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TITLE: Nanoink Printed Amperometric Immunosensor for Rapid and Inexpensive Screening of Tuberculosis

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Fort Detrick, Maryland 21702-5012

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5e. TASK NUMBER

5f. WORK UNIT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The project aims to develop a point-of-care diagnostic platform for tuberculosis diagnosis. The sensor detects Mycobacterium tuberculosis (MTB; H37Ra strain) cells and MPT-64 antigen spiked in human sputum samples. Single walled carbon nanotubes are used as a sensing element for detection of target analytes. In the project period, various SWCNTs-based immunosensors were designed and fabricated. The fabrication and detection protocol was optimized. For specific detection, antibodies to MPT-64 were generated and characterized. Through affinity purification, the antibodies became more sensitive and specific to target analytes. Using the sensor, the detection limit was 10^6 CFU/mL for BCG and 100 ng/mL for MPT-64. In the next project period, the detection protocol will be further optimized to achieve detection limit of 10^3 CFU/mL (or equivalent concentration to MPT-64).

15. SUBJECT TERMS
Tuberculosis, Carbon nanotubes, Point-of-care diagnosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	16	

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1. **INTRODUCTION:**

Subject: This project is to develop a point-of-care platform for tuberculosis diagnosis. The diagnostic sensor identifies both Mycobacterium tuberculosis (MTB) cells (H37Ra strain) and MPT-64 antigen spiked in human sputum samples. This film-type amperometric immunosensor detects the target analytes using single walled carbon nanotubes (SWCNTs) printed on a plastic film. Upon binding of target to the sensor surface, the electric current is monitored and the changes are correlated to the binding of target analytes-MTB cells and MPT-64 spiked in human sputum samples. To enhance the specificity, additional antibodies specific to the TB antigen MPT-64 were raised and tested. This project also involves the optimization of the processing of human sputum samples prior to detection with the biosensor.

Purpose: The purpose of the project is to develop a more rapid and high performance assay that is also low cost. The assay, faster and cheaper than smear microscopy and PCR, will facilitate the development and validation of new TB prevention and treatment methods for military use. The proposed immunosensor will provide a practical solution that addresses the current need for rapid, inexpensive and accurate TB screening in field settings where resources are limited.

Scope: In the project, we aim to achieve a detection limit of 1,000 CFU/mL (or equivalent detection limit: 125 pg/mL for MPT-64) with a detection time of 20 minutes. The sample is benign sputum samples spiked with MTB and MPT-64. Changes in electric current through SWCNTs upon binding of analyte are monitored to detect the target without culture or PCR amplification. In the first year, the sensor was optimized with both BCG and MPT-64 to detect target analytes spiked in phosphate buffer saline buffer (PBS). In the 2nd year BCG was replaced with MTB. Antibodies were raised against MPT-64 to evaluate the specificity. In the 2nd year, the sensor was evaluated for sputum samples spiked with MTB and MPT-64.

2. **KEYWORDS:** MTB, BCG, MPT-64, IgY, Carbon nanotubes, Printing, Diagnosis, Immunoassay.

3. **ACCOMPLISHMENTS:**

The major goals and the planned dates in the SOW for the project period (May 1, 2018~October 31, 2018) are described as below.

<u>Aim 1 (completed: 100%)</u>

Task 1: Design and fabricate SWCNTs-based immunosensors (Original planned dates: May 15, 2017).

A1: Fabrication of various dimensions of SWCNT based sensors

A2: Study of SWCNTs doped with various concentrations of PEI

Task 2. Sensor optimization for specificity and sensitivity using pure samples (Original planned dates: September 15, 2017).

B1: Preparation and evaluation of antibodies for BCG and MPT-64

B2: Fabrication of antibody immobilized sensors

B3: Evaluation of sensor to sensor variation.

<u>Aims 2a (Task 3).</u> Demonstrate the prototype device with pure samples (Original planned dates: March 15, 2017): 90% completed.

C1: Preparation of cells and MPT-64 (completed in the previous period)

C2: Demonstration of sensor performance for MTB (BCG) in PBS buffer (90% completed).

C3: Demonstration of sensor performance for MPT64 in PBS buffer (50% completed).

<u>Aims 2b (Task 4).</u> Demonstrate the prototype device with simulated sputum sample (Original planned dates: October 31, 2018): 30% completed.

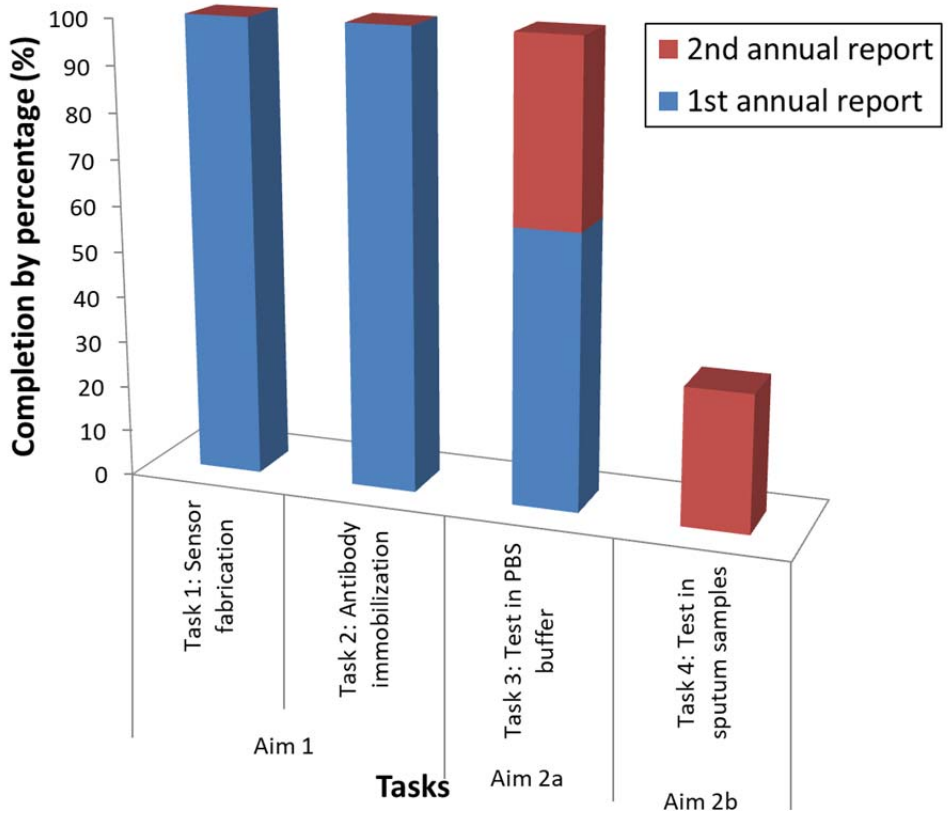


Fig. 1: Tasks for the project and the completion rate by percentage.

The accomplishment and the actual completion dates are described below.

Fig. 1 shows the progress of each task according to the specific aims and tasks for report period 1 (May 1, 2017~April 30, 2018) and report period 2 (May 1, 2018~October 31, 2018). This report is based on the work in period 2. A rapid and simple method for TB screening was developed using nanoink printed sensors functionalized with polyethylenimine (PEI), single-walled carbon nanotubes (SWCNTs) and antibodies. The novelty of this biosensor is based on its simplicity and low production cost. The stamping of silver ink defined an array of electrodes on a polyethylene terephthalate (PET) film. An area of the silver electrodes was functionalized with PEI, SWCNTs and antibodies sequentially. The PEI coating enabled SWCNT doping for electrical detection and ionic binding of antibodies for immobilization. The sensor was characterized by assaying for detection of BCG cells. The sensitivity for BCG detection was 1,000 CFU/mL. Sputum was liquefied to reduce the viscosity. Several liquefaction protocols were tested, and characterized with a UV spectrophotometer. This report focuses on the progress of the 2nd period (orange bars in Fig. 1), Task 3 and Task 4.

Task 3. Demonstration of sensor performance for MTB (BCG) in PBS buffer (90% completed).

The protocols in the project period 1 were further optimized to improve the signal to noise ratio. The optimized fabrication protocols are as below.

1. Single walled carbon nanotubes (SWCNTs; 5mg/mL in 1% SDS-deionized water) were spin-coated on a polyethylene terephthalate (PET) film. After spin-coating, the film was cured at 100°C for 10 min.
2. 0.05%-PEI was spin-coated on the SWCNT film and cured at 100°C for 10 min.
3. Resistance was measured after PEI coating.
4. Silver electrodes were stamped on the PEI/SWCNT film, which was cured at 80°C for 30 min.
5. Antibodies (0.7mg/mL) were incubated and bound on the PEI/SWCNT film at 4°C for 24 min.
6. After rinsing with DI water, the sensor was ready for detection.

The optimized detection protocols are as follows:

1. Resistance was measured after antibody binding.
2. Sample solution (20μL) was placed on the sensor surface for immunocomplex formation for 2 min at room temperature.
3. The sensor was rinsed with DI water.
4. The resistance was measured for control and target solutions.
5. The resistance ratio between the resistance after antibody binding and the resistance after target binding was compared for control and target analytes.

Fig. 2a shows the fabricated sensor on a PET film. The grey silver electrodes were patterned to make 500μm-gap electrodes. SWCNT/PEI/antibodies were sequentially patterned between two silver electrodes. The major difference from the previous sensors was that SWCNTs were exposed between two electrodes. The interfacial area between SWCNTs and silver electrodes was completely covered with silver electrodes to eliminate the resistance change by the Schottky effect (Fig. 2b). In addition, the current measurement in the previous report was changed into resistance measurement to make the detection protocol simple. In other words, resistance was measured using a multimeter instead of current-voltage (I-V) measurement.

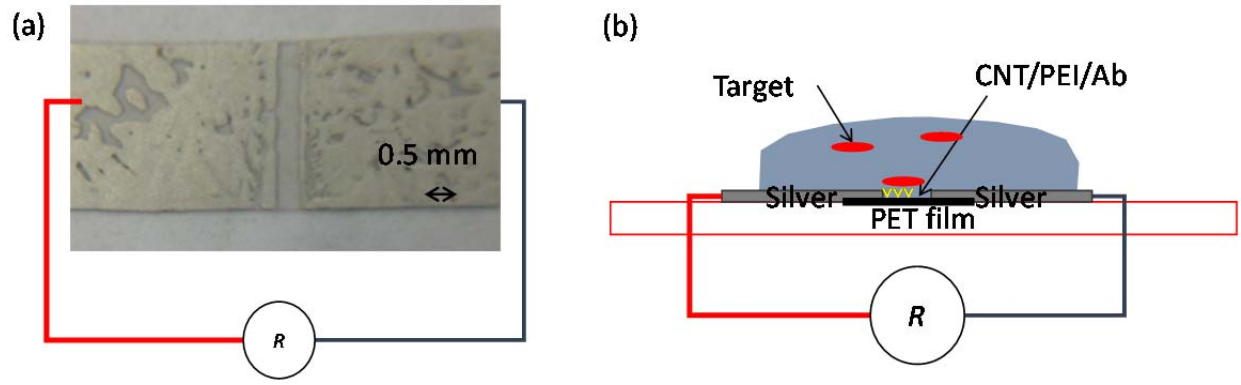


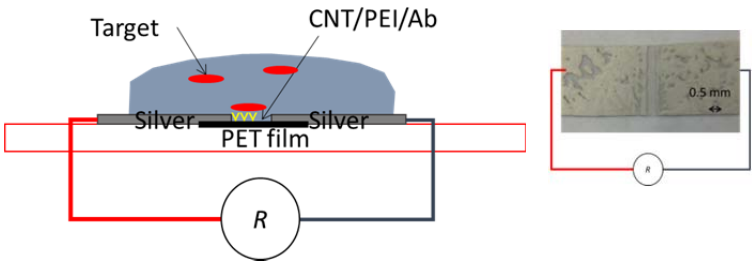
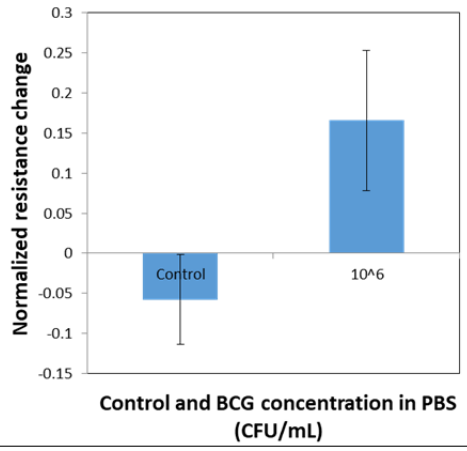
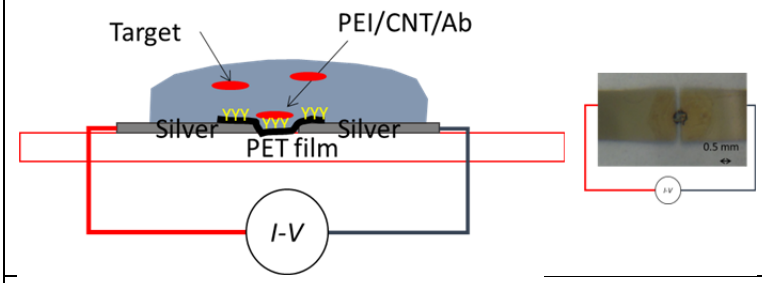
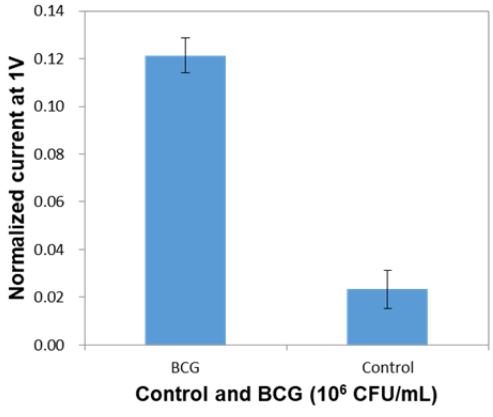
Fig. 2 (a) Sensor image; resistance is measured between two silver electrodes. (b) Cross section of a sensor; the interfacial area between SWCNTs and silver electrodes is covered with silver electrodes. The resistance is changed by only an electrostatic gating effect not the Schottky effect at the interface.

In the 2nd project period, we tested two sensor platforms; one was to expose only SWCNT region and the other was to expose both SWCNT and SWCNT/silver interface regions. Table 1 compares the sensor configuration. In the case of the previous sensor configuration, only SWCNTs were exposed to the target analytes. The interfacial area was completely covered with silver electrodes. In the latter case, only an electrostatic gating effect affected the resistance change. It was found that the former sensor configuration was more reproducible than the latter format. In the latter format, SWCNTs were deposited on gold electrodes, which exposed both SWCNTs and the SWCNT/gold electrodes to the target analyte. Using the latter sensor, we could obtain a signal for 10^6 CFU/mL-BCG in comparison to control. However, the signal was not reproducible possibly due to the hysteresis at the interface region.

-Immobilization of antibodies.

According to the optimized protocol, antibodies were immobilized at 4°C for 24 hours. The resistance before and after antibody binding was measured. When antibodies were immobilized at the sensor surface, the average resistance change was 50%-increase (Fig. 3a).

Table 1. Testing results for various configurations of SWCNT sensors

	Sensor configuration, picture and data	Working principle*	Pros & cons
1. Current sensor (July 2018 ~present)	 	**Electrostatic gating effect** ; measurement in 1xPBS buffer.	Simple fabrication Simple detection protocol. **Reproducible results**
2. Previous sensor (May 2018~June 2018)	 	**Electrostatic gating effect + Schottky effect;** Measurement in DI water	Simple fabrication Simple detection protocol. **Not reproducible**

-Demonstration of sensor performance for BCG in PBS buffer.

The detection of BCG in 1x PBS buffer was demonstrated using an optimized protocol. We used 20µl of 1x PBS and various concentrations of BCG cells from 10^2~10^6 CFU/mL for negative and positive controls, respectively. The resistance before and after target binding was measured and compared in Fig. 3b. *I-V* measurement was conducted in between steps and compared (Fig. 3b). According to the dose response, the developed sensor qualitatively detected BCG cells as low as 10^3 µg/mL in 1x PBS.

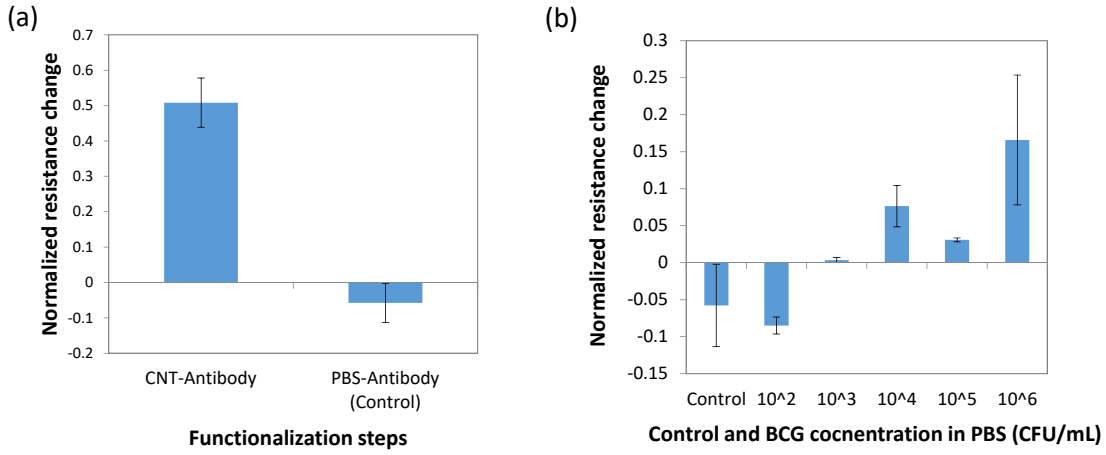


Fig. 3 (a) Normalized resistance change in the SWCNT and antibody steps (N=6) and in the antibody and control test steps (N=6). (b) Dose response results with M. BCG. When target BCG cells are bound, the resistance is increased. Without BCG cells (control) and 100 CFU/mL of BCG cells, the resistance is decreased (N=3 for BCG cells in PBS, N=6 for control).

(Task 4). Demonstrate the prototype device with simulated sputum sample (Original planned dates: October 31, 2018): 30% completed.

In this task, we aim to liquefy and decontaminate sputum samples for detection of target analytes. We tested two protocols for sputum liquefaction. The main purpose was to dilute sputum samples and to achieve homogeneous medium physically. The uniform ion concentration using the high ionic reagents will result in stable control signal of the sensors because electrostatic interaction is a key for the sensing mechanism. The total volume after adding reagents became 4 times the original sputum volume. The addition of reagents will offer a relatively uniform electrical resistivity to obtain more consistent measurement of resistance.

The two kinds of sputum samples tested were human sputum samples and synthetic sputum samples. Sputum samples were obtained from BioReclamation, Inc. The samples were deidentified from the company. Synthetic sputum was prepared according to Sanders *et. al.* (Sanders, N. N., Van Rompaey, E., De Smedt, S. C. & Demeester, J. Structural alterations of gene complexes by cystic fibrosis sputum. *American Journal of Respiratory and Critical Care Medicine* **164**, 486-493, doi:10.1164/ajrccm.164.3.2011041, 2001). Briefly, to a 21.74 ml buffer solution (85mM NaCl, 3 mM CaCl2, 20 mM HEPES, pH7.4) was added 500 mg mucin (sigma #M3895), 91.35mg DNA

(sigma #D1626), BSA 543.7 5 mg (sigma #A7030), 57.55 mg DPPC (sigma #P0763) and 8.1 mg DPPG (sigma #42627) and vortexed, creating a very viscous mucus like consistency.

To reduce viscosity and clarify the sputum, the following protocols were tested. 100 µL sputum was first mixed with 100 µL PBS followed by either 100 µL NALC (4 mg mL^{-1} *N*-acetyl-L-cysteine) or 100 µL 0.4M NaOH. 3mm glass beads were added to the tube and the solution was vortexed for 5 minutes. After vortexing a 4% SDS solution (sodium dodecyl sulfate, 100 µL) was added, followed by additional vortexing for 5 minutes for complete liquefaction. Liquefaction and clarity of sputum was tested by measuring OD at 600nm.

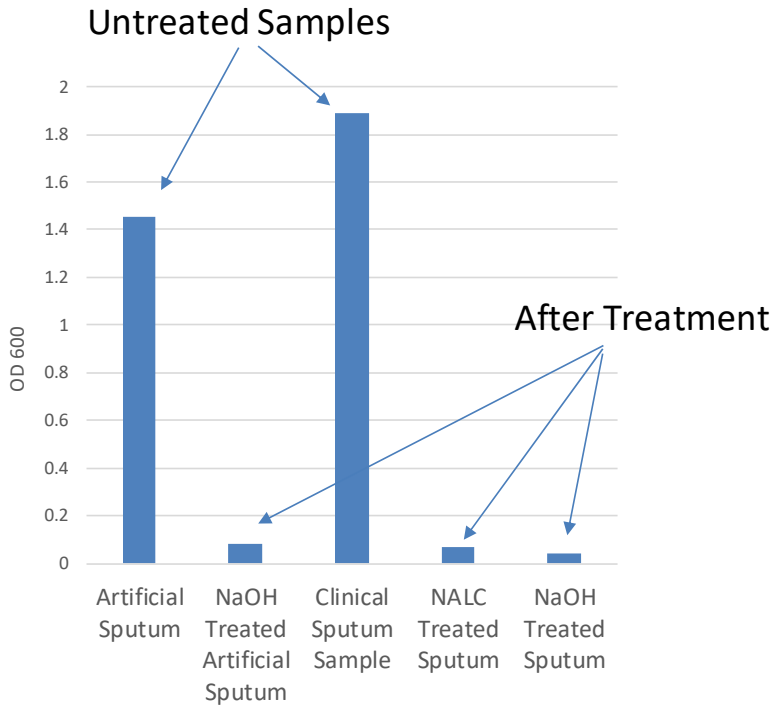


Fig. 4 OD 600nm Results of Sputum Processing. Both NALC +SDS and NaOH+SDS are effective in liquefying the sputum samples. Low OD measurement values show successful liquefaction of sputum samples.

Fig. 4 shows the results of the OD measurement. The results show a significant reduction on OD600 after treatment. The sputum samples were also significantly less viscous (a qualitative observation when pipetting the sample). Both the NaOH and NAC treatments worked to reduce the OD600, and the procedure worked on both the human samples as well as the artificial sputum samples. The artificial sputum samples were tested with NaOH only. Both sputum protocols will be used for detection of target analytes in sputum. In addition, a solution of Triton X-100 detergent was used in place of SDS and showed similar results to SDS containing samples, which may be better for antibody containing samples (preliminary data not shown).

In conclusion, the fabrication process for the sensor was accomplished with optimization of the fabrication and detection protocols. Two sensing platforms were tested using BCG cells in PBS. One platform exposed only the SWCNTs to detect the target by an electrostatic gating effect. The other platform exposed both SWCNTs and SWCNT/gold electrode regions to detect the target by an electrostatic gating effect and a Schottky effect. After repeated experiments, it was found that the sensor using just an electrostatic gating effect was more reproducible.

For the dose response test using various concentrations of BCG in PBS, BCG cells could be detected down to 10^3 CFU/mL. Our plan for the next period will be described in the plan section.

In summary, the followings are the major accomplishments of the project as outlined in the proposal plan,

A. Demonstrate the prototype device with pure samples (Completed dates, October 31, 2018, 90% completed).

- Optimization of sensor fabrication and detection protocols.
- Dose response test for BCG in PBS (10^3 CFU/mL)

- **What opportunities for training and professional development has the project provided?**

Graduate student training: The PI is working closely with a graduate student on the project. The experimental and multidisciplinary nature of this project allows the student to learn and gain various hands-on experiences and develop extensive laboratory skills.

At the University of Washington, graduate student, Seong-Joong Kahng was trained with support from this project. He fabricated the sensors and ran the bioassays.

At Washington State University, a graduate student, Anwarul Karim, was trained in the period 2 (May 1, 2018~October 31, 2018). He fabricated the stamp and the electrodes.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

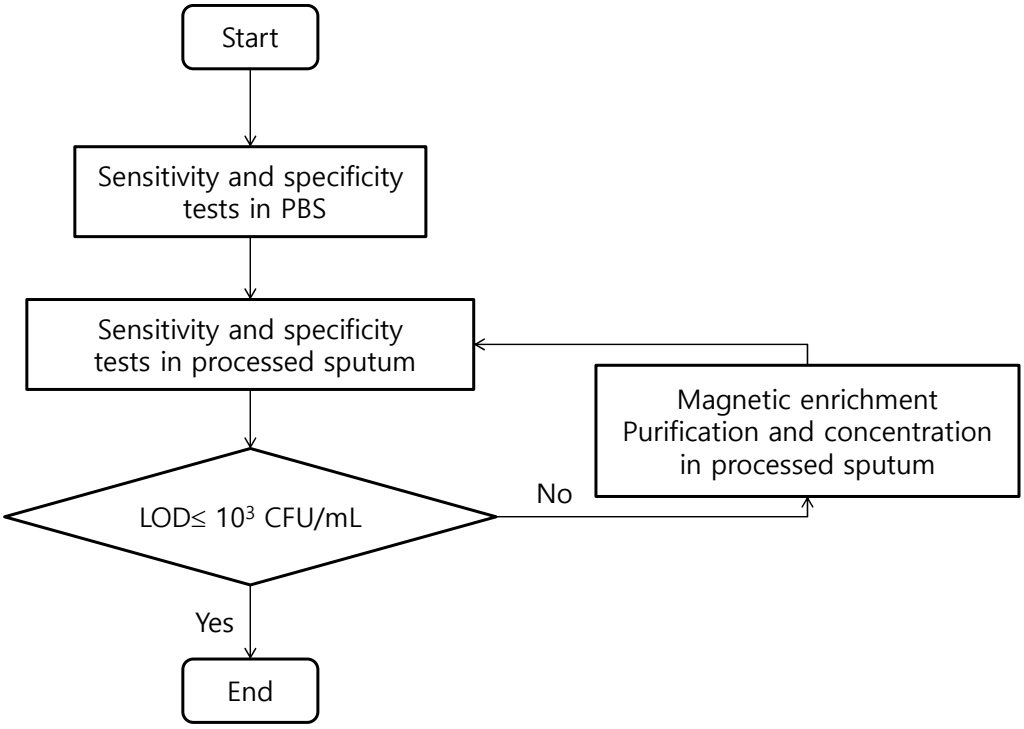


Fig. 5 Flow chart for the project conducted in the period of November 1, 2018 ~October 31, 2018.

In the next project period, the detection protocol will be applied to detection of both MTB and MPT64 at a sensitivity of 10^3 CFU/mL. Specificity test will also be conducted using *E. coli K1* and *S. aureus* spiked samples for *MTB* detection and *NTM* (i.e. *M. avium*) in PBS buffer. The following flow chart (Fig. 5) shows the remaining tasks for the next project period.

Once the test for the detection limit using targets in PBS is completed, Task 4 (detection limit test in sputum samples spiked with target analytes) will be conducted. The sputum will be processed as described in the report. If the limit of detection (LOD) of 10^3 CFU/mL is achieved, all the goals set out for this project will have been achieved. If the LOD is greater than 10^3 CFU/mL, enrichment using magnetic beads will be attempted. After the enrichment and purification, the enriched targets will be suspended in a 1 x PBS. The targets in PBS will be detected by the SWCNT immunosensor. Note that Co-PI, Dr. Furlong is an expert using magnetic beads for concentration and purification of target bacteria, protein and nucleic acids from human samples. We believe the magnetic enrichment will offer the LOD of 10^3 CFU/mL.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

- **What was the impact on the development of the principal discipline(s) of the project?**

This innovative approach based on nanoink printed amperometric immunosensor will be critical in point of care diagnosis of tuberculosis (TB). The proposed work will establish a reproducible nanoink printing method for cost-effective manufacturing of the amperometric immunosensor. The immunosensor will be capable of detecting MTB and protein biomarkers (MPT64) in sputum samples with detection limit as low as 1,000 CFU/mL in 20 minutes.

- **What was the impact on other disciplines?**

The printing technology of SWCNTs will have impact on large scale fabrication of physical, chemical and biosensors. In addition, the fabrication steps will offer a stepping stone for scalable nanomanufacturing. See the attached manuscript in the appendix.

- **What was the impact on technology transfer?**

Nothing to report

- **What was the impact on society beyond science and technology?**

In this project, we are developing a diagnostic assay that can be used at the point of care to rapidly and accurately diagnose TB. With the nanoink printed amperometric immunosensor, we aim to achieve a detection limit of 1,000 CFU/mL with the detection time of 20 minutes in sputum samples, which will be faster and at significantly lower cost than smear microscopy and PCR, and more accurate than skin tests. All this will help to provide patients with the right treatment without delay. In addition, we will be able to reduce the occurrence of false positives, which will spare the patient inconvenience, cost and potential toxicity from unnecessary drug treatments. Therefore, the immunosensor can offer an immediate solution that can address the current challenge of rapid, inexpensive and accurate TB diagnosis in low resource settings.

5. **CHANGES/PROBLEMS:**

There is no significant change in the proposed plan. To successfully accomplish the proposed plan, we requested no cost extension of the project. The newly approved end date is October 30, 2019, which is one more year from the original termination date. The present annual report was described according to the changed plan.

- **Changes in approach and reasons for change**

  Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  As mentioned in the conclusion, the screening effect of high concentration ions in PBS significantly lowered the sensitivity of the SWCNT sensor, which was resolved by introducing deionized water for washing before electric measurement. We will optimize the detection protocol further to obtain a reliable protocol.

- **Changes that had a significant impact on expenditures**

  Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Not applicable.

- **Significant changes in use or care of human subjects**
- **Significant changes in use or care of vertebrate animals.**
- **Significant changes in use of biohazards and/or select agents**

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

  Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

Name:	*Jae-Hyun Chung*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-9861-8559
Nearest person month worked:	*1*
Contribution to Project:	*Chung has organized meetings among the project individuals, analyzed the data, and led the project.*
Funding Support:	
Name:	*SeongJoong Kahng*
Project Role:	*Research Assistance*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12 months with 50% effort*
Contribution to Project:	*Mr. Kahng has fabricated and tested the sensors.*
Funding Support:	
Name:	Scott Soelberg
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0001-8010-7753
Nearest person month worked:	2
Contribution to Project:	Mr. Soelberg has performed work in the area of antibody characterization and assay development
Funding Support:	
Name:	Clement Furlong
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-6489-7211
Nearest person month worked:	1
Contribution to Project:	Professor Furlong has served as PI for this subproject.
Funding Support:	

Name:	*Dr. Jong-Hoon Kim*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-6088-7676*

Nearest person month worked:	*1*
Contribution to Project:	*Dr. Kim has designed the experiments and managed the project activities and progress based on the planned timeline*
Funding Support:	
Name:	*Anwarul Karim*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*2*
Contribution to Project:	*Mr. Karim has fabricated electrodes.*
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to report

- **What other organizations were involved as partners?**

  Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**

   Nothing to report

9. **APPENDICES:** Nothing to report.