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14. ABSTRACT During year 2 we successfully met nearly all benchmarks proposed in our statement of work. Strong team interactions (face-to-face meetings, videoconferences, numerous email communications and lab visits) allowed for useful discussions and insured rapid research progress. The Johnson group successfully fabricated, tested, and fully characterized biosensors consisting of nanotube transistors with covalently bound scFv antibodies developed by the Robinson group specific for osteopontin, one of our proof-of-concept biomarkers. We found that the sensor response was in excellent agreement with theory with a detection limit of ~ 1pg/mL, approximately 100-1000 fold more sensitive than standard ELIAS assays. This technology thus has the potential to open up biomarker detection to proteins found at very low levels in serum. This data supports our efforts to transition into testing patient samples in year 3. Our ultimate goal is the development of a prototype biosensor that will be used to retrospectively test patient samples during year 3 of this award. The research accomplishments achieved in years 1-2 have positioned us well for meeting this goal. By the end of this funding period we aim to have generated sufficient data to support the development of this sensor platform.					
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Year 2 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 this three year funding period encompass the generation of swCN and single-chain antibody (scFv) reagents necessary to carry-out initial proof-of-principle experiments (year 1), optimizing the biosensors for use in biologic fluids (year 2), and performing a retrospective analysis of patient samples to determine the sensitivity and specificity of the biosensors (year 3).

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 2 were focused on validating our first generation biosensors. It is recognized that compounds in serum, such as salts and albumin, can negatively impact biosensor function and were of concern based on studies performed in year 1. To address these issues, SWCNs were functionalized with anti-OPN scFv (23C3) using a covalent bonding approach based on a diazonium chemistry that results in random scFV orientation since coupling may occur through an amide bond to one of several possible residues (Figures 1.1 and 1.2). We are the first to demonstrate the use of the diazonium method for SWCNs integrated in transistor devices.¹ Series of tests were performed to evaluate the sensitivity and specificity of our first generation biosensors under increasingly complex conditions (Figures 1.2 – 1.3). During year 2 we established an upper concentration limit where phosphate buffer saline had no impact on the electronic transport of 23C3-functionalized SWCN devices (data not shown). This was followed by the addition of the target antigen (osteopontin, OPN) to the PBS buffer. As seen in Fig 1.3B, addition of OPN to the buffer resulted in a change in the electronic transport of the devices (specifically, an increase in the on-state current) consistent with an antibody/antigen interaction occurring at the surface of the SWCN device. Under the conditions defined in Fig 1.3B we demonstrated that 23C3-functionalized SWCN can detect OPN in a dose-dependent manner with a detection limit of 1 pg/mL, a factor of 100-1000 less than conventional ELISA (Fig. 1.3C). Non-specific absorption of serum proteins such as albumin to the SWCN is a major concern in the development process. During year 2 we established that bovine serum albumin (BSA) at levels of 450 ng/mL had no impact on the ability of the 23C3-functionalized SWCN to detect OPN (90 ng/mL) in simulated serum (Fig 1.4), thereby demonstrating that the biosensor is both sensitive and selective for OPN.

The results obtained during year 2 demonstrate that the SWCN is approximately 100 – 1000 fold more sensitive than standard ELISAs and therefore has the potential to open up biomarker detection to proteins found at very low levels in serum. This data supports our efforts to transition into testing patient samples in year 3. In addition we will continue to address aspects of sensitivity and specificity of the biosensors. Specifically, experiments will be carried out to address: 1) the upper limit at which BSA has no impact on biosensor function, 2) the impact of oriented coupling of antibodies to the surface (see Aim 2), and moving to additional biomarkers (uPAR and PSA).

Aim 2. Optimizing antibodies to enhance biosensor capabilities

In year 1 we isolated single chain Fv (scFv) antibodies against each of the target biomarkers. We have hypothesized that instrumental to the success of the antibody-functionalized swCN is the ability to conjugate scFv antibodies in a manner that results in attachment of reproducible levels of active scFv on the sensor surface. Therefore year 2 goals focused on modifying the structure of the scFvs to optimize their attachment to the biosensor surface via site-directed conjugation (Figure 2.1 and Figure 2.2). We proposed to conjugate scFv antibodies to the swCN in a site-directed manner through incorporation of a free cysteine residue into the scFv to facilitate either thiol- or maleimide-based coupling strategies (Figure 2.3). Free cysteine residues were incorporated into two locations, the C-terminal tail and the linker connecting the Vh and Vl domains, in the ATN-615 and 23C3 scFv. Both locations are predicted to result in scFv coupling in a manner that oriented the antigen-binding site away from the SWCN surface (Figure 2.2). Unexpectedly, incorporation of the free cysteine residues into the 23C3 and ATN-615 scFv resulted in complications associated with purification and activity. An example of this is detailed in Figure 2.4. ATN-615 was purified from bacterial expression system at approximately 0.5 mg/L and chromatographed over a size exclusion column as a single peak consistent with its predicted molecular weight of 26kDa. In stark contrast, incorporation of a free cysteine at the C-terminal tail of ATN-615 to create ATN615-cys resulted in lower levels of expression and aggregation of the scFv (Figure 2.4B&C). Similar results were seen with 23C3 as well (data not shown). The behavior of the 23C3 and ATN-615 scFv is contrasted by the behavior of the 4D5-cys scFv that was created as a control (Figure 2.5). 4D5 scFv was modified to incorporate a free cysteine at the C-terminus (4D5-cys). This protein was expressed at approximately 0.5 mg/L and migrated as a single 26kDa species. Purified 4D5-cys was capable of immunoprecipitating its target antigen (HER2). Although initially developed as a control antibody, the 4D5-cys scFv will be used to both establish conjugation conditions for thiol-based coupling and to test our hypothesis related to distance (see below) while additional antibody engineering steps are undertaken to stabilize the 23C3 and ANT-615 scFv frameworks.

As part of other work we have developed the ability of to conjugate proteins to SWCN through the 6xHIS purification tag present at the C-terminus of our scFv (Figure 2.1 and 2.3). In this scenario device fabrication incorporates NTA and is completed with the addition of Ni ions, which are chelated by the NTA complex. Use of this platform provides us with a universal strategy to couple scFv in an oriented fashion without the need for additional modification steps that could deleteriously affect the stability or antigen binding capabilities, as was seen with both 23C3 and ATN-615. Although we expect this approach to be ideal for detection of biomarkers in biological samples, preclinical testing is hampered by the universal nature of the 6X-HIS purification tag, commercial forms of biomarkers all contain 6X-HIS tags. During year 2 we built an OPN expression construct with a cleavable 6X-HIS tag. We established conditions for efficient expression, purification, and removal of the HIS-tag (data not shown) and are now ready to begin functionalizing SWCN with 23C3 and testing for the impact on biosensor sensitivity. These results will be directly comparable to the results described in Aim 1.

We hypothesized that decreasing the distance between the antigen-binding domain and the SWCN surface will increase the sensitivity of the sensor. Our initial proposal focused on the use of thiol-based coupling via chemical linkers of different lengths to increase this distance. Because of issues associated with the production and stability of the cysteine-tagged 23C3 and ATN-615 scFv we will carry out the initial studies with the hu4D5-cys scFv and a hu4D5 Fab'. As depicted in Figure 2.6 the 50 kDa Fab (comprised on variable and CH1 domains) doubles the distance between the SWCN and antigen binding

as compared to the scFv. We have purified hu4D5 Fab' (Figure 2.7) and are now ready to use the hu4D5 antibody/antigen system to facilitate testing of the overall hypothesis. When combined with results obtained in testing of 6X-HIS coupling strategies results obtained with the hu4D5 system will drive the continued engineering of 23C3 and ATN-615 scFv.

In conclusion, all reagents are now in hand to test the impact of oriented coupling of scFv through both thiol and 6X-HIS approaches.

Aim 3. Validation of swCN biosensors in patient samples

Renewal of the exempt IRB approval covering this work was applied for and granted.

Key Research Accomplishments

- Demonstrated attachment of scFv to SWCN by atomic-force microscopy and measurement of electronic characteristics.
 - Attached scFv via standard amine-based coupling approach.
 - AFM figures demonstrate that attached proteins are consistent with the size of 25 kDa scFv
- Demonstrated a concentration-dependent response to OPN using 23C3-functionalized SWCN biosensors. The experimental detection limit is approximately 1 pg/mL. This is predicted to beat current ELISA detection limits (1 ng/mL) by a factor of approximately 1000. These studies were performed with non-oriented scFv. We anticipate an increase in the percentage of scFv capable of binding antigen when oriented coupling strategy is employed. This may in turn increase the dynamic range of the sensors by expanding the level of increase in ON state current
 - Signal saturated at concentrations above 10 ng/mL
 - Concentration at half-filling is ~ 500 pg/mL)
 - Percent occupation at lowest detectable concentration is ~20%
- Demonstrated that OPN detection is specific
 - Addition of 450 ng/mL BSA to PBS does not increase on-state current as compared to PBS alone. Both result in <5% increase in on-state current.
 - At concentrations of OPN that saturate the sensor response (80 – 100 ng/mL) the addition of 450 ng/mL of BSA to PBS buffer did not affect response. All conditions tested resulted in approximately 25 – 30% increase in on-state current.
- Site-directed conjugation of scFv to swCN surface.
 - At end of YR1 we had identified two potential strategies for site-directed conjugation (thiol-based coupling and coupling through Ni-NTA/6X-HIS interaction. Goal was to take both strategies forward to retire risks associated with this critical step in sensor development
 - Thiol-coupling
 - Cysteine residues were incorporated into either the linker peptide between the Vh and Vl domains or at the C-terminus of the protein to facilitate thiol-based coupling.

- Incorporation disrupted antigen binding and/or protein stability.
- As a control anti-HER2 scFvs were constructed at same time. These retained both antigen binding and stability. Implies issues with 23C3 and ATN-615 were intrinsic to scFv not the approach.
- Use of this strategy for these scFv will require additional engineering steps to stabilize the proteins. These could include either Fab' generation or stabilized frameworks for the scFv.
- Ni-NTA conjugation
 - Utilizes the 6xHIS purification tag present on the C-terminus of scFv.
 - Protocol for Ni-NTA based attachment of scFv to swCN is being optimized
 - Constructed OPN with a cleavable 6xHIS tag, optimized expression and cleavage. Will be used for testing the Ni-NTA coupling strategy and directly comparing to results obtained during year 2 with randomly coupled scFv.

Reportable Outcomes

- Renewal of the required IRB approval was sought and granted for the clinical protocol to obtain CaP samples from the FCCC biosample repository (January 2011)
- Research results were presented at the IMPaCT conference in two posters given by Drs. Johnson and Robinson, Orlando, FL (March 2011).
- Research results were presented in poster format at the American Physical Society Meeting, Dallas, TX (March 2011)
- Research results were presented in poster format at 16th Annual Postdoctoral and Graduate Student Research Symposium, FCCC (June 2011).

Conclusions

The ultimate goal of this research is the development of a prototype biosensor that will be used to retrospectively test patient samples during year 3 of this award. During year 2 of this synergistic idea award we built upon our year 1 successes and were able to demonstrate that 23C3-conjugated SWCN biosensors are capable of specifically detecting the OPN biomarker at pg/mL levels in simulated serum samples. This result provides the initial proof-of-concept that we were looking for to begin transitioning toward retrospectively analyzing patient samples with our biosensors. Additional advances in antibody design and coupling strategies were made during year 2 that are predicted to improve the overall performance of the sensors. These improvements will be tested in year 3. The success seen this year was the direct result of close interactions between the Johnson and Robinson groups. Interactions throughout the year included numerous phone and email correspondence, as well as face-to-face meetings, between members of the FCCC and UPenn groups. Amongst those meetings were two that included both PIs. In addition, a member of the FCCC group spent one week at the UPenn site performing research. This was instrumental for transfer of technology in both directions. We are well positioned to meet the goals outlined in our initial proposal of testing our biosensor platform in patient samples before the end of the grant period.

References

1. B. R. Goldsmith, J. J. Mitala, J. Josue, A. Castro, M. B. Lerner, T. H. Bayburt, S. M. Khamis, R. A. Jones, J. G. Brand, S. G. Sligar, C. W. Luetje, A. Gelperin, P. A. Rhodes, B. Discher and A. T. C. Johnson, ACS Nano **5**, 5408-5416 (2011).

Supporting Data

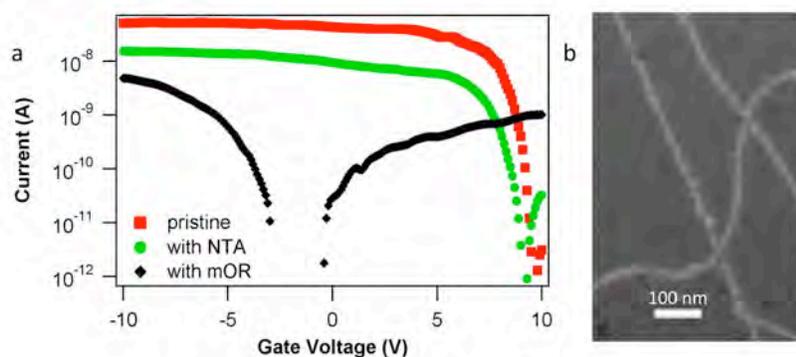


Figure 1.1 I(Vg)s of a carbon nanotube device in its pristine state (red squares), after diazonium-NTA-Ni functionalization (green circles) and after attachment of His-tagged mouse olfactory receptors (black diamonds). Bias voltage is 100 mV in all cases. (b) SEM image demonstrating attachment of His-tag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

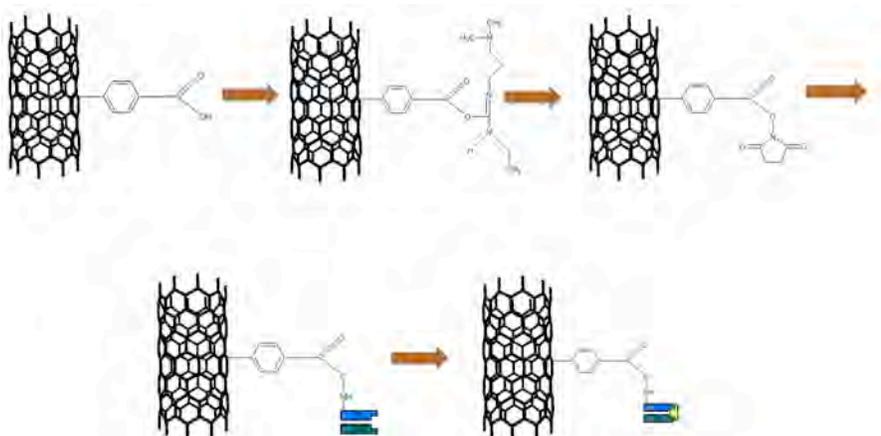


Figure 1.2: Functionalization scheme for OPN attachment. First, a defect is created on the nanotube sidewall using a carboxylated diazonium salt. The defect is then activated by EDC and stabilized with NHS. ScFv protein displaces the NHS, with random orientation. OPN binds preferentially to the scFv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

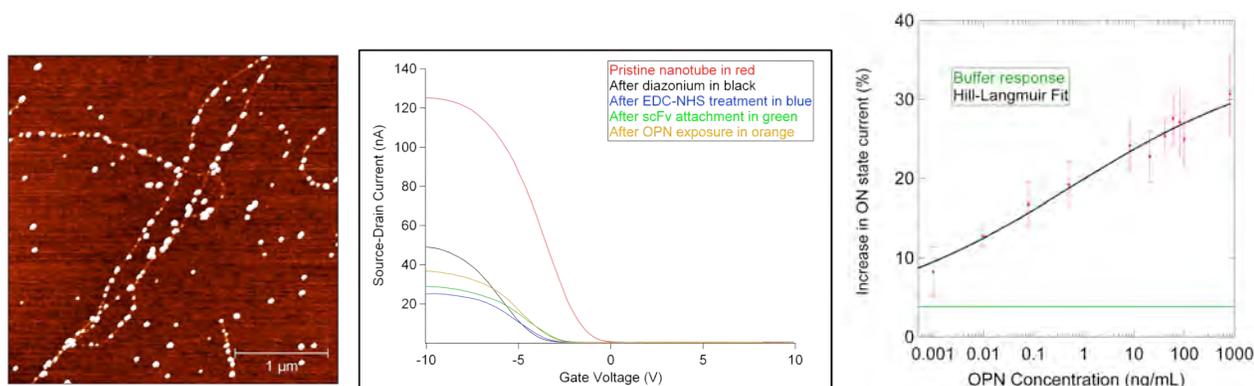


Figure 1.3 a) AFM image showing attachment of proteins to carbon nanotubes using the diazonium carboxylated diazonium salt/EDC/NHS chemistry. Lateral range 2.5 μm , height scale, 10 nm. b) I-Vg characteristics of an as-grown carbon nanotube transistor (red); after treatment with a diazonium salt (black) 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

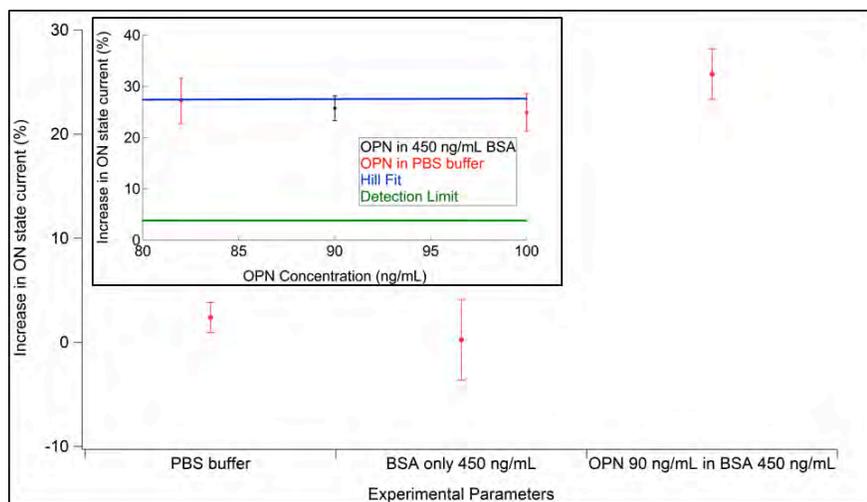


Figure 6: Control data for the functionalized SWCN FET. Response to unspiked, diluted PBS buffer is shown in green. Response to diluted PBS spiked with bovine serum albumin (BSA) at 450 ng/mL is indistinguishable from response to unspiked PBS. Inset: 90 ng/mL OPN in BSA background produces a response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

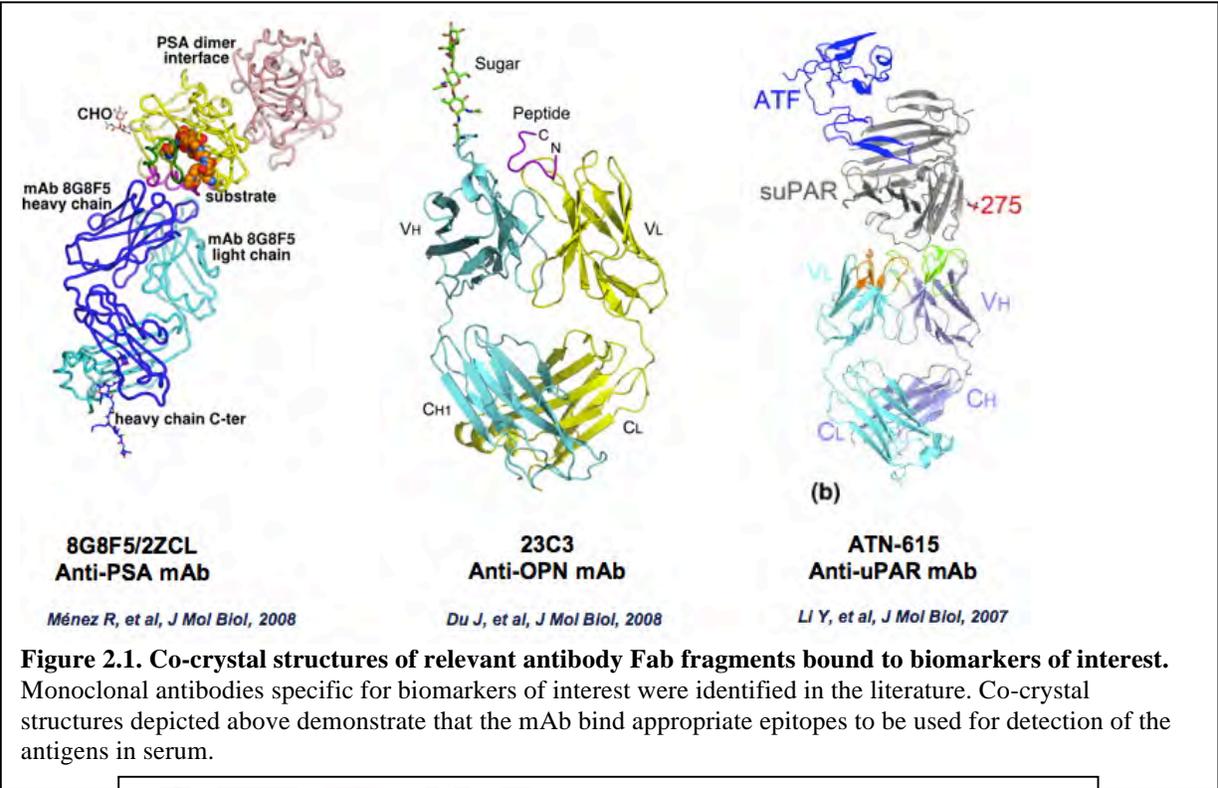


Figure 2.1. Co-crystal structures of relevant antibody Fab fragments bound to biomarkers of interest. Monoclonal antibodies specific for biomarkers of interest were identified in the literature. Co-crystal structures depicted above demonstrate that the mAb bind appropriate epitopes to be used for detection of the antigens in serum.

