SURFACE MODIFICATION OF CARBON STRUCTURES

FOR BIOLOGICAL APPLICATIONS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Engineering

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Elizabeth Irene Maurer ENTITLED Surface Modifications of Carbon Structures for Biological Applications BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Engineering

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ABSTRACT

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Carbon substrates have a wide variety of applications, many of which are enabled by appropriate surface modifications. In particular, the use of carbon-based substrates for biological devices can be quite advantageous due to their relative inertness and biocompatibility. Moreover, graphitic carbon can take many forms ranging from flat sheets to foams, fibers, and nanotubes. In this project, larger carbon substrates such as microcellular foam and flat graphite have been modified with carbon nanotubes, and their potential use in two types of biological applications was tested. The first study involved an investigation of the growth and proliferation of osteoblast cells on carbon, so that such structures can be evaluated for possible use as a scaffold for *in-vivo* tissue regeneration. The surface modifications that were compared are a collagen coating, a silica film, and a strongly adhered carbon nanotube layer. It was seen that the attachment of carbon nanotubes led to the highest density and viability of osteoblast cells on the surface indicating their potential benefit in implant and cell scaffolding applications. In the second study, carbon nanotubes were attached on the graphite, and subsequently decorated with gold nanoparticles and a ribonucleic acid (RNA) sequence. These nano-structures show advantages in detecting the DH5α *E. coli* bacterial strain, indicating potential use as a biosensor. Proof-of-concept results indicate increased attachment of gold nanoparticles coated with an RNA capture element compared to uncoated particles onto the *E. coli*. This demonstrates the potential use of this concept in creation of a

multi-array sensor for fast and sensitive detection of many types of pathogens. These results clearly show that attachment of carbon nanotubes on larger carbon substrates can provide the basis for several unique biological devices.

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Finally, I wanted to express my gratitude towards Wright State University for the excellent quality of education and research experience I received there which will greatly help me in my future as a Materials Science Engineer.

DEDICATION:

I would like to dedicate this work to my parents, Jack and Deb, as well as to my siblings, Eric, Tony, and Trisha. Without their support, encouragement, occasional sarcasm, and much optimism, I would never have been able to accomplish so much.

1. Introduction

Carbon is a group 14 element, which is nonmetallic, forms different allotropes, and is quite abundant in nature [(1)]. At high temperatures it can react with oxygen, sulfur, silicon and other metals, though at low temperatures it is stable and chemically inert. It has been used in applications ranging from pencil lead and printing ink, to graphene sensors [(2)] and nanofiber devices [(3)]. It can take many forms due to unique carbon bonding, one of which is carbon nanostructures [(4)]. These nano-structures can take many forms and have unique properties such as small size, attractive electronic properties, high surface area, strength, and chemical inertness [(5)]. It is also known to be biocompatible in devices [(6)]. Because of this, carbon is an ideal material for biology based applications.

For this thesis project, carbon structures were utilized to enhance their function in biological applications. This was done through modification of the surface with carbon nanotubes, nano-layers, and nanoparticles. Two applications and their platforms' development will be discussed in detail. The first application, tissue regeneration, was conducted on a microcellular carbon foam substrate that was modified with carbon nanotubes, which has recently been published [(7)]. The toxicity of this substrate was compared to other surface modifications as well as the uncoated foam to determine if enhancement of the structure could be observed. This work is quite significant compared to other tissue scaffold studies in that not only is a porous substrate one that can facilitate nutrient flow throughout the substrate to the cells, but the addition of carbon nanotubes,

which could increase the overall strength of the scaffold, has not yet been adequately investigated.

The second study was on the development of a biosensor platform for the detection of the bacterial strain, *Escherichia coli*. This was accomplished through modifying the surface of graphite with carbon nanotubes decorated with gold nanoparticles as well as a RNA capture element. The novel aspect of this study was utilizing nano-materials to not only enhance the electrical properties of the overall sensor, but also to greatly enhance the surface area to allow for more attachment sites, aiding in the sensitivity of pathogen detection.

From these two studies, it will be shown that with the modification of carbon structures with carbon nanotubes, the overall substrate is enhanced for a promising future in biological applications.

2. Literature Review

2.1 Biological Applications of Carbon Substrates

Carbon has been used in many types of biological applications [8] [9]. This is due to its relative inertness as well as biocompatibility. The ability for carbon to be utilized in many types of applications is due to the wide variety of structures that carbon atoms can form. Sheets of carbon atoms that are arranged in a honeycomb-like pattern that are oriented in parallel with one another form the structure called graphite. Graphite is a very inert material, which has led to its use as a base material for various biological coatings, such as graphite coated with pyrolitic carbon for use in mechanical heart-valve prostheses [10].

Graphitic carbon foam is a relatively new material with potential applications in heat sinks, radiators, and net-shape aerospace composites due to its interconnected porosity and low density, coupled with controllable thermal and electrical properties [11] [12]. Another configuration that has been developed is carbon fibers. These can be woven into long threads for commercial use because of their high strength. One such study performed on carbon fiber/carbon composite was for implant use [13] because of this enhanced strength property.

A structure, which has been fast emerging in technology recently, is carbon nanotubes. Iijima was one of the first to report on this structure back in 1991 [14]. This formation is a unique configuration of carbon atoms in a tubular arrangement and is on the nanometer scale, thus giving it the name "nanotube". This configuration consists of a single sheet of carbon rolled into a tube to form a single-walled carbon nanotube. Concentric sheets of graphene tubes create what is known as a multi-walled carbon nanotube. Because of this unique arrangement of carbon atoms, small size, and high surface area, strength, and conductivity, carbon nanotubes have been widely researched in the biological and biomedical fields, which will be described in detail in section 2.2.

Surface modifications of these carbon structures are being used for the advancement of materials in a wide array of studies using various techniques. For use in absorbing organic and inorganic pollutants, activated carbon was studied by Chingombe and colleagues [15].

2.2 Biological Applications of Nanotubes

As described previously, nanotubes have advantageous properties which make them extremely valuable in biological applications. Because of its hollow nature, the nanotube has the potential for use in drug delivery. In one study, carboplatin, a cancer treatment agent, was encapsulated in carbon nanotubes [16]. These nanotubes then were introduced into cancer cells and subsequently cell viability was measured. They

observed an inhibition of the cancer cell growth in those exposed to the filled nanotubes with respect to those exposed to unfilled nanotubes.

Not only do nanotubes have the potential to be filled, but because of their high surface area to volume, there are more attachment sites for chemicals, biochemicals, and even other nanoparticles to their surface. This can be exploited in many ways ranging from electronics and filtration to biological systems and biomedical devices.

Kam and colleagues utilized single walled carbon nanotubes in their study to selectively destruct cancerous cells [17]. In this study, they exploited the property that carbon nanotubes absorb in the near infrared range while inside cancerous cells. Because of this absorbance, the nanotubes' temperature increases, and ultimately induces cell death.

2.3 Porous Carbon Used as Tissue Scaffold

Osteoblasts are derived from the mesenchymal stem cell, and differentiate into osteoclasts or osteocytes to eventually produce bone or cartilage if the necessary growth factors are present. Although these cells are able to produce healthy bone, in the case of osteoporosis or arthritis, bones cannot function correctly or be produced fast enough, therefore requiring some sort of scaffolding to support and enhance cell growth and function.

Porous materials infiltrated with bone cells can be very beneficial as future implants which can facilitate in-growth of human bone and promote osteo-integration.

Bioengineering of these substrates includes designing and modifying new materials to promote and enhance bone cell growth, while remaining non-toxic to surrounding tissues. Surface modifications will facilitate a more stable bone to implant interface without becoming loose over time because of the variation in surface structure. Recent studies have been performed on a range of materials that have been manipulated to be porous such as silicon and various polymers [18], [19], [20]. Porous materials are currently under investigation in different laboratories in order to simulate human bone and promote osteointegration, and their preliminary studies show that surface roughness plays a large part in this process [21], [22].

In addition to substrate structure, coatings on substrate surfaces play a large role in cell growth. For example, collagen has been shown to increase bone formation [23], in addition to providing ductility and adding toughness to the structure [24]. This shows that surface modifications can be utilized in order to improve cell adhesion [25] as well as enhancing many other properties.

Although there are many varieties of implants being researched, there have not been any studies conducted on microcellular carbon foam as a potential scaffold. This is an important material to consider because carbon has been shown to be biocompatible [26] and is presently available as porous foam [27]. Several surface modification techniques on these shapes have been developed for multiple applications [28], but whether they facilitate bio-compatibility and cell growth is not known. Recently, a few studies have appeared on the possible use of bare carbon foam [29] and titanium-coated carbon foam [30] as cell implant materials, both of which indicate reasonable biocompatibility and absence of toxicity.

At this time, a more detailed study is possible, which can identify possible surface modification approaches tailored for specific biological environments. Such an investigation will provide effective assessment and development of this material for specific implants. This study is aimed at addressing their interaction with bone cells, and the main goals are the following: (i) determine if the cellular graphitic foam facilitates osteointegration and aids in healthy, stable bone growth with the differentiation of osteoblasts, and (ii) investigate the effects of carbon nanotube coated foam and compare cell growth with other various coatings on microcellular foam. Monitoring of cell morphology has been performed via fluorescence imaging and scanning electron microscopy, and changes in cell viability were obtained through a biochemical assay.

2.4 Carbon Nanotubes in Bacterial Sensing

Many pathogens are found in our everyday environment. Some of which are not harmful, but occasionally infectious bacteria contaminate surfaces which can spread rapidly. Common types of infections that affect thousands of people are from *E. coli* (70,000 infections a year) [(31)], as well as another well known bacterium *Salmonella* (40,000 cases a year) [(31)]. These are just two of the many types bacteria that the potential to spread quickly. One less common bacteria that has the potential to be used as biological weapon is *Bacillus anthracis* which causes the disease Anthrax. Though this pathogen does not spread rapidly, it is much more harmful. In 2001 one half of all inhalation victims died [(31)].

Because of the likelihood that the pathogens could spread quickly and/or be extremely harmful, early detection is needed. Current methods are slow with culture times taking around approximately 48 hours before proper identification of the pathogen can be determined. Experimentally this culturing time has been able to be reduced to 6 hours and Fourier Transform Infrared Spectroscopy conducted to detect *E. coli* [32]. Although this is quite a breakthrough in detection time, advances still need to be made in the development of a sensor in order to prevent infection from these quickly spreading diseases.

It is well known that carbon nanotubes have excellent electrical properties [33] which make it so appealing for use in sensing systems. Because most, if not all, of the atoms in a carbon nanotubes are on or near the surface, any absorption of molecules to its surface will change the electrical properties of it [34]. This unique property makes these structures suitable for bio-sensing applications.

A biosensor, as described by the International Union of Pure and Applied Chemistry, is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element [35].

There are numerous types of sensors currently being developed in the detection of various analytes. Carbon nanotubes are one platform being widely investigated. One such application is in gas detection through changes in the dielectric constant [36]. Nanotubes have also been utilized for use as a reagentless biosensor through electron

transfer of glucose oxidase [37]. Not only have nanotubes been used in detection of chemicals, whole-cell detection is essential for the development of a bacterial sensor. When real-time detection is needed, having to do little to no sample preparation before loading the analyte on the sensor is quite essential. One study done on whole-cell detection was conducted through deposition of antibodies specific for *Salmonella* on a piezo-electric crystal [38]. Utilizing novel materials such as carbon nanotubes and combining it with biological detection agents, a unique and novel biosensor can be developed for bacterial sensing.

For this study, a carbon substrate, graphite, is used as the base component for the sensor. It is then coated with a silica layer which is the foundation for carbon nanotube growth. Gold nanoparticles were synthesized onto the surface of the nanotubes to increase surface area as well as provide an attachment point for a ribonucleic acid (RNA) sequence. This sequence was found to attach specifically to the surface of the bacterial strain DH5α *E. coli*, though this capture element could be extended to different pathogens and placed on separate sections to produce a multi-arrayed sensor. The sensor composite is shown in Fig 1.

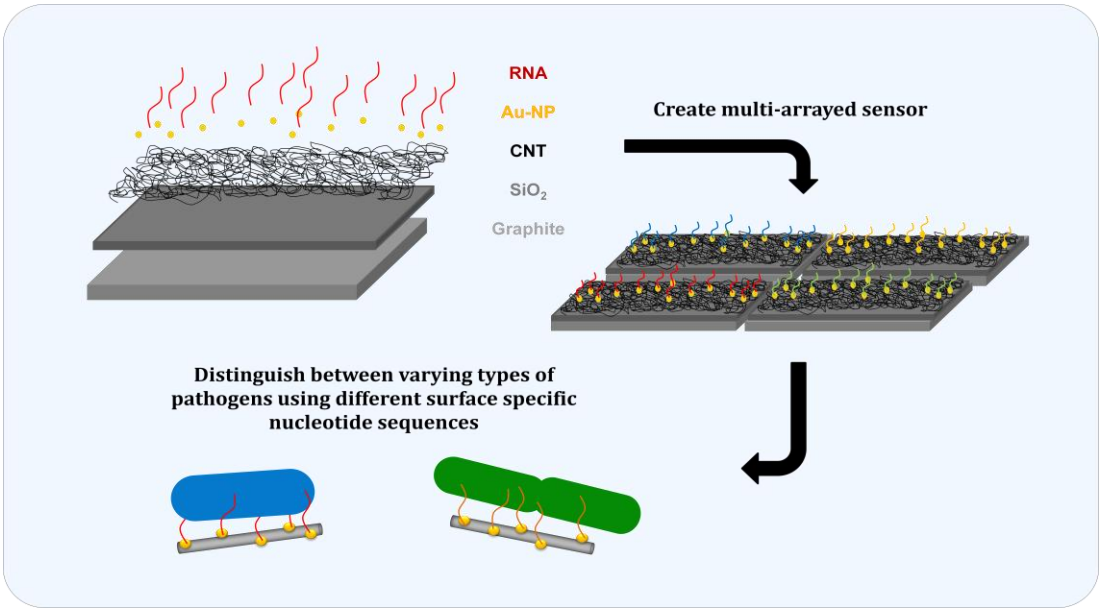

Fig1. Schematic showing each component/layer of the sensor as well as end result of research project.

3. Experimental

3.1 Carbon Substrates

3.1.1 Preparation of Microcellular Carbon Foam

Microcellular carbon foam samples were generously supplied by Koppers Inc. (Pittsburgh, PA) in large blocks and then cut down to 1x1x0.2 cm cubes. The foam used (L1a) has a pore diameter of ~ 500µm, 78% porosity, and a density of 0.34 g/cc. They were rinsed with Phosphate Buffered Saline (PBS, Invitrogen, Carlsbad, CA), an isotonic water based salt solution in order to remove any debris, and were then sterilized under ultraviolet light prior to the seeding of cells.

The foam samples were then placed in 24-well culture plates and adhered to the bottom of each well with double sided carbon tape to keep the foam submerged in cell media during incubation periods.

3.1.2 Preparation of Graphite

Highly oriented pyrolitic graphite sheets (Molecular Imaging) were cut and sanded into 1x5x0.2cm rectangles. Before coating the surface with silica, the top of the substrate was rubbed with lint-free paper to remove any non-adherent graphite which will

ensure a strong bond between the silica and graphite. The coating process was done in a microwave plasma reactor at 300W power in which a mixture of oxygen and HMDSO ($C_6H_{18}OSi_2$) were exposed to the graphite sample. The silica coated graphite was then placed in a chemical vapor deposition furnace for carbon nanotube growth.

3.2 Nanotube Growth

A nano-hair type layer of strongly grafted carbon nanotubes (CNT) used to create a hierarchical structure was fabricated on these surfaces using a two-step process developed recently at Wright State University [39]. This involves activation of the foam surface with plasma-derived silica, followed by Chemical Vapor Deposition (CVD) of CNT using a Xylene-Ferrocene solution as well as Hydrogen in a two-stage furnace.

To prevent oxidation at high temperature, argon was flowed through the furnace throughout the growth process. The temperature was increased to 800°C, and a mixture of Ferrocene ($C_{10}H_{10}Fe$) and Xylene ($C_6H_4C_2H_6$) were injected at a temperature of 235°C with the addition of hydrogen gas. The sample was kept at 800°C for a specific length of time and then left to cool to room temperature while still being kept in the argon atmosphere. Scanning electron microscopy images of these samples are shown in Fig 2 and Fig 3 on carbon foam and Fig 5 on graphite.

3.3 Cell Growth in a Porous Microcellular Structure: Influence of Surface Modification and Nanostructures

3.3.1 Preparation of Films

Three types of surface modifications were investigated in this study: (i) Inorganic hydrophilic coating (Silicon dioxide), (ii) Organic macromolecular coating (Collagen), and (iii) Carbon nanotubes grown on the foam surface.

Silicon dioxide (SiO_2) on the surface of the microcellular foam was prepared at Wright State University via plasma coating process. Details of this deposition process, as well as chemistry and morphology of resulting nanolayer have been discussed in earlier publications [(40)].

Collagen solution was purchased from Upstate Biotechnology (Lake Placid, NY), and diluted (.4% solution of 3.75 mg/mL) in sterile phosphate buffered saline (PBS, Invitrogen). The uncoated foam samples were immersed in the diluted collagen solution under UV light for about 20 min. After a one hour incubation at room temperature the collagen solution was removed and the samples were left to air dry for 1-4 hours in a sterile hood.

Typical morphology of these coatings on the foam is shown in Figures 2 and 3.

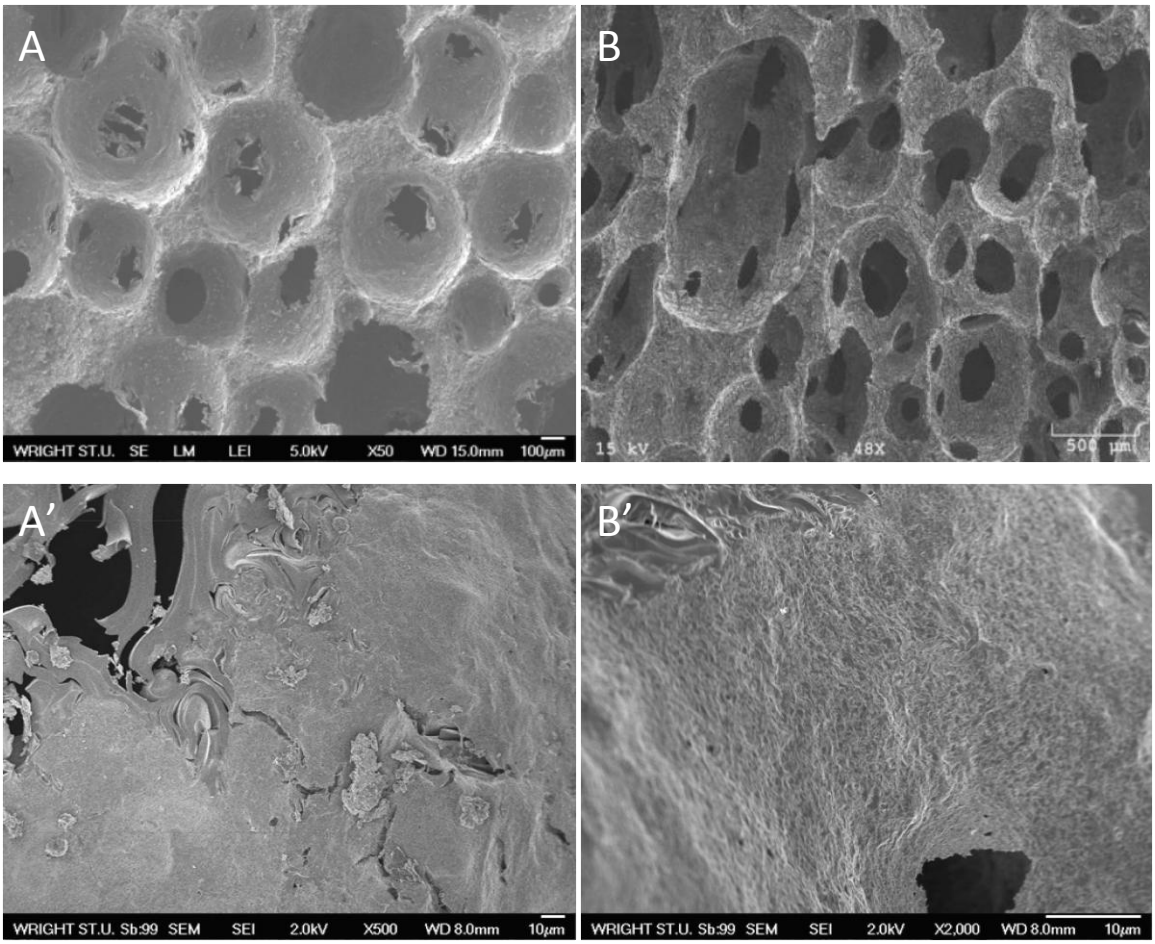

Fig 2. SEM images of Uncoated foam (A), and Silicon Dioxide (B) coated foam. Images with Standard low magnification of 50 X are in the upper level, while higher magnification pictures of Uncoated (A') and Silicon Dioxide (B') showing texture of pore walls are shown below.

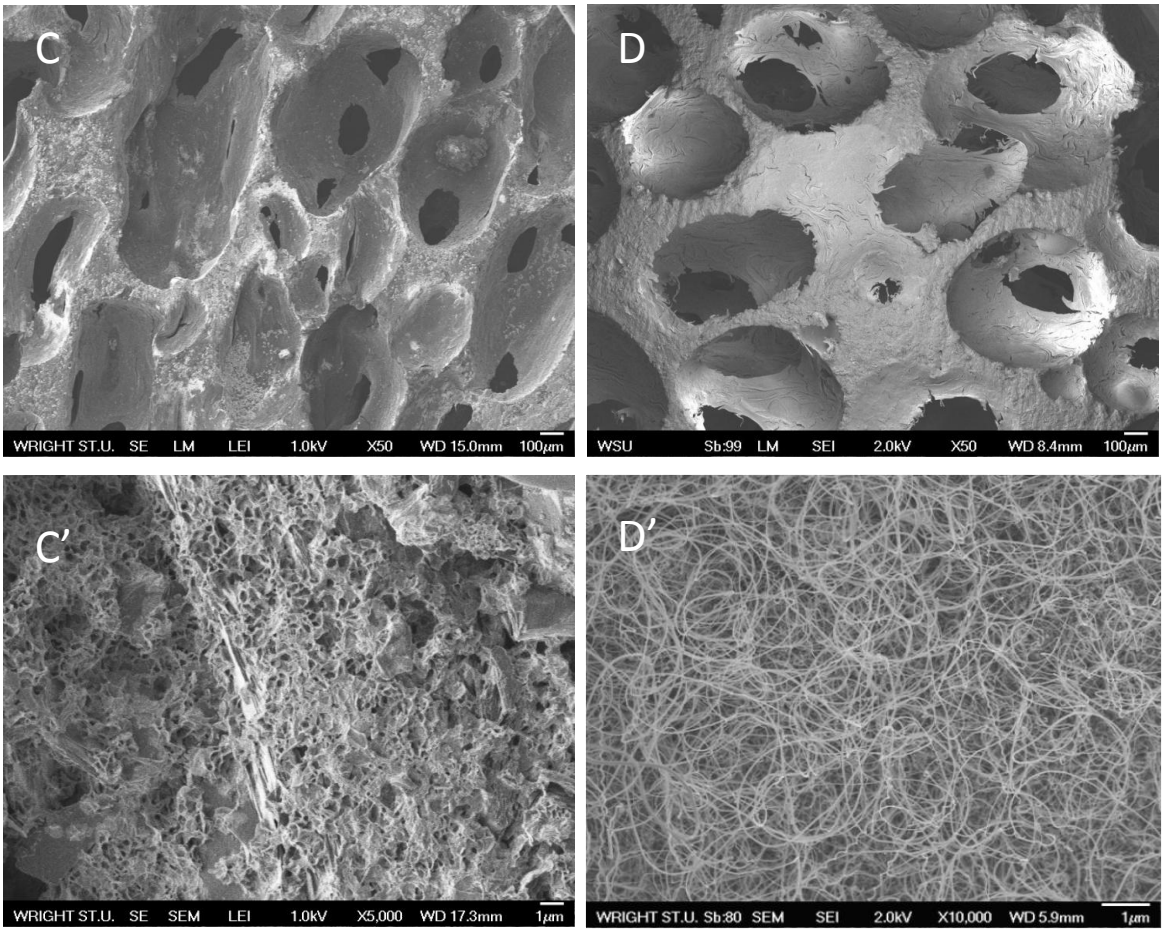

Fig 3. SEM images of Collagen Coated (C) foam, and Carbon Nanotube (D) Coated foam. Images with Standard low magnification of 50 X are in the upper level, while higher magnification pictures of Collagen (C') and Carbon Nanotubes (D') showing texture of pore walls are shown below.

3.3.2 Cell Culture

Human fetal osteoblast cells (hFOB 1.19, ATCC, CRL-11372, Manassas, VA) were cultured in 25-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 in air. Media for these cells consisted of a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium(DMEM, Gibco, BRL, Gaithersburg, MD) which was supplemented with 10% fetal bovine serum (FBS, Gibco BRL), and .3 mg/mL of G418 disulfate salt(SIGMA, St. Louis, MO). Fresh media was added approximately every two days. When the Osteoblasts were confluent, they were trypsinized with 0.25% trypsin (Sigma) diluted in PBS. To determine cell density, the cells were counted in a standard hemocytometer. The suspended cell solution was then centrifuged at 1000 rpm for 5 minutes and re-suspended in fresh medium.

For each following assay, a 1.5 mL of hFOB cell suspension containing 1 x 10^6 cells/ml was seeded in 24 well plates containing the uncoated and coated microcellular foam samples as well as an empty well which will later be used as the "control". Fresh culture medium was replaced on the second day of the incubation period during these experiments. After a 72 hour incubation time, the medium was removed and the samples rinsed twice with PBS.

3.3.3 Cell Viability/Cell Function

Cell viability was measured using the CellTiter 96 ®AQueous One Solution assay from Promega(Madison, WI). The solution contains a tetrazolium compound [3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). After the cells were grown for 72 hours, the MTS solution was added to each well containing FBS deprived medium. This was used so the FBS did not interfere with the reading. They were then incubated for one hour in which the media turned a reddish color which was due to a reduction of the chemical in the solution. This solution was removed and placed in a 96-well plate and positioned in a BioTek plate reader to record the absorbance. The quantity of formazan product is measured by the amount of 490 nm absorbance and is directly proportional to the number of living cells in culture.

3.3.4 Cell Morphology

To view the overall cell morphology as well as substrate, microscopy preparation was conducted. After the samples were incubated with cells for 72 hours and rinsed twice with PBS, the cells were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) diluted in PBS, and then dehydrated through a series of ethanol dilutions (50, 70, 90, and 100% ethanol in water for 15 minutes each). Following drying, the samples were mounted on a brass specimen holder with carbon tape and viewed at various magnifications on a JOEL JSM – 7401 Field Emission Scanning Electron Microscope (Tokyo, Japan).

In order to study nuclear sizes and density of cells, fluorescence labeling was performed. For fluorescence imaging, the cells were grown on the carbon foam samples for 72 hours and rinsed twice with PBS. The cells were then fixed with 4%

paraformaldehyde (Electron Microscopy Sciences) at room temperature for 10 minutes. The cells were rinsed with PBS and permeabilized with 0.1% Triton X-100(Fischer Scientific, Pittsburgh, PA) for five minutes. After rinsing twice the cells were immersed in 1% Bovine Serum Albumin (BSA, Thermo-Fisher Scientific) for 20 minutes. The actin filaments were then stained with Alexa Fluor 555-phalloidin from Invitrogen for 45 minutes. The samples were then rinsed with PBS and a few drops of Prolong Gold Reagent with DAPI nuclear counter stain (Invitrogen) were added. The foam samples were then flipped upside down into a separate empty well so as to view the cells after curing for 1 day at 2-6°C in the dark and imaged on the Olympus IX71 Inverted Fluorescence Microscope.

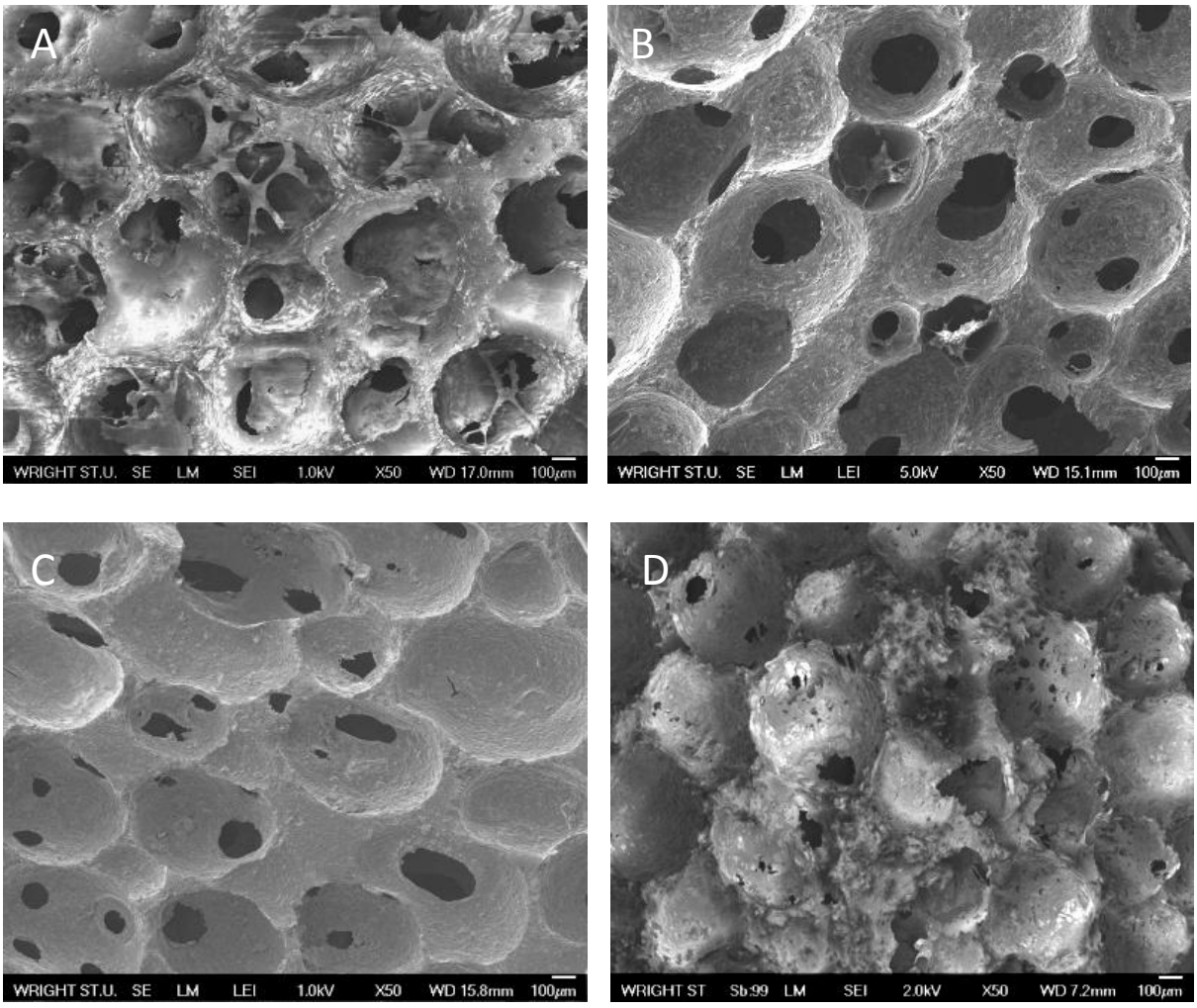

Fig 4. SEM images of cells grown on various coatings on foam: (A) Uncoated foam, (B) Silicon Dioxide coated foam, (C) Collagen Coated foam, and (D) Carbon Nanotube coated foam. Pictures are of the cells grown on each of the coated foams for 72 hours.

3.4 Functionalization of Nanomaterials for Biosensor Applications

3.4.1 Gold Nanoparticle Attachment to Carbon Nanotubes

Graphite samples with carbon nanotubes grown on the surface were cut into 1x5x0.2mm pieces. They were then attached to the sides of a 150ml beaker with double sided carbon tape. This was conducted so that the solution could still be mixed with a magnetic stir bar on the bottom of the beaker without contacting the samples. Next, 43.75mL of water was then added to the beaker and heated to room temperature while stirring for 3 minutes. The heat was then turned off and 3mL of a 0.4% $HAuCl_4$ (MP Biomedicals LCC) in water solution was added to the heated water. After approximately one minute, a 5mL solution of 0.1% $Na_3C_6H_5O_7$ (Fischer Scientific) and 0.01% $NaBH_4$ (Fischer Scientific) was also combined, which instantly turned the solution from a pale yellow, to maroon in color and was continuously stirred for 2 minutes. The gold colloid solution was removed from the beaker and the samples, still attached to the side of the beaker, were rinsed with distilled water for 3 min. After removal of the water, the samples were then left to dry. SEM images of the gold nanoparticle coated nanotubes are shown in Fig. 5

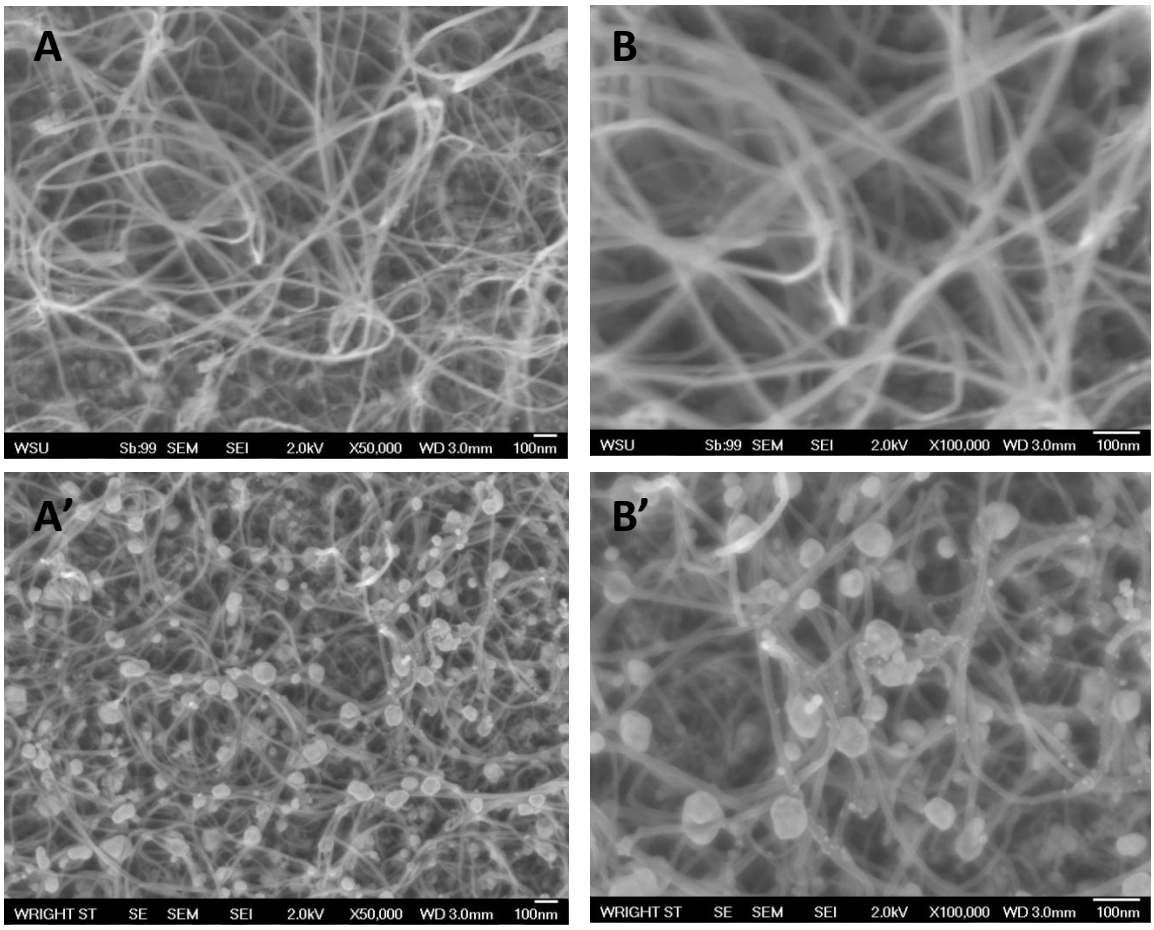

Fig 5. Scanning Electron Microscopy images of carbon nanotubes from the chemical vapor deposition process: (A&B) before, and (A'&B') after gold nanoparticle attachment.

3.4.2 DNA Transcription

The surface-specific RNA sequence used in this study was discovered by Hye-Mi So and colleagues [41] using a systematic evolution of ligands by exponential enrichment (SELEX) process. The sequence is shown in Fig 6.

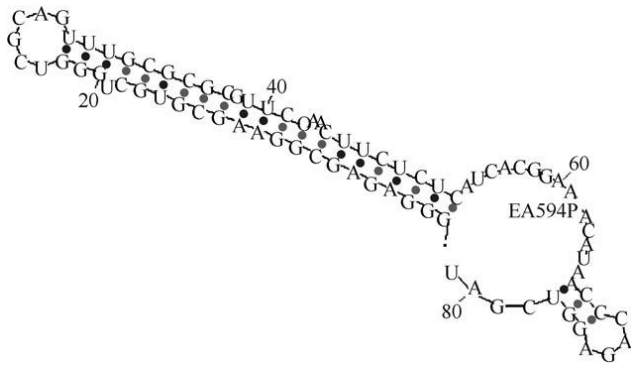

Fig 6

To obtain the exact RNA sequence needed, DNA transcription was carried out. Transcription is a method in which DNA is used as a template to build RNA sequences off of. DNA transcription was performed using the DuraScribe® T7 Transcription kit (Epicentre® Biotechnologies). This was chosen for its unique ability to produce RNA that is resistant to Rnase A degradation by replacing Cytidine triphosphate (CTP) and Uridine-5'-triphosphate (UTP) with 2'-Fluorine-dCTP and 2'-Fluorine-dUTP in the reaction solution. The DuraScribe polymerases utilize the same standard T7 transcription promoter sequence recognized by other T7 RNA polymerases. Another advantage to using this kit is that only 1µg of control DNA template is needed for the reaction to produce 30-50µg of RNA product. It should be noted that the promoter sequence location will determine if sense-RNA or anti-sense-RNA will be produced. For this

study, sense-RNA is created which is a copy of the DNA template in RNA form; whereas anti-sense RNA would be a compliment strand. The procedure was followed per manufacturer's instructions.

The DNA template used is shown below. The portion shown in bold is double stranded for the first 17 nucleotides, which is necessary for polymerase recognition during transcription to produce sense RNA. Its compliment for this section is also shown below. These were annealed together at 94°C for 2 minutes using the same concentration of strands each so that there were not an excess of long or short strands of DNA in solution.

5' - **TAA TAC GAC TCA CTA TA**G GGA GA GGG AGA GCG GAA GCG TGC TGG GTC GCA GTT TGC GCG CGT TCC AAG TTC TCT CAT CAC GGA ATA CAT AAC CCA GAG GTC GAT GGG GGG GGG GGG – 3'

5' – **TAT AGT GAG TCG TAT TA** – 3'

In brief, in an RNAse/DNase free eppendorf tube 7.1µl of sterile dionized water was added. To this 4.9µl of 1µg DNA template as well as 4µl of: 10X reaction buffer, adenosine triphosphate(ATP), guanosine triposphate(GTP), 2'-Fluorine-dCTP, 2'-Fluorine-dUTP, dithiothreitol (DTT), and T7 enzyme mix. This 40µl total volume was incubated on a heat block at 37°C for 6 hours. This final product solution was stored in a -20°C freezer to prevent degradation.

After successful transcription of the DNA to RNA, a thiol group is hybridized on the end of the RNA, which is necessary for its attachment to gold nanoparticles. This is

due to the high affinity Thiol has to gold and was accomplished through a compliment nucleotide hybridization of cytosine and guanine. A 12 cytosine chain with a thiol group on the 3' end was annealed to the 12 guanine spacer on the RNA transcribed product sequence. This was conducted in an eppendorf tube which was placed in a heat block at 94°C for 2 minutes and then cooled.

For verification of successful transcription as well as concentration determination of the RNA, absorbance was recorded using a spectrophotometer, NanoDrop. In brief, 1µl of RNA sample is pipetted onto the end of a fiber optic cable. The second fiber optic cable is put in contact with the liquid sample so that it bridges between the two cables. A pulsed xenon flash is used as the light source and scans wavelengths from approximately 220nm to 750nm. The absorbance is then recorded and displayed as a wavelength vs. absorbance graph. The signature peak for nucleic acids which is around approximately 260nm, is monitored.

3.4.3 RNA Attachment to Gold Nanoparticles

For proof-of-concept experiments in determining if RNA will bind to the gold, RNA was first attached to colloidal gold nanoparticles. These particles were synthesized using the same process as the particles that were created on the carbon nanotube samples, described previously. To determine concentration of the particles before RNA loading, UV-Vis was conducted. Using the Beer-Lamber Law shown below, the concentration can be determined. A = absorbance in optical density units, ε =

exstinction coefficient(at the surface plasmon wavelength), ℓ = path length through sample, and c = concentration of nanoparticles. (A= obtained from UV-Vis spectrum, ε = $1.935*10^{10}$ M^{-1} cm^{-1}, ℓ = 1cm)

$$A = \varepsilon c \ell$$

After successful synthesis of the 44nm particles, 1 mL of the colloid was pipetted into a scintillation vial. 1 mL of thiolated RNA was added, stirred briefly, and left to rest in the dark for 20 minutes. Next, 246 µl of 100mM Phosphate buffer and 40 µl of 2M Sodium Chloride (NaCl) was added to the RNA/Au-NP mixture and stirred for 20 minutes on a lab shaker. 40µl of 2M NaCl was then added to the solution and stirred for another 20 minutes. This was repeated a final time and left to sit overnight in the dark. The next day the particles are centrifuged at 12,000xg for 15 minutes to form a pellet. The supernatant was removed and the pellet was re-suspended in 100µl of 100nM NaCl, 25nM Tris Acetate. This mixture was vortexed and centrifuged again at the same speed two more times. This entire coating process was conducted on gold nanoparticles without the addition of RNA in order to verify attachment on the surface.

To further confirm attachment, Dynamic Light Scattering (DLS) and Zeta Potential measurements were taken. DLS is a process in which a laser is focused on the particles which then scatter this light. From this scattering the size can be determined. Zetapotential of a particle is the charge on the surface of a particle, which can be an

indicator of attached molecules on the surface as well as the potential for settling of the particles.

3.4.4 Cell culture

DH5α *E. coli* bacterial strain was purchased from ATCC. To prepare for exposure to nanoparticles, 10µl of frozen stock solution of the cells were diluted in 6ml of Luria Bertani (LB) broth which was made from 20g of powder dissolved in 1 Liter of water and autoclaved. The cells were then incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day, the cells were washed by centrifugation and re-suspended in PBS. Bacteria concentration was determined by a spectrophotometer, where an absorbance of the solution that is 0.250 at a 600nm wavelength is equivalent to 10^8 cells/ml, which was the concentration used throughout this study.

3.4.5 Proof-of-Concept

In order to determine enhanced attachment via RNA/*E. coli* binding, a proof-of-concept study was conducted using uncoated and RNA coated gold colloids. The particles were nano-size gold spheres that were citrate stabilized and RNA coated. After conducting DLS and Zeta potential the particles were incubated with *E. coli*, centrifuged, and the UV-Vis spectra were obtained. The procedure is described as follows.

After coating the particles, they were then exposed to *E. coli* to view RNA attachment. All experiments were conducted in triplicate to verify findings. First, at room temperature, 400µl of 10^8 concentration of DH5α in phosphate buffered saline was combined with 100µl of the coated particles. This was run in parallel with 100µl of bare

gold nanoparticles with 400µl of bacteria also at room temperature. These were under constant movement for 20 minutes on a lab shaker. After the exposure time was complete, the samples were spun down at a relatively low speed, 5.2g. This was conducted at this speed so that the bacteria would spin out, but any unbound particles would not. The supernatant was removed and they were resuspended in sterile water for UV-Vis measurements.

4. Results & Discussion

4.1 Cell Growth in a Porous Microcellular Structure: Influence of Surface Modification and Nanostructures

To observe cell morphology on each substrate, after 72hours of cell-growth, the cells were prepped for imaging. After fixing and dehydrating the cells, Scanning Electron Microscopy was conducted. Figure 4 shows SEM images of osteoblasts cultured on various surfaces. On the uncoated foam image it can be seen that the cells spread well, and span across complete pores which are 500-600 micrometers in diameter. As can be seen in the fluorescence images in Fig 7, these structures are actually a network of cells. On the silicon dioxide coating, the cells formed tight clusters close to the pore walls, which is expected since this coating is hydrophilic, and enhances wetting of the walls with the culture medium. The number of cell networks that span across open pores in the given incubation period is significantly reduced with the silica coating. Seen in the control pictures for collagen coated foam, collagen was confirmed to be on the surface, which was verified in SEM images by the porous network it created. After growing the cells on this sample, the SEM images had shown them to be fully coating some of the pores (Fig. 4) which was later confirmed with fluorescence. For the carbon nanotube attached foam, cell growth was more prolific in all respects. Cell walls were completely covered, and it can be seen from images such as Fig 4 that cell networks spanning across open pores were increased, and were noticeably more prevalent than seen in uncoated foam.

Fluorescence microscopy was utilized in imaging individual cells on the foam with DAPI and TRITC filters which made the nucleus and actin fluoresce blue and red respectively. As stated earlier, this was able to help in determining the cells that spread across pores were actually a complete network of individual cells. The images from each coating: uncoated, collagen, and carbon nanotube coated foams, are shown in Fig 7 for comparison. The collagen and carbon nanotube coated samples showed the most actin present which could be a sign of a stronger bond between the cells and foam (Fig. 7). To confirm the prediction of the silicon dioxide coated foam, cells appeared to be densely packed proving the coating to be hydrophilic. It is worth noting that the cell's actin filaments on the carbon nanotube coated foam (Fig 7) are seen to be elongated and directional which may enhance strength as well as bonding. This indicates that the CNT structures may provide a suitable template that not only increases surface area for cell growth, but also may provide a directional template in future implant applications.

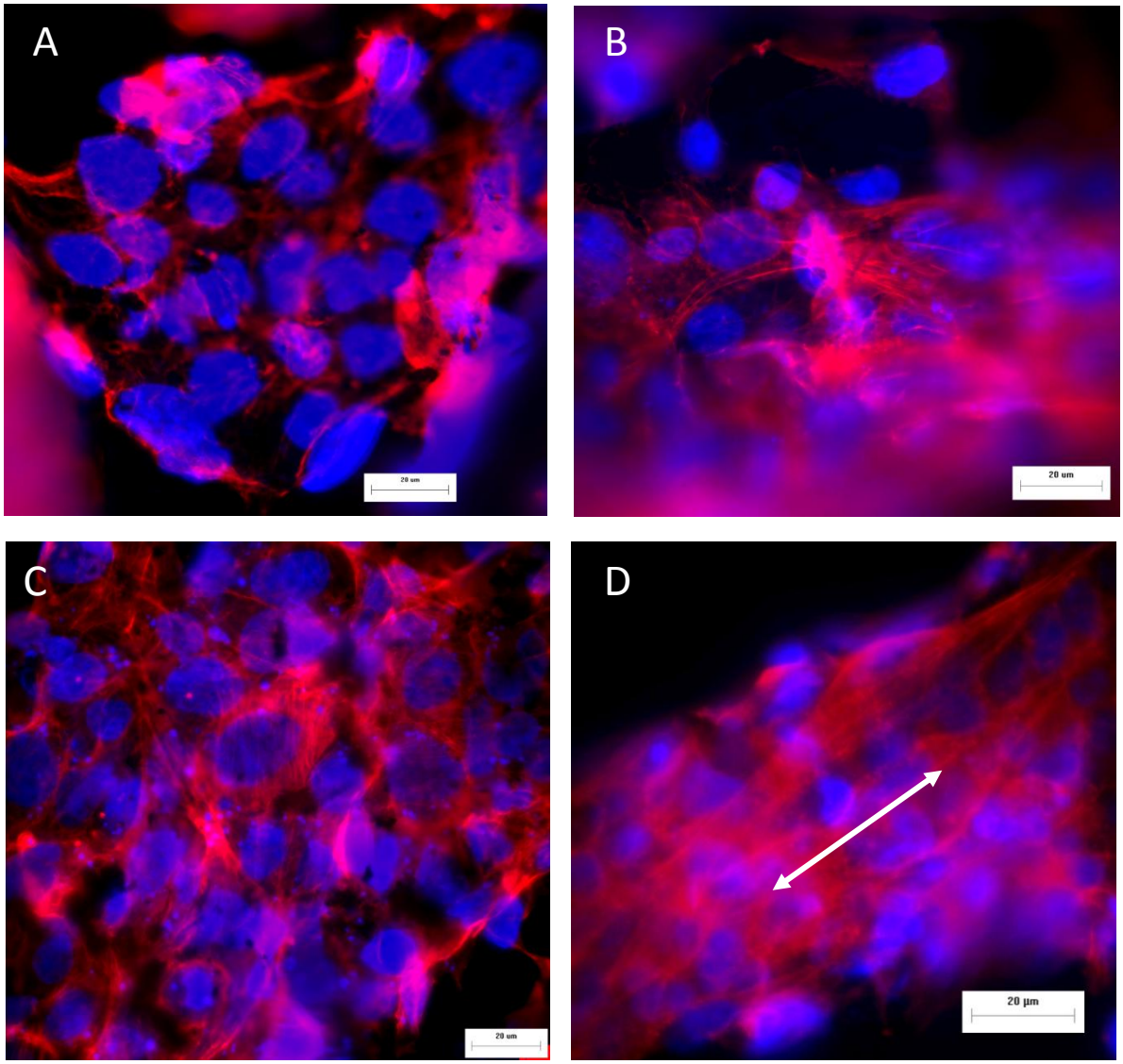

Fig 7. Fluorescence images of: (A) Uncoated, (B) Silicon dioxide coated, (C) Collagen coated, and (D) Carbon nanotube coated foam samples. Nuclei are stained blue, while the actin is stained red. Pictures are of the cells grown on each of the coated foams for 72 hours.

Sample Type	Nuclear Density ($x10^{-3}$ Cells /μm^2)	Nuclear Size (μm)	MTS (% Control)
Uncoated Foam	2.2	15.4	102.9
SiO_2 Coated Foam	2.8	12.6	92.4
Collagen Coated Foam	2.4	16.4	96.6
CNT attached Foam	4.2	12.5	107.1

Table I. Summary of cell morphology and viability MTS values (compared to control) and average cell parameters. Nuclear density was calculated by averaging over 3-4 images, the number of nuclei seen in an area of the fluorescent images. *Cells were grown on each of the coated foams for 72 hours.*

Multiple images from the same sample were used to get estimates of average cell sizes and densities summarized in Table I. It must be noted that the images are two-dimensional representations of curved three-dimensional surfaces. Therefore, the actual quantitative values are not expected to be accurate. However, comparison of these numbers obtained on identical foam geometries can provide some very important insight about how the different surface modification techniques influence cell growth. The biological implication of average cell size is not clear at this point, but nuclear density may be a good indicator of how well they divide. Thusly, if a substrate is seen to contain fewer cells per area, the surface modification could be hindering their ability to proliferate.

MTS results averaged over three or more assays have been summarized in Table I along with cell sizes and densities seen from fluorescent images. It must be noted that all samples yield MTS assays above 90% of control, implying acceptable biocompatibility. Silica and collagen-coated foams were seen in SEM to be adhering to the surface of pores in the foam. They appear to have marginally higher nuclear densities, but marginally lower MTS viability compared to uncoated foam. On the other hand, the CNT grafted foams are more enhanced in all aspects: they have increased adhesion to pore walls and increased cell network spanning on open pores as seen in SEM, significantly higher nuclear density as seen in Fluorescence imaging, along with some increase in mitochondrial activity as seen by MTS assays. This shows that the coatings, as well as the uncoated carbon foam, are non-toxic to the hFOB cell line based on biochemical assays conducted in this study. However, further long term studies with additional

biochemical end points are needed to confirm these materials are biocompatible without significant toxicity.

4.2 Functionalization of Nanomaterials for Biosensor Applications

4.2.1 Gold Nanoparticle Attachment to CNT

To view carbon nanotube growth, as well as gold nanoparticle size and dispersion, Scanning Electron Microscopy was conducted. As seen from the images in Fig 5 carbon nanotubes were successfully grown on the surface of silica coated graphite. Minimal amounts of excess iron particles were seen on the surface of the nanotubes, which is important so that they would not be confused with gold particles. After gold nanoparticle synthesis, imaging was performed to ensure attachment to the surface of the nanotubes as well as even distribution. In the SEM images, well dispersed gold nanoparticles were seen adhering to the carbon nanotube surfaces showing low agglomeration.

4.2.2 DNA to RNA Transcription

After the Transcription process, concentration measurements were taken by spectrophotometry. As seen from Fig 8 the concentration was approximately 4.2 μg/μl. After dilution and thiol attachment, the concentration was 240ng/μl.

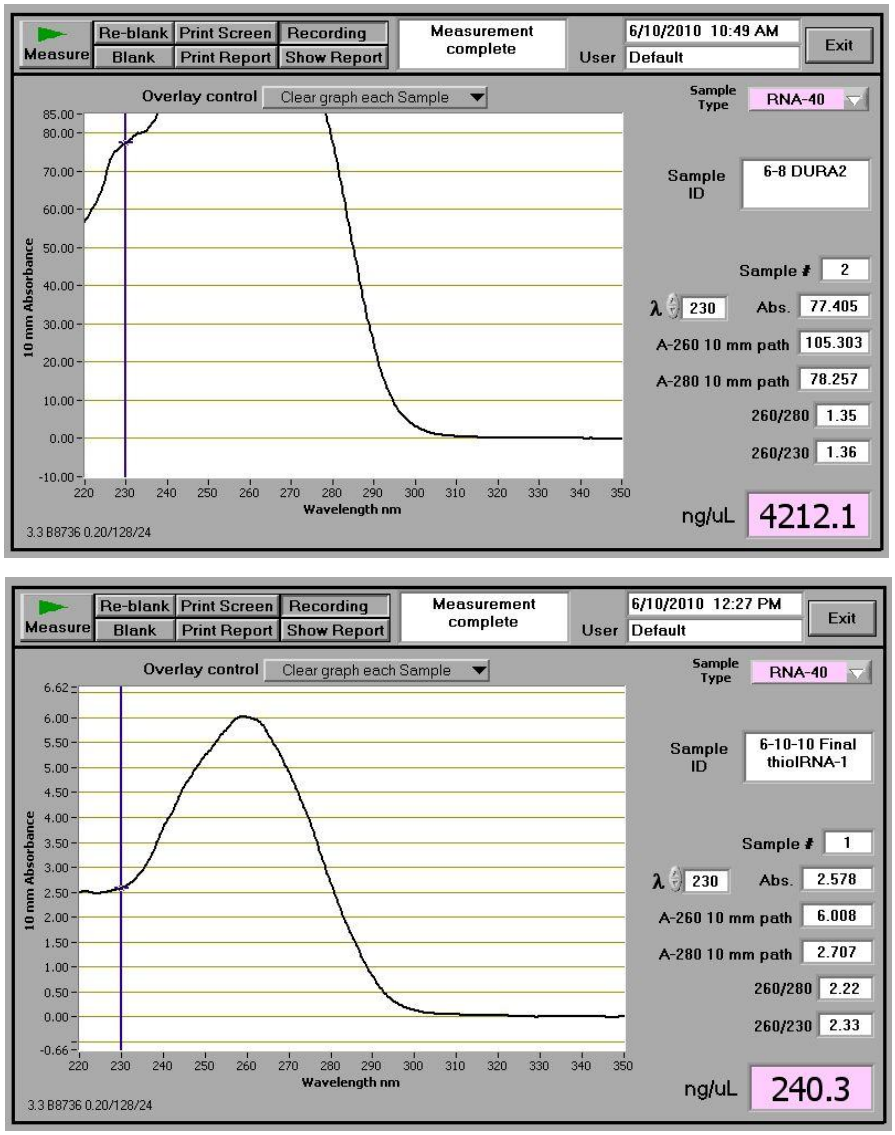

Fig 8. Spectrophotometer graphs of RNA concentration: (A) after transcription and (B) after thiol attachment and dilution.

4.2.3 Proof-of-concept

For proof-of-concept studies to verify attachment of the RNA to the *E. coli*, gold particles were coated with the thiolated RNA sequences. This was conducted in liquid solution so gold colloid solutions were used at a concentration of 6.67E+09 nanoparticles/mL. To determine the diameter of the synthesized gold nanoparticles in solution, Dynamic Light Scattering and surface charge measurements were taken. The average diameter of the citrate stabilized uncoated nanoparticles, was approximately 44nm, while its surface charge was -41.8mV. The RNA-coated gold nanoparticles had a higher average diameter of approximately 55nm and a more positive surface charge of -32.8mV. Error in size measurements and surface charge are within 2% according to Malvern Instruments for well dispersed and suspended particles. From these two measurements, it is shown that the particles were successfully coated with RNA. This is due to the increased hydrodynamic diameter from the layer of RNA as well as the change in surface charge which further indicates attachment. This is depicted in Fig 9.

4.2.4 RNA coated particle exposure to E. coli.

After exposing the nanoparticles to the bacteria for 20 minutes and resuspended in water (Fig 10), the solutions were then put into a UV-Vis spectrometer and the absorbance was recorded. One milliliter of the sample is placed in a quartz cuvette and absorption is measured from ~200nm to 800nm. The graph in Fig11 shows the signature peak of gold nanoparticles at approximately 525nm with a much higher absorbance and slight peak shift seen with the RNA-coated particles as compared to the uncoated

particles. The increased amount of absorbance is due to higher concentration amounts of gold nanoparticles. This significant data confirms enhanced attachment with the addition of *E. coli* specific RNA compared to uncoated particles.

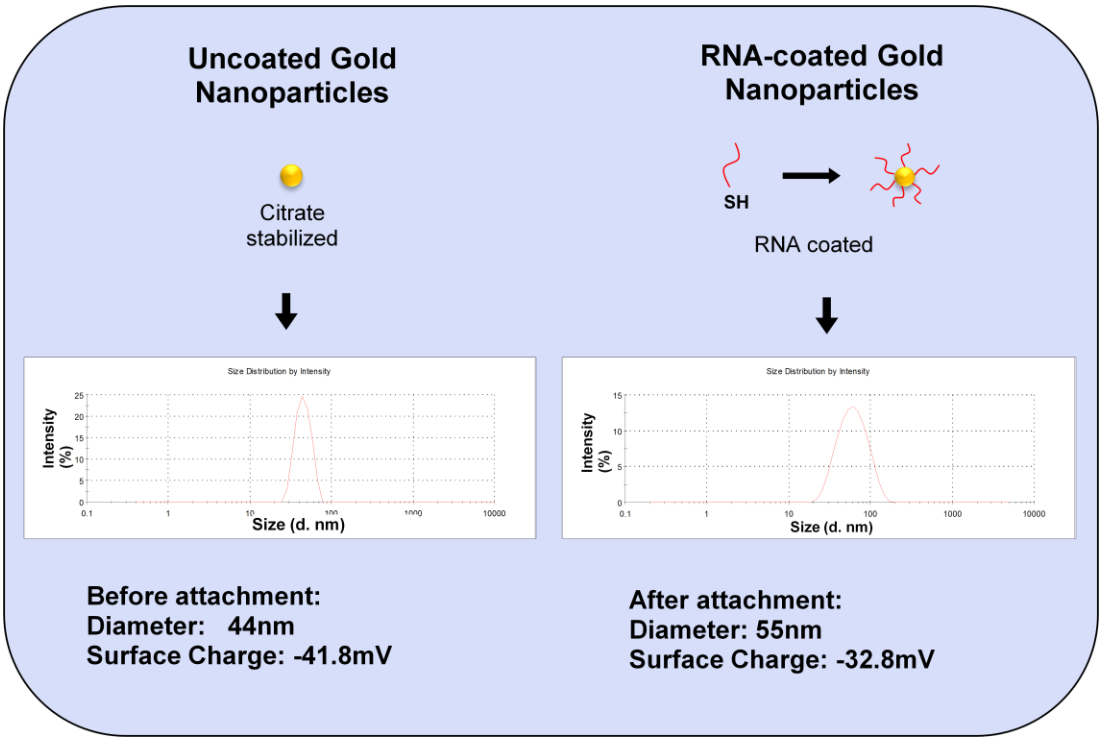

Fig. 9. Dynamic light scattering data to determine nanoparticle diameter and Zeta potential to determine surface charge of uncoated and RNA-coated gold nanoparticles.

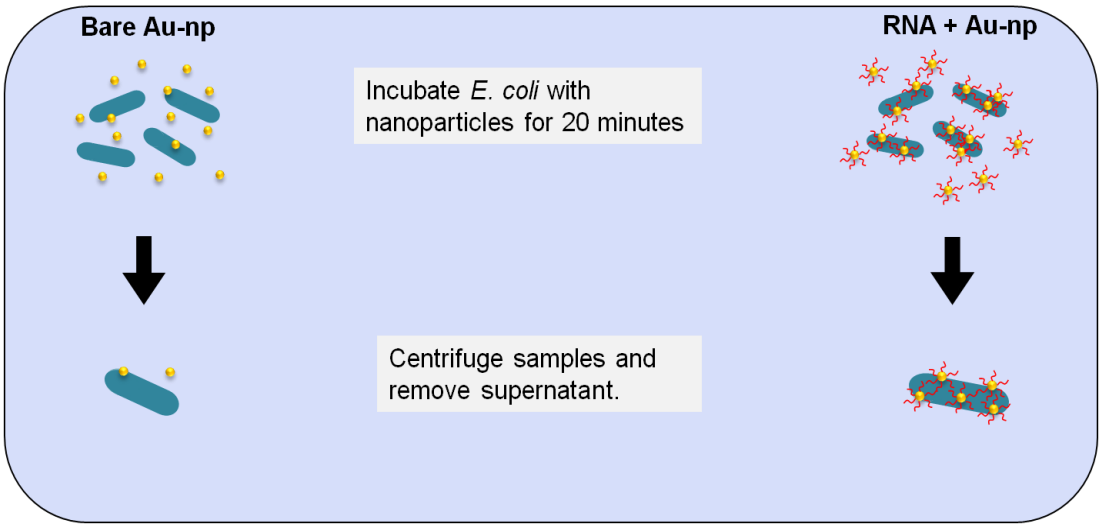

Fig. 10. Illustration of Proof-of-Concept procedure in which E. coli is incubated in solution with uncoated and coated nanoparticles for 20 minutes. These samples were then centrifuged, the supernatant was removed, and the pellet was rinsed.

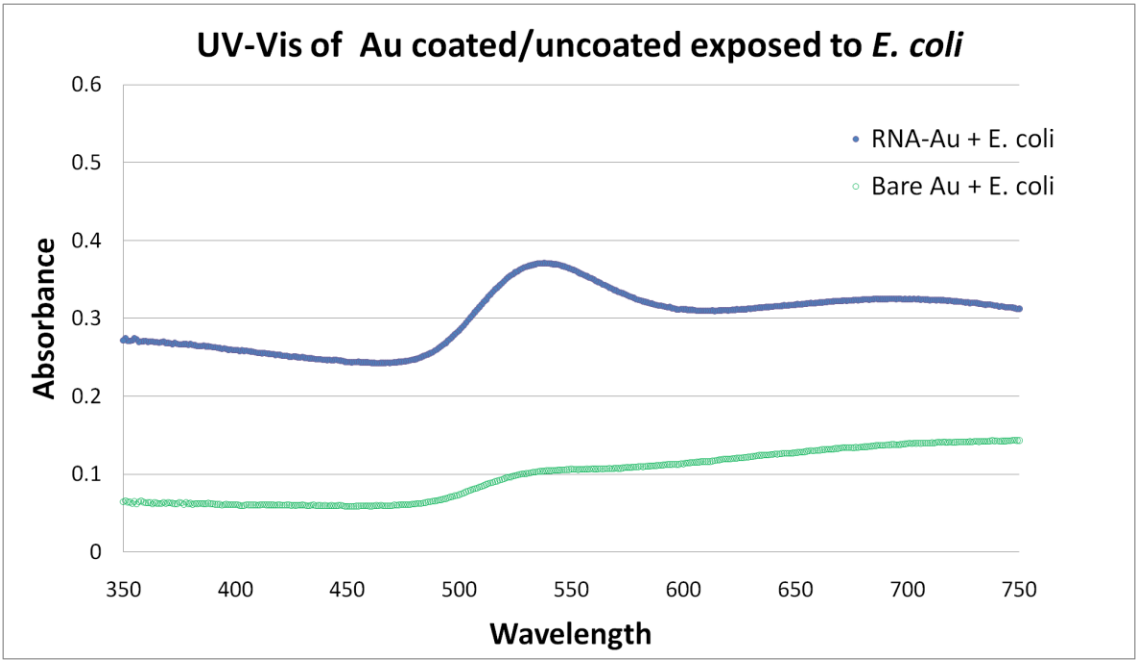

Fig 11. **UV-Vis measurements were conducted on the *E. coli* samples containing uncoated and RNA-coated gold nanoparticles. The graph above shows the signature peak of gold nanoparticles at approximately 525nm with a much higher absorbance and slight peak shift seen with the RNA-coated particles. The increased amount of absorbance confirms enhanced attachment with the addition of *E. coli* specific RNA.**

5. Summary & Conclusions

This study has shown that different surface modifications on graphite provide a novel foundation for biological applications. Two such platforms were explored and discussed in detail. The first study investigated a carbon foam scaffold modified with carbon nanotubes for use in tissue regeneration. In the second study, carbon nanotubes grafted on a graphite substrate was developed as a platform for pathogen detection. From these two investigations, it has been shown surface modifications on carbon can be used in an array of biological applications.

5.1 Cell Growth in a Porous Microcellular Structure: Influence of Surface Modification and Nanostructures

This porous type of carbon substrate can be useful as a scaffolding material for tissue growth, but their biocompatibility has not been adequately investigated in the past. The influence of various surface treatments was studied: an inorganic hydrophilic coating (silica), an organic coating (collagen), and grafting of carbon nanotubes (CNT). The results have shown that all foams have acceptable biocompatibility. Silica and collagen coatings tend to have more cell growth along pore walls, but the collagen did not have any significant influence on overall nuclear density or cell viability, whereas silica had a slight decrease in cell density. On the other hand, nanotube attachment resulted in simultaneous increase of cell proliferation, density, and viability.

Biocompatibility studies were performed on cultured cells using microscopy and biochemical techniques to assess morphology as well as cell proliferation. From this study it can be shown that microcellular carbon foam is biocompatible and that varying surface modifications affected the cell proliferation and attachment of osteoblasts. More specifically, attaching carbon nanotubes on surfaces of future implants may provide a hierarchical nanostructure with increased biocompatible surface area for improved cell attachment.

In conclusion, it has been shown that various coatings on carbon foam can hinder or promote various cell functions and growth. From this observation, different coatings could be utilized for specific needs. If a stronger bond of the cells to the foam is needed, carbon nanotube coated microcellular foam would be the most fitting of the tested coatings because of the increased amount of actin that is formed. In addition, directional growth may play a large role in strength which was shown also in the nanotube coated foam samples. As for mitochondrial functioning, the CNT and uncoated foams proved to function better than the control, which implies an increase in amount of cells present that are still alive and healthy. When compared to one another, the carbon nanotube coated foam is seen to be the most promising coating with increased viability and aligned growth, while silicon dioxide, although non-toxic, did not perform as well overall compared to the other samples. This study was able to show the varying effects on the growth of osteoblast cells on the structure of microcellular carbon foam, and showed differing effects on cellular function and growth from the grafted carbon nanotubes to other coatings.

5.2 Functionalization of Nanomaterials for Biosensor Applications

It has been shown in this study that a sensor platform was developed utilizing carbon nanotubes to enhance the capture of a bacterial strain, *E. coli*. From this research, carbon substrates, in the bulk and nano-scale, have the potential to be developed into a novel sensor to detect pathogens in the environment.

The present study was aimed at developing a novel sensor to detect bacteria based on carbon nanotubes functionalized with gold nanoparticles with a ribonucleic acid (RNA) sequence attached as a capture element. The gold nanoparticles were successfully synthesized on multi-walled carbon nanotubes which were grown on a substrate via a chemical vapor deposition process. Highly oriented pyrolitic graphite was used as the base carbon structure due to its ability to withstand high temperatures, for nanotube growth, and ease of use. Carbon nanotubes have been utilized due to their unique electrical properties and high surface area. Gold nanoparticles were exploited for their ease in functionality and to enhance the sensitivity of the overall structure. Their dispersion on the carbon nanotubes, shape, and composition have been characterized through Scanning Electron Microscopy (SEM) and Energy Dispersive x-ray Spectroscopy (EDS).

After confirming gold attachment and dispersion, RNA that is specific to a surface protein of the DH5α *E. coli* bacterial strain, was attached to gold nanoparticles through a thiol (SH) terminated RNA chain which was validated through dynamic light scattering (DLS) as well as its zeta potential value. Further, the DH5α strain was exposed to the RNA modified gold nanoparticles in a liquid solution, and was successfully

captured. This was confirmed using Ultraviolet-Visible Spectroscopy (UV-Vis). After this proof-of-concept study was completed, the next step was to carry out this process on the carbon nanotube/gold nanoparticle substrate and conduct microscopy measurements. The same concept can be applied to other bacterial surface-specific RNA sequences, attaching them in the same manner, and ultimately creating a multi-array sensor to determine the strain of bacteria as well as the concentration. From this research, it has been shown that utilizing novel nanostructures for the application of a bio- sensor in bacterial detection and identification is possible.

6. Future Outlook

This study has shown two basic applications of surface modifications on carbon substrates. Through the attachment of nanotubes, enhancement of the overall structures was observed. This research can be expanded in many ways to corroborate the current findings as well as assist with various projects and applications.

6.1 Cell Growth in a Porous Microcellular Structure: Influence of Surface Modification and Nanostructures

For *in-vitro* cell studies, in order to understand enhanced growth mechanisms due to carbon nanotubes, various alternate biomarkers could be investigated. Alkaline phosphatase, an enzyme that is produced by osteoblasts, could be measured for increased production as well as osteocalcin, which is also produced by the osteoblasts during normal cell growth.

In addition to biomarker production, this porous structure could be expanded to other cell lines, or antimicrobial studies could be performed for the application of filtration systems because of the carbon foams' interconnected porosity.

6.2 Functionalization of Nanomaterials for Biosensor Applications

For future biosensor applications, optimization of the RNA attachment as well as *E.coli* capture can be performed. Enhanced attachment of the RNA could further increase the overall capture capability of the system. This can also be extended into other types of bacteria to determine specificity so that a multi-arrayed sensor can be developed. This is important for detection and identification of unknown samples in the environment. Lastly, this sensor platform can be tested through electrical measurements to quantify, in real-time, the amount of bacterial attachment. This fast detection is necessary so the amount of infections can be drastically reduced with quickly spreading diseases.

As was shown in the preliminary study of gold nanoparticles on carbon nanotubes, there was no significant attachment of uncoated gold nanoparticles compared to RNA coated gold nanoparticles. Though the CNTs may have had an effect on the RNA attachment, bacteria attached nevertheless. This unique find could be utilized in filtration systems to remove pathogens in water supplies. In addition, because of the unique composite structure and surface area exposed, this could also be used in neutralizing chemicals in the atmosphere or in liquid form.

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