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TITLE: ANTIBODY-FUNCTIONALIZED CARBON NANOTUBE TRANSISTORS AS
BIOSENSORS FOR THE DETECTION OF PROSTATE CANCER

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Prostate cancer (CaP) is the most commonly diagnosed cancer and second leading cause of cancer deaths among American men and as such represents a major public health issue. CaP displays a range of clinical behaviors, from indolent to aggressive, with development of overt metastatic disease being arguably one of most significant events in the progression of prostate cancer. At present digital rectal exams (DRE) and PSA screening are the "gold-standard" for detection of prostate cancer. In stark contrast to outcomes seen when diagnosed at advanced stages, detection of early-stage, localized, disease often results in successful treatment, with long-term disease-free survival in 60-90% of patients. The work associated with a this grant proposal is focused on the development of a novel biosensor platform, using validated CaP biomarkers as proof-of-concept, that we hypothesize will have increased sensitivity over currently available technologies. Such a device has the potential to improve detection, leading to better patient outcomes.					
15. SUBJECT TERMS Biosensor, Single-wall carbon nanotube, Field Effect Transistor, Osteopontin, OPN, Prostate Specific Antigen, PSA, uPAR, antibody, single-chain Fv, scFv, oriented coupling					
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Year 3 Report for Contract W81XWH-09-1-0206

ANTIBODY-FUNCTIONALIZED CARBON NANOTUBE TRANSISTORS AS BIOSENSORS FOR THE DETECTION OF PROSTATE CANCER

PI – A.T. Charlie Johnson, Jr.

PCRP SYNERGISTIC IDEA DEVELOPMENT AWARD, JOINT PROPOSAL WITH PROPOSAL LOG NUMBER PC080542, FOX CHASE CANCER CENTER, DR. MATTHEW ROBINSON. THE LINKED GRANT AWARD IS W81XWH-09-1-0205.

Introduction

Early detection and differential diagnosis are critical components for the successful treatment of prostate cancer (CaP). The research being carried out under this Synergistic IDEA Award is focused on the development of a novel biosensor platform for the detection of CaP biomarkers in biologic fluids, such as

serum. The proposed biosensors are comprised of antibody-functionalized single-wall carbon nanotube (swCN) transducers. We hypothesize that the specificity inherent in antibody-antigen interactions, when combined with the swCN platform, will create a novel biosensor platform with increased sensitivity over currently available technologies. The goals for project encompassed the generation of swCN and single-chain antibody (scFv) reagents necessary to carry-out initial proof-of-principle experiments, testing first generation biosensors using simulated serum samples and optimizing the biosensors, and performing a retrospective analysis of patient samples to determine the sensitivity and specificity of the biosensors.

Body

Research carried out during the second year of the grant period focused on testing our first generation SWCN biosensors in simulated serum in preparation for moving into patient samples in year 3. We also used this time period to generate antibodies optimized for site-specific conjugation to the biosensors with the goal of improving sensitivity of the biosensors.

Aim 1. Construction and initial validation of a single-wall carbon-nanotube biosensor for detecting prostate cancer

Experiments in year 3 were focused primarily on validating our first generation biosensors and successfully preparing the data for publication. The published results demonstrate that biosensors comprised of the 23C3 anti-osteopontin single-chain Fv antibody conjugated to single-wall carbon nanotube transistors were approximately 100 – 1000 fold more sensitive than standard ELISAs for detecting the prostate cancer biomarker osteopontin (OPN) in synthetic serum. Extrapolating that sensitivity to other antibody/antigen systems would provide the potential to open up biomarker detection to proteins found at very low levels in serum. However, the levels of serum albumin (450 ng/mL) at which we were able to detect OPN were far below the levels seen in normal serum. Studies suggest that strategies to remove albumin from serum need to be developed to fully exploit the sensitivity of the SWCN-based biosensors. Experiments to address this issue will be carried out during the unfunded extension that was granted for this work.

Aim 2. Optimizing antibodies to enhance biosensor capabilities

Prostate specific antigen (PSA) represents the most widely used biomarker for prostate cancer screening and as such represents the gold standard for testing of our devices in prostate cancer patient serum samples. Initial efforts to develop an anti-PSA scFv based on the 8G8F5 IgG failed due to instability of the antibody when engineered into the scFv format (Figure 1). Expression of the scFv in a bacterial expression system resulted in yields of 0.15 mg/L of culture and that scFv tended to form higher order multimers based on the multiple peaks seen in the elution profile when the scFv was chromatographed over a gel filtration column. Appropriate framework residues were identified in the Vh and Vl domains of the 8G8F5 scFv and site-directed mutagenesis was performed to introduce cysteine residues. The resulting disulphide bond was hypothesized to stabilize the monomeric form of the scFv and increase production yields. The gene encoding the 8G8F5:S=S scFv was subcloned into the pSEC-Tag2 mammalian expression vector, expressed in HEK293T cells, and purified from culture medium containing low levels of fetal bovine serum. As seen in Figure 1 (right panel) the disulphide-stabilized 8G8F5 migrated as a single peak on the gel filtration column. Higher molecular weight shoulder peak represents carry over serum albumin present in the mammalian expression medium. When visualized by SDS-PAGE the albumin and scFv peaks are well separated. Yields were increased from 0.15mg/L to 6mg/L with the optimized construct. Activity of the 8G8F5:S=S scFv was verified by

immunoprecipitation to confirm that introduction of the disulphide bond did not disrupt the ability of the scFv to bind PSA in solution. To test this we took advantage of the LNCaP prostate cancer cell line that secretes PSA into the culture medium. LNCaP cells were grown to confluence, cell supernatant was harvested and clarified for use as a source of PSA. The 8G8F5:S=S scFv was non-covalently coupled to Ni-NTA agarose beads through its 6XHIS affinity tag present at the C-terminus of the protein (an anti-HER3 scFv was used as a control in the experiments). When incubated with LNCaP conditioned media the 8G8F5:S=S scFv was able to immunoprecipitate PSA from the media (Figure 2). The anti-HER3 scFv failed to immunoprecipitate PSA, demonstrating the selectivity of the assay. This scFv is now ready for use on the SWCN-based biosensors for PSA detection.

Aim 3. Validation of swCN biosensors in patient samples

Renewal of the exempt IRB approval covering this work was applied for and granted. This will be instrumental in carrying out testing of patient samples during the unfunded extension.

Key Research Accomplishments

- Repeated studies to confirm a series of results related to detection of OPN via 23C3-functionalized SWCN.
 - Concentration-dependent response to OPN
 - Response is selective for OPN
 - Response could be seen in the presence of serum-albumin.
- Prepared and submitted a manuscript related to the findings outlined above. This manuscript was accepted to the journal *ACS nano* (Impact factor 10.774). Accepted version of manuscript is attached as part of this report.
- Identified need for developing a method to robustly remove serum albumin from patient samples without altering biomarker concentration
 - Developing this method will be a major focus during the unfunded extension that was granted for this work.
- Optimized expression and stability of the 8G8F5 anti-PSA scFv through incorporation of a disulphide bond between the Vh and Vl frameworks
 - Improved overall stability, resulting is essentially all monomeric scFv, as judged by elution pattern on gel filtration column
 - Improved total yield from 0.15 mg/L to 6 mg/L
 - Stabilized scFv retained ability to bind PSA as measured by immunoprecipitation of native PSA out of LNCaP conditioned medium.
- Developing method to produce sufficient quantities of PSA for carrying out proof-of-concept studies with 8G8F5:S=S functionalized SWCN-based biosensors in preparation for testing patient samples.

Reportable Outcomes

- Research results were published in the journal *ACS Nano* (Impact factor: 10.774). The article is: Lerner, M., D'Souza, J., Pazina, T., Dailey, J., Goldsmith, B., Robinson, M.K*, Johnson, A.T.* Hybrids of a genetically engineered antibody and a carbon nanotube transistor for detection of prostate cancer biomarkers. *ACS Nano* 6(6):5143-5149, 2012. PMID: PMC3383883 * = co-corresponding authors

- Renewal of the required IRB approval was sought and granted for the clinical protocol to obtain CaP samples from the FCCC biosample repository (January 2012)

Conclusions

As a direct result of close interactions between the Johnson and Robinson groups over the course of this synergistic IDEA award we have been able to make significant progress toward reaching the ultimate goal of this project, validating the SWCN-based biosensors in prostate cancer patients serum samples. During this year we achieved a major milestone toward that goal through validating the utility of these sensors in simulated serum. The results of those studies were recently published in a high-impact journal (ranked 5/66 in the field of nanoscience & nanotechnology and 6/134 in the field of chemistry;physical by Thomson Reuters). During this year we also identified hurdles to validating the biosensors in patients samples, specifically the impact of serum components such as albumin. Work in the upcoming unfunded extension will focus on developing methods to overcome those hurdles. We also optimized the expression and stability of an anti-PSA scFv that will be used for validation of the sensors in the patient samples.

References

Appendices and Supporting Data

Figures taken from Lerner, M., D'Souza, J., Pazina, T., Dailey, J., Goldsmith, B., Robinson, M.K.* Johnson, A.T.* Hybrids of a genetically engineered antibody and a carbon nanotube transistor for detection of prostate cancer biomarkers. *ACS Nano* 6(6):5143-5149, 2012.

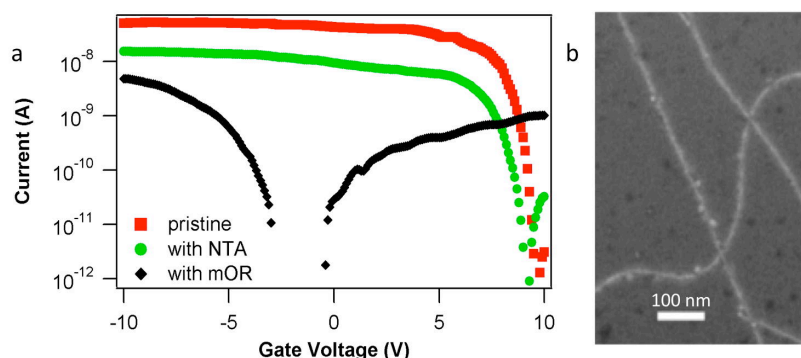


Figure 1.1 $I(V_g)$ s of a carbon nanotube device in its pristine state (red squares), after diazonium-NTA-Ni functionalization (green circles) and after attachment of His-tagged mouse olfactory receptors (black diamonds). Bias voltage is 100 mV in all cases. (b) SEM image demonstrating attachment of His-tag labeled 30nm gold nanoparticles to carbon nanotubes using diazonium-NTA-His chemistry.

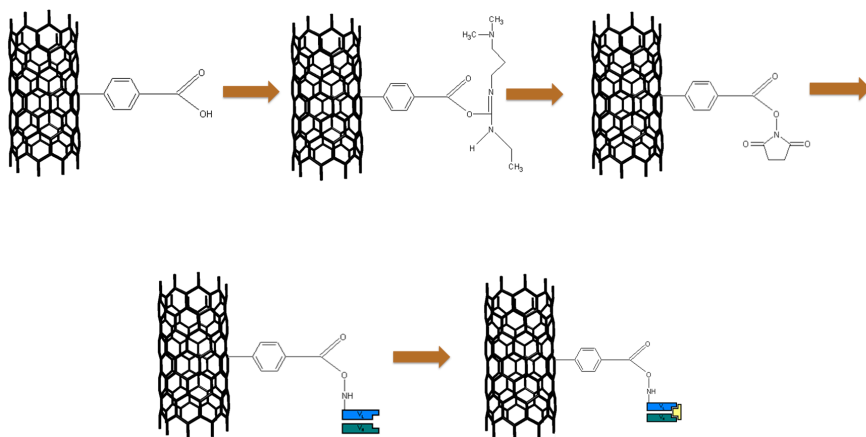


Figure 1.2: Functionalization scheme for OPN attachment. First, a defect is created on the nanotube sidewall using a carboxylated diazonium salt. The defect is then activated by EDC and stabilized with NHS. ScFv protein displaces the NHS, with random orientation. OPN binds preferentially to the scFv in the detection step.

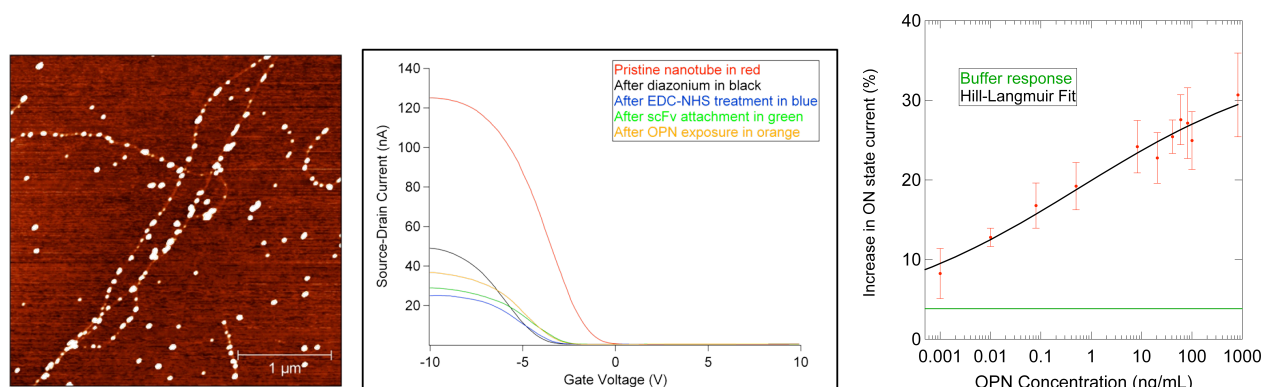


Figure 1.3 a) AFM image showing attachment of proteins to carbon nanotubes using the diazonium carboxylated diazonium salt/EDC/NHS chemistry. Lateral range 2.5 μm , height scale, 10 nm. b) I-Vg characteristics of an as-grown carbon nanotube transistor (red); after treatment with a diazonium salt (black) and EDC-NHS (blue); after functionalization with scFv to OPN (green) and upon exposure to a solution of OPN at a concentration of 30 ng/mL (orange) in 0.5M phosphate buffer solution diluted 1:100 with deionized water. A clear response is observed (increase in the “on-state current” of the transistor), suggesting that the detection scheme is effective. c) Response versus concentration data show excellent agreement with the prediction of Hill-Langmuir adsorption theory. From this data, the scFv affinity is approximately 500 pg/mL, and the detection limit is 1 pg/mL

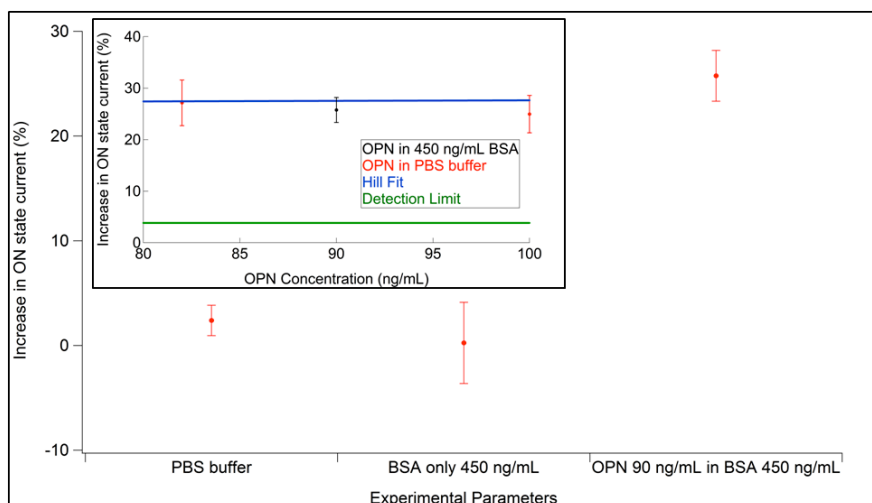


Figure 6: Control data for the functionalized SWCN FET. Response to unspiked, diluted PBS buffer is shown in green. Response to diluted PBS spiked with bovine serum albumin (BSA) at 450 ng/mL is indistinguishable from response to unspiked PBS. Inset: 90 ng/mL OPN in BSA background produces a response equal to that found for OPN in plain buffer.

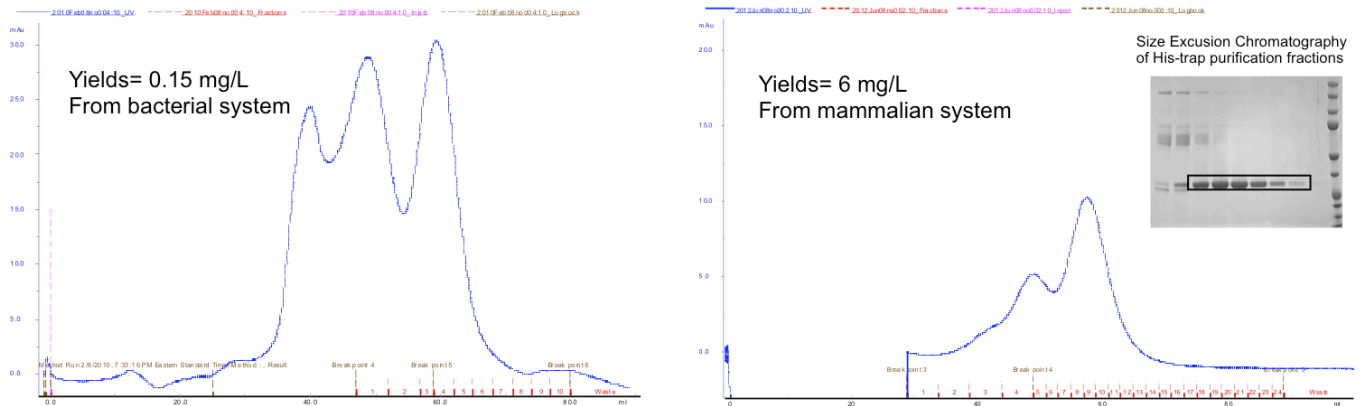


Figure 7: Stability engineering of anti-PSA scFv

Left panel: gel filtration chromatograph of 8G8F5 scFv purified from bacterial expression system shows higher order multimers consistent with inherent instability of scFv. Yields were 0.15mg/L of culture. Right panel: gel filtration chromatograph of 8G8F5:S=S scFv. Yield were increased to 6 mg/L and majority of scFv migrated as monomer. Shoulder peak corresponds to albumin observed in SDS-PAGE analysis.

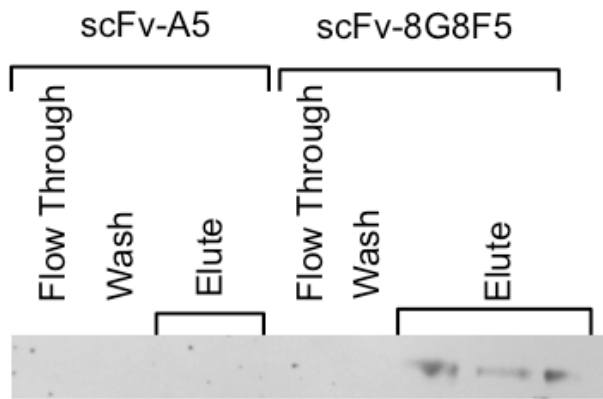


Figure 8: 8G8F5:S=S retains ability to bind PSA

Disulphide stabilized 8G8F5 immobilized on Ni-NTA beads through its 6XHIS affinity tag was capable of immunoprecipitating PSA from LNCaP conditioned medium as judged by Western blot analysis. The control anti-HER3 A5 scFv failed to IP PSA, demonstrating the specificity of 8G8F5.