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Reagentless electrochemiluminescent detection of protein biomarker using graphene-based magnetic nanoprobes and poly-L-lysine as co-reactant

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ABSTRACT

This work described the construction of a reagentless and ultrasensitive electrochemiluminescence (ECL) immunosensor using poly-L-lysine as a co-reactant with $Ru(bpy)_3^{2+}$ for signal amplification and magnetic Fe_3O_4 loaded graphene nanosheet as nanoprobes, which can achieve an impressive detection limit of 0.03 pg/mL human total 3,3′,5-triiodothyronine (T3), a kind of diagnostic markers of thyroid disease. The bionanoprobes were prepared based on the coimmobilization of $Ru(bpy)_3^{2+}$ and T3 detection antibody on the Fe_3O_4 loaded graphene nanosheet and the sensing interface was achieved by assembling T3 capture antibody on the gold nanoparticles (AuNPs) loaded electro-deposited L-lysine film modified bare glass carbon electrode (GCE). ECL responses were generated from the modified electrodes described above by just immersing them in phosphate buffer solutions (PBS) based on the sandwich-type immunoreactions. T3 was measured quantitatively in the range from 0.1 pg/mL to 10 ng/mL, which exhibits sufficiently high sensitivity and stability. The reagentless ECL immunoassay is a promising approach for the detection of a wide range of molecular analytes.

1. Introduction

Recently, thyroid disease has been listed as the second largest disease of the endocrine field. It is reported total 3,3′,5-triiodothyronine (T3) is the most reliable and widely used diagnostic marker for thyroid function (Tai et al., 2004; Park et al., 2011; Mulder et al., 2012; Manna and Mugesh, 2012). Normally if the observation of an increase or decrease in serum total T3 level exceeds the normal value range (typically 0.5–2.0 μg/L for total T3), it may induce permanent functional abnormalities (Duggan and Craik, 1996; Severino et al., 2011). Therefore, highly efficient and reliable analytical techniques are necessary for T3 determination in serum.

There are various methods and strategies that have been applied for the detection of T3, such as enzyme-linked immunoassay (ELISA) (Silvaieh et al., 2002), chemiluminescence immunoassay (CLIA) (Sun et al., 2010), immunoradiometric assay (IRMA) (Navakouski et al., 2012) and liquid chromatography (Vacek et al., 2010; Wang and Stapleton, 2010). However, improvements are still required, as these methods remain cumbersome, time-consuming, and harmful to the operator's health. Electrochemiluminescence (ECL) immunosensors are of great interest because of their high sensitivity, controllability of ECL reaction, simple instrumentation, and low cost. One of the most extensively studied ECL luminophore is Ruthenium(II) tris(2,2′-bipyridyl) $\left(Ru(bpy)_3^{2+}\right)$ or its derivatives, for the reason that $Ru(bpy)_3^{2+}$ has high quantum yields and long excited state lifetimes as well as strong luminescence (Deiss et al., 2009; Damrauer et al., 1997). Recently, much effort has been devoted to improve the detection sensitivity of the $Ru(bpy)_3^{2+}$ ECL system by adding additives as effective co-reactants, such as oxalate (Rubinstein and Bard, 1981), peroxydisulfate (Suk et al., 2011; Cheng et al., 2012), or tripropylamine (TPA) (Fan et al., 2009; Kanoufi et al., 2001) to the electrolyte. Blackburn et al. developed ECL immunoassays and DNA probe assays using TPA as co-reactant dissolved in electrolyte and $Ru(bpy)_3^{2+}$ as the label (Blackburn et al., 1991). Yang et al. have designed a simple method for fabricating a boron-doped diamond electrode by enhancing the ECL intensity of the $Ru(bpy)_3^{2+}$ by adding TPA into working buffer solution as a co-reactant (Yang et al., 2010a,b). It can be seen that the $Ru(bpy)_3^{2+}$/TPA system has been studied widely because of its high ECL efficiency and commercial usefulness. However, with strong water-solubility, it is difficult to immobilize the TPA into the electrode. Thus, the TPA was usually added into the electrolyte as additives, which might lead to a more complex assay system and increase the analytical steps and expense. Therefore, to choose a suitable co-reactant of $Ru(bpy)_3^{2+}$ and immobilize it on the electrode surface is one of

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the most important points to be considered in ECL biosensor design.

Recently, magnetic nano-materials specially as immobilizing carrier of biomolecules have aroused great interest in current researches due to their large surface area, high bioactivity, and excellent stability and especially easy preparation. More importantly, it was also convenient for concentrating biological molecules by applying an external magnetic field. Tang et al. reported magneto-controlled graphene as immunosensing probes and multifunctional nanogold hollow microspheres as distinguishable signal tags (Tang et al., 2011). Zhao and co-workers described a novel ECL biosensor based on the magnetic beads technology and signal enhancement of gold nanoparticles for protein kinase activities and inhibition monitoring (Zhao et al., 2012). We have also designed bienzyme functionalized three-layer composite magnetic nanoparticles for electrochemical immunosensor construction (Zhuo et al., 2009). Therefore, the design of magnetic nano-material as biomolecule carriers should be highly potential in biosensor fabrication.

Inspired by those perspectives, we presented a reagentless ECL immunosensor based on the graphene-based magnetic nanoprobes using the Fe_3O_4 loaded graphene nanosheet as carries and $Ru(bpy)_3^{2+}$ as ECL labels in this work. The sensing interface was achieved by assembling T3 capture antibody (anti-T3) on the gold nanoparticles (AuNPs) loaded electro-deposited L-lysine film modified bare glass carbon electrode (GCE). A sandwich immunoassay format was employed to detect T3 with the bionanoprobes as tracer, which were prepared based on the coimmobilization of $Ru(bpy)_3^{2+}$ and T3 detection antibody (Ab_2) on the Fe_3O_4 loaded graphene nanosheet $\left(Ab_2/Ru(bpy)_3^{2+}/Fe_3O_4@GO\right)$. Herein, the poly-L-lysine containing many primary amino and secondary amino groups could act as the co-reactant to significantly enhance the ECL of $Ru(bpy)_3^{2+}$. To the best of our knowledge, the poly-L-lysine as co-reactant of $Ru(bpy)_3^{2+}$ has rarely been discussed. This strategy avoids the addition of the co-reactant to the electrolyte and significantly simplifies the immunoassay procedure, shortens the analytical time, and thus provides a new promising platform for clinical immunoassay.

2. Experimental

2.1. Reagents and material

$Ru(bpy)_3Cl_2 \cdot 6H_2O$, Nafion (5 wt%), gold chloride ($HAuCl_4$) and BSA (96–99%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). T3, goat-anti-T3 thyroxine (T4) and anti-T4 were obtained from Biocell Company (Zhengzhou, China). 3,4,9,10-perylene tetracarboxylic acid (PTCA) was received from Lian Gang Dyestuff Chemical Industry Co. Ltd. (Liaoning, China). Nafion was diluted to 1 wt% with ethanol solution. Phosphate buffer solutions (PBS) with pH 7.4 was prepared by mixing standard stock solutions of 0.1 M K_2HPO_4, 0.1 M NaH_2PO_4, and 0.1 M KCl and adjusting the pH with 0.1 M HCl or NaOH, then diluting with double distilled water. All chemicals were of analytical grade and used without further purification. All solutions were prepared with double distilled water and stored in the refrigerator (4 °C).

2.2. Apparatus

The ECL emission was monitored by a model MPI-A electrochemiluminescence analyzer (Xi'An Remax Electronic Science & Technology Co. Ltd., Xi'An, China). A conventional three-electrode system was used with Ag/AgCl (saturated KCl) as the reference electrode, a platinum wire as auxiliary electrode and a modified glass carbon electrode (GCE, Φ=4 mm) as the working electrode in the experiment. Cyclic voltammetric (CV) measurements were carried out with a CHI 610A electrochemistry workstation (Shanghai CH Instruments, China). A three-electrode electrochemical cell was composed of a modified glass carbon electrode (GCE, Φ=4 mm) as the working electrode, a platinum wire as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode. The morphologies of different nanocomposites were characterized by scanning electronmicroscopy (SEM, S-4800, Hitachi, Tokyo, Japan) at an acceleration voltage of 20 kV. X-ray photoelectron spectroscopy (XPS) measurements were carried out using a VG Scientific ESCALAB 250 spectrometer (Thermoelectricity Instruments, USA) and using Al Ka X-ray (1486.6 eV) as the light source.

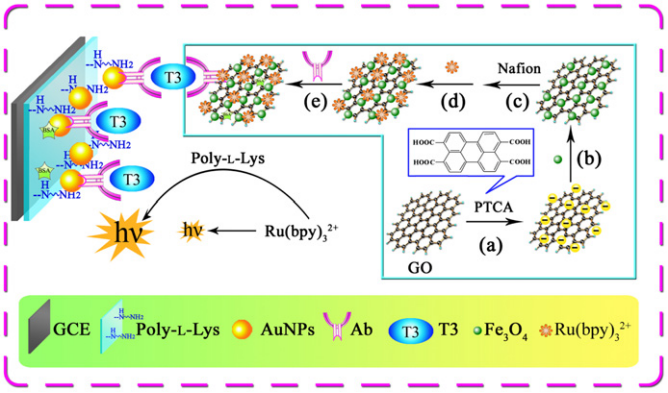
2.3. Preparation of Fe_3O_4@GO compounds

The Fe_3O_4 nanoparticles modified graphene oxide (GO) nano sheets (Fe_3O_4@GO) were synthesized with a one-step method under base condition by the following procedure. First of all, the GO nanosheets were modified with –COOH groups accordingly by hydrophobic interaction. Briefly, 2.0 mg GO and 0.5 mg PTCA were dissolved in 5.0 mL double distilled water by continuous ultrasonication and then stirred at room temperature for overnight to decorate the surface of GO with PTCA via π–π stacking. The structural formula of PTCA is shown in Scheme 1. The resulting targets were washed for several times to remove the free PTCA, and then it was dispersed in 2 mL 0.1 M PBS (pH 7.4) and stored at 4 °C.

Next, the obtained 2 mL GO solution was added into 5.0 mL 0.8 mM iron ion solution containing Fe^{2+} and Fe^{3+} with the molar proportion of 1:2 and stirred to form well-dispersed mixture, then it was added dropwise into 50 mL 2 mol/L NaOH simultaneously under vigorous mechanical stirring for 30 min at 80 °C. The precipitate was washed and collected by applying an external magnetic field until the supernatant solution turned neutral. Finally, magnetic graphene oxide (Fe_3O_4@GO) was obtained by centrifugation at 10,000 rpm for 15 min, washing with ethanol several times and then stored at 4 °C when not in use.

2.4. Preparation of $Ab_2/Ru(bpy)_3^{2+}/Fe_3O_4$@GO probe ($Ab_2$ bioconjugates)

To prepare the $Ru(bpy)_3^{2+}/Fe_3O_4$@GO composite, Nafion (Nf) was used as a cross-link agent to modify the surface of Fe_3O_4@GO



Scheme 1. Schematic diagrams of the immunosensor and the signal amplification mechanism. The insets of (a) displayed GO nano sheets were decorated with PTCA, (b) Fe_3O_4 nanoparticles were loaded on the functionalized GO nano sheets, (c) cross-linking agent, Nafion, was introduced, (d) and (e)$Ru(bpy)_3^{2+}$ and T3 detection antibody were immobilized.