Supplementary Information (ESI)

for

Femtomolar detection of a cancer biomarker protein in serum with ultralow background current by anodic stripping voltammetry

Muhammad J. A. Shiddiky,[a,c] Prakash H. Kithva,[a,c] Sakandar Rauf[a] and Matt Trau*[a,b,c]*

[a]Australian Institute for Bioengineering and Nanotechnology, [b] School of Chemistry and Molecular Biosciences, [c] Centre for Biomarker Research and Development, University of Queensland, Brisbane, QLD 4072, Australia. Fax: +61-33463973; Tel: +61-33464178; E-mails: m.shiddiky@uq.edu.au (MJAS); m.trau@uq.edu.au (MT)

Chemicals

Human EpCAM/TROP-1 monoclonal antibody (produced in mouse), human EpCAM/TROP-1 biotinylated polyclonal antibody (produced in goat), and recombinant human EpCAM antigen were purchased from R & D Systems (USA). *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-(*N*-morpholino) ethanesulfonic acid (MES) hydrate, sodium choloroacetate, 3-aminopropryl-triethoxysilane (APES), and 3-phosphonopropinic acid (PPA) were purchased from Sigma-Aldrich (USA). Single layer graphene oxide aqueous suspension was obtained from Nanocs, Inc. (Boston, MA). Qdot® 525 ITK™ amino (poly ethylene glycol) quantum dots, streptavidin, and bovine serum albumin (BSA) were purchased from Invitrogen (Australia). All other reagents were obtained from Sigma-Aldrich (USA) and used without further purification. The phosphate buffer saline (PBS) solution consisted of 0.01 M potassium phosphate, 0.138 M NaCl, and 0.0027 M KCl (pH 7.4). The MES buffer comprised of 100 mM MES hydrate (pH 5.2).

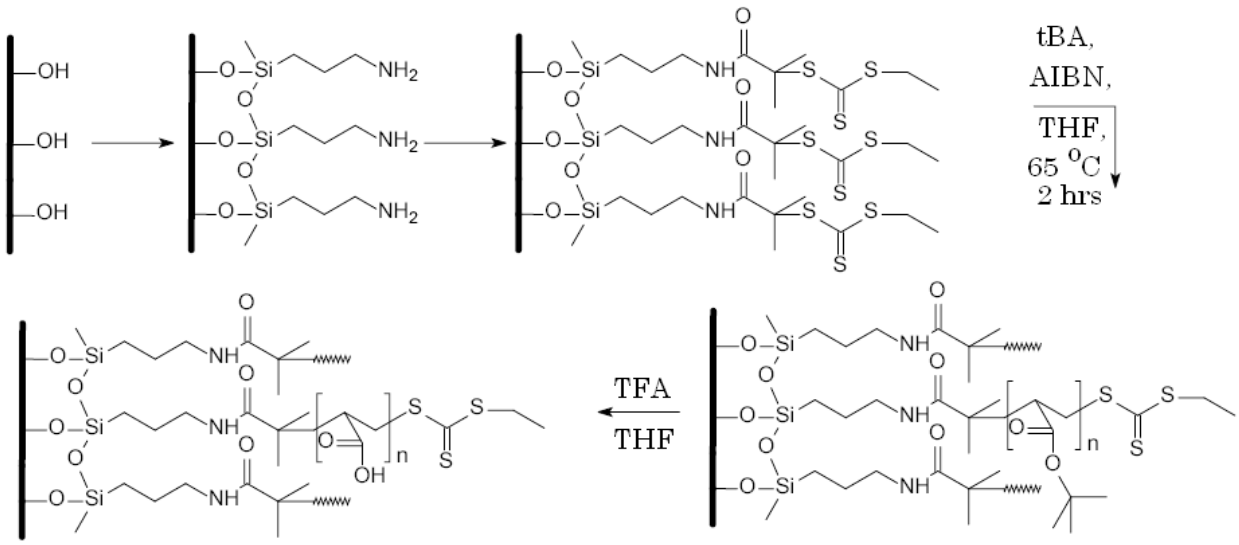
'pAA Brush' preparation on ITO substrate and thickness measurement

Glass slides coated with ITO (Nanocs, Inc., Boston, MA) were cut into 3 mm × 20 mm pieces and cleaned with ethanol, acetone, 2-propanol and water, successively, followed by drying in air at 60 °C. The effective working area of the clean electrodes (0.58 cm^2) electrode was determined under linear sweep voltammetric conditions for the one-electron reduction of $K_3[Fe(CN)_6]$ (1.0 mM in water (0.5 M KCl))[S1] and use of the Randles-Sevcik relationship.[S2] The cleaned substrates were then treated with a mixture of water, hydrogen peroxide (30%), ammonia solution (30%), in a 5: 1: 1 ratio at 70 °C for 2 h to ensure the presence of active hydroxyl groups on the surface. Subsequently, amine functionalization of the substrates were performed by reacting them with aminopropyl triethoxy silane (APES) solution (3 vol.% in methanol) for 36 h at room temperature. 2-ethyl sulfonyl thiocarbonyl 2-methyl propionic acid (EMP) chain transfer agent (CTA), synthesized according to the procedure outlined in Convertine *et al.*[S3] was then immobilized on

amine-functionalized substrates by activating the carboxylic acid group of EMP using equal molar quantity of N, N'-diisopropylcarbodiimde (DIC) and N, N'-diisopropylethylamine (DIPEA) in DCM. The covalently attached CTA on the substrates were then washed with DCM and THF and dried under nitrogen flow.

8.5 mL *tert*-butyl acrylate (*t*BA) monomer (by passing through base activated alumina column to remove inhibitor) and 1.28 mg 2,2'-azobis(isobutyronitrile) (AIBN) initiator were added to 15 mL THF in a 50 mL round-bottom flask equipped with magnetic stirrer bar. The CTA-functionalized substrates were placed in the flask and purged with Argon gas for 30 min under slow stirring, followed by heating at 65 ^{o}C for 4 h under an Argon atmosphere. The polymerization reaction was stopped by quenching the flask in an ice bath and the polymer coated substrates were washed with THF. The *tert*-butyl groups of the poly(*t*BA) chains were cleaved by treating the slides with TFA (tri-fluoro acetic acid) in THF for 2 h to form poly(acrylic acid) (pAA) (Scheme S1).

The thickness of the pAA brush was measured using Dektak 150 surface profiler. First a line was drawn on the surface of the polymer by pressing a needle gently on the substrate. The stylus of the Dektak was placed just before the start of the line and travel across the line to measure the depth. The depth of the line was then used to calculate the thickness of the polymer.



Scheme S1: Schematic illustration of the sequential reaction steps involved in the preparation of pAA brush on ITO coated glass slides.

Preparation of the 2D-GR-QDs-anti-EpCAM bionanoconjugate

The bionanoconjugate was fabricated according to the procedure described previously.[S4,S5] Briefly, an aqueous suspension of two-dimensional graphene oxide (2D-GR) nanosheet was first treated with excess quantity of NaOH and $ClCH_2COONa$ (1:1 wt. ratio), followed by acidification with 0.1M HCl to convert ester, hydroxyl, and epoxide groups of 2D-GR nanosheet into carboxylic acid groups. The acid functionalized nanosheets were repeatedly washed with water (by centrifugation at 12000 rpm for 7 mins)

until disperses in water and then dialyzed against water for 72 h. Amine-functionalized quantum dots (QDs) (50 µL amino-QDs at 8µM) and streptavidin (50 µL streptavidin at 10 µg/mL) were then simultaneously coupled on carboxylic acid groups functionalized-GR nanosheet, using EDC/NHS chemistry following the standard protocol. The 2D-GR/QDs-streptavidin conjugate was then incubated in a 500 µL of 50 µg/mL biotinylated EpCAM antibody solution to immobilize them on the conjugate. This biotinylated anti-EpCAM/GR/QDs conjugate (hereafter defined as 2D-GR-QDs-anti-EpCAM) was used as labels for the detection of EpCAM antigen by SWASV.

Antibody immobilization and immunoreaction procedure

The carboxylic groups of the polymer brush were activated by immersing the substrates in a mixture of 400 mM EDC and 100 mM NHS in pH 5.2 MES buffer for 1.5 h. This was immediately followed by 1.5 h incubation at 37 °C with 50 µg/mL monoclonal anti-EPCAM antibody in PBS buffer containing 0.05% Tween-20. The antibody-immobilised ITO/APES/pAA substrates were incubated at 37°C for 1 h in PBS buffer and in 98% human serum containing increasing concentration of spiked EpCAM antigen (5 fg/mL to 10000 ng/mL in PBS, and 150 fg/mL to 10000 ng/mL in serum), followed by washing the substrates 3 times with PBS containing 0.05% Tween-20. Spiked serum samples were made by adding 10 µL EpCAM solution (in PBS) in to 500 µL of serum. Next, they were incubated in 100 µL as-prepared 2D-GR-QDs-anti-EpCAM bionanoconjugate solution for 2 h at at 37 °C to complete the sandwich-type immunoreactions.

Confocal microscopy

Confocal microscopy images for the ITO/APES/pAA/anti-EpCAM/EpCAM/bio antiEpCAM/GR/QDs immunosensors were taken using Zeiss LSM 710 confocal microscope. The fluorescence signal of QDs 525 was acquired using the LSM software defined excitation (405nm) and emission wavelengths for QDs 525. The objective lens LD Plan-Neofluar × 20 with N.A. 0.4 was used to acquire fluorescence images of quantum dots. A JEOL 1010 Transmission electron microscope (TEM) operating at 100 kV was used to observe the morphology of bionanoconjugates. The samples were prepared by dropping 10 µl of the 2D-GR-QDs-anti-EpCAM on the copper grid and allowed to dry.

Electrochemical measurements

The voltammetric and electrochemical impedance spectroscopy (EIS) experiments were conducted at room temperature (22 ± 1°C) in a standard three-electrode electrochemical cell arrangement using an electrochemical analyzer CHI 650D (CH Instruments, Austin, TX). In EIS experiments, the electrochemical cell consisted of a modified-ITO substrate as a working electrode, a Pt wire counter electrode, and a Ag/AgCl (3 M NaCl) reference electrode. The EIS spectra were recorded in a 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) in 1.0 M KCl electrolyte solution using an alternating current voltage of 5.0 mV, with the frequency range of 0.1 Hz – 10 kHz.

To perform SWASV measurements, the surface attached QDs were dissolved by immersing the ITO/APES/pAA/anti-EpCAM/EpCAM/2D-GR-QDs-anti-EpCAM immunosensor in 250 µL of 0.1 M HCl solution for 2 h, and the resulting solution was then transferred in an electrochemical cell containing 1.75mL of 100 mM MES (pH 5.2) buffer. The three-electrode arrangement used for the SWASV experiments consisted of a glassy carbon working electrode (diameter = 3 mm), a Pt wire counter electrode, and a Ag/AgCl (3 M NaCl) reference electrode. The effective working area of the GCE (0.068 cm^2) electrode was determined under linear sweep voltammetric conditions for the one-electron reduction of $K_3[Fe(CN)_6]$ (1.0 mM in water (0.5 M KCl))[S1] and use of the Randles-Sevcik relationship.[S2]. The stripping experiments involved a 60 s pretreatment at +600 mV, a 600 s accumulation at -1200 mV (with stirring), 15 s rest period (without stirring) and square-wave voltammetric scan from -1200 to -250 mV using a step potential of 4-mV, amplitude 25 mV, and frequency 25 Hz.

Square wave anodic stripping voltammetric response:

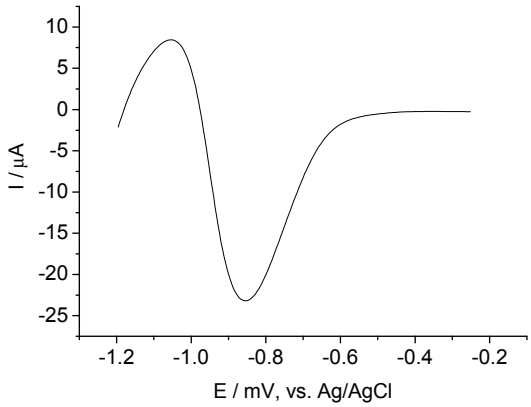


Fig. S1 SWASV profiles for the immunosensor at 1 ng/mL EpCAM in PBS.

References

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