PLATINUM NANOPARTICLE LABEL MEDIATED DEPOSITION OF PLATINUM CATALYST FOR ULTRASENSITIVE ELECTROCHEMICAL IMMUNOSENSORS

FIELD OF INVENTION

The invention relates to a method for detecting biomolecules, such as DNA and protein tumor markers, in a sample, and in particular, to an electrochemical method therefor. The method is suitable for use in diagnostic kits for DNA and protein tumor markers.

BACKGROUND TO THE INVENTION

The following discussion of the background to the invention is intended to facilitate an understanding of the present invention. However, it should be appreciated that the discussion is not an acknowledgment or admission that any of the material referred to was published, known or part of the common general knowledge in any jurisdiction as at the priority date of the application.

There is an increasing need for the development of simple, rapid and ultrasensitive immunobiosensors for the detection of proteins. These sensors would be immensely beneficial for applications such as early stage disease diagnosis, detection of biological threats, and drug screening. The advantage of using an immunosensor over other types of sensors in these applications lies in their high selectivity, which originates from the high selectivity and binding affinity of the antibody to its antigen. Consequently, the need for analyte enrichment and purification may be bypassed. However, since the antibody–antigen binding only causes minimal physiochemical changes, this binding event cannot be detected by most analytical instruments. Typical detection methods employ antibodies that are conjugated with a substance that can be read out easily by spectrophotometric, piezoelectric or electrochemical methods. Nanoparticles have been widely used for this purpose.

In the field of ultrasensitive electrochemical immunosensors, nanoparticles can be used to increase the loading of electrochemically detectable species so as to enhance the sensitivity of the sensor. Both metallic nanoparticles and

semiconductor quantum dots have been employed. In most cases, after the labeling step, a strong oxidant would be used to dissolve the nanoparticles to produce a solution containing a large quantity of ions (e.g. Ag^+, Cd^{2+} and Cu^{2+}), and then stripping voltammetry or ion selective electrode would be used to detect the dissolved ions. To avoid this complicated pre-oxidation step, it has been developed an electrochemical magnetic immunosensor based on magnetic beads and gold nanoparticle labels. The captured gold nanoparticle labels on the immunosensor surface were directly quantified by electrochemical stripping analysis. Poly(guanine)-functionalized silica nanoparticles have been used as biological labels for the sensitive electrochemical immunoassay. After a complete sandwich immunoassay, the concentration of the target analyte-dependent guanine was detected electrochemically using electrogenerated $Ru(bpy)^{3/3+}$ as an electron transfer mediator. To advance biosensor sensitivity, nanoparticle labels have also been used as seeds to catalyze the precipitation of Au or Ag to further increase the loading of Au or Ag. Previously, it has been reported an electrochemical immunoassay based on silver growth upon colloidal gold labels. After silver metal dissolution in acid, stripping voltammetry was used for detection. It has further been reported an amplified electrochemical immunoassay for the quantitative detection of rabbit immunoglobulin G analyte based on square-wave stripping voltammetric detection of Au from the mediated deposition of Au nanoparticle labels after Au was dissolved in HBr/Br_2 solution. Recently, an ultrasensitive electrochemical immunosensor for PSA has also been reported. The detection antibody was conjugated with Ag nanoparticle labels, which allowed the implementation of a silver enhancement approach based on a seeded-mediated nucleation and growth mechanism. Following that, a unique solid-state Ag/AgCl process facilitated detection by significantly amplifying the signal.

It is desirable to provide an alternative ultrasensitive electrochemical method for detecting DNA and other biomolecules (such as protein markers).

SUMMARY OF THE INVENTION

Throughout this document, unless otherwise indicated to the contrary, the terms "comprising", "consisting of", and the like, are to be construed as non-exhaustive, or in other words, as meaning "including, but not limited to".

Herein it is described a novel signal amplification strategy for the development of ultrasensitive electrochemical immunosensors. The amplification strategy is based on platinum catalyzing the hydrogen evolution reaction. To demonstrate its advantage, the electrochemical signal enhancement strategy has been applied as a novel prostate-specific antigen (PSA) immunosensor. The immunosensing protocol utilized a gold electrode with the PSA capture antibodies bound to its surface via covalent bonding. After PSA was bound to the electrode surface, a secondary platinum nanoparticle-labeled detection antibody was used to complete the sandwich immunosensor. The resulting electrode was then dipped in a platinum developer solution containing 1 mM of $PtCl_4^{2-}$, 0.1 M of formic acid (reductant) and 0.5% Tween 80 (pH 6.5) to generate bare platinum catalysts in close proximity to the Au electrode surface through a seed-mediated nucleation and growth mechanism. The signal readout was obtained electrochemically via a Pt-catalyzed hydrogen evolution reaction in an acidic aqueous medium containing 10 mM of HCl and 1 M of KCl. A detection limit of 1 fg/ml was achieved.

BRIEF DESCRIPTION OF THE DRAWINGS

In the figures, which illustrate, by way of example only, embodiments of the present invention,

FIG. 1 shows a schematic of the electrochemical PSA biosensor based on Pt-catalyzed hydrogen evolution for signal amplification.

FIG. 2 shows TEM images of Pt nanoparticles synthesized with (a) 0.25 g/100 ml and (b,c) 0.10 g/100 ml of capping agents. (d) Pt nanoparticle cluster–antibody conjugates. Scale bars in these images are 5 nm, 0.5 μm, 10 nm and 0.2 μm, respectively.

FIG. 3 shows cyclic voltammograms of proton redox process obtained in an acidic solution containing 10 mM of HCl and 1 M of KCl with different electrodes (2 mm diameter): (—) bare Au electrode, (—) Au electrode modified with a mixed thiol monolayer (10% MUOH, 90% MHA), (—) bare Pt electrode, and (○) our PSA immunosensor, when the PSA analyte concentration is 10 nM.

FIG. 4 shows (a) effect of Pt enhancement time (from the top curve to the bottom curve: 0, 2, 5, 10, 15, 30, 45 and 60 min) on the voltammetric response of PSA immunosensors in an aqueous solution containing 10 mM of HCl and 1 M of KCl. (b) Dependence of the steady-state diffusion controlled limiting current and peak current (data obtained from (a)) on the enhancement time for (■) 0.1 pg/ml and (□) 0 pg/ml of PSA.

FIG. 5 shows calibration curves of the PSA immunosensor obtained with a Pt enhancement time of (Δ) 10 min and (□) 30 min.

FIG. 6 shows formation of disulfide-based polyamidoamines (mixed oligomers) **2**.

DETAILED DESCRIPTION

Detection of biomolecules such as DNA and protein tumor markers is important in diagnostic tests for cancer and other diseases. The purpose of this invention is to detect low concentrations of these biomolecules by incorporating significant signal amplification. Specifically, this invention utilizes a unique Pt enhancement process to grow Pt catalyst based on the principle of seed-mediated nucleation and growth, using target-selective probe molecule-conjugated Pt nanoparticle labels as seeds. The resulting Pt catalyst accelerates the hydrogen evolution reaction in a proton-rich electrolyte medium for signal amplification, improving the sensitivity of electrochemical biosensors. As Pt can effectively catalyze hydrogen evolution in an acidic solution, considerable signal amplification can be achieved. The basic principle of the sensing strategy involves several key steps: (1) immobilization of spacer molecules and target-selective probe molecules on the electrode surface, (2) assembly of target analytes on the modified electrode, (3) labeling of the target analytes with Pt nanoparticles conjugated with target-selective probe molecules, (4) Pt enhancement based on seed-mediated nucleation and growth in a unique Pt developer solution consisting of 1 mM of $PtCl_4^{2-}$, 0.1 M of formic acid (reductant) and 0.5% of Tween 80 (pH 6.5), (5) electrochemical detection of Pt-catalyzed hydrogen evolution reaction in an acidic solution containing 10 mM of HCl and 1 M of KCl. The signal obtained is related to the concentration of the biomolecules, allowing for proper quantification of the

biomolecules. The Pt enhancement step and the Pt-catalyzed hydrogen evolution step represent the key innovation for this biosensor, leading to a detection limit that is amongst the lowest reported thus far.

Herein it is reported an ultrasensitive electrochemical detection strategy using Pt-catalyzed hydrogen evolution for signal amplification in the sensing of PSA illustrated in **FIGURE 1**. In this case, analyte PSA was captured by the capture antibodies covalently bound on the electrode surface. Subsequently, the captured antigens were sandwiched with a Pt nanoparticle-labeled PSA detection antibody. The resulting electrode was then dipped into a platinum developer solution containing 1 mM of $PtCl_4^{2-}$, 0.1 M of formic acid and 0.5% of Tween 80 (pH 6.5). Pt nanoparticle labels catalyzed the reduction of $PtCl_4^{2-}$ by formic acid to form Pt particles on the electrode surface. This Pt-modified electrode was then placed into an acidic solution for proton reduction using a voltammetric method. The current measured depended on the coverage of Pt catalyst on the electrode surface, which could be correlated to the PSA concentration.

The advantages of using Pt nanoparticle-catalyzed H^+ reduction include: (1) the H^+ reduction is electrochemically reversible on Pt surface, and (2) the Pt surface is much more active than other conductive surfaces, e.g. carbon, ITO, Au or Ag. Thus, the interference from the proton reduction at the uncovered electrode surface is minimal. Consequently, a clean background can be expected during voltammetric experiments when non-Pt electrodes are used. Additional advantages include: (3) the diffusion coefficient for protons is approximately 1×10^{-4} $cm^2\,S^{-1}$, which is at least an order magnitude higher than any other species, and (4) inorganic acids are normally stable and highly soluble in water. Pt nanoparticle labels have been used as catalysts for the electrochemical reduction of H_2O_2 in amplified electrochemical detection of DNA by others previously. However, using Pt nanoparticle labels in protein detection is more challenging. The electrode and the Pt nanoparticle labels conjugated to the detection antibody are separated by the antibodies, which are typically 10 nm in diameter. The electron tunneling through such a long distance is too slow to be detected. Therefore, a Pt enhancement step by seed-mediated nucleation and growth is required to introduce a Pt catalyst in close proximity to the electrode surface. Seed-mediated Pt deposition has been attained using a Pt developer solution consisting of Pt salt

and ascorbic acid. However, it normally takes a few hours for Pt to grow with this developer solution. Thus, it is not suitable for application to rapid protein detection.

Experimental

Materials and reagents

PSA from human serum (P-3338) was purchased from Sigma-Aldrich. Monoclonal antibodies to PSA were obtained from Meridian Life Science Inc., Biodesign International (M86433M as capture antibody and M86111M as detection antibody). O,O'-bis[2-(N-succinimidyl-succinylamino)ethyl] polyethylene glycol 3,000 (NHS-PEG-NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-succinimidyl ester (NHS), 16-mercapto-1-hexadecanoic acid (16-MHA), 11-mercapto-1-undecanol (11-MUOH), ethylamine, $NaBH_4$, H_2PtCl_6, K_2PtCl_4, formic acid, Tween 80, NaOH, boric acid, sodium tetraborate, Tris hydroxymethyl (aminomethane) (TRIS), Na_2HPO_4, NaH_2PO_4, KCl and HCl were obtained from Sigma-Aldrich. Nanopure water (resistivity > 18 KΩ cm) was used in the studies.

Apparatus

Cyclic voltammetry was performed with a CHI 760C electrochemical workstation (CH Instruments, Texas). A conventional 3-electrode system was employed. A 2 mm-diameter gold electrode (CH Instruments), a platinum wire and an Ag/AgCl (3M KCl) electrode (CH Instruments) were employed as the working electrode, counter electrode, and reference electrode, respectively. TEM experiments were performed on a JEOL JEM-3010 electron microscope (200 kV). Centrifugation was conducted with an Allegra 64R Centrifuge (Beckman Coulter, California).

Synthesis of Pt nanoparticles and Pt nanoparticle-antibody conjugates

1 mM of H_2PtCl_6 and 2.5 mg/ml of disulfide-based polyamidoamines were dissolved in 200 ml of nanopure water, and stirred for 10 min. Disulfide-based polyamidoamines contained both thiol groups for strong Pt nanoparticle stabilization, and primary amine groups for further bioconjugation (see the following paragraphs below). The solution turned brown upon the addition of 48 mg of $NaBH_4$ dissolved in 2 ml water, suggesting the formation of Pt nanoparticles. The Pt nanoparticles were then concentrated to 10 ml via water evaporation. 30 ml

of acetone were added to the solution to precipitate the Pt nanoparticles via centrifugation. The precipitate was collected and re-dissolved in 10 ml of nanopure water. This procedure was repeated twice to obtain pure Pt nanoparticles. TEM image shown in **FIGURE 2a** showed that the size of the Pt nanocrystals was approximately 3 nm. However, sedimentation of these Pt nanocrystals in a pure aqueous solution via centrifugation was impossible even at 21,000 rpm. For ease of centrifugation and separation of the Pt nanoparticle-antibody conjugates from the excess antibodies after conjugation, less capping reagent (1 mg/ml) was used while keeping the amount of the other reagents unchanged. The resulting Pt nanoparticles were purified using the same protocol as described above. Low-resolution transmission electron microscopy (TEM) showed that the Pt particles have a diameter of 40 nm as shown in **FIGURE 2b**. However, high-resolution TEM image shown in **FIGURE 2c** illustrated that these particles were aggregates of 3-nm Pt nanocrystals.

The Pt nanoparticle clusters were conjugated with detection antibodies using NHS-PEG-NHS linker, which reacted with the primary amines from both Pt nanoparticles and detection antibodies. Pt nanoparticle clusters (2.5 mg) were diluted in 1 ml of borate buffer (pH 7.5), and mixed with an excess amount of NHS-PEG-NHS 3000 (10 mg dissolved in 100 μl of dimethyl sulfoxide (DMSO)). After 15 min of incubation, the NHS-PEG-NHS-conjugated nanoparticles were sedimented by centrifugation at 13,000 rpm to remove excess NHS-PEG-NHS. The recovered activated particles were immediately dissolved in 1 ml of borate buffer (pH 7.5) containing 0.1 mg of M86111M detection antibodies, and incubated for 2 h under shaking. 100 μl of TRIS buffer (1 M, pH 7.4) were added to block any free NHS groups. The final Pt nanoparticle–antibody conjugates were separated by centrifugation at 13,000 rpm to remove excess detection antibodies. TEM image indicated that the Pt nanoparticles were stable upon conjugation (see **FIGURE 2d**). The conjugated nanoparticles were kept at 4°C.

Preparation of sandwich immunosensor

Au electrode of 2 mm-diameter for the immunosensor was first polished carefully using 0.3-μm alumina slurry, and electrochemically cleansed in a H_2SO_4 solution (0.5 M) by cycling the potential between -0.2 V and 0.8 V vs. a Pt wire

quasi-reference electrode for 5 min. The electrode was then washed with nanopure water, and dipped into 100 μl of an ethanolic solution containing 0.1 mM of 16-MHA and 0.9 mM of 11-MUOH overnight. 11-MUOH was used as a spacer to obtain an optimal density of the COOH groups on the electrode surface and to prevent non-specific adsorption. The electrode was then soaked for 2 h in 100 µl of phosphate buffered saline (PBS) (0.05 M, pH 7.5) containing 5 mM of NHS, 20 mM of EDC and 100 μg/ml of M86433M capture antibodies. The antibodies were covalently bound to the electrode through the conjugation of amine groups from the antibodies with the activated carboxyl groups on the electrode surface. Next, 10 µl of ethanolamine solution (1 M) were added to block any free activated carboxyl groups. The resulting antibody-modified electrode was washed with nanopure water, and then with 10 mM of glycine (pH 2.2) to remove any non-covalently bound antibodies. The electrode prepared was exposed to PSA analyte concentrations of 0–10 ng/ml for 30 min. It was washed with PBS before dipping into the Pt nanoparticle-labeled detection antibodies. After 15 min, the electrode was thoroughly washed with 0.01 M of TRIS buffer (pH 7.4) containing 0.15 M of NaCl to remove non-specifically bound Pt nanoparticle-labeled detection antibodies. The electrode was then carefully washed with nanopure water. It was next dipped into the Pt developer solution containing 1 mM of $PtCl_4^{2-}$, 0.1 M of formic acid and 0.5% of Tween 80 (pH 6.5) for a certain amount of time. The electrode was washed again with nanopure water before conducting cyclic voltammetric measurements in an aqueous solution containing 01 M of KCl and 0.01 M of HCl.

Results and Discussion

Formulation of the Pt developer solution

An important step in this immunosensing strategy was the rapid seed-mediated generation and deposition of bare Pt particles on the electrode surface in the aqueous phase at room temperature under physiological pH using a Pt developer solution. We found that a Pt developer solution containing 1 mM of $PtCl_4^{2-}$ and 0.1 M of formic acid reductant was stable for approximately 30 min, and that the Pt

nanoparticle seeds could efficiently catalyze the reduction of $PtCl_4^{2-}$ by formic acid to produce Pt. To increase the stability of the Pt developer solution from approximately 30 min to more than 1 day without significantly decreasing the rate of seed-mediated deposition of Pt, 0.5% of Tween 80 was added to the developer solution. The pH of the developer solution was adjusted to 6.5 to avoid the detachment of PSA or detection antibody from the electrode surface in an acidic environment (due to the presence of 0.1 M of formic acid in the Pt enhancement step).

FIGURE 3 shows the cyclic voltammograms of proton redox processes obtained at different electrodes in an acidic solution containing 10 mM of HCl and 1 M of KCl. Since the reduction of H^+ occurred at a potential close to that of O_2 reduction, 10 mM of HCl was used so that the signal due to O_2 reduction was negligible compared to that of H^+ reduction. Consequently, removal of O_2 was not required during the experiments. The results showed that the bare Pt electrode was much more active for facilitating the hydrogen evolution process. A well-defined voltammogram was obtained with a formal potential of -0.417 V vs. Ag/AgCl (taken as the average of the anodic peak potential, -0.384 V vs. Ag/AgCl, and the cathodic peak potential, -0.449 V vs. Ag/AgCl). In contrast, proton reduction at the bare Au electrode surface was much slower with a very large overpotential. The peak potential of -0.741 V vs. Ag/AgCl for proton reduction at a bare Au electrode was almost 0.3 V more negative than that at the Pt electrode. The overpotential of proton reduction was even higher at the Au electrode modified with a mixed thiol monolayer. In contrast, the voltammogram obtained using the PSA immunosensor when the analyte PSA concentration was 10 nM, was essentially identical to that obtained at a bare Pt electrode. This suggested that the Pt particles generated with the developer solution have good activity for proton reduction.

Effect of Pt enhancement time

The amount of Pt deposited onto the electrode surface during the Pt enhancement step, and hence the electrochemical response of the immunosensor was time-dependent. Experiments were therefore conducted to investigate the effect of the Pt enhancement time on the voltammetric response of the PSA

immunosensor. The transition from a steady-state sigmoidal-shaped voltammogram to a transient peak-shaped voltammogram is clearly illustrated in **FIGURE 4a**, as expected. When the enhancement time was short enough (2–5 min) and the Pt particle density and/or size was sufficiently small, (i.e. the distance between the particles was 10 times their diameter), every individual Pt particle behaved like an independent microelectrode. Subsequently, the mass transport was governed by radial diffusion, and sigmoidally shaped steady-state voltammograms were observed. When the enhancement time was longer than 1 h, the electrode surface would be almost entirely covered with Pt particles. The mass transport to the electrode was then governed by planar diffusion. The voltammogram obtained after Pt enhancement treatment resembled that obtained at a Pt disc electrode (see **FIGURE 3**). When the enhancement time increased from 10 min to 45 min, the Pt particle density and/or size increased, causing the diffusion layer of each Pt particle microelectrode to overlap. Therefore, the transition of steady-state voltammogram to an ideal transient voltammogram of a macro-disc electrode was observed. Controlled experiments conducted in the absence of PSA showed that background currents were small. This suggested that thiol-modified Au electrodes were almost inert towards the catalytic growth of Pt, and that the non-specific adsorption of PSA or detection antibody was minimal. Values of the steady-state diffusion-controlled limiting currents or peak currents obtained from **FIGURE 4a** are summarized in **FIGURE 4b**.

Calibration curves

The results in **FIGURE 4** implied that a higher sensitivity would be expected if a longer Pt enhancement time was applied. However, longer enhancement time might also decrease the dynamic concentration response range of the immunosensor. To examine this, two calibration curves were obtained using different Pt enhancement times (**FIGURE 5**). In both cases, the PSA immunosensors have a good response to PSA over a wide concentration range. When a Pt enhancement time of 10 min was used, the steady-state diffusion limiting current or peak current increased when the PSA concentration increased from 10 fg/ml to 10 pg/ml. The peak current approached a plateau when the PSA concentration was higher than 0.1 ng/ml. The results also showed that the sensitivity of the PSA immunosensor increased and its dynamic response range

shifted towards a lower concentration range (1 fg/ml to 1 pg/ml) when a longer Pt enhancement time of 30 min was used.

Synthesis and characterization of disulfide-based polyamidoamines

1. Materials and methods

1.1. Materials

Methyl-3-mercaptopropionate, tris(2-aminoethyl)amine (96%), pentaethylene-hexamine, dimethylsulfoxide (DMSO) and tetrahydrofuran (THF) were all purchased from Sigma-Aldrich and used as received.

1.2. Methods

^{1}H and ^{13}C NMR spectra. Spectra were recorded on a Bruker AVANCE 400 at 400 MHz and 100 MHz, respectively, using the deuterated solvent indicated. CS ChemNMR Pro version 6.0 (Upstream Solutions GbmH Scientific Software Engineering CH-6052 Hergiswil, Switzerland) was employed to analyze the spectra.

Gel permeation chromatography (GPC). The molecular weight (M_w) of polyamidoamine was analyzed by GPC (Waters 2690, MA, USA) with a differential refractometer detector (Waters 410, MA, USA). The mobile phase consisted of 0.5 M of sodium acetate and 0.5 M of acetic acid solution with a flow rate of 1 mL/min. A Shodex OHpak SB-803 HQ (8.0 mm × 300 mm) column was used. Number and weight average molecular weights (M_n and M_w) as well as polydispersity indices were calculated from a calibration curve using a series of dextran standards (Aldrich, Missouri, USA) with molecular weights ranging from 667 to 778000.

Elemental analysis. The nitrogen content of the polyamidoamine was determined by elemental analysis using Perkin-Elmer Instruments Analyzer 2400 CHN/CHNS and Eurovector EA3000 Elemental Analyzers.

2. Synthesis and characterization of disulfide-based polyamidoamines (mixed oligomers)

*2.1 Formation of amine monomer (**1**)*

10 ml of tris(2-aminoethyl)amine (0.067 mol), 60 ml of methanol and 17 ml of epichlorohydrin (0.22 mol) were placed in a flask under nitrogen. The mixture was left to stir in an ice bath for 24 h. Next, 250 ml of pentaethylenehexamine (1.08 mol) were added to the contents of the flask in conjunction with 100 ml of DMSO. The mixture was left to stir at 90°C for 24 h. The contents of the flask were then dialyzed against flowing water using dialysis tubing with a molecular weight cut-off of 500 Da (Spectrum Laboratories, USA) for 1 day, followed by freeze drying. δ_H(400 MHz, D_2O) 3.70 (1H, m, -CH_2-C***H***(OH)CH_2-); 3.10–2.20 (m, -NH-C***H***$_2$-C***H***$_2$-NH-, -C***H***$_2$-CH(OH)C***H***$_2$-, and -NH-C**H**$_2$-C**H**$_2$-N(-C**H**$_2$-C**H**$_2$-NH-)$_2$). δ_C(100 MHz, D_2O) 67, 58, 56, 55, 53, 52, 49, 48, 47, 45, 44, 39, 38 and 37.

*2.2 Formation of disulfide-based polyamidoamines (mixed oligomers) (**2**)*

6.5 g of the amine monomer (**1**) (0.0081 mol) were added to a flask with 150 ml of methanol and 0.70 ml of methyl-3-mercaptopropionate (0.0064 mol). The mixture was then left to stir at 60°C for 24 h. The excess methanol was removed under vacuum, and then the mixture was precipitated in THF and dried using a vacuum oven. δ_H(400 MHz, D_2O) 3.70 (1H, m, -CH_2-C***H***(OH)CH_2-); 3.30–3.20 (2H, t, -NH-CH_2-C***H***$_2$-NH-C(=O)-CH_2-CH_2-S-); 3.10–2.20 (m, -NH-C***H***$_2$-C***H***$_2$-NH-, -NH-CH_2-CH_2-NH-C(=O)-C***H***$_2$-C***H***$_2$-S-S-, CH_2-CH_2-NH-C(=O)-C***H***$_2$-C***H***$_2$-SH, -C***H***$_2$-CH(OH)C***H***$_2$-, and -NH-C**H**$_2$-C**H**$_2$-N(-C**H**$_2$-C**H**$_2$-NH-)$_2$). δ_C(100 MHz, D_2O) 165, 67, 58, 56, 55, 53, 52, 49, 48, 47, 45, 44, 39, 38, 37, 27 and 21.

3. Results and discussion

3.1. Synthesis and characterization of disulfide-based polyamidoamines (mixed oligomers)

The oligomeric polyamidoamine was prepared via a two-step procedure. The first step involved a ring-opening mechanism in conjunction with nucleophilic substitution. For this simple procedure, pentaethylenehexamine was employed as the main amine as shown in **FIGURE 6**. Briefly, we initially formed the amine monomer (**1**) compound by reacting the core amine, tris(2-aminoethylene)amine with epichlorohydrin in methanol via ring opening in an ice bath for 24 h. This was

followed by reacting the core with excess pentaethylenehexamine via nucleophilic substitution in an oil bath at 90°C for 24 h. Subsequently, the amine monomer (**1**) compound was purified via dialysis in water. We then reacted the purified amine monomer (**1**) with the thiol ester, methyl-3-mercaptopropionate, via nucleophilic substitution at 60°C for 24 h in methanol. Lastly, the excess methanol was removed under vacuum, and the disulfide-based polyamidoamines (mixed oligomers) were obtained as a crude mixture via precipitation in THF. The oligomeric mixture was then dried in a vacuum oven.

The chemical structure of both the amine monomer (**1**) and the disulfide-based polyamidoamines (mixed oligomers) (**2**) were confirmed using ^{1}H and ^{13}C NMR spectroscopy. In addition, with the aid of GPC analysis, the weight average molecular weight of the disulfide-based polyamidoamines (mixed oligomers) was determined to be 2.8 kDa with a polydispersity index of 1.4.

Following from the above description, it has been demonstrated that a new Pt developer solution containing 1 mM of $PtCl_4^{2-}$, 0.1 M of formic acid and 0.5% of Tween 80 (pH 6.5) was very effective for the seed-mediated growth of Pt particles. The Pt particles generated using this method were active towards electrochemical hydrogen evolution. The advantage of using this Pt enhancement strategy and the Pt-catalyzed hydrogen evolution for the development of ultrasensitive immunosensors has been successfully demonstrated in the example of PSA detection. A detection limit of 1 fg/ml has been achieved. Further advantages include: (i) simplicity, as the approach does not require complex and expensive optical systems; and (ii) high sensitivity, associated with the high catalytic activity of Pt towards the hydrogen evolution reaction as compared to other metals, high solubility and high diffusion coefficient of proton in water.

Besides providing for amplified biomolecules detection and diagnostic kits for DNA and protein tumor markers, the above description is also suitable for the fabrication of Pt-modified surface for catalytic reactions (e.g. fuel cell applications).

Although the foregoing invention has been described in some detail by way of illustration and example, and with regard to one or more embodiments, for the purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes, variations

and modifications may be made thereto without departing from the spirit or scope of the invention as described in the appended claims.

I CLAIM:

1. A method for providing an ultrasensitive electrochemical immunosensor, comprising:

   – using a platinum (Pt) enhancement process to grow Pt catalyst based on the principle of seed-mediated nucleation and growth;

   – using target-selective probe molecule-conjugated Pt nanoparticle labels as seeds;

   wherein the resulting Pt catalyst accelerates a hydrogen evolution reaction in a proton-rich electrolyte medium for signal amplification, thereby improving the sensitivity of the electrochemical immunosensor or biosensor.