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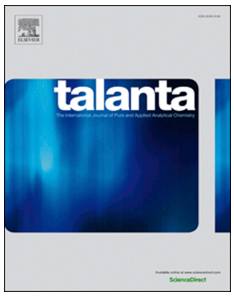

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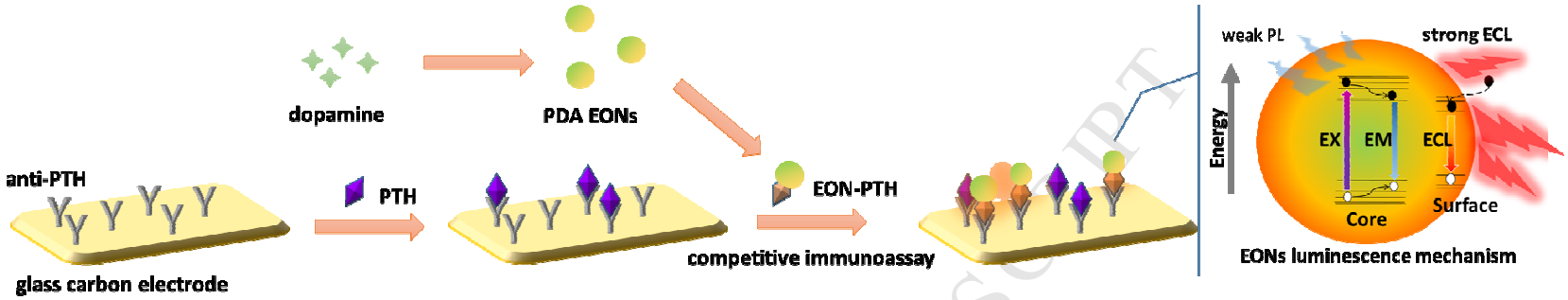

The polydopamine electrochemiluminescence (ECL)-organic nanoparticles (EONs) based immunosensing strategy was designed for parathyroid hormone detection.

A novel polydopamine electrochemiluminescence organic nanoparticle-based biosensor for parathyroid hormone detection

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Abstract

In this work, polydopamine electrochemiluminescence (ECL)-organic nanoparticles (EONs) based immunosensing strategy was designed for parathyroid hormone (PTH) detection. Dopamine is oxidized and polymerized to form polydopamine organic nanoparticle via self-polymerization process. Unlike the low photoluminescent efficiency and unsatisfactory fluorescence characters of the fluorescent organic nanoparticles (FONs), the polydopamine EONs do not only show unique physicochemical properties and excellent biocompatibility, but also provide ideal electrochemical properties and bright ECL signals, which can be employed as high-quality ECL luminophores. The ECL-related properties and performance of the EONs are further discussed in this paper. The sensing method has a linear response in the range of 0.05-8ng/mL with a detection limit of 17 pg/mL. The applicability of this method is evaluated through the determination of PTH in human plasma samples with satisfactory results. To our best knowledge, this was the first time about the exploration of polydopamine organic nanoparticles as ECL luminophores in the biosensing application.

Keywords: ECL organic nanoparticle; polydopamine; parathyroid hormone detection

1. Introduction

Parathyroid hormone (parathormone, PTH) is secreted by the chief cells of the parathyroid glands as a polypeptide which contains 84 amino acids [1]. The changes in the PTH-level within serum can directly indicate the parathyroid gland disease. For example, hyperparathyroidism can result in excessive production of PTH [2]. PTH can not only regulate calcium and phosphorous levels in extracellular fluids, but also stimulate the multiple intracellular signals of cyclic adenosine mono-phosphate (cAMP) and both protein kinases A and C [3, 4]. Moreover, the PTH level is also associated with cardiovascular and chronic kidney diseases, and several types of cancer. PTH is involved in the metastatic pathway through the growth and differentiation of neoplastic cells. When the breast and prostate cancer metastasized to the bone, PTH concentrations in serum have significantly increased. So, PTH detection in the human blood is very important to monitor osteoporosis, parathyroid disorders, malignant-associated hypercalcemia and the effectiveness of treatment in cancer patients. Until now, only a few analytical methods for PTH detection have been developed, including fluorescence method, electrochemical analysis, chromatographic analysis [1-6]. Because PTH has very short half-life of only a few minutes and is serum normal levels of 11–54 pg/mL after excision of the parathyroid tissue [6], it is essential to further develop a simple, rapid and accurate method for the determination of PTH, especially intraoperative PTH analysis.

Electrogenerated chemiluminescence technology (ECL) inherited and incorporated the distinct analytical advantages of electrochemistry and spectroscopy. Compared to conventional photoluminescence based analytical method, ECL emission light does not require an external light source. So, there is no derived background noises (e.g. autofluorescence and scattered light) in ECL sensing system. As a result, the sensitivity and the signal-to-noise ratio of ECL are improving dramatically. Until now, many kinds of NPs with reliable ECL signals has been employed in the exploration of highly sensitive ECL sensing system in the clinical diagnosis, such as quantum dot, carbon nanodots, g-C_3N_4 nanosheets, and noble metals nanoclusters [24, 25]. So, it was worth developing highly efficient ECL nano-luminophores. Recently, organic

nanoparticles (FONs) with nontoxic nature and high biocompatibility have attracted more and more attention. Compared with low ECL efficiency inorganic nanoparticles (e.g. semiconductors quantum dots, silicon quantum dots, carbon dots and metal clusters), FONs possess high ECL efficiency, structural variability, lower toxicity, high biocompatibility and biodegradable potential, which can overcome the limitations of fluorescent inorganic nanoparticles [12-14]. In the past few decades, different kinds of FONs have been reported based on small organic dyes, conjugate polymers and aggregation induced emission dyes [15-16]. For example, as kind of dopamine derivative, polydopamine (PDA) has been employed to construct new organic nanoparticles. Zhang reported the prepared PDA-based FONs exhibited excitation-wavelength-dependent emission and was used for cell imaging [17]. Lu et al. have reported that colloidal PDA nanospheres can work as novel photothermal therapeutic agents for in vivo cancer therapy [18]. Up to now, the development of organic nanoparticle-based sensing research is still in its preliminary stage. Only a few reports focused on the application of organic nanoparticles in the biosensing system and cell imaging. Some problems still hinder the development of FONs, such as the limited conventional organic dyes, the complex and time-consuming synthesis routes, and low photoluminescent efficiency [19-23]. Therefore, the exploration of organic nanoparticles with novel excellent sensing properties is greatly desirable and significant.

In this work, we reported for the first time the novel PDA-based ECL-organic nanoparticles (EONs) were prepared in the biosensing application. As shown in Fig. 1, the novel EONs-based biosensor possessed several advantages: (i) According the oxidation of catechol, PDA-based EONs can be prepared via dopamine self-polymerization under alkaline conditions through covalent bonding, hydrogen bonding, $\pi-\pi$ interactions, and so on [26, 27]. Compared with the traditional ECL nano-luminophores, EONs have unique physicochemical properties, superior biocompatibility, and non-toxicity. (ii) The high proportion of carbonyl and carboxyl groups on the EONs surface have contributed to the ECL reaction with oxygen-containing intermediate radicals and high ECL efficiency. And the stronger

π–π stacking interaction between the oligomeric units in PDA EONs can induce electron injection. (iii) The PDA has high specific surface area, good thermodynamic stability, and stable chemical properties on the electrode surface. [28, 29] So, PDA EONs are ideal luminophore for ECL sensing application. The prepared biosensor was applied to the rapid and efficient detection of PTH in human blood with high selectivity and sensitivity and high stability.

2. Experimental

2.1 Materials and chemicals

All chemical reagents in this experiment were of analytical grade. $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, sodium citrate, and other chemical reagent were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Ammonia solution, PTH, anti-PTH and BSA were purchased from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Dopamine and chloroauric acid were obtained from Xilong Chemical Co., Ltd. (Guangdong, China). Water used in the experiments was ultrapure deionized water (resistance 18MΩ cm).

2.2 Apparatus

The photoluminescence measurements were performed on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Co., Kyoto, Japan) equipped with a xenon lamp using right-angle geometry and a quartz cell. FT-IR spectra were recorded with a Bruker IFS66V FT-IR spectrometer equipped with a DGTS detector (32 scans). Ultraviolet–visible (UV–vis) absorption spectra were obtained using a GBC Cintra 10e UV–vis spectrometer. The pH value of the buffer solution used was measured with a PHS-3C pH meter (Tuopu Co., Hangzhou, China). Transmission electron microscopy (TEM) images were obtained with a Hitachi electron microscope operating at 200 kV. The ECL detection system consisted of a BPCL ultraweak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China) and a CHI 660B electrochemical workstation (Shanghai Chenhua Instrument Co., China). The voltage of the photomultiplier tube (PMT) was set at 900 V during the whole process. Electrochemical impedance spectroscopic (EIS) analysis was performed on a CHI 660B electrochemical workstation in 2.5mmol/L

$K_3[Fe(CN)_6]/K_4[Fe(CN)_6$ with 0.1 mol/L KCl as the supporting electrolyte. The impedance spectra were recorded in the frequency range of 0.01 Hz–100 kHz with an amplitude of 5 mV. A conventional three-electrode system was used in this system. A glassy carbon electrode (GCE) was used as the wording electrode. A platinum wire was employed as the counter electrode and an Ag/AgCl (saturated KCl) electrode as the reference electrode.

2.3 Synthesis of PDA EONs

EONs were prepared by aqueous phase-based oxidation polymerization. Firstly, 100mg dopamine was added in the 250mL bottom flask and diluted with 140mL ultrapure water. After mixed, the 0.1mL Tris Buffer (pH=8.5) was quickly added into the suspension. The suspension was heated in the water bath at 30 ^oC and stirred for 7.5 hours. Then the EONs were separated from the aqueous phase. The resulting EONs were washed with purified water twice to remove superfluous dopamine molecules, following by drying under vacuum. Quantum yield of EONs was measured according to the reported quantum yield of quinine sulfate (0.54) in 0.1 mol/L H_2SO_4. The QY of the resultant QDs was calculated according to the follow equation:

$$Y_u=Y_sI_u/I_s\ A_s/A_u\ n_u^2/n_s^2\ [30]$$

where Y represents the quantum yield, I represents the measured integrated emission intensity, A represents the absorbance intensity, n is the refractive index of the solvent, u represents the S-doped BN QDs, and s represents the quinine sulfate standard sample. The ECL efficiency (Φ_{ECL}) of QDs was calculated according to the follow equation:

$$\Phi_{ECL}=\Phi^0_{ECL}\ I/I^0\ Q^0/Q\ [31]$$

Where Φ^0_{ECL} is the ECL efficiency of the standard $(Ru(bpy)^{3+}$, 5%), I and I^0 are the integrated ECL intensity of the species and the standard, and Q and Q^0 the faradic charges (in coulombs) for the investigated and the standard species, respectively.

In the preparation of PTH-EONs, 0.125g EONs were dispersed in 16 mL of PBS

(pH 7.4) buffer solution under stirring for 30 min at room temperature. Then, 2 mL of PTH solution (20μg/mL, pH=7.4) were added and incubated at room temperature for 2 h. The PTH-EONs was isolated and washed with PBS (pH 7.4) buffer solution. After that, 2 mL of 2wt % BSA was added in and incubated at room temperature for 2 h to block the excess nonspecific group on the EONs. The excess BSA was moved off and washed with PBS (pH 7.4) at least three times. At last, the final PTH-EONs nanoprobe was dispersed into 15 mL PBS (pH 7.4) and stored at 4□ until use.

2.4 ECL Immunoassay protocol

The GCE was first carefully polished with the 1.0 and 0.05 mm alumina slurry followed by rinsing thoroughly with double distilled water, and then ultrasonically cleaned in ethanol and double distilled water to obtain a mirror-like surface. Finally, it was dried under nitrogen atmosphere ready for use. The polymelamine (pMel)/ GCE was prepared by electro-polymerization. In the electro-polymerization process, GCE was immersed in 0.1 mol/L H_2SO_4 containing 1.0 mmol/L melamine with the potential range of 0–1.6 V for 20 cycles. As a result, GCE can be coated by pMel layer. [32]. The purpose of modifying pMel at the electrode was to immobilize the anti-PTH. PMel displays good adhesion to the GCE surface. For the modified GCE, the PMel can improve the stability of ECL sensor. Then the anti-PTH was dropped on the pMel/GCE for further experiments. 5μL of anti-PTH (10μg/mL) was dropped onto PMel/GCE and dried in air. The modified electrode was blocked with 2wt % BSA solution.

The ECL electrode was utilized to capture target PTH in the sample solutions for 10 min. After immunoreaction, the electrode was rinsed with washing buffer and dried in air. Finally, the ECL electrode were further reacted with complementary 0.5 mg PTH-EONs for 10 min. Then, the electrode was rinsed with washing buffer. All electrochemical measurements were carried out with the three-electrode configuration at room temperature. A potential from 0.0 V to -2.5 V was applied to the working electrode, and the ECL signal was recorded simultaneously with 0.1 mol/L $K_2S_2O_8$ as coreactant

3. Results and discussion

3.1. The EONs synthesis and characterization

As shown in Scheme 1, the new protocol of ECL competitive immunoassay was designed for the detection of PTH based on the PDA EONs. The dopamine is oxidized and polymerized to form PDA EONS and provide strong luminescence signals. In the the competition immunoassay, target PTH was captured by the PTH antibody on the electrode surface. Then the unreacted sites of PTH antibody were bound by PTH-EONs and generated the ECL signals of EONs. Topography and the size of the EONs can be directly visualized by TEM. As shown in Fig. 2, the prepared EONs had a uniform particle size distribution and the size is consistent. They showed a spherical shape with mean particle size of 15 nm. In the aqueous phase-based oxidation polymerization, the EONs had uniformly packaged PDA shell structure.

To further investigate EONs surface properties, FT-IR spectrum was shown in Fig.2 B, the peak at 1620 cm^{-1} implied the existence of residual phenyl ring like structure, supporting the PDA polymerization on the MNPs. The wide peak at 3400 cm^{-1} was assigned to stretching vibration of N-H, indicating that there were lots of amino groups on the surface of EONs. In order to study intrinsic luminescence properties of EONs, the photoluminescence excitation and emission spectrum are shown in Fig.3. The fluorescence excitation and emission peak of the PDA EONs were at ca.270nm and ca.477nm, respectively. And the QY of EONs was ca.5%, which is quite low as fluorescence luminophore for the reliable bioanalysis. The similar fluorescence limitation of organic nanoparticles is also reported by other reference. [19-23] The ECL peak of EONs is at 620nm. Compared to the fluorescence emission peak, the significant red shift (ca.150nm) was attributed to active PDA surface states and surface defects on the EONs surface. Unlike the photoluminescence generation process with photo absorption, the electron transfer occurred at the surface of EONs in the ECL reaction. The ECL generation way required to provide the excited state of EONs. The high proportion of carbonyl, carboxyl and catechol groups on the EONs surface can form narrower band gaps than the core. As a result, a significant red shift of the ECL peak from the photoluminescence can be observed. [29] Moreover, the electron transfer on the EONs surface can provide enough energy

to generate ECL emission light. The ECL efficiency of EONs is ca. 2.9% (Φ_{ECL} of commercial [$Ru(bpy)_3^{2+}$] is 5%), which appropriate to the ECL biosensing application.

Because the synthesis condition had the large effect on the PDA EONs structure and luminescence properties. Therefore, the effects of the EONs synthesis condition were investigated. Following the increased synthesis time, the dopamine solution color changed from colorless to orange-yellow in the synthesis process, which indicated that dopamine was oxidized to its quinone derivative and successfully formed the PDA EONs. As shown in Fig. 2 C, the highest PL intensity of the EONs was obtained at 7.5 hour. In Fig.2 D, experimental results further demonstrated that the synthesis time and temperature played a key role in PDA polymerization. The effects of synthesis temperature on the luminescence intensity of the EONs were more obvious, which can decide the structure formation of PDA net structure. Therefore, 30^{o}C and 7.5 hours were selected as the optimized synthesis condition for the following experiments.

3.2. Electrochemical characterization of EONs

As is shown in Fig. 4 A, the cyclic voltammetry of EONs was performed with a supporting electrolyte of 0.1 mol/L KCl and 1.0 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. Because of many oxidizing (e.g. -COOH) and reductive groups (e.g. -OH) on the surface of EONs, there are a oxidation peak at 0.152V and a reduction peak at 0.275V. On the CV graph, EONs showed good electrochemical activity with a pair of symmetrical reversible redox peaks. The cyclic voltammetry of EONs was also measured in PBS and PBS containing 0.1 mol/L $K_2S_2O_8$. As shown in the Fig 4(B), the reduction peaks of EONs in PBS was -1.06V. When 0.1 mol/L $K_2S_2O_8$ was added, the wide reduction peaks of EONs shifted to-1.125V. The obvious change in the cyclic voltammetry confirmed the ECL reaction process of EONs and $K_2S_2O_8$. It can be seen from Fig. 4 (C) that the ECL signal can be generated at -1.8 V and reach the maximum intensity at -2.1V. The ECL mechanisms of EONs were formation of the excited state EONs* with the existence of the $K_2S_2O_8$ co-reactant. The mechanism for

ECL generation can be described with the equations below:

$$EONs + e^- \rightarrow EONs^{\cdot-} \quad (1)$$

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{\cdot-} \quad (2)$$

$$EONs^{\cdot-} + SO_4^{\cdot-} \rightarrow EONs^* + SO_4^{2-} \quad (3)$$

$$EONs^* \rightarrow EONs + h\nu \quad (4)$$

Electrochemical impedance spectroscopy (EIS) provided the electroconductibility of EONs on the electrode. As is shown in Fig. 4 C, the cleaned GCE showed a mass diffusion limiting process. There were slight increases at 43.23 Ω when EONs were connected on the electrode. The EIS data were fitted to an equivalent circuit (inset of Fig. 4 (D)) and the charge transfer resistance (Rct) values of the modified electrodes were obtained by calculating the diameter of the semicircles in the plot (99.14 Ω). It indicated that PDA EONs have good electroconductibility.

3.3 Determination of PTH

As is shown in Fig. 5, with the concentration of target PTH increasing, the ECL intensity decreased. Fig. 5 B showed there was a good linear correlation between the ECL intensity and the PTH concentration in the range of 0.05~8 ng/mL. The linear equation between the concentration of target PTH and ECL intensity was I= -12323 C_{PTH} + 155507 with the limit of detection (LOD) of 0.017 ng/mL and the limit of quantification (LOQ) of 0.05 ng/mL. The correlation coefficient was 0.9868. The LOD and LOQ are calculated in accordance with previous reports. [33] The LOD of the sensor was calculated according to the follow equation:

$$X=3sB/b$$

where X indicates LOD, sB indicates standard deviation of blank signal (testing frequency is 10), b indicates the slope of the linear calibration curve. Some other reported methods for the determination of PTH were compared with the proposed method. The comparison was summed up in Table 1. It is clearly showed that the EONs-based biosensor had a relatively wide linear range and low LOD.

Fig. 6(A) showed the reliability of PDA EONs. The stability of the EONs on GCE was tested by a serious of measurements (n=12) in phosphate buffer solution (pH=7.4) 0.1 mol/L $K_2S_2O_8$. The relative standard deviation (RSD) of EONs was 5.76%. These results revealed that the ECL sensing system based on EONs was feasible. To further investigate the reproducibility, intra and inter-assays were performed (Figure 6 B); the relative standard deviations (ECL response) of intra- and inter-assays were 1.94% and 7.31%, respectively, which demonstrated an acceptable repeatability of the proposed biosensor for the detection of PTH. The effect of some other potentially coexisting species was studied in the presence of PTH and presence of excess amounts of interfering species. The concentration of Zn^{2+}, Co^{2+}, Cl^-, Br^-, NO_3^-, SO_4^{2-}, Cu^{2+}, Ba^{2+}, L-His, acetic acid, citric acid, GSH, creatinine was 10 mmol/mL, the concentration of dopamine was 20 mmol/L, the concentration of Na^+ was 50 mmol/L, and, the concentration of L-Cys was 100 mmol/L. It indicated that these interfering substances had little interference on the detection of PTH. The applicability of the proposed ECL sensor was evaluated by detecting the PTH in human plasma with the standard addition method. Recovery studies were carried by spiking various amounts of PTH to the samples. A summary of the calculated mean TPH concentration for each sample is shown in Table 2. Recoveries range from 94% to 108% was obtained, which indicated the feasibility of EONs-ECL sensing system for the determination of TPH in real samples.

4. Conclusion

In summary, an EONs-based ECL sensor has been developed. Its utility has been established for the detection of PTH in real human plasma samples. The EONs displayed bright ECL signals and can be used to detect PTH with high sensitivity, good precision, and acceptable storage stability. In the competitive immunoassay, the EONs ECL system with $K_2S_2O_8$ as a co-reactant was employed to increase the sensitivity and precision of the sensor. On the one hand, the new type of organic nanoparticle-based ECL sensor has great potential for future biological application and diagnostics. On the other hand, the new PDA EONs open up attractive perspectives for biosensing system and can be a powerful functional ECL

nanomaterial.

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Caption：

Fig.1. Schematic representation the EONs-based ECL sensor for PTH detection.

Fig.2 (A) TEM image of EONs, (B) the FT-IR of EONs, (C) The effect of the PDA polymerized time on the PL intensity of EONs in 30^{o}C. (D) The effect of synthesis temperature and time on the PL intensity of EONs. (a) 2.5h, (b) 5h, (c) 7.5h, and (d) 24h

Fig.3 (A) The PL excitation spectrum, (B) the PL emission spectrum, and (C) the ECL spectrum of EONs.

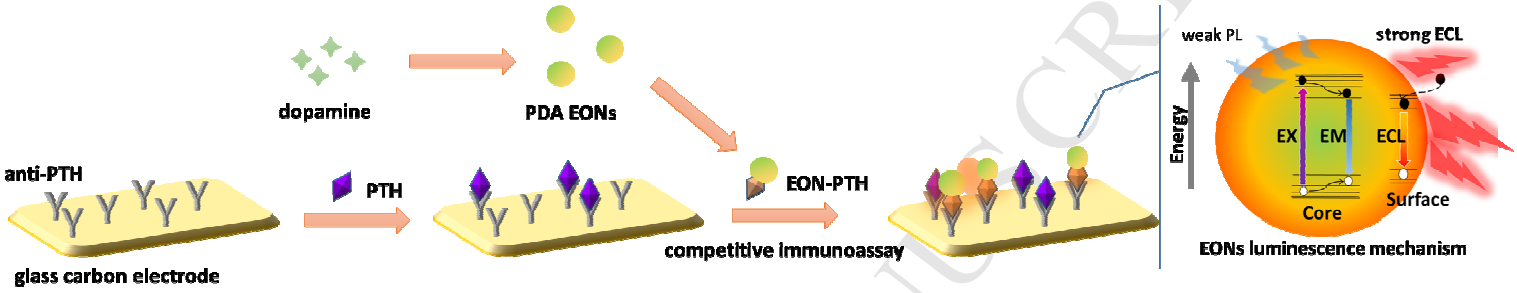
Fig.4 (A) Cyclic voltammetry of 3mg/mL EONs and bare GCE in 0.1 mol/L KCl solution containing 1.0 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. (B) Cyclic voltammetry of 3mg/mL EONs in in PBS and 0.1 mol/L $K_2S_2O_8$. (C) ECL behavior of PDA EONs with 0.1 mol/L $K_2S_2O_8$. (D) EIS spectrum of EONs and bare GCE in 0.1 mol/L KCl solution containing 2.5 mmol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The impedance spectra were recorded in the frequency range of 0.01 Hz–100 kHz with an amplitude of 5 mV.

Fig.5 (A) ECL traces of different concentrations of PTH. (From left to right: 0.05, 0.1, 0.5, 1.0, 3, 5, 8 ng/mL. (B) Calibration curve for quantification of PTH.

Fig.6 (A) The ECL stability of PDA EONs. (B) The ECL reproducibility of the proposed biosensor for PTH Detection.

Table 1 Comparison of different methods for the detection of PTH.

Table 2 Determination of PTH in EDTA-treated human plasma samples.



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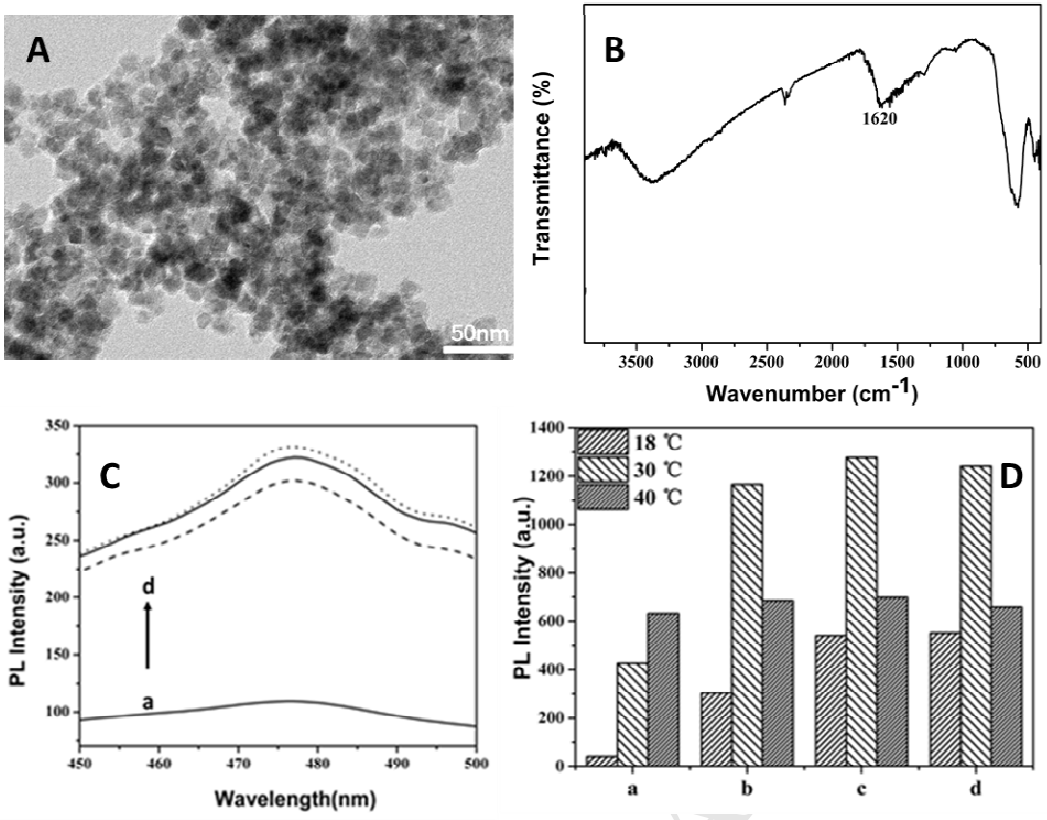

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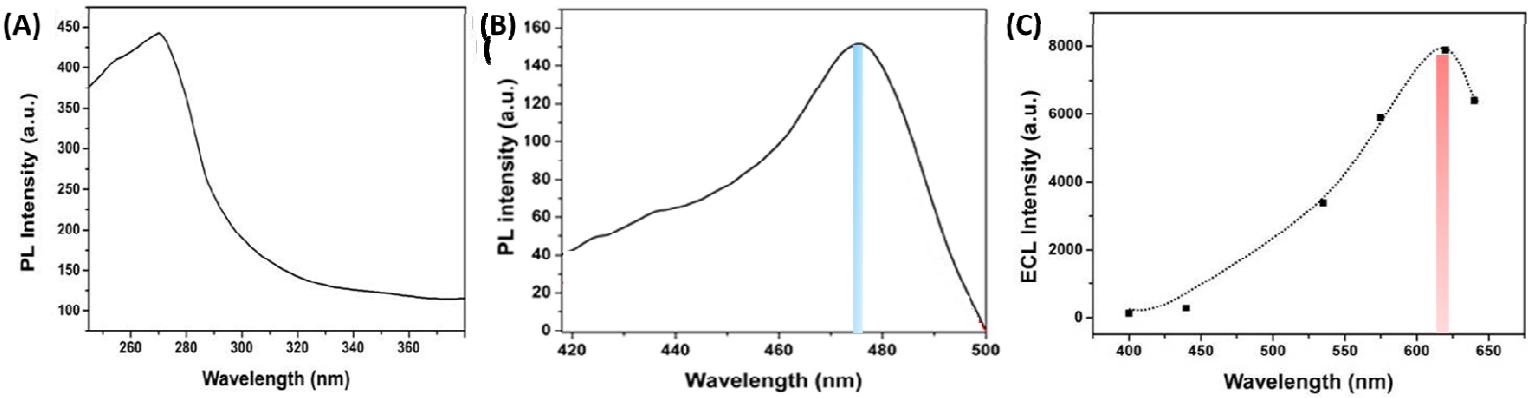

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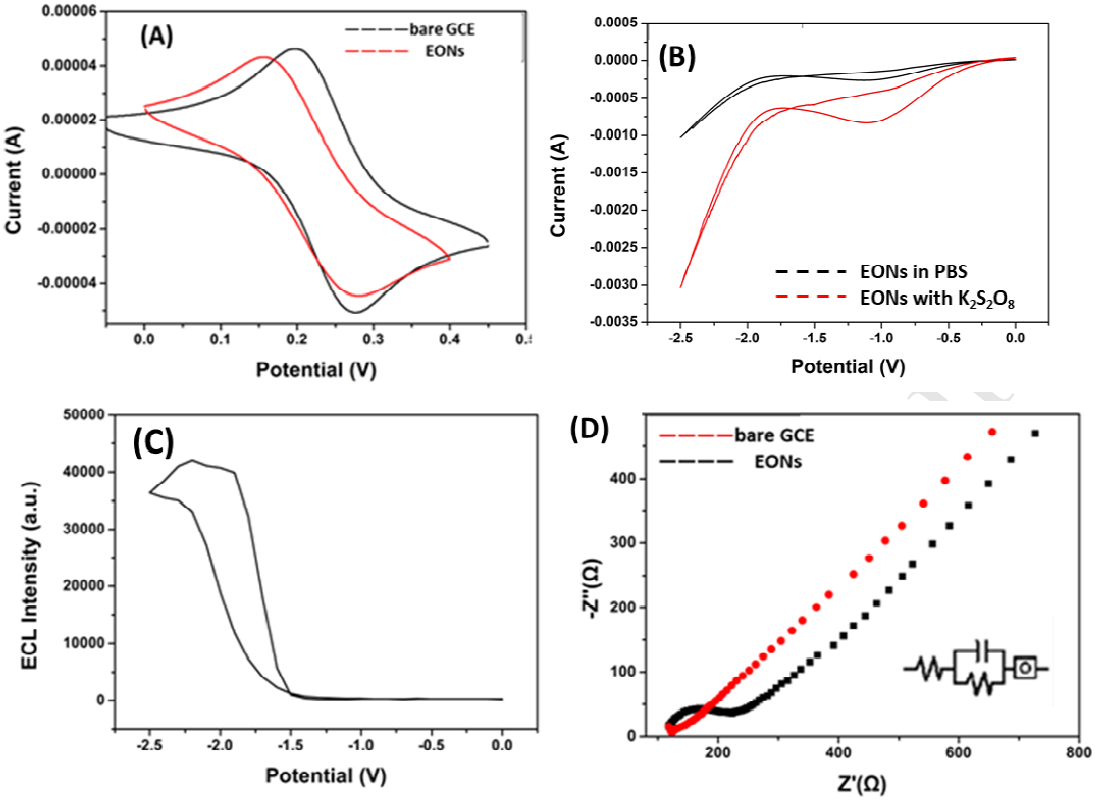

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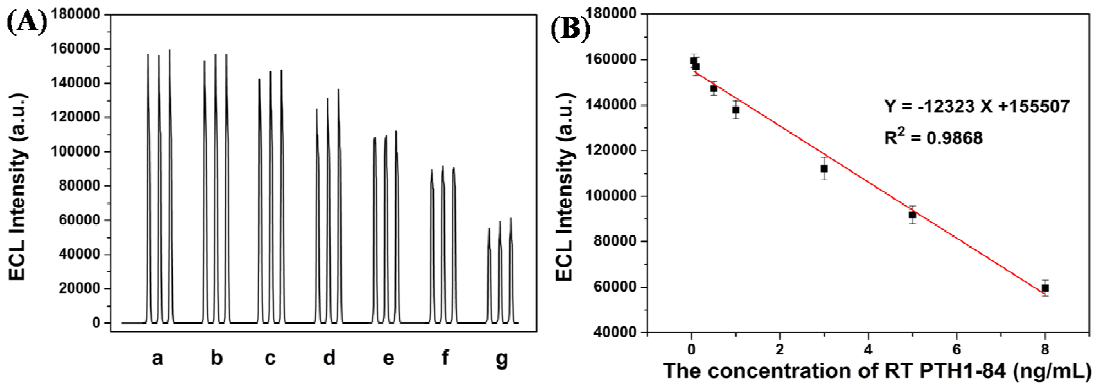

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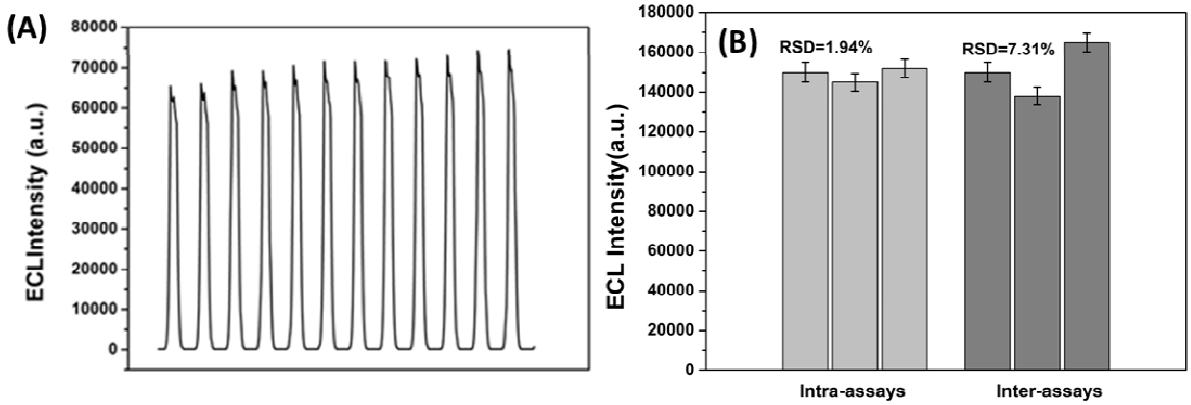

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Table 1 Comparison of different methods for the detection of PTH

Methods	The linear range	LOD	References
Electrochemical immunosensor	10–50 pg/mL	7.65 pg/mL	1
Nanoporous membrane	60-400 ng/mL	55 ng/mL	2
Electrochemical immunosensor	50-500 ng/mL		3
Acoustic wave biosensor	61 ng/mL-100 μg/mL	61 ng/mL	4
Impedimetric biosensor	0.1–0.6 pg/mL	0.1 fg/mL	6
Capillary zone electrophoresis method	0.25–250 μg/mL	0.25 μg/mL	34
EONs-based ECL sensor	0.05~8ng/mL	0.017ng/mL	This work

Table 2 Determination of PTH in EDTA-treated human plasma samples

Sample	Original (ng/mL)	Spiked (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (%, n=3)
1	0.054	0.050	0.108	108.0	3.33
		0.100	0.151	97.0	2.54
2	0.073	0.050	0.120	94.0	4.20
		0.100	0.169	96.0	1.48
3	0.080	0.050	0.128	96.0	2.89
		0.100	0.183	103.0	1.28

The EONs provide ideal electrochemical properties and bright ECL signal sbased on the surface defect state emission.

The high proportion of carbonyl and carboxyl groups on the EONs surface have contributed to the ECL reaction with oxygen-containing intermediate radicals and high ECL efficiency.

EONs have unique physicochemical properties, superior biocompatibility, and non-toxicity.