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Research Article

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In-situ Polymerization of N-(3-aminobenzyl)-N, N-dimethyl-N-dodecyl Ammonium Bromide on Glassy Carbon Electrode for Estradiol Detection

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Abstract

Estradiol is a common endocrine disrupting chemicals. Therefore, it is necessary to develop simple, sensitive and rapid methods for testosterone bioanalysis. In this work, poly (N-(3-aminobenzyl)-N,N-dimethyl-N-dodecyl ammonium bromide) (PAS), a novel cathodic organic electrochemiluminescence (ECL) emitter, was synthesized by in-situ polymerization of the corresponding monomer on glass carbon electrode. The PAS ECL system was constructed with the PAS modified electrode, and potassium persulfate (KPS) as oxidizing agent and co-reactant. It was applied to the ECL detection of estradiol. According to the results of fluorescence and dynamic light scattering (DLS), mixed micelle of PAS and estradiol was formed. The combination of PAS with estradiol intensified the fluorescence of the ECL system and enlarged the colloidal size. Hydrogen bonds and electrostatic interaction may play a role in the combination. The linearity for estradiol quantification is 6 pM to 1.5 nM with a LOD of 0. 1 pg/mL. The modified electrode can be easily renewed by washing with ethanol and water and the relative error in estradiol measurement does not exceed ± 7% in 7 time recycling. The detection of estradiol in urine sample qualified the ECL system with a recovery of 94.5 to 105% and the calculated coefficient of variation of 3.8%. This indicates that the PAS ECL system has the potential to detect estradiol in urine.

Keywords: Poly[(N-(3-aminobenzyl)-N, N-dimethyl-N-dodecylaminium Bromide)], in-situ polymerization, Electrochemiluminescence, Estradiol detection

Introduction

The hormone estradiol, also known as 17β-estradiol, is a naturally occurring estrogen with high biological activity and is secreted mainly by female ovaries or male testes. Estradiol plays a vital role in many physiological processes [1]. Estradiol is an indicator of human sexual maturity. Monitoring estradiol levels in humans can help doctors diagnose and treat sexual hormone imbalances. For women, increasing of estradiol concentration in the systemic circulation can promote breast development, genital growth, and change the body fat distribution in adolescent women [2]. Estradiol is not only related to the growth and development of female body, but also has very important significance for middle-aged and elderly people. Cooke et al. reported in their recent review the important role of estradiol in male physiological regulation [3]. They pointed that early studies found that estradiol exists in many male tissues in the form of binding state of ESR1 and ESR2 with proteins, and each shows a unique distribution and effect. In animal exogenous estrogen therapy experiments, Exogenous estradiol can promote body growth and development of male mice with reproductive diseases, especially during development. Estradiol in ESR1 helps reduce the appearance of abnormal sperm. In addition, the loss of ESR1 or the corresponding aromatase also affects non-reproductive targets such as brain, fat, skeletal muscle, bone, cardiovascular and immune tissues. For example, the decrease of estradiol levels can lead to bone loss [4]. However, unlike females, the concentration of estradiol in serum does not change along with men age. Therefore, in elderly men, osteoporosis is closely related to the bioavailability of binding state estradiol [5]. Overall, men face the problem of decreased levels of estradiol recycling as they age, and this decline may lead to osteoporosis in men and women. Therefore, it is necessary to develop a highly sensitive and specific estradiol detection method.

At present, many sensitive human serum estradiol detection methods has been used in clinical diagnosis, including high performance liquid chromatography-mass spectrometry (HPLC-MS), chemiluminescence immunoassay, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay cross-linking. Immunosorbent assay (ELISA). Although the detection limit of mass spectrometry can reach ~10 pg/mL. This method is too complicated to samples and the instrument is expensive. Therefore, mass spectrometry is not applicable to routine clinical practice. Although RIAs can perform rapid, sensitive and inexpensive in detection of a large number of

clinical samples, the use of radioisotopes and scintillation fluids limits their practical application for the detection of estradiol in serum. ELISA quantifies target molecules by observing changes mediated by the enzyme. Although the estradiol ELISA assay has been widely used for the detection of estradiol, and various ELISA kits have been commercialized for the detection of serum estradiol, this method cannot induce the target molecular antigen in low concentration to generate sufficiently discernible color changes, and the enzyme labeling instrument does not have the function of distinguishing the color change. Therefore, a single ELISA kit cannot detect estradiol below a concentration of 10 pg/mL. In order to solve these problems, many alternative testing methods have been developed in recent years. N. Yildirim et al. developed an aptamer-based folded fluorescence sensor that detects 2.1 nM of estradiol by fluorescence. Zhang et al. used the label-free competition ECL method to detect, with a detection limit of 1.1 pM, and can detect estradiol at concentrations ranging from 0.01 to 10 nM. X. Hao et al. constructed a label-free aptamer sensor using NiHCNFe NPs as the probe, which can induce 0.8 pM of estradiol by electrochemical method. Its detection range is 1 to 600 pM [6].

Electrochemiluminescence (ECL) is an analytical assay that has much attention in recent years. ECL is a kind of energy relaxation when the emitter returns to the ground state, after electrochemical oxidation and reduction [7,8]. This new detection method has high sensitivity, simple operation, and strong specificity. It has been widely used in biological analysis, organic reaction mechanism research and other fields. The modified electrode has the advantages of increased stability, increased sensitivity, enhanced signal, and specificity [9-12]. More importantly, the modified electrode-based ECL system is not limited by the dispersion of the material in the medium [13,14]. Therefore, it has become the main form of design strategy of ECL detection system. The most commonly used method of modified electrode ECL bioanalysis is to modify the specific compound or biomacromolecule to a clean glassy carbon electrode, which can increase the signal of the ECL system and also enhance the specificity of the system. Compared with the solution, modifying the ECL analysis probe or emitter onto the electrode can make the system more stable to the target, and can also reduce external interferences such as temperature and dust [14]. Since most organic molecules, such as nanoparticles, quantum dots, used for ECL analysis have poor conductivity, the signal intensity of ECL system may be greatly reduced or even disappear [15]. Therefore, many modified electrodes must first use a conductive material as a foundation and then assemble functional materials. For example, Guangpeng Liu et al. modified gold particles to electrodes by electrodeposition, and assemble organic probes to this basement [16]. This ECL system use hemin/G-body deoxyribozyme to mimic the catalytic activity of horseradish peroxidase, which can catalyze H_2O_2 to produce large amounts of ROS for enhancing the ECL signal of luminol. Although organic probes and DNA macromolecules are used to impart electrode specificity, this ECL system is used to detect silver ion concentrations in natural water bodies. This shows organic material modification motor is not limited to the detection of organic molecules. Jingjing Xu and his team also ensured the conductivity of the ECL system by depositing gold nanoparticles on electrode [17]. The g-C_3N_4 nanosheets were then modified on the electrode to detect the cholesterol in individual cells on the electrode surface. It is worth noting that even if a conductive material is used as a substrate to ensure the conductivity of the ECL system, too many modified organic functional materials will hinder the electron transport and reduce the ECL signal intensity.

In this work, we will introduce a new compounds as the electrode modification material and ECL emitter. Poly [N-(3-aminobenzyl)-N,N-dimethyldodecyl-1-ammonium bromide] (PAS) is a positively charged polycationic quaternary ammonium salt that can be effectively adsorbed to the negative charged surface of the glassy carbon electrode. The in-situ polymerization of its monomer on the electrode can place PAS firmly to the glassy carbon electrode surface, resulting in PAS modified electrode of strong stability. PAS on the modified electrode is not easily eluted or dissolved in the case of solvent rinsing, and has the potential for reuse [18]. We also increased the intensity signal of PAS ECL system by optimizing the polymerization time.

Experimental Section

Chemicals and Apparatus

Bromodecane and tert-butyl 3-((dimethylamino)methyl) phenylcarbamate were purchased from Xiya Chemical Co. (Shandong, China). Chloroform, light petroleum, ethyl acetate, dichloromethane, methanol, anhydrous magnesium sulfate, ammonium peroxydisulfate, potassium peroxydisulfate, and silica gel (200–300 mesh) were obtained from Kelong Fine Chemical Co. (Liaoyang, China). Estradiol was purchased from Alfa Aesar (Ward Hill, MA, USA). Double-distilled water was used in all of the experiments. Glassware was obtained from Synthware Glass Co. (Beijing, China).

The degree of polymerization was measured by Shanghai Boyan Testing Technical Service Co. (Shanghai, China) using gel permeation chromatography (GPC). A Cary Eclipse Fluorescence Photometer (Agilent, Santa Clara, CA, USA) was used to obtain fluorescence emission spectra. ECL experiments were carried out using an MPI-E electrochemiluminescence detector (Remex, Xi'An, China).

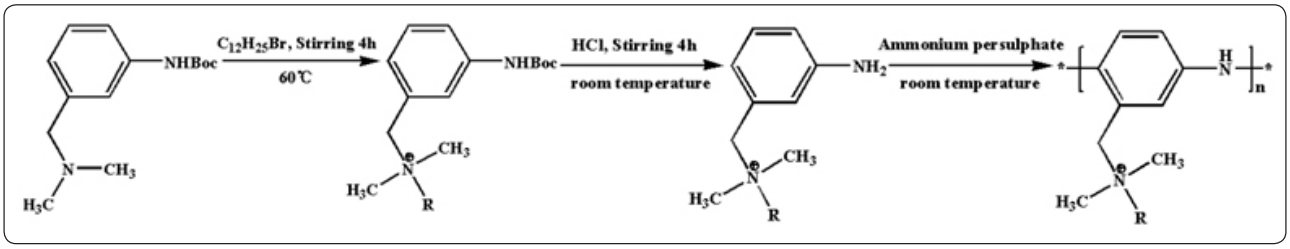
A three-electrode system was employed in all of the electrochemical and ECL experiments, consisting of a glassy carbon electrode (GCE, Φ = 4 mm) as the working electrode, Ag/AgCl (saturated KCl) as the reference electrode, and Pt wire as the counter electrode.

Preparation of Monomer and poly (N-alkyl aniline)

The preparation of the monomer was as shown in Scheme 1. The tert-butyl 3-((dimethylamino)methyl)phenylcarbamate and dodecyl bromide (molar ratio=1:1.2) were dissolved in acetonitrile, respectively. The two solutions were mixed and stirred at 60 °C for 4 h. After the reaction was completed, unreacted bromododecane in the product was removed by column chromatography. 0.2g obtained product was dissolved in ethyl acetate, and mixed with 2 mL 5M hydrochloric acid. Then, the mixture was stir for 4h. The pH was adjusted to 7.0 with saturated sodium hydroxide solution to terminate the reaction. The monomer was extracted with ethyl acetate, and dried by adding anhydrous magnesium sulfate. The desiccant was removed by suction filtration. The filtrate was evaporated under reduced pressure to get monomer N-(3-aminobenzyl)-N, N-dimethyl-N-dodecyl ammonium bromide. The product was stored in a cool, dry environment.

Monomer N-(3-aminobenzyl)-N,N-dimethyl-N-dodecyl ammonium bromide and ammonium persulfate (molar ratio 1:1.2) were dissolved in a small amount of methanol and distilled water, respectively. The two solutions were mixed and stirred at room temperature overnight

to ensure the polymerization of monomers into polymers. The polymerized product was then washed sequentially with water and ethyl acetate to remove the initiator and unreacted monomers. The product, Poly [N-(3-aminobenzyl)-N, N-dimethyl-N-dodecyl ammonium bromide] was finally vacuum dried and characterized by FTIR.



Scheme 1: Process of syntheses of monomer and its polymerization

The process of electrode modification

The GCE was polished with 0.05 μm alumina slurry and sonicated in acetone, ethanol and distilled water for 5 minutes, respectively. After dried in air, the washed electrode was placed in 1 mL of a 0.4 mM monomeric ethanol solution for 5 min. Then 1 mL 0.2 M PBS (pH 7.4) containing 0.48 mM ammonium persulfate aqueous solution was added, and uniformly mixed. The reaction was carried out at room temperature for days. The electrode was took out, and washed with ethanol and distilled water 3 times. At last, the modified electrode was dried in a cool place for electrochemical and ECL detection.

Electrochemical and ECL evaluation of PAS

500 μL of anhydrous ethanol was mixed with 3 mL of 0.2 M PBS (pH 7.4) containing 75 mM potassium persulfate and 2 mM potassium nitrate. The modified electrode was placed in the mixed solution at room temperature for 5 minutes, and then the electrochemical and ECL evaluation of the modified electrode was carried out. Unless otherwise specified, all electrochemical and ECL experiments were performed in a mixed solution of ethanol and water (volume ratio = 1:6). The potential range of electrochemical scan was -1.8 to 0 V. The scan direction was positive, and scan rate was 0.1 V/s. The photomultiplier tube voltage is 800 V.

DLS measurement of the solution of PAS and estrodiol

The blank solution was composed of 500 μL of 8.8 μM PAS ethanol solution, 500 μL ethanol and 3 mL of 0.2 M PBS (pH 7.4). The test solution was above blank solution mixed with 500 μL of 8.8/17.6/26.4 μM estradiol ethanol solution and the molar ratio of PAS to estradiol was 1:1/1:2/1:3, respectively. The solutions were left at room temperature for 4 hours before DLS measurement.

Procedures for estradiol determination in nature samples

The estradiol ethanol solution with different concentrations of estradiol was prepared by dissolving estradiol into 0.2 M PBS (pH 7.4) solution containing ethanol (The volume ratio of ethanol to water is 1:3), 53 mM potassium persulfate, and 2 mM potassium nitrate. After the solution was thoroughly mixed, it was placed at room temperature for 5 minutes before ECL experiments. When ECL experiment was ended, the modified electrode was rinsed three times with ethanol, distilled water, then air-dried overnight for the next ECL experiments.

Natural water samples was collected from four corners of the pool in our university. The urine sample was from a 27-year-old female at different times. The samples were placed in PE tubes for one day without any pretreatment. 500 μL of the sample supernatant was mixed with 2.25 mL 0.27 M PBS (pH 7.4) containing 75 mM potassium persulfate and 2.7 mM potassium nitrate. After the solution was standed for 5 minutes, the estradiol in the samples was measured.

Results and Discussion

Characterization of PAS

The ^{1}H and ^{13}C NMR spectra of the monomer are shown in Figures 1. The absorptions are ascribed as follows.

^{1}H NMR (400MHz, $CDCl_3$, TMS, ppm): δ7.04-7.01(m, 2H), 6.72-6.63 (m, 2H), 4.55(s, 2H), 3.31-3.27(t, 2H), 3.07(s, 6H), 1.67(s, 2H), 1.24-1.20(d, 20H) 0.85-0.81(t, 3H).

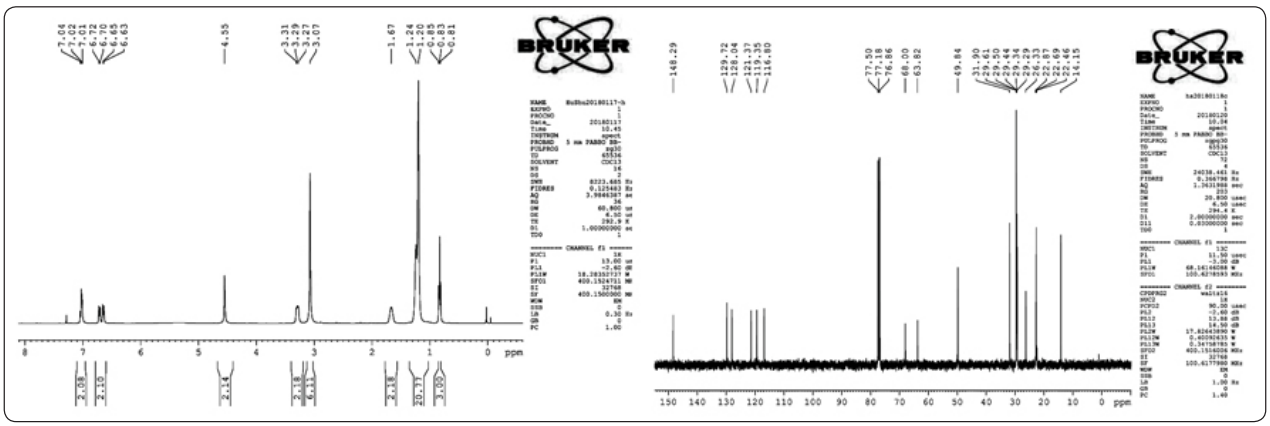


Figure 1: ^{1}H NMR and ^{13}C NMR spectra of monomer

^{13}C NMR (100 MHz, $CDCl_3$, TMS, ppm): δ148.29, 129.72, 128.04, 121.37, 119.35, 116.80, 68.00, 63.82, 49.84, 31.90, 29.61, 29.50, 29.44, 29.34, 29.29, 26.33, 22.87, 22.69, 22.46, 14.15.

Figure 2 shows the FTIR spectra of the synthesized PAS and its monomer. It can be seen from the infrared absorption of monomer that the strong broad peak at 3500 cm^{-1} belongs to the stretching vibration of N-H and the stretching vibration of O-H in the adsorbed water molecule. The strong peaks at 2925 and 2855 cm^{-1} are the stretching vibration of methyl and methylene groups in the alkyl side chain of monomer molecule. The 1627.83 cm^{-1} peak is attributed to the amine group on the benzene ring, and the 1460.96 cm^{-1} peak is due to N-CH_3 vibration absorption; The 1120.99 cm^{-1} peak is ascribed to C-N stretching vibration; The adsorption of β-CH_2 connecting on N^+ appears at 1044.11 cm^{-1} and that of benzene ring at 653.90 and 583.36 cm^{-1} [19-21].

Comparing with the infrared absorption of the monomer, it is seen that the basic absorption peaks are different in position and strength. The infrared absorption of PAS around 1679-500 cm^{-1} is stronger than that of its monomer. It can be explained that the number of the same functional groups in oligomer is increased due to polymerization. The peak at 1679.92 cm^{-1} is obvious, which is attributed to the C=N vibration of the anthracene ring on the main chain [22]. The peak at 1204.52 cm^{-1} is the vibrational motion of C-N on the main chain [23].

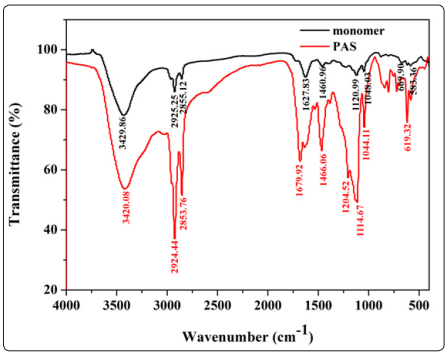


Figure 2: FTIR spectra of the monomer and PAS

Figure S1 shows the results of GPC analysis of polymer with THF as the eluent. The peak at 9 min belongs to PAS. And the peaks of 10.1, 10.3 and 11 min are due to the solvent THF. The degree of polymerization was calculated to be 9, and the average molecular mass (Mw) was 2669 g/mol. There is a strong mutual repulsion between the nitrogen cations on the branch, which is not conducive to the mutual proximity of the molecules and hinders the polymerization reaction, so the degree of polymerization of the obtained product is small, and the so-called polymer is actually an oligomer.

Electrochemical and ECL Performance of PAS

Figure 3 shows the ECL intensity and polymer molecular weight of PAS at 2 to 8 days of electrode modification. Both the ECL signal and polymer molecular weight increase with the increase of in-situ polymerization time of modified electrode. When the electrode is modified to the sixth day, the ECL signal of electrode is at the maximum and stable. When the modification time is longer than six days, the ECL intensity decreases, while the polymer molecular weight continued growing. Therefore, the time for electrode modification is set at 6 days.

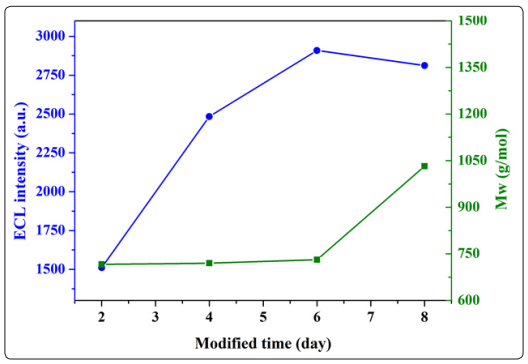


Figure 3: Average molecular mass of PAS and ECL intensity of electrodes modified in 2 to 8 days

Curve a in Figure 4 is the CV and ECL curves of clean glassy carbon electrode in 0.2 M PBS / 0.01 M KCl solution at pH 7.4 in the potential range of 2.6 to -2.6V. There seems no ECL signal, which shows that ethanol does not interfere with the system under this condition. Curve b the CV and ECL curves of the PAS-modified electrode. It can be seen that there are two weak cathode and anode ECL signals at -1.9V and 2.5V and two very weak oxidation peaks at 0.7V and 1.9V. This can be explained that PAS is electrochemically oxidized during the positive potential scanning, and then electrochemically reduced during the negative potential scanning, producing ECL signal. There is a delay between the ECL initial potential and the redox initial potential of the CV. This indicates that PAS is capable of generating an ECL signal, which can be an ECL emitter [7]. The ECL intensity of PAS is greatly enhanced from 90 a.u. to 3200 a.u. in the presence of KPS, and its ECL initial potential is changed from -1.9 V to -2.0 V (curve c), hinting a reaction happening between PAS and KPS. The strong peak at -1.2V is the reduction peak of $S_2O_8^{2-}$ [24].

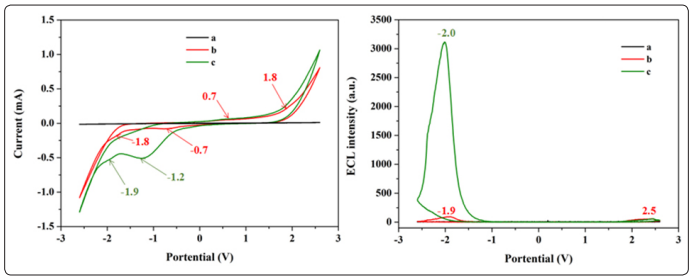


Figure 4: CV and ECL curves of (a) blank, (b) PAS and (c) PAS / 0.07 M KPS (0.2 M PBS, 0.01 M KCl, pH 7.4,)

ECL scan potential range of PAS modified electrode is optimized (Figure 5). When scan range is -2.6 to 0 V, ECL signal is extremely unstable. The recycling number of the electrode in this potential scan range is extremely limited. When scanning potential range is -2.0 to 0 V, the ECL signal is still not stable enough. When potential scanning range is -1.8 to 0 V, the ECL signal is almost as strong as the previous two scan ranges, and the signal is quite stable during electrochemical reduction. So, the scan potential range for PAS ECL system is set to -1.8 to 0 V.

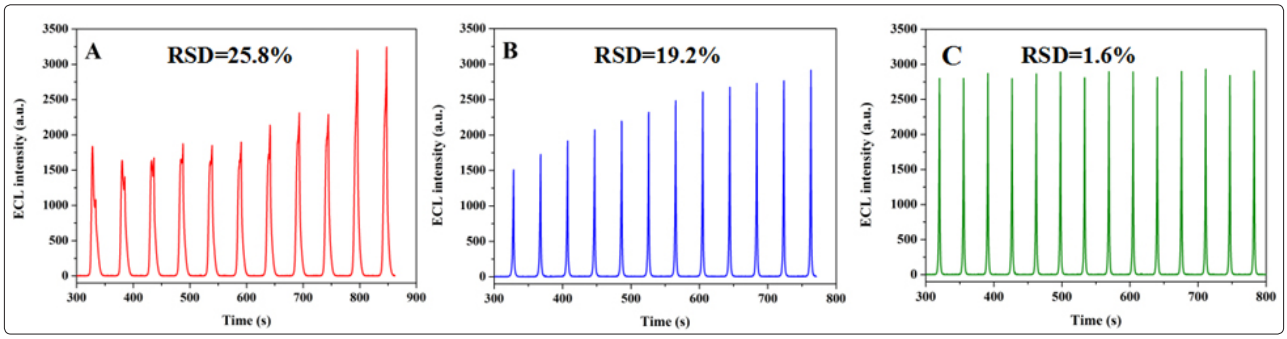


Figure 5: Relationship between scanning range and ECL signal stability of PAS modified electrodes [(A) -2.6 V to 0 V, (B) -2.2 V to 0 V, and (C) -1.8 V to 0 V]

When $S_2O_8^{2-}$ is present, the current intensity of PAS-modified electrode is drastically increase, and the oxidation peaks of PAS-modified electrode are hardly observed (Figure 4). In order to understand the impact of $S_2O_8^{2-}$ on PAS, the fluorescence of PAS was measured. Curve a in Figure 6 illustrates that the emission of PAS is quite weak in 280-400 nm. With the addition of KPS, the position is significantly red shifted, the fluorescence intensity is greatly increased and the emission spectrum is obviously broadened (Curve b). This may indicate that there is oxidation of PAS by $S_2O_8^{2-}$, which causes the conjugated backbone of PAS to grow, enhancing the conjugated system of PAS. In ECL experiments, the oxidized PAS can be electrochemically reduced during the ECL scan to produce an ECL signal.

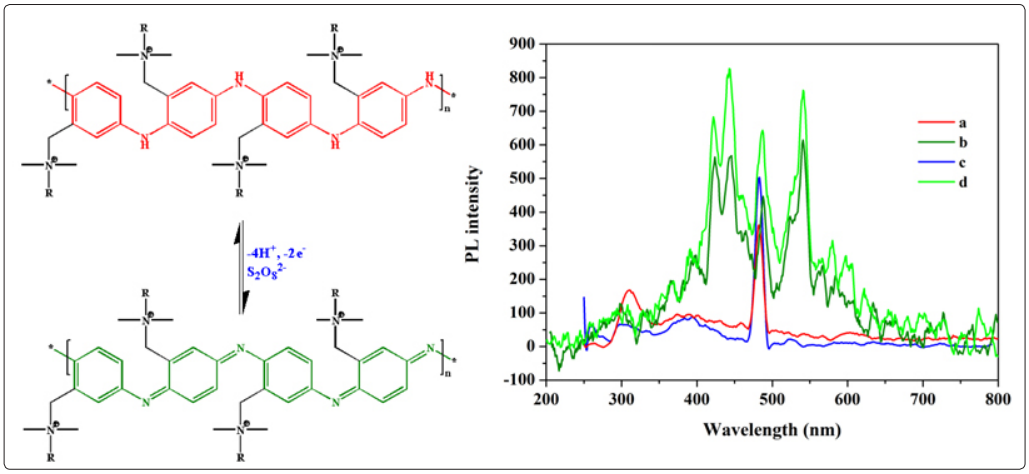


Figure 6: Normalized PL spectra: (a) PAS; (b) PAS/ KPS; (c) estradiol; (d) PAS/ estradiol/ KPS

As shown in Figure 7, the ECL intensity of PAS-modified electrode increases along with the increase of KPS concentration. When KPS concentration is 0.05 M, the ECL signal intensity is at maximum. When this concentration is further augmented, ECL signal begins to drop. Therefore, KPS concentration of the subsequent ECL experiment is set to 0.05 M, at which the ECL efficiency of PAS at the highest.

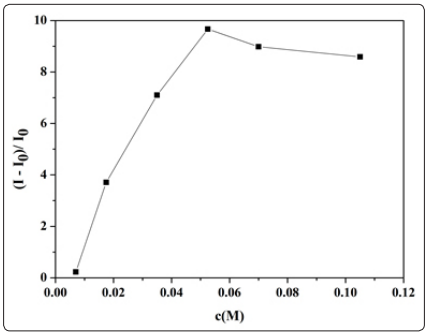


Figure 7: The relationship between KPS concentration and ECL enhancement rate of PAS modified electrode

It can be seen in Figure 8, the pH value of the solution will affect the ECL signal intensity of PAS modified electrode. At pH 8.0 the ECL signal intensity is the strongest. The pH 8.0 of phosphate buffer may be chosen for the next experiments. However, it has been found that under the condition of pH 8.0, the ECL signal of PAS modified electrode shows a significant decrease during the second scan, and the ECL signal intensity is less than 1000 a.u. in the third scan. Therefore, pH 7.4 is as the pH of ECL experiments.

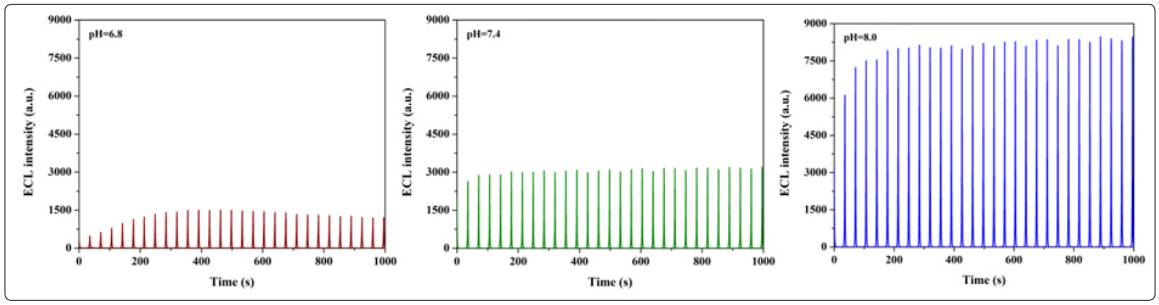


Figure 8: The dependence of ECL intensity of PAS modified electrode on pH of the solution

Interaction between PAS and estradiol

Figure 6 also illustrate that the fluorescence intensity of estradiol is very weak in 250-480 nm (Curve c in Figure 6). In estradiol/PAS/KPS system, the fluorescence is slightly enhanced and the fluorescence waveform resemble very much that of PAS/KPS (Curve d in Figure 6). Thus it means that estradiol may not form a covalent bond with PAS on the modified electrode and cannot alter the PAS conjugate backbone. Considering that PAS has positive charges and C=N structural units, and the estradiol molecule has a phenolic hydroxyl group and a hydroxyl group, the phenolic hydroxyl group may be dissociated and exist as an oxygen anion, thereby forming a salt bond with the nitrogen cation in the PAS (electrostatic interaction), while the hydrogen in hydroxyl may form a hydrogen bond with the conjugated main chain C=N, as shown in Figure 9 [25,26]. This weak connection cannot change the fluorescence emission of PAS, however, it may be disassociated by washing with solvent and offer the chance of multiple uses of the modified electrode in estradiol measurements.

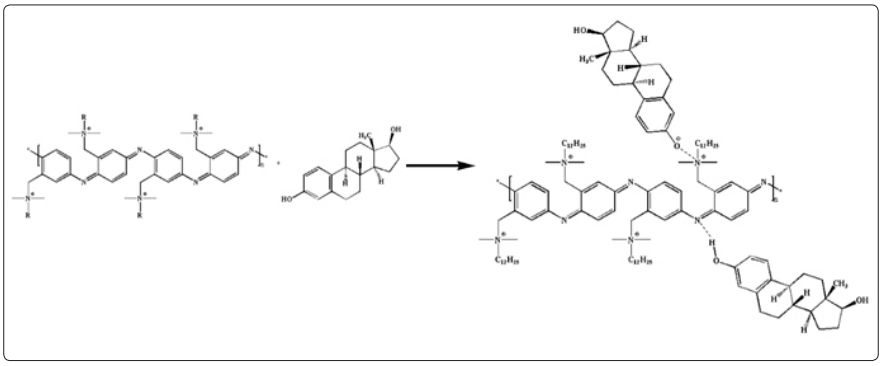


Figure 9: The possible binding of estradiol onto PAS

According to Figure 9, the connection of PAS with estradiol will bring a large volume particle of PAS/estradiol. So critical aggregation concentration (CAC) measurement and DLS analysis were carried out.

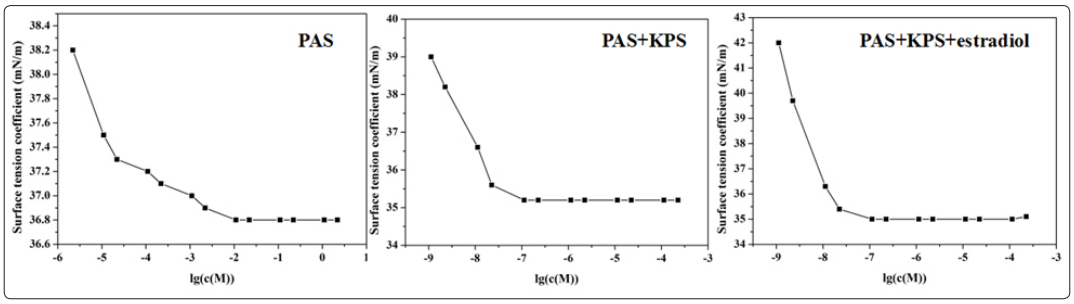


Figure 10: The relationship of surface tension with concentration of PAS (pH=7.4)

Figure 10 demonstrates the dependence of surface tension of the solution on PAS concentration. It is clear that PAS exhibits the character of polycationic surfactants: lowering the surface tension of water and self assembling into aggregates. The surface tension – concentration curve for PAS shows a few turns, suggesting that PSA is made up of homologues with different polymerization degree. The curve becomes smoother by the addition of KPS, indicating that homogeneous high molecules are generated by the oxidation of KPS. Besides, the addition of KPS decreases further the surface tension of the solution (about 35.2 mN/m). However, the introduction of estradiol in to PAS/KPS system brings almost no change in the solution, meaning that estradiol takes part in forming micelles instead of inserting into the surface PAS film. Therefore, there is not obvious change in fluorescence and in surface tension of estradiol/PAS/KPS solution (about 35 mN/m). The CAC of PAS colloidal solution is obtained according to the curves. It is 51 μM for PAS colloidal solution, 0.024 μM for PAS/KPS and 0.023 μM for estradiol/PAS/KPS, respectively.

Figure 11 is the particle size distributions of mixed solution of PAS and estradiol. It can be seen that the main particle size of PAS aggregation in solution is about 1400 nm. The particle size is enlarged by estradiol, and the main particle size is about 1750 nm for PAS/estradiol (1:1), 2200 nm for PAS/estradiol (1:2) and 2700 nm for PAS/estradiol (1:3). The more estradiol is added, the larger the mixed micelle size.

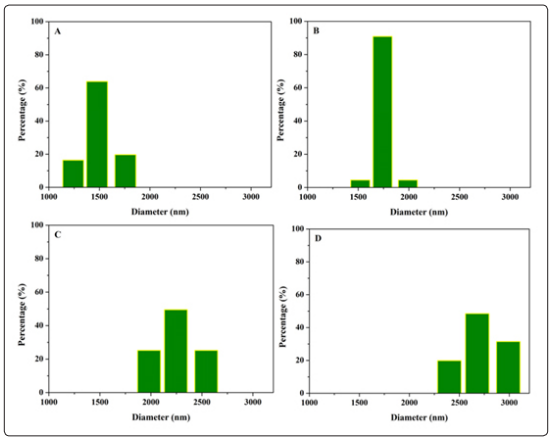


Figure 11: The particle size distributions of 8.8 μM PAS (A), 8.8 μM PAS mixed with 8.8 μM estradiol (B), 17.6 μM estradiol (C), and 26.4 μM estradiol (D)

The concentration of PAS solution employed in the determination of DLS is 8.8 μM, which is much larger than the CAC of PAS/KPS (0.023 μM). Thus, the micelles of the oligomer molecules are certainly formed in the test condition. It is the reason for such a large size aggregates existing in the solution. A possible ECL luminescence mechanism for the combination of PAS and estradiol is as shown below [24]:

PAS-estradiol + e- → PAS-estradiol•-

$S_2O_8^{2-} + e^- \rightarrow S_2O_8^{\bullet 2-}$

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

PAS-estradiol•- + $SO_4^{\bullet -}$ → PAS-estradiol* + SO_4^{2-}

PAS-estradiol* → PAS-estradiol + hν

The performance of PAS ECL system for estradiol detection

The curves in Figure 12 (right hand) specify that the ECL intensity of PAS/KPS is enhanced with increasing estradiol concentration. A linear relationship between the logarithm of estradiol concentration and the ECL signal intensity is illustrated in Figure 12. The linear formula is $I = 1555.4\log(c) + 20174.8$, R = 0.9940. Wherein I is the ECL signal intensity (a.u.) of PAS ECL system, and c is the concentration (M) of estradiol in ethanol solution. The linear range of estradiol concentrations is from 6 pM to 1.5 nM. So PAS ECL system may have an ideal application potential in the quantitative analysis of estradiol.

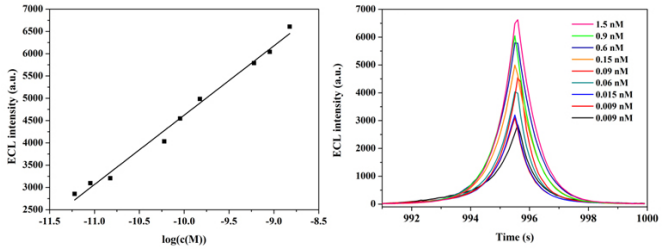


Figure 12: Calibration curve for estradiol determination and ECL intensity at different estradiol concentrations

Considering that PAS and estradiol are combined by hydrogen bonding or salt bonding, the estradiol and other adsorbate on the modified electrode may be removed by washing with distilled water and ethanol due to the weak nonchemical connection of PAS and estradiol. So PAS modified electrode can be reused in estradiol detection. After rinsing three times with distilled water and ethanol, respectively, the electrode was dried overnight and reutilized in estradiol analysis. To test the reuse performance, the estradiol solution of 90 pM was detected by washed PAS modified electrode. As shown in Figure 13, the ECL signal of PAS modified electrode can maintain the original intensity until the 7th recycling in estradiol analysis.

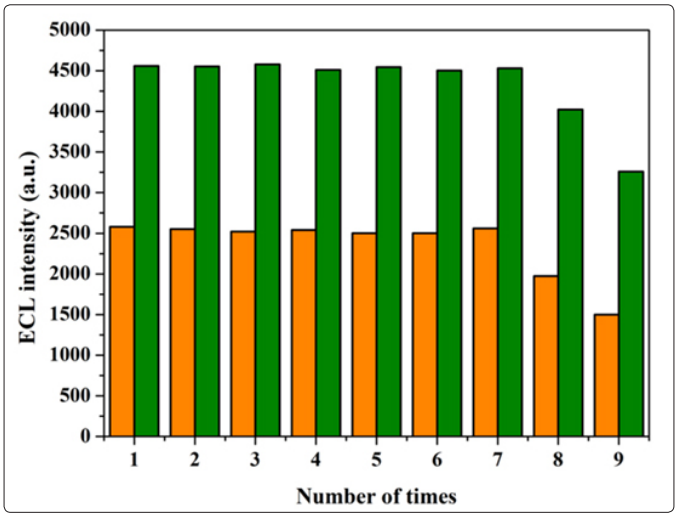


Figure 13: The reusability of PAS modified electrode

Table 1: Experimental results of repeated use of PAS modified electrodes

Number of times	1	2	3	4	5	6	7
ECL intensity (a.u.)	4559.8	4552.1	4578.6	4512	4546	4503.3	4531.0
Measured value (pM)	91.4	90.3	93.9	85.1	89.5	84.0	87.5
Relative error (%)	1.6	0.3	4.3	-5.4	-0.6	-6.7	-2.8

Table 1 lists the measurement results of PAS modified electrode on the 90 pM estradiol solution for 7 times. The data in this table demonstrate that the ECL intensity is substantially maintained in the first seven runs, and the relative error of the estradiol measurement does not exceed ± 7%. It is shown that PAS modified on the glassy carbon electrode by in-situ polymerization does bind tightly to the surface of the negatively charged electrode and has good stability in application. PAS modified electrode can be reused by simple cleaning with water and ethanol.

PAS modified electrode was utilized to the detection of estradiol in natural waters and urine. As shown in Table 2, the PAS modified electrode can detect estradiol in the urine samples. Comparing the concentrations of estradiol added and the total estradiol concentration, the absolute error of the estradiol concentration in sample and added is no more than ±0.1 nM. The recovery of the sample recovery experiment is 94.4%~104.5%, and the calculated coefficient of variation is 3.8%. PAS ECL system does not detect estradiol in the water samples of the school pool, or the estradiol in the sample is below the detection range of PAS ECL system. Comparing the concentrations of estradiol added and the total

estradiol concentration, the absolute error of the estradiol concentration in sample and added is no more than ±8 pM. The recovery is 97.7%~108%, and the calculated coefficient of variation is 8.6%.

Table 2: The detection of estradiol in Urine and Natural Water

Sample type	Sample number	Estradiol in sample (nM)	Added estradiol (nM)	Total estradiol (nM)	Recovery (%)	Coefficient of Variation
Urine sample	1	1.2	1.0	2.3	104.5	3.8%
Urine sample	2	0.8	1.0	1.7	94.4	
Urine sample	3	2.0	1.0	3.0	100.0	
Urine sample	4	1.0	1.0	1.9	95.0	
Natural Water	5	<0.0006	0.015	0.0162	108.0	8.6%
Natural Water	6	<0.0006	0.015	0.0159	106.0	
Natural Water	7	<0.0006	0.030	0.0301	100.3	
Natural Water	8	<0.0006	0.030	0.0293	97.7	

A comparison is made among the estradiol detection results by PAS modified electrode and by the reported method. As can be seen from Table 3, the sensitivity of PAS modified electrode ECL system is not higher than ultrasensitive immunoassay, molecular imprinting, electrochemical sensor etc., but the performance is superior to the reported ECL method. Therefore, we believe that this system is valuable for the detection of estradiol.

Table 3: Comparison of analytical properties by different methods for estradiol detection

method	Linear range (ng/mL)	LOD (ng/mL)	CV(%)
Surface-enhanced Raman scattering[2]	0.01−100	0.065	20
Label free immunosensor [27]	0.5–20	0.1	51
Ultrasensitive amplification immuneassay [28]	5×10^{-5}-1	6.37×10^{-6}	8.1
Molecularly imprinted [29]	3×10^{-9}-3×10^{4}	1×10^{-7}	3.5
electrochemical sensor [30]	3×10^{-6}-3×10^{3}	3×10^{-7}	5
Electrochemiluminescence [31]	0.03-30	0.003	12
Electrochemiluminescence	0.003-3.0	1×10^{-4}	3.8

Coefficient of Variation (CV) = (standard deviation/average) ×100

Conclusions

The PAS modified electrode is achieved by in-situ polymerization of N-(3-aminobenzyl)-N,N-dimethyl-N-dodecyl ammonium bromide. PAS ECL luminescence system is constructed by PAS modified electrode together with potassium persulfate as oxidant and initiator and applied to the estradiol detection. The following conclusion can be drawn according to the experiment.

1. PAS on the electrode is first oxidized by potassium persulfate and then reduced to produce an ECL signal upon ECL negative potential scanning. PAS ECL system is at the highest luminous efficiency and most stable under the conditions of 0.05M potassium persulfate, pH value of 7.4 and scanning potential range of -1.8 to 0V.
2. PAS self-assembles into micelles in the solution due to its character of polycationic surfactants. At the presence of KPS, the CAC value and the surface tension of the solution decrease; and with the addition of estradiol the CAC value and surface tension almost unchanged. With the addition of estradiol, the mixed colloidal particles forms and the particle size increases.
3. PAS modified electrode can be easily regenerated by rinsing with ethanol and dried for the next 7 times measurement, and the relative error of each tests did not exceeds ± 6%.
4. PAS ECL system can be utilized to quantify estradiol in natural water and urine. The linear range of 6 pM to 1.5 nM for estradiol detection. The recovery rate is 94.5%~105%, and the coefficient of variation is 3.8% for estradiol detection in urine.

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