Electronic Supplementary Information

The Solid-State Ag/AgCl Process as a Highly Sensitive Detection Mechanism for an Electrochemical Immunosensor

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Detailed Experimental Procedures

1. Reagents

Prostate-specific antigen (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), sodium borohydride (NaBH₄), silver nitrate (AgNO₃), ascorbic acid, Tween 80 were obtained from Sigma-Aldrich. Reagents for the synthesis of the capping agent, including methyl-3-mercaptopropionate and tris(2-aminoethyl)amine (96%), were also obtained from Sigma-Aldrich. Phosphomolybdic acid sodium salt hydrate (Na₃PMo₁₂O₄₀), which was used as interference agent, was obtained from Sigma-Aldrich.

2. Characterization

¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 at 400 and 100 MHz, respectively, using the indicated deuterated solvents. CS ChemNMR Pro version 6.0 (Upstream Solutions GmbH Scientific Software
Engineering CH-6052 Hergiswil, Switzerland) was employed to analyze various protons and carbons.

Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 Fourier-transform infrared (FTIR) spectrometer and a Perkin-Elmer Spectrum One FTIR spectrometer. Samples were prepared with KBr in a disk prior to analysis.

Molecular weight of polyamidoamine was analyzed by gel permeation chromatography (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, USA) with molecular weights ranging from 667 to 778000.

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

Cyclic voltammetry of the assay was performed with CHI 400 Electrochemical Analyzer (CH Instruments, Texas, USA). Gold electrode (CH Instruments), a platinum wire, and Ag/AgCl (3 M of KCl) electrode (CH Instruments) were used as the working electrode, counter electrode and reference electrode, respectively.

3. Electrode Surface Modification

To form a mixed self-assembled monolayer (SAM) on the electrode surface, the Au electrodes were first polished carefully using 0.3-μm alumina slurry, and cleaned electrochemically in a H_2SO_4 solution (0.5 M) by cycling the potential between -0.2 V and 0.8 V vs. Pt wire quasi-reference electrode for 10 min. These electrodes were then washed with deionized (DI) water, and dipped into 100 μl of ethanol solution containing a mixture of 0.1 mM of 16-MHA and 0.9 mM of 11-MUOH overnight.

4. Synthesis and Characterization of Branched Disulfide-based Polyamidoamine (Capping Agent Used for Ag Nanoparticle Synthesis)
4.1 Formation of Branched Disulfide-Based Polyamidoamine

20 mL of methanol, 25 mL of tris(2-aminoethyl)amine (0.17 mol), 38 mL of methyl-3-mercaptopropionate (0.34 mol) and 100 mL of dimethyl sulfoxide (DMSO) were placed in a flask. The flask was left to stir for 3 h at 120°C. Next, the contents of the flask were cooled to 30°C. The crude product was precipitated into tetrahydrofuran (THF), and then dialyzed against water for 1 day with a continuous flow by a membrane dialysis method using dialysis tubing with a molecular weight cut-off (MWCO) of 1 kDa (Spectrum Laboratories, USA). The branched disulfide-based polyamidoamine was harvested by freeze drying, and characterized by IR, and \(^1\)H and \(^{13}\)C NMR spectroscopies. Disulfide-based polyamidoamine: \(v_{\text{max}}\) (KBr disk) (cm\(^{-1}\)) 3400 strong (broad) \([u(\text{N-H})]\); 2950 medium \([u(\text{C-H})]\); 2360 weak \([u(\text{S-H})]\); 1640 strong \([u(\text{C=O})]\). \(\delta\)\(^1\)H (400 MHz, D\(_2\)O) 3.20–3.10 (2H, t, N-CH\(_2\)-CH\(_2\)-NH-C(=O)-CH\(_2\)-CH\(_2\)-S-, signal B); 2.90–2.20 (m, -NH-C(=O)-CH\(_2\)-CH\(_2\)-SH; -NH-C(=O)-CH\(_2\)-CH\(_2\)-S-S-; NH\(_2\)-CH\(_2\)-CH\(_2\)-N(-CH\(_2\)-CH\(_2\)-NH-C(=O))\(_2\)-, signal A). \(\delta\)\(^{13}\)C (100 MHz, D\(_2\)O) 175, 54, 52, 38, 37, 35 and 27.

4.2 Analysis of Branched Disulfide-Based Polyamidoamine

The branched disulfide-based polyamidoamine was prepared in a one-pot reaction via nucleophilic substitution and thiol oxidation between tris(2-aminoethyl)amine and methyl-3-mercaptopropionate as shown in Scheme 1. The reaction was performed in air in DMSO so as to allow the thiol groups to be oxidized rapidly to form the disulfide bonds in the polymer backbone.
Scheme S1. Formation of branched disulfide-based polyamidoamine (a representative form).

The chemical structure of the disulfide-based polyamidoamine was characterized by \(^1\)H and \(^13\)C NMR and IR spectroscopies. The \(^1\)H NMR peaks at 2.90–2.20 ppm were assigned to some of the methylene protons (NH\(_2\)-CH\(_2\)-CH\(_2\)-NH-C(=O))\(_2\)-) of tris(2-aminoethyl)amine, as well as the methylene protons (-NH-C(=O)-CH\(_2\)-CH\(_2\)-S-) from the thiol ester methyl-3-mercaptopropionate (signal A) (Figure S1). The peaks at 3.20–3.10 ppm for the remaining methylene protons (NH\(_2\)-CH\(_2\)-CH\(_2\)-N(-CH\(_2\)-CH\(_2\)-NH-C(=O))\(_2\)-, signal B) of the tris(2-aminoethyl)amine moiety demonstrated that the amine group next to these methylene protons was part of a conjugated system, such as that from an amide bond, indicating the successful formation of polyamidoamine. IR spectroscopy further confirmed the structure of polyamidoamine as proposed in Scheme 1. The IR spectrum displayed a strong absorption at 1640 cm\(^{-1}\) assignable to \(\nu\)(C=O) of the amide unit. The expected broad absorption due to \(\nu\)(N-H) was observed at 3400 cm\(^{-1}\). There was a weak absorption at 2360 cm\(^{-1}\), which was attributed to \(\nu\)(S-H) of the thiol unit. The \(M_w\) of the branched disulfide-based polymer was determined by GPC to be 4.3 kDa with a polydispersity index of 2.0. The nitrogen content of the polyamidoamine was \(\sim\) 19%, as expected.
Figure S1. $^1$H NMR spectrum of branched disulfide-based polyamidoamine in D$_2$O, with a representative portion of the polymer’s structure.

5. Synthesis and Bioconjugation of Ag Nanoparticles

The water-soluble Ag nanoparticles were synthesized by using the branched disulfide-based polyamidoamine, which contained both thiol group for strong Ag nanoparticle stabilization and primary amine group for further bioconjugation. 1 mM of AgNO$_3$ and 0.5 mM of branched disulfide-based polyamidoamine (capping agent) were dissolved in 200 ml of DI water and stirred for 10 min. 2 mM of NaBH$_4$ dissolved in 2 ml of water were added dropwise until the Ag solution turned dark brown. The Ag nanoparticles were then concentrated by evaporating water to 10 ml. The nanoparticles were washed with acetone, precipitated at 21,000 rpm, and re-dissolved in 10 ml of DI water.

To complete the bioconjugation between the Ag nanoparticles and the detection antibodies, NHS-PEG-NHS 3000 was used as the long-arm linker. The two long ends of the NHS-PEG-NHS 3000 binded to amine groups through a well-established chemical reaction. Ag nanoparticles (0.850 mg) were first diluted in 1 ml of borate buffer (pH 7.5) and mixed with NHS-PEG-NHS 3000 (20 mg dissolved in 100 μl of DMSO) to achieve
linkage between NHS-PEG-NHS and the Ag nanoparticles. An excess amount of NHS-PEG-NHS was used to prevent aggregation between the Ag nanoparticles. After 15 min of incubation, the NHS-PEG-NHS-conjugated Ag nanoparticles were passed through a Sephadex column to remove excess free NHS-PEG-NHS that was not bound to the Ag nanoparticles. The recovered activated Ag nanoparticles were immediately mixed with M86111M detection antibody (1 ml of 0.2 mg/ml antibody) and incubated for 2 h under shaking. 100 μl of Tris hydroxymethyl (aminomethane) (TRIS) (pH 7.4) buffer were added to block any free NHS groups. The conjugated nanoparticles were kept at 4°C.

6. Sandwich Immunoassay

The COOH groups on the surface of the mixed monolayer-modified electrodes were activated with 20 mM of NHS and 100 mM of EDC for 15 min. The electrodes were washed with DI water, and immediately dipped in 100 μg/ml of M86433M capture antibody (antibody was diluted to the desired concentration with 10 mM of acetate buffer (pH 6.0)). After 1 h of incubation, the electrodes were washed with DI water, and immersed for 10 min in 1 M of ethanolamine (pH 8.5) to block any free activated NHS groups. The electrodes were washed with 10 mM of glycine (pH 2.2) to remove any non-covalently bound antibodies.

The electrodes prepared were exposed to PSA analyte at different concentrations from 0–100 ng/ml for 1 h. The electrodes were washed with phosphate buffered saline (PBS) before dipping into the Ag nanoparticle-labeled detection antibody. After 1 h, the electrodes were washed thoroughly with 0.01 M of TRIS buffer (pH 7.4) containing 0.15 M of NaCl to remove non-specifically bound nanoparticles. The electrodes were then vigorously washed with DI water. The Ag nanoparticles were developed in Ag enhancement solution (1 mM of AgNO₃, 0.5 mM of ascorbic acid and 0.5% of Tween 80) for 10 min. The electrodes were then washed again with DI water, and a cyclic voltammetry was applied to read the signal response.