Bioapplications of Carbon Nanotubes and Carbon Nanotube Assemblies

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Abstract

As new materials are discovered, their potential and applications are investigated widely across the various scientific disciplines for general or highly specialized applications. While new nanomaterials such as carbon nanotubes have received the greatest interest for electronics, optics, and structural composites, their applications have also been explored for biological applications such as sensing, selective cell destruction, cellular growth scaffolds, and intracellular delivery of bioactive cargos. Carbon nanotubes are unique materials particularly suited for these applications as they possess characteristic optical and electronic properties in conjunction with large aspect ratios and massive surface areas. The work of this thesis explores the use of carbon nanotubes for cellular growth scaffolds in Chapters 3, tailoring the various properties of these scaffolds in Chapter 4, and their cellular internalization and intracellular locations in Chapter 5.

The aim of Chapters 3 and 4 are to create a surface that mimics a cell’s natural environment by varying characteristics such as roughness, pore size distribution, wettability, and chemical functionalization of the carbon nanotubes surface. Such variations can have beneficial, detrimental or abnormal effects on the tested cell line as a cell’s natural environment within the body consists of a three dimensional mesh of extracellular matrix proteins which is not at all replicated by the commonly used polystyrene tissue culture flask. Carbon nanotubes possess diameters ranging from 0.7 to several nanometers and lengths that can range up to several microns thereby allowing certain types of CNTs to scale with these extracellular matrix proteins and thus impart a nanoscale textured topology that more closely resembles a cell’s in vivo environment. Additionally, the replacement of extracted extracellular matrix proteins for coating cellular growth surfaces with synthetic carbon nanotubes eliminates any risk of pathogen contamination and batch-to-batch variability of biological specimens. Fundamental understanding of the interactions between carbon nanotube surfaces and adhered cell cultures will provide a foundation for carbon nanotube applications in 3-dimensional cellular growth scaffolds and tissue implantation devices.

Chapter 5 explores the interactions between designed peptides with slight variations in their amino acid sequences and the consequential effects of these peptide interactions
with carbon nanotubes for cellular internalization and intracellular location. The
efficacy of pharmaceutical drugs and the cellular responses to biomacromolecules
depends heavily upon their abilities to transverse the cellular plasma membranes, and
exploring the interactions with designed biomolecules such as synthetic peptides
provides simple methods for increasing the cellular internalization of carbon nanotubes
and altering the intracellular delivery location. The results and methods investigated
within these chapters can then be easily applied to other carbon nanotube transporter
schemes.
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Chapter 1
Bioapplications of Carbon Nanotubes

1.1 Introduction

Individual carbon nanotubes (CNTs) and materials based on carbon nanotubes have been widely investigated for a diverse array of applications.\textsuperscript{[1]} In recent years, focus on CNTs has grown to include their possible biological applications such as drug delivery transporters, selective-cell destruction agents, biosensors, cellular growth substrates, and prosthetic implant materials.\textsuperscript{[2]} Several physical properties including high surface area, nanoscopic dimensions, a rigid platform structure, good electrical conductivity and excellent mechanical properties make them appealing for such diversified biological end goals. In addition to these intrinsic properties, CNTs can be derivatized with various chemical or biological molecules to further tailor them for specific aims. These derivation schemes can be accomplished through covalent chemical functionalization or through strong physical interactions with hydrophobic and aromatic regions of biological molecules.\textsuperscript{[3-7]} Furthermore, CNTs have demonstrated the ability to traverse cellular membranes at high efficiencies while carrying an assortment of cargos that retain their biological activities without incurring acute toxicity to most cell types rendering them highly applicable for intracellular sensing and delivery applications.\textsuperscript{[8-10]}

Despite this unquestioned potential there are many issues that must be resolved. In particular there are contradictory opinions in the literature relating to internalization mechanisms and toxicity. These contradictions undoubtedly arise as a result of variations in synthesis and preparation methods used. Thus, as will be highlighted below, it is absolutely essential for researchers to pay close attention to preparative methods. Other problems also exist; pristine carbon nanotubes are hydrophobic in nature and are found in mesoscopic aggregates as a result of inter-tube Van der Waals attraction. As a result, the solubility in an aqueous environment is low and processing is thus cumbersome. However, these problems are overcome through direct chemical functionalization or through physical interaction with an amphiphilic agent. However, chemical functionalization may deleteriously affect the electrical and optical properties of CNTs by destroying/disrupting $sp^2$ conjugation of the
carbon lattice. Additionally, CNTs when synthesized and prepared by various methods contain metal catalysts, defect sites, or possess biopersistent lengths which trigger toxic responses somewhat complicating their applications in biological environments. These shortcomings should not be viewed as an end for bioapplications as several processing remedies exist for each arising shortcoming.

1.2 Carbon nanotubes as cellular growth substrates and tissue scaffolds

Cell-substratum interactions rely heavily upon topological and chemical cues, and while it is well established that microscale topographies and patterns influence cellular behaviors only recently have nanoscale features and their effects been examined. To date, variations in nanoscopic surface characteristics such as roughness, architecture, and elastic modulus demonstrate alterations to a variety of cellular behaviors. New insights and control over the nanoscale topography of cellular substrates will optimize medical implantation devices and will facilitate new investigations into biological processes including embryogenesis, angiogenesis, and pathological conditions. Nanotextured surfaces can be created by an assortment of methods including but not limited to electron beam lithography, ion beam lithography, electrochemical etching, electrospinning, nanoparticle adhesion, laser ablation, molecular self-assembly, polymer phase separation, and more. Macromolecules and nanoparticles are inherently suited for nanoscale surface modifications by simple incorporation within or deposition onto a material’s surface and among these nanomaterials exist CNTs which have a fibrillar shape and an array of versatile optical, electrical, and mechanical characteristics opening new dimensions for cellular substratum applications. The depth and complexity of cell-substratum interactions should not be underestimated as explanations for several of these biological mechanisms remain elusive; however, fundamental understanding of cellular responses to artificially designed surfaces begins with a look into naturally occurring cellular growth matrices.

In vivo mammalian tissue consists not only of cells but also of an interconnecting meshwork of macromolecules constituting what is called the extracellular matrix (ECM).
The two principal classes of extracellular macromolecules include polysaccharide chains, specifically glycosaminoglycans, and fibrous proteins such as collagen, elastin, fibronectin, and laminin. These polysaccharides and proteins vary widely in terms of their monomer composition, surface charge, structural orientation, size, and occupied volume allowing different bodily tissues to adapt specific functions such as transparent shielding for the cornea, calcified robustness necessary for teeth and bones, and molecule-specific filtration for the kidneys. Generally, these macromolecules possess molecular weights ranging from the tens of thousands up to several million Daltons and can adopt fibrillar, globular, or gelatinous phase structures. These ECM components interact with adjoining cells in a multitude of ways and are by and large, depending upon the tissue, neither a simple structural support for cell anchorage dependence nor a permanent fixture.

In several types of tissues, interactions between cells and the ECM sway, if not completely govern, a series of cellular behaviors and attributes including survival, adhesion, proliferation, migration, differentiation, development, metabolism, morphology, and orientation. Elegant mechanisms for the interplay between cells and their surrounding ECM come into play and highlight the importance of selecting a substratum material both adapted and tailored for a multitude of cellular interactions. For instance, certain cell types employ contractile, cytoskeletal actin and myosin filaments in conjunction with bound integrin transmembrane adhesion proteins to exert tensile stress upon fibronectin fibrils attached to the exterior of the cellular membrane. The exerted stress along the fibronectin axes aligns the fibronectin fibrils with the cytoskeletal filaments and exposes periodically spaced binding sites for other fibronectin molecules, ECM proteins, and cells. Thus, the intracellular cytoskeleton can influence the assembly, organization, and alignment of the surrounding ECM; however, the relationship between extra- and intracellular organizational proteins is a two way street as the ECM can in turn orientate cellular morphology in an anisotropic manner. In terms of applications, certain implantation grafts such as cardiac, muscular, or vascular tissues must perform under load or fluid shear stress as well as match the elasticity of surrounding tissue, and alignment of cells by an underlying anisotropic architecture has increased the ultimate tensile stress of such an implant several fold imparting the necessary mechanical strength. Any material tailored for a specific cellular response by offering a patterned surface, parallel arrays in this
example, must nonetheless accommodate several other requisites associated with viable cellular substrata.

Any material that adds only one piece to the jigsaw of medical implantation device requirements will never see preclinical trials, but CNTs are not restricted to their as produced form and are easily adapted to remedy one or many obstacles by functionalization with molecular accessories. Such accessories include organic groups to alter surface charge,\(^{[39]}\) binding ligands to signal cellular adhesion,\(^{[40]}\) or a surrounding dynamic layer of ions.\(^{[41]}\) Underlying all of these adaptations remains a rigid starting platform with a high aspect ratio and fibrillar shape scaling with the physical dimensions of several of the basic biological components naturally occurring within the ECM. CNTs provide an additional, fundamental advantage for replicating cellular environments owing to their ability to form a porous meshwork with adjustable pore diameter distributions.\(^{[42]}\) Many reports have successfully demonstrated proof of principle experiments emphasizing CNT utility for cellular growth surfaces.

Several investigations have reported on high binding affinities that a variety of cell phenotypes have for CNT surfaces indicating the range of tissue implantation devices or novel substrata for which CNTs may prove advantageous. Thin films of CNTs increased adherence, proliferation, and improved cell-cell communications of mouse fibroblast cells compared to cell culture plates, polyurethane, or carbon fibers,\(^{[43]}\) and a CNT coating overlayed upon silicone promoted the adherence and proliferation of Saos-2 cells (osteoblast-like cells) which was virtually non-existent on the bare silicon.\(^{[44]}\) Another study reported massive Saos-2 cell spreading and proliferation in conjunction with numerous filopodia attachments to such an extent that standard enzymatic treatment with trypsin-EDTA could not detach the cells from the CNT surface.\(^{[45]}\) Neuronal cells also exhibited preferential adherence to CNTs by migrating off of the surrounding SiO\(_2\) surface onto the CNT fabricated islands.\(^{[46]}\) The CNT surfaces reported in [38-41] received little post-synthesis processing and no advanced modifications to enhance cellular adherence underscoring the innate suitability of CNTs for supporting cellular growth.
As mentioned, CNTs and consequently CNT surfaces can be functionalized with a variety of molecular accessories to enhance cellular viability or function. Neurons require substrata highly permissive to axon and neurite extension, and these neural processes can be enhanced or diminished depending upon the underlying surface chemistry. Simply coating the CNT sidewall by physiosorption with the bioactive molecule, 4-hydroxynonenal, more than doubled average neurite length, number of neurites per cell, and branches per neurite compared to the uncoated CNT surface.\textsuperscript{(47)} Chemical treatment of MWNTs prepared in one study produced MWNTs with carboxylic groups, poly(aminobenzene sulfonic acid) (PABS), or ethylenediamine imparting negative, zwitterionic (two opposite and neutralizing charges in a molecule of multiple resonance structures), and positive surface charges, respectively. Neurons grown on the positively charged CNT surface possessed longer neurites and more branching than neurons grown on negatively or neutrally charged surfaces.\textsuperscript{(48)} Modern bone and dental implants are often coated with hydroxyapatite (HA) to facilitate structural and functional connections between the tissue and load-bearing implant. In one study, CNTs functionalized with different types of negatively-charged organic groups induced HA crystallization in varying amounts with one type of functionalization aligning the plate-like HA crystals along the CNT axes.\textsuperscript{(49)} In addition to forming sufficient amounts of HA for artificial bone materials, the study shows that CNTs can be functionalized in multiple steps and fashions before use in application.

CNTs lying within a plane can be produced in highly or semi-aligned arrays to afford an anisotropic surface. Surfaces made from highly-aligned MWNT sheets drawn from MWNT growth forests following CVD synthesis have supported long-term growth on assorted cell types including fibroblasts, Schwann cells (a specialized neural cell), and primary neural cells.\textsuperscript{(50)} The parallel-lying MWNTs induced cytoskeletal orientation and increased motility of fibroblast cells, promoted adhesion and axon extension of dorsal root ganglia cells, increased axonal length, and directed the outgrowth of the neuronal growth cone responsible for steering the direction of axonal growth. Highly aligned yarns drawn from these sheets also demonstrated similar cellular effects giving rise to potential implant devices for ligamentous skeletal or muscular tissue. Semi-aligned patterns of CNTs have also been constructed by deposition between patterned self-assembled monolayers of a masking agent. Mesenchymal stem cells grown on these SWNT monolayers of aligned
SWNTs demonstrated orientation, stretching, and directed growth.\textsuperscript{[51]} Aligned CNT substrata have further relevance in stem cell technology as slight variations in the \textit{in vitro} niche of adult and embryonic stem cells can have profound effects over self-renewal and differentiation behaviors. Sridharan and co-workers aligned Type I collagen and collagen dispersed CNTs along a plastic surface by dip-coating slides into solutions and studied the differentiation responses of cultured human embryonic stem cells.\textsuperscript{[52]} While cells on the aligned collagen and collagen-CNTs matrices displayed phenotypic morphologies of ectodermal lineages in contrast to the control, remarkably higher levels of an early neural progenitor marker were expressed on the collagen-CNT matrix alone.

CNTs substrata need not be thought of in only 2D terms as different methods have created variations in the microscale roughness of CNT surfaces. Convex and concave structures can be formed by acid treatment of perpendicularly aligned MWNT forests. The concave structures formed pits which varied in diameter depending upon the length to which the MWNTs were grown. These substrates acted as a cell-seeding device with adjustable dimensions that promoted extensive growth, spreading, and adhesion of mouse fibroblasts.\textsuperscript{[53]} Correa-Duarte et al. coated polystyrene particles with functionalized MWNTs imparting a nanotextured surface to microscale polymer beads which can be produced with different sized diameters.\textsuperscript{[54]} Firkowska and co-workers expanded on this method and were able to create free-standing 3D hexagonally-shaped networks by CNT incorporation within the polystyrene interstitial sites and subsequent removal of the polystyrene by dissolution in THF and a reactive ion etching process.\textsuperscript{[55]} Such a structure may one day serve as the basis for a 3D implantation device with controllable porosity and mechanical properties. Smooth muscle cells have already been incorporated into a 3D collagen-CNT matrix gel where the CNTs slightly improved the overall mechanical properties of the matrix by delaying gel compaction.\textsuperscript{[56]} These instances demonstrate that CNTs and their advantages for 2D cell substrata may act in combination with their mechanical strength, flexibility, and low density to simultaneously resolve many drawbacks commonly encountered with implantation devices.

Specialized cell types, such as neurons, have seen novel uses of CNT substrata or have specific requirements setting them apart from generic cellular applications. Several papers
have reported CNTs to be conducive to neuronal adhesion and permissive to neural processes outgrowth suggesting that CNT are fundamentally biocompatible with neurons. Neural compatibility in combination with their electrical and mechanical properties constitutes CNTs as a candidate for neural prosthesis and interfacing. The mere presence of CNTs below neuronal cells has induced higher frequencies of synaptic transmissions compared to glass coverslips suggesting that CNTs boost neuronal signaling,157 and layer-by-layer assembled films of a SWNT-poly(ethyleneimine) complex demonstrated equal viability with neural stem cells to poly-L-ornithine, one of the most commonly used growth substratum for neural stem cells.158 These layer-by-layer films also promoted neural stem cell differentiation into mature neurons, astrocytes, and oligodendrocytes. While neuronal interfacing may have no immediate clinical benefits, improvements in this area may elucidate biological mechanisms and neural interactions relevant to injury and disease. Preferential adhesion of neural cells for CNTs allows for the study of axonal outgrowth and connection between neural clusters spaced distances apart on patterned CNT islands159 as well as directed growth and migration along CNT surface architectures.60 CNT surfaces have also doubled as electrodes allowing simultaneous detection of spontaneous electrical activity of the adhered neuronal clusters across the patterned array of the chip.46

Neurons are not the only cells for which CNTs are fundamentally suited as CNTs are envisioned materials for permanent bone or dental implanted prosthetics. Compared to titanium, a leading material for bone prosthetics, CNTs are stronger,61 lighter,62 and have excellent flexibility as well as forming surfaces that naturally mimic an ECM morphology with the capability to facilitate hydroxyapatite crystallization. Additionally, bone cell proliferation and viability have been reported for several CNT surfaces fabricated through varying methods. Saos-2 cells are an immortalized bone cell line that have demonstrated higher metabolic activity on small-diameter CNT surfaces compared to the control surfaces or large-diameter CNT surfaces, although these cells displayed a disorganized cytoskeleton on any of the CNT surfaces.63 This negative correlation between CNT diameter and metabolic activity may be due to smaller-diameter tubes having dimensions closer to naturally occurring Type I collagen fibrils possessing diameters of a few nanometers. As with other cell types, patterned CNT lanes coated with fibronectin demonstrated orientational alignment of osteoblast-like cells which may significantly increase the overall
mechanical strength of a bone implantation prosthetic. Electrical stimulation of osteoblast cells may not seem intuitive for any practical advantage, but exposure to AC currents increased bone cell proliferation and extracellular calcium production of osteoblasts grown on CNT-polylactic acid composites demonstrating application for accelerated bone repair. The use of CNTs in these in vitro experiments of osteoblast and Saos-2 cells exemplify novel and alternative strategies arising from a newly investigated material for the tissue engineering field.

1.3 Carbon nanotubes as intracellular transporters of biological cargos

CNTs have demonstrated the ability to traverse cellular membranes on likely account of their nanoscopic diameters and favorable interactions with the phospholipid membrane. In addition to this fundamental necessity of intracellular transporters, CNTs also possess a large surface area capable of high cargo-loading abilities through covalent and non-covalent attachments with various proteins, peptides, imaging tags, DNAs, RNAs, and pharmaceutical drugs. In several instances, CNTs showed the ability to transport these attached biological moieties into different mammalian cell lines with the cargos retaining their biological efficacies in either proof-of-principle demonstrations or investigations into CNT delivery efficiency compared to other transporter agents.

Intracellular delivery of proteins has been facilitated through non-covalent or covalent functionalization schemes. Many proteins spontaneously adsorb onto the sidewalls of CNTs allowing for simplistic CNT intracellular delivery schemes. Kam and Dai showed that acid-oxidized SWCNTs transported fluorescently labeled bovine serum albumin and cytochrome C into non-adherent HL60 and Jurkat cells as evidenced by fluorescent microscopy; however, the higher molecular weight protein, human immunoglobin G, was not internalized on account of its size. The internalized cytochrome C in this study was able to induce apoptosis through the activation cascade of signaling enzymes that induce cell death demonstrating retained biological functionality of a protein cargo delivered by SWCNTs.
Transporting DNA into living cells can be a cumbersome task as the negative charges arising from the phosphate-deoxyribose backbone repel the negative charges present on the cellular membrane. Conventional methods for transporting DNA into cells have included using cationic polymers,\(^7\) viral transduction,\(^7\) or electroporation.\(^4\) In recent years, several investigations have reported successful intracellular transportation of functional DNA through a variety of CNT conjugate schemes. Pantarotto and co-workers used CNTs covalently functionalized with a pyrrolidine ring bearing a free amino-terminal oligoethylene glycol moiety to impart positive charge to the overall CNT-DNA complex, and demonstrated that the charge ratio of the positive amine groups to the negative charges on the DNA affected the efficiency of the delivered gene's expression.\(^5\) Kam and co-workers demonstrated SWCNT delivery of fluorescently labeled DNA by simply sonicating the SWCNTs in the presence of the DNA.\(^9\) Liu and co-workers used polyethylenimine (PEI) grafted on MWCNTs to significantly increase expression efficiency compared to PEI or DNA alone. PEI acts to rupture endosome and lysosome vesicles through osmotic swelling allowing for increased escape of the cargo DNA.\(^6\) These studies demonstrate fundamental proof-of-principle delivery of DNA through CNT conjugates; however, the efficiencies are much lower compared to other conventional transfection methods.

In contrast to the delivery efficiency with DNAs, CNT-siRNA complexes have equaled and surpassed several conventional siRNA transfection agents including Lipofectamine®.\(^8\) RNAs are small single-stranded nucleic acids possessing a ribose backbone chain in place of DNA's deoxyribose chain. These properties of RNA result in an overall smaller negative charge than DNA and act to increase cellular permeability explaining the greater success of CNT-siRNA complexes. Kam and co-workers employed a phospholipid-poly(ethylene glycol) (PL-PEG) copolymer as a dispersing agent for CNTs with a cross-linker containing a sulfide group that reversibly bonds to an attached sulfide group on synthesized short-interfering RNA (siRNA). The disulfide bond formed between the cross-linker and siRNA dissociates at low pH environments found in late stage endosomes and lysosomes allowing for release from the PL-PEG-CNT conjugate within these vesicular compartments. siRNA then escapes the vesicular compartment while the PL-PEG-CNT conjugate remains trapped for exocytosis.\(^7\) Zhang and co-workers demonstrated efficient delivery of telomerase reverse transcriptase (TERT) siRNA to \textit{in vivo} tumors resulting in
significant reduction and thus demonstrating relevant clinical application for CNT conjugates. TERT acts by silencing the telomerase reverse transcriptase RNA which otherwise would repair the telomeres found at the ends of DNA coils\cite{78}. Continuous repair of telomere degradation results in tumorigenesis and can be use to immortalize primary cell lines, therefore the silencing of TERT results in continuous degradation of telomeres and tumor remission. Liu and co-workers silenced the HIV-specific cell-surface receptor, CD4, and its co-receptors, CXCR4/CCR5\cite{69} which when silenced are no longer present to facilitate the HIV virus' cellular entry mechanism\cite{79}. These studies demonstrate great potential for CNTs to facilitate siRNA induced silencing both in vivo and in vitro.

CNTs have also demonstrated success as delivery transporters for several bioactive, pharmaceutical drugs for both in vivo and in vitro studies. The earliest demonstration of pharmaceutical delivery was by Wu and co-workers who first covalently functionalized SWCNTs with amphotericin B\cite{80}, a drug administered intravenously and considered to be the most effective antibiotic to cure fungal infections\cite{81}; however, amphotericin B is highly toxic as a result of its aqueous insolubility. Covalent attachment of amphotericin B to functionalized SWCNTs offers the advantages of increased solubility, decreased aggregation of amphotericin B, dual functionalization with a fluorescent tag for imaging and tracking, and higher efficacy owing to high amounts of internalization. This study demonstrated the retention of amphotericin B's antifungal abilities when conjugated to SWCNTs and administered to Jurkat cells exposed to different fungal species. In vivo studies of CNT-drug conjugates have shown promising results thus moving CNTs closer to clinical applications.

SWCNTs also possess distinct advantages as selective-cell destruction agents without delivery of any biological cargos owing to their ability to absorb near-infrared radiation (NIR) of wavelengths to which most biological tissue is transparent. Kam and co-workers demonstrated this effect by heating SWCNT dispersions through NIR laser exposure and then applied this effect to destroy cell lines incubated with SWCNTs\cite{80}. Gannon and co-workers used a non-invasive radiofrequency field to thermally induce cytotoxicity in three human cancer lines exposed to a polymer-CNT conjugate\cite{82}. These studies show that
SWCNT can function as selective-cell destruction agents with no loading of therapeutic agents.

The large surface area of CNTs not only allows for high loading but also accommodates multifunctional conjugate schemes. One distinct advantage of multifunctional conjugation schemes has been the addition of a signaling tag to the CNT conjugate to allow for targeted delivery and higher payload efficiencies. Kam and coworkers attached a folate moiety to SWCNTs for enhanced uptake in cells with higher expression of folate receptors over a control co-culture. This demonstrated selective targeting as a proof of principle experiment as folate receptors are highly expressed tumor markers on the cellular membrane surface of various cancer cells which act to facilitate receptor-mediated endocytosis of folate containing species. McDevitt and co-workers covalently attached tumor specific antibodies which specifically bind to tumor specific cellular membrane receptors demonstrating targeted delivery in vivo. Chen and co-workers covalently attached both anticancer agents and tumor recognition modules to SWCNTs and demonstrated negligible toxicity to in vitro cells lines that do not express the recognized membrane receptor.

These studies demonstrate the CNTs can be functionalized in a variety of ways to facilitate intracellular delivery of various biological cargos that retain their bioactive behaviors and highlight the versatile functionalization schemes available to accomplish specific delivery aims.

1.4 Toxicity of carbon nanotubes

Much attention has been given to the versatile and potential uses of CNTs in biological and non-biological applications; relatively few studies in comparison to the whole of CNT literature have investigated the health hazards that might arise from this novel material. This trend of lacking toxicological investigations has begun to reverse over the years, with in vitro and in vivo studies showing detrimental effects to cell cultures and bodily tissues with the most alarming studies demonstrating asbestos like behavior. Such a material
should hardly seem suitable then for clinical or medicinal applications; however, CNTs come in a variety of forms spanning differences in their dimensions, surface properties, contaminants, preparation methods and much more. Each of these variations in a given CNT batch can singularly or synergistically in combination with other parameters render CNTs benign or toxic. Toxicity studies have been further complicated by the CNT surface area and hydrophobicity. These surface attributes allow CNTs to interact with an assortment of cell-growth media components and commonly used toxicity assay dyes leading to either indirect cytotoxic effects or false results. The specific properties of CNTs and the dangers they pose to mammalian health will be assessed below.

Before understanding how individual CNT characteristics influence toxicity issues, a quick overview of CNT toxicity studies and their methods will be described. In vitro studies have investigated the effects on CNT exposure to lung, skin, and immune system cell cultures because these tissues are the most likely to be exposed to CNTs within a workplace environment or are responsible for clearance and detection of foreign body materials. In terms of methods, in vitro studies have covered the basics of population viability in terms of proliferation, changes in cell morphology, apoptosis, and necrosis. Most of these generally report decreases in proliferation with various results in terms of increased cell death. Decreases in proliferations may arise from the physical presence of CNTs on the cellular membrane acting to hinder adhesion mechanisms, induce down-regulation of extracellular matrix proteins, increase oxidative stress, or act with a combination of these. Other in vitro studies have investigated genotoxic effects and reported increases in mutation frequency, increases in DNA helix breaks and changes in gene expression resulting from CNT treatment. The majority of in vivo studies have explored the effects arising from CNTs within animal lungs and generally concluded that CNTs induce mild to severe toxic responses.

A CNT's length can span from the nanometer to millimeter length scales depending upon the synthesis method but the comparatively small diameter of a typical CNT in comparison to its length often draws a comparison between CNTs and other carcigenic materials such as synthetic vitreous fibres and asbestos. Exposure to fiber-derived free
radical generation often induces hazardous amounts of DNA damage and genetic mutations over periods of exposure giving rise to a highly malignant form of cancer, mesothelioma. Common symptoms of these bio-persistent fibers include alveolar lesions termed granulomas, signs of oxidative stress, and excessive fibrous tissue within the examined specimen.\textsuperscript{120} Macrophage cells are responsible for the removal of foreign materials from their host, and high aspect ratio fibers with lengths exceeding 20 µm encumber complete phagocytosis making them extremely resistant to bio-clearance mechanisms.\textsuperscript{121} Regarding CNTs, Pollard et. al. demonstrated CNTs instilled as spherical or stellate shape agglomerates with entire populations less than 20 µM produced no statistically significant adverse reactions compared to samples with individually suspended MWNTs, agglomerates, and ropes of MWCNTs with lengths exceeding 20 µM.\textsuperscript{120} Tajaki et. al demonstrated actual mesothelioma formation in mice genetically susceptible to cancer and with treatments containing excessive amounts of MWNTs possessing significantly large lengths.\textsuperscript{122} Bio-applications that CNTs may be well-suited for will generally not require such lengths especially since long CNT transporters are entirely unpractical for drug-delivery while shortening CNT samples batches is easily achieved by altering synthesis conditions or processing by extended sonication in combination with centrifugation.\textsuperscript{123,124}

While CNT length factors heavily into bio-clearance issues, the size of the CNT diameter raises concerns of their potential to migrate throughout the body, traverse through tissue membranes, and accumulate within organs. Several types of particles with nanoscopic diameters similar to CNTs cross protective linings within the body demonstrating that various forms of nano-meter sized foreign body material may gather in regions separate from the administration site.\textsuperscript{125-128} CNTs under varying conditions and preparation methods have demonstrated the ability to traverse to various organs throughout the body following various routes of administration.\textsuperscript{129,130} Labeled CNTs possessing a ligand targeting specific cell types have shown increased rates of internalization for in vitro culture populations expressing the corresponding membrane receptors\textsuperscript{9} while covalent or non-covalent modifications to the CNT exterior involving large polymers or bio-molecules easily tailor the size and bio-retention time of the entire CNT delivery scheme.\textsuperscript{131} Such
CNT modifications, can effectively deflect unwanted passage across particular or general biological barriers.

The high surface area per unit volume of the CNT coupled with their hydrophobic nature can facilitate interactions with a host of biological molecules. Numerous examples of dispersion-forces based or aromatic-stacking interactions with proteins, RNA, DNA, and enzymes have been shown. Casey and co-workers have also reported toxic effects indirectly arising from the adsorption of growth media nutrients thereby indirectly causing cytotoxicity. CNTs have also demonstrated the ability to interact with several commonly employed toxicity assays; the results arising from MTT, Neutral Red, Comassie Blue, Alamar Blue™, and WST-1 assays have been questioned further complicating issues with CNT toxicity trials. Additionally, the large surface area provided by each CNT can cause strong adhesion to and can even distort membrane configurations thus decreasing permittivity and hindering proliferation. In addition to directing migration within the body, functionalization schemes used to coat or modify the CNT surface must appropriately cover the CNT surface to reduce unwanted biological interactions.

Commonly used functionalization and purification schemes often begin by acid refluxing treatments of CNTs thereby attaching soluble functionalities while dissolving exposed catalyst particles remaining after CNT synthesis. Increasing amounts of catalysts have been shown to generate significant amounts of free radicals and increase inflammatory responses within cellular environments necessitating the removal or reduction of exposed particles. Pitfalls of these schemes may include redeposition of the catalyst particles on the CNT surface or development of defect sites along the CNT surface. Any redeposited metals will remain bio-reactive for generation of free radicals. Acid-oxidation treatments may also increase the number of CNT defect sites which in turn increases toxicity. In a companion study, Fenoglio et al. and Muller et al. used high-temperature annealing to eliminate residual catalysts, carbon impurities, and defect sites which in turn reduced the amount of free-radicals in solution generated by the Fenton reaction and photolysis of H₂O₂. Earlier work by Fenoglio et al. demonstrated this CNT quenching effect and proposed that a radical scavenging mechanism was responsible
for the toxicity observed in vivo. Muller then confirmed that a decrease in defects corresponding to non-radical quenching CNTs led to less acute pulmonary toxicity and genotoxicity.

Despite the negative effects attributed to CNTs in vitro and in vivo, CNTs may still find many prominent and useful roles in biological applications. CNTs possess inherently unique properties that not only make them advantageous for the applications described within this thesis, but also introduce a variety of complications when placed into bodily tissues or cellular environments. Fortunately, several viable solutions exist to mitigate or eliminate adverse effects arising from these individual problems. In summary, CNTs should be short, appropriately functionalized for the intended purpose, and free of metal catalysts and defects to reduce side-effects arising from any CNT treatment. As with any clinical therapy, the pros must be weighed and balanced against the cons arising from the potential therapies involving CNTs.

1.5 Conclusions

This thesis seeks to build upon the investigations described above and explore the possible biological applications that CNTs may hold as cellular growth substrates and to investigate underlying interactions that biological molecules have upon CNT cellular internalization. Chapter 2 provides an overview and background of the various materials used including CNTs, human embryonic stem cells, and designed synthetic peptides in addition to reviewing the foundations of various instrumental methods including Raman spectroscopy, absorption spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, atomic force microscopy, scanning electron microscopy, and contact angle measurements. Chapter 3 details an investigation into the use of CNTs as human embryonic growth substrates and examines how varying the surface features of these substrates can affect various behaviors of the human embryonic stem cells. Chapter 4 closely examines how changing CNT dispersion parameters shapes the physical attributes of the fabricated CNT films to which adherent cell cultures respond. Chapter 5 shifts from the use of CNTs as cellular growth substrates and examines how the interactions between designed synthetic
peptides and SWCNTs affect cellular internalization in mammalian cells. Finally, Chapter 6 concludes with overviews of each of these Chapters and highlights aspects of possible future investigations.
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Chapter 2
Materials and Techniques

2.1 Introduction

Several materials and methods will be employed repeatedly throughout this thesis and will be described within this chapter. The principle materials include carbon nanotubes (CNTs), synthetic peptides, and human embryonic stem cells (hESCs). The methods employed investigate the properties and characteristics of each of these materials and their interactions with one another. Overviews of the materials and methods and principles of operations for these methods are presented in this chapter.

2.2 Carbon nanotubes

Carbon nanotubes are promising materials in many fields of research due to their intrinsic optical, electrical, mechanical, and dimensional properties with potential applications as conductive and high-strength composites, energy storage and energy conversion devices, sensors, field-emission displays and radiation sources, hydrogen storage media, and nanometer-sized semiconductor devices and interconnects. As described in Chapter 1, CNTs have several potential opportunities to aid in biological applications such as cellular growth substrata, drug delivery transporters, selective cell destructive agents and biosensors.

Nanotubes are cylindrically shaped molecules with diameters spanning from less than one nanometer to approximately one hundred nanometers and with lengths ranging from a few hundred nanometers to several microns. Conceptually, a nanotube which has been severed from end to end on one edge of its circumference and then unrolled and flattened into a single sheet will possess an atomic lattice identical to a graphene sheet.

Graphene, like carbon nanotubes, is comprised of sp² hybridized carbon atoms. This hybridization delocalizes π-orbital electrons within the lattices of graphene and carbon nanotubes giving these materials their conductive properties and increasing their atomic
bond strength. While semi-conducting graphene possesses a zero band gap,[12] carbon nanotubes may be either metallic or semi-conducting with a range of band gaps depending upon their diameters and chiral properties.

Nanotube chirality depends upon the angle at which the opposite edges of the flattened nanotube sheet connect. Each type of nanotube possesses a chiral vector that indicates specific carbon atoms lying on opposite edges of the nanotube sheet that join when hypothetically rolled into a cylinder. The chiral vector is comprised of two types of unit vectors denoted as ‘a1’ and ‘a2’ lying within each hexagonal unit cell of the sheet lattice as shown in Figure 1. The chiral vector, C, is defined by Equation 2.1 where n and m are the integer numbers for each type of unit vector required to form the chiral vector.

\[ C = n a_1 + m a_2 \]

*Equation 2.1* Formula for a CNT chiral vector in terms of unit lattice\[11\]

Each type of nanotube chirality is classified by the \((n, m)\) notation. If the sum of \(n\) and \(m\) unit vectors is divisible by three than the nanotube will have a zero band gap and behave metallically, but if the sum of these unit vectors is not divisible by three then the nanotube will be semi-conducting.\[11\]

*Figure 1* Chiral vector conceptually connecting two ends of a carbon nanotube along a graphene sheet (left) and single walled carbon nanotubes with different chiralities (right)
Depending upon synthesis conditions, carbon nanotubes can possess one or more shells. In the latter case, multiple smaller diameter nanotubes sit concentrically inside of larger nanotubes. Nanotubes having only one, two, or multiple shells are termed single-walled carbon nanotubes (SWCNT), double-walled carbon nanotubes (DWCNT), and multi-walled carbon nanotubes (MWCNT), respectively. SWCNTs, DWCNTs, and MWCNTs have different surface areas, conductivities, and solubilities rendering each type of nanotube more suitable for specific applications.

Several synthetic methods exist for production of carbon nanotubes with each method offering its own advantages and disadvantages, but all methods require a high energy source, a carbon feed, and transition metal catalysts. Commonly employed synthetic methods include laser ablation, arc discharge, and chemical vapor deposition. All synthesis methods result in residual contamination by the catalysts and other carbonaceous impurities and also generate a distribution of nanotube diameters and chiralities. A number of limitations hinder processing carbon nanotubes into a functional form from their crude postproduction state including universally poor solubility, synthesis contamination, bundle aggregation, and chirality distribution; however, these problems can be overcome to some extent with the aid of dispersants, chemical purification, sonication, centrifugation, and processing CNTs at low concentrations.

2.3 Peptides

Bio-incompatibility and dispersion instability at high CNT concentrations of standard surfactants such as Triton-X 100 and sodium dodecyl sulfate have led researchers to search for new dispersing agents of biological origin. Many naturally occurring proteins possess both hydrophobic and hydrophilic regions that allow for aqueous dispersion of CNTs; however, many proteins only mildly interact with and solubilize CNTs due to structural conformations, steric hindrances, minimally exposed contact areas, and high amounts of molecular charge. Optimization of biological dispersing agents can be achieved by designing amphiphilic peptides with secondary structures to functionally disperse CNTs which can later be used as a starting foundation for other CNT bioapplications. Zorbas and
co-workers have extensively researched the interactions of a specific peptide sequence termed Nano-1 and its derivatives with SWCNTs and determined that increasing the number of aromatic residues within the sequence enhanced SWCNT dispersing ability of the peptide chain.\textsuperscript{[14-16]} In a related study, Chin et al. reported that the use of Nano-1 as a SWCNT dispersing agent enhanced intracellular uptake of SWCNTs in HeLa cells compared to SWCNTs dispersed in growth media alone.\textsuperscript{[17]} Adsorption interactions of the Nano-1 peptide and its derivatives with SWCNTs and consequential effects upon cellular internalization are explored further in Chapter 5.

Nano-1 adopts either a random coil or an \( \alpha \)-helical secondary protein structure in water depending upon its concentration, the pH, or the presence of SWCNTs.\textsuperscript{[15,18]} The \( \alpha \)-helix shown in Figure 2 is a common structural motif adopting a right-handed coiled conformation with a 1.2 nm diameter, 3.6 amino acids per turn, 0.54 nm per helical rise, and typically possesses between 4 to 40 amino acids.\textsuperscript{[19]} \( \alpha \)-helices form because apolar regions of the amino acid main chain act to minimize contact with surrounding polar aqueous environment. Hydrogen bonds between main chain atoms systematically form along the helical axis between rises substantially adding to the overall conformational stability. Depending upon their polarity, functional groups of the individual amino acids will extend from the helical core and interact with the surrounding environment.

The folded conformation of the \( \alpha \)-helix and all proteins depend upon the free energy difference, \( \Delta G \), between the folded and unfolded states. \( \Delta G \) depends upon the enthalpy contributions arising from the hydrophobic interactions, hydrogen bonds, and ionic bonds found within the protein chain and upon entropic contributions. The formal relation between \( \Delta G \) and changes in enthalpy and entropy is given by Equation 2.2 where \( \Delta H \) is the change in overall enthalpy, \( T \) is temperature, and \( \Delta S \) is overall change in entropy. For the folding of most proteins, \( \Delta G \) ranges between 5-15 kcal/mol which is not much more than the free energy contribution of a single hydrogen bond highlighting the delicate stability of a folded protein.\textsuperscript{[19]}
\[ \Delta G = \Delta H - T \Delta S \]

*Equation 2.2* Enthalpic and entropic contributions to free energy difference between the folded and unfolded states of protein

![Diagram showing the structure of an α-helix](image)

*Figure 2* Structure of the α-helix visualized from the side showing hydrogen bond interactions between helical pitches acting to stabilize the conformational structure

Nano-1 has twenty-nine hydrophobic and hydrophilic amino acid residues. Selective positioning of each amino acid within the designed α-helix sequence affords Nano-1 a hydrophobic and hydrophilic surface when folded into the helical conformation. Additionally, opposing faces of the Nano-1 peptide have opposite charges to promote favorable electrostatic interactions between peptides adsorbed onto SWCNT surfaces. Peptide primary sequences are often denoted in single letter symbols which represent one of the twenty commonly occurring amino acids, and in the case of α-helices, conformational structures can be shown along their helical axis as shown in *Figure 3* to better illustrate the position of various residues within the helix.
Efforts to increase the binding affinity of the Nano-1 for SWCNTs have led researchers to exchange various amino acids within the Nano-1 sequence with phenylalanine amino acids which will increase the overall hydrophobicity of the SWCNT interaction face and promote \(\pi-\pi\) stacking interactions with the nanotube surface.\(^\text{14}\) Three Nano-1 derivatives have been made with 0, 4, or 8 phenylalanine amino acids and are referred to within this thesis as 0F, 4F, or 8F, respectively. Each derivative also has a fluorescently labeled analogue with a fluorescein fluorophore attached to the first lysine residue in the peptides' primary sequences to aid with investigations concerning intracellular location and cellular internalization.

![Figure 3 Axial cross-section view of the Nano-1 peptide with amino acids represented by their 1 letter symbols and labeled apolar and polar faces of the Nano-1 peptide when folded into the \(\alpha\)-helical conformation\(^\text{15}\)](image)

2.4 Human embryonic stem cells

Stem cells are specialized cells possessing two significant abilities to propagate new stem cells or to mature into other adult cell types of the body. These processes of stem cell propagation and of stem cell maturation are termed self-renewal and differentiation, respectively. Stem cells, because of these two abilities, can potentially provide an unlimited source of new cells and tissues that otherwise could not be created. Potential applications arising because of this include regenerative therapies for Parkinson's disease,
Alzheimer’s disease, stroke damage, cardiovascular damage, Type 1 Diabetes, cancer therapies, burn injuries, and entire organ replacement. In each of the above diseases, non-replenishing tissue is either permanently damaged, non-existent, or cannot regenerate quickly enough necessitating a high-input source of renewable tissue. While theoretically possible to provide cures for these diseases, understanding and manipulating crucial differentiation processes, overcoming a patient’s immune responses following implantation, and engineering logistical aspects of stem cell harvesting and culturing methods remain as blockades to clinical treatments.

Several problems remain for embryonic stem cells. Factors controlling their differentiation fates are not entirely understood and in cases where some control over differentiation fate has been demonstrated, small fractional proportions of the entire cell population remain undifferentiated or differentiate into an unintended cell type. Upon therapeutic implantation, these unsuccessfully differentiated cells can render unintended and detrimental consequences upon the patient, namely tumorigenesis. Conversely, retention of self-renewal properties for an entire hES cell population under optimized in vitro conditions remains incomplete resulting in spontaneous differentiation. Understanding the factors and variables responsible for these occurrences begins with an overview of embryogenesis.

The development of an embryo, serves as a foundation to comprehending key points of hESC differentiation and behavior in both in vitro and in vivo environments. Five days
after fertilization of an egg, the zygote develops into a spherical structure, termed a 
blastocyst, between 100-200 microns in diameter. A blastocyst consists of 70-100 cells in 
total arranged in outer layer that acts a sheath surrounding an inner platelet of cells. The 
outer layer, termed a trophoblast, will develop into a large part of the placenta while the 
inner platelet of cells, termed the inner cell mass (ICM) will develop into the fetus. As the 
ICM develops, it arranges itself into three layers called the endoderm, the mesoderm, and 
the ectoderm. The ectoderm is the outermost layer of the ICM resting against the 
trophoblast and develops into the skin and nervous system. The mesoderm layer lies 
between the other two layers and develops into the skeleton, blood, and muscle. The 
endoderm is the innermost layer and gives rise to various organs including the lungs, liver, 
pancreas, stomach, and intestines. As in the early stages of in utero development, under in 
vitro conditions, the next cell type a pluripotent embryonic stem cell differentiates into 
along a given differentiation pathway will be either an ectodermal, mesodermal, and 
endodermal cell. The arrangement of ICM cells into three differentiating layers reveals 
that an ICM cell’s and its neighbors have interplaying effects.

When hESC are grown in vitro, they are commonly grown in co-culture with mitototically 
inactivated embryonic fibroblast cells of human or mouse origin. These fibroblasts release 
growth factors and secrete extracellular matrix (ECM) proteins that maintain pluripotency 
to a large extent and aid hESC adhesion. hESC can grow on a variety of ECM proteins 
alone including laminin, collagen, vitronectin, fibronectin, or Matrigel® without a co-
culture of embryonic fibroblasts; however, the growth media used to nourish the hESC 
must be previously incubated with MEFs in order to acquire their excreted growth 
 factors. Matrigel® is the second most common hES growth substrata consisting of a 
rich assortment of ECM proteins extracted from mouse tumors. In addition to the 
growth factors released by the MEF co-cultures, other growth factors must be individually 
added to the growth media aimed to retain pluripotent behavior.

Quality and reliability of MEFs can vary from batch to batch if received frozen from a 
company, and Matrigel® and other ECM proteins do not prove to be as good a substrate as 
embryonic fibroblasts in terms of cell viability. Additionally, all of these substrates are 
derived from animals and pose a risk of contamination that would elicit an immunogenic
response if implanted into a patient. An entirely synthetic growth media has been fabricated to overcome the problem of nourishing the cells from non-animal sources; however, an entirely synthetic substrate to date remains elusive.

A synthetic substrate seeking to replace conventional biological growth substrates for hES cells should emulate an ECM in terms of three-dimensionality and nanofibrous topography offering cells a pliable mesh as opposed to a flat and rigid surface. Methods for generating these nanofibrous scaffolds include electrospinning, self-assembling peptides, and methods based on polyelectrolyte complex coacervation and phase separation techniques. Another possible avenue, for three dimensional synthetic scaffolds are carbon nanotubes (CNTs). Much success has already been demonstrated for growth of various specific cell lines including neuronal cells, mesenchymal stem cells, and osteoblast cells. In addition, CNT substrates can be varied in terms of roughness which will be shown later in this thesis to play a crucial role in hESC cellular behavior.

### 2.5 Raman scattering of carbon nanotubes

CNTs exhibit high Raman scattering intensities due to their extended $\pi$-electron structures making Raman spectroscopy an extremely useful technique for investigations involving small quantities of CNTs. The Raman spectral features of CNTs also shift with changes in the CNTs local environment thereby serving as an indicator of surrounding conditions. Several biomolecules including peptides also exhibit Raman scattering and shifts in spectral peaks upon changes in secondary structure conformations.

Matter interacts with incident radiation by transmission, absorption, reflection, or scattering processes. Scattering processes affect a fraction of the total radiation and proceed by elastic or inelastic mechanisms. Elastic scattering, also known as Rayleigh scattering, disperses radiation with an equal frequency to the incident radiation while inelastic processes scatter radiation at higher or lower energies. The difference in energy between the inelastically scattered radiation and the incident radiation corresponds to vibrational transitions. Inelastic scattering or Raman scattering is detected in the visible and NIR region of the EM spectrum corresponding to these vibrational transitions.
Raman scattering occurs when radiation induces a change in a molecule's polarizability and excites the molecule to a virtual electronic state with an energy lying between the lowest excited electronic state and the ground electronic state. After Raman scattering, the molecule returns to the ground electronic state but will not return to the vibrational level from which it originated. When the molecule returns to a different vibrational level, the scattered radiation imparts or extracts an amount of energy equal to the difference between the initial and final vibrational levels. Imparting and extracting vibrational energy is termed Stokes and anti-Stokes scattering, respectively. These processes are illustrated in Figure 5.

Figure 5 Electronic and vibrational transitions involved in Rayleigh and Raman scattering

The intensities of Raman scattered radiation to incident radiation are approximately 0.001% necessitating a high amplitude radiation source such as a laser focused onto the sample through an objective lens. Scattered radiation from the sample diffracts from a grating into a photon multiplier tube or charge coupled device which detects the scattered radiation's intensities and frequencies as shown in Figure 6.
Carbon nanotubes exhibit high Raman intensities because their extended π-electron structures allow for extensive electron delocalization over the surface of the nanotube increasing the polarizability of the nanotube lattice. Additionally, excitation laser frequencies will often closely match the transition energy between the valence and conducting bands of specific nanotubes with certain diameters leading to a condition known as resonance enhanced Raman scattering.\(^{11}\) Resonance enhancement increases vibrations by a factor of \(10^2\) to \(10^6\) allowing for detection of analyte concentrations as low as \(10^{-8}\).\(^{16}\) Figure 7 shows the transition level energies for SWCNTs with different diameters and chiralities.\(^{31}\)

*Figure 6 Schematics of a Raman spectrometer showing how elastically scatter light travels from the samples to detector*
The main Raman spectral peaks for carbon nanotubes occurring between 250-400 cm\(^{-1}\), 1280-1400 cm\(^{-1}\), and 1500-1650 cm\(^{-1}\) are termed the radial breathing modes (RBM), the disorder-induced D-band, and the G-band, respectively. 2\(^{\text{nd}}\) order peaks for the D-band are located between 1700 cm\(^{-1}\) and 1900 cm\(^{-1}\).

**Figure 7** Transition energies for single-walled carbon nanotubes with various diameters, lattice chiralities, and electronic character\(^{31}\)

**Figure 8** Raman spectrum with 785 nm excitation of an isolated single-walled carbon nanotube deposited on silicon (asterisks denote features arising from the silicon substrate)\(^{28}\)
RBM modes correlate to lattice vibrations where the carbon atoms vibrate in phase in the radial direction. The wavenumbers of the RBM peaks vary inversely with the diameter of the tube and can determine the diameter of an isolated nanotube, \( d \), by Equation 2.3 where \( \omega_{\text{RBM}} \) is the wavenumber at which the RBM peak occurs and \( A \) is a constant equal to 248 cm\(^{-1}\).\(^{[32]}\)

\[
\omega_{\text{RBM}} = \frac{A}{d}
\]

*Equation 2.3 Raman spectral wavenumber for the RBM mode of an isolated SWCNT of a given diameter\(^{[32]}\)*

The resonance enhancement effect insures that observed RBM signals only arise from CNTs in resonance with the excitation laser, thus characterization of the distribution of nanotube diameter improves with increasing the number of excitation lasers or through the use of tunable lasers.\(^{[33]}\) RBM modes can also distinguish isolated SWCNTs from bundled SWCNTs by Equation 2.4 where \( A \) equals 234 cm\(^{-1}\) and \( B \) is an upshift arising from interactions between bundled tubes.

\[
\omega_{\text{RBM}} = \frac{A}{d} + B
\]

*Equation 2.4 Determination of isolated vs. SWCNT bundles by shifts in RBM spectral features\(^{[32]}\)*

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The disorder-induced D-peak results from disruptions in sp² hybridized carbon lattice which can arise from sp³ hybridized carbon atoms, vacancies, or kinks in the nanotube lattice.\cite{32}

The G-peak between 1500-1650 cm⁻¹ is comprised of six Raman allowed modes corresponding to lattice vibrations in the tangential and circumferential directions.\cite{32}

Graphite, for which the G-peak is named, exhibits a single feature at 1582 cm⁻¹ corresponding to its tangential vibrational mode. Unlike graphite, carbon nanotubes possess six vibrational modes in this region because they belong to different classes of symmetry.
Two specific features in the G-band domain termed G\textsuperscript+t and G\textsuperscript{-} correspond to the tangential and circumferential phonons, respectively. Metallic carbon nanotubes are more symmetrical than chiral semi-conducting tubes. These differences in symmetry give rise to a slight difference in wavenumber for the G\textsuperscript{+} feature and a significant spectral shift for the G\textsuperscript{-} feature. In metallic CNTs, the G\textsuperscript{+} and G\textsuperscript{-} features occur at 1587 cm\textsuperscript{-1} and 1550 cm\textsuperscript{-1} respectively, while semi-conducting CNTs exhibit these spectral peaks at 1592 cm\textsuperscript{-1} and 1570 cm\textsuperscript{-1}, respectively.

The wavenumber of the G\textsuperscript{-}-peak corresponding to the circumferential phonons decreases proportionally with CNT diameter regardless of symmetry, while the wavenumber of the G\textsuperscript{+}-peak is virtually independent of diameter. Thus, the difference in wavenumber between the G\textsuperscript{+}-peak and the G\textsuperscript{-}-peak can be used to determine the diameter of a single SWCNT or the ensemble average diameter of a nanotube population. The nanotube diameter is given by Equation 2.5 where \( \omega_0 \) equals 1592 cm\textsuperscript{-1}, \( \omega_G \) is the observed wavenumber of the G\textsuperscript{-}-peak, and \( \beta \) equals -45.7 cm\textsuperscript{-1} or -79.5 cm\textsuperscript{-1} for semiconducting or metallic CNTs, respectively.\[ d = \frac{\beta}{\omega_G - \omega_0} \]

*Equation 2.5 Nanotube diameter determination by the G\textsuperscript{-}-band Raman spectral feature of a SWCNT\[32]\]

2.6 Absorption spectroscopy

In this study, absorption spectroscopy is used to detect the dispersion states and concentrations of carbon nanotubes and peptides. When isolated in dispersion, SWCNTs absorb light proportionally to concentration, but upon aggregation the SWCNT extinction coefficient diminishes making absorbance disproportional to SWCNT concentration. A plot of extinction coefficient vs. concentration reveals the concentration at which the onset of bundling occurs.\[34\]
Electronic transitions between ground and excited states of molecules correspond to absorptions of electromagnetic radiation in the region between 200 and 1100 nm \(^{30}\). A sample's absorption spectrum over these wavelengths can reveal its electronic transition energies, concentration, extinction coefficient, and interactions with its environment. Absorption spectrometers work by monitoring the transmitted intensity of radiation through a sample compared to the transmitted intensity through a blank reference sample. The intensity of the absorption is then given by Equation 2.6 where \( I, T_s, \) and \( T_0 \) are the intensity of the absorption peak, transmission intensity through the sample, and the transmission intensity through the reference, respectively.

\[
I = -\log \frac{T_s}{T_0}
\]

*Equation 2.6 Absorption intensity given in terms of the negative logarithm of the measured transmissions of the sample and reference\(^{30}\)*

Absorption generally corresponds linearly to the amount of sample that lies within the radiation beam path at low concentrations; however, at high concentrations, typically greater than 0.01 M for small molecules, charge effects arise between neighboring molecules which lessens the change in absorption at an individual wavelength but broadens the overall absorption peak. \(^{30}\) Within certain concentration ranges, absorption, \( A \), follows the Beer-Lambert law which states that the intensity of absorption is proportional to the concentration of the sample, \( C \), the path length of radiation through the sample, \( B \), and the intrinsic molar extinction coefficient of the sample, \( \varepsilon \).

\[
A = \varepsilon BC
\]

*Equation 2.7 Beer-Lambert law relating the absorbance to a sample's concentration, path length, and molar extinction coefficient*
Since incident radiation can be scattered, reflected, or absorbed upon contact with a sample, many spectrometers employ dual beam radiation paths to counteract any attenuation effects. Most absorption spectrometers employ a monochromator to selectively diffract individual wavelengths from the light source as shown in **Figure 11**.

![Figure 11 Schematics of a dual-beam absorption spectrometer showing the beam path from radiation source to dual detectors](image)

**Figure 11 Schematics of a dual-beam absorption spectrometer showing the beam path from radiation source to dual detectors**

### 2.7 Fluorescence spectroscopy

Fluorescence spectroscopy is used in a number of chemical and biological applications and is especially applicable to low concentration range studies as a result of its inherent sensitivity. Fluorescence spectroscopy often quantifies unknown concentrations, determines excitation lifetimes, and is used to detect fluorescent species. In biology, modifying a species of interest with a fluorescent tag can aid microscopic studies of cells or tissues. Other notable biological applications include gel electrophoresis, fluorescent activated cell sorting, DNA detection, and fluorescence recovery after photobleaching. In this work, fluorescence is used to highlight the intracellular location of Nano-1 peptides and associated SWCNTs after intracellular internalization.

Molecular emission occurs when a molecule in an excited electronic state radiatively decays to its ground electronic state. To excite to the higher electronic state, molecules must first receive an amount of energy equal to the difference between excited and ground...
state energy levels. Emitted light occurs at longer wave lengths than the excitation light because while the molecule is in the excited electronic state, relaxations between the vibrational energy levels of the excited state transfer energy to their environment through heat.

Figure 12  Electronic and vibrational transitions involved in fluorescent excitation, decay, and emission mechanisms

A number of deactivation processes may hinder the quantum yield of a molecule where quantum yield is simply defined as the number of molecules in their excited states that fluoresce divided by the total number of excited molecules. Quantum yield generally decreases with increasing temperature because the increase in frequency of collisions with the surrounding solvent and other solutes improves the probability for deactivation by external conversion whereby energy is transferred from the excited state molecule to the surrounding solvent or other solutes. pH changes that induce resonance destabilization by charge effects will also reduce quantum yield because the lifetime of the excited state molecule diminishes as a result of this destabilization. Dissolved oxygen may oxidize the fluorescing species or may promote a transition to an excited triplet state of lower energy as a consequence of oxygen's paramagnetic properties.\[30\]
Figure 13  Schematics of a luminescent spectrometer showing a light source travelling to the sample and the emitted fluorescent light of the sample

A simple luminescent spectrometer contains basic spectroscopic components. A light source passes through an excitation filter or diffracts off a monochromator to allow one wavelength region of light to irradiate the sample which then emits in all directions. Emission filters or a subsequent monochromator then selects light of the wavelength of interest and directs it to the emission detector. Emission detectors are usually placed at right angles to reduce detection of scattered light emanating from the solvent and cuvette.

Fluorescence generally occurs in molecules with an extended aromatic system containing a fused benzene ring. Aromatic systems generally possess a low $\pi \rightarrow \pi^*$ transition energy and also possess high molar absorptivities and excited state lifetimes. Chemical modification of the aromatic system can alter the wavelengths and intensities of the excitation and emission peaks and affect quantum yield. Structural rigidity within the aromatic network of the molecule stabilizes an extended $\pi$-electron system while in the excited state. Such molecular characteristics are common in cellular imaging dyes.

Luminescent emission from fluorescent dyes that are covalently or non-covalently attached to cellular components signals the intracellular location of these components within the cell. Dyes may be generic and simply diffuse across the cellular membrane into the cytoplasm and nucleus or dyes can be specific and only associate with one type of cellular component.
Multiple dyes can be used in conjunction to highlight several areas of interest provided that their emission peaks do not significantly overlap. Special dyes can offer unique advantages to intracellular tracking and targeting. Some dyes will only fluoresce in low pH ranges making the acidic environments of lysosomes ideal for labeling. Primary antibodies possess specific binding conformations for membrane proteins found on organelles and will attach and remain to these components. A secondary antibody with a fluorescent tag introduced to the cell will bind to the primary antibody thus completing this detection scheme; however, common problems arising from dyes include toxicity and photooxidative decay upon excitation.\[^{136}\]

2.8 Circular dichroism spectroscopy

Zorbas et al. have shown by circular dichroism (CD) spectroscopy that Nano-1 (4F) folds into the α-helix conformation above 200 mM concentrations but remains in a random coil conformation at lower concentrations, and that SWCNTs induce α-helical folding in Nano-1 below 200 mM.\[^{115}\] The extent to which SWCNTs can lower the onset concentration at which folding occurs for each Nano-1 derivative will serve as a qualitative indicator for SWCNT-peptide binding affinities. CD spectroscopy will also aid investigations into the fluorescent emission dependence of the fluorescently labeled Nano-1 derivatives upon folding.

Unpolarized light propagates through space three-dimensionally while linearly polarized light propagates within a single plane. Circularly and elliptically polarized light propagate forward with the magnitude of the electronic component spiraling about the axis. Left (LCP) and right (RCP) circularly polarized light spiral about their axes in the counterclockwise and clockwise direction, respectively. Circular dichroism (CD) spectrometers do not differ significantly from the design of typical absorption spectrometers except that they employ polarizers and polarization detectors to measure the difference in absorbed intensities of LCP and RCP light, \(\Delta A\), as it passes through optically active samples. Most CD spectra are given in units of ellipticity, represented as \(\theta\), vs. wavelength as given in Equation 2.8.
ΔA = \frac{4\pi(\text{degrees})}{180 \ln 10} = \frac{\alpha(\text{millidegrees})}{32,982}

Equation 2.8 Relation of ellipticity to difference in intensity of measured LCP and RCP polarized light

Biological molecules with secondary structures impart distinct spectral features for their CD peaks. This occurs because CD effects arise from the interactions of polarized light with transition dipoles that are heavily influenced by spatial conformations of the polypeptide. Electronic transitions in the peptide chromophore of polypeptide chains produce absorption peaks in the UV spectral region between 180 nm and 250 nm. These transitions correspond to the transitions of non-bonding π-electrons which are delocalized to some extent over the carbon, oxygen, and nitrogen atoms of the peptide main chain. The lowest energy transition of the peptide chromophore is an n→π* transition of the carbonyl bond occurring in the 210-230 nm range and depends heavily upon the hydrogen bonding of the oxygen atom. A second higher energy π→π* transition also originating from the carbonyl bond occurs near 190 nm and is heavily affected by the involvement of the nitrogen atom.

Spectral peaks for a standard absorption spectrum and a CD spectrum of an optically active, in terms of producing a CD signal, sample will generally occur at the same or nearby wavelengths. These wavelengths corresponding to electronic transitions give rise to standard CD spectral features including α-helices, β-sheet structures, and random coils as shown in Figure 14. The CD spectra of α-helices are characterized by two negative peaks occurring at 208 nm (π→π*) and 222 nm (n→π*) while the random coil spectrum has a negative CD signal below 200 nm and a positive band around 218 nm. β-sheet structures (not present in any of the Nano-1 derivatives) have a negative peak at 216 nm.

Most analyses of CD spectra weigh the contributions from each standard secondary structure spectral feature until the modeled data reaches a minimum in the mean square
difference from the sample data. The percentage of each conformation found within the polypeptide chain equals the weighted percentage of each spectral feature.

Figure 14 Standard circular dichroism spectra for common secondary structures including the α-helix, the β-sheet, and the random coil

2.9 Atomic force microscopy

Atomic force microscopy (AFM) is a surface analysis technique capable of examining the nanoscopic dimensions and characteristics of the outermost layer of a material. The materials for an AFM sample need not be extremely bulky and can be a single atomic layer or individual molecules deposited on another molecularly flat surface. AFM offers visual depictions over which an atomic-size probe scans and acts in a similar manner as a stylus does on a record. In addition to a visual depiction, AFM can also offer information pertaining to the frictional, viscoelastic, magnetic, and electronic characteristics of a surface. AFM offers some advantages over scanning tunneling microscopy (STM) and scanning electron microscopy (SEM) which also offer nanoscopic resolution. AFM examines all materials regardless of conductivity while both scanning electron microscopy and scanning tunneling microscopy require samples to be conducting, and AFM can also operate at room temperature on a simple desktop and also function under specialized environments including liquids and gases.
Basic components of an AFM include a laser, a probing tip attached to a several-hundred micron-scale wide cantilever with a reflective back-side coating, and photodiode detector composed of quartered spatial sections tuned to the frequency of the laser. The laser is aligned and focused onto the backside of the cantilever reflecting into, focusing on, and centering upon the four quadrants of the photodiode detector. As the tip comes in to contact with the surface or begins to interact with any surface forces, vertical and torsional distortions of the cantilever reflect the incident laser onto new positions within the different quadrants of the photodiode detector. The four sections of the photodiode detector designated as $A$, $B$, $C$, and $D$ are depicted in Figure 15. Each quadrant receives a different proportion of the entire laser beam and the signal intensity reported from each quadrant determines the distortion of the cantilever thus revealing a sample’s morphological surface features. The tip is raster scanned along the surface of the material by either driving the tip or the stage with a piezo-electric ceramic. These ceramics actuate upon applied directional voltage allowing for nanoscopic control over the tip’s or stage’s movement. A feedback control loop acts over the entire process to insure the voltage is adjusted to the piezo-electric ceramic when the tip is too far or too close to the sample surface.

![Photodiode Detector Diagram](image)

**Figure 15** *Fundamental operations of basic components in an atomic force microscope*

AFM tips come in a variety of sizes and shapes but usually scale tens of microns high by a few microns wide and all tips become extremely narrow towards their end point giving
nanoscopic scale size dimensions to the tip's apex. Wear and tear of the tip is inevitable even in experienced user's hands while mishandled AFM operation can break a tip instantly. Fortunately, tips can be easily replaced within less than a minute. Tips are commonly made from silicon nitride and other silicon materials owing to their high material strength, and can be tailored to specific operational modes and techniques for and beyond basic AFM operation including contact mode, non-contact mode, magnetic force mode, electrostatic force mode, and more.

The detector acquires three different signals including the overall laser intensity from the summation of the four quadrants \((A+B+C+D)\), the difference in intensities from quadrants \(A\) and \(D\) \((A-D)\), and the difference in intensities from quadrants \(B\) and \(C\) \((B-C)\). These signals contain information which relates to the normal deflection and torsional deflection of the cantilever. Normal deflection (DFL) is measured as the difference in intensities from the top and bottom halves of the detector \(((A+B)-(C+D))\) while torsional deflection (LF) measures the difference in intensities from the left and right halves \(((A+B)-(C+D))\).

Two principle modes of AFM techniques exist including contact mode AFM and non-contact mode AFM (also referred to as tapping mode AFM, dynamic force mode AFM, and intermittent mode AFM). Contact mode AFM engages the tip directly to the surface with a constant force of \(-10^{-7}-10^{-10}\) N and holds the tip in direct contact to the surface for the entire scan under the guidance of the feedback control loop. The main signal of interest in contact mode is DFL as it relates directly to the heights of features present upon a sample's surface. A set point value for the DFL signal is chosen by the operator and the feedback loop acts to maintain the DFL signal measured from the photodiode at user defined set point as the tip moves over the sample's surface. More specific methods of contact mode AFM include the contact error method, the lateral force imaging, the force modulation method, and the spreading resistance imaging method and each of these methods of contact mode AFM provide unique and specialized information. The contact error method treats the DFL deviations from the set point as an error and maps this change over the scan area sometimes providing more detailed surface reconstructions of the sample's topography. In the lateral force imaging method, the LF signal coming from the photodiode arises because of torsional twists of the cantilever arising from different frictional coefficients present upon
the surface. The spreading resistance imaging method holds a conductive probe with applied voltage in contact with the surface and measures electrical currents to the surface during the scan offering information on the conductivity of localized regions.

Non-contact AFM does not hold the tip at a specified force with the surface but instead measures vertical oscillations of the resonant frequency of the cantilever as the tip interacts with the surface. A piezo current in contact with the chip part of the probe drives oscillations in the cantilever giving resonant frequencies greater than 100 kHz and amplitudes falling between 1 nm and 100 nm. The laser reflects off the oscillating cantilever into the photodiode detector producing changes in the signal intensity between the top and bottom halves of the detector creating a variable component of the DFL signal. The oscillating tip interacts with the surface by briefly striking the surface and either absorbing energy from the surface or dissipating energy accumulated during the rest of the oscillation. As the tip strikes the surface, features in the sample’s surface will distort the amplitude of the variable component of the DFL signal revealing those features’ heights. The tip’s interaction with the surface also produces a frequency shift and a phase shift of the tip’s resonant frequency which can be mapped over the scan and is primarily used in the phase imaging method. Phase imaging detects the attractive, repulsive, and capillary forces arising between the tip and surface which reveal surface adhesion and viscoelastic properties of localized surface regions. Non-contact AFM employs cantilevers with higher resonant frequencies than contact AFM cantilevers, and non-contact AFM cantilevers generally possess a high quality-factor (Q-factor) which characterizes the retention of stored energy within the cantilever dampened with each oscillation.

AFM can investigate several properties pertaining to CNT sample quality, dispersion state, substrate morphology, as well as to living cells. As produced CNT batches possess varying size distributions in terms of individual CNT diameters and lengths, and dispersion processing methods like ultra-sound sonication can shorten CNT lengths with enough power while centrifugation separates CNT bundles from individually suspended CNTs. Drop-cast and spun-cast droplets of a CNT dispersion onto flat substrates such as mica can be analyzed with AFM to reveal a CNT population’s diameters, the extent of CNT aggregation within the dispersion, and effects of various dispersion processing conditions.
CNT substrate morphology like any surface can vary accordingly to fabrication method, and various properties including roughness, height distribution, and local frictional forces can be obtained from across such a surface. A few applications of liquid AFM of cells include detecting membrane features, examining the membrane integrity, and determining the stage of mitosis. When used in tandem with a confocal microscope or Raman spatial scanning system, a liquid AFM can provide physical information correlating to various cell surface features identified by their biomolecular or chemical composition.

2.10 Scanning electron microscopy

Scanning electron microscopy (SEM) examines a sample's surface by raster scanning a high energy electron beam across an examined area. SEM offers higher resolution (as low as 0.4 nm) than optical microscopy on account that the wavelengths of ejected electrons depend upon the accelerating voltage whereas the minimum wavelength achievable with optical microscopy is limited by the Bragg diffraction limit. Electrons emitted from the beam gun, termed primary electrons, penetrate and elastically scatter within the material's surface being deflected by atomic nuclei. Primary electrons never lose energy in elastic scattering therefore their speed never changes; however, their trajectories do. The paths of primary electrons within a material travel within a region termed the interaction volume, and a higher accelerating voltage produces a larger interaction volume which can span from less than 100 nm up to 5 µm giving rise to a variety of detectable signals.\textsuperscript{[10]}
These detectable signals emanate from within the sample's surface and include emitted secondary electrons, scattered primary electrons, Auger electrons, X-rays, and cathodaluminescence. Secondary electrons can originate from within ~50 nm of the sample's surface and arise from inelastic collisions between primary electrons and either loosely bound conduction band electrons or tightly bound valence band electrons. When these inelastic collisions occur, primary electrons lose an amount of kinetic energy equal to the binding energy of the band (typically less than 50 eV) from which the secondary electron originates. Primary electron collisions can also be elastic and result in back scatter of the primary electrons. Electrons in outlying orbitals emit X-ray radiation when they drop to inner-shell orbital vacancies left by inelastic collisions with primary electrons. The emitted X-ray radiation can be detected or can be absorbed by other orbital electrons resulting in the emission of Auger electrons. Cathodaluminescence occurs when excited orbital electrons return to their ground states by emitting radiation.
A scanning electron microscope employs a field emission gun which emits electrons through thermoionic heating. Tungsten is typically chosen as the filament material on account of its high melting temperature and low vapor pressure. Magnetic condenser lens focus the electron beam to a spot size of a few nanometers as two pairs of magnetic coils act as an objective lens to direct the beam spot in the x- and y-directions of the raster scan.

2.11 Contact angle measurements

Contact angle measurements investigate the wetting energies between a liquid phase droplet and a solid surface indicating the relative hydrophobicity or hydrophilicity of that surface. On hydrophilic surfaces with high surface energies, water droplets will spread out through adhesive forces to lower the overall surface energy of that surface. A water droplet on a hydrophobic surface will lower its surface tension by adopting a geometric formation of the lowest possible surface area resulting in a contact angle greater than 90° as shown in Figure 18.
Contact angles arise from the tensions of the interfaces of the three present phases termed the solid-liquid interface, $\gamma_{SL}$, the solid-vapor interface, $\gamma_{SV}$, and the liquid-vapor interface, $\gamma_{LV}$. The liquid-gas interface is defined as the tangential line of the water droplet originating at the point where all three interfaces intersect as shown in Figure 19. The contact angle is defined as the angle formed between the solid-liquid interface and the liquid-gas interface and is given by Equation 2.9. A surface is considered hydrophobic if $\Theta$ is greater than 90° and hydrophilic if $\Theta$ is less than 90°.

$$\cos \Theta = \frac{\gamma_{SV} - \gamma_{SL}}{\gamma_{LV}}$$

*Equation 2.9 Young’s Equation rearranged to solve for contact angle in terms of the three interface tensions arising between phases present in the droplet system*
2.12 Conclusions

The materials and methods presented in this chapter have specific uses for meeting the aims of the following chapters. Chapter 3 seeks to optimize a nanotube surface such that it is suitable for sustained hESC growth under culture conditions while Chapter 4 searches for the effects upon on CNT surfaces that result from processing of CNT dispersions. To accomplish these goals, AFM of the CNT surfaces quantifies the roughnesses of the various fabricated CNT films relative to the sizes of hESC colonies and individual cells while SEM will provide images of various CNT surface features ranging from several hundred microns down to several hundred nanometers. Contact angle measurements are employed to investigate the overall hydrophilicity of the resulting CNTs films and determine their suitability as cellular growth substrates as surface wettability factors heavy into cellular adhesion and proliferation. Chapter 5 explores interactions between the synthetic Nano-1 peptide and its two derivatives with SWCNTs where absorption spectroscopy, CD spectroscopy, and fluorescence spectroscopy constitute the majority of investigations; however, AFM is used to determined exfoliation of SWCNT aggregates after association with the peptides. Chapter 5 will also explore the effects that the Nano-1 peptide and its derivatives have upon SWCNT cellular internalization where Raman scanning spectroscopy and confocal microscopy are the main tools for examining SWCNT internalization. The
various methods presented within this Chapter will also have minor roles in the work presented in subsequent chapters as well.

2.13 References

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Chapter 3
Carbon Nanotube Surfaces as Human Embryonic Stem Cell Substrata

3.1 Introduction

Carbon Nanotubes (CNTs) are finding a more prominent role as scaffolds to grow various cell types owing to their nanoscopic dimensions, mechanical robustness, and electronic properties as discussed in detail in Chapter 1. The work presented in this chapter explores the responses of human embryonic stem cells (hESC) to varying attributes of a CNT surface including roughness, chemical functionalities, and surface energy. The CNT surfaces described herein were constructed by filtration of CNT dispersions onto a polymer membrane and subsequent deposition onto a glass Petri dish surface. Properties of these CNT surfaces and their corresponding dispersions were investigated by Raman scattering, scanning electron microscopy (SEM), and atomic force microscopy (AFM). Investigations into cellular responses are specific to the cell type and will be described in detail below but generally focus upon adherence, proliferation, viability, and the display of cell-specific biomolecular markers. The goal of the work presented in this section seeks to appropriately tailor a CNT surface to human embryonic stem cell cultures in terms of adherence, viability, proliferation, and retention of pluripotent behavior.

Current hESC substrata consist of materials of biological origin that are not entirely reliable from batch to batch and may fail a researcher or clinician on critically important occasions. Additionally, these biological substrata run the risk of containing pathogens or contaminants that will void their use for clinical therapies owing to complications that may arise from viruses and immunogenic epitopes. For example, a co-culture of mouse embryonic fibroblasts (MEFs) is a standard substratum to sustain hESC growth but contains Neu5Gc, a sialic acid that induces an immune response by complement activation in most humans. If exposed to Neu5Gc, hESCs will metabolically incorporate substantial amounts of this pathogen rendering them useless for implantation therapies. With the success CNTs have demonstrated for growing other highly specific cell types, one can consequentially conceive the idea of using this versatile material in hESC technology to
provide an entirely reliable and contaminant free surface to sustain and promote cellular growth.

3.2 Procedures for preparing standard hESC substrata and hESC tissue culture maintenance

Conventional growth substrata for hESCs include a co-culture of MEFs plated onto a layer of gelatin deposited onto tissue culture treated polystyrene (PS) dishes or a rich assortment of extracellular matrix proteins extracted from mouse tumor cells termed Matrigel. MEFs used in all experiments were treated with mitomycin C to inactivate their proliferation in order to prevent consumption of nutrients and space reserved for hESCs. A digital image of a MEF feeder layer and SEM image of Matrigel are shown in Figure 1. Deposition of each of these hESC growth substrata onto tissue culture treated PS dishes is as follows.

![Figure 1 Optical image of mouse embryonic fibroblasts (left) and scanning electron microscopy image of Matrigel](image)

**MEF co-cultures** To establish an *in vitro* co-culture of MEFs, type A gelatin from porcine skin (Sigma-Aldrich®) is added to MilliQ® water at a 0.01% concentration and then autoclaved under standard conditions to sterilize the solution and dissolve the gelatin. This gelatin solution is then deposited onto tissue culture treated plates and left to sit in a cell
incubator for 1 hour. The gelatin solution is then aspirated and the cell dish is washed twice with phosphate buffered saline (PBS) before plating with MEFs. MEFs (Chemicon®) are stored at -80°C before thawing in a water bath at 37°C before use. Freshly thawed MEFs are then dispersed in designated volumes of MEF growth media to afford plating densities of ~200 cells/cm². MEF media is composed of ~90% Dulbecco's Modified Eagle Medium (Gibco®), ~10% fetal bovine serum (Clontech®), 1 mM L-glutamine (Gibco®), and 1% nonessential amino acids (Gibco®). MEFs deposited onto tissue culture treated PS dishes are left overnight in a cellular incubator before plating with hESCs the following day.

**Matrigel** To prepare Matrigel coated surfaces, Matrigel stock solutions (BD Falcon®) stored at -20°C are thawed overnight on ice at 4°C and diluted to half of the stock concentration in DMEM-F12 (Gibco®), portioned into aliquots for later use, and frozen at -80°C for storage. When ready for use, aliquots of Matrigel are further diluted with DMEM-F12 under chilled conditions and deposited onto tissue culture dishes and left to polymerize in a cellular incubator for 1 hour. Matrigel is handled and stored below room temperature at all times prior to coating to prevent any polymerization before deposition onto the desired surface.

**hESC tissue culture** Live hESCs are maintained under standard cellular incubator conditions consisting of a sealed, humidified chamber containing at 5% CO₂ atmosphere at 37°C. hESCs are incubated in freshly replaced growth media on a daily basis consisting of 80% DMEM-F12, 20% KNOCKOUT serum replacement (Gibco®), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol (Fisher®), 1% nonessential amino acids, and 4 ng/ml of human basic fibroblast growth factor (R&D systems®). hESC cultures grown in the absence of a MEF co-culture are incubated in conditioned hESC growth media because soluble factors released by MEFs are crucial for sustainment of hESC pluripotent capabilities. Conditioned hESC growth media is produced by overnight incubation of a MEF culture with hESC growth media, collection of the conditioned media next day, and storage at -20°C until needed for use. Conditioned media is supplemented with 4 ng/ml of human basic fibroblast growth factor before use.
Passaging hESCs

hESCs are passaged beginning with gentle enzymatic treatment of previously grown and confluent hESC cell cultures. Collagenase Type IV (BD Falcon®) is routinely used to partially cleave hESCs adhered to the surrounding extracellular matrix and is prepared by dissolution in DMEM-F12 at a concentration of 1 mg/mL followed by filtration through a 0.22 µm pore size filter for sterilization purposes (BD Falcon®). The collagenase solution is then incubated for 5 minutes with hESC cultures that have been washed with PBS. Following collagenase treatment, hESC colonies are scraped from the dish with the tip of disposable plastic pipet, collected into a centrifuge tube, centrifuged at 200 RCF for 5 minutes, and then dispersed in fresh hESC growth media at an appropriate volume to plate cells at a density of ~1,000 cells/cm². To plate cells at the appropriate density, detached cells are diluted in fresh growth media to a predetermined passaging ratio which is defined as the ratio of cells per area present on the previous substratum to the number of cells per area plated onto the new substratum. Maintenance of a hESC tissue culture tracks the number of passages that a hESC culture has received because primary cell lines have a limited lifetime. hESC tissue culture protocols employ a standard notation that denotes the hESC line and number of passages that a hESC culture has received since its original extraction and initial in vitro culture. As an example of this notation, a hESC culture labeled H9 P22 indicates that the culture originated from the H9 hESC line and has been passaged 22 times.

3.3 Procedures for stains, fluorescence microscopy, and flow-cytometry

Alkaline phosphatase (AP) staining, Hoechst staining, immunostaining, and preparation for flow cytometry are common techniques in stem cell biology and have been employed repeatedly for experiments described within this chapter; therefore, protocols of these methods will be given in this section.

Alkaline phosphatase staining AP is a hydrolyse enzyme responsible for the removal of phosphate groups from several biomolecules and is present in all cell types in the human body. In hESCs, AP exists as a membrane marker with elevated expression levels signifying pluripotent character compared to differentiated cells.¹³ As retention of
pluripotent capabilities is crucial for a hESC substratum seeking to replace MEF cocultures or Matrigel. AP staining is used in several of the following experiments to check for differentiation. Fast Red reagent and napthol phosphate buffered solution (Invitrogen®) were used for AP detection in all experiments. To stain for AP, hESC growth medium is removed from the hESC growth plates which are then washed once with PBS. hESCs are then fixed in a 4% paraformaldehyde solution in MilliQ® water for 30 minutes and washed three times with MilliQ® water. Fast Red reagent is dissolved in MilliQ® water at a concentration of 1 mg/mL. 40 µL of napthol phosphate buffered solution is added for every 1 mL of the Fast Red solution immediately before addition to the hESC growth plates. The staining solution is removed after 15 minutes and hESCs are washed three times with MilliQ® water to produce visibly red hESC colonies as shown in Figure 2. MilliQ® water is added to the hESC growth plates for storage at 4°C.

**Figure 2 Optical image of human embryonic stem cell colonies stained with alkaline phosphatase signifying pluripotent behavior**

**Hoechst staining** To fluorescently stain the nuclei, Hoechst staining reagent (Invitrogen®) is prepared as a stock solution in MilliQ® water at a concentration of 10 mg/mL and stored at 4 °C. Following fixation and permeabilization described in the AP staining procedure, hESCs are treated with a 5 µg/mL Hoechst solution for 5 minutes before washing three times with PBS.
Oct-4 antibody labeling  Oct-4 goat primary antibodies (R&D Systems®) and fluorescently labeled rabbit secondary antibodies (Invitrogen®) bind to Oct-4, a transcription factor protein present in hESCs that signifies pluripotency.\textsuperscript{11,12} Following washing and fixation as described in the AP staining procedure, hESCs are permeabilized with a 0.1% Triton X-100 solution (Sigma-Aldrich®) at room temperature for 5 minutes before washing three times with PBS. A blocking solution consisting of 10% rabbit serum and 0.5% Triton X-100 in PBS is then applied to the hESCs for 1 hour to reduce non-specific binding of the primary antibody. Permeabilized hESCs are then treated overnight at 4°C with a ~2 µg/mL primary antibody PBS solution containing 1% rabbit serum. The following day, hESCs are washed three times with PBS before addition of the ~5 µg/mL secondary antibody PBS solution supplemented with 1% goat serum for 1 hour under complete darkness at 4°C to complete the immunostaining procedure.

Preparation for flow cytometry  Flow cytometry examines the scattered and luminescent light emitted from individual microscopic particles such as hESCs as they flow through a microfluidic channel. Preparation for flow cytometry begins by enzymatic treatment with trypsin-EDTA (Gibco®) for 3 minutes not only to remove the hESCs from the growth plates but to also split colonies into individually suspended cells. Trypsin inhibitor (Gibco®) dissolved in DMEM-F12 at 1 mg/mL is added to each well dish to prevent further damage to the hESCs by trypsin. hESCs and MEFs are collected and passed through a 35 µm pore size nylon cell strainer (BD Falcon®) to separate the smaller hESCs from the larger MEFs. The hESCs are then collected in flow cytometry analysis tubes which are then centrifuged at 200 RCF for 5 minutes to resuspend the hESCs in buffered solution specific for flow cytometry. To fluorescently label the SSEA-4 pluripotent marker present on the hESC cellular membrane, SSEA-4 primary antibody (Developmental Studies Hybridoma Bank®) is added to the hESC buffered suspensions chilled on ice at a concentration of ~1 µg/mL for 20 minutes. The buffered cell suspensions are centrifuged and resuspended twice in fresh buffered solution to remove any residual primary antibody. The fluorescently labeled secondary antibody is then added to the buffered cell suspensions for 20 minutes at a concentration of ~5 µg/mL under darkened lighting conditions. During the labeling treatment with the secondary antibody, propidium iodide (PI) (Sigma-Aldrich®) is added to the buffered suspensions at a concentration of 1E-5 µg/mL. PI
becomes fluorescent after diffusing through a degrading or totally compromised cellular membrane and binding to the intracellular DNA of an apoptotic or necrotic cell thus serving as a marker for cellular death. Finally, the buffered cell suspensions are ready for flow cytometry analysis after being centrifuged and resuspended twice in fresh buffered solution to remove any residual secondary antibody and PI.

3.4 Initial trials with as-produced and oxidized SWCNT surfaces as hESC substrata

Single-wall CNT (SWCNT) surfaces were created by filtration deposition of SWCNT dispersions onto filter membranes and subsequent removal of the membrane by solvent dissolution. This method deposits a film of SWCNTs onto a glass Petri dish surface which can span from hundreds of nanometers to a few microns in thickness depending upon the filtered volume of the SWCNT dispersion. To begin investigations into CNT surfaces as possibly viable hESC substrata, CNT surfaces were coated with gelatin and plated with MEFs to replace the conventional tissue culture treated PS. Briefly, the hESCs responded poorly to the as-produced SWCNT surfaces plated with MEFs while they grew and thrived upon acid-oxidized SWCNT surfaces plated with MEFs. In ensuing experiments without MEFs, both types of surfaces were neither able to facilitate significant hESC adhesion and growth nor promote healthy colony morphologies.

SWCNTs (Carbon Nanotechnologies Inc.) were produced by the high pressure carbon monoxide (HiPCO©) process over Fe catalyst particles. The amount of synthesis catalysts present in the as-produced SWCNTs was evaluated by thermogravimetric analysis (TGA) to check for excessive amounts of residual iron present upon the SWCNT sidewalls which might induce detrimental effects upon hESC cultures. 1.5 mg of the as-produced SWCNTs were seated into a platinum TGA pan and heated in air to 800°C to oxidize all carbonaceous material thus leaving only the synthetic SWCNT catalyst. As shown in the combustion profile in Figure 3, the as-produced SWCNT batch contained 40% residual iron catalysts by mass. This amount of iron may account for the poor viability of hESCs grown on as-produced SWCNTs described later in this chapter as iron generates toxic free radicals by the Fenton reaction.[16]
As-produced SWCNTs were dispersed in NMP at a concentration of 0.0075 mg/mL for spin casting and AFM analysis. This concentration was chosen for NMP to limit aggregation of CNT bundles to accurately determine the dimensions of individual CNTs.\textsuperscript{(17)}

The AFM phase image displayed in Figure 4 shows the as-produced SWCNTs deposited onto freshly cleaved mica following spin casting of 10 µL of the dispersion at 3000 rpm for 30 seconds. Each feature resembles an isolated nanotube with nanometer sized diameters and with lengths ranging from ~0.5 µm to 1.5 µm matching values reported previously.\textsuperscript{(18)}
A cellular membrane’s exterior consists mainly of phosphate groups with a zwitterionic or negative charge and transmembrane proteins with exposed hydrophilic regions that are held in contact against the cellular substratum. Thus, surface wettability factors heavily into cellular adhesion and growth as non-polar surfaces are generally unsatisfactory as cellular substrata.\textsuperscript{19}

To counter the hydrophobic nature of a pristine SWCNT surface, SWCNTs were refluxed in a strong oxidizing acid to afford covalent functionalization with carboxylic acid groups along the CNT sidewall as described in the literature.\textsuperscript{20} Acid-oxidation treatment proceeded by refluxing 10 mg/mL of as-produced SWCNTs in 2.5 M nitric acid for 36 hours. The SWCNT acid dispersion was probe sonicated (Branson Sonifier 450) at an output power of 10 Watts for 30 minutes before proceeding to another 36 hour reflux period. The resulting SWCNT dispersion was then sonicated as before and filtered onto a 100 nm pore size Whatman anodisc filter (Fisher\textregistered). The SWCNT filtrate was washed with copious amounts of MilliQ® water, dried, and weighed to determine the recovered mass of acid-oxidized SWCNTs to be 0.881 g. The filtrate was scraped from the anodisc filter and deposited into a small volume of MilliQ® water before reweighing the filter to determine the recovered amount of oxidized SWCNTs to be 0.699 g. The solution containing the oxidized SWCNTs was then diluted to a concentration of 1 mg/mL before resuspension by an identical round of probe sonication.
The Raman spectra generated by 30 second exposure times at 473 nm excitation for the as-produced and acid-oxidized SWCNTs are shown in Figure 5. The spectrum for the as-produced SWCNTs is normalized such that the intensities of the G-peaks for both samples are equal. A significant increase in the intensity of the defect band at \(-1347 \text{ cm}^{-1}\) relative to the G-peak for the oxidized SWCNTs demonstrates the destruction of the pristine \(sp^2\) hybridized carbon lattice during acid refluxation.\(^{21}\) The increased aqueous solubility of the resulting oxidized SWCNT material combined with the relative increase in intensity of the defect band suggests successful SWCNT functionalization primarily with carboxylic acid groups.

Figure 5 Raman spectra with 473 nm excitation of as-produced and acid-treated single-walled carbon nanotubes showing an increase in the D-band of the acid-treated single-walled carbon nanotubes
For the creation of a SWCNT surface, both types of SWCNTs were suspended at a concentration of 0.01 mg/mL in 0.01% gelatin solutions by probe sonication of the chilled dispersions at an output power of 15 Watts for 45 minutes. Gelatin was chosen as a dispersing agent because an additional coating of gelatin would be applied to the SWCNT surfaces before plating with MEFs. Immediately following probe sonication, 75 mL of the resulting dispersions were filtered onto a 0.22 µm pore size mixed-nitrocellulose membrane (Fisher®) to produce a film of SWCNTs as shown in Figure 6. The SWCNT filtrate and adjoined nitrocellulose membrane were dried at 60°C for 1 hour before rewetting and pressing the SWCNT surface against the bottom of a glass Petri dish. Six acetone baths at 15 minute intervals dissolved the nitrocellulose membrane leaving a semi-transparent SWCNT film adhered to the glass Petri dish.

SWCNT surfaces were sterilized by overnight exposure to ultraviolet radiation in a tissue culture hood before following standard gelatin coating and MEF plating procedures. Confluent H9P59 hESCs were passaged onto SWCNT surfaces at a 2:1 passaging ratio and examined by inverted bright field microscopy 5 days after plating. shows representative areas of the SWCNT surfaces compared to a control dish of H9P59 hESCs cultured on MEFs. The images reveal that majority of all MEFs had died on both as-produced and acid-oxidized SWCNT surfaces; however, robust hESC colony growth with healthy
morphologies were observed on the oxidized SWCNT surface, but only apparently unhealthy colonies remained on the as-produced SWCNT surface. These results demonstrate that oxidized SWCNTs may temporarily support hESC growth but are likely to have damaging effects upon adhered cells due to necrotic effects observed for the MEFs. These results also imply an advantage of SWCNT surfaces functionalized with hydrophilic groups over pristine SWCNTs for sustaining hESC growth.

![Figure 7 Inverted bright field images of H9P59 cells grown on mouse embryonic fibroblasts (left), as-produced single-walled carbon nanotube surfaces with mouse embryonic fibroblasts (middle), and acid-treated single-walled carbon nanotube surfaces with mouse embryonic fibroblasts (right)]

To further explore the suitability of as-produced SWCNTs and acid-oxidized SWCNTs as possibly viable hESC substrata, H9P61 hESCs were plated in the absence of a MEF co-culture onto gelatin-dispersed SWCNT surfaces at a 2:1 passaging ratio and cultured in conditioned media. 4 days after plating, the few remaining colonies on both surfaces exhibited unhealthy appearances, smaller sizes, and cells that possessed stretched morphologies along the colonies' perimeters as shown in Figure 8 definitively demonstrating that SWCNT surfaces prepared in this fashion cannot support hESC growth.
3.5 Amide and amine functionalized MWCNT surfaces promote hESC growth and proliferation

Based upon the limited success demonstrated for SWCNTs covalently functionalized with carboxylic acid groups, subsequent experiments attempted to answer if another type of functional group attached to the CNT sidewall would promote hESC adhesion and growth. Polyllysine has induced adhesion for suspension cell cultures and increased adhesion for various cell types. Polylysine contains amine organic groups that possess a positive charge at neutral pH providing favorable electrostatic interactions with the cellular membrane. Thus, CNTs functionalized with amine organic groups seemed a likely candidate for experiments seeking to improve hESC growth by altering the CNT surface chemistry.

Multi-Wall CNTs (MWCNTs) functionalized with amine groups were purchased from Nanocyl® because SWCNTs functionalized with amine groups were not offered as a product. Nanocyl® reports these MWCNTs as being ~10 nm in diameter and <1 µm in length. The chemical reactions Nanocyl® employs to functionalize MWCNTs proceed first by acid oxidation treatment to attach carboxylic acid groups to MWCNT sidewall and then by the Schmidt reaction that produces an approximately half and half mixture of resulting
amine and amide organic groups.\textsuperscript{[24]} Henceforth, this particular type of functionalized MWCNT will be referred to as NH\textsubscript{2}-MWCNT.

NH\textsubscript{2}-MWCNT surfaces were constructed by the same processing conditions and parameters as the SWCNT surfaces and are henceforth referred to as Gel-NH\textsubscript{2}-MWCNT surfaces. NH\textsubscript{2}-MWCNTs do not disperse well in water nor in the presence of 0.01\% gelatin as aggregates, such as those shown in Figure 9, will always remain unless removed by centrifugation or by sedimentation. When incorporated into a NH\textsubscript{2}-MWCNT surface, these aggregates impart a semi-opaque nature in terms of transparency to the NH\textsubscript{2}-MWCNT film to the extent that visibility of adhered hESC colonies by inverted bright field microscopy was significantly hindered if not eliminated as shown in Figure 10.

![Figure 9 Scanning electron microscopy image showing spherical NH\textsubscript{2} functionalized multi-walled carbon nanotube aggregates drop-cast onto silicon](image_url)

\textit{Figure 9 Scanning electron microscopy image showing spherical NH\textsubscript{2} functionalized multi-walled carbon nanotube aggregates drop-cast onto silicon}
H9P46 hESCs were passaged at a 2:1 ratio onto a Gel-NH2-MWCNT surface. 1 day after plating 115 semi-visible colonies were counted by inverted bright field microscopy; however, AP staining 4 days after plating allowed 122 colonies to be counted by eye suggesting that NH2-MWCNT surfaces might be suitable to sustain hESC growth. To test whether gelatin in the NH2-MWCNT film was solely responsible for hESC growth and adhesion, NH2-MWCNTs were prepared in a 0.01% solution of bovine serum albumin (BSA) (Sigma-Aldrich®). BSA has no known binding functions to promote cellular adhesion or growth and proved to be a suitable replacement for gelatin as a dispersing agent. H9P46 hESC were passaged at a 2:1 ratio onto this BSA-NH2-MWCNT surface, and 81 semi-visible colonies were counted by inverted optical microscopy 1 day after plating while 113 colonies were counted by eye following AP staining 4 days after plating. To prove that hESCs were not adhering to the BSA-NH2-MWCNT surface only on account of the BSA present within the BSA-NH2-MWCNT film, H9P82 hESCs were split at a 2:1 passaging ratio onto glass dishes coated with BSA by a 0.01% aqueous BSA solution for 1 hour similar to the procedure prescribed for gelatin coating of tissue culture plates. H9P82 hESCs were also plated onto another glass Petri dish that had been filled with a 1% BSA solution and allowed to dry thereby leaving a visibly yellow layer of BSA on the surface. No colonies adhered to the dish coated by the 0.01% BSA solution while very few unhealthy and small colonies with unhealthy morphologies were seen adhered to the dried BSA film 1 day after plating. 4 days after plating, all adhered colonies and the deposited

Figure 10 Brightfield (left) and corresponding fluorescent image (right) of a human embryonic stem cell colony on a NH2-functionalized multi-walled carbon nanotube surface
BSA film had been washed away with the daily media changes. These H9P82 hESCs were also plated onto NH$_2$-MWCNT surfaces constructed from aqueous dispersions with neither gelatin nor BSA to test whether hESCs adhered to the Gel-NH$_2$-MWCNT and BSA-NH$_2$-MWCNT films only on account of the NH$_2$-MWCNTs present upon the surface. No colonies were observed by eye upon the dispersant-free NH$_2$-MWCNT surface following AP staining 4 days after plating.

hESC colonies grown on these BSA-NH$_2$-MWCNT surfaces were then tested for viability and expression of the pluripotent marker SSEA-4 by flow cytometry as described in section 2. Results for these viability and pluripotent assays are shown in Figure 11 where the abscissa axis in these flow-cytometry plots represents the emission intensity for each cell counted while the ordinate axis represents the percentage of cells counted at singular emission intensity relevant to the greatest number of cells emitting at a given intensity for the entire sample. H9P82 hESCs were passaged at a 2:1 ratio onto a MEF co-culture, Matrigel, or BSA-NH$_2$-MWCNT surface and examined by flow cytometry 4 days after plating. Four H9P82 hESC populations were stained with PI of which one was grown on a MEF co-culture and exposed to UV-radiation for 15 minutes to induce cellular apoptosis and necrosis. One population grown on a MEF co-culture was left unstained to demonstrate that the majority of detected emission originates from the PI stain. Two maxima occur within the plot for the PI stained populations at approximately 10 and 10$^3$ emission intensities where the maximum occurring at 10 emission intensity represents healthy viable cells and the maximum occurring at 10$^3$ represents fully necrotic cells. Emission intensities between these two peaks represent apoptotic cells with partially degraded membranes allowing for slower diffusion of the PI stain than the fully necrotic cells. The plot demonstrates that the hESC population grown on the BSA dispersed NH$_2$-MWCNT surface contained approximately the same amount of apoptotic and necrotic hESCs as the population grown on the MEF co-culture and fewer apoptotic and necrotic cells than the population grown on Matrigel. H9P82 hESCs grown on these surfaces were also immunostained for the membrane marker, SSEA-4, which serves as indicator of pluripotency. Populations grown on the NH$_2$-MWCNT surface demonstrated equal SSEA-4 expression as the population grown on Matrigel and more SSEA-4 expression than the population grown on the MEF co-culture. These results indicate that the NH$_2$-MWCNT
surface serves equally as well as MEF co-cultures or Matrigel surfaces in terms of short-term viability and retention of pluripotency.

![Flow Cytometry of Propidium Iodide Stain](image)

**Flow Cytometry of Propidium Iodide Stain**

- Unstained
- NH$_2$-MWCNT Film
- Matrigel
- MEFs
- Induced Cell Death

![Flow Cytometry of SSEA-4 Antibody Stain](image)

**Flow Cytometry of SSEA-4 Antibody Stain**

- NH$_2$-MWCNT Film
- MEFs
- Matrigel

*Figure 11 Flow cytometry of H9P82 human embryonic stem cells grown on a mouse embryonic fibroblast co-culture, Matrigel®, or BSA dispersed NH$_2$ functionalized multi-walled carbon nanotube film stained with propidium iodide for viability (top) or SSEA-4 antibody marker for pluripotency (bottom)*

3.6 Roughness of the BSA-NH$_2$-MWCNT surface affects hESC morphology and adhesion

hESC colonies grown on BSA-NH$_2$-MWCNT surfaces were then examined by upright fluorescence microscopy to avoid the transparency drawback arising from the semi-opaque nature of the NH$_2$-MWCNT film. H9P79 hESCs were plated onto NH$_2$-MWCNT at a 2:1 passaging ratio and stained with Hoechst and Oct-4 antibodies 4 days after plating. H9P79
hESC colonies are shown below in Figure 12 and possessed 3D morphologies similar to that of embryoid bodies. Embryoid bodies are spherical aggregates of hESCs that have been detached from the substratum to allow for spontaneous differentiation.\textsuperscript{[25]} In addition to the embryoid body-like formation centered in each colony, hESCs growing away from the embryoid body-like center were spaced further apart from one another and possessed larger nuclei than cells grown on MEF co-cultures or Matrigel. Increased spacing between hESCs and larger nuclei serve as a indicative precursor to differentiation.\textsuperscript{[26]} These features of embryoid body-like formations and increased cell spacing observe in these colony morphologies indicate abnormalities in terms of hESC morphologies and therefore these particular BSA-NH$_2$-MWCNT surfaces are not suitable as hESC growth substrata.
To investigate why these BSA-NH2-MWCNT surfaces supported colony adhesion and growth but induced abnormal colony morphologies, NH2-MWCNT films were examined by SEM and are shown in Figure 13. Large aggregates are shown on the BSA dispersed NH2-MWCNT surfaces ranging from 5 to 30 µm which are likely to obstruct normal hESC colony outgrowth and expansion as hESCs average approximately 10-20 µm in diameter and 2.5 µm in terms of height.\textsuperscript{[27]} As stated in section 3.4, these aggregates shown in Figure 9 arise from poor aqueous dispersion conditions which do not completely debundle NH2-MWCNTs.

Figure 12 Hoechst (left column) and Oct-4 (right column) fluorescent images of H9P79 human embryonic stem cell colonies 4 days after plating on Matrigel (top) and on BSA dispersed NH2-functionalized multi-walled carbon nanotube surfaces (bottom)
To eliminate the presence of these large aggregates to allow for higher transparency and proper hESC colony growth, 1 liter of an aqueous dispersion containing only 10 mg/mL of NH$_2$-MWCNTs were probe sonicated at the lowest power output setting of 4W for 15 minutes and allowed to sediment for 3 days. The minimal applied sonication power favored dispersal of only the most soluble NH$_2$-MWCNTs which remained in solution during the sedimentation period. Following the sedimentation period, dispersions appeared transparent with clearly visible NH$_2$-MWCNT aggregates settled onto the bottom surface of the glass container as shown in Figure 15.
900 mL of this dispersion was decanted without disturbing the sediment and probe sonicated at 10W for 30 minutes in the presence of 0.01% BSA following similar sonication treatments reported in the literature. The entirety of the resulting dispersion was then deposited onto a glass Petri dish as described above. Although this greatly improved the transparency of the NH$_2$-MWCNT film, no hESCs colonies adhered to this NH$_2$-MWCNT surface. SEM images for this surface are shown in Figure 16 and demonstrably show a comparably smoother surface to the BSA dispersions that were not allowed to sediment. These smoother surfaces were the result of a dispersion consisting mostly of individually dispersed NH$_2$-MWCNTs and lacking large aggregates.
The results for the rough and smooth BSA-NH$_2$-MWCNT surfaces suggest that some amount of surface roughness is required for hESC adhesion, but too much roughness will incur abnormal colony morphologies. Thus, a surface possessing a roughness in between those of the two BSA-NH$_2$-MWCNT surfaces described above might facilitate proper hESC adhesion and growth. To construct such as surface, hereafter described as semi-rough, NH$_2$-MWCNTs were probe sonicated in 0.01% BSA aqueous solutions chilled on ice at an output power of 15 Watts for 45 minutes. The darkness and turbidity of this dispersion exposed to higher sonication energy indicated that NH$_2$-MWCNT aggregates were present at high quantities in solution. Dispersions were allowed to sediment for 3 days to remove excessively large aggregates that may hinder normal colony formation and growth. Following the sedimentation period, dispersions appeared more transparent with visible NH$_2$-MWCNT aggregates resting on the bottom surface of the container. The resulting natant was decanted and sonicated at the same conditions as the initial sonication treatment. 75 mL of the sonicated natant was then filtered to form the BSA-NH$_2$-MWCNT surface followed by deposition onto a glass Petri dish by procedures described above.

SEM images of this semi-rough BSA-NH$_2$-MWCNT surface are shown in Figure 17 demonstrating that this semi-rough surface contains NH$_2$-MWCNT aggregates that impart microscale roughness absent in the smoothest BSA-NH$_2$-MWCNT surface but lacks the excessively large aggregates present within the roughest surface. Despite sedimentation
and decantation to remove aggregates, these semi-rough surfaces still exhibited a semi-opaque optical nature.

To test the hESC growth efficacy of these semi-rough surfaces, H9P82 hESCs were plated onto the BSA-NH₂-MWCNT surfaces at a 2:1 passaging ratio. 4 days after plating, colonies were AP stained and fluorescently labeled with Hoechst and Oct-4 antibody for examination by upright fluorescence microscopy. Figure 18 shows that AP stained colonies exhibited extensive cell growth across the entire BSA-NH₂-MWCNT surface and exhibited a cellular density similar to that of hESCs grown on MEF co-cultures or Matrigel. It should be noted, however, that the darkly stained regions of the colonies slightly resemble, although to a significantly lesser extent, the EB-like formations seen on the roughest BSA-NH₂-MWCNT surface indicating that NH₂-MWCNT surfaces may require further tuning in terms of surface chemistry and topology before they can be considered fully viable hESC growth substrata. The AP stain also serves as a partial indicator that H9P82 hESCs retained pluripotent capabilities.
Figure 18 Digital image of alkaline phosphatase stained colonies grown on a semi-rough BSA dispersed NH\(_2\) functionalized multi-walled carbon nanotube film

Figure 19 displays fluorescent images of the H9P82 hESCs 4 days after plating and stained with Hoechst and Oct-4 antibody. Diameters of the hESC nuclei within the colonies grown on the BSA-NH\(_2\)-MWCNT surface matched those of hESCs grown on MEF co-cultures or Matrigel, and cells within the colonies on the BSA-NH\(_2\)-MWCNT surfaces also exhibited normal spacing. Oct-4 fluorescent images were taken of the H9P82 hESCs grown on BSA-NH\(_2\)-MWCNT surfaces with the same exposure settings as the corresponding hESCs grown on Matrigel. An equal amount of emission intensity observed for the colonies grown on the two different substrates indicates approximately equal amounts of Oct-4 expression in both sample populations further demonstrating short-term retention of pluripotent capabilities for hESCs grown on BSA-NH\(_2\)-MWCNT surfaces.
3.7 Quantifying the roughness of the BSA-NH$_2$-MWCNT surfaces

The roughness of each surface was analyzed by AFM. Ten 50x50 µm scan areas recorded with resolutions of 256x256 points were sampled on each of the three surfaces and analyzed by Nova 959 image analysis software (NT-MDT®). The roughness of each scanned surface area is defined by equation 3.1 where $R$ is the roughness, $n$ is the number of points, and $y_i$ is the difference between the height of an individual point and the average height for every point within the scan.

$$ R = \frac{1}{n} \sum_{i=0}^{n} |y_i| $$

*Equation 3.1 Definition of surface roughness employed by Nova® image analysis software*

Average values and standard deviations for the ten sample scans taken over the smooth, semi-rough, and rough BSA-NH$_2$-MWCNT surfaces are 151 ± 22 nm, 313 ± 16 nm, and 432 ± 132 nm, respectively. SEM images are shown in Figure 20 for comparison.
Figure 20 Scanning electron microscopy images of the 3 different BSA dispersed NH₂ functionalized multi-walled carbon nanotube films with the smooth (left), the semi-rough (middle), and the rough (right) surface each having an average roughness of 151 ± 22 nm, 313 ± 16 nm, and 432 ± 132 nm, respectively.

3.8 Conclusions and Future Work

This work demonstrates great potential for CNT surfaces to replace conventional hESC growth substrata thus eliminating the use of unreliable and possibly contaminated surfaces. The results reported in section 3.4 indicate that NH₂-MWCNTs alone are not enough to facilitate hESC adhesion but require an associated agent which may act to soften the overall tensile modulus of the surface or alter the surface chemistry of the NH₂-MWCNT sidewalls. To provide an entirely synthetic hESC growth substratum, synthetic CNT dispersing agents such as amphiphilic polymers possessing varying mechanical properties and wettabilities should be investigated as possible replacements for the gelatin or BSA employed here in this work. To investigate the role of MWCNT sidewall chemistry, corresponding MWCNTs lacking the amine and amide functionalization groups should be dispersed with the reported gelatin and BSA solutions in an attempt to create MWCNT substrata with similar topographies in terms of roughness to the successful Gel-NH₂-MWCNT and BSA-NH₂-MWCNT surfaces. This work also demonstrates the crucial role that surface topology exerts upon hESC adhesion, morphology, and growth. To optimize hESC viability and proliferation, all current and potential substrata should engineer surfaces of optimal nano- and microscale topologies. Finally, the effects that CNT surfaces may exhibit upon hESC sustainability should be investigated for several months and many passages in terms of viability, pluripotency, and differentiation.
3.9 References


[23] D. Carrier, M. Pezolet; *Biophys J*: **1984**, *46*, 497


Chapter 4
Controlling the Properties of Multi-Walled Carbon Nanotube Surfaces

4.1 Introduction

A detailed study of the control and tuning of a multi-wall carbon nanotube (MWCNT) surface by altering dispersion processing parameters is presented within this chapter. The aggregate state of any carbon nanotube (CNT) dispersion not only factors heavily into a resulting surface produced from a CNT dispersion, but also into any CNT based materials that begin with dispersion processing. CNT surfaces can be fabricated by a variety of methods including layer-by-layer assembly,\textsuperscript{[1]} spin casting,\textsuperscript{[2]} drop casting,\textsuperscript{[3]} spray deposition,\textsuperscript{[4]} electro-spinning,\textsuperscript{[5]} and CNT synthesis upon a catalyst substrate.\textsuperscript{[6-8]} Filtration deposition of a CNT dispersion onto a porous polymer membrane was chosen for these investigations not only to carry on from the work presented in Chapter 3, but also because the effects arising from altering dispersion processing parameters are mitigated if not lost entirely with the other above mentioned CNT surface fabrication methods.

Because different cell types respond with individual behaviors to any given extracellular matrix or synthetic substratum, the aim of this work is to produce a versatile range of MWCNT surfaces in terms of microscale roughness, wettability, and pore size distribution to appropriately tailor any given MWCNT surface to a specified cell type or other relevant application. Altering the surface properties of a MWCNT surface may find applications for filtration devices,\textsuperscript{[9]} sensors,\textsuperscript{[10]} electrodes,\textsuperscript{[11]} and actuators.\textsuperscript{[12]}

The multi-wall carbon nanotubes functionalized with amine and amide chemical groups (NH\textsubscript{2}-MWCNT) used in Chapter 3 were chosen for all film production in this work because of the success demonstrated for the NH\textsubscript{2}-MWCNTs films with human embryonic stem cells. Additionally, excellent dispersion stability is necessary to proceed with further investigations into alterations of CNT dispersions and their resulting surfaces, and NH\textsubscript{2}-MWCNTs have demonstrated adequate dispersion stability in water and have shown to be highly stable in several organic solvents. The foundation of the work presented in this chapter will investigate how to modify the aggregate state found within NH\textsubscript{2}-MWCNT
dispersions through exposure to sonication energy, filtration volume, dispersion concentration, solvent, centrifugation, and filtration volume. Surface properties including roughness, wettability, surface area, and these properties’ relation to one another are investigated by atomic force microscopy (AFM), scanning electron microscopy (SEM), contact angle measurements, and gas adsorption measurements combined with Brunauer-Emmet-Teller (BET) analysis.

4.2 Changing the roughness of MWCNT surfaces by altering dispersion parameters

4.2.1 AFM analysis

The roughness of each NH2-MWCNT surface was determined by AFM as described in Chapter 3, Section 7. Ten 50 µm x 50 µm scan areas recorded with resolutions of 256x256 points were sampled on each of the three surfaces and analyzed by Nova® image analysis software (NT-MDT®). 50 µm x 50 µm scan areas were chosen to scale approximately with the size of cells and the size of newly passaged human embryonic stem cell colonies to make these investigations relevant to the work presented in Chapter 3 and future cellular substrata studies. The roughness of each scanned surface area is defined by equation 4.1 where R is the roughness, n is the number of points in the scan, and \( y_i \) is the difference between the height of an individual point and the average height of all points within the scan.

\[
R = \frac{1}{n} \sum_{i=0}^{n} |y_i|
\]

*Equation 4.1 Definition of surface roughness used by Nova® image analysis software*
4.2.2 Sonication time

The effects of sonication time were first investigated on NH$_2$-MWCNTs dispersed in chloroform (CHCl$_3$) as sonication is the first step in NH$_2$-MWCNT dispersion processing. NH$_2$-MWCNTs were prepared in chloroform at concentrations of 0.005 mg/mL in 500 mL volumetric flasks. Volumetric flasks were filled to the mark and then bath sonicated in chilled water. Figure 1 shows the same dispersion sonicated for 20 minutes and 100 minutes and demonstrates that additional sonication time darkens a NH$_2$-MWCNT-CHCl$_3$ dispersion by breaking apart dispersion agglomerates. Figure 2 show absorption spectra for the same dispersion bath sonicated with 150 W output power at 20, 40, 60, 80, 100, 200, and 300 minutes. Absorbance increases at all wavelengths with additional sonication time further demonstrating that sonication breaks apart dispersion aggregates.

![Figure 1 Photographs of NH$_2$-MWCNT chloroform dispersions sonicated for 20 minutes (left) and 100 min (right) showing decreasing transparency with increasing sonication exposure](image)
Figure 2  UV-vis spectra for different sonication times of 0.005 mg/mL NH₂-MWCNT chloroform dispersions showing decreasing transparency with increasing sonication exposure

Figure 3 shows the absorbance at 400 nm for the absorption spectra shown in Figure 2. After 100 minutes of bath sonication, the absorbance no longer increases indicating that the sonication treatment has reached its maximum effect on the agglomerates.
Figure 3: Absorbance at 400nm for different sonication times of 0.005 mg/mL NH$_2$-MWCNT chloroform dispersions showing decreasing transparency with increasing sonication exposure.

Figure 4: SEM images of drop cast dispersions on silicon from 0.005 mg/mL NH$_2$-MWCNT chloroform dispersions sonicated for 20 (left) and 100 (right) minutes demonstrating the dissolution of NH$_2$-MWCNT aggregates.

Figure 4 shows silicon surfaces that were spin-coated with 10 µL droplets of the NH$_2$-MWCNT-CHC$I_3$ dispersions bath sonicated for 20 and 100 minutes. Large agglomerates approximately 10 µm in diameter and individual NH$_2$-MWNTs appear on the surface spin-coated with the dispersion that was bath sonicated for 20 minutes while smaller agglomerates less than 2 µm in diameter along with individual NH$_2$-MWNTs appear on the
surface spin-coated with the dispersion that was bath sonicated for 100 minutes definitively demonstrating that bath sonication disintegrates dispersion aggregates.

NH₂-MWCNT surfaces were fabricated by filtering 50 mL of 0.005 mg/mL NH₂-MWCNT-CHCl₃ dispersions that were sonicated for various amounts of time onto 100 nm pore size Whatman anodisc filters (Fisher®). Figure 5 shows AFM height images scanned over 50 µm x 50 µm areas of surfaces fabricated from dispersions sonicated for 20 and 100 minutes. AFM was chosen as a technique to quantifiably characterize surface roughness. Large agglomerates are noticeably visible upon the NH₂-MWCNT surface created from the dispersion sonicated for 20 minutes but are not present upon the NH₂-MWCNT surface created from the dispersion sonicated for 100 minutes.

Figure 5 AFM height images of surfaces from 0.005 mg/mL NH₂-MWCNT chloroform dispersions sonicated for 20 minutes (left) and 100 minutes (right) showing a decrease in surface roughness as a result of fragmenting NH₂-MWCNT aggregates by increasing sonication exposure

Figure 6 shows the average roughness of ten 50 x 50 µm AFM scans taken over NH₂-MWCNT surfaces fabricated from NH₂-MWCNT-CHCl₃ dispersions bath sonicated with 150 W output power for at 20, 40, 60, 80, 100, 200, and 300 minutes. Surfaces created from NH₂-CHCl₃ dispersions sonicated for 60 minutes or more are not significantly different in terms of average roughness. The error bars represent the standard deviations of
the roughnesses measured for each of the ten AFM scans and a plot of standard deviations vs. sonication times for the 10 AFM scans sampled over each surface is shown in Figure 7.

![Figure 6](image)

**Figure 6** Plot of average roughness for 10 AFM scans sampled over each NH$_2$-MWCNT surface vs. sonication time of the 0.005 mg/mL NH$_2$-MWCNT chloroform dispersion showing a decrease in surface roughness with increasing sonication exposure

![Figure 7](image)

**Figure 7** Plot of standard deviations for the 10 AFM scans sampled over each NH$_2$-MWCNT surface vs. sonication time of 0.005 mg/mL NH$_2$-MWCNT chloroform dispersion

The standard deviation of the ten AFM scans sampled for surfaces created from NH$_2$-MWCNT-CHCl$_3$ dispersions of lower bath sonication times are significantly larger than surfaces created from NH$_2$-MWCNT-CHCl$_3$ dispersions of higher bath sonication times. This effect arises from the random deposition of the large agglomerates upon the filter.
membrane during filtration for surfaces created from NH$_2$-MWCNT-CHCl$_3$ dispersions of lower bath sonication times, and increased bath sonication time disintegrates dispersion agglomerates thus homogenizing the dispersion which in turn prevents the random filtration deposition of large agglomerates.

4.2.3 Filtration volume

Any agglomerates remaining in solution are likely to contribute to surface roughness even if embedded deep within the NH$_2$-MWCNT thick film; therefore, increasing the filtration volume should augment the number of agglomerates deposited within the film. Therefore, the effects of filtration volume upon the surface roughness were investigated by filtering different amounts of a NH$_2$-MWCNT-CHCl$_3$ dispersion. The dispersion was prepared at concentration of 0.005 mg/mL of NH$_2$-MWCNT and bath sonicated in chilled water for 100 minutes. These processing conditions still allow for smaller agglomerates to remain in solution as shown in Figure 4. Volumes of 15, 25, 50, 75, and 100 mL were filtered and analyzed by AFM as described above. Figure 8 shows the average roughness for ten AFM scans plotted against filtration volume. As expected, the average roughness increases with filtration volume. The standard deviations for the ten scans taken over each NH$_2$-MWNT surface also appear to not vary widely from another suggesting that the agglomerate state for the dispersion is homogeneous in terms of the smaller agglomerates remaining in solution following extensive bath sonication.
4.2.4 NH$_2$-MWCNT concentration

Other reports on dispersions of CNTs have reported higher stabilities and greater amounts of individually suspended CNTs when processed at lower concentrations$^{[13]}$; therefore, CHCl$_3$ dispersions of various concentrations were prepared by bath sonication in chilled water for 100 minutes and used to fabricate NH$_2$-MWCNT films. To insure the resulting films possessed a similar thickness to one another, the absorbance of each dispersion was determined by absorption spectroscopy at 400 nm and then used to determine the amount of volume to be filtered necessary to insure that an equal amount of NH$_2$-MWCNTs are deposited in each film. \textit{Figure 9} shows the roughness for ten AFM scans sampled over each NH$_2$-MWCNT surface prepared at 0.005, 0.0025, 0.001, and 0.0005 mg/mL of NH$_2$-MWCNTs. The point representing the average roughness for the surface prepared from the 0.0005 mg/mL NH$_2$-MWCNT-CHCl$_3$ dispersion in \textit{Figure 9} possesses an average roughness significantly higher than the other surfaces prepared from NH$_2$-MWCNT-CHCl$_3$ dispersions at higher concentrations. However, a second 0.0005 mg/mL dispersion was bath sonicated for 200 minutes and a third dispersion sonicated for 300 minutes possessed an average roughness of 247 $\pm$ 37 nm and 250 $\pm$ 23 nm, respectively. This data
demonstrates that the concentration of filtered NH$_2$-MWCNT-CHCl$_3$ dispersion does not significantly alter the roughness of the resulting NH$_2$-MWCNT film.

![Figure 9 Plot of average roughness for ten AFM scans sampled over each NH$_2$-MWCNT surface roughness vs. concentration](image)

4.2.5 Dispersing solvent

The choice of dispersing agent or solvent factors heavily into the stability of a CNT dispersion.$^{[14]}$ N-Methylpyrrolidone (NMP) ranks highly among several organic solvents reported to disperse CNTs at practical concentrations as it is able to suspend individual nanotubes at concentrations higher than other solvents. Therefore, the effects the dispersing solvent or agent employed to suspend NH$_2$-MWCNTs upon surface roughness were investigated by preparing dispersions consisting of NMP, CHCl$_3$, and 0.01% bovine serum albumin (BSA). BSA was chosen to compare with NH$_2$-MWCNT surfaces reported in Chapter 3. Figure 10 shows a typical AFM height image for NH$_2$-MWCNT surfaces prepared by filtration of 0.005 mg/mL NH$_2$-MWCNT dispersions in NMP, CHCl$_3$, and 0.01% BSA. The NH$_2$-MWCNT surface prepared in 0.01% BSA displays visibly rougher surface features indicating that BSA does not disintegrate NH$_2$-MWCNT agglomerates as well as NMP or CHCl$_3$. The NH$_2$-MWCNT surface prepared from the NH$_2$-MWCNT-NMP dispersion displays slightly smaller surface agglomerates than the surface prepared from the NH$_2$-MWCNT-CHCl$_3$ dispersion.
Table 1 displays the average roughness for ten AFM scans of NH$_2$-MWCNT surfaces prepared from CHCl$_3$, NMP, and 0.01% BSA NH$_2$-MWCNT dispersions. The NH$_2$-MWCNT-NMP dispersion produces the smoothest surface of the three dispersions and possessing a surface nearly twice as smooth as the NH$_2$-MWCNT-CHCl$_3$ dispersion and approximately three and a half times as smooth as the NH$_2$-MWCNT-BSA dispersion. The results demonstrate that the choice of dispersing medium heavily affects the roughness of the resulting NH$_2$-MWCNT film. Although the NH$_2$-MWCNT-NMP dispersion provides a smoother surface than the NH$_2$-MWCNT-CHCl$_3$ dispersion, NMP possess a high boiling point of 202 °C and is known to strongly adsorb onto CNT sidewalls.$^{13}$ Therefore, subsequent experiments were still performed with NH$_2$-MWCNT-CHCl$_3$ dispersions to
avoid the issue of removing any residual NMP from the NH$_2$-MWCNT surface which may affect applications.

**Table 1 Average roughness for ten AFM scans of NH$_2$-MWCNT surfaces prepared from CHCl$_3$, NMP, and 0.01% BSA NH$_2$-MWCNT dispersions**

<table>
<thead>
<tr>
<th></th>
<th>Average Roughness</th>
<th>Standard Deviations of Roughness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>220 nm</td>
<td>±17 nm</td>
</tr>
<tr>
<td>NMP</td>
<td>125 nm</td>
<td>±10 nm</td>
</tr>
<tr>
<td>0.01 mg/mL BSA</td>
<td>430 nm</td>
<td>±32 nm</td>
</tr>
</tbody>
</table>

4.2.6 Centrifugation

Centrifugation of CNT dispersions is known to remove nanotubes containing large amounts of catalyst impurities and CNT agglomerates; therefore, high speed centrifugation was performed on 0.005 mg/mL NH$_2$-MWCNT-CHCl$_3$ dispersions sonicated for 100 minutes in chilled water to obtain smoother NH$_2$-MWCNT films than films produced by processing methods consisting of bath sonication only. NH$_2$-MWCNT-CHCl$_3$ dispersions were centrifuged twice for one hour at 12,100 g, 27,200 g, 48,400 g, and 75,600 g. Supernatants were carefully removed without disturbing the sediment and analyzed by absorption spectroscopy to determine the absorbance at 400 nm. Figure 11 displays the absorption spectra of NH$_2$-MWCNT-CHCl$_3$ dispersion supernatants and shows that increased centrifugation speed reduces the amount of NH$_2$-MWCNTs in solution by likely removal of large NH$_2$-MWCNT agglomerates.
A 10 μL droplet of the NH$_2$-MWCNT-CHCl$_3$ from the supernatant removed from NH$_2$-MWCNT-CHCl$_3$ dispersions centrifuged at 75,600 g was spin-coated onto a freshly cleaved mica surface and analyzed by AFM. Figure 12 shows a representative AFM height image of this sample and displays individual NH$_2$-MWCNTs demonstrating that the supernatant is free of large NH$_2$-MWCNT-CHCl$_3$ agglomerates.
NH$_2$-MWCNT-CHCl$_3$ supernatants were then filtered in predetermined volumes to afford NH$_2$-MWCNT films of approximately equal thickness which were then analyzed by AFM. Figure 13 shows the average roughness for ten AFM scans of NH$_2$-MWCNT surfaces prepared from supernatants removed from NH$_2$-MWCNT -CHCl$_3$ dispersions centrifuged at different speeds vs. centrifugation speed and demonstrates that high-speed centrifugation successfully removes large NH$_2$-MWCNT agglomerates that contribute to the roughness of NH$_2$-MWCNT films’ surfaces.
Figure 13  Average roughness for ten AFM scans of NH$_2$-MWCNT surfaces prepared from supernatants removed from NH$_2$-MWCNT chloroform dispersions centrifuged at different centrifugal forces.

Figure 14 shows representative AFM height images from NH$_2$-MWCNT surfaces prepared from supernatants removed from NH$_2$-MWCNT–CHCl$_3$ dispersions centrifuged at the various centrifugal forces. The NH$_2$-MWCNT surface prepared from the supernatants removed from NH$_2$-MWCNT–CHCl$_3$ centrifuged at 75,600 g is visibly smoother than the other surfaces with contain randomly scattered, smaller agglomerates. An AFM height image of an area measuring 5 µm x 5 µm of this smoothest surface is shown in Figure 15 and when analyzed for roughness measures 16 nm. No nanotube-like features like those shown in Figure 12 appear in Figure 14 on account of the low resolutions chosen for the AFM scanner for the images in Figure 14.
Figure 14 AFM height images NH$_2$-MWCNT surfaces prepared from supernatants removed from NH$_2$-MWCNT chloroform dispersions centrifuged at 12,100 g (top left), 27,200 g (top right), 48,400 g (bottom left), and 75,600 g (bottom right)
4.3 Pore size distribution by BET and SEM image analysis

Pore size distribution and increased surface area of the NH$_2$-MWCNT films will factor heavily into the wettability of these films as more exposed surface area allows for higher cohesive wetting. Brunauer-Emmett-Teller (BET) analysis of an isotherm of nitrogen adsorption into porous materials is the standard method for determining pore size distribution; however, BET analysis is not suited for determination of pore size distribution in NH$_2$-MWCNT films because BET requires approximately 10 mg of material and each NH$_2$-MWCNT film weighs approximately 250 µg. Therefore, Nova® image analysis software was used to model hundreds of fitted circles of various diameters to the pores visible within SEM image of the NH$_2$-MWCNT films as shown in Figure 16. Circles are fitted to regions within the SEM image possessing a certain level of contrast above a chosen threshold for their inner, darker regions and their circumferences. The Nova® software then produces a histogram of the number of fitted circles in terms of their diameter. A histogram against pore size diameter for the fitted image shown in Figure 16 appears in the left of Figure 17 against the BET analysis shown in the right of Figure 17 for the same NH$_2$-MWCNT paper weighing over 10 mg.
The algorithm for fitting circles to the pores within an image produces a large quantity of noise for pores of smaller diameters (<30 nm) compared to pores of larger diameters (>30 nm). This noise can be partially corrected by subtracting a fitted Lorentzian curve from the entire pore size distribution generated by the Nova® software. This distribution that has been corrected for noise appears in the right of Figure 17 with the BET pore size distribution for the same sample. Each distribution is normalized to its statistical mode.
SEM images of three samples of NH$_2$-MWCNT films prepared by different processing methods were then analyzed by the Nova® software. These samples consisted of one film produced by filtering 15 mL of a 0.005 mg/mL dispersion sonicated for 100 minutes, a second produced by filtering 150 mL of the same dispersion, and a third by filtering the supernatant of 0.005 mg/mL dispersion produced by centrifugation at 75,600 RCF. SEM images and corresponding pore size distributions generated by the Nova® software of these samples appear in Figure 18. The weighted averages for each pore diameter distribution are 40 nm, 73 nm, and 26 nm for the films produced by 15 mL of the dispersion, 150 mL of the dispersion, and the supernatant of the dispersion, respectively.

Figure 18  SEM images (top) of NH$_2$-MWCNT films prepared by filtering 15 mL (left) and 150 mL (middle) of a 0.005 mg/mL NH$_2$-MWCNT dispersion and by filtering the supernatant of that dispersion at 75,600 RCF. Corresponding pore size distributions (bottom) generated for each NH$_2$-MWCNT sample

Figure 18 shows an increase in population for pores with diameters greater than ~60 nm for the NH$_2$-MWCNT film produced from 150 mL of a 0.005 mg/mL dispersion compared to
the other two samples. This increase in population for larger pore sizes may occur due to mechanical stresses applied to the film during the drying process following the acetone baths. During the drying process, large aggregates present within the film produced by the 150 mL dispersion may have induced these stresses within an otherwise flat layer of isolated NH$_2$-MWCNTs like that of the film produced from the centrifuge supernatant. An increase in the pore size diameter distribution for the film produced from 15 mL of the dispersion also occurs when compared to the film produced from the supernatant of the dispersion. Fewer NH$_2$-MWCNT aggregates are present within the film produced from 15 mL of the dispersion compared to the film produced by 150 mL of the dispersion. This lesser amount of aggregates within the thinner film may in turn lead to a lower value of total stress induced within the NH$_2$-MWCNT film during acetone drying explaining the lower pore diameter distribution of the film produced from 15 mL of the dispersion compared to the film produced from 150 mL of the dispersion.

4.4 Contact angle measurements

Contact angles determined by DASI® image analysis software of digital images of droplets resting upon a material's surface indicate the surface tensions between the liquid-surface interface, the liquid-air interface, and the surface-air interface. Higher contact angles for water droplets indicate a more hydrophobic surface than lower contact angles existing under the same ambient conditions. A 5 µL droplet of water possessing a contact angle of 141 degrees is shown deposited upon a hydrophobic NH$_2$-MWCNT surface in Figure 19. Applications for contact angle dependent phenomena include self-cleaning surfaces,$^{[16]}$ filtration apparatuses,$^{[17]}$ and cellular growth scaffolds,$^{[18]}$ therefore, all NH$_2$-MWCNT film processing methods should investigate the resulting effects on the film’s wettability.
NH$_2$-MWCNT films analyzed for roughness and pore size distribution in previous sections were tested with contact angle measurements as described in Chapter 2 to determine how the roughness and pore size distribution alters the wettability of an NH$_2$-MWCNT film, and the results for these samples appear in Figure 20. These NH$_2$-MWCNT film samples include those from section 4.2.3 prepared by filtering different volumes of the NH$_2$-MWCNT dispersion and those from section 4.2.6 prepared by centrifuging NH$_2$-MWCT dispersions at different centrifugal forces.

The results show that within each sample set of the NH$_2$-MWCNT papers prepared with different dispersion volumes and centrifugal forces, contact angle decreases with increasing roughness as shown in the top left and top right of Figure 20; however, as shown in the bottom left of Figure 20, the contact angle does not decrease significantly until an average roughness of ~350 nm is obtained with NH$_2$-MWCNT film samples prepared by filtering large volumes of the NH$_2$-MWCNT dispersion. Additionally, some of the samples prepared at lower centrifugal forces exhibit a lower average roughness yet lower contact angle than half of the samples prepared with low filtration volumes. These results suggest that average roughness is not the predominant factor for altering the wettability of an NH$_2$-MWCNT film but does to a limited extent affect the contact angle of a given NH$_2$-MWCNT film. The bottom right of Figure 20 shows three samples with different contact angles in terms of the weighted average of their pore size diameter distribution. With this limited number of data points, one cannot definitively conclude the effect that pore size distribution...
may have upon the wettability of a NH$_2$-MWCNT film, but this plot suggests that an increase in pore size diameter distribution may be the predominate factor for the wettability of an NH$_2$-MWCNT film.

Figure 20 Contact angles and average roughness of NH$_2$-MWCNT films prepared by filtering different volumes of a NH$_2$-MWCNT dispersion (top left), contact angles and average roughness of NH$_2$-MWCNT films prepared by centrifugation at different forces (top right), contact angles in terms of average roughness for samples prepared by different volumes and centrifugal forces (bottom left), and contact angles in terms of the weighted average of the pore size distribution for samples prepared by different volumes and centrifugal forces (bottom right)
4.5 Conclusions and future work

The work presented in this chapter demonstrates that the processing a NH₂-MWCNT dispersion in terms of sonication exposure time, filtration volume, dispersing medium, or centrifugation all greatly affect the roughness of a NH₂-MWCNT film. The effects that these processing parameters may have upon pore size diameter distribution and wettability are not fully elucidated and will need further analysis by SEM and the Nova® image processing software. With a greater understanding of how these parameters affect the pore size distribution of a NH₂-MWCNT film, one can then look to prove or disprove a correlation between pore size distribution and surface wettability. An understanding of all of these processing parameters will allow for further studies into the interactions between CNT film cellular growth scaffolds and the adherent cell culture such as those reported in Chapter 3.

4.6 References

367, 1631
129
Chapter 5
Cellular Internalization of Carbon Nanotubes and Adsorbed Peptides

5.1 Introduction

As described in Chapter 1, carbon nanotubes (CNTs) are highly investigated as drug delivery transporters for various pharmaceutical agents and biological cargos owing to their large surface areas and ability to traverse cellular membranes. While most of this work to date has focused on proof of principle experiments for delivery of various biological cargos, little has been done in terms of understanding the fundamental interactions between CNTs and enhancement of their internalization into cells. To augment the efficacy of a delivered payload, CNT transporters should be prepared with functional groups or dispersing agents that boost cellular internalization, efficiently release the cargo at target destinations, and speed the exiting rate from the cell or body. While this can be accomplished by attaching functionalities with specific binding receptors for various cellular cues, simpler modifications to an overall CNT transporter scheme should be investigated to avoid overly complex modifications and high production costs. The aim of this chapter's work seeks to develop methods employed with a spatial scanning Raman spectrometer termed the NTEGRA Spectra and to investigate how the interactions with synthetic peptides (discussed in Chapter 2) which vary in their primary amino acid sequences, mainly in the number of phenylalanine residues, affect CNT internalization and intracellular location. The primary amino acid sequences for 0F, 4F, and 8F in single letter notation where hyphens separate α-helical heptad secondary structural repeats are:

0F: E-VEALEKK-VAALESK-VQALEKK-VEALEHG

4F: E-VEAFEKK-VAAFESK-VQAFEKK-VEAFEHG

8F: E-FEAFEKK-FAAFESK-FQAFEKK-FEAFEHG

Original aims of the designed 4F peptide included controlling the diameter distribution of SWCNT aggregates and controlling the morphology of hierarchical self-assembled SWCNT structures by designing the 4F peptide with various characteristics that would
respond to ion concentrations within the peptide-SWCNT dispersion and drying conditions. Upon folding into an α-helical structure, the 4F peptide seen along its axis resembles the depiction shown in Figure 1.

![Helical wheel diagram of the 4F peptide showing primary amino acid positions within the α-helix secondary structure](image)

Figure 1 Helical wheel diagram of the 4F peptide showing primary amino acid positions within the α-helix secondary structure

The notable design features of the 4F peptide derive from the amino acid properties located at various positions in the helix and include a hydrophobic face represented by the α and δ positions of each heptad repeat, a hydrophilic face represented by the β, γ, ε, ζ, and θ positions of the heptad repeat, negatively charged amino acids in the ε position and positively charged amino acids in the θ position to promote favorable electrostatic interactions between helices adsorbed onto the SWCNT sidewalls. When folded in this conformation, the hydrophobic face strongly adsorbs onto the SWCNT sidewalls while the hydrophilic face solubilizes the peptide-SWCNT complex. Thus, variations among the 0F, 4F, and 8F peptides can alter peptide self-aggregation and SWCNT interactions which in turn affect SWCNT cellular internalization.

Few techniques exist to gauge cellular internalization of CNTs. Fluorescent microscopy can proceed by detecting the emission from either the near-infrared fluorescence of semiconducting single-walled carbon nanotubes (SWCNT)\(^{10,11}\) or the emitted frequencies of an adjoined fluorophore.\(^{1,5,12}\) However, SWCNT aggregation or adsorption of...
intracellular species quenches the SWCNT near-infrared fluorescence\textsuperscript{[11]} insuring a cutoff in accurate quantifiable detection above a certain amount of internalized SWCNTs. Similar effects follow for attached fluorophores. While these quenching effects can be mitigated to some extent by employing a biocompatible and highly efficient dispersing agent, a technique that can measure the amount of internalized CNTs regardless of dispersing agent or intracellular concentration and aggregation state will be less limited in scope in terms of investigations into cellular reactions to various CNT functionalization schemes. The high intensity of the G-band of a SWCNT Raman spectrum allows for simple detection by a Raman spectrometer and when used in conjunction with a piezo-electric scanner allows for efficient quantification and determination of intracellular location of internalized SWCNTs on a cell by cell basis.

5.2 Raman scattering of CNTs, materials, and cells

5.2.1 Crucial parameters of the NTEGRA Spectra for spectral mapping

Investigations into cellular internalization and intracellular locations of SWCNTs in this work are performed primarily on the NTEGRA Spectra system (NT-MDT\textregistered). This system combines a NANOFINDER 30 (Solar TII\textregistered) Raman spectrometer with a scanning probe microscope which allows for the operation of confocal Raman scattering and fluorescent microscopy limited in spatial resolution only by the Bragg diffraction limit. Thus, applications of the NTEGRA Spectra extend to 3-dimensional Raman and luminescent imaging, 3-dimensional chemical analysis, quantification of a detectable material over a known volume and much more. The NTEGRA Spectra is especially suited for the study of SWCNT interactions with cells as SWCNTs possess an exceptionally high Raman cross-section enhanced even further by the resonance Raman effect to allow for the detection of small quantities of CNTs within cells. Additionally, the NTEGRA Spectra can image all cellular organelle features in conjunction with a fluorescent stain on par with conventional confocal fluorescent microscopes to study the intracellular location of internalized CNTs.
For any spectrum or spectral map taken with the NTEGRA Spectra, several parameters must be considered and recorded as adjustments in any of these can vastly alter an experiment’s results and the image produced as a spectral map. The ultimate aim of scanning with the NTEGRA Spectra is to produce spectral maps over given regions that represent all chemical signals of interest mapped with the lowest resolution possible. Minimum spatial resolution is achieved by closing an aperture to the light detector, the pinhole, to a diameter approximately the size of the focused light beam to allow for confocal microscopy. If as much signal is to be gathered from large volumes of the mapped space for quantification purposes, then the pinhole should be removed or opened to the maximum distance possible to allow for wide field Raman microscopy.

The Chinese hamster ovarian (CHO) cells used in this work range from 20 to 40 µm in diameter and up to 2.5 µm in height. Therefore, spectral maps must be sized appropriately to cover an entire cell with the mapped points spaced at appropriate distances to provide excellent spatial resolution but not create excessive scan times. The lateral resolution for a point of focused light and objective lens is given by

Equation 5.1 where $r$ is the minimum distance between two points of light to be resolved by the Rayleigh criterion, $\lambda_0$ is the wavelength of the excitation laser, and $NA_{obj}$ is the numerical aperture of the objective.[13]

$$r = 0.61 \frac{\lambda_0}{NA_{obj}}$$

Equation 5.1 Radius of the central disk of an Airy disk diffraction pattern for a point of light in terms of the wavelength of that light and numerical aperture of the objective[13]

For all experiments in this chapter, either a 473 nm or 633 nm excitation laser was used in conjunction with a 100x oil immersion objective lens with a 1.40 numerical aperture. Different laser excitations sources can sometimes reveal different Raman features of molecules, affect the absorption efficiency, and highlight different types of SWCNTs within a population based upon their electronic transition energies. The lateral resolution is
then determined to be 206 nm and 275 nm for the 473 nm and 633 excitation sources, respectively. The axial resolution for wide field microscopy is given by Equation 5.3 where $r$ is again the minimum distance for two points of light to be resolved, $n$ is the refractive index of the medium, and $NA$ is the numerical aperture of the objective lens.\[[13]^{131}

$$r_{axial} = 1.4 \frac{\lambda_0 n}{NA^2}$$

*Equation 5.2 Axial resolution of wide field microscopy*\[[13]\]

Thus for wide field microscopy, the axial resolution is 510 nm and 683 nm for the 473 and 633 nm excitation sources, respectively. For confocal microscopy, the resolution of the axial field is given by Equation 5.3 where $K$ is a scalar correction factor equal to 0.95 for a pinhole microscope.\[[13]\]

$$d = K \frac{\lambda_0}{n \left[ 1 - \sqrt{1 - \left( \frac{NA}{n} \right)^2} \right]}$$

*Equation 5.3 Axial resolution for confocal scanning*\[[13]\]

The axial resolution is then determined to be 424 nm and 568 nm for the 473 nm and 633 nm excitation sources, respectively.

In addition to spatial resolution, spectral resolution and spectral range is also a vital factor for spectral mapping with the NTEGRA Spectra system. Spectral resolution and range is altered by changing the diffraction grating within the spectrometer and the focal distance. Since luminescence is one of the important signals of interest for this work, a diffraction grating with 100 grooves per millimeter was used to capture a spectral region of 500 nm spanning the entire emission range of most fluorophores. After the appropriate grating has
been chosen for a given spectral range, it must be calibrated to a known standard such as
the 520.5 cm\(^{-1}\) peak of a silicon wafer as shown in Figure 2. Also shown in Figure 2 are
overtone features of the 520.5 cm\(^{-1}\) peak appearing at higher wavelengths.

![Raman spectra of a silicon wafer with 473 and 633 nm excitation](image)

**Figure 2** Raman spectra of a silicon wafer with 473 and 633 nm excitation

### 5.2.2 Spectra of specimens

Spectra for all specimens were gathered to identify their signature peaks and the positions
of those peaks for spectral mapping. SWCNTs (Unidym\textregistered) were examined with the 473
nm and 633 nm lasers as shown in Figure 3. The radial breathing modes (150-350 cm\(^{-1}\)),
defect band (1290 cm\(^{-1}\)), G-band modes (1490-1750 cm\(^{-1}\)) appear in the 473 nm and 633
nm excitation spectra and the G' band (2670 cm\(^{-1}\)) appears only in the 473 nm excitation
spectrum because of the limited spectral range. The origins of these modes are discussed in
Chapter 2.
Figure 3 Raman spectra of as produced SWCNTs with 473 nm excitation (left) and 633 nm excitation (right)

All spectra and spectral maps in this chapter are performed on cells either grown or deposited upon glass coverslips and submersed in phosphate buffered saline (PBS) solution. To establish a background and confirm that no significant peaks will arise within the spectral regions corresponding to the SWCNT G-band or any fluorophore's spectral emission range, Raman spectra were taken of a glass coverslip, PBS, and a CHO cell and are displayed in Figure 4. While various spectral features can be observed, it should be noted that these spectra were taken at high exposure times of 60 seconds which is unnecessary for the detection of the SWCNT G-band or the fluorescence of an organelle stain. The prominent spectral features shown in Figure 4 for the glass coverslip appearing just below 500 cm\(^{-1}\) correspond to the D\(_1\) and D\(_2\) vibrational modes of SiO\(_2\)\(^{[14]}\) and the feature at 920 cm\(^{-1}\) corresponds to silica borate groups.\(^{[15]}\) The features for PBS can be attributed to the \(-\text{OH}\) bending mode at 1645 cm\(^{-1}\) and the \(-\text{OH}\) stretching mode in water at 3450 cm\(^{-1}\).\(^{[16]}\) The prominent spectral features arising solely from the cell can be attributed to bending modes of the CH\(_2\) and CH\(_3\) bonds at 1290 cm\(^{-1}\) 1440 cm\(^{-1}\) of proteins and lipids and the stretching modes of CH\(_2\) and CH\(_3\) bonds of proteins and lipids in the 2900 cm\(^{-1}\) region.\(^{[17]}\)
Raman spectra were obtained from cells incubated with peptide-SWCNT dispersions to confirm that SWCNT spectral features can be detected. Peptide-SWCNT dispersions were prepared with concentrations of 0.0065 mg/mL of SWCNTs and 0.0195 mg/mL of the 0F, 4F, or 8F peptide in PBS. Peptide-SWCNT dispersions were bath sonicated with chilled water for 1 hour immediately before incubation. For incubation, 500 µL of the peptide-SWCNT dispersion was added to 2 mL of cellular growth media and administered to \textit{in vitro} CHO cells 24 hours after plating. Following the 24 hour period, CHO cells were washed twice with cellular growth media, twice with PBS, treated with trypsin, and centrifuged twice in PBS at 100 RCF for 4 minutes before examination by Raman scattering. A CHO cell incubated with an 8F-SWCNT dispersion for 24 hours and its corresponding Raman spectra with 473 nm and 633 nm excitations are shown in Figure 5.
The SWCNT Raman features shown in Figure 5 only confirm that SWCNTs are present within the confocal volume of the examined cell but do not confirm that SWCNTs are internalized within the CHO cell. The possibility exists that SWCNTs are merely embedded upon the cellular membrane surface.

To confirm cellular internalization as well as intracellular location, spectral mapping of cellular organelle fluorescent dyes can be used in conjunction with the observed SWCNT Raman spectral features. The emission spectra of CHO cells stained with a cell nucleus marker termed Draq5 (Biostatus Limited®) and a cellular membrane marker termed FM 4-64 (Invitrogen®) were acquired separately and are shown in Figure 6. To stain cells with Draq5, the Draq5 stain is prepared in CHO cellular growth media at a concentration of 0.0025 µM and then added to the CHO cells for 5 minutes before fixation with 4% formaldehyde in PBS for 10 minutes. To stain cells with FM 4-64, a coverslip with as-grown CHO cells was immersed in a FM 4-64 staining solution at a concentration of 5
μg/mL in ice cold Hank’s Buffered Salt Solution (HBSS) (Invitrogen®) for 40 seconds, rinsed three times with HBSS, and then fixed in a 4% formaldehyde-HBSS solution for 10 minutes.

Figure 6 Emission spectra for the FM 4-64 plasma membrane stain (left) and the Draq5 nucleus stain (right) used in later experiments for fluorescent spatial mapping

5.3 SWCNT interactions with peptides

The extent to which the 0F, 4F, or 8F peptide can suspend and isolate SWCNTs factors heavily into how cells will internalize the peptide-SWCNT conjugate as will be shown in sections 5.4 and 5.5. AFM was used to investigate SWCNT features from spun-cast samples of dispersions to determine whether SWCNTs were suspended mainly as aggregates or individual tubes, and circular dichroism (CD) was employed to investigate the changes in secondary structures for each peptide when used as SWCNT dispersing agents.

Peptide-SWCNT dispersions were created by adding equal mass ratios of the 0F, 4F, or 8F peptides and SWCNTs to PBS followed by bath sonication treatment for 20 minutes under chilled conditions followed by probe sonication treatment for 30 seconds at an output power of 5 Watts. Deviating from aqueous preparation of peptide-SWCNT dispersions by Dieckmann and co-workers, peptide-SWCNT dispersions were prepared in PBS
because PBS mimics cellular growth media conditions in terms of ion concentration and pH.

5.3.1 AFM height profiles to assess the amount of individually suspended SWCNTs in dispersion

10 µL droplets of 0F-SWCNT, 4F-SWCNT, and 8F-SWCNT dispersions were spun-cast onto freshly cleaved mica at 3000 rpm for 30 sec to assess the extent of SWCNT aggregation in these dispersions. AFM height scans of spun-cast samples from dispersions with higher proportions of SWCNT bundles will display features of greater height compared to dispersions consisting mainly of individually suspended SWCNTs. An AFM height image of a 4F-SWCNT dispersion at a concentration of 0.35 mg/mL spun-cast onto a mica surface is shown in Figure 7. Large and relatively long bundles of SWCNTs as well as some individually suspended SWCNTs are visible in the AFM height image of Figure 7. These large aggregate features in Figure 7 are present in dispersion and upon the mica surface on account of the high SWCNT concentration of the sample and charge screening effects of the PBS solution that act to decrease the charge-charge interactions between adjacently adsorbed peptides on dispersed SWCNTs.\textsuperscript{[21]} The cross-sectional profile shown in the right of Figure 7 shows the heights of the various features observed in the corresponding AFM height image and is used to measure the SWCNT feature's diameter.
Height profiles of SWCNT features in the AFM height images can be acquired to determine their diameter distributions in order to assess the extent of debundling at various SWCNT concentrations and with the different peptides. For all peptide-SWCNT dispersions, extensive debundling occurs at lower concentrations below 0.035 mg/mL as shown by the histograms of SWCNT features’ diameters in Figure 8.

The average diameters and standard deviations (n = 50 for higher concentrations and n < 25 for 0.0035 mg/mL concentration) for SWCNT features examined by AFM height images for all peptide-SWCNT dispersions are displayed in Table 1. At the lowest dispersion concentration of 0.0035 mg/mL, all peptide-SWCNT dispersions appear to have only individually suspended SWCNTs considering that Unidym® SWCNTs have diameters between 0.7 and 1.4 nm and the peptide coating can add 1-3 nm. At the 0.035 mg/mL concentration, the 4F and 8F peptides significantly debundle more SWCNTs than the 0F peptide with the 8F peptide exhibiting the greatest debundling, and at the highest dispersion concentration of 0.35 mg/mL concentration, the 8F peptide slightly debundles more SWCNTs than the 4F peptide which in turn slightly debundles more SWCNTs than the 0F peptide. These results suggest that the 4F and 8F peptides are significantly better than the 0F peptide at producing SWCNT dispersions of individually suspended SWCNTs.
Figure 8 Height distributions of SWCNT features in AFM height images of spun-cast dispersions onto freshly cleaved mica for 0F-SWCNT (top left), 4F-SWCNT (top right) and 8F-SWCNT (bottom) dispersions at different concentrations

<table>
<thead>
<tr>
<th>Dispersion Type</th>
<th>Concentration 1</th>
<th>Concentration 2</th>
<th>Concentration 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0F-SWCNT dispersions</td>
<td>1.25 ± 0.37 nm</td>
<td>3.5 ± 1.05 nm</td>
<td>10.04 ± 4.14 nm</td>
</tr>
<tr>
<td>4F-SWCNT dispersions</td>
<td>0.94 ± 0.31 nm</td>
<td>1.86 ± 0.96 nm</td>
<td>9.58 ± 3.40 nm</td>
</tr>
<tr>
<td>8F-SWCNT dispersions</td>
<td>1.20 ± 0.94 nm</td>
<td>1.48 ± 0.58 nm</td>
<td>8.11 ± 2.87 nm</td>
</tr>
</tbody>
</table>

Table 1 Average diameters and standard deviations for SWCNT features examined by AFM height images
5.3.2 Circular dichroism spectroscopy of peptide solutions and peptide SWCNT dispersions

The secondary structures of the three peptides with and without the presence of SWCNTs were examined by circular dichroism (CD) spectroscopy to investigate whether SWCNTs induce a conformational change in the secondary structure of the adsorbed peptides. An overview of CD spectroscopy and the various spectral features is given in Chapter 2. Dieckmann et al. reported such a conformational change for the 4F peptide when sonicated in the presence of SWCNTs.[8] The CD experiments presented herein look to quantify the change in \( \alpha \)-helical content of the three peptides before and after sonication with SWCNTs by analysis of the recorded CD spectra with CDPro® software analysis. Peptide dispersions were prepared at equal mass ratios of peptide and SWCNTs at concentrations of 0.035 mg/mL, 0.065 mg/mL, 0.11 mg/mL, 0.20 mg/mL, and 0.35 mg/mL in phosphate buffered saline solution (PBS). With the eventual goal of understanding how peptide-SWCNT interactions affect the cellular internalization of SWCNTs, PBS was chosen as the dispersion medium because its salt concentrations and pH mimic that of cellular growth media. Figure 9 shows the molar ellipticities of the three peptides without SWCNTs at a concentration of 0.35 mg/mL. Characteristic \( \alpha \)-helical features appear in the 0F and 8F spectra at 208 nm and 222 nm[221] while the 4F spectrum exhibits spectral behavior of a random coil conformation. The percentage of \( \alpha \)-helical content for the 0F, 4F, and 8F peptides shown in Figure 9 are 78%, 8.5%, and 33% as calculated by the CDPro® spectral analysis software. CDPro® spectral analysis software functions by assuming that the CD spectrum for any molecule with a secondary structure can be represented by a linear combination of the spectra of secondary structural elements. The software then fits the sample’s spectrum with an appropriate percentage of secondary structural elements seeking to minimize the mean square difference from the sample data and the weighted average model.
Figure 9 Molar ellipticities of the 0F, 4F, and 8F peptides at 0.35 mg/mL in PBS

The molar ellipticities for the three peptides at different concentrations in PBS are shown in Figure 10 and demonstrate that the primary amino acid sequences directly affect the peptides’ self-interactions. The phenylalanine residues in the $d$ position of the helical wheel representation are known to strongly limit peptide aggregation\textsuperscript{[19]} which in turn stabilizes the $\alpha$-helical conformation. Because all phenylalanine residues are replaced by leucine residues in the 0F peptide, 0F tends towards self-aggregation giving rise solely to the $\alpha$-helical features seen in Figure 10 at all concentrations. Figure 10 also shows that 8F tends towards a more $\alpha$-helical conformation than 4F as was reported by Zorbas et al.\textsuperscript{[19]}

Figure 10 CD spectra for the 0F (left), 4F (middle) and 8F (right) peptides at different concentrations in PBS showing varying amounts of secondary structures
When SWCNTs are added to the peptide solutions at equal mass ratios, no conformational changes occur for the 0F-SWCNT dispersion while more α-helical content is induced in the 4F-SWCNT dispersions and 8F-SWCNT dispersions as shown in Figure 11. These conformational changes for the 4F and 8F peptides immediately suggest a higher degree of interaction between the 4F and 8F peptides and SWCNTs than for the 0F peptide and SWCNTs on likely account that self-aggregation of the 0F peptide with itself prevents association with SWCNTs. Because 8F already exists largely in an α-helical conformation, less of a percentage change in α-helical content is to be expected than for the unfolded 4F peptide. The changes in α-helical content upon dispersion treatment with SWCNTs closely resemble the results first reported for the 4F peptide and SWCNTs by Dieckmann et al.[8]

![Figure 11 Molar ellipticities of 0F (left), 4F (middle) and 8F (right) peptides with and without SWCNTs at equal mass ratios and concentrations of 0.35 mg/mL in PBS](image)

The CD spectra for the peptide solutions and peptide-SWCNT dispersions were then analyzed by the CDPro® spectral analysis software to quantify the percentage of α-helical content. Figure 12 shows the percentage change in α-helical content by subtracting the α-helical content of the peptides alone from the α-helical content of the peptides in the presence of SWCNTs divided by the α-helical content of the peptides alone expressed as a percentage. As shown in Figure 12, more α-helical content is present within the 4F and 8F samples containing SWCNTs than those without while approximately the same percentage of α-helical content exists in the 0F samples regardless of the absence or presence of SWCNTs. Greater changes in α-helical content are observed for the lowest peptide and SWCNT concentrations of 0.035 mg/mL on likely account that exfoliation of the SWCNT
aggregates expose more surface area to interact with the peptides and induce conformational changes. Points at the highest concentration are likely to be skewed as the darkness and turbidity of the SWCNT dispersions may affect the observed CD spectra. These results reaffirm that the adsorption interactions between the 4F and 8F peptides and the SWCNTs induce conformational changes in the secondary structures of the adsorbed peptides while inducing no changes in the structure for the 0F peptide.

Figure 12 Percentage change in α-helical content for the 0F, 4F, and 8F peptide samples with and without an equal mass ratio of SWCNTs at different concentrations

5.4 Spectral mapping of the SWCNT G-band and fluorescent stains

5.4.1 Spectral maps of fluorescent organelle stains

To confirm that the NTEGRA Spectra can produce fluorescent maps of stained organelle features, as-grown CHO cells were prepared as described above for the FM 4-64 stain and then mapped with the 473 nm laser in 0.5 μm steps in wide field mode. The resulting image for the FM 4-64 stained cells is shown in Figure 13.
As-grown CHO cells were then stained with the Draq5 nucleus stain as described above and then mapped with 633 nm laser in 0.5 µm steps in wide field mode. The resulting image for a Draq5 stained cell is shown in Figure 14.
5.4.2 Confocal spectral mapping of SWCNTs, FM 4-64, and Draq5

To insure maximum internalization of SWCNTs by the CHO cells when incubated with cellular growth media containing SWCNTs or with peptide-SWCNT dispersions, CHO cells were incubated with cellular growth media containing SWCNTs at a concentration of 1.3µg/mL, well below the SWCNT aggregation concentration, or with peptide-SWCNT dispersions as described in section 5.2 and then stained with the FM 4-64 membrane marker. Cells were then examined by confocal spectral mapping.

To insure that all confocal planes of a CHO cell are mapped and that the internalization analysis discussed later in section 5.4 covers all of the depth of the cell, the height of a CHO cell must be determined. As-grown CHO cells were examined by AFM in a liquid cell as shown in Figure 15 and determined to never exceed 2.5 µm in terms of height. As discussed in section 5.1, the axial resolution for confocal spectral mapping for the 473 nm and 633 nm excitations are 424 nm and 568 nm, respectively.

Confocal spectral maps were generated with the 633 nm excitation source by mapping the average intensity of the 640-920 nm spectral region covering the emission window shown in Figure 6. This spectral region covers the entire emission from the FM 4-64 stain and also contains the G-band and other SWCNT features as shown in Figure 17. Figure 16
shows four of these spectral maps at different focal planes along the axial direction separated by 0.5 µm distances of a single fixed CHO cell incubated with an 8F-SWCNT dispersion for 24 hours and stained with the FM 4-64 membrane marker as described above. The regions of highest contrast in each map shown in Figure 16 correspond to the presence of internalized SWCNTs as confirmed by the corresponding Raman spectrum shown in Figure 17.
Figure 16 Confocal fluorescent and Raman images of a FM 4-64 stained cell incubated with 8F-CNT dispersions for 48 hours showing the bottom confocal plane (top left), second from bottom (top right), third from bottom (middle left), and top image in the axial direction (middle right) and corresponding optical image and mapped area marked in blue of the cell (bottom). The step between each focal plane is 0.5 μm.
Figure 17  Fluorescent and Raman spectrum taken from point within a CHO cell (identified with a blue box in the image on the left) showing the CNT G-band and D-band for cells incubated with an 8F-CNT dispersion

Figure 18 shows the spectral maps for as-grown CHO cells incubated with media containing SWCNTs or the 0F-SWCNT and 4F-SWCNT dispersions. These maps confirm internalization for the different dispersions; however, while internalized SWCNTs were easily detected for all peptide-SWCNT dispersions upon the first cell examined, several cells incubated with the media containing SWCNTs were examined before any detectable G-band signal was observed.
Figure 18 Fluorescent and Raman spectra (right) taken from points within the cells showing the CNT G-band and D-band for cells incubated with cellular growth media containing SWCNTs (top), 0F-CNT dispersion (middle), and 4F-CNT dispersion (bottom)
To insure that SWCNTs are responsible for cellular internalization and not the peptides, fluorescently labeled analogues of the peptides with an attached fluorescein fluorophore were used to disperse the SWCNTs and then incubated with CHO cells for 24 hours. Results were then compared to what was found when the cells were incubated with the fluorescently labeled peptides without CNTs. Fluorescently labeled peptide solutions were prepared at a concentration of 0.01125 mg/mL and used to disperse SWCNTs at a concentration of 0.0075 mg/mL. Fl-peptide-SWCNT dispersions were bath sonicated for 1 hour with chilled water before incubation with CHO cells. Fl-peptide solutions of equal peptide concentration were also incubated with CHO cells for 24 hours. Figure 19 shows the resulting confocal fluorescent images and demonstrates that Fl-peptides do not enter into cells by themselves but require the presence of SWCNTs for cellular internalization. All images in Figure 19 were captured with the same exposure settings and integration times to allow for fair comparison between images. Figure 19 also demonstrates that only a small amount if any of the Fl-0F peptide associated with SWCNTs enters into cells.
Figure 19 Confocal fluorescent images of cells incubated with Fl-0F solution (top left), Fl-0F-CNT dispersion (top right), Fl-4F solution (middle left), Fl-4F-CNT dispersion (middle right), Fl-8F solution (bottom left), and Fl-8F-CNT dispersion (bottom right)
For colocalization studies of the SWCNT G-band with the luminescent Draq5 nucleus stain, the mapped spectral regions excited by the 633 nm laser were set to determine the average signal intensity from 50 to 5000 cm\(^{-1}\) (640 to 920 nm) and the maximum signal intensity from 1570 to 1630 cm\(^{-1}\). An image for each of these spectral maps is shown in Figure 20. Features corresponding to internalized SWCNTs appear in the spectral map of the maximum intensity from 1570 to 1630 cm\(^{-1}\) that do not appear in the spectra of the maximum intensity from 50 to 5000 cm\(^{-1}\).

Figure 20  Raman spectrum showing the mapped spectral regions (left), spectral map produced by mapping the average intensity in the spectral window from 150-5000 cm\(^{-1}\) marked with blue lines in the corresponding spectrum (middle), and spectral map produced by mapping the maximum intensity in the spectral window from 1570-1610 cm\(^{-1}\) marked with red lines in the corresponding spectrum (right). The maps are obtained from a fixed CHO cell stained with Draq5 to highlight the nucleus.

Spectral maps can then be outputted into 2-dimensional numerical arrays where intensity values are normalized to the same intensity scale regardless of the chosen spectral region or whether a maximum or average intensity is recorded. By subtracting the normalized maximum signal in the spectral window from 1570-1610 cm\(^{-1}\) from the normalized average intensity in the spectral window from 150-5000 cm\(^{-1}\), a digitized array representing the intracellular presence of the SWCNTs can be produced as shown in Figure 21.
Figure 21 Digitized recreations of spectral maps from Figure 20 showing the mapped spectral region of the maximum intensity in the spectral window from 1570-1610 cm\(^{-1}\) (left), the mapped spectral region of the average intensity in the spectral window from 150-5000 cm\(^{-1}\) (middle), and digitized representation of the spectral map of average intensity in the spectral window from 150-5000 cm\(^{-1}\) subtracted from the spectral map of maximum intensity from 1570-1610 cm\(^{-1}\) (right).

The digitized recreation of the spectral map primarily showing the intracellular presence of SWCNTs is then overlayed with the original spectral map of the average intensity in the spectral window from 150-5000 cm\(^{-1}\) corresponding mainly to the Draq5 nucleus stain to produce the images shown in Figure 22.

Figure 22 shows that SWCNTs cannot be detected by this analysis when dispersed in cellular growth media alone in good agreement with the observation reported for the FM 4-64 colocalization experiment. Qualitatively, the 0F peptide seems to internalize fewer SWCNTs than the 4F or 8F peptide suggesting that a dispersing agent’s ability to stabilize and isolate SWCNTs in suspension as discussed in section 5.3 factors heavily into the amount of internalized SWCNTs. Figure 22 also shows that internalized SWCNTs are localized into clusters and not spread evenly throughout the cell. Some possible overlap with the nucleus can be seen in several of the images where a portion of the nucleus appears damaged or missing; however, this may be a result of the SWCNTs quenching the Draq5 stain or a cluster of SWCNTs bending into the nucleus.
5.5 Measuring internalization of SWCNTs by Raman mapping

The efficacy of any delivered cargo through a CNT transportation scheme will depend heavily upon the amount of the internalized cargo and associated SWCNTs; therefore, a means to quantify the amount of internalized CNTs will bridge a gap in understanding.
between the amount of internalized CNTs and the cellular effect observed after delivery of
the cargo. This section discusses the method and analysis used for quantifying the
internalization of SWCNTs by the NTEGRA Spectra and compares the amounts of
internalized SWCNTs when dispersed in standard cellular growth media or by the 0F, 4F,
and 8F peptides.

A spectral map generated by the NTEGRA Spectra contains a specified number of points
representing the maximum or average intensity over a chosen spectral region. Each map
can be digitized as discussed above in section 5.3 and transformed into a histogram for
further analysis. *Figure 23* shows a spectral map of the average intensity between 1570-
1640 cm\(^{-1}\) corresponding to the CNT G-band signal over a cell incubated with an 8F-SWCNT dispersion for 30 hours. *Figure 23* also shows the corresponding histogram for
the Raman map fitted with a Gaussian curve using Origin 6.1® software. The majority of
points (>~95%) within these spectral scans will correspond to regions where SWCNTs are
not present and therefore constitute background noise arising from locations of the
surrounding PBS or immersed cell. Since a large proportion of the mapped points represent
noise, a Gaussian curve can be fitted to this population to represent the overall noise of each
spectral map. The point within the histogram at which the fitted Gaussian curve levels off
is taken as the cutoff between background noise and true signal arising from SWCNTs
present within the mapped region. Every point’s value above the Gaussian cutoff is then
normalized such that the Gaussian curve cutoff value is set to zero and then summed to give
the total intensity of G-band signal arising from each cell.
An equal number of points and distance between points must used in each spectral scan to insure that a comparison between cells of different samples is fair. CHO cells posses an elongated and irregular morphology in addition to growing next to one another as shown in Figure 24. These morphological characteristics make individual analysis of as-grown cells inconsistent from cell to cell. Therefore, cells are cleaved from their substrates with trypsin-EDTA (Gibco®) and deposited onto glass coverslips to give a consistently rounded morphology as shown in Figure 24.
All spectral maps for quantifying internalized CNTs are done in wide field microscopy mode by setting the pinhole to its maximum diameter to gather signal from as many confocal planes spanning the depth of the examined cell as possible. Wide-field scanning comes with the drawback that it cannot distinguish signal arising from SWCNTs that might be deposited on the cellular membrane surface and internalized SWCNTs; therefore, accurate quantification of internalized CNTs is skewed.

To test the validity of this method, CHO cells were incubated with the supernatant following centrifugation of cellular growth media containing SWCNTs (Unidym®) at a high concentration of 1 mg/mL. This dispersion was centrifuged for two 30-minute cycles at 3000 RCF. Cells were then examined and analyzed according to the above method at five different time points. The results are displayed in Figure 25 and show an upward trend in overall G-band intensity per cell vs. incubation time as is to be expected.
Quantification of internalized SWCNTs was then examined with the CHO cells incubated for 30 hours with media containing SWCNTs and the peptide-SWCNT dispersions. The results are shown below in Figure 26. While the average values for the internalized SWCNTs for the 4F and 8F dispersions exceed those for the media containing SWCNTs and the 0F dispersions, their standard deviations do not allow their average values to be considered statistically significant. To improve this experiment, cells should be treated with mitomycin C that would act to stop cell cycle division and thus avoid unintended internalization side-effects that may arise from cellular growth. The higher average values for the 4F-SWCNT and 8F-SWCNT dispersions are in agreement with the qualitative observations reported for the Draq5 colocalization study. While signal was observed for cells incubated with media containing SWCNTs, this signal may have arisen from SWCNTs lying upon the cellular membrane surface as these spectral maps were performed in wide field mode. As reported for the internalization experiment with the FM-464 membrane marker, the majority of cells incubated with media containing SWCNTs did not exhibit G-band signal from within the cell interior.
The likely reason for higher internalized amounts of SWCNTs with the 4F-SWCNT and 8F-SWCNT dispersions lies in the 4F and 8F peptides’ abilities to disperse and exfoliate large bundles of SWCNTs compared to the 0F peptide. The principle components in cellular growth media adsorbing onto SWCNTs have been reported as fibrinogen, apolipoproteins, albumin, phenol red, and riboflavin.[23-25] Other publications have reported that cell growth media does not isolate single SWCNTs from bundles and therefore fits with the hypothesis that the smaller diameter of a single SWCNT allows for greater cellular internalization than the large diameter of a bundle of SWCNTs.[24,26] Additionally, this size-dependent effect has been reported in the literature for the internalization of other nanoparticles.[27,28]

5.6 Conclusions and future work

The results in this chapter demonstrate that highly dispersed and isolated SWCNTs with small diameters internalize into CHO cells in greater quantities than bundles of SWCNTs with large diameters as shown by the colocalization studies with the FM 4-64 and Draq5 stains, the greater fluorescence emanating from within CHO cells incubated with Fl-4F-
SWCNT and Fl-8F-SWCNT dispersions, and the histogram analysis for the media containing SWCNTs and associated peptides. Thus, SWCNT delivery schemes should employ dispersing agents or covalent functionalization strategies that insure the isolation of SWCNTs in suspension for optimized internalization into target cells; however, functionalization schemes should also effectively coat the sidewalls of the SWCNTs preventing media components adsorbing onto SWCNTs resulting in a secondary toxicity effect. While the histogram analysis does offer insight into the quantities of internalized SWCNTs, the method may need refinement to reduce the large standard deviations observed within each population of sample cells and comparison with another method that determines the actual mass or quantity of internalized SWCNTs. This might be accomplished by high temperature annealing to burn away any biological material while leaving behind SWCNTs that survive the combustion or by cellular lysis to release the internalized SWCNT material. Additionally, the SWCNTs embedded upon the cellular membrane complicates matters of determining the actual amount of internalized SWCNTs by wide field Raman mapping. This problem can be eliminated by 3D confocal spectral mapping in conjunction with a cellular stain; however, the luminescence from the cellular stains may offset and distort the signal arising from internalized SWCNTs. Biological variability also plays significantly into the quantification of internalized SWCNTs as some cells within a population may simply internalize great quantities of SWCNTs while others may not. These methods for quantifying SWCNT internalization and intracellular location should then be applied to the leading SWCNT functionalization schemes reported in the literature to optimize variables for greater SWCNT internalization.

5.7 References

Chapter 6
Summary and Future Work

6.1 Summary

The work presented within this thesis has investigated the use of carbon nanotubes (CNT) as cellular growth scaffolds, controlling the morphological and physiochemical properties of CNT substrates, and exploring the interactions among carbon nanotubes, synthetic peptides, and cellular internalization.

The use of a carbon nanotube surface for replacing mouse embryonic fibroblast co-cultures and Matrigel® layers for supporting human embryonic stem cell (hESC) growth has proven highly promising and should be able to fully replace these conventional surfaces with slight modifications. While as-produced single-walled carbon nanotubes (SWCNT) and carboxylic acid functionalized SWCNTs did not support hESC growth in any long term viable manner, surfaces made from bovine serum albumin dispersed multi-walled carbon nanotubes (MWCNT) functionalized with amine and amide groups (NH₂-MWCNT) proved highly successful for supporting human embryonic stem cell growth with full retention of several pluripotent markers suggesting that an entirely synthetic carbon nanotube substrate should prove viable for sustained hESC growth when optimized to an appropriate roughness and wettability. The roughness of each of these NH₂-MWCNT surfaces were crucial factors for the hESC adherence and colony morphology with the smoothest surface promoting no cellular adherence and the roughest surface forcing the hESC colonies to grow with an abnormal spheroid shaped center uncharacteristic of flat and well-spread hESC colonies. The results discovered in this work suggest that CNTs may play crucial roles for differentiation and pluripotent behaviors of stem cells in general and may prove that CNT surfaces are optimal for several primary cell lines.

Expanding on the work presented in Chapter 3, Chapter 4 investigated in detail the changes in NH₂-MWCNT surface characteristics including roughness, pore size distribution, and wettability in response to various dispersion processing parameters including sonication exposure times, dispersing agent or solvent, filtration volume of the processed dispersion,
NH₂-MWCNT concentration, and centrifugation speed. Altering any of the processing parameters except concentration was found to greatly affect surface roughness in microscale dimensions while not changing the nanoscale topography. Changing the filtration volume or centrifugation speed was also shown to affect the pore size distribution and wettability of the NH₂-MWCNT surface; these observed characteristics are heavily interlinked with the surface roughness of each film's surface. The results shown in this chapter may not only affect the development of CNT surfaces for cellular growth scaffolds, but may also find applications in CNT filtration devices, sensors, and actuators.

Chapter 5 then develops the use of the NTEGRA Spectra® micro Raman scanning instrument for cellular investigations with fluorescently marked cellular organelles in conjunction with the observed Raman peaks of internalized SWCNTs. Methods for exploring the intracellular location and quantifying the amount of internalized SWCNTs have proven successful with room for improvement in terms of quantification. These methods were then applied to investigate how the interactions between the 0F, 4F, and 8F peptides affected SWCNT internalization and intracellular location and found that while all three peptides increased the amount of internalized SWCNTs compared to SWCNT dispersions made solely with cellular growth media, the 4F and 8F peptides increased SWCNT internalization more than compared to the 0F peptide on likely account that these peptides are able to better disperse and isolate SWCNTs than the 0F peptide. These findings suggest that any SWCNT delivery schemes should seek to isolate carbon nanotubes of the smallest diameter possible to increase the amount of internalized SWCNTs and adjoined biological cargo.

6.2 Future work

Future investigations of carbon nanotubes as primary cell culture growth scaffolds should seek to find an appropriate biocompatible polymer that can replace the biologically derived bovine serum albumin dispersing agent to render a fully synthetic substrate free of any pathogenic contamination or forego the addition of such a polymer and tailor these CNT surfaces in such a manner as to modify their surface wettabilities to a hydrophilic character
capable of supporting cell culture growth in vitro. The use of CNTs for cellular growth scaffolds should not stop at the second dimension but look to incorporate CNTs into three-dimensional scaffolds for use in implantation devices and tissue regeneration. Incorporation of CNTs could greatly modify the elastic and compressive moduli of such devices as well as reduce the overall mass and allow for expanding modification to the pore size distribution to allow greater or less cellular motility and vascular flow within these devices.

The central focus of modifying NH$_2$-MWCNT surface characteristics from the endpoint of Chapter 4 should seek to clarify how the various processing parameters of sonication exposure time, CNT concentration, dispersing agent or solvent, filtration volume, and centrifugation specifically affect the pore size distribution within these films as the author believes that while the surface roughness does factor into the wettability of these films, it does not weigh as heavily as the pore size distribution. A clear understanding between these dispersion processing parameters and pore size distribution will elucidate the final mysteries between pore size distribution, wettability, and cell adhesion and proliferation. Pore-size distribution may be the greatest determining factor for controlling hESC behavior and final elucidation of all physical properties of a CNT surface in conjunction with biological functionalization may provide for an optimal hESC growth substrate. Control over the pore size distribution of these NH$_2$-MWCNT films and other CNT films will also greatly affect the applications for membrane filtration devices, chemical sensors, and actuators.

Chapter 5 provides methods for determining the intracellular location and amounts of internalized SWCNTs and can be applied to more commonly used SWCNT functionalization schemes reported in the literature. Such applications can be applied for altering the delivery location of the payload cargo and optimizing functionalization scheme parameters to raise the efficacy of the delivered cargo. Following this work, the author seeks to investigate how molecular characteristics of phospholipid dispersing agents such as tail length, tail saturation, and electrostatic charge affect SWCNT cellular internalization. The author also hopes to investigate the internalization amounts of smaller diameter SWCNTs compared to larger MWCNTs. Additionally, no toxicity studies were carried out
on the effects peptide-CNT dispersions might have on mammalian cells and should be included in future investigations as well as toxicity studies for phospholipid CNT dispersions.