PDDA-AuNCs) composite was used as a plat form to immobilize OA. The CMCNT-PDDA increased electrode conductivity and surface area , while AuNCs enhanced conductivity and served as OA binding site. By doing so, the sensiti vity of the OA sensor was improved.

As a result, we designed a competitive ECL immunosensor that enabled the rapid and highl y sensitive detection of OA, as demonstrated through the analysis of certified shellfish stand ards containing OA (i.e., the CRM-DSP-Mus-c quality control standard). In addition, we provi de novel insights into the N,S-GQDs/Ru(bpy)3 2+-based self-enhanced ECL system, which w e hope will spur the development of innovative ECL markers and broaden their applications for bioanalysis.

Experimental methods

Synthesis of RuSiNPs@N,S-GQDs

RuSiNPs were produced using a previously published method. Then, 2 mL of RuSiNPs suspe nsion (2.5 mg mL−1) was mixed with 30 mL of PDDA (poly(diallyldimethylammonium chlori de)) solution (1%) and stirred for 3 h in the dark. The obtained mixture underwent centrifug ation at a speed of 12,000 rpm for a duration of 15 min in order to eliminate the supernatant . Following this step, the resulting precipitate was subjected to three rounds of washing wit h water. The washed precipitate was then suspended in 5 mL of water to yield PDDA-functio nalized RuSiNPs.

RuSiNPs (referred to as RuSiNPs-PDDA) were maintained at 4 °C and then sonified for 10 mi n before each use. Finally, RuSiNPs@N,S-GQDs were synthesized. Specifically, 2 mL of RuSi NP-PDDA was added to 500 µL of N,S-GQDs (8 mg mL−1), and the compound was agitated a t room temperature for 3 h. The RuSiNPs@N,S-GQDs nanocomposite precipitates were conc entrated by centrifugation, bathed three times with water, and resuspended in 4 mL of wate

r. The RuSiNPs@N,S-GQDs were then contained at 4 °C.

ECL immunoassay for OA

To initiate the experiment, BSA (100 µL, 1 wt%) was incubated with OA-CMCNT-PDDA-AuNCs /GCE for a period of 1 h at room temperature. This step was crucial in order to prevent any n on-specific binding sites from being activated. Subsequently, the OA-CMCNT-PDDA-AuNCs/ GCE was dipped in a solution of RuSiNPs@N,S-GQDs-Ab (20 µL in phosphate buffer (pH 7.4, 10 mmol L−1)) and OA standard solution, the CRM-DSP-Mus-c sample, or spiked samples (1 0 µL each) and incubated at 37 °C for 50 min. ECL tests were performed in phosphate buffer (pH 8.0, 1.0 mol L−1) with a photomultiplier tube at −800 V.

Using the experimental results, the binding rates of RuSiNPs@N,S-GQDs-Ab and fixed OA at various free OA concentrations were computed. The binding rates between fixed OA and fre e OA were calculated as ECL free OA / ECL fixed OA × 100%, where ECL free OA and ECL fixe d OA are the intensities of ECL with and without free OA, respectively. The S-curve plot of th e binding rate versus OA concentration was obtained to determine the half maximal inhibito ry concentration (IC50) and limit of detection (LOD).

Results and discussion

Characterization of N,S-GQDs

The transmission electron microscopy (TEM) images of the N,S-GQDs are shown in Fig. 2A. A s shown, the N,S-GQDs were well dispersed and uniform in size, having an evenness particle size of approximately 2.5 nm. The elemental composition and chemical structure of the N,S -GQDs were also analyzed using X-ray photoelectron spectroscopy (XPS). The XPS full spectr um scans of the N,S-GQDs showed four typical peaks at 400.0, 168.1, 258.1, and 531.2 eV, res pectively, corresponding to N1p, S2p, C1s, and O1s. The four atomic percentages of N, S, C, and O were calculated separately to be 3.97%, 2.33%, 49.01%, and 44.69%, showing that N and S had been resoundingly doped into the GQDs framework.

The high-resolution N1s spectrum contains three peaks at 398.2, 399.3, and 400.4 eV, which are ascribed to pyridinic nitrogen (nitrogen-containing six-membered ring), pyrrolic nitroge n (nitrogen-containing five-membered ring), and graphitic nitrogen (N-(C)3), respectively. T he high-resolution S2p spectrum contains peaks at 167.6 and 168.7 eV, both corresponding to the C-S(O)2-C sulfone bridge. These results further verify the successful doping of N and S into the GQD structure.

Next, the optical properties of the N,S-GQDs were investigated using UV–vis absorption and fluorescence mapping. The N,S-GQDs yielded two absorption peaks at 232 and 300 nm, whi ch are attributed separately to the $\pi \rightarrow \pi^*$ transition of the C = C bond and the $n \rightarrow \pi^*$ transit ion of the C = O bond. Fluorescence mapping showed a maximum emission peak at 533 nm for the N,S-GQDs when excited at 300–440 nm. The synthesized N,S-GQDs were dark yellow in daylight and emitted yellow-green fluorescence under a UV lamp at 365 nm.

RuSiNPs have poor electrical conductivity because of their surface silica layer. Therefore, RuSiNPs/CS/GCE (810 Ω) presents a larger impedance than CS/GCE (280 Ω), whereas the impedance of the RuSiNPs@N,S-GQDs/CS/GCE (430 Ω) is significantly lowered after modification of N,S-GQDs, which implies that the interfacial charge transfer between the RuSiNPs@N,S-GQDs/CS/GCE and the electrolyte is faster, and the synthesized N,S-GQDs have good electrical conductivity.

Characterization of OA fixed electrodes and ECL immunoprobes

Unlike our previous direct modification method, we adopted the mixing and stirring method before modification, which increased the contact between CMCNT-PDDA and AuNCs, allowing more AuNCs to bind to the CMCNT-PDDA surface and providing more binding sites for OA immobilization at the modified electrode. UV–vis spectra were used to characterize the spectral changes after the assembly of the CMCNT-PDDA-AuNCs. The CMCNT-PDDA-AuNCs nanocomplex shows a characteristic absorption peak of AuNCs at 275 nm.

Following this, we synthesized an ECL immunoprobe referred to as RuSiNPs@N,S-GQDs-Ab through an amidation reaction involving N,S-GQDs and anti-OA-MAb. The characterization process included TEM analysis, zeta potential measurements, and UV–vis absorption spectroscopy. Numerous black particles were uniformly distributed on the surface of the nanospheres, and the morphologies of RuSiNPs-PDDA and N,S-GQDs were combined, reflecting that the N,S-GQDs were uniformly dispersed on the outside of RuSiNPs-PDDA.

The adsorption of N,S-GQDs caused the positively charged RuSiNPs-PDDA to become negatively charged, which further confirms the successful attachment of RuSiNPs to N,S-GQDs; the zeta potential of the formed RuSiNPs@N,S-GQDs complex was −9.9 mV, whereas, after linking with anti-OA-MAb, the zeta potential of the couples shifted positively to approximately −0.57 mV, possibly because the antibody is a positively charged protein.

The OA-CMCNT-PDDA-AuNCs/GCE produced a significantly increased ECL signal (approximately 19.3-fold) when incubated in the RuSiNPs@N,S-GQDs-Ab solution, whereas the ECL signal produced by non-specifically adsorbed RuSiNPs@N,S-GQDs was almost negligible. Taken together, these results confirm that the ECL immunoprobe RuSiNPs@N,S-GQDs-Ab had been produced successfully.

Determination of OA

To confirm the viability of the designed ECL immunosensor for OA detection, we established the ECL curves in response to different concentrations of OA on the OA-CMCNT-PDDA-AuNCs. The binding between the OA and CMCNT-PDDA-AuNCs/GCE and free OA resulted in fewer RuSiNPs@N,S-GQDs-Ab being immobilized on the electrode surface, which led to a reduction in the ECL intensity. However, the immunosensor exhibited high sensitivity to OA concentration because of the large amount of N,S-GQDs loaded outside the core–shell structure of the RuSiNPs@N,S-GQDs immune beacon, which not only enhanced the signal intensity of R

uSiNPs but also provided more binding sites for anti-OA-MAb, improving the chance of specific binding of antigen OA to the antibody.

Under optimal detection conditions, standard antibody binding rate curves were obtained to determine OA concentrations by competition between fixed OA and free OA using the probe. The S-shaped four-parameter logistic equation fitted standard curve obtained for the ECL immunosensor in phosphate buffer showed a good linear relationship between the concentration of free OA and the antibody binding rate in the range of 0.003–40 ng mL−1, yielding a corresponding linear equation of $y = -7.54 \ln(x) + 36.16$, $R^2 = 0.99$, IC50 of 0.14 ng mL−1, and LOD (corresponding to 85% antibody binding rate, S/N = 3) of 0.001 ng mL−1, indicating that the proposed ECL immunosensor has a wide linear range and high sensitivity for OA detection.

Table 1: Recovery tests of the developed ECL immunoassay for spiked Solen gouldi samples

Sample	Spiked OA (ng mL⁻¹)	Found (ng mL⁻¹)	Recovery (%)	RSD (%) (n=3)
Solen gouldi	0.10	0.096	96.00	3.60
Solen gouldi	0.20	0.207	103.50	3.20
Solen gouldi	0.40	0.392	98.00	2.60

Specificity, stability, and repeatability

Theoretical accuracy is crucial for the practical use of sensors. The root-square deviation (RSD) of the ECL response signal was only 3.0% after 15 consecutive scans, indicating the high stability of the ECL immunosensor. Next, six electrodes prepared in parallel were used to detect the same concentration of OA six times. The corresponding RSD value was 5.3%, indicating that the prepared OA immunosensing electrodes have good reproducibility.

Dinophysistoxin-1 (DTX-1), saxitoxin (STX), and 13-desmethylspirolide C (SPX-1) standards were selected to investigate the specificity of this method for OA detection. When DTX-1, STX, and SPX-1 were added alone, the binding rate of the immunosensor decreased by 38%, 5%, and 6%, respectively, relative to the blank control; after adding equal concentrations of OA to the above solutions, the binding rate of the blank group decreased by 67%, compared to 65% and 64% for STX/OA and SPX-1/OA, while the bonding rate of DTX-1/OA dropped by 69%. This indicates that the ECL immunosensor did not cross-react with STX and SPX-1, whereas the presence of DTX-1 resulted in some interference with the sensor.

Application for real sample analysis

The practical applicability of the designed OA immunosensor was ascertained through spiked recovery experiments conducted on shellfish samples contaminated with OA. Precise concentrations of OA were introduced into uncontaminated Solen gouldi samples, resulting in final OA concentrations of 0.1, 0.2, and 0.4 ng mL−1 in the shellfish samples. Subsequently, these spiked samples were analyzed using the ECL immunosensor. The recoveries ranged from 96.0 to 103.5%, and the measured OA concentrations closely corresponded to the spike

d OA concentrations. This observation underscores the high reliability of the experimental i mmunosensor in the context of OA detection in shellfish samples, affirming its potential for quantitative determination of OA within authentic samples.

Conclusions

In this paper, N,S-GQDs characterized by uniform particle size and favorable dispersion wer e synthesized employing electrochemical methodologies. The obtained ECL results reveale d the pronounced sensitization effect of N,S-GQDs on both Ru(bpy)3 2+ and RuSiNPs. This st udy elucidated the ECL sensitization mechanism intrinsic to the Ru(bpy)3 2+/N,S-GQDs syst em. By encapsulating N,S-GQDs onto the surface of RuSiNPs, an ECL self-enhanced RuSiNP @N,S-GQDs complex with a core–shell architecture was constructed. An immunoprobe boa sting exceptional ECL performance characteristics was formed by efficiently coupling this se lf-enhanced ECL complex with anti-OA-MAb.

A novel ECL immunosensor was conceived by employing the CMCNT-PDDA-AuNCs/GCE as a detection platform and RuSiNPs@N,S-GQDs as potent ECL markers. This immunosensor wa s demonstrated through the competitive reaction that involves immobilized OA and free OA . Crucially, the sensor presented an IC50 of 0.14 ng mL−1, a linear range of 0.003–40 ng mL− 1, and an LOD of 0.001 ng mL−1. Thus, an immunosensor for the assessment of OA in quality control standards, spiked recovery experiments, and real samples was successfully develop ed.

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