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Single Conducting Polymer Nanowire Chemiresistive Label-Free Immunosensor for Cancer Biomarker

Mangesh A. Bangar, Dhammanand J. Shirale, Wilfred Chen, Nosang V. Myung, and Ashok Mulchandani*

Department of Chemical and Environmental Engineering and Center for Nanoscale Science and Engineering, University of California, Riverside, California 92521

A simple and cost-effective, all-electrochemical method to fabricate and assemble single conducting polymer nanowire based biosensors was developed. Polypyrrole (Ppy) nanowires were synthesized by electrochemical polymerization using an alumina template. The single-nanowire chemoresistive sensor device was assembled using ac dielectrophoretic alignment followed by maskless anchoring on a pair of gold electrodes separated by 3 μm. To establish an efficient covalent surface biofunctionalization route, glutaraldehyde (GA) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) chemistries were compared. EDC was established to be the most effective chemistry and was used to surface-functionalize a single Ppy nanowire with cancer antigen (CA 125) antibody to fabricate a nanoimmunosensor for CA 125 biomarker detection and quantification. The immunosensor had excellent sensitivity with a lower detection limit of 1 U/mL CA 125 and dynamic range up to 1000 U/mL in 10 mM phosphate buffer. Furthermore, there was no loss of performance upon exposure to CA 125 in spiked human blood plasma. This demonstrates the clinical importance of these sensors for cancer marker detection with cost benefits and great portability for diagnosis of patients at the point of care.

The detection of clinically relevant antigens has been a subject of intense research in recent years due to their importance in diagnostics and cure. For example, detection of cancer antigens has a very big impact on the diagnosis and treatment of cancer patients and also in assessing the condition of patients during or after a treatment. Current techniques for definitive tumor detection include ultrasound, biopsy, or hysteroscopy.[1] Also, monitoring tumor marker protein on a frequent basis gives progress of the patient's health. For example, cancer marker protein CA 125 is recommended for clinical use in ovarian cancer screening of high-risk women with ovaries and tracking the disease progression or relapse.[2−4] The normal level of CA 125 in blood is less than 35 U/mL,[5,6] and besides ovarian cancer it is also elevated in a number of other malignant conditions such as breast cancer, mesothelioma, non-Hodgkin's lymphoma, gastric cancer, and leiomyoma and leiomyosarcoma of gastrointestinal origin, and benign conditions like early pregnancy, uterine fibroid, and pelvic inflammatory disease.[7−9] The tumor marker proteins are detected using antibody assays against the cancer antigen. The first monoclonal antibody against CA 125 was discovered in 1981[10] and is used in sandwich enzyme-linked immunosorbent assay (ELISA).[6,11] The protocol involves several incubation and washing steps making the process slow, indirect, and difficult for real-time detection. Also, the portability of the system is limited due to the requirement of an appropriate transduction system/facility and trained personnel.

Recently, label-free detection of different target analytes (proteins, viruses, oligonucleotides) using a chemiresistive or field effect transistor (FET) sensor has gained tremendous research interest. These sensors require a minimal or no sample preparation and make real-time monitoring possible due to faster transduction mechanism. The portability, sensitivity, and overall performance of these devices were improved by using nanostructures such as silicon nanowires[12−15] or carbon nanotubes.[16−18] Due to its small cross-sectional area, nanowires/nanotubes show

* To whom correspondence should be addressed. E-mail: adani@engr.ucr.edu.

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no lateral current shunting resulting in maximum response compared to thin-film counterparts.[19] However, fabrication of these nanostructures requires harsh conditions and is expensive.[20−23]

Conducting polymers have shown great promise as a material for sensor application.[24−26] Their excellent magnetic, optical, and tunable electrical properties[27−29] along with biocompatibility[25−30] and benign electrochemical[31,32] or chemical[26,33] synthesis route offer several advantages over other nanostructures. When integrated as a semiconducting channel in an FET, change in the surface potential caused by specific binding of targeted charged analyte to a biorecognition molecule (such as antibody) on the surface causes change in the electrical properties (e.g., conductance) of the conducting polymer. The use of conducting polymer nanowires for biosensing application, however, has been limited due to their incompatibility with traditional microfabrication processes such as lithography and focused ion beam (FIB) owing to its possible thermal damage during these processes.[26,34] Simpler and less costly methods of assembling conducting polymer nanowire devices have relied on bottom-up geometry in which nanowires are deposited on the top of prefabricated electrodes. A limitation of this method is the difficulty in eliminating the change in contact resistance due to physical disturbances/movements introduced during liquid phase sensing. Also, to achieve sensor to sensor reproducibility, it is necessary to have precise number of individual nanowires electrically connected between the electrodes, which is lacking during drop-casting method of fabrication.[26]

To address these issues we have developed a simple and cost-effective all-electrochemical approach to fabricate, assemble, and anchor a single conducting polymer (polypyrrole, Ppy) nanowire to fabricate a functional device. Glutaraldehyde (GA) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) chemistries were evaluated for their efficacy in surface functionalization of Ppy nanowires. The devices were further functionalized with antibodies toward cancer biomarker, cancer antigen (CA 125) using EDC chemistry. With the use of a simple chemiresistive transduction mode, these biosensors were used to detect CA 125 over concentration range of 1 to 1000 U/mL in phosphate buffer (PB). Finally, detection of CA 125 in spiked human blood plasma was demonstrated to show its potential application for real clinical samples without major sample preparation.

EXPERIMENTAL SECTION

Nanowire Synthesis. Ppy nanowires were electrochemically synthesized using the well-established template-directed electrodeposition.[31,35] Alumina template with 200 nm nominal pore diameter and about 60 μm thickness (Whatman International Ltd., Maidstone, England) was used as a scaffold for nanowires fabrication. Briefly, one face of the template was sputter-coated with ~200 nm thick gold using the Emitech K550 (Emitech Ltd., Kent, England) sputter coater. The electrodeposition was carried out in a three-electrode cell with Ag/AgCl (3 M KCl) as a reference electrode, platinum-coated titanium strip as a counter electrode, and alumina template with the gold bottom-seed layer as a working electrode using an EG&G PAR VMP2, (Princeton Applied Research, Tennessee) potentio/galvanostat. The electrolyte consisted of 0.5 M pyrrole and 0.2 M $LiClO_4$ at pH 6.3 and was purged with 99.99% nitrogen for 30 min before deposition. Ppy nanowires were deposited potentiostatically at 0.9 V versus Ag/AgCl until the template was overdeposited (~15 min). The overdeposited Ppy was mechanically polished away, the template was washed with water and acetone to remove any residues, and the gold seed layer was removed using 0.15 M KI in 0.1 M I_2 gold-etchant solution. After washing the template with water, alumina template was dissolved in 30% (v/v) H_3PO_4 acid and briefly sonicated to free the nanowires from the template to form a suspension. The nanowires were subsequently washed with water three times before suspending them in water. The suspension was diluted 10-fold for further use.

Single Nanowire Based Device Assembly. Prefabricated gold microelectrodes made up of an array of 16 pairs of electrodes with ~70 μm separation between adjacent pairs with each pair containing rectangular electrodes of ~55 μm width separated by a 3 μm gap were used as contact electrodes for device assembly. The two sides of 16 pairs were shorted to form two terminals. An alternating current (ac) field of 5 MHz and 1 V peak-to-peak voltage was applied between these terminals. A 2 μL drop of Ppy nanowire suspension was dispensed on it, and alignment was carried out until the drop was completely evaporated. To achieve single-nanowire connection between a pair of contact electrodes, excess nanowires were physically/manually removed using a probe tip made out of 25 μm diameter gold wire under a 1000× magnification optical microscope.

Gold was selectively electrodeposited on the 16 pairs of contact electrodes to anchor the nanowires. A three-electrode configuration electrochemical cell consisting of the 16 pairs of contact electrodes with a single Ppy nanowire as the working electrode, Ag/AgCl as a reference electrode, and a platinum-coated metal strip as a counter electrode was used. The electrolyte was Technigold (Technic Inc., California) at pH of 7.0, and the deposition potential was −0.5 V versus Ag/AgCl at room temperature for 10 min for built up of an ~300 nm thick gold layer on the gold electrode surface.

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Fluorescein Isothiocyanate Labeling of Bovine Serum Albumin. An amount of 100 μL of 10 mg/mL fluorescein isothiocyanate (FITC) solution in dimethylformamide (DMF) was added to 1 mL of 2 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich Inc., Missouri, USA) solution in 0.1 M, pH 9 sodium bicarbonate buffer, and the reaction mixture incubated at room temperature for 1 h with continuous stirring. The resulting BSA−FITC conjugate was purified by dialysis against pH 7.0 phosphate buffer saline (PBS).

Nanowire Covalent Surface Biofunctionalization. 1. GA Chemistry.[36] Ppy nanowires were incubated in 4% GA solution in pH 7.0 PBS buffer for 1 h. After removal of excess reagent and subsequent washing with buffer, nanowires were incubated with FITC-tagged BSA solution (2 mg/mL) for another 1 h. The excess BSA−FITC solution was removed, and the nanowires washed with 0.1% Tween-20 in PB buffer (PBT) followed by PB buffer alone. Nanowires sample without the GA treatment was exposed to BSA−FITC solution as a negative control and washed with PBT followed by PB.

2. EDC Chemistry.[37] Ppy nanowires were incubated in FITC-tagged BSA solution (2 mg/mL) and 60 mM EDC solution in 0.1 M MES buffer at resulting pH of ~5.5. To this reaction mixture excess of *N*-hydrosuccinimide (NHS) in dimethyl sulfoxide (DMSO) (2 mg/mL) was added. The reaction was carried out for 3 h. The excess reagent was subsequently removed, and nanowires were washed with PBT followed by PB. The control sample mentioned above in GA chemistry section also served as a negative control for EDC chemistry.

To establish the yield of individual surface chemistry, nanowire samples were observed under a fluorescence microscope at 100× magnification with/without green FITC filter. The presence of green fluorescence indicated the presence of BSA−FITC bound to nanowires either covalently or noncovalently.

Biosensor Construction for CA 125 Detection. 1. Sensor Construction. Single-nanowire devices were biofuncationalized with antibodies toward cancer marker protein CA 125 using EDC chemistry by using the anti-CA 125 monoclonal antibody (Fitzgerald Inc., Massachusetts) solution (~3 mg/mL) instead of BSA−FITC. The biofunctionalization was skipped for control samples. To block nonspecific interactions between biomolecules and the nanowire, devices were incubated in BSA (1 mg/mL) solution in PB at physiological pH of 7.0. The excess BSA was removed, and the devices washed with PBT followed by PB.

2. Sensing Measurement. Sensors were characterized in terms of their current−voltage ($I-V$) response measured using a semiconductor parameter analyzer (model 4155A, Agilent Technologies Inc., California) with a 30 μL drop of PB on the nanowire. The voltage was swept from −200 to +200 mV, and the current was recorded. The nanowire conductance was measured as the slope of $I-V$ near zero voltage in the linear range of ±100 mV. Conductance changes of the sensor upon exposure to different CA 125 (Fitzgerald Inc., Massachusetts) concentrations in either 10 mM PB or spiked human blood plasma (Sigma-Aldrich Inc., Missouri) were measured in wet condition (with a 30 μL drop of PB on the nanowire) after the sensor was incubated in respective analyte solution for 5 min followed by washing with PBT followed by PB.

RESULTS AND DISCUSSION

Nanowire Synthesis. The use of alumina template for nanowires fabrication has been well studied. With the use of 200 nm pore diameter and 60 μm thick alumina templates long Ppy nanowires were fabricated by overdepositing Ppy inside and out of the pores (figure not shown). The excess deposit was easily removed by mechanical polishing. This ensured the length of electrodeposited nanowires was about 60 μm. Approximately 10^8 (which equals the pore density of alumina template[38]) nanowires were produced from ~0.64 cm^2 (~8 × 8 mm^2) of alumina template. To obtain uniform suspension of these nanowires after seed layer removal and template etching, the sample was sonicated, which broke the nanowires further into smaller fragments resulting in more number of nanowires and very dense suspension. However, the length of the resulting nanowires (20.8 ± 8.6 μm, n = 130) was still sufficient to fabricate functional devices with electrical resistance in the desired range.

Alternating Current Dielectrophoretic Alignment and Single Ppy Nanowires Device Fabrication. A 2 μL of diluted suspension was dispensed on the prefabricated array of 16 pairs of electrodes under the influence of applied ac field. In presence of ac field the charged Ppy nanowires were attracted toward the high electrical gradient, which is present near the edges and the gap of the contact electrodes. Also, as the nanowires aligned themselves in the direction of the applied electric field, they aligned perpendicular to the gap between the electrodes.[39] As shown in Figure 1A, multiple nanowires were aligned between a pair of electrodes establishing electrical contact between them. Furthermore, nearly all the electrode pairs in an array of 16 were connected with at least a single nanowire, in a single alignment attempt. This is attributed to a high concentration of nanowires in the suspension. In order to get single-nanowire connection, excess nanowires were removed manually by scrapping them away using a 25 μm diameter gold wire probe. The tip of the probe was substantially smaller and could remove nanowires separated by as small as 5−6 μm (Figure 1B). Preparation of a single device using this method took a maximum of a couple of minutes depending upon complexity of the nanowires' placement. Furthermore, as shown in Figure 1C, the technique provided a high yield (15 out of 16) of arrays of single-nanowire connections. The combined ac alignment and nanowires removal methodology is a facile high-throughput and cost-effective technique that can be easily adopted for other nanostructures.

Maskless Electrodeposition for Nanowire Anchoring. Single-nanowire devices fabricated using this approach can severely suffer from changes in contact resistance due to motion or surface tension of liquid during biosensing. Ensuring a firm contact between nanowire and contact electrodes is essential to alleviate this problem. Recently, our group introduced a maskless electrodeposition technique in which nickel was selectively deposited on the contact gold electrodes so that the ends of the conducting

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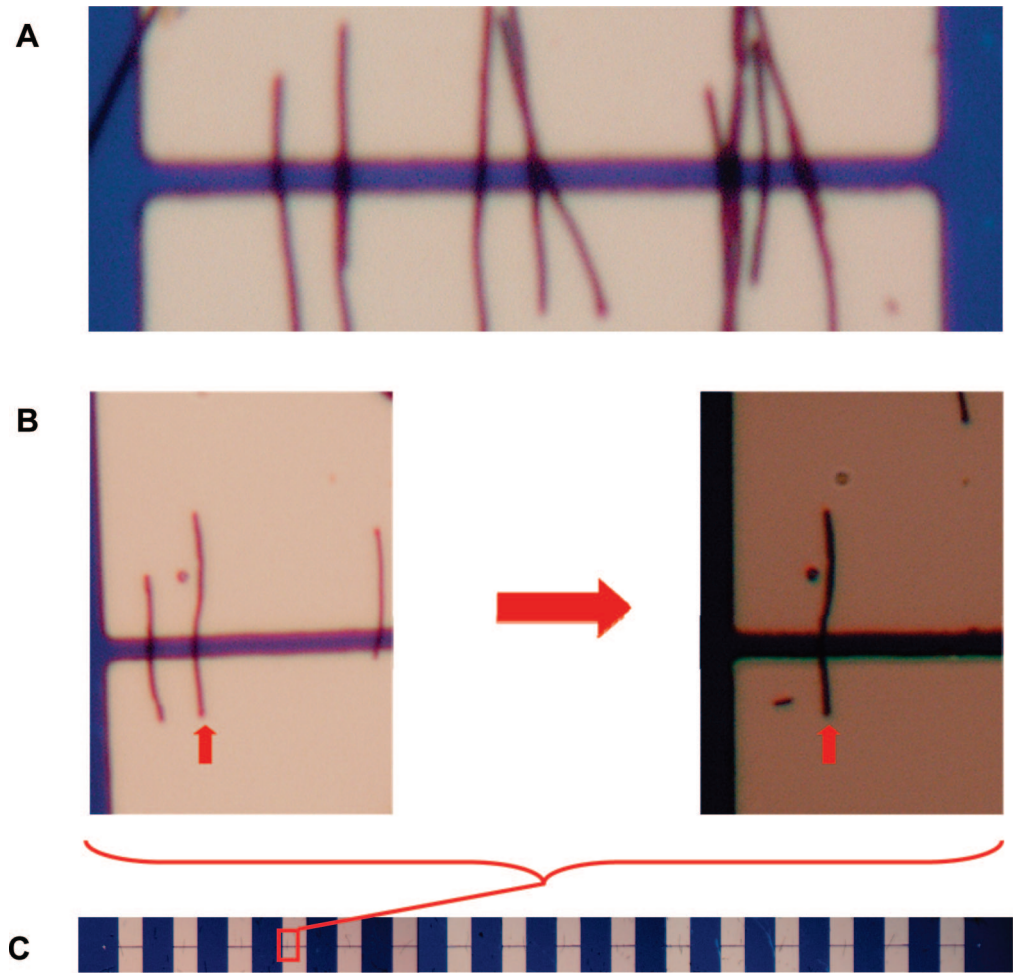

Figure 1. (A) Optical image of ac dielectrophoretically aligned Ppy nanowire across a pair of gold electrodes separated by a gap of 3 μm showing perpendicular alignment of Ppy nanowires across the electrode gap. (B) Optical images of a pair of electrodes with a 3 μm gap before and after physical removal of excess nanowires showing nanowires as close as 5−6 μm can be removed using a micrometer-sized gold wire probe. (C) Array of 15 single Ppy nanowire connections out of 16 pairs of ~55 μm wide gold electrodes separated by ~70 μm with a gap of 3 μm between each pair.

polymer nanowire were anchored.[34] This resulted in stable contact resistance. In the present study, we have used gold instead of nickel. The choice of gold was based primarily on in its inertness toward most biochemicals. The success of the proposed technique depends on the ability to selectively deposit gold on contact electrodes and not on the nanowire. To determine the deposition conditions to achieve this, linear sweep voltammetry on 0.5 cm^2 of gold and Ppy film was studied. As shown in Figure 2A, the gold reduction rate on Ppy was lower than that on gold surface for potentials more negative than −0.38 V, reaching a maximum at about −0.5 V (vs Ag/AgCl reference electrode) above which the reduction on Ppy increased drastically. Thus, to minimize gold deposition on the nanowire surface compared to large gold electrodes, reduction of gold on Ppy was kept at possible minimum by performing deposition at −0.5 V. A partial to complete anchoring of the nanowires with no deposition on the nanowire in the gap was achieved in 10 min of deposition (Figure 2, parts B and C), which resulted in a stable contact resistance upon exposure to liquid medium.

Surface Biofunctionalization of Ppy Nanowires. Entrapment during polymer synthesis and covalent attachment post polymerization are the two widely used methods of biofunctionalizing conducting polymer. The major disadvantages of the entrapment method are loss of biological activity due to adverse synthesis conditions and inaccessibility of the biomolecule as it is buried inside the polymer matrix. These can reduce the sensitivity and increase the response time of the biosensor. Covalent functionalization, on the other hand, immobilizes biomolecule on the polymer surface making it fully accessible for interaction with its complementary target giving higher sensitivity and a faster responding biosensor. We investigated biofunctionalization of Ppy nanowires by GA and EDC that have been employed widely for covalent immobilization of biomolecules.[36,37] Figure 3, parts A and B, shows the reaction mechanism for covalent biomolecule immobilization on Ppy by GA and EDC chemistries, respectively. Briefly, GA or EDC is first attached to the secondary amine groups on the Ppy nanowire or carboxylic groups on the biomolecules and the other end of the linker molecule is attached to the amine groups of the biomolecules or the secondary amine groups on the Ppy nanowire surface, respectively. To select the best route for covalent biofunctionalization of Ppy nanowire sensors, surface immobilization of BSA−FITC on nanowires suspension was first studied. Biomolecules immobilization on Ppy nanowires without GA and EDC treatment (control) showed fluorescence, attributed to nonspecific adsorption of BSA−FITC due to hydrophobic interaction. How-

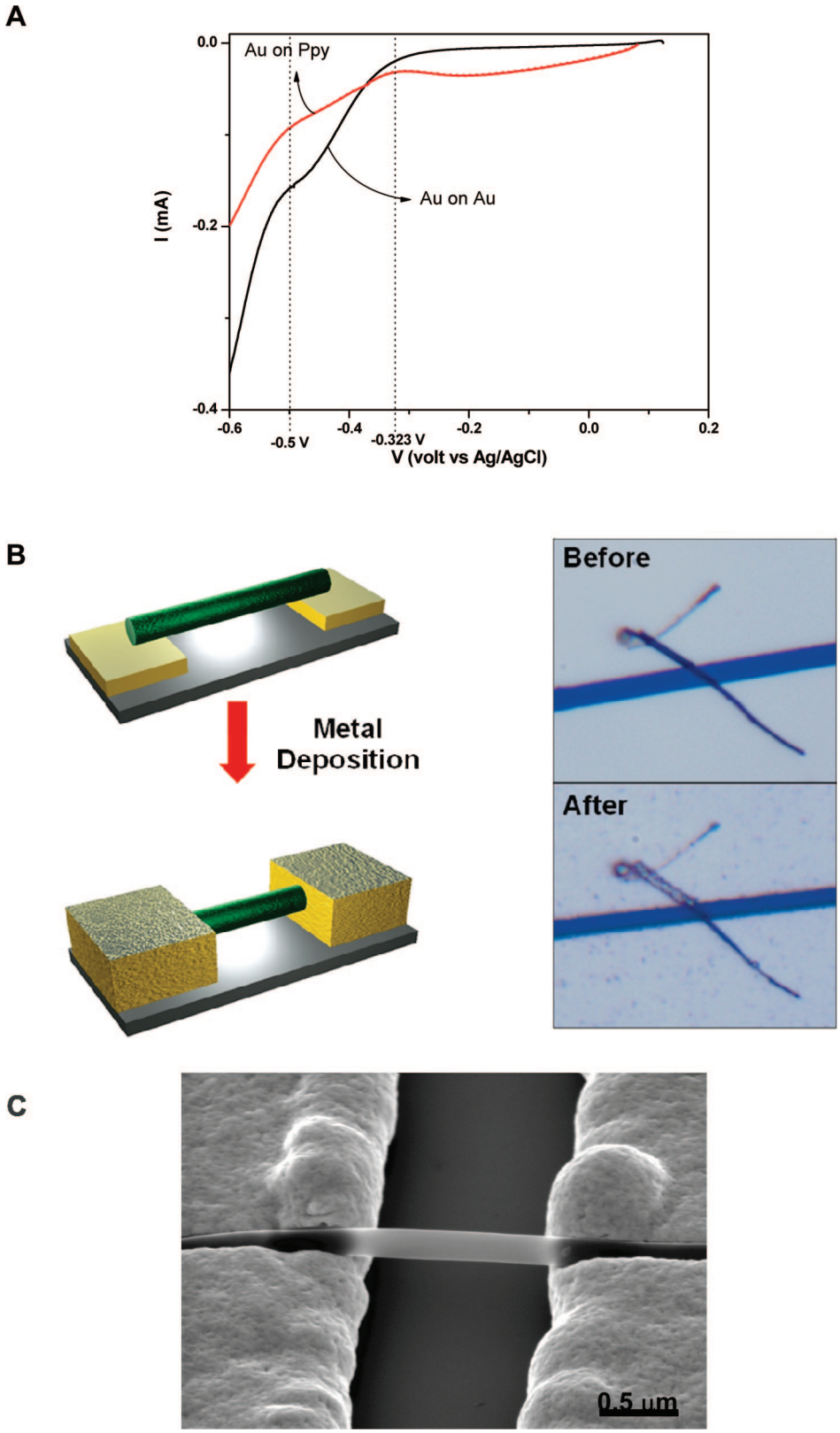

Figure 2. (A) Linear sweep voltammograms of gold electrodeposition on gold and Ppy surfaces (~0.5 cm^2 each). Scan rate 1 mV/s. (B) Schematics of maskless electrodeposition of gold along with optical images of before and after selective gold electrodeposition on gold microelectrodes separated by a 3 μm gap connected with a single Ppy nanowire. (C) Scanning electron micrograph (SEM) of a maskless electrodeposited single Ppy nanowire device showing selective gold electrodeposition on the electrodes resulting in partial anchoring of the nanowire.

ever, over 70% of the original fluorescence signal was reduced on washing the nanowires with PBT (data not shown). Nanowires functionalized with GA and EDC exhibited both nonspecific adsorption and covalent attachment of BSA−FITC as evidenced by the decrease in the single-nanowire fluorescence line scan peak intensity from the original (Figure 3C, upper panels) upon PBT

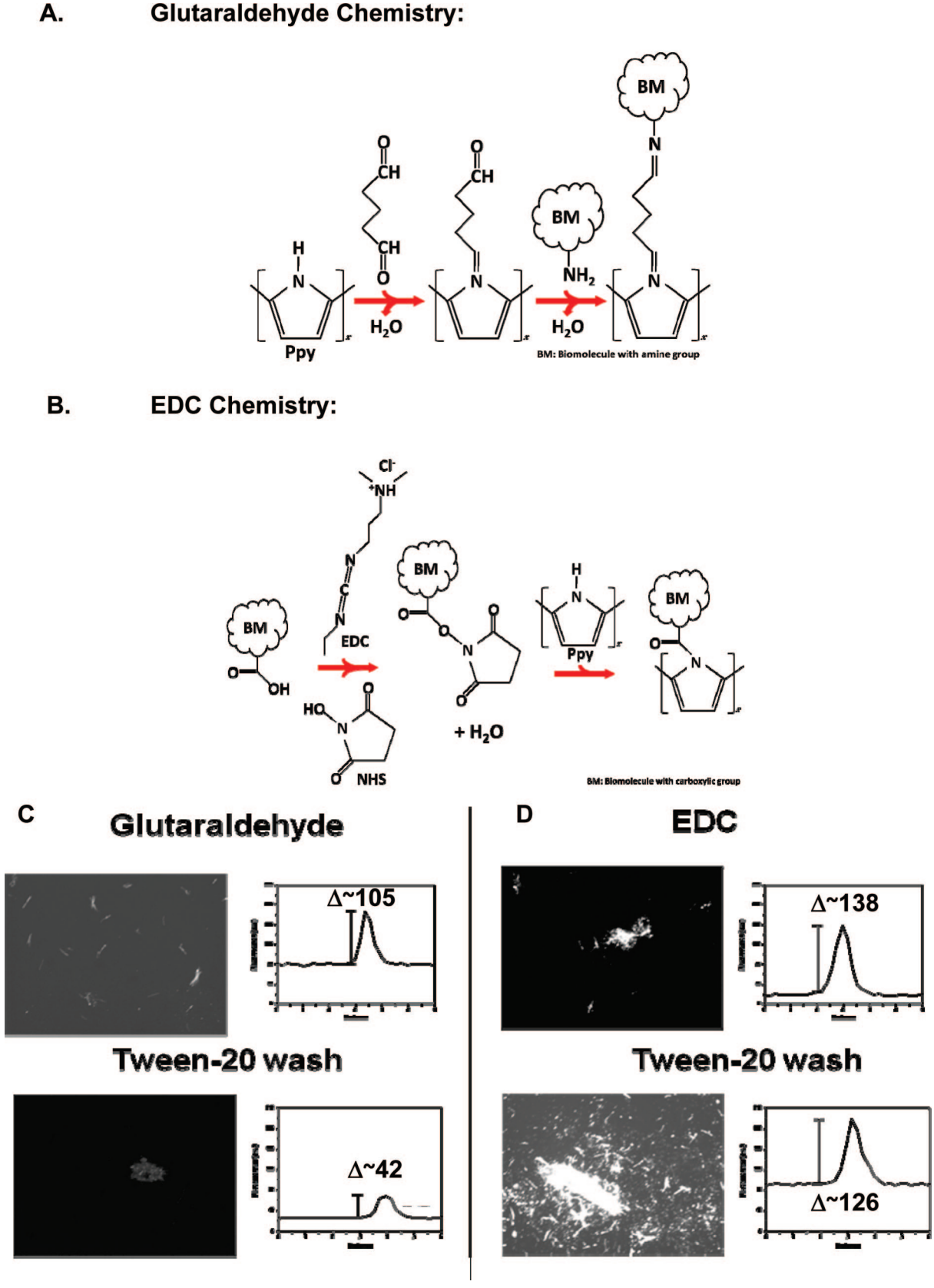

Figure 3. Reaction mechanism of (A) GA chemistry and (B) EDC chemistry. Fluorescence microscope images of a Ppy nanowire functionalized with BSA−FITC conjugate using GA chemistry (C) and EDC chemistry (D) before and after Tween-20 washing which removed physically adsorbed BSA−FITC conjugate molecules. Representative fluorescence intensity line scans across a single nanowire diameter are shown next to the respective images.

washing (Figure 3C, lower panels). The fluorescence intensity reduction in case of GA-functionalized nanowires, however, was higher (similar to the nonspecific adsorption, i.e., control) when compared to the EDC functionalization route indicating a higher

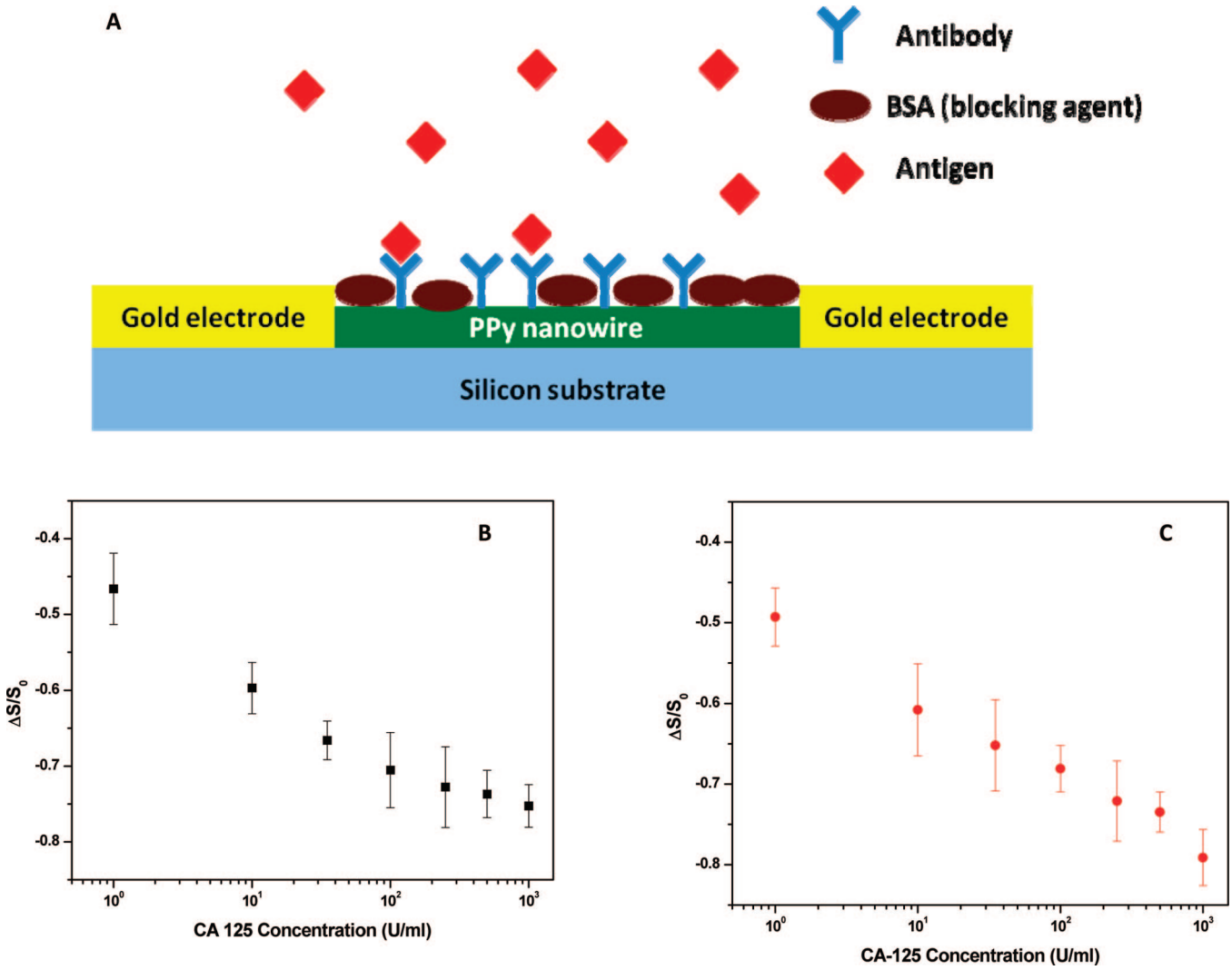

Figure 4. (A) Schematic of a single Ppy nanowire sensor covalently modified with anti-CA 125 using EDC chemistry and blocked by BSA to reduce nonspecific binding. Calibration curve in terms of normalized conductance change of a single Ppy nanowire biosensor for CA 125 detection in 10 mM phosphate buffer (B) and spiked human blood plasma (C). Each point represents an average of three (B) or four (C) measurements, and the error bars represent ±1 standard deviation.

yield of covalent attachment for EDC over the GA method. Therefore, EDC chemistry was used for further sensor fabrication.

Anti-CA 125 Functionalized Single Ppy Nanowire Biosensor. Single Ppy nanowire biosensors were fabricated using EDC chemistry and antibodies against cancer antigen (CA 125) as recognition molecule. The devices were incubated with BSA to further block the nonfunctionalized sites on the nanowire (Figure 4A). Figure 4B shows the nanowire immunosensor response $[(S - S_0)/S_0$, where S is the conductance after exposure to CA 125 and S_0 is conductance after exposure to buffer] as a function of logarithmic CA 125 concentration in 10 mM PB. As shown, the sensor was extremely sensitive with greater than 45% change in conductance even at the lowest tested concentration of 1 U/mL. Further, it had a wide dynamic range spanning from 1 to 1000 U/mL. This observation was consistent with recent theoretical work on silicon nanowire biosensors.[40] Control devices without antibodies and blocked with BSA showed about 17% ± 3% change in the conductance toward the highest concentration of CA 125 (1000 U/mL), indicating a low nonspecific adsorption of the biomarker to the nanowire itself.

The utility of the developed sensor for detection of CA 125 in real samples was evaluated by measuring different CA 125 concentrations spiked in human blood plasma (Figure 4C). As shown in the figure, the lower detection limit, dynamic range, and sensitivity of the biosensor for CA 125 in human blood plasma was quantitatively comparable to PB. Response of the sensor toward unspiked human blood plasma was about 32% ± 3% indicating either very low nonspecific interaction between the sensor and the blood plasma constituents or presence of CA 125 in as-purchased human blood plasma at very low concentration (<1 U/mL). This demonstrates the applicability of this single-nanowire biosensor for detection of the biomarker in real samples with minimal or no preparation. More importantly, these results indicate the applicability of the sensor to detect CA 125 concentrations well below and above 35 U/mL which has been established as the maximum normal blood level of CA 125 with minimal or no sample preparation.[5,6]

To summarize, a simple and cost-effective methodology for fabrication of single Ppy nanowire chemiresistive immunosensor devices was developed and demonstrated for highly sensitive and selective detection of cancer biomarker CA 125. The methodology developed in this study can be readily extended for fabrication of nanoimmunosensors for detection of other proteins, viruses, oligonucleotides, etc. by using an appropriate recognition molecule. More importantly, this methodology can be applied for fabricating an array of sensors with different recognition molecules on a single silicon substrate for multiplex sensing in a cost-effective manner. Thus, even though detection of CA 125 alone may not be definitive for malignancy detection in all cases,[41,42] detection of a panel of different cancer markers (e.g., CA 72-4, M-CSF, HE4, mesothelin, B7-H4 along with CA 125 for ovarian carcinoma detection)[43−47] is possible with an array for more definitive diagnosis. Due to their small size, easy and fast electrical transduction mechanism, and ability to integrate modern day electronics, detection at the point of care/use without the need for extensive sample preparation and trained professionals is possible with these single nanowire based biosensors. Further, integrating the sensor to a microfluidics platform will improve fluid handling and make real-time detection feasible. Such sensor arrays can find applications in detecting target analytes in the fields of health care, environmental monitoring, homeland security, food quality and safety, etc.

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