Project Number:

TEMPLATE ASSISTED LAYER-BY-LAYER FABRICATION OF PROTEIN NANOTUBES

A Major Qualifying Project Report:

Submitted to the Faculty

Of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

_____.

Keith Harrison

Date:

Approved:

_____.

Prof. Jianyu Liang, Advisor

1 Table of Contents

2 Table of Figures

3 Table of Tables

4 Acknowledgements

This project was completed with the help of the Mechanical Engineering department of Worcester Polytechnic Institute. Special thanks to Jianyu Liang and Shelley Dougherty for all of their help, which this project could not have been completed without.

5 Abstract

This paper investigates the process variables involved in layer-by-layer (LbL) fabrication of protein nanotubes. Three main process variables were identified as having potential to significantly affect end results. These included protein concentration, solution volume used per layer, and storage time. The effects of each variable on protein activity were studied through controlled experiments and then rationalized in this report.

6 Executive Summary

***Index Terms*—Avidin, Glucose Oxidase, Layer-by-Layer, and Nanotube.**

INTRODUCTION

THIS document summarizes our research investigating the process variables involved in creating layer-by-layer (LbL) protein nanotubes. Nanotubes have great potential in medical applications. They have shown ability to carry proteins into cell nuclei [1] and have potential for transporting genetic material across cell membranes as well [2].

Nanotubes are extremely versatile and can be made from numerous materials. Currently the most popular nanotubes are made from carbon. Carbon nanotubes can be functionalized to increase biocompatibility, but still pose risks of cytotoxicity [2].

Many proteins are already FDA approved. This has sparked an interest in fabricating nanotubes entirely from protein. Researchers have established two major methodologies for protein nanotube synthesis. The first is self-assembly [3-6]. The downfall of this method being that the end result is limited to the chemistry and functionality of the biomolecule. Alternatively, layer-by-layer techniques offer more control to the researcher [7-9]. This method is also compatible with a greater number of proteins than self-assembly.

METHODOLOGY

Protein Preparation

Layer-by-layer methodologies for protein nanotube fabrication are based on electrostatic charges holding positive and negative layers of proteins together. We chose avidin (isoelectric point pH 10) and glucose oxidase (isoelectric point pH 4.2) as our proteins. Each was suspended in phosphate buffered saline (PBS) to maintain a neutral pH, providing avidin with a positive charge and glucose oxidase a negative charge.

Layer Deposition

Protein layers were obtained by vacuum filtering PBS, avidin, and glucose oxidase, respectively, through a 200 nm anodized aluminum oxide (AAO) template followed by a 50 nm polycarbonate membrane. Filtering all three reagents through once was considered one nanotube layer. In each test three layer nanotubes were used. After all layers were deposited 1 mL PBS was filtered through to remove any loose proteins from the templates. The nanotubes were left in the AAO templates for characterization.

Variable Testing

Protein concentration – To test the effect of protein concentration on nanotube formation, solutions of both proteins were made in concentrations of 0.1, 0.5, and 1 mg/mL. A volume of 200 µL of solution was used each time a protein was filtered through the membranes. Characterization of the nanotubes created from each solution was then performed via glucose activity assay.

Solution volume – Three different volumes (100, 200, and 300 µL) of 1 mg/mL protein solution were tested in making the nanotubes. These were characterized via glucose activity assay.

Storage time – The effect of storage on protein functionality in nanotubes was investigated by checking the glucose activity rates initially, after one day, and after eleven days.

RESULTS AND DISCUSSION

The transmission electron micrograph (TEM) seen in Fig. 1 confirms the presence of the avidin-glucose oxidase nanotubes being created by LbL technique.

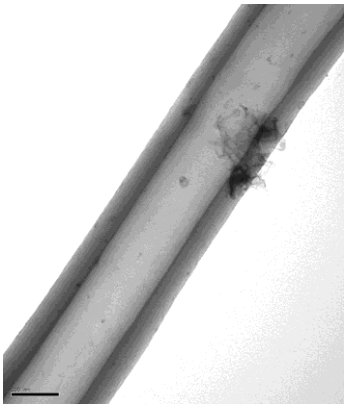

Fig. 1 TEM image of avidin-glucose oxidase nanotube (scale bar represents 100 nm.)

Protein concentration's effect on nanotube formation remains inconclusive. Difficulty with the glucose oxidase activity assay prevented proper characterization of the nanotubes created by different protein concentrations. For unknown reasons the absorbance measured were notably higher than our expectation. We are still seeking an explanation. However, the absorbance measured in the following two experiments were within a reasonable range.

The volumetric effect of solution showed an increase in protein activity from 100 µL to 200 µL. There was no notable change from 200 µL to 300 µL. Since increasing solution volume increases production time, we consider 200 µL the ideal solution volume.

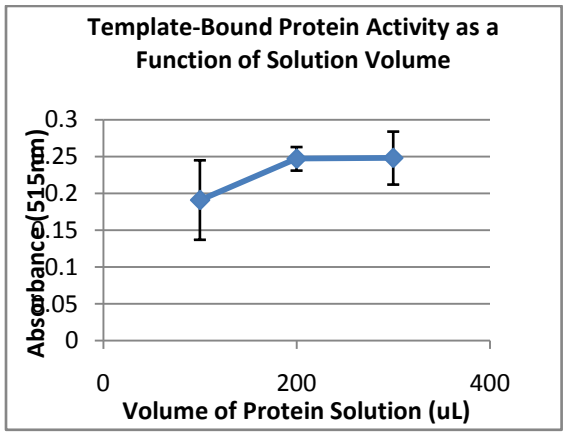

Fig. 2: Nanotube protein activity as a function of solution volume.

Storage time had a notable effect on the bioactivity of the protein nanotubes. Fig. 3 shows the absorbance measured using a glucose activity assay. Repeated absorbance measured by UV-vis spectroscopy on the same samples shows a clear decrease in enzymatic activity for the glucose oxidase as a function of storage time.

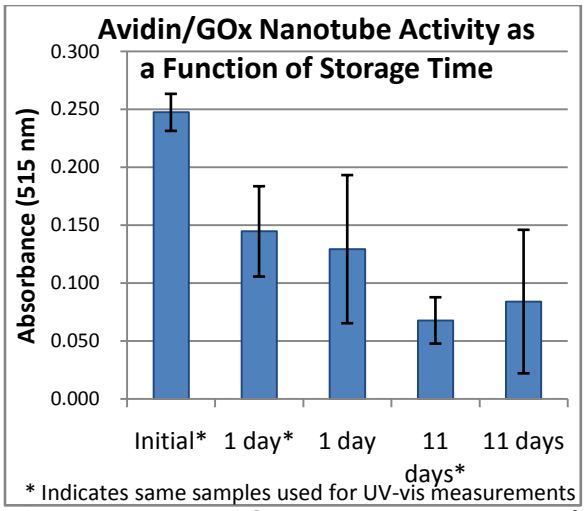

Fig. 3 Protein Nanotube Activity as a Function of Storage Time

Conclusions and Recommendations

Protein nanotube fabrication is a complex process with many variables. Further work should be done to investigate the effect of protein concentration on nanotube formation. The negative effect of storage on protein nanotubes indicates that storage technique and time need to be a consideration in protein nanotube applications.

Acknowledgment

K. T. Harrison thanks J. Liang and S. Dougherty for all of their help, which this project could not have been completed without.

References

[1] Shi Kam NW, Dai H. Carbon nanotubes as intracellular protein transporters: Generality and biological functionality. J. Am. Chem. Soc. 2005; 127: 6021-5026.

[2] Biano A, Kostarelos K, Prato M. Applications of carbon nanotubes in drug delivery. Current Opinion in Chemical Biology. 2005; 9:674-679.

[3] Spector MS, Selinger JV, Singh A, Rodriguez JM, Price RR, Schnur JM. Controlling the morphology of chiral lipid tubules. Langmuir. 1998;14:3493-3500.

[4] Audette GF, van Schaik EJ, Hazes B, Irvin RT. DNA-binding protein nanotubes: learning from nature's nanotech examples. Nano Letters. 2004; 4:1897-1902.

[5] Matsumura S, Uemura S, Mihara H. Construction of biotinylated peptide nanotubes for arranging proteins. Mol. BioSyst. 2005;1:146-148.

[6] Kol N, Adler-Abramovich L, Barlam D, Shneck RZ. Self-assembled peptide nanotubes are uniquely rigid bioinspired supramolecular structures. Nano Letters. 2005; 5: 1343-1346.

[7] Tian Y, He Q, Cui Y, Li J. Fabrication of protein nanotubes based on layer-by-layer assembly. Biomacromolecules. 2006; 7: 2539-2542.

[8] Lu G, Ai S, Li J. Layer-by-layer assembly of human serum albumin and phospholipids nanotubes based on a template. Langmuir. 2005; 21: 1679-1682.

[9] Hou S, Wang J, Martin CR. Template –synthesized protein nanotubes. NanoLetters. 2005; 5:

7 Introduction

As the field of pharmaceuticals is growing rapidly, so is the technology for drug delivery. Nanocapsules and nanotubes possess a variety of traits that make them attractive drug carrier candidates.

For example, carbon nanotubes have already proven effective in transporting proteins to selected cells [1]. Their small size allows for easy cellular uptake, and the proteins they carry have shown in vitro functionality [1]. Nanotubes possess great potential as a delivery system for small drug molecules and genetic material as well, the potential advantage over other delivery methods being the ease of fabrication and diversity as a nanocarrier [1]. Such technology could help overcome many of the difficulties associated with nano-scale medicine.

Carbon nanotubes pose the problem of biocompatibility. As a result there has been an increased interest in the use of bionanomaterials in the fabrication of nanotubes. The term bionanomaterial refers to nanoscale materials that are considered to be compatible with the living system. Proteins, peptides, liposomes, and polymers are examples of bionanomaterials that have been approved for use within the human body by the Federal Drug Administration (FDA) [5]. By fabricating nanostructures from biomaterials the problem of biocompatibility delaying FDA approval is circumvented, adding to the attractiveness of biomaterials for fabricating nanostructures.

The main objective of this study is to demonstrate the layer by layer (LbL) fabrication of protein nanotubes with controlled diameter, wall thickness and length using anodized aluminum

oxide (AAO) membranes as a template. By suspending proteins in solutions above or below their isoelectric point we could manipulate the protein's charge. By alternately filtering positively and negatively charged proteins through a template, layers are expected be held together by an electrostatic charge on the inner walls of the template pores. This would form a structure that could be quickly and easily fabricated. Two proteins (avidin and glucose oxidase) were chosen for their different isoelectric points. With avidin having an isoelectric point of pH 10 [11] it would be positively charged when suspended in a pH 7 buffer solution. On the contrary, glucose oxidase having an isoelectric point of 4.2 [12] would be negatively charged in a pH 7 buffer solution. It is expected that wall thickness would be a function of how many layers were deposited on the pore walls. Length is usually limited to the thickness of the template [6]; however, certain reports have shown difficulty in controlling the length of protein nanotubes [5]. Originally, we also proposed to investigate a controlled cleaving process to trim the nanotubes to a uniform length. Unfortunately, time and budget constraints did not allow us to explore the controlled cleaving, in the project we focused on investigating the process variables for forming layer-by-layer protein nanotubes. These variables included storage time and technique, volume of solution filtered through template, and concentration of proteins in solution.

8 Literature Review

Gene therapy has recently become a pharmaceutical hotspot with the mapping of the human genome. One of the present problems is how to efficiently, safely, and cost effectively deliver the genetic material to the target cells. Viral vectors have shown potential in delivery of genetic material to cells. Unfortunately, they face problems of scalability and biocompatibility [13]. Another vector, plasmid DNA, has considerable difficulty penetrating the cell and reaching the target nucleus on its own [2]. Nanotubes, nanocapsules, and nanoparticles all have potential to efficiently deliver genetic material across a cell membrane and even into the nucleus. In one study plasmid DNA expressing β-galactosidase was attached to functionalized carbon nanotubes (*f*-CNT). Gene transfer experiments were then performed comparing the effectiveness of *f*-CNT delivered DNA versus the cell uptake of DNA without the *f*-CNT. Gene expression was five to ten times higher when the DNA was delivered by *f*-CNT [2].

There is much debate upon which mechanism the cell employs to uptake nanotubes. Some studies suggest that endocytosis is the main mechanism for cellular uptake of nanotubes [1]. This theory is supported by findings of *f*-CNT-protein conjugates within endosomes [1]. Phagocytosis has also been suggested by some studies [3] and insertion and diffusion by others [2]. Transmission electron microscopy (TEM) can be used to visualize CNT within cells. TEM images have shown CNT perpendicular to the cell membrane, which would suggest the insertion and diffusion theory [2]. This theory proposes that the CNT perforate and diffuse through the lipid bilayer without seriously damaging the cell [2]. In spite of the uncertainty regarding the uptake mechanism there is evidence to show that *f*-CNT have a high propensity to cross cell membranes [14, 15]. This has been shown by attaching fluorescently labeled proteins to the

nanotubes and comparing the fluorescence of their target cells before and after the nanotubes are introduced [1].

In protein nanotube technology, two major fabrication techniques have evolved; self-assembly and template-assisted assembly. Self-assembly techniques are somewhat limited because the end result is unique to the chemistry and functionality of the biomolecule [16, 17, 18, 19]. Studies of chiral lipid tubules have shown the ability to somewhat manipulate nanotube morphology [16]. By altering molecular structure, lipid concentration, and solution conditions the researchers were able to control the number of bilayers in the tubule walls. However, they had no control over diameter. Forming the tubules in water and mixtures of alcohol provided unique morphologies were formed [16]. It is possible that similar techniques could be used to control the morphologies of protein nanotubes as well.

Another group engineered protein nanotubes from type IV pili (fiber-like protein polymers produced by many bacteria) from *Pseudosomonas aeruginosa* [17]. The nanotubes created from pili were found to bind to single stranded DNA with moderate affinity, adding to their potential as a vector for gene delivery. Thus it is important to note that proteins are naturally involved in forming nanostructures with unique functions, and that natural phenomenon are an important place to look for nanotech inspiration.

Alternatively, the template-assisted method of nanotube fabrication is highly versatile and controllable. The methodology is relatively simple and typically relies on either electrostatic charges to hold the protein layers together (layer-by-layer method) [6, 7] or the chemical cross-linking of proteins (alternate immersion method) [5, 8, 9].

Many of the current methods for template assisted fabrication of protein nanotubes rely on chemical cross-linking to strengthen the weak bonds of proteins [5, 6, 8, 9]. Specifically, alternate immersion techniques involve preparing the template with a cross-linking agent such as glutaraldehyde before the template is immersed in a protein solution. To lessen the non-specific binding of proteins to the face of the template (instead of the pore walls), the faces are often sputter coated with gold (Au). Sputter coating with Au prevents the glutaraldehyde from attaching to the template face and thus lessens the chances of proteins adhering where they are not wanted [8].

In alternate immersion techniques the number of layers is determined by the number of times the template is immersed in the cross-linking agent and then protein solution. Other dimensional qualities of the nanotubes are largely dependent on the pore structure of the template, with the outside diameter always being defined by template pore size. It stands to reason that template thickness would define overall nanotube length; however, at least one report has found otherwise [5]. Some advantages of this approach would be the control over dimensional qualities afforded to the user, while offering a wide variety of proteins to choose from. This technique also creates very strong bonds due to the protein immobilizing unit glutaraldehyde. Disadvantages would be the amount of time wasted soaking the template in different solutions, and lack of automation capability. Also an unnecessary amount of protein may be used in creating enough solution to fully submerge the template each time a layer is created.

Layer-by-layer techniques involve alternately filtering proteins through templates using either pressure or vacuum filtration. This approach can be used in conjunction with cross-linking to strengthen the bonds or simply using electrostatic charges. One study fabricated layer-by-

layer nanotubes by alternately filtering cytochrome-c and glutaraldehyde [6]. Glutaraldehyde is often used to immobilize proteins by forming covalent bonds. The same study also made protein nanotubes formed by alternately adsorbing layers of cytochrome-c and poly-(sodium styrenesulfonate) (PSS) onto membrane templates [6]. The cytochrome-C and PSS protein nanotubes were held together with electrostatic charges. Cytochrome-c has an isoelectric point of pH 10.4 and is thus positive at pH 7.0, while PSS is negatively charged at pH 7.0 [6]. This methodology most closely reflects the one used to create protein nanotubes from avidin and glucose oxidase in this paper. However, the study involving cytochrome-c and PSS nanotubes sputter coated their membranes with gold before protein deposition to prevent protein from remaining on the surface of the template. The most notable advantage of utilizing a layer-by-layer approach would be the ease of fabrication. This process is less time consuming than alternate immersion methods and affords the user more control than self-assembly. Protein waste could be minimized by determining minimum solution volume and solution concentration for optimum protein adsorption. The process also possesses great potential for automation as it largely involves repetitive pipetting of solutions.

As nanotechnology develops and potential medical applications begin to become realities, regulatory agencies such as the FDA will face new challenges. Current FDA regulations may not effectively cover the new challenges of nanotechnology. This gives rise to the new field of nanotoxicology. The small size of nanoparticles also poses potential risk to researchers and anyone involved in their fabrication. Their size lends to easy crossing of epithelial and endothelial layers, into the lymph and circulatory systems. Nanoparticles could then reach potentially sensitive target sites such as bone marrow, lymph nodes, spleen and heart [20]. Although, it can be argued that nanoparticles are a naturally occurring phenomenon that

we have been exposed to all of our life (especially with the increase in pollution) researchers will need to perform serious risk assessments regarding nanoparticle fabrication.

Another new area of study that will arise from the interest in nanoparticles as a drug delivery system will be in regards to how the nanoparticles are delivered. Now it is unclear if nanotubes would be delivered orally, intravenously, transdermally, via implantation, etc. Therefore, the pathway that different nanoparticles take throughout the body will need to be extensively studied. Some studies with carbon nanotubes have shown the specific targeting of tumors with antibody-functionalized radio-labeling [21]. However, there is always the possibility of nanotubes ending up where they are not wanted.

9 Project Approach

9.1 Initial Approach

The layer-by-layer approach had shown promising results when used with cytochrome C (cyto-c) and Glutaraldehyde (GA) or PSS [6]. We initially felt that by alternately filtering avidin (isoelectric point – pH 10) suspended in solutions of pH 5 and pH 12 through 200 nm pore AAO templates, the opposite charges would be enough to hold together structures the size and shape of template pores. Attempted characterization of the tubes via UV-vis showed questionable results (discussed in the Results section). This led us to try soaking the templates in GA overnight after depositing the proteins on the pore walls. The GA would supposedly act as a crosslinking agent and strengthen the bonds. This caused dissolving of the templates to be extremely difficult and left an undesirable precipitate in the solution.

We then hypothesized that the alternate filtration of solutions of acidic then basic pH may actually just be changing the pH and consequently the charge of the protein inside the pores every time a solution is filtered through. As a result, no electrostatic bonding was taking place and any measurements on the spectrometer indicating a peak at 282 nm was likely the result of loose protein, not nanotubes.

9.2 Project Approach

These results lead us to rethink the project approach. The opposing pH values had quite likely ruined the chances of maintaining opposite charges on the proteins to induce electrostatic bonding. Thus, if we approached the same problem with solutions of neutral pH we would not disrupt the charges of the protein. By selecting one protein that had an isoelectric point above pH 7 and another with an isoelectric point below pH 7 we felt we could effectively maintain opposite charges while alternately filtering the two protein solutions through the template. We

then proposed to suspend avidin (isoelectric point pH 10) and glucose oxidase (GOx) (isoelectric point pH 4.2) in phosphate buffer saline (PBS), (pH 7) and alternately filter them through the same 200 nm AAO templates.

Using two different proteins in layer-by-layer fabrication of nanotubes is a more widely documented approach, which has shown success [6]. Since controlling the process variables of protein nanotube fabrication was our goal, we decided to stay with the more promising methodology for the rest of our studies.

10 Design

Upon deciding to use two proteins with opposite isoelectric points suspended in neutral solution it was necessary to design a simple yet effective technique for fabricating the nanotubes. There were many design choices that had to be made, and they will be detailed in the following sections.

10.1 Protein Choice Justification

The proteins for use in this project were chosen based on a few factors. First, they are both readily available at a relatively low cost. They are commonly used in labs and are available in a number of forms. For example, avidin can be purchased in a fluorescent form, which could prove useful in further investigations of the nanotubes in living systems. Their isoelectric points on opposite sides of the pH scale also were essential to the design of this process. By maintaining a neutral pH in both solutions the avidin would maintain a negative charge and the glucose oxidase would be positive.

10.2 Template Choice Justification

Anodized aluminum oxide membranes were chosen as a template for their availability in a wide array of pore sizes, thicknesses, and pore densities. They can be fabricated in the lab using a well established two-step anodization procedure, which produces more uniform pores than the commercially available templates. However, time was a major constraint in this project, so commercially available AAO discs were used as received from Whatman Corporation. They had average pore size of 200 nm and a thickness of approximately 60 μm. For any further continuations of this project it is recommended that templates are fabricated to the most uniform specifications possible.

10.3 Choosing the Proper Nanotube Liberation Solvent

Once the nanotubes are formed on the inner pores of the membrane, we needed a method of liberating them without damaging their structure. To determine the appropriate chemical to dissolve the templates a simple experiment was setup with three AAO templates suspended in different chemicals and the time until the entire inner portion of the template was dissolved was recorded. Some of the chemicals had been used to liberate nanotubes from templates in previous published works. Each AAO template was soaked in a petri dish of 10 mL of either Sodium Hydroxide (NaOH), Phosphoric Acid (H_3PO_4.), or Hydrochloric Acid (HCl) and total dissolve time was recorded. A template was considered totally dissolved when the clear plastic ring around the AAO was all that was left of the template. In certain cases there was precipitate left on the bottom of the petri dish and this was noted in the results for it is an undesirable byproduct for the next step of the process. The solvent that most rapidly and completely dissolved the templates was the NaOH.

10.4 Storing the AAO Templates with Nanotubes

As a result of the difficulties with nanotube liberation, we decided to characterize the nanotubes within the AAO templates. Originally we had tried storing the templates dry in small containers in the lab refrigerator. Since proteins are a biological material we questioned the effect that dry storage for any extended period of time would have on the proteins. This led to our later study of protein activity as a function of dry storage time. For all other studies this variable was circumvented by performing activity assays within a few hours of initial nanotube fabrication.

10.5 Choosing the Process Variables to Study

Initially, the main variable we sought to correct was non-uniform nanotube length. Given the constraints of the project we needed to rethink the variable and decided to focus on process

variables instead. The variables we found to cause the most inconsistency in the process were chosen with regard to how they could affect the end product.

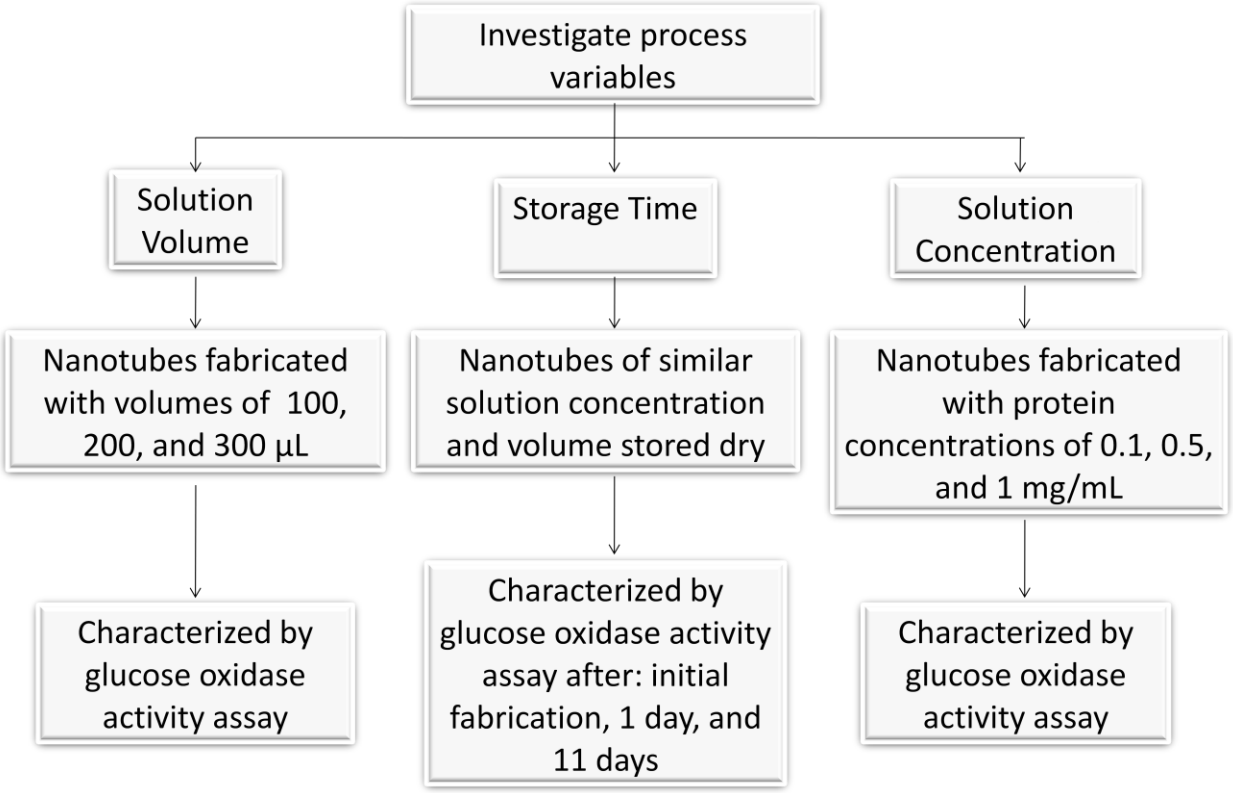

Figure 1: Process variables

10.5.1 Volumes Filtered

In the beginning we assumed that 200 µL was a sufficient volume of each protein solution to filter through the template for each layer. The benefit of using such a small volume was that each time a protein needed to be deposited it only took approximately three minutes to vacuum filter through. Seeing as the inside of each pore has a limited surface area only a certain amount of protein can adhere to the walls of the channel. If we were depositing too much protein we could possibly be wasting time and expensive protein. If we were using too little protein we could possibly be leaving gaps in the cylindrical structure of the NTS thus leaving it

incomplete. Beyond these problems there was also the chance that too much solution may create too much flow through the channels and prevent proteins from properly adhering.

10.5.2 Protein Concentration

For similar reasons to finding the correct volume of solution to use, we needed to make sure the concentration of that solution was right. To begin with we had used 1 mg/mL solutions for both Avidin and GOx. Both proteins are expensive and therefore had to be used sparingly. But if the solution was not concentrated enough the proteins might not have the proper exposure to the channel walls. On the other hand if it was overly concentrated we would have risked blocking the channels while unnecessarily wasting protein.

11 Methodology

11.1 Avidin Preparation

To employ our design of the layer-by-layer method of protein nanotube fabrication it was necessary to have a solution of avidin at a pH of 7. At a pH below the isoelectric point (pH 10) the avidin will carry a net positive charge. PBS was chosen as the buffer solution because it is widely accepted for use of maintaining constant pH without denaturing proteins.

A 1 mg/mL concentration was made for the initial stock solutions. Initially 10 mL of PBS were mixed with 10mg of avidin.

11.2 Glucose Oxidase Preparation

The GOx was prepared with the same basic technique as the avidin solution. PBS was again used to maintain the pH without denaturing the protein, giving the GOx a negative charge. The 1 mg/mL concentration was again used to prepare 10 mL of solution using 10 mg of glucose oxidase.

11.3 Peak Absorbance Determination

After creating each stock solution peak absorbance was measured using a UV-vis spectrophotometer. The peak absorbance could later be used to ensure the proteins had not denatured and determine approximate batch sizes. This is all possible because of the Beer-Lambert Law [10] which relates concentration as directly proportional to absorption.

A 2 mL sample of each solution was scanned separately on the spectrophotometer with wavelengths from 190 nm – 400 nm. Then a 50:50 mixture (1 mL of each) was run through a similar scan and the results from all three scans were compared (Figure 2).

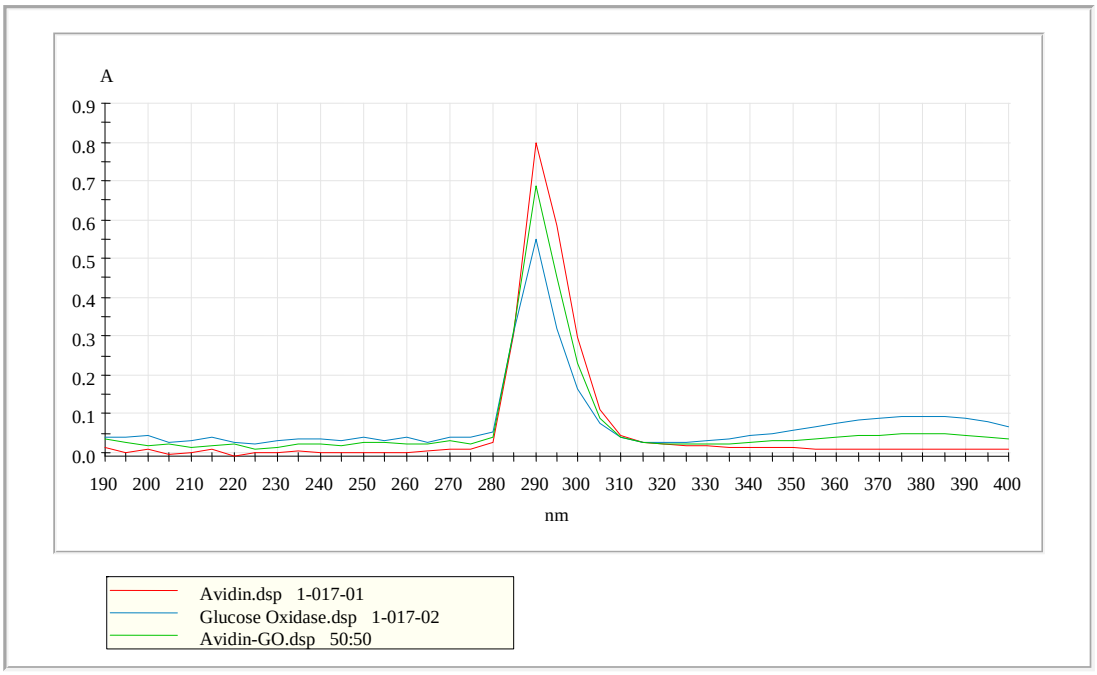

Figure 2: Avidin, GOx, 50:50 Scans

11.4 Avidin-Glucose Oxidase Nanotube Fabrication

The layer-by-layer method was employed to fabricate the avidin nanotubes. This method utilizes the protein's electrostatic charges. By alternately filtering positively and negatively charged proteins through a 200 nm anodized aluminum oxide (AAO) template one protein layer is formed.

First the filtration is set up as seen in Figure 3, where it can drain into a flask which is connected to a vacuum pump. The membranes used were commercially available filters made by Whatman.

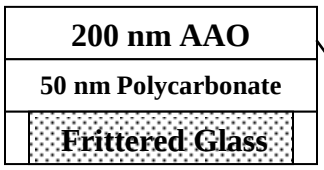

Figure 3: Vacuum Filter Template Setup

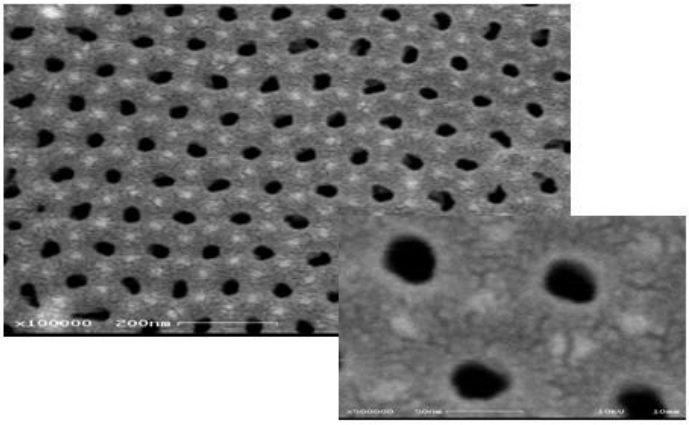

Figure 4: SEM Images of AAO Template

Then 200 µL of PBS is filtered through to remove any loose material from the inner pores, followed by 200 µL avidin solution, and then 200 µL GOx. This creates one layer on the 200 nm pore walls of the AAO template (Figure 5). By repeating this process multiple times the thickness of the nanotube walls can be controlled.

The 50 nm polycarbonate membrane below the 200 nm AAO template serves to slow the flow of solution through the 200 nm AAO template pores. This allows more time for binding than would be possible if the solution drained straight through the AAO template pores.

Upon reaching the desired number of protein layers 1 mL of PBS was flushed through the filtration setup to remove any loose proteins.

11.5 Avidin- Glucose Oxidase Nanotube Liberation

The AAO template was then dissolved in 10 mL of 0.1M Sodium Hydroxide (NaOH). Once completely dissolved, the NaOH was vacuum-filtered through a similar setup as Figure 3, but without the 200 nm AAO template. This collected the 200 nm outer diameter (OD) tubes, while allowing the NaOH to pass through the 50 nm polycarbonate filter. Filtration often took longer than 24 hours.

The polycarbonate filter was then placed in 2 mL of DI water to re-suspend the nanotubes into solution. Upon collecting the nanotubes the solution was then scanned by UV-vis to verify the presence of the nanotubes. Previous tests had determined peak absorbance of avidin and GOx to be around 290 nm. Thus, the scan should show a peak around this wavelength as shown in Figure 2.

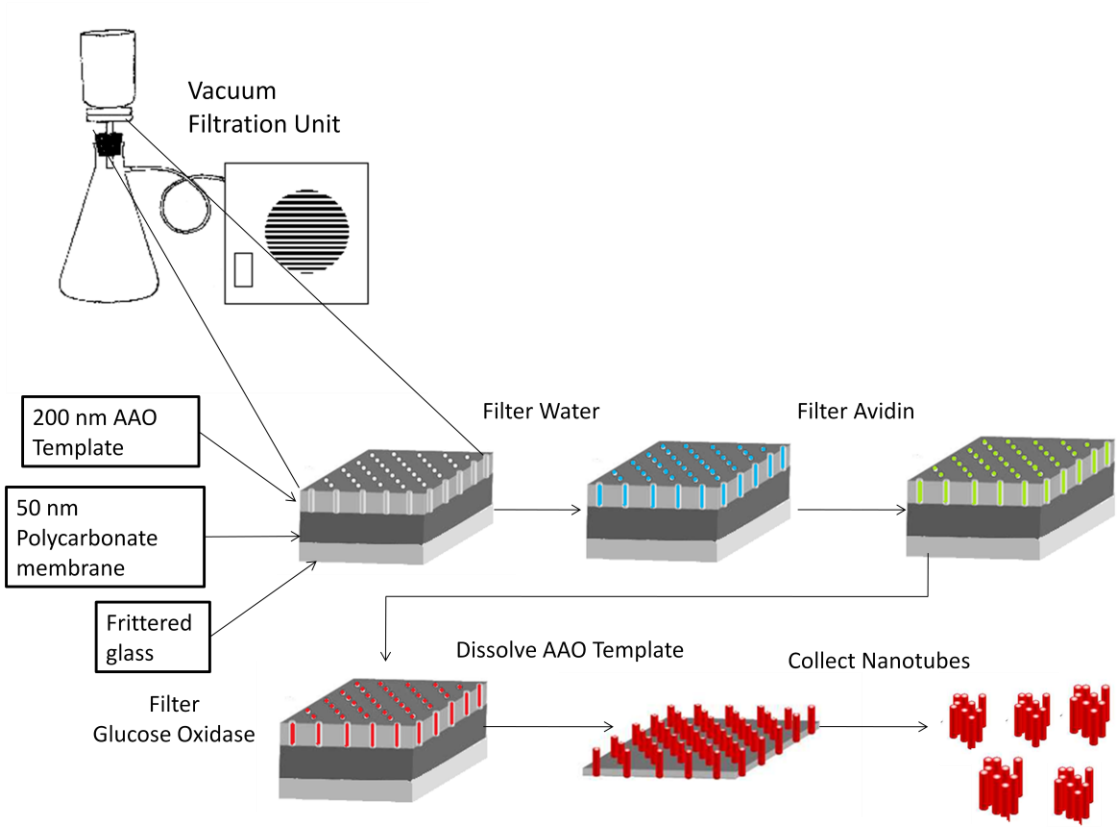

Figure 5: Av-GOx NTS Fabrication Schematic

11.5.1 Determining the Best Solvent

After problems arose with NaOH two other solvents were tried at the following concentrations:

Solvent (10 mL each)
0.1M NaOH
0.1M HCl
0.5M H_3PO_4

Table 1: Solvent Concentration

10 mL of each solvent was pipetted into separate petri dishes and one AAO template was submerged in each. Each dish was marked as to its contents and then observed over 48 hours as often as possible. It was noted whether the template completely dissolved and whether or not there was a precipitate.

11.6 Characterization

A variety of techniques were used to characterize the nanotubes. When the nanotubes were initially being liberated from the templates and suspended in solution, UV-vis spectroscopy was used to measure the absorbance of the solution. Absorbance could theoretically be translated to nanotube density in solution if compared to previously made Absorbance vs. Concentration graphs of the proteins (see Figure 8 & Figure 9). Another technique used to characterize the nanotubes was glucose activity assays, which is another type of absorbance measurement. Finally, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to measure and photograph the nanotubes. It should be noted that most characterization was done while the nanotubes were still template bound as the effect of the NaOH on the nanotube structure was in question.

11.6.1 Glucose Activity Assay

The glucose activity assay sought to confirm that the GOx molecules had not been significantly altered during the nanotube fabrication. Glucose oxidase is an enzyme which catalyzes the decomposition of glucose to gluconic acid [5]. By measuring the change of absorbance with UV-vis spectroscopy over time the reaction can verify the enzymatic activity of GOx is retaining its function.

To perform this assay the following solutions were made:

Reagent	Molar Concentration
Phenol	13 mM
4-aminoantipyrine	0.7 mM
Peroxidase	10 units
Glucose	0.1 M

Table 2: Glucose oxidase activity assay reagants

One part of each of the above solutions was then mixed in a petri dish. The template bound tubes were then submerged in the dish and incubated at 37ºC for 5 minutes. Then 2 mL of the solution was pipetted into a cuvette and absorbance was measured at 515 nm over 5 minutes with measurements being recorded every 10 seconds. The data was then plotted in Microsoft Excel for a visual representation of the change in absorbance over time.

12 Results

12.1 Best Chemical for Dissolving Templates

Solvent (10 mL Each)	**Time to Completely Dissolve (Hours)**	**Precipitate After Dissolving (Yes/ No)**
0.1M NaOH	< 3 (48+ with NTS crosslinked w/ GA)	No (Yes with NTS crosslinked w/ GA)
0.1M HCl	48+	No
0.5M H_3PO_4	24+	No

Table 3: Chemical results for dissolving templates

Given the results from these tests, 0.1M NaOH was chosen as the best chemical for dissolving the AAO templates. The only times it failed to completely dissolve the template was in the presence of avidin NTS crosslinked with GA. Additionally, other instances outside of this test had shown a precipitate to form when using 0.1M NaOH.

12.2 Avidin LbL Nanotubes Crosslinked with GA

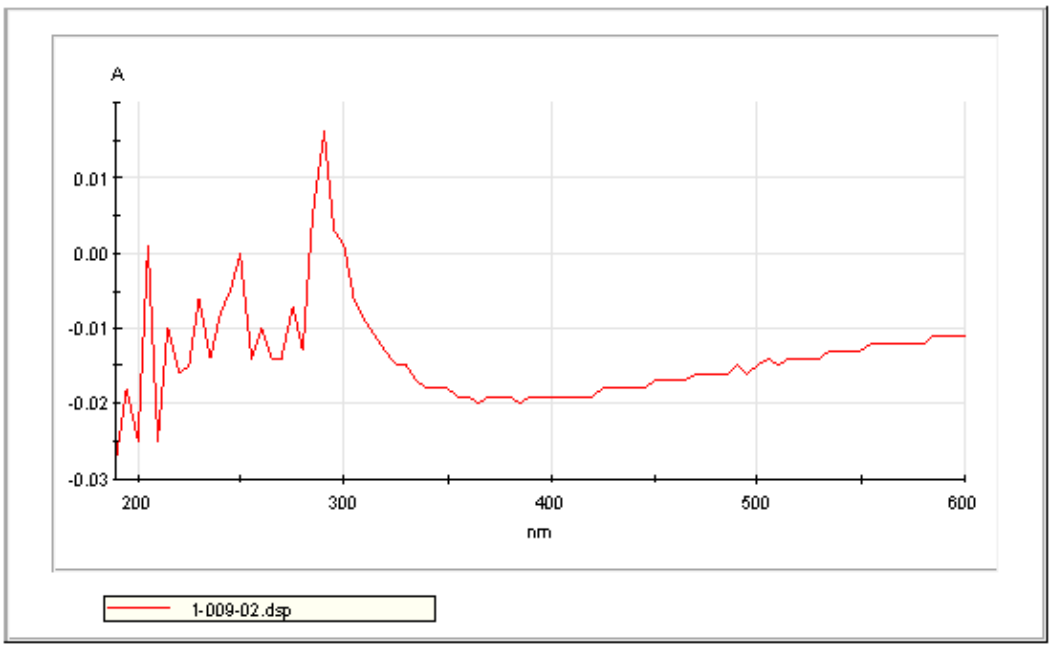

Figure 6: 5 Layer Avidin NTS Absorbance Scan

The peak absorbance at 290 nm does indicate the presence of avidin in this early attempt at avidin nanotubes. The peaks preceding the 290 nm peak are in stark contrast to the nearly flat area before the 290 nm peak in Figure 2. The peaks could be the result of loose proteins rather than avidin nanotubes. SEM images of the templates indicate no avidin nanotubes.

12.3 Avidin-Glucose Oxidase Nanotubes

Figure 7 shows a TEM image of an avidin-glucose oxidase nanotube. The template used for this particular nanotube was soaked in binding agents (phosphonic acid followed by glutaralydehyde) to protect against the NaOH used to dissolve the AAO template.

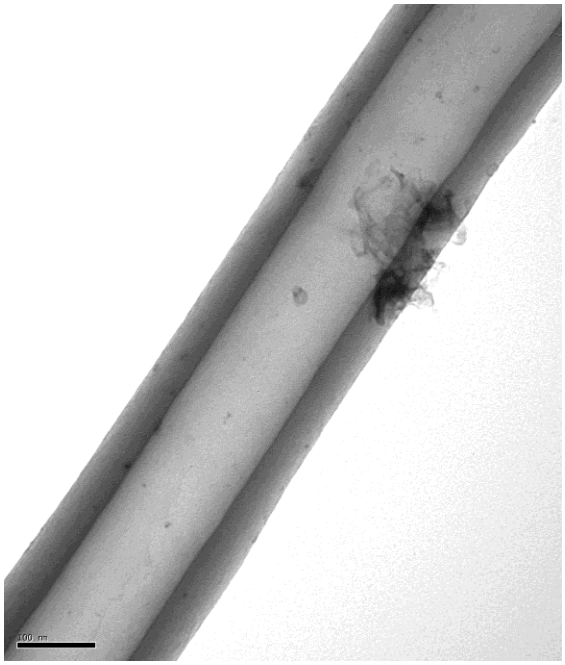

Figure 7: TEM image of avidin-glucose oxidase nanotube (scale bar represents 100 nm)

12.3.1 Concentration Test

The effects of protein concentration in the stock solution on nanotube formation were determined by UV-vis. Each solution was scanned before use between wavelengths of 250nm-500nm. The graphs generated from these scans are seen below. A scan was also performed on a 50% Avidin – 50% Glucose solution to represent the ratio of proteins in the nanotubes.

The peaks around 280 nm clearly indicate the presence of the proteins in solution. Since the Beer-Lambert Law states that absorption is directly proportional to concentration the higher peaks should correspond to higher concentrations. However, the graphs below clearly indicate a different pattern, which appears to be almost random. The 0.5 mg/mL solutions should have been between the other two curves but appear as the lowest in the scans of the individual protein solutions and then the highest in the 50-50 Avidin Glucose mix. This is contrary to any logic provided by the Beer-Lambert law. These scans should be repeated with disposable cuvettes to

check for repeatability of the results. It is possible that even though the cuvettes were thoroughly cleaned between each use there may have been some residue that affected the absorbance.

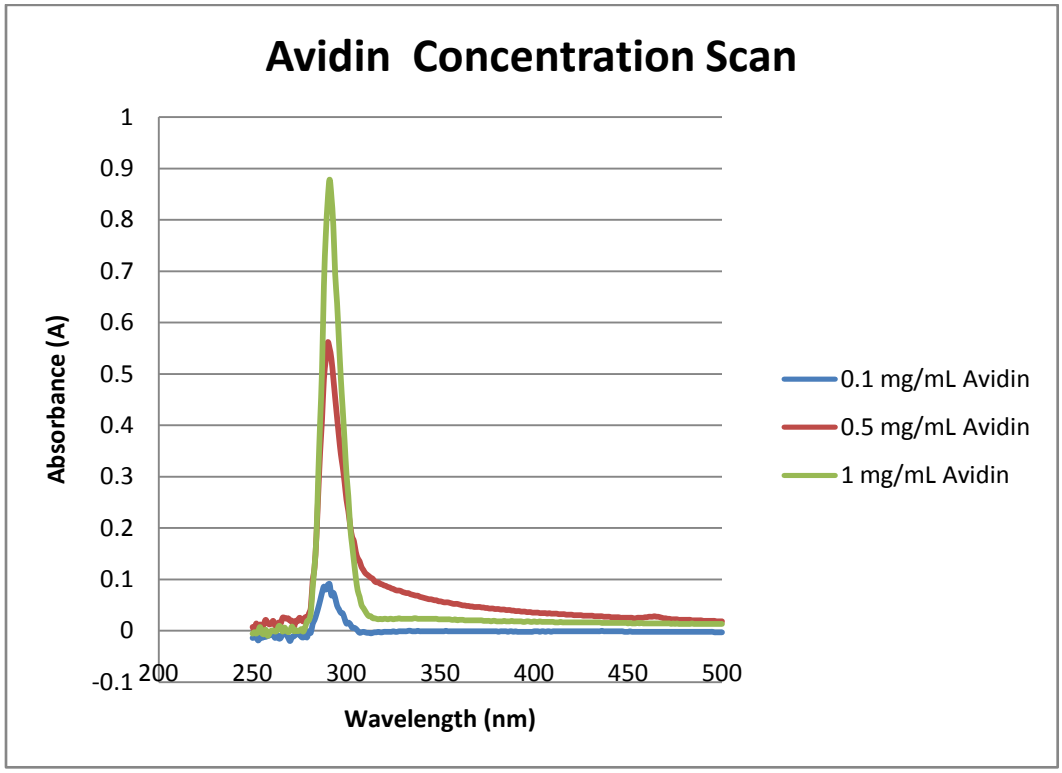

Figure 8: Avidin Stock Solution Scan of Three Concentrations

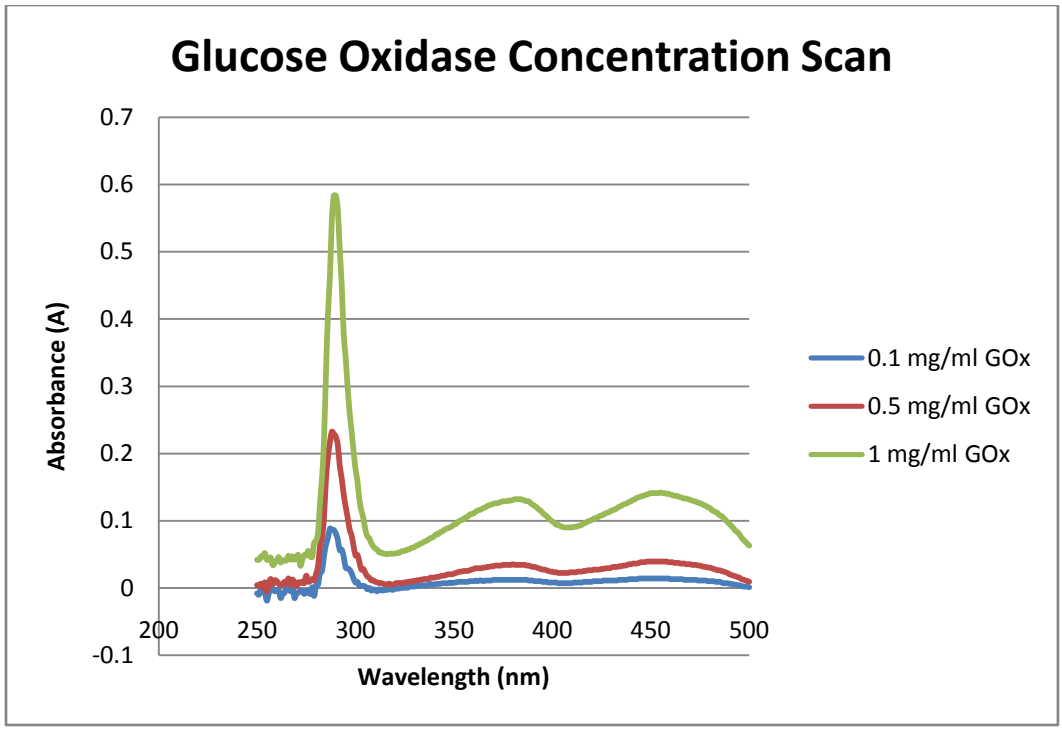

Figure 9: Glucose Oxidase Solution Scan of Three Concentrations

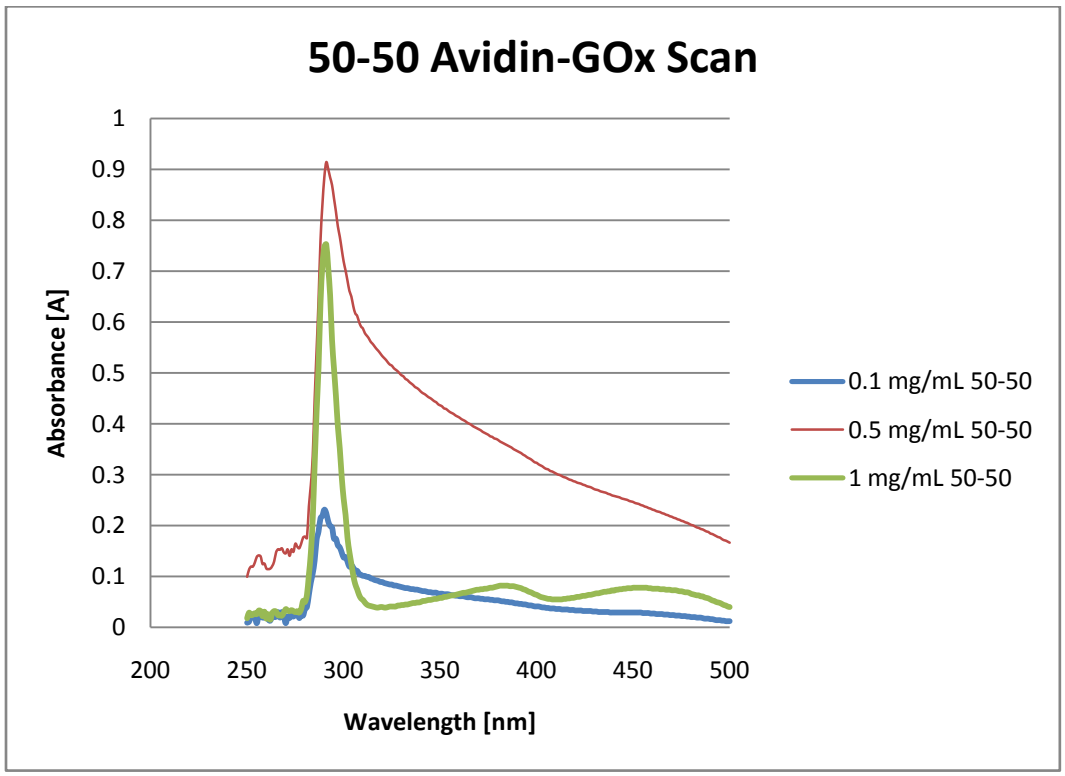

Figure 10: 50% Avidin - 50% Glucose Oxidase Scan of Three Concentrations

The absorbance rates (A/min) shown in Figure 11 are all similar, with the curve of 1 mg/ml having a slightly larger slope than the other two, thus the fastest absorbance rate. Even though its absorbance is the lowest, having the fastest absorbance rate means that the glucose oxidase catalyzed the oxidation of glucose the fastest. Therefore, the proteins in these 1 mg/mL maintained the best functionality and/or had the greatest number of exposed proteins.

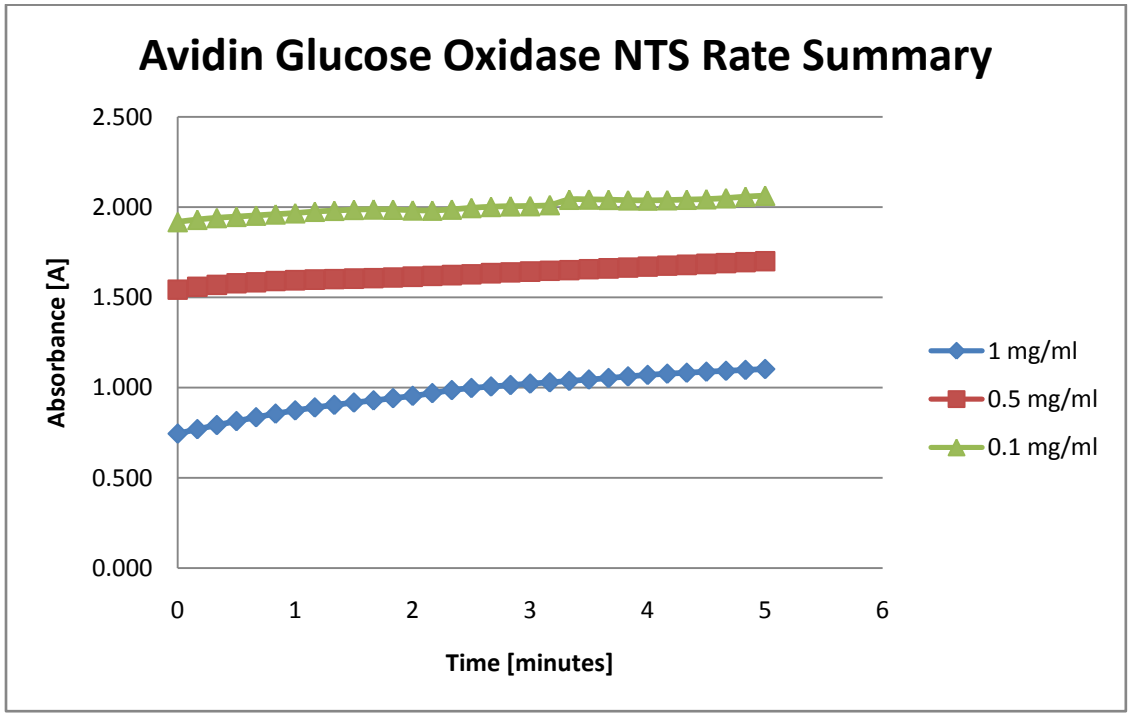

Figure 11: Nanotube Concentration Study (Note: Each curve represents the averages of rates from three templates filled with nanotubes from solutions of the same concentration.)

12.3.2 Solution Volume Test

As can be seen in Figure 12 there is an increase in absorbance from 100 µL to 200 µL. This shows that at 100 µL the protein did not have enough time to fully bind to the inner pore surface, resulting in incomplete nanotubes. However, between 200 µL and 300 µL there is a plateau representing protein saturation in the nanochannels occurring at 200 µL.

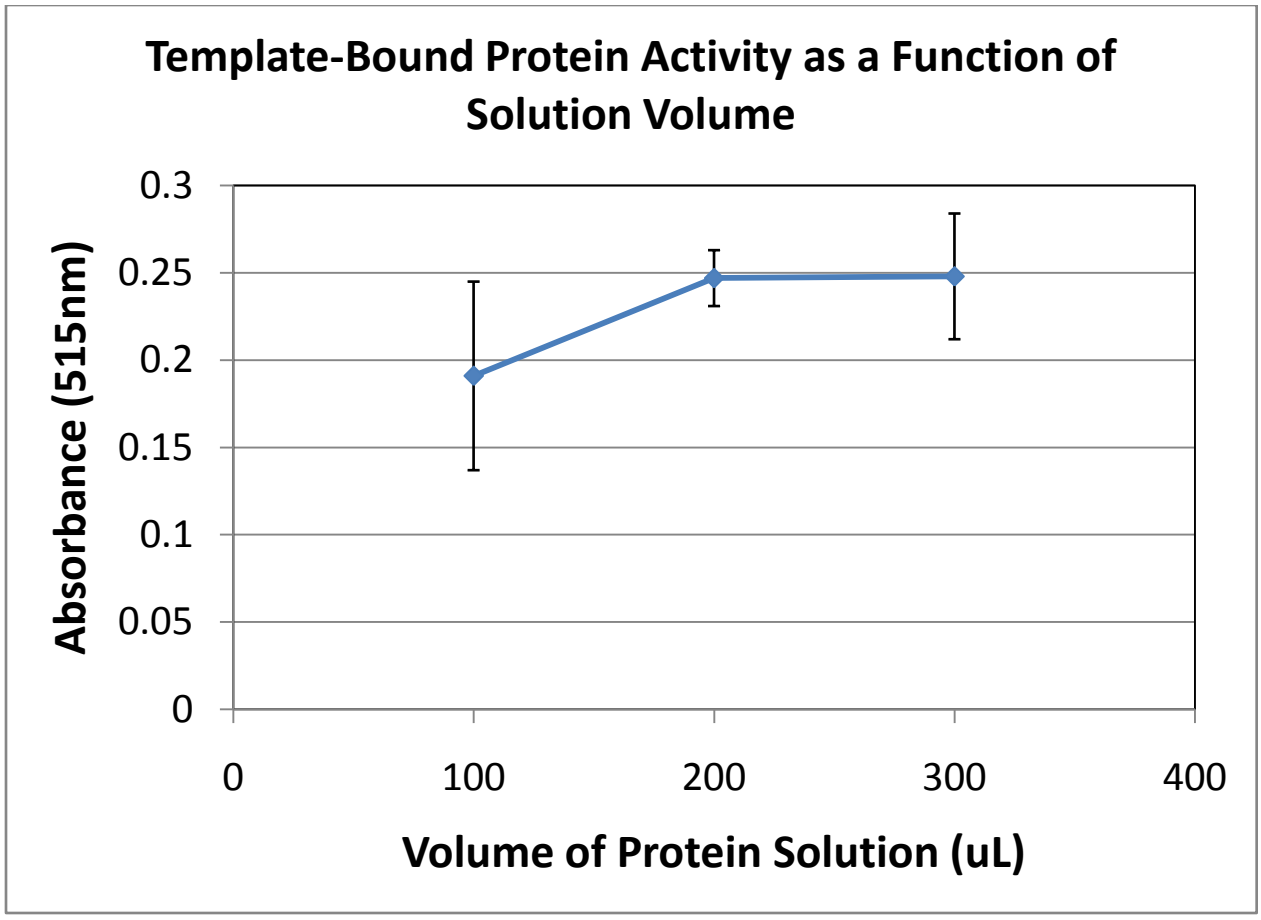

Figure 12: Solution Volume vs. Protein Activity of template bound Av-GOx NTS

12.3.3 Dry Storage Time Test

Protein activity rates show a clear decrease in the nanotubes when being stored dry. Figure 13 shows that after eleven days of being stored dry the protein nanotubes are at less than half of the activity they were directly after initial fabrication.

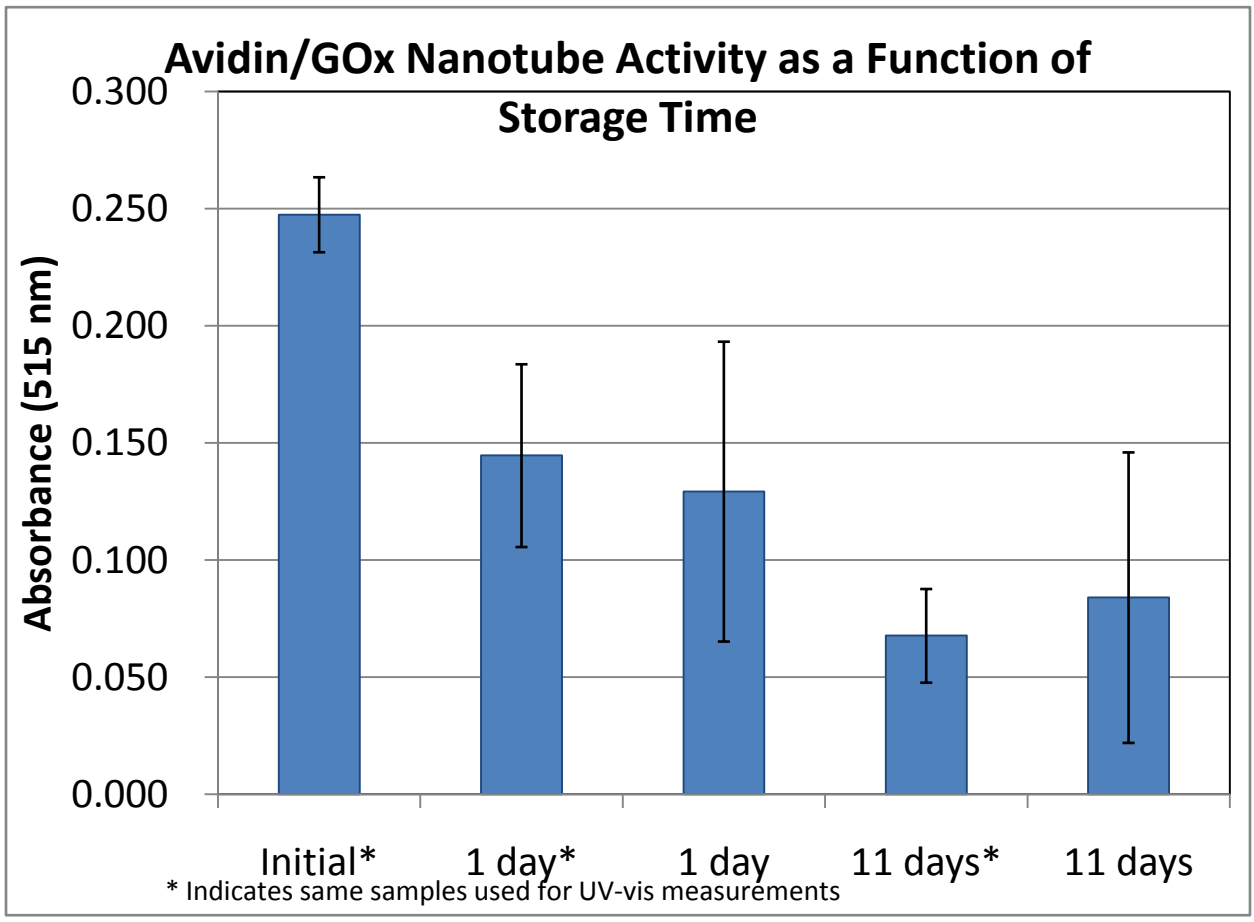

Figure 13: Av-GOx NTS activity as a function of storage time

13 Analysis and Discussion

13.1 Template Dissolving

The tests performed here indicated that NaOH was the best chemical for dissolving the AAO templates in a reasonable amount of time. However, the NaOH was too strong for the proteins and was most likely destroying the electrostatic bonds holding the protein layers together.

One approach to this problem would be to investigate new template materials. Finding the correct combination of template and solvent would be crucial in liberating the nanotubes.

Another approach would be soaking the templates in a crosslinking agent such as GA after protein deposition. This should support the weak electrostatic bonds in holding up to strong solvents.

13.2 Avidin Nanotubes

The early attempts at manipulating the charge of one protein (avidin) alone to create electrostatic bonds showed unfavorable results. At this point it is suggested that a more successful approach is manipulating the charge of two proteins with opposite isoelectric points in a solution of similar pH.

13.3 Avidin-Glucose Oxidase Process Variables

13.3.1 Solution Concentration

Absorbances measured in characterization of variable solution concentration exceeded the expected range. As a result, the data collected here was seen as invalid. The most likely cause of the flawed data was an undetermined source within the glucose oxidase activity assay

chemicals. There is a wide array of methods for calculating glucose oxidase activity. It is suggested that an alternative activity assay be used in determining the effect of solution concentration on the nanotube formation.

13.3.2 Solution Volume

It was important to determine the ideal volume of protein solution to filter through the templates each time a layer was deposited. Filtering too much solution would waste time and valuable protein; whereas, not filtering enough solution would leave the nanotubes incomplete. As seen in Figure 12, protein activity increases from 100 µL to 200 µL. This shows that the nanotubes were incomplete when 100 µL was being used. However, from 200 µL to 300 µL there is a plateau showing that the nanopores were saturated with protein at 200 µL. Thus 200 µL was seen as the ideal volume at 1 mg/mL concentration.

13.3.3 Storage Time

If protein nanotubes are to enter commercial applications their shelf-life needs to be understood. When storing the nanotubes dry within a template, our tests showed that protein activity severely decreases as a function of time (Figure 13). For any commercial application such a short shelf-life is unacceptable. Thus better storage techniques need to be investigated. Temperature and medium (liquid, gas, etc.) both need to be considered for maintaining the activity of the protein NTS.

14 Conclusions

In conclusion, fabrication of protein nanotubes is possible through a relatively simple procedure. Electrostatic charges are significant enough to initially bind protein layers together; however, they may not stand up to the rigors of strong chemicals used to dissolve AAO templates. This problem may be effectively circumvented by the use of binding agents such as GA.

It is necessary to note the effect of process variables on the final product as uniformity will be necessary for any commercial applications. Both solution volume and storage time had notable consequence on the final product.

Finally, the methodologies developed here afford the user with great control. This methodology can be applied to nearly any two proteins with significantly different isoelectric points. Also, dimensions can be controlled easily by choice of template.

15 Recommendations

It is recommended that future studies on template assisted layer-by-layer fabrication of protein nanotubes investigate the following:

1. The effect of chemicals used to liberate the nanotubes
    - It is likely that the chemicals used are too severe for protein nanotubes held together solely by electrostatic charge. New combinations of template, chemicals, and binding agents should be investigated in a manner that keeps the overall procedure simple, quick and inexpensive.
2. The scalability of this approach
    - One of the major advantages to layer-by-layer methodology is its relative speed and simplicity. It should be investigated how these qualities could aid in the large scale production of protein nanotubes if this technology were to be taken on for major commercial applications.
3. Characterization of nanotube properties
    - The net charge, solubility, cell internalization capability, and drug attachment potential of these specific nanotubes should all be investigated. Another major benefit of layer-by-layer approaches being the control that the user has over these properties (specifically dimensions). Therefore, once the properties of these specific nanotubes are known they can be tweaked to better suit their potential applications.
    - Developing a controlled cleaving process to regulate nanotube length was one of the original goals that did not come to fruition in this study. Future

studies should investigate if this is necessary and how it could be performed (mechanical shearing, etc.).

16 Glossary

Avidin *(n.)*: A protein, found in the white of egg that combines with and prevents the action of biotin, thus injuring the animal that consumes it in excess by producing biotin deficiency. (http://dictionary.com)

Glucose Oxidase *(n.)*: An enzyme which converts glucose into gluconic acid and hydrogen peroxide (H2O2). It is used to help diagnose diabetes. (http://cancerweb.ncl.ac.uk/cgi-bin/omd?glucose+oxidase)

Glutaraldehyde *(n.)*: A compound $C_5H_8O_2$ that contains two aldehyde groups and is used as a disinfectant and in fixing biological tissues. (http://dictionary.com)

Isoelectric point *(n.)*: The pH at which a protein would have a net neutral charge.

17 Bibliography

[1] Shi Kam NW, Dai H. Carbon nanotubes as intracellular protein transporters: Generality and biological functionality. J. Am. Chem. Soc. 2005; 127: 6021-5026.

[2] Biano A, Kostarelos K, Prato M. Applications of carbon nanotubes in drug delivery. Current Opinion in Chemical Biology. 2005; 9:674-679.

[3] Cherukuri, P.; Bachilo, S. M.; Litovsky, S. H.; Weisman, R. B. J. Am. Chem. Soc. 2004, 126, 15638

[4] Bianco, A.; Hoebeke, J.; Godefroy, S.; Chaloin, O.; Pantarotto, D.; Briand, J.-P.; Muller, S.; Prato, M.; Partidos, C.D. J. Am. Chem. Soc. 2005, 127, 58.

[5] Dougherty, S.; Liang, J. Template-assisted Fabrication of Protein Nanocapsules. Worcester Polytechnic Institute.

[6] Tian Y, He Q, Cui Y, Li J. Fabrication of protein nanotubes based on layer-by-layer assembly. Biomacromolecules. 2006; 7: 2539-2542.

[7] Lu G, Ai S, Li J. Layer-by-layer assembly of human serum albumin and phospholipids nanotubes based on a template. Langmuir. 2005; 21: 1679-1682.

[8] Hou S, Wang J, Martin CR. Template –synthesized protein nanotubes. NanoLetters. 2005; 5:231-234.

[9] Baker LA, Jin P, Martin CR. Biomaterials and biotechnologies based on nanotube membranes. Crit. Rev. Solid State Matter. Sci. 2005; 30: 183-205.

[10] K Fuwa, BL Valle. Physical Basis of Analytical Atomic Absorption Spectrometry. The Pertinence of the Beer-Lambert Law. Analytical Chemistry, 1963 - pubs.acs.org

[11] Avidin Product Sheet. Sigma-Aldrich.
http://www.sigmaaldrich.com/sigma/product%20information%20sheet/a9275pis.pdf

[12] Glucose Oxidase Product Sheet. Sigma-Aldrich.

http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/G6641

[13] Putnam D. Polymers for gene delivery across length scales. Nature Materials. 2006; 5:439-451.

[14] Pantarotto D, Briand JP, Prato M, Bianco A: Translocation of bioactive peptides across cell membranes by carbon nanotubes. Chem Commun (Camb) 2004;16-17.

[15] Shi Kam NW, Jessop TC, Wender PA, Dai H: Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into mammalian cells. J Am Chem Soc 2004, 126: 6850-6851.

[16] Spector MS, Selinger JV, Singh A, Rodriguez JM, Price RR, Schnur JM. Controlling the morphology of chiral lipid tubules. Langmuir. 1998;14:3493-3500.

[17] Audette GF, van Schaik EJ, Hazes B, Irvin RT. DNA-binding protein nanotubes: learning from nature's nanotech examples. Nano Letters. 2004; 4:1897-1902.

[18] Matsumura S, Uemura S, Mihara H. Construction of biotinylated peptide nanotubes for arranging proteins. Mol. BioSyst. 2005;1:146-148.

[19] Kol N, Adler-Abramovich L, Barlam D, Shneck RZ. Self-assembled peptide nanotubes are uniquely rigid bioinspired supramolecular structures. Nano Letters. 2005; 5: 1343-1346.

[20] Oberdörster, Gunter, Jan Oberdörster, and Eva Oberdörster. "Nanotoxicology: an Emerging Discipline Evolving From Studies of Ultrafine Particles." Environmental Health Perspectives 113 (2005): 823-839.

[21] Mr, McDevitt, Chattopadhyay D, Kappel Bj, Jaggi Js, Schiffman Sr, and Antczak C. "Tumor Targeting with Antibody-Functionalized, Radiolabeled Carbon Nanotubes." J Nucl Med. (2007) 48(7):1180-9.