Evaluation of Thin Film Nanotube-Antibody Matrix Materials for Potential Integration into Immunodiagnostic Biosensors*

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Abstract

Most quantitative immunodiagnostic assay products require large laboratory equipment, controlled lab environments, and highly skilled technicians, making them expensive and impractical for use in other environments such as physician offices, the home and field clinics of developing countries. The long-range goal of this work is to attempt to develop an inexpensive quantitative hand-held immunobiosensor-based technology that can easily be applied to any antibody:antigen combination and used in any setting. A new approach to measure antibody:antigen binding in the presence of nanotubes has been described in several recent studies. Using conductive polymer/nanotube/antibody solution-impregnated filter paper, these researchers showed a change in filter paper conductivity (resistance) upon application of the antigen-containing sample (1, 2). This change, measured as a chemical potential change in the test strip, was hypothesized to be due to antigen binding to the impregnated antibody that altered the electrical contact between adjacent conductive nanotubes. In contrast to these studies using imprecisely-loaded filter paper test strips, our work aims to develop and evaluate an immunoresistive biosensor comprised of a precisely formulated thin film composite antibody:nanotube micro-expandable matrix mesh-like conductive material ("matrix"). This matrix is coated and then cured between two electrical contacts printed on flexible non-porous film. Initial experiments to demonstrate proof of concept of our design resulted in improvements in both the design of the test system components and in the formulation of the matrix. In this study, a test system and sensor design is described which performs with the accuracy necessary to enable fine-tuning of matrix formulations to prove the concept. Test results of the latest formulation and future directions are discussed.

Introduction

In 2009 paper test strips impregnated with an antibody:nanotube dispersion were successfully used in a standard three electrode chemical cell, with the test strip as one of the electrodes, to correlate chemical potential change of the strip with concentration of a specific toxin (antigen) in water [1]. It was hypothesized that as antigen bound to the antibody, the conductive nanotubes were pushed apart from each other, thus reducing the conductivity of the paper strip. The most significant challenge and time spent to complete the work was in fabricating the paper test strips by dipping them in a nanotube/poly(sodium 4-styrene-sulfonate) dispersion containing antibody (specific for binding microystin-LR) and then freeze-drying them. This dipping and freeze-drying process was repeated 13 times to achieve an appropriate conductivity for the paper test strip. Although cumbersome to produce the filter paper construct, work by the authors established this as a potentially useful method for environmental testing of harmful chemicals (those chemicals that are antigenic and to which specific antibodies can be created and used in the biosensor design). Based on the work that was published in 2009 [1], work was initiated in this research area in our laboratory at WMU in 2010 [3, 4].

At the 2012 Intel Science Fair for high school students, a poster presentation describing a similar filter paper test strip approach but using an antibody directed at detecting the pancreatic cancer biomarker mesothelin (molecular weight 40,000 daltons) in blood or urine was announced at the INTEL American Ingenuity Awards for Youth Achievement [2,5]. The student was given support in a Johns Hopkins University laboratory to develop and test his idea. The youth won the national competition's $75,000 grand prize and a guest spot on the "Cobert Report". Similar to the study in 2009 [1], the filter paper pancreatic cancer test strips were prepared by repeatedly dipping filter paper in nanotube/polymer solutions containing antibody in order to coat the filter paper, but in contrast to the 2009 work that studied only antigen in water, the 2012 study showed that the treated filter paper test strips could detect target antigen in patient blood samples, which consist of more complex biological media containing high levels of many proteins.

These researchers hypothesized that the mechanism of resistance change using a matrix system composed of nanotubes, dispersants, and antibody protein species is due to expansion that occurs due to charge/structural forces driving the conductive elements -- the antibody-coated nanotubes -- further apart upon the impregnated antibody's binding with the applied target antigen molecule.

In our laboratory, we developed for evaluation an immunoresistive biosensor comprised of a precisely formulated thin film composite antibody:nanotube micro-expandable matrix mesh-like conductive material ("matrix") and constructed utilizing thin film printing and deposition technologies.

We are designing formulations based on the hypothesized-mechanism by incorporating components into the matrix that should facilitate such a physicochemical response in the matrix.

Figure 1a shows a conceptual drawing of the carbon nanotube matrix containing associated capture antibody. Figure 1b shows a depiction of a thin layer of nanotubes with capture antibody (shown in blue) adsorbed on the nanotube and intercalated between different nanotubes at two different junctions. Figure 1c shows the expansion that is hypothesized to occur when the capture antibodies (shown in blue in Figure 1b) bind antigen (antigen is shown as green). Note that the nanotubes shown in light gray have moved apart as a result of the antigen binding. When capture antibody that is intercalated between two different nanotubes binds antigen, the binding event perturbs or forces the two nanotubes further apart, thus lowering their electrical charge transfer capability from one nanotube to the next. This increases the overall resistance to current flow through the matrix within the test channel (located between silver carbon-coated contacts at the two ends of the test channel). The test channel within which the matrix material is coated is ~ 2.5 cm long. The nanotubes used in these studies are ~1 µm long. As such, there will be far more than 25,000 nanotube-to-nanotube overlap points along any straight line between the ends of the test channel. When antibody is present at a junction point, binding of antigen to the antibody can affect current flow through that region if the nanotube-nanotube separation distance is affected. The effective change in separation distance is measured as a change in the matrix channel resistance.

Test Plate and Test System

An initial plate design allowed for 4 matrix channels (A, C, A, C) to be used at a time on one test plate (see Figures 2 and 3), allowing for two specific binding tests and two associated non-specific binding reference tests to be run side by side. Figures 2 and 3 represent this plate configuration (currently we are using a 5 channel per plate design). Matrix composition for channels A and C are the same except matrix channels A contain test capture antibody and matrix channels C contain reference non-capture antibody. All component concentrations including antibody concentration are the same for all matrix channels for a particular matrix formulation being tested. The test plate design is shown from a top-angled viewing perspective in Figure 2. A side view looking parallel with the matrix channels on the plate showing the inside schematic of the deposited layers is shown in Figures 3 and 4.

The following steps were used in the construction of the single use 4-channel disposable plates, layer by layer (see Figure 2):

1. To the PET (Polyethylene terephthalate, Dupont Teijin Films Melinex® ST505) base layer, conductive carbon-coated silver ink (Henkel Electrodag 479SS coated with Henkel Electrodag 965SS) was applied as electrical contacts. These were applied sequentially by screen-printing and drying to result in 4 separate unfilled squares on each side of the plate (ultimately at the ends of each channel).
2. Using a Sonoplot plotter, non-wetting polymer (flouropolymer 3M™ Novec™ 2702 Electronic Grade Coating) was applied as unfilled rectangular boxes overlapping each electrical contact at the sides of the glass plate to create a total of 4 channels and then dried (the carbon-coated silver contact is exposed to the interior of each end of each channel and directly contacted by the matrix). Drying was followed by UVO treatment.
3. The previous layer was then laminated to a ~2"x3" glass base slide.
4. Matrix material was added to each channel (matrix is contained due to the non-wetting polymer barrier) and then cured by drying.
5. After applying an adhesive spacer to each end of the glass plate, a 1"x3" glass cover slide was attached to the double stick adhesive spacer so the slide was positioned directly above the matrices (see Figure 4). This slide has eight parallel lines of non-wetting polymer that lie directly above the 8 parallel lines of non-wetting polymer on the PET below. This allows the liquid sample to migrate into the gap above each of the matrix layers.

With the spacer providing a narrow air gap (4 mils or less depending on the spacer, <100,000 nm) above the matrices and the glass cover slide (see Figure 4 side view), a controlled volume of antigen containing test liquid sample can be applied that will precisely coat each matrix with test sample.

Figures

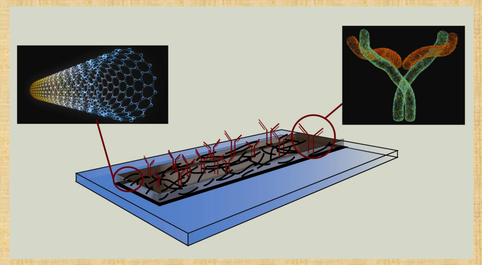

Figure 1a. Conceptual drawing of matrix containing nanotubes and capture antibody.

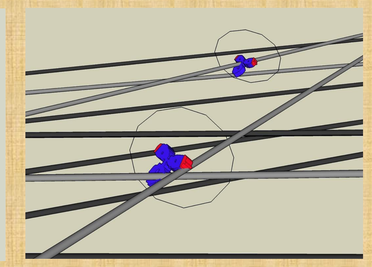

Figure 1b. Depiction of capture antibody (blue) associated with nanotubes within matrix.

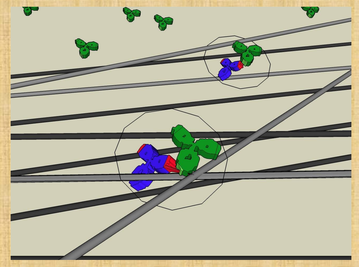

Figure 1c. Depiction of capture antibody (blue) binding antigen (green) from solution. Note the change in light gray nanotube-nanotube contacts from Figure 1b to 1c within the circled areas. Such a spacial expansion change has been hypothesized as contributing to resistance changes in filter paper-based nanotube/polymer/antibody test strips upon antigen binding to capture antibody.

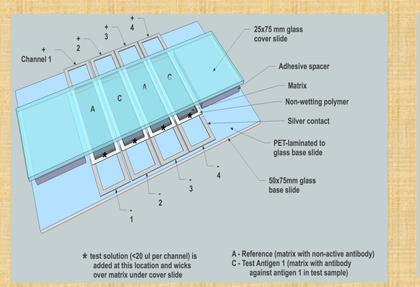

Figure 2. Schematic of a four-channel test plate for testing matrix formulations. Once connected to the data acquisition system, the resistance of each matrix channel is measured every 10 sec upon addition of ~20 microliters of the antigen-containing test solution to each matrix channel A, C, A, C.

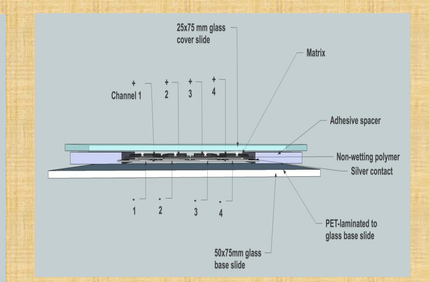

Figure 3. Side view of a four channel test plate. The narrow gap between the bottom of the top glass slide and the top of the matrix for the four channels, through surface tension, draws the antigen containing test solution under the top glass slide when applied at the edge of the slide (see asterisk on Figures 1 and 3). The bottom of the top glass slide has lines of anti-wetting polymer strategically deposited to contain the applied liquid test sample over each matrix.

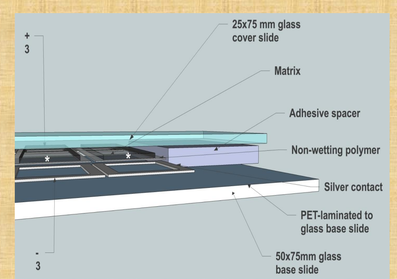

Figure 4. Enlarged view of the various layers that are affixed to the glass base slide of a test plate.

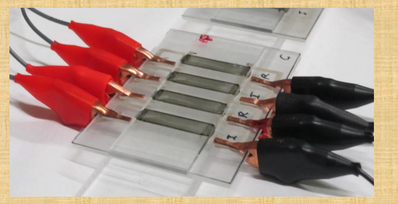

Figure 5. As an example, this four channel plate is attached to a flat piece of acrylic, raising the plate contacts above the surface to allow attachment of alligator clips leading to Keithley 2700/7700 data acquisition system.

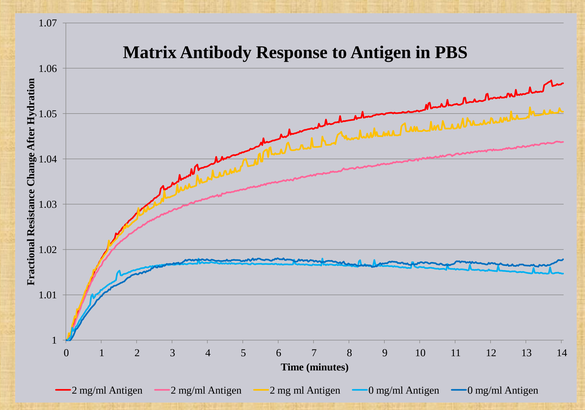

Figure 6. Matrix formulation containing the active antibody showing resistance change to 2mg/ml antigen solution relative to buffer response.

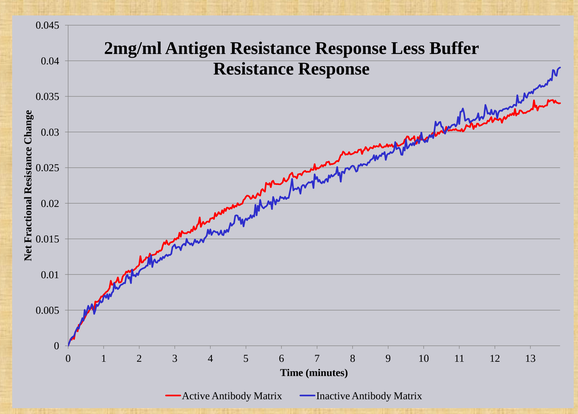

Figure 7. The red curve shown above represents the average of the 2mg/ml antigen response minus the average of the 0 mg/ml antigen response for the matrix with active antibody shown in Figure 6. The blue curve above represents the same calculation, but for the matrix containing inactive antibody (graph not shown). The nearly identical curves (blue and red) indicates that binding is dominated by non-specific binding and significant adjustment to the formulation is necessary to minimize non-specific binding and maximize specific binding.

Testing and Measurements

Prior to adding test liquid to the test channels, the plate is affixed to a raised level strip support to allow positive and negative alligator clips to be attached to channels of a Keithley 2700/7700 Data Acquisition System (having a 20 channel multiplexing capability and enabling a 2-wire constant current resistance measurement) for measuring resistance of each channel as a function of time (see Figure 5).

Once data acquisition is started, liquid sample is applied to each channel at the point shown by the asterisks (Figures 2 and 4). The narrow gap between the bottom of the top glass slide and the top of the matrix for the four channels (see Figure 3) allows the antigen containing test solution to draw under the top glass slide through surface tension. The bottom of the top glass slide has lines of anti-wetting polymer strategically deposited to contain the applied liquid test sample over each matrix. As a result, liquid sample moves underneath the glass to completely cover and contact the matrix (and only the matrix in each channel). Approximately 25 microliters per channel is required. The resistance change within each channel is monitored as a function of time. Data is used to model biosensor resistance response (rate and extent of change) to antigen binding as a function of the amount of applied antigen (antigen concentration).

Matrix Formulation Materials

Different matrix formulations consisting of different concentrations of nanotubes, dispersants, and antibody (binding/capture or non-binding species) were coated onto the prepared test channels at different film thicknesses.

Single-walled nanotubes (SWNT) were purchased from SWeNT. SG 76 High Conductivity single walled nanotubes with a 1,000 aspect ratio to tube diameter (0.92+/-0.27nm) and processed under different sonication energy and/or centrifugation/separation conditions to obtain dispersed solutions.

Dispersants that have been examined included carboxymethylcellulose, poly(sodium 4-styrenesulfonate), and glycerol from Sigma/Aldrich.

The antibody, of the antibody:antigen pair used in this study, was goat anti-rabbit IgG antibody (Sigma) and was used in the active (capture/binding) matrix. Its antigen was rabbit anti-mouse IgG ((Sigma) molecular weight ~150,000 daltons).

The reference channel matrix contained the rabbit anti-mouse IgG antibody that does not react with antigen in solution (for these studies it is the same as the antigen in solution).

All matrix formulations examined were aqueous based. All matrix-coated plates were tested following 18-24 hours of curing/drying at room conditions to reduce moisture to less than 10% by weight of the matrix. Test matrix active antibody resistance response change was assessed alongside matrix with inactive antibody (reference) when exposed to a solution containing antigen and 0.01M phosphate buffered saline.

Results and Discussion

The current plate design and test system was put into use this spring and is capable of monitoring resistance change for each channel in the test plate every 2 seconds. The gap above a channel draws antigen in 0.01M phosphate-buffered saline over the matrix. This fluid has a resistance of 30kΩ-60kΩ, and therefore the resistance of the matrix is intentionally kept less than 3000Ω to minimize the effect of gap variation to less than 1% of matrix resistance. Approximately 12 formulations have been tested where nanotube, nanotube dispersants, fluidization components and antibody concentrations were varied. From these studies we found that higher nanotube concentrations decrease resistance, higher concentrations of nanotube dispersants create a more uniform matrix and increase resistance (but may impart too much rigidity to the cured matrix to be able to expand with antibody binding), and higher levels of nanotube fluidity agents do not increase dried resistance as much as expected but still may be important for improving stability of the antibody in the cured matrix and porosity of the matrix.

Figure 6 shows the resistance change of our latest formulation containing active capture antibody as a function of time when exposed to two different concentrations of antigen (0 mg/ml and 2 mg/ml). The same experiment was done with the same formulation containing inactive antibody (the results are not shown in the poster due to space limitations, but the graph is very similar to Figure 6). Figure 7 shows the average resistance response of the matrix containing active antibody to antigen at 2 mg/ml less the average resistance response of the matrix containing active capture antibody to buffer (red curve). The blue curve was generated using the data referred to above (not shown) with the matrix containing the inactive antibody. The difference between these curves in Figure 7 represents specific antigen:antibody binding. Because these two curves are nearly identical, the binding we are observing is predominately non-specific binding, not our objective, but encouraging in that we can quantitatively measure non-specific binding. Protein mass buildup on the nanotube matrix through non-specific binding increases resistance by 3.5% (Figure 6). The increase in resistance, not the magnitude of change, is consistent with reported behavior of physical protein adsorption onto nanotubes. Our aim is to attain a 10% to 20% resistance change due to specific binding of the antigen to the antibody incorporated between the nanotubes within the matrix mesh. A demonstrable change in resistance of 10% to 20% due to structural changes between nanotube-nanotube contact caused by antibody:antigen binding is necessary for the concept to be proven.

This formulation will be further modified by adding non-specific binding blocking agents and matrix fluidity-enhancing agents to make it more micro-expandable, more porous, and to reduce antibody denaturation potential during curing in an attempt to maximize specific binding. The current formulation exhibits visible nanotube flocculation and it is hypothesized that this will also need to be reduced to improve coating characteristics and reproducibility.

Future work includes formulating a matrix that has less rigid structural properties, so that the matrix:antibody mesh structure will rapidly expand (or dissociate) upon exposure and binding to antigen and thus elicit a rapid and large resistance change. By eliminating any excess SWNT dispersant within the matrix and increasing the concentration of a lubricating and antibody-biocompatible stabilizer, we should generate a less structurally rigid and more porous matrix with a higher potential of responding to antigen. Other agents (certain amino acids, bovine serum albumin, surfactants and antimicrobials) may need to be added to impart stability and reduce non-specific binding.

The test plate and test system developed for this work allows for suitable evaluation of multiple formulations to support formulation development. These will be used in evaluating a range or formulations over the coming months to show proof of this immunobiosensor concept.

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