Single-Wall Carbon Nanotube Forest Arrays For Immunoelectrochemical Measurement of 4 Protein Biomarkers for Prostate Cancer

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Supporting Information:

The **biomaker proteins** in this work were Prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), Platelet factor-4 (PF-4) and Interleukin-6 (IL-6). Prostate specific antigen is a 34 kDa protein produced by the cells of the prostate gland. PSA is present in small quantities in the serum of normal men, and is often elevated in the presence of prostate cancer. Normal concentration of PSA in healthy men is less than 4 ng mL^{-1} where as in cancer patients its greater than 4 ng mL^{-1}. Prostate specific membrane antigen is a 100 kDa cell surface glycoprotein expressed by prostate epithelial cells [2,3] and is over expressed during prostate cancer.[4] Normal PSMA levels in healthy persons are 200 – 300 ng mL^{-1}. Abnormal levels of PSMA are also associated with solid tumors such as breast, lung, ovary, bladder, and intestinal tract cancers.[4,5] Platelet factor 4 (PF4) is a 7.8 kDa cytokine belonging to the CXC chemokine family. As PF4 is released upon platelet stimulation, it serves as a useful marker for measuring platelet activation and secretion.[6] PF4 levels in plasma are 2–10 ng/ml. Plasma concentration is used as a marker of platelet activation. [7] PF-4 was also revealed to be a marker of new vessel formation in xenografts of human breast cancer. [8] Interleukin-6 (IL-6), is a 20 kDa cancer biomarker protein. [9] Overexpression of IL-6 is associated with several different cancers, including head and neck squamous cell carcinoma (HNSCC).[10] Mean serum IL-6 levels in healthy individuals are 6 pg mL^{-1} or less while its levels in patients with HNSCC are 20 pg mL^{-1} or greater.[10] Serum IL-6 is also elevated in colorectal,[11] gastrointestinal [12] and prostate cancers.[13,14]

EXPERIMENTAL DETAILS

Chemicals and Materials. Bovine serum albumin (BSA), Casein and Tween-20 were from Sigma-Aldrich. Monoclonal (Mouse) primary anti-human prostate specific antigen (PSA) antibody (clone no. CHYH1), tracer secondary anti-PSA antibody (clone no. CHYH2) with HRP conjugation, monoclonal (Mouse) primary anti-human prostate specific membrane antigen (PSMA) antibody (clone no. CHYH2), secondary anti-PSMA antibody (clone no. CHYH1) with HRP conjugation were obtained from Anogen/Yes Biotech Lab, Ltd. PSA standard (Ag) were obtained from Sigma-Aldrich, PSMA standards were obtained from national institute of health (NIH). Monoclonal anti-human Interleukin-6 (IL-6) antibody (clone no. 6708), biotinylated anti-human IL-6 antibody, recombinant human IL-6 (carrier-free) in calf serum and Streptavidin-Horseradish peroxidase (HRP) were obtained from R&D systems. Monoclonal anti-human CXCL4/PF-4 antibody, recombinant human PF-4 (carrier-free), and biotinylated anti-human PF-4 antibody were obtained from R&D systems. Human serum samples from patients were

obtained from the Capital biosciences, 10 samples were from male serum who were diagnosed with prostate cancer and 4 samples were from normal female serum. Single-walled carbon nanotubes (HiPco) were from Carbon Nanotechnologies, Inc. 2,2′-Azino-Bis(3-ethylbenzthiazoline- 6-sulfonic acid) was from Sigma. Immunoreagents were dissolved in pH 7.0 phosphate saline (PBS) buffer (0.01 M in phosphate, 0.14 M NaCl, 2.7 mM KCl) unless otherwise noted. 1-(3-(dimethylamino)- propyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHSS) were dissolved in water immediately before use.

To obtain pure PSMA, the human coding sequence of PSMA was sub-cloned into a pEF1/His A, B, C expression vector (Invitrogen, Carlsbad, CA). Next, HEK293T cells grown overnight in DMEM supplemented with 10% fetal bovine serum (10 cm plates), were transfected with 10 μg/plate of the pEF1/His A, B, C PSMA DNA using PolyFect Transfection Reagent (Qiagen, Valencia, CA), which was then followed by growth for an additional 48 h prior to lysis with RIPA buffer. Purification of epitope tagged (polyhistidine; 6xHis) containing PSMA from the total cellular proteins was carried out using the ProBond Purification System (Invitrogen), and by following the provided instructions only recombinant proteins containing a 6xHis sequence can be enriched. Next, the specificity of eluted proteins was assessed by Western blot and, then 5□10 □L fractions were resolved on a SDS gel together with BSA standards, and scanned after staining with Coomassie blue. Using NIH Image software, only the band corresponding to the molecular weight of 6xHis PSMA was quantified against the BSA standards.

Instrumentation. A CHI 1030 electrochemical workstation was used for cyclic voltammetry and amperometry at ambient temperature (22 ± 2 ^{o}C) in an electrochemical cell which accommodated 4 working electrodes. Amperometry was done at -0.3 V vs SCE with the SWNT immunosensor working electrode and solution was stirred at 2500 rpm using a magnetic stirrer.

Fabrication of SWNT Immunoarrays. The 4-electrode arrays consisted of 4 demountable bundled, abraded disks (Momentive Performance Materials, A= 0.16 cm^2) of ordinary pyrolytic graphite embedded in heat shrinkable tubing, similar to those reported for a toxicity screening array.[15] SWNT forests were assembled on each electrode from aged, oxidized SWNT dispersions in DMF on these disks as reported previously for individual sensors.**Error! Bookmark not defined.** Briefly, SWNTs were first carboxyl-functionalized and shortened by sonication in 3:1 HNO3/H2SO4 for 4 h at 70 °C. These dispersions were filtered, washed with water, dried, and dispersed in DMF. Abraded, ordinary basal plane pyrolytic graphite (PG) disks (*A*= 0.16 cm2) were prepared for forest assembly by forming a thin layer of Nafion and $Fe(OH)_x$ on their surfaces. After immersion of these substrates into aged DMF dispersions of shortened SWNTs, vertically assemblies of SWNTs are formed (forests), which were then dried in a vacuum for 18 h.

For primary antibody attachment, 30 μL of freshly prepared 400 mM EDC and 100 mM NHSS in water were placed onto the SWNT forest sensor units, and washed with water after 10 min. This was followed by treatment for 3 h at room temperature with 20 μL of 2 nmol mL^{-1} primary anti-PSA, anti-PSMA, anti-PF-4 or anti-IL-6 antibody (Ab1) in pH 7.0 PBS buffer. The arrays were then washed with 0.05% Tween-20 in PBS buffer and PBS buffer to remove unreacted antibodies. Array elements were then incubated for 1 h at

room temperature with 20 µL of 0.4% saturated casein + 0.05% Tween-20, followed by washing with 0.05% Tween-20 in PBS buffer and PBS buffer for 3 min. Sensitivity of the immunosensor was improved by minimizing non-specific binding (NSB) by employing washing steps to block free antibody binding surface on the electrode. After washing the electrodes thoroughly with PBS Tween-20 and PBS buffer for 3 minutes, 10 µL of the analyte containing PSA (0 – 40 ng mL^{-1}), PSMA (10 - 250 ngmL^{-1}), PF-4 (0 – 40 ng mL^{-1}), IL-6 (0 – 2 ng mL^{-1}) in undiluted calf serum was incubated with each sensor unit for 1.25 h at room temperature.

For patient serum samples, 10 µL of sample was incubated with each immunosensor unit. After washing with 0.05% Tween-20 and PBS buffer, the PSA and PSMA immunosensor electrodes were incubated with single labeled Ab_2-HRP conjugates for additional 1.25 h, while as PF-4 and IL-6 immunosensor units were incubated with biotinylated-Ab_2 conjugates for 1 h. The electrodes were then rinsed thoroughly with PBS-Tween 20 and PBS buffer. The PF-4 and IL-6 units were incubated with streptavidin-HRP conjugates, washed after 0.5 h and the 4-unit arrays were stored at 4 ^oC till further use.

After fabricating the immunosensor array and binding the labeled secondary antibody system, the array was placed in an electrochemical cell containing pH 7.0 buffer and 1 mM hydroquinone mediator. The amperometric signal was developed at -0.3 V vs SCE by injecting H_2O_2 was to 0.4 mM while stirring the solution at 2500 RPM.

ELISA. Biomarkers in the serum samples were also measured using standard ELISA assays. PSA ELISA kit was from Anogen, PF-4 and IL-6 ELISA kits were from R&D systems, while for PSMA, an ELISA developed in-house was employed.

Primary anti-human PSMA antibody (clone no. CHYH2) were diluted to 200 µg/mL in 0.2 M $NaHCO_3$, pH 9.4. ELISA plate wells were incubated with 50 µL of 200 µg/mL of primary anti-PSMA (Ab1) overnight at room temperature. Incubation mixture was removed by aspirating the contents into a waste container. All wells were filled with PBS Tween-20 and the contents were aspirated to waste container. This was done 3 times followed by washing with buffer. Then ELISA wells were incubated with the blocking solution (saturated casein) for 1 h at room temperature and washed using automated ELISA plate washer for 3 times using wash buffer. Then, the ELISA wells were incubated with 50 µL of 10, 25, 50, 100, 200, 250 ng /mL standard PSMA and the clinical serum samples. All samples and standards were subjected in parallel to same analysis in triplicate. Samples were incubated at 37 °C for 1 h followed by automated washing with buffer. Then, 50 µL of biotinylated anti-human PSMA antibody (clone no. CHYH1) were added to all wells and incubated for 1 h at 37 °C followed by washing with buffer for 3 times. 50 µL of streptavidin-HRP (1:200 times diluted) were added to each well, incubated for 30 min. followed by washing with buffer for 3 times. 50 µL of substrate solution (1:1 mixture of tetramethylbenzidine and hydrogen peroxide) were added to each well and incubated at 37 °C for 30 minutes. Then 50 µL of stop solution (2 N sulfuric acid) were added to each well and mixed well. Optical density was recorded at 450 nm using ELISA plate reader.

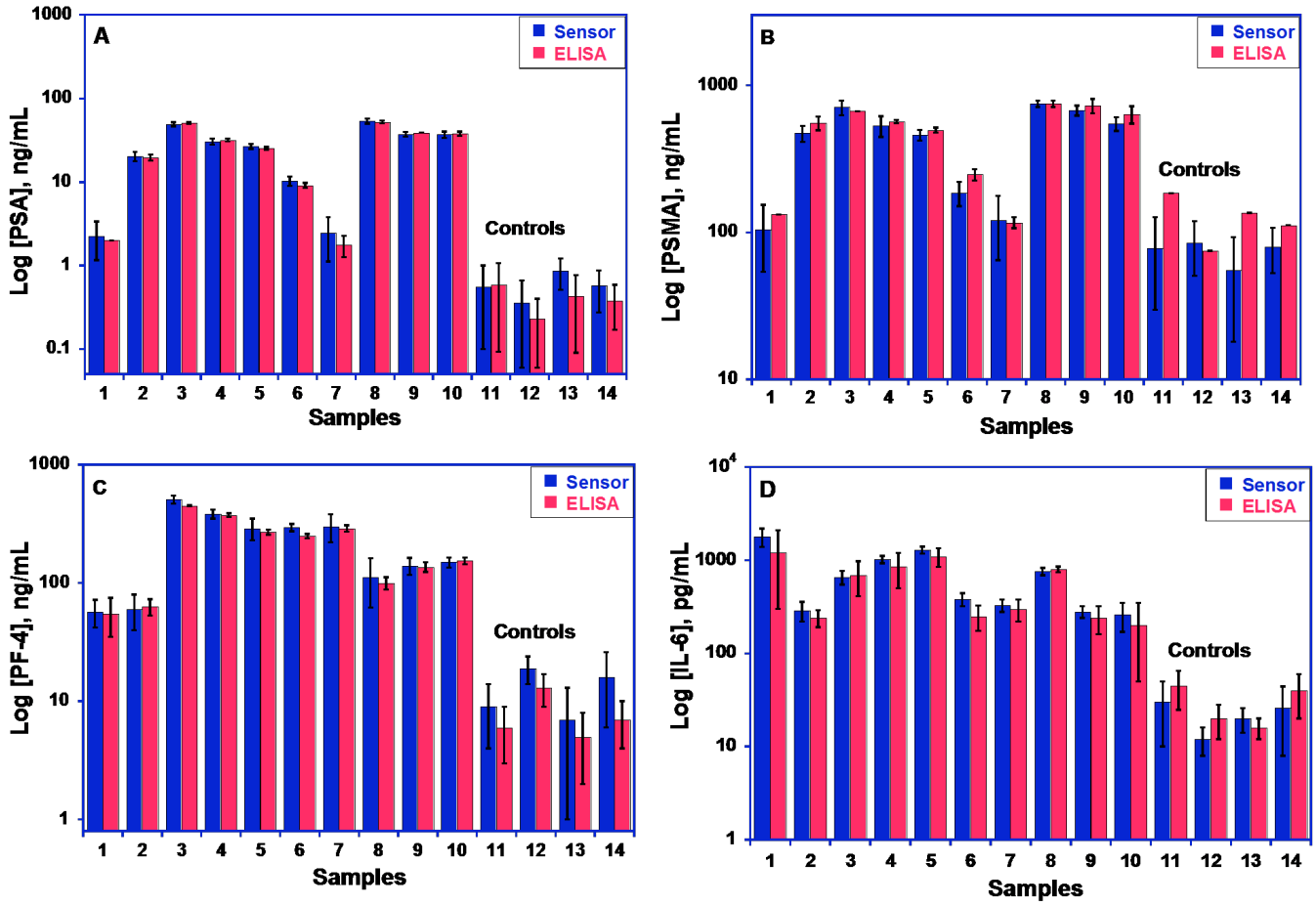

Figure S1. Validation of SWNT immunoarray results for the human serum samples by comparing against results from ELISA determinations (RSD ± 10%): (A) PSA, (B) PSMA, (C) PF-4, (D) IL-6.

TABLE S1. Slopes and Intercepts of Correlation plots of SWNT immunoarray results for human serum samples against results from ELISA determinations for the same samples

Biomarker	Slope ± sd	Intercept ± sd
PSA	0.98 ± 0.01	0.46 ± 0.40
PSMA	1.01 ± 0.05	-43.1 ± 21.3
PF-4	1.08 ± 0.02	-0.7 ± 5.4
IL-6	1.10 ± 0.05	5.3 ± 27.6

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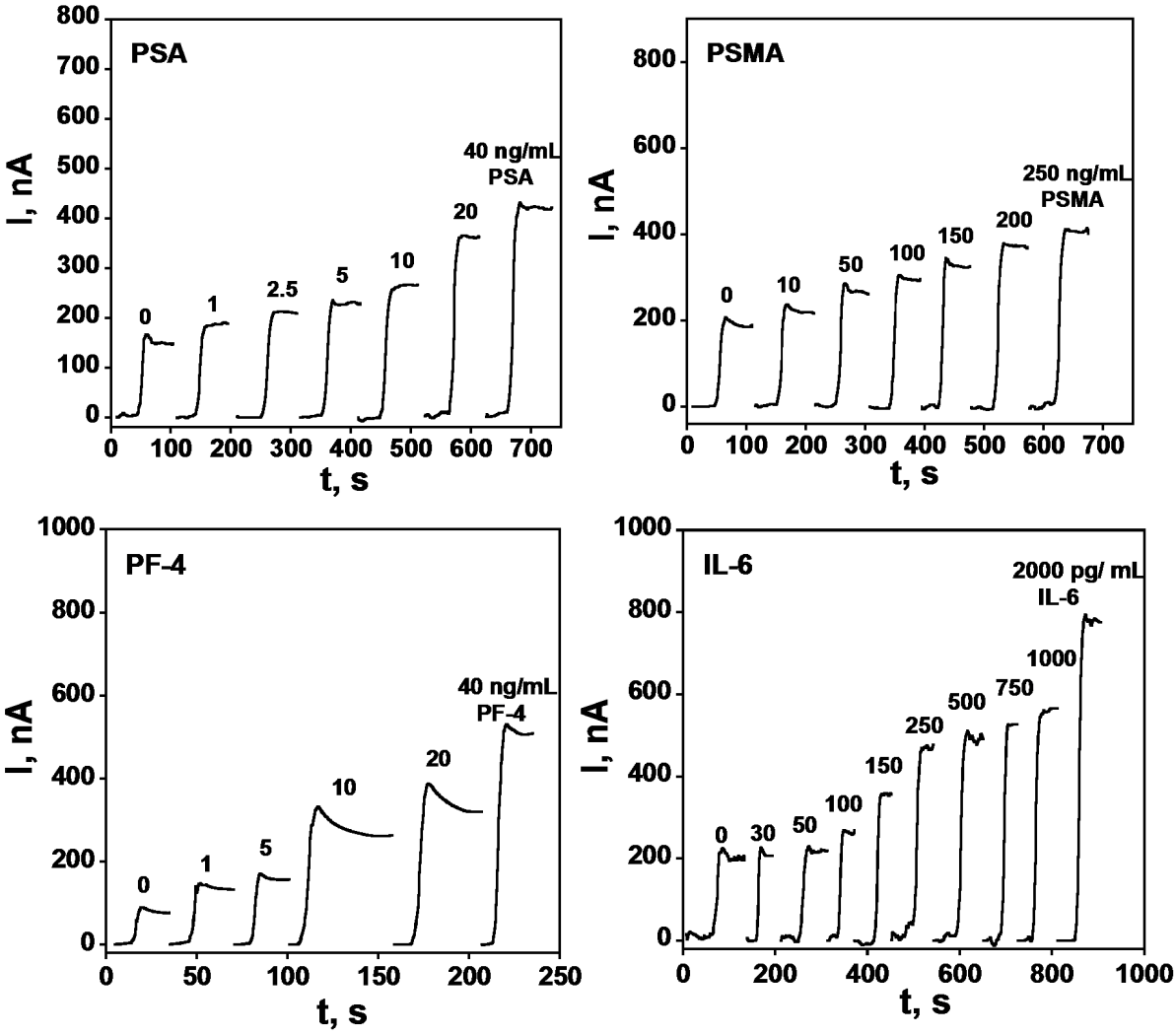

Figure 1. Amperometry at -0.3 V and 2500 rpm after placing arrays in buffer containing 1 mM hydroquinone and then injecting H_2O_2 to 0.4 mM for SWNT immunoarrays incubated with antigen standards in 10 µL undiluted calf serum for 1.25 h followed by Ab_2-HRP (PSA & PSMA) or Ab_2-streptavidin-HRP (PF-4 & IL-6) in 10 µL 0.4% w/v casein and 0.05% tween 20 PBS buffer.