5-2011

A Study on the Applications and Toxicity Assessments of Carbon Nanotubes in Tissue Engineering

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A STUDY ON THE APPLICATIONS AND TOXICITY ASSESSMENTS OF CARBON NANOTUBES IN TISSUE ENGINEERING

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biological Engineering

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2011

ABSTRACT

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by

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Utah State University, 2011

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Carbon nanotubes (CNTs) are one of the most popular nanomaterials. There has been increasing interest in the development and applications of carbon nanotubes due to their huge potential in industrial and medical applications. Recent applications of carbon nanotubes include development of scaffolds and drug delivery systems. Despite rapidly emerging applications of CNTs, little is known about the impact of CNTs on cellular processes, especially mesenchymal stem cell (MSC)'s differentiation. Also, the effects of nanoparticle exposure under different conditions on cellular responses have not been well characterized yet.

To characterize the effects of CNTs on creating nanoscale scaffolds for tissue engineering, we incorporated multi-walled CNTs (MWCNTs) into reconstituted type I collagen, and evaluated proliferation, differentiation, mineralization and inflammatory response of MSC on those scaffolds. MWCNTs were homogeneously distributed in collagen matrix, and strongly entrapped in collagen at the concentrations below 100 ppm. Alkaline phosphatase (AP) activity and mineralized nodules of extracellular matrix (ECM)

were monitored as osteogenic differentiation markers. AP activity was significantly increased in 12 days after being replaced by differentiating media. Collagen enhanced AP activity, and MWCNT-collagen scaffolds induced additional increase in AP activity. The MSC released a significantly higher level of AP on MWCNT-collagen scaffolds than the plastic surface did at day 16. An increasing percentage of ECM mineralization was seen at day 16 after being replaced by differentiating media in the presence of MWCNT-collagen scaffolds. This study indicated the possibility of enhancement in MSC differentiation in the MWCNT-collagen scaffolds. The increased level of differentiation markers was due to the increased stiffness of the scaffolds for MSC. Our data indicated that the collagen-MWCNT scaffolds might have the potential application to create nanoscale scaffold materials for tissue engineering.

To illustrate the effects of interleukin-8 (IL-8) expression in human alveolar epithelial cells (A549) under various exposure conditions of CNT, we measured the level of IL-8 expression in the presence and absence of serum following exposure of SWCNTs. The results demonstrated that the IL-8 expression was enhanced in the presence of serum. The IL-8 expression kept increasing at low concentration even after removing SWCNTs from the media. Further studies are required to characterize biological functions and toxicological potentials of nanomaterials.

(99 pages)

ACKNOWLEDGMENTS

This thesis would not exist without the support of several influential people. I wish to express sincere gratitude to my advisor, Dr. Soonjo Kwon. Thanks for his guidance and instruction on my research in those years, his enthusiastic encouragement on my study and my entire research. I am also very grateful for the mentorship of Dr. David Britt and Dr. Tapas Kar in my advisory committee, who spent lots of time helping me with the problem I faced in my projects. In addition, thanks to everyone in the Integrated Tissue Engineering Laboratory (ITEL) who played some role in my research. I would like to express my appreciation to Hemang Patel, who helped me a lot during those years. Hemang has enlightened me not only on my research, but also on the development of insight into living. Most importantly, I would like to thank the department of Biological Engineering for their supporting and help during those years. Especially, I would like to thanks to Ms. Anne Martin for all her help during those years.

I have been blessed with supportive family and friends who have helped me on the road to this degree. I have incredible parents, who have always being positive and uplifting, and always supporting (and cheerleading) whatever choices I made for my personal and educational goals. I would certainly not be where I am today without their love and encouragement. I am so grateful for my sister Reyhan Baktur, who provided me not just a house to live in for three years, but also love and encouragement throughout my years at USU. Also, I am grateful to all my friends in China, especially to Greece, who has always encouraged me to be strong. I love them all.

Rena Baktur

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LIST OF ABREVIATIONS

CNTs……Carbon Nanotubes

SWCNTs……Single-Walled Carbon Nanotubes

MWCNTs……..Multi-Walled Carbon Nanotubes

AP……Alkaline Phosphatase

β-GP……...β-glycerophosphate

CIAP……Calf Intestinal Alkaline Phosphatase

Dex……...Dexamethasone

DMEM..... Dulbecco's Modified Eagle's Medium

ECM……Extra Cellular Matrix

EtOH…….....Ethanol

FBS……Fetal Bovine Serum

IL-6…….....Interleukine 6

IL-8…….....Interleukine 8

L-AA…….....L-Ascorbic Acid

MSC……..Mesenchymal Stem Cells

NF-κB……....Nuclear Factor κB

PBS…….....Phosphate Buffered Saline Solution

RANK…….Receptor Activator of NF-κB

RANKL……Receptor Activator of NF-κB ligand

CHAPTER 1

INTRODUCTION

1.1 Background

Over the past decade, rapid development in nanoscience and nanotechnology has resulted in the successful synthesis and characterization of various inorganic nanomaterials.[1-3] The unique physical properties of the nanopaticles, such as their extremely small size, make them highly suitable for a wide range of biological and medical applications. In all those nanoparticles, carbon nanotubes (CNTs) have been the focus of intense research that was motivated by their unique physical, chemical and mechanical properties, since they were discovered by Iijima in 1991.[4]

Carbon nanotubes (CNTs) are a new generation of materials possessing superior mechanical, thermal, and electrical properties.[5,6] They are mosaics of carbon atoms that form graphene sheets and curl into seamless tubules. CNTs are classified as single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs) based on the number of graphene layers. CNTs exhibit unique properties that have made them an attractive material for the application in molecular electronics,[7] medical diagnostics, biosensors,[8] and drug delivery carriers.[9]

The CNTs have been proposed for biomedical applications, such as cell tracking and labeling, nano-sensors, and vehicling for controlled release of drugs, and vehicling for delivery of bioactive agents. Synthetic polymers and CNTs have demonstrated promise for these materials in neural and orthopedic tissue engineering applications. When CNTs are used as scaffold materials in regenerative medicine, especially in bone tissue engineering, they are in direct contact with the bone. These included implants for

arthroplasty, bone fracture, dental treatment and scaffolds for bone tissue regeneration. The reaction of the bone cells to CNTs is critical for functional maintenance of the biomaterials for a long time in the tissues. Recently, there have been several reports on the application of CNTs for scaffolds. For example, CNTs coated with bioactive molecules were used as substrates for nerve cell growth.[10] Also in Webster's study they reported that carbon nanofibers increased osteoblast functions. MacDonald reported a collagen-SWCNT composite for cell culture substrate.[11] However, there is a limit amount of information about the CNT and collagen mixture scaffold for stem cell growth. This give researchers a new idea to use collagen-CNT composite to check stem cell biocompatibility.

On the other hand, enthusiasm for CNTs use has been tempered by relevant concerns regarding their toxicity. The high aspect ratio of CNTs made them easily become airborne and inhaled. The evaluation of their pulmonary effects has received a considerable amount of interest and emerge studies investigate the toxicity of CNTs *in vivo* and *in vitro*. Most of the emerging literatures on the toxicity of CNTs on the lung tissue of various animal models have been reported and appared to be inconsistent amony the research findings. Some of the researches confirmed that CNTs can have toxic effects, and some of the toxic effects in mammals have been attributed to high concentration of CNTs. There also have been a range of *in vitro* studies using human or mammalian cell lines to investigate the toxicity mechanisms, which suggest that oxidative stress, inflammation reactions, and immune toxicity may be key features of CNT toxicity. Most of the studies that suggest CNT toxicity used a high concentration of CNT to expose the cell line or to inject the animal. However, there was no specific number about how much

CNT will get inhaled in to human body and in the environment. Small amount of CNTs inhaled effect is still unknown and the machines of CNTs to airway inflammation reactions need to be indicated.

1.2 Motivation and Objectives of Research

Carbon nanotubes (CNTs) and other carbon nanomaterials are of interest for biological and medical applications because of their high chemical durability, mechanical strength and electrical properties. Studies on the applications of carbon nanomaterials have been carried for the substrate of cell culture,[12-14] drug delivery systems[15,16] and medical implant materials.[17] Several studies have shown that cells have high affinity to CNTs.[18-20] Other studies that showed the bone cell affinity to CNTs and bone formations on sintered MWCNTs[21]. During those studies, MacDonalds provided a good idea for using collagen and CNTs to make a good composite material, which show high mechanical strength and good cell viability. However, until now there are no studies that use collagens - CNTs composite materials to make a scaffold to culture mesenchymal stem cells (MSC), especially a good composite material with good optically transparent. Thus, in our study, we

a) Designed CNTs-collagen composite scaffolds using different types of MWCNTs (functionalized and non-functionalized) with good optically transparent.

b) Estimated mesenchymal stem cell's viability, differentiation, mineralization and inflammatory response on those types of CNTs-collagen scaffolds.

c) Measured the mechanical properties of those CNTs-collagen scaffolds.

Carbon nanotubes have gained enormous popularity due to a variety of potential applications which will ultimately lead to increased human and environmental exposure

to these nanoparticles. The lung is regarded as one of the main portals of entry for nanoparticles, which emphasizes the importance of pulmonary toxicity evaluation. The lung epithelium plays an important role in the modulation of inflammatory processes by releasing inflammatory cytokines such as IL-8 and nitric oxide [22-24]. However, the effects of concentration, exposure time, media component and aggregation of CNTs on cellular toxicity and their mechanisms have not been well defined. In the second study, we estimated effect of exposure conditions on SWCNT-induced inflammatory response in human alveolar epithelial cells. This study was spurred by the following fundamental questions:

a) What other environmental factors (e.g. exposure time, media components) may affect the SWCNTs related inflammatory responses or cellular toxicity?

b) Is SWCNTS induced inflammatory response alleviated by removing SWCNTS following exposure?

Chapter 1 describes general introduction and motivations of studies on the applications and toxicity assessments of carbon nanotubes in tissue engineering. In chapter 2, Literature Review introduced researches about the CNTs biomedical applications, especially in bone tissue engineering applications. It also looked at the toxicity study of CNTs, both *in vivo* and *in vitro,* especially on human alveolar epithelial cells. Chapter 3 exhibited the research related to effect of Carbon nanotubes –collagen composite scaffold on mesenchymal stem cell proliferation, differentiation, mineralization and inflammatory response. Chapter 4 contained the study about the effect of exposure conditions on SWCNT-induced inflammatory response in human alveolar

epithelial cells. Based on the experimental data in chapter 3 and chapter 4, conclusions and recommendations were given in chapter 5.

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CHAPTER 2

LITERATURE REVIEW

2.1 Carbon nanotubes

Carbon nanotubes (CNTs) are one of the new class materials for industrial and biomedical applications. Sumio Iijima discovered multiwall CNT (MWCNTs) in 1991 by an arc-discharge method.[1,2] After two years, he made the observation of single-walled nanotubes (SWCNTs).[3] Since then, CNTs have captured the attention of researchers worldwide.

CNTs are allotropes of carbon with a cylindrical nanostructure. They are a seamless cylindrical sheet of graphite whose diameter is nano scale and its aspect ratio (diameter verses length ration up to 132000000:1) is significantly larger than any other materials.[4] CNTs not only exhibit extraordinary strength but also are the stiffest materials have been found on the earth yet.

CNTs are categorized as SWCNTs and multi-walled nanotubes (MWCNTs). A SWCNT is a nano size tube formed by bonded carbon atoms arranged in a hexagonal pattern. An individual SWCNT is a very thin tube at least several micrometers long, the diameter of the SWCNTs are vary between about 0.7 and 3 nm,[5] with an aspect ratio greater than 1000.[6] Structurally, it resembles a rolled up single layer of graphite sheet. MWCNTs have two or more concentric layers with various diameters (generally range from 10 to 200 nm)[7] and lengths. MWCNTs generally exist in single tubes and form very few bundles. Both of SWCNTs and MWCNTs have van der Waals forces between the molecules, this causes them to aggregate into microscopic bundles or topes. However, the MWCNTs are less effective than the SWCNTs.[8]

On the other hand, the lack of solubility and the difficult manipulation in any solvents have imposed great limitations to the use of CNT. They can be dispersed in some solvents by sonication, but precipitation immediately occurs when this process is interrupted. It has been demonstrated that CNT can interact with different classes of compounds[9-11]. Extensive research on CNTs focuses on modifying CNTs by adding specific chemical groups, leading to a significant change in their properties (Like –OH, -COOH, $-NH_2$).

Commercially, CNTs are produced from carbon atoms found in graphite by arc discharge , by pulsed laser vaporization[12], and by a chemical-vapor deposition (CVD) process[13]. Nowadays, the most common synthesis method is the HiPco process which was developed in the Smalley's laboratory[14]. CNTs samples invariably contain some residual impurities. Usually they are metals, organics, and support materials. The most commonly used metals in CNT synthesis are Co, Fe, Ni and Mo. Post production processing removes the majority of metal catalyst. However, CNTs may still contain some residual metal by mass. In our research, we used many different types and different sizes of CNTs. All CNTs we bought are made by HiPco method. The Table 2.1 showed the detail information about the MWCNTs we used in our research.

2.2 Carbon Nanotubes Applications

The nano scale of nature has logically given rise to the interest in using nanomaterials for tissue engineering and biomedical field. In the medical field, biomaterials are expected to be developed using CNTs for clinical use. Since 2000, with CNT high aspect ratio, electrical and physical properties, there has been an approximate doubling each year of articles related to CNT for use in biomedical application. Also the

research have increased rapidly from 2005 and continue to increase (Figure 2.1).[15] However, there have been only a few cases of a truly clinical application to date, and therefore the use of CNTs in biomaterials must be considered to be at an early experimental stage.

2.2.1 Application in biomedical: cancer, drug, and matrix

Applications of CNTs in the field of biotechnology have recently started to emerge, raising great hopes. CNTs have been proposed as a component for DNA and protein biosensors [16,17], ion channel blockers [18] and bioseparators and biocatalysts [19]. Concerning the biomedical applications of CNTs, their use is becoming relevant in neuroscience research and tissue engineering. Cell tracking and labeling, sensing cellular behavior, augmenting cellular behavior and enhancing tissue matrices are the four areas that CNT can be used in tissue engineering [20]. CNTs have also been used as a new platform to detect antibodies associated with human autoimmune diseases with high specificity [21]. In a similar context, CNT covalently have been modified at their open ends with DNA and RNA, and have led to innovative systems for hybridization of complementary DNA strands allowing for ultrasensitive DNA detection [22].

Carbon nanotubes have potential novel application in nanomedicine as biocompatible and supportive substrates, and as pharmaceutical excipients for creating versatile drug delivery systems. The applications of CNTs as drug excipients are of interest, given their capacity to interact with macromolecules such as proteins and DNA [23]. There are some research showed that functionalized carbon nanotubes used as vectors for *in vitro* delivery of small molecules [24], a potential vector for transporting proteins,

peptides, and genes in to the nucleus [25-27]. Efforts are continuing to develop novel systems for the delivery of protective antigens.

Table 2.1 Carbon Nanotubes (CNTs) detail information

Types	Outside diameter	Inside diameter	Length	Components Contents (%)			
				C	Cl	Fe	Ni
MWCNT	20-30nm	5-10nm	10-30 μm	98.35	0.45	0.26	0.94
Short MWCNT	20-30nm	5-10nm	0.5-2.0 μm	98.34	0.47	0.24	0.94
MWCNT-OH	20-30nm	5-10nm	10-30 μm	98.37	0.47	0.23	0.93
Short MWCNT-OH	20-30nm	5-10nm	0.5-2.0 μm	98.32	0.48	0.25	0.94
MWCNT-COOH	20-30nm	5-10nm	10-30 μm	98.39	0.45	0.23	0.93
Short MWCNT-COOH	20-30nm	5-10nm	0.5-2.0 μm	98.34	0.46	0.25	0.94

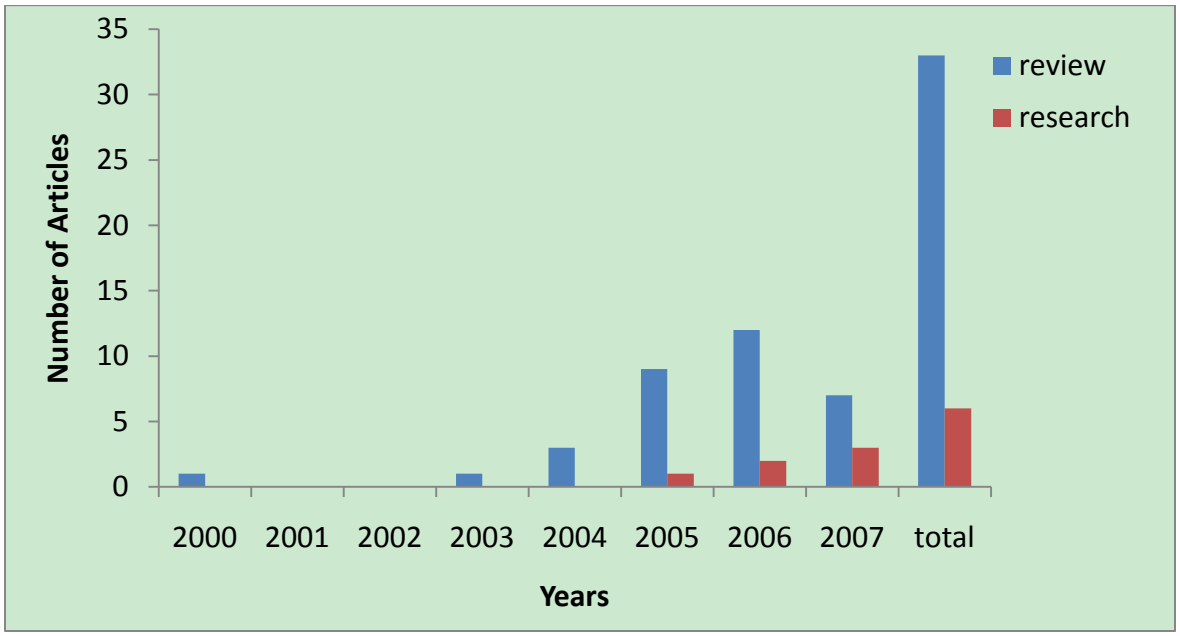


Figure 2.1 Number of publications on CNTs applications in biomaterials by year

CNTs are also potential biocompatible materials that can serve as a supportive matrix. The matrix plays a critical role in tissue engineering. It is responsible for defining the space the engineered tissue occupies and aiding the process of tissue development. Compared to the polymers, CNTs can easily functionalized and have good mechanical strength. CNTs have the potential for providing the need structural reinforcement for tissue scaffolds. Adding CNTs in to polymer can significantly improve the mechanical strength of the materials. In Wang et.al research they found MWCNT blended with chitosan showed significant improvement in mechanical properties compared with those without chitosan [28].

In vitro studies have shown that CNT as a scaffold has successfully grown several different types of cells. For instance, smooth muscle cell have been successfully grown on the SWCNT with collagen scaffold [29]. CNT scaffold support L929 mouse fibroblasts growth [30]. CNT are widely used as a mimic neural fibers scaffold to guide neurite growth [30-34]. Nowadays, researchers try to use CNT as a scaffold for bone cell growth.

2.2.2 CNTs applications in bone tissue engineering

A major goal in bone bioengineering is to create artificial scaffold materials that have the capacity to sustain bone cell growth, proliferation and increment or replace bone tissue. When CNTs are used in biomaterials to contact with bone, the reactions of cells including bone tissues to CNTs are critical. These reactions are especially important when CNTs are used for bone tissue engineering. These cells are mainly osteoblasts, osteocytes, osteoclasts, and chondrocytes. Osteoblasts, the bone forming cells, proliferate on the bone surface, produce and secrete bone matrix proteins. As they progress through stages of cell differentiation under hormonal control, they mineralize the matrix by means

of production of hydroxyapatite crystals. There have been several studies on the influence of CNTs on osteoblastic cells which are representative of these cells.

There are some published studies on the application of CNTs to biomaterials associated with bone. What they did basically was expose bone cell to different types of the CNTs, then checked cell proliferation and differentiation. For examples, in Zhang *et al* investigated effect of SWCNT, double walled carbon nanotubes (DWNTs) and MWCNT on the proliferation, differentiation, adipocytic transdifferentiation and mineralization of primary osteoblasts. They found the treatment of CNTs could reduce the viability of primary osteoblasts and inhibit the mineralization of osteoblasts in a dose-dependent manner. Also CNT reduced the adipocytic transdifferentiations. However, the inhibition was not strong enough to reverse the cytotoxicity and suppression on viability and mineralization of primary osteoblasts [35]. Similar results were found by Zanello group. They concluded that CNTs sustain osteoblast growth and bone formation. CNTs showed promising biocompatibility with osteoblast cells and appeared to modulate the cell phenotype. Thus CNTs represent a potential technological advance in the field of bone bioengineering[36]. In Narita et al's study they showed that MWCNTs inhibit osteoclastic bone resumption *in vivo*.MWCNTs inhibit osteoclastic differentiation and suppressed a transcription factor essential for osteoclastogenesis *in vitro* [37]. Recently, the main aim of many studies is to examine the effects of CNTs on mesenchymal stem cell viability, proliferation, and phenotype. Mooney *et al'*s study revealed that functionalized CNTs were easier to disperse in human mesenchymal stem cell media. Study clarified the COOH- functionalized SWCNT were least toxic to the cells, and the CNT had no adverse effects on cell biocompatibility, proliferation, or differentiation [38].

Since attention has been paid to the use of CNTs as biomaterials in contact with bone, it is critical to understand the reaction of bone cell to CNTs. Some of the studies used CNT films to test the effects of CNTs on bone cells. Kalbacova's groupindicated that differently prepared SWCNT films are not toxic for human osteoblasts and could be used for biomedical applications [39]. In Aoki et al's study they made CNT scaffold by vacuum filtration. And cell proliferation and morphology were investigated using a scanning electron microscopy. Osteoblast cells on CNTs showed excellent proliferation with extension of cell morphology in all directions [40]. After two years, Aoki's group investigated the dependence of biocompatibility of carbon materials on crystal structure with the aim of developing biomedical applications. They used SWCNT and MWCNT as scaffolds for cell culture and compared with graphite (GP). They discovered the osteoblast cell responses in terms of cell morphology, cell proliferation, and ALP activities were quite different between those carbon atom materials. With CNTs scaffolds, cell growth, proliferation, and functions were higher than GP. In particular, SWCNT cell proliferation and ALP activity were higher than on MWCNT. They thought those results could be due to the surface topology and chemical properties of the substrates [2]. However, the SWCNT films showed different results to MSC. SWCNT films and carbon fibers reduced the metabolic activity of both investigated cell types in different ways, as the reduction is more produced for carbon fibers. The high quality SWCNT films are apparently not toxic for the MSC under the conditions used. The observed reductions of metabolic activities can be occasioned by the surface morphology of the carbon materials [41]. Park's results clearly showed that MSC exhibited preferential growth on CNT patterns, and the cell culture results suggested that the CNT patterns didn't have a

harmful effect on the MSC. Those results investigated CNT patterns have enormous potential as a new platform for basic research and applications using stem cells [42].

All those studies discussed above are using pure CNTs, however, reinforcement of naturally derived polymers with carbon nanotubes is another promising area that is just beginning to be explored. In 2006, Chlopek *et al*. fabricated MWCNT with polysulfone PSU (which is currently used in medicine) and investigated their effect on osteoblast. The cellular test performed in this study confirmed a good biocompatibility of nanotubes. Like high level of viability of the examined cells in contact with the nanotubes, the unchanged level of oteocalcin released from osteoblasts, the lack of proinflammatory IL-6;these effects point out a good cellular biocompatibility [43]. Type I collagen is one of the most studied molecules in biology because of its very important physical and biochemical functions in the extracellular matrix of many tissues, and has shown promise as a matrix for regenerative medicine [44,45]. MacDonald *et al.* reported a collagen-SWCNT composite for the cell culture substrate. The SWCNTs were strongly entrapped by collagen and the composite showed high mechanical strength. And the cell showed good cell viability. The affinity between collagen and CNTs was expected [29]. Recently, Terada *et al* .developed a transparent MWCNTs coating for cell culture dishes and estimated its properties for cell culture. In this research, they found rat osteoblast-like cells cultured on the MWCNT-coated dish which showed slightly lower viability and proliferation compared to the collagen-coated dish. The cell adhesion on the MWCNT coated dish was much higher than that on the collagen-coated dish. Therefore, CNT-coating for dishes will be a useful new material for cell culture [46]. However, there is only limit research about collagen-CNT composite materials, especially on stem cell research.

2.3 Mesenchymal Stem Cells

It is commonly believed that stem cell is involved in continuous maintenance and repair of most tissue types. Taken into consideration that stem cells are capable of self renewal and they share the ability to differentiate in multiple lineages. This class of cells is of paramount importance for an organism, not only during development, but also during adulthood with respect to cellular homeostasis. Stem cells for non-hematopoietic tissues, which can be derived from bone marrow are commonly termed mesenchymal stem cell (MSC). MSCs are considered a readily accepted source of stem cells because such cells have already demonstrated efficacy in multiple types of cellular therapeutic strategies, especially in bone tissue regeneration strategies [47].

MSC differentiation, morphology, markers expression and inflammatory response

The multiline differentiation potential of MSC populations derived from a variety of different species has been extensively studied *in vitro* since their first discovery in the 1960s [48]. MSC has the capacity to differentiate into all connective tissue cell types, including bone, cartilage, tendon, muscle, marrow, fat and dermis. *In vitro* studies showed successfully differentiation in a variety of cell lineages, including osteoblasts, chondrocytes, adipocytees, fibroblasts, myoblasts and cardiomyocytes, hepatocytes, tenocytes, cenocytes, and even neurons.[49-52]

The differentiation can be simulated *in vitro* by adding the proper differentiating supplements to MSC, including dexamethasone (Dex), L-ascorbic acid (L-AA), and β-glycerophosphate (β-GP), each of them play a unique role in the differentiation process.[53-56] For convenience, the differentiation process of the osteoblast committed

MSC is divided into several stages, like osteoprogenitors, preosteoblasts, osteoblastes, osteocytes, and bone lining cells (Figure 2.2).

Osteoprogenitor cells: it is a mainly functionalized cell type. Osteoprogenitors are able to clonally generate cells of osteoblast lineage but lack self-renewal capacity [57]. The cell morphology is spindles shaped and reside in close proximity to the preosteoblast layer with a distance from bone formation surface in the periosteal layer of bone [58].

Preosteoblasts: this type of cell is considered as a precursor of the oteoblasts and indentified in part by their localization in the adjacent one or two cell layers' distance from the osteoblasts lining bone formation surfaces. Although preosteoblasts resemble osteoblasts histologically and stain for ALP acitivity, they have not yet acquired many of the other characteristics of fully mature cells, and unlike osteblasts, preosteoblasts are thought to possess a limited proliferation potential.

Osteoblasts: it is a post proliferative, strongly alkaline phosphatase positive cell lining bone matrix at sites of active matrix production. This type of cell has a large nucleus, enlarged Golgi apparatus, and extensive endoplasmic reticulum, which are typical features of cells highly active in protein production.[59]

Osteocytes: It is a non-proliferative, terminally differentiated cell of the osteoblasts lineage. They are non-migratory cells, locked inside small lacunae in the mineralized bone matrix or the newly formed osteoid. Compared with mature osteoblasts, the osteocytes are smaller and have decreased ALP activity and cytoplasmic organelles. However, there is an emerging consensus that osteocytes play an important role in the maintenance of bone structure.[60-62]

Bone lining cells: These types of cells are flat, thin and elongated, covering most if not all the non-remodeling bone surfaces. Bone lining cells are considered postproliferative, inactive in matrix production and may take part in mechanotransduction and protection of the bone matrix from osteoclastic resorption.[63] Another feature of the bone lining cells is their potential to be reactivated to cuboidal, which can active osteoblasts in response to particular stimuli.[64]

The cells change dramatically in morphology from their *in vivo* state. However, this compartmentalization is problematic for *in vitro* studies. Therefore, using molecular markers to evaluate the differentiation of MSC is most practical. The MSC differentiation is associated with temporal modification in the expression of a set of macromolecules. These molecules include membrane-associated enzymes (such as Alkaline phosphatase), bone matrix protein (such as type I collagen), and different cytokine receptors. Those macromolecules not only play important roles in regulating cell proliferation and function but also provide a panel of markers that reflect the transitional stages in osteoblast development (Figure 2.3).

Type I collagen: It is a primary product of osteoblasts during bone matrix formation, constituting approximately 90% of the total organic matrix in mature bone [65]. In osteoblast lineage cells, type I collagen is clearly expressed before the preosteoblast stage, and it has been documented that up-regulation of type I collagen occurs prior to up-regulation of any other matrix molecules and prior to ALP.[66]

Alkaline phosphatase (ALP): Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. It is the most frequently used phenotypic marker to

evaluate bone activities [67]. The major role of ALP in bone is to facilitate mineralization. It is abundantly presents in membranes of osteoblasts and matrix vesicles when it covalent bounds to glycosylphosphatidylinositol (GPI).[68] In addition, ALP can also be detected in subpopulations of osteoprogenitors and preosteoblasts, well before mineralization and prior to the expression of the noncollagenous matrix molecules.[69] That is why ALP plays as a membrane-bound receptor involved in osteoprogenitor osteoblast adhesion, migration, and differentiation.[70]

Osteocalcin: Osteocalcin is a carboxylated bone protein also known as bone Gla protein. It is the most abundant non-collagenous protein of bone and has a very narrow expression pattern. Osteocalcin is undetectable in preosteoblasts and expressed only by the mature osteoblasts and osteocytes. Therefore, the latest expressed osteoblast makers is osteocaclin.[71,72]

Interleukin-6 (IL-6): A variety of publications suggest that treating inflamed cells with nano-particles decreases the cells' inflammatory responses.[73,74] Cytokine expression has been measured for MSC isolated from rat bone; results indicated that MIF, IL-8, Serpin E1, GROα, IL-6, MCP-1, and SDF-1 were the most highly expressed [4]. IL-6 is a multifunctional cytokine that can activate target genes involved in proliferation, differentiation, survival and apoptosis in a variety of cells.[72,75] Indeed, researchers have shown that the production of interleukin-6 by bone marrow cells is regulated by sex steroids.[76-78] Upon loss of gonadal function, osteoclast and osteoblast formation in the marrow increase. Both changes are apparently mediated by increase in the production of IL-6.[79] A pathogenic role for IL-6 and its receptor has been also demonstrated in other systemic diseases with increased bone remodeling, such as hyperparathyroidism and

hyperthyroidism.[80] IL-6 activates nuclear factor κB (NF-κB) through the mitogen-activated protein kinase (MAPK) signal transduction pathway, which regulates expression of the receptor activator of NF-κB ligand (RANKL).[81] through the IL-6-gp130-STAT3 signaling pathway.[82] RANKL then binds to receptor activator NF-κB (RANK) on the surface of osteoclast progenitor cells, inducing the formation of a mature osteoclast.[83] Therefore, IL-6 cytokines stimulate osteoblast differentiation and bone formation.

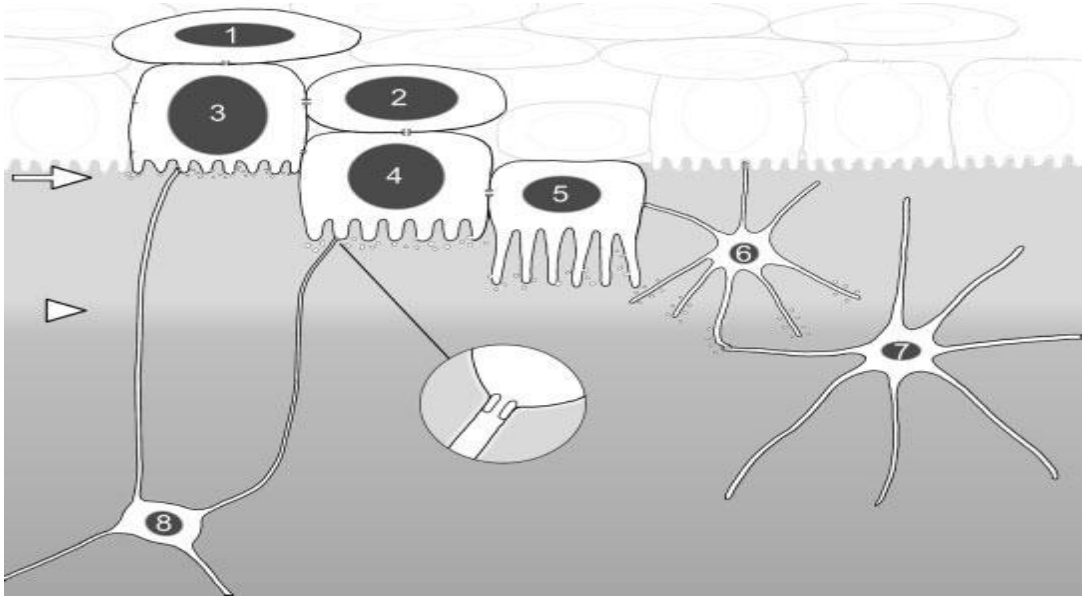
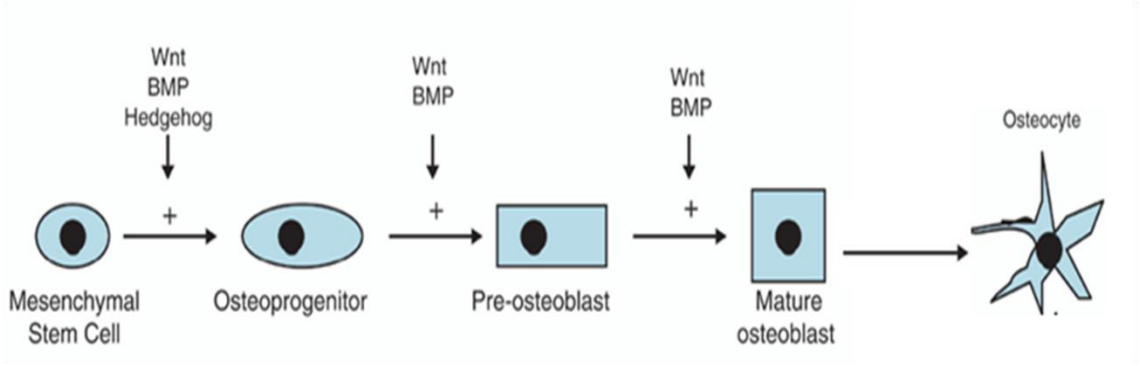


Figure 2.2 1. preosteoblast, 2. preosteoblastic osteoblast, 3. osteoblast, 4. osteoblastic osteocyte (Type I preosteocyte), 5. osteoid-osteocyte (Type II preosteocyte), 6. Type III preosteocyte, 7. young osteocyte, 8. old osteocyte. Diagram drawn by Tim Fedak (www.figs.ca).[61]



Lineage commitment	Proliferation	Matrix Maturation	Mineralization
	CD44	Alkaline Phosphatase	E11/p38/OF45
ALCAM	Histone	Bone Salio Protein	Osteocalcin
CD105	Collagen1	Collagen1	Osteopontin
CD63	TGF		Collagenase
RunX2	Osteopontin		

Figure 2.3 Biomarker produced during different stages of MSC differentiations.

2.4 Toxicity Assessment of Carbon Nanotubes

CNTs are useful materials with numerous technological potentials. When CNT synthesis moved from laboratories to factories and the products became commercial commodities, CNTs began to come under toxicological scrutiny.

2.4.1 *In vivo toxicology studies: rat, pig, fish*

Most of the emerging literatures on the toxicity of CNTs have shown the model of respiratory exposure with consequent concerns about lung injury, inflammation and possible early signs of tumor formation in the lung (Table 2.2).[84]

Table 2.2 Pulmonary toxicity studies of CNTs in animals [84]

Test materials	Metal content	Animal species	References
Soot containing CNTs	Co/Ni No info on %	Guinea pig	Huczko et al.,2001
SWCNTs	Ni:10%	Mouse	Lam and McCluskey, 2000 (unpublished report)
SWCNTs	Fe:26.9% Mo:0.95% Ni:0.8%	Mouse	Lam et al., 2004
SWCNTs, purified	Fe:2.1%	Mouse	Lam et al., 2004
SWCNTs	Ni:26% Y:5.0% Fe:0.5%	Mouse	Lam et al., 2004
SWCNTs	Ni:5% Co:5%	Rat	Warheit et al., 2004
SWCNTs, purified	Fe:0.23%	Mouse	Shvedova et al., 2005
MWCNT	Co:0.95% Fe:~1%	Rat	Muller et al., 2005

The first study of a carbon nanotube product in Chunea pigs conducted by Huczko' et al of Warsaw University was published in 2001.[85] The results of this exploratory study showed that the intratracheal instillation of fibrous carbon nanostructures does not change pulmonary function and does not induce any measurable inflammation in bronchoalveolar space in CNTs-exposed guinea pigs. Thus, based on this research, they thought working with soot containing carbon nanotubes is unlikely to be associated with any health risk.

However, after three years, the first study of SWCNT toxicity , in which lung histopathology in exposed mice was examined,[86] was conducted to address NASA's concern that workers in occupational settings could be exposed to the airborne dust of this novel materials of unknown toxicity. In Lam, C. W's study they used three products which were made by different methods and contained different types and amounts of residual catalytic metals. Mice were intratracheally instilled with 0, 0.1, or 0.5 mg of carbon nanotubes, a carbon black negative control, or a quartz positive control, and then they were euthanized 7 days or 90 days after the single treatment for a histopathological study of the lungs. All nanotube products induced dose-dependent epithelioid granulomas and, in some cases, interstitial inflammation in the animals of the 7 days groups. These lesions persisted and were more pronounced in the 90 days groups; the lungs of some animals also revealed peribronchial inflammation and necrosis that had extended into the alveolar septa. The lungs of mice treated with carbon black were normal, whereas those treated with high-dose quartz revealed mild to moderate inflammation. These results show that, for the test conditions described here and on an equal-weight basis, if carbon nanotubes reach the lungs, they were much more toxic than carbon black and can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures.

Not only in the mouse, Warheit and his research group of Du Pont Company also conducted toxicity study in rats of a SWCNTs product made by their company.[87] In this study the lungs of rats were instilled either with 1 or 5 mg/kg of the SWCNT, quartz particles (positive control), carbonyl iron particles (negative control), phosphate-buffered saline (PBS) + 1% Tween 80, and graphite particles (lung tissue studies only). Following

exposures, results from the lung histopathology component of the study indicated that pulmonary exposures to quartz particles (5mg/kg) produced dose-dependent inflammatory responses, concomitant with foamy alveolar macrophage accumulation and lung tissue thickening at the sites of normal particle deposition. Pulmonary exposures to carbonyl iron or graphite particles produced no significant adverse effects. Pulmonary exposures to SWCNTs in rats produced a non-dose-dependent series of multifocal granulomas, which were evidence of a foreign tissue body reaction and were nonuniform in distribution and not progressive beyond 1 month post exposure. The physiological relevance of SWCNT-induced multifocal granulomas findings should ultimately be determined by conducting an inhalation toxicity study. MWCNTs have been shown to produce lung lesions similar to those observed in studies with SWCNTs. The Muller's research group tested two forms of MWCNTs, and they found that 60 days after rats were each given a single ITI does of 0.5, 2, or 5mg MWCNT, their lungs showed inflammation, granulomas, and fibrosis.[88]

The concerns about the toxicity of carbon nanotubes on mammalian studies have increased, and there is even one article showing the toxicity of SWCNT on aquatic life using rainbow trout.[89] They used a semi-static test system to expose rainbow trout to a freshwater control, solvent control, 0.1, 0.25 or 0.5 mg/L SWCNT for up to 10 days. From this study they found that SWCNTs are respiratory toxicant in trout.the fish were able to manage oxidative stress and osmoregulatory disturbances, but other cellular pathologies raised concerns about cell cycle defects, neurotoxicity, and some unidentified blood borne factors that possibly mediate systemic psthologies.

2.3.2 *In vitro toxicology studies*

Human exposure to nanoparticles in the air is a complex process. Particle sizes can range from individual particles of around 50 nm or much less in vehicle exhausts.[90] The growth of larger aggregates of particles is associated with coagulation or condensation processes in the urban air [91]. Another approach used in toxicological investigations is to study the responses of individual cell types with a view of obtaining evidence on the mechanisms of toxicity. Toxicological studies are assessing respiratory exposure of nanoparticles *in vitro.* These raise some concerns for health effects, but without measurements of manufactured nanoparticles in the atmosphere, it is difficult to precisely quantify the current risk to the general public. That is why many researchers tried the different dose and exposure time of CNT on different cells. Several *in vitro* assessments of the toxicity effects of manufactured nanomaterials have been carried out (Table 2.3).

Overall, these studies suggest that oxidative stress is one possible mechanism of toxicity. This may be also linked to inflammation responses. Shvedova *et al.*[94] showed that SWCNT cause oxidative stress and cellular toxicity to HEK. There are also results that come from skin cell culture,[94] like Monteiro-Riviere *et al.* found that MWCNT caused a time dependent release of the pro-inflammatory cytokine, Interleukin 8 (IL-8),[93] from human epidermal keratinocytes. Other toxicity effects at the cellular level include changes in cell growth and turnover. In Cui *et al*.[92] study they found the inhibition of HEK 293 cell proliferation, induced cell apoptosis, and decreased cellular adhesive ability.

Table 2.3 Examples of the toxic effects of CNTs from *in vitro* studies

Nanomaterial	Dose/Exposure time	Cells	Study
SWCNT	Serial dilutions from 0.78 to 200ug/ml	Human HEK 293 (human embryo kidney) cells	Cui *et al*. 2005 [92]
MWCNT	Serial dilutions of 0.2-0.1 mg/ml for 1, 2, 4, 8, 12, 24 and 48 hour	Human epidermal keratinocytes (HEK)	Monteiro-Riviere *et al.* 2005 [93]
SWCNT	0.06-0.24mg/ml for up to 18 hour	HEK cells in culture	Shvedova *et al.* 2003 [94]
SWCNT	0, 400,800 μg/ml For 24 hour	A549 cell	Davoren *et al.* 2007 [95]
SWCNT	30 and 60 μg/ml for 1, 24 and 48 hour	Human macrophage cells	Fiorito *et al*. 2006 [96]
SWCNT	1mg/ml for 4 days	Human epithelial-like Hela cells	Yehia *et al*. 2007 [97]

The lungs as a whole have been subjected to testing for the potential hazards of inhalation exposure to carbon nanotubes. The cytotoxicity effect of CNTs has been evaluated *in vitro* on culturing lung cells. Kisin *et al.* [98] have reported loss of viability in lung fibroblast cell line in a concentration and time dependent manner after the exposure of cells to SWCNT. The serum supplementation to the cell culture medium probably made the CNT non toxic, as demonstrated by Yehia *et al.*[97] Human epithelial-like lung cell line (A549) has been widely used in the *in vitro* cytotoxicity assessment of SWCNT. Maria Davoren's[95] study revealed that the SWCNT have low acute toxicity after exposure

of A549 cells to a wide dose range of SWCNT (1.56-800 ug/ml) for 24 hours. And in this study, they found greater SWCNT toxicity was observed in absence of serum. Casey *et al.*[99] demonstrated that the cause of cytotoxicity to the A549 cells to be due to media constituent depletion and referred to it as a case of false positive result. On the other hand, some studies showed the effect of the CNTs to A549 cells. For example , the research group in Korea found little cytotoxic effects on the proliferation and viability of A549 and other three cell lines expose to the SWCNT concentrations (<250-500ug/ml) within 48 hours. However, exposing A549 cells to high SWCNT concentrations (250-500ug/ml) for 72 hours resulted in an inflammatory response with oxidative stress and membrane damage [100]. Herzog *et al.* showed that exposing A549 cells to SWCNT samples can lead to the suppression of a variety of inflammatory mediators including IL-8, IL-6 and MCP-1 *in vitro* [101]. At the same time, researchers demonstrated that MWCNTs are able to induce inflammatory responses *in vitro*. Oxidative stress and NF-κB activation seem to play an important role in MWCNTs-induced cytokine production, which suggests that MWCNTs exert proinflammatory effects by a mechanism that is mediated by ROS and NF-κB [102].

Despite these details on effects in cell culturesand respiratory effects *in vitro*, our knowledge on CNTs toxicity is incomplete. Some results indicated CNTs to be highly toxic [77,103-105], and others showed low toxicity or no signs of toxicity at all [106-108]. In addition, many of these experiments used a "proof of principle" approach using relatively high doses to ensure a clear demonstration of toxicity effect. However, these types of experiments do not generally give data on "no effect" levels that are of interest in risk assessment. More detailed studies are needed.

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CHAPTER 3

CARBON NANOTUBE-COLLAGEN SCAFFOLDS FOR MESENCHYMAL STEM CELLS' DIFFERENTIATION: CHANGES IN ALKALINE PHOSPHATASE EXPRESSION AND MINERALIZATION

3.1 Abstract

Studies of the application of carbon nanotubes (CNTs) have been carried out for the substrates of cell culture, drug delivery systems, and medical implant materials. However, little is known about the impact of CNTs on cellular processes such as adhesion, proliferation, and differentiation. We hypothesized that augmenting the properties of naturally derived polymers (collagen, in this study) through the incorporation of CNTs might enhance osteogenic differentiation of MSCs *in vitro*. In this study, we incorporated different concentrations and types of multi-walled CNTs (MWCNTs) into reconstituted type I collagen, and evaluated proliferation, differentiation, mineralization, and inflammatory response of mesenchymal stem cells (MSCs) on these MWCNT-collagen scaffolds. MWCNTs were distributed in collagen matrix, and strongly entrapped in collagen at the concentrations below 100 ppm. Alkaline phosphatase (AP) activity and mineralized nodules of extracellular matrix (ECM) were monitored as osteogenic differentiation markers. Collagen itself enhanced MSCs differentiation and mineralization due to its nanoscale feature. MWCNTs added to collagen scaffolds induced an additional increase in MSCs differentiation and mineralization. MWCNT-collagen scaffolds induced significantly higher level of AP activity than plastic surface and increased ECM mineralization 12 days after replacement with differentiating media.

Additional increase in MSC differentiation and mineralization may be due to the increased stiffness and tensile strength of MWCNT-collagen scaffolds.

3.2 Introduction

There have been significant advances in cell-based therapy and tissue engineering for the repair and replacement of damaged tissues and organs. Nanotechnology has emerged showing great potential for the creation of the next generation of materials. Recent researches have more focused on the role of nano-structure and nano-scale materials for tissue engineering applications.[1] Carbon nanotubes (CNTs) were first introduced by Oberlin et al. in 1976[2] and their development and applications have been dramatically increasing in biosensors, tissue engineering, and biomedical devices, because of their unique electronic, chemical and mechanical properties[3-6]. Studies of the application of carbon nanomaterials have been carried for the substrate of cell culture[7-9], drug delivery systems,[10,11] and medical implant materials. The applications of CNTs have tremendous potentials for them as substrates of cell cultures, drug delivery systems, and medical implantable materials.[10-13] Nanomaterial-based scaffolds can provide many critical features of the microenvironments for cell adhesion, proliferation, and differentiation. However, little is known about the impact of CNTs on cellular processes such as adhesion, proliferation, and differentiation. Nanomaterial-based scaffolds can provide many critical features of the microenvironments for cell adhesion, proliferation, and differentiation.

A variety of new approaches and new materials have been developed for creating improved scaffolds with engineered tissues.[14,15] The advent of the field of nanotechnology has stimulated an interest in creating nanocomposite materials for a

variety of purposes, including biomedical applications. Initial studies using composites of synthetic polymers and CNTs have demonstrated promise for these materials in neural and orthopedic tissue engineering applications. The reinforcement of naturally derived polymers (e.g. ECM) with carbon nanotubes has been attempted to compensate for ECM scaffold limitations such as weak mechanical properties, lack of electrical conductivity, the absence of adhesive and microenvironment-defining moieties.[16] CNTs have diameters ranging from 0.7 to 2.0 nm (typically around 1.0 nm), with the lengths that are often hundreds or thousands of times greater than their diameter.[17] This high aspect ratio of CNTs plays an important role as in fiber-reinforced composite materials additives.[18] CNTs has similar viscoelastic property to that observed in soft-tissue membrane, so they have been used to icrease the Yong's modulus and tensile strength of the hybrid biomaterials.[16,19,20]

In this study, we used MWCNT-reinforced collagen scaffolds to investigate the osteogenic differentiation of MSCs. The overall goal is to develop scaffolds that provide enhanced mechanical and functional properties in a variety of tissue engineering applications, by augmenting the properties of naturally derived polymers through incorporation of carbon nanotubes. Our study was spurred by the following questions: 1) How CNT-based materials affect cellular processes (e.g. renewal, metabolic activity, and differentiation) of MSCs? 2) Does this addition of CNTs into naturally derived polymers result in creating stiffer environment in which osteoprogenitor cells may prefer to be differentiated into osteocytes? To address the above issues, we incorporated different concentrations and types of multi-walled CNTs (MWCNTs) into reconstituted type I collagen, and evaluated proliferation, differentiation, mineralization, and inflammatory

response of mesenchymal stem cells (MSCs) on these MWCNT-collagen scaffolds. Alkaline phosphatase (AP) activity and mineralized nodules of extracellular matrix (ECM) were monitored as osteogenic differentiation markers. Collagen itself enhanced MSCs differentiation and mineralization due to its nanoscale feature. MWCNTs added to collagen scaffolds induced an additional increase in MSCs differentiation and mineralization. MWCNT-collagen scaffolds induced significantly higher level of AP activity than plastic surface and increased ECM mineralization 12 days after replacement with differentiating media. Additional increase in MSC differentiation and mineralization may be due to the increased stiffness and tensile strength of MWCNT-collagen scaffolds.

3.3 Materials and Methods

3.3.1Materials and Reagents

HiPco derived MWCNT, MWCNT-OH and MWCNT-COOH were purchased from Cheap Tubes Inc (Brattleboro, VT). Rat tail collagen type I was purchased from BD bioscience (Rockville, MD). Alamar Blue™, Quant-iT™ PicoGreen® dsDNA detection kit, and rat interleukin 6 (IL-6) CytoSet™ was purchased from Invitrogen Corporation (Carlsbad, CA).

3.3.2 Isolation and Culture of rat MSCs

The rat femurs were removed under aseptic conditions and the connective tissue was excised. The epiphyses were removed from the femurs and the cavity was washed twice with phosphate buffered saline (PBS) solution with 1% penicillin/streptomycin to isolate bone marrow. The solution was centrifuged at 1000 rpm for 10 minutes, and the PBS was extracted. The MSCs were resuspended in Dulbecco's Modified Eagle's

Medium (DMEM) with other supplements: 10% fetal bovine serum (FBS), 5 ml of penicillin/streptomycin, and 1 ml of amfotericin. Most of MSCs were at or between osteoprogenitor and pre-osteoblast stage. The MSCs were seeded at a density of 4×10^4 cells/well on MWCNT-collagen scaffolds and incubated at 37℃ and 5% CO_2. The MSCs can be differentiated to the osteoblastic phenotype by the addition of 10 mM of β-glycerophosphate, 50 μg/ml of ascorbic acid, and 10 nM of dexamethasone.

3.3.3 Dispersion of MWCNTs

Three different types (MWCNT, MWCNT-OH, MWCNT-COOH) of MWCNTs (20 - 30 nm in diameter) with two different length (long; 10-20 m and short; 0.5 - 2.0 m) were added to distilled water and sonicated for 10 minutes with a 10 second cooling time per 1 minute sonication cycle using a Sonicator 3000 manufactured by Misonix (Farmingdale, NY). After sonication, a nontoxic surfactant polyvinylpyrrolidone (PVP) (Fisher Scientific, PA) was added to the SWCNT solution to inhibit the aggregation of MWCNTs in the solution.

3.3.4 Preparation of MWCNT-collagen scaffolds

Different concentrations (10, 50, 100, and 855 ppm) of MWCNTs was formulated into a fixed concentration of collagen type I (1 mg/ml) with 10% $NaHCO_3$. 1000 μl of MWCNT-collagen mixture were transferred to each well of 24 well plates, and cell incubated for 30mins at 37 ^oC.

3.3.5 Cell viability assay

The Alamar blue assay (Biosource International, Camarillo, CA, USA) is designed for the measurement of cell viability. It incorporates a fluorometric indicator

based on detection of metabolic activity. The oxidized form of Alamar blue (AB), which has little intrinsic fluorescence, becomes reduced in viable cells, and the reduced form of the AB is highly fluorescent. The viability of the cells can be quantified by the extent of this conversion [21]. As negative control, AB was added to the medium without cells. The plate was further incubated for 1hour at 37.0 ^{0}C. To examine cell viability, MSCs were loaded with Alamar blue at the final concentration of 10%. Under each experiment, fluorescence of Alamar blue was measured on three separate cell monolayers at an excitation wavelength of 545 nm and an emission wavelength of 590 nm by a fluorescence microplate reader (Synergy HT Multi-Mode Microplate Reader, Bio-Tek). The cell viability was calculated as the ration of fluorescence intensity in treated to untreated cells.

3.3.6 Quantification of DNA contents

The Quant-iT PicoGreen assay is a method used to quantify DNA. It is a nonspecific method that relies strictly on the total amount of DNA present rather than the presence of a specific gene. Briefly, cells were washed once with phosphate-buffered saline (PBS) after removal of culture medium andlysed in 200 ml of 25mM Tris/0.5% Triton X-100 (pH 8) solution followed by three freeze/thaw cycles. Quantification of DNA contents was conducted according the manufacturer's instructions (Molecular Probe, TX). Fluorescence intensity was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm by a fluorescence microplate reader (Synergy HT Multi-Mode Microplate Reader, Bio-Tek).

3.3.7 Alkaline phosphatase (AP) activity

The alkaline phosphatase (AP) detection is designed for the measurement of cell differentiation.Calf intestinal alkaline phosphatase (CIAP) for AP Detection was purchased from Promega (Madison, WI). The substrate (0.4mM of CSPD with Sapphire II, CD100RX) for AP was purchased from Applied Biosystems (Carlsbad, CA). Preparation method of cell lysates was same as a quantification method of DNA contents. Chemiluminescence of AP activity in each sample was detected by a chemiluminrscence microplate reader (Synergy HT Multi-Mode Microplate Reader, Bio-Tek). The light output from each sample was integrated for 10 seconds after a 2 second delay. Chemiluminescence data were calibrated with a CIAP standard curve to present AP activity.

3.3.8 Interleukin 6 expression

Levels of IL-6 were determined by specific sandwich ELISAs, according to the manufacturer's instructions. IL-8 human antibody pair and buffer kit were purchased from Invitrogen (Carlsbad, CA).

3.3.9 Von Kossa Staining

The cell cultures were washed twice with PBS, fixed in phosphate buffered formalin for 10 min, washed once with de-ionized water, and serially dehydrated in 70%, 95%, and 100% ethanol two times and dried. The cells were then rehydrated in 100%, 95%, 80% EtOH, and de-ionized water. After addition of 5% silver nitrate solution, the wells were exposed to sunlight for 20 minutes (or light for 2 h) after which the plate was rinsed with water. After addition of sodium thiosulfate (5%) for 3 minutes, the cells were then rinsed in water. Acid fuchsin counterstain (5 ml of 1% acid fuchsin + 95 ml of picric

acid + 0.25 ml of 12N HCL) was added to cell layers for 5 minutes. The cells were washed with de-ionized water, twice with 95% EtOH, twice with 100% EtOH, and dried for image analysis .The number of mineraized nodules was quantified using a 10 X objective.[22]

3.3.10 Measurement of mechanical and surface property of MWCNT-collagen scaffolds

The surface properties of MWCNT-collagen scaffolds were investigated by water contact angle (WCA) measurement. A 2 μL water droplet was dripped on the dried surface of a MWCNT-collagen scaffold. The image was captured 6 seconds after the water droplet was dripped on the surface. Contact angles (left and right) were measured by an optical contact angle meter (VCA Optima, AST products Inc.).

Compressive testing was performed on MWCNT-collagen scaffolds in wells of a 24 well plate. The height of each sample from the bottom of the well was approximately 5 mm, and the surface are of the bottom of the well was 1.9 cm^2. The diameter of the probe that was used was 10 mm. The test was performed at and extension rate of 10 mm/min until failure was reached, using an Instron 5542 apparatus (Instron, Norwood, MA). Yong modulus was determined from the elastic regime slope of stress–strain curves (<40% strain).

3.3.11 Statistical Analysis

Statistically significant differences were set $p \leq 0.05$. Statistically significant differences were set $p \leq 0.05$. Statistical analyses were carried out using one-way analyses of variance (ANOVA) followed by Dunnett's multiple comparison tests.

3.4 Results

3.4.1 Effect of concentration of MWCNTs on MSC' differentiation in MWCNT-collagen scaffolds

Different concentrations of MWCNTs were added to collagen scaffolds. MSCs were seeded onto the MWCNT-collagen scaffolds. When MSCs reach confluence, the cell culture media was replaced with differentiating media. AP activity was measured at four day interval. Collagen itself enhanced MSCs differentiation. MWCNTs added to collagen scaffolds induced an additional increase in MSCs differentiation. MWCNT-collagen scaffolds induced significantly higher level of AP activity than plastic surface 12 days after replacement with differentiating media. MWCNT-Collagen scaffold-induced AP activities were not dose dependent (Figure 3.1). Although AP levels were higher in MWCNT-scaffolds at all concentrations of MWCNTs, transparency of the scaffolds reduced as the concentrations of MWCNTs increased (Figure 3.2). Transparency of the MWCNT-collagen scaffolds was good at lower MWCNT concentrations (<100ppm of MWCNTs), but was significantly reduced at higher MWCNT concentrations (>100 ppm of MWCNTs).

3.4.2 Effects of different types of MWCNT-collagen scaffolds on MSC' differentiation

Three different types (MWCNT, MWCNT-OH, MWCNT-COOH) of MWCNTs with two different length were added to collagen scaffolds at 10 ppm of MWCNTs. When MSCs reach confluence, the cell culture media was replaced with differentiating media. AP activity was measured at four day interval. AP activity increased as the cells were differentiated on all different types of MWCNT-collagen scaffolds. The level of AP activity of MSCs was higher on all different types of MWCNT-collagen scaffolds. At the

day 12, all types of MWCNT-collagen scaffolds induced the higher level of MSC differentiation, compared to the plastic surface and collagen controls (# $p<0.05$) (Figure 3.3). However, different types of MWCNTs did not show significantly differences in AP activity on MWCNT-collagen scaffolds.

3.4.3 Effects of different types of MWCNT-collagen scaffolds on the formation of mineralized matrix nodules

Three different types (MWCNT, MWCNT-OH, MWCNT-COOH) of MWCNTs with two different length were added to collagen scaffolds at 10 ppm of MWCNTs. When MSCs reach confluence, the cell culture media was replaced with differentiating media. As the days increased, MSCs induced the increased mineralized nodules. The area of mineralized matrix nodules was significantly higher at day 15 (Figure 3.4). Especially, MSCs on both MWCNT-collagen (short) and MWCNT-OH-collagen (short) scaffolds induced significant increase in mineralization of matrix nodules (Figure 3.4).

3.4.4 Effects of MWCNT-collagen scaffolds on cell viability and cell proliferation

There was no significant change in cell viability among different types of MWCNT-collagen. Significant decrease in cell viability on MWCNT-collagen scaffolds was observed only at day 4 (Figure 3.5A). All types of MWCNT-collagen scaffolds did not induce a detrimental effect on cell proliferation (Figure 3.5B). In most cases, cell proliferation was increased during this period.

3.4.5 Effects of MWCNT-collagen scaffolds on inflammatory response

The levels of IL-6 expression did not change significantly as the days increased. However, IL-6 levels were significantly lower on all types of functionalized MWCNT-

collagen scaffolds, except MWCNT (long)-collagen scaffolds, than plastic surfaces (Figure 3.6) (# $p<0.05$).

3.4.6 Mechanical properties of MWCNT-collagen scaffolds

Contact angles of collagen scaffolds are higher than those of plastic surface, but they are not significantly different. There was no significant difference in contact angles among different types of MWCNT-collagen scaffolds (Figure 3.7). The elastic modulus increases with the addition of MWCNTs to the collagen gels. The stiffness was varied as we increased the concentrations of MWCNTs in collagen scaffolds. 50 ppm of MWCNTs in MWCNT-collagen scaffolds showed significantly higher stiffness than others (Figure 3.8).

3.5 Discussion

All cells or tissues are exposed to the specific microenvironments that are generated locally at the micro- or nano-scale level by cell–cell, cell-soluble factor, or cell–extracellular matrix interactions and that influences cell functions in their tissues. Especially, each cell type is specifically tuned to the specific microenvironments in which it resides. For example, the brain tissues are softer than bone tissues. Consequently, neural cell growth, survival and differentiation are favored by a highly compliant matrix. By contrast, osteoblast differentiation and survival occurs preferentially on stiffer extracellular matrices with material properties more similar to newly formed bone [23]. ECM or ECM-like structure can create cellular microenvironment for specific cells or tissues. In addition to creating cellular microenvironment using ECMs, nanomaterials can be used to compensate for ECM scaffold limitations such as weak mechanical properties, lack of electrical conductivity, and the absence of microenvironment-defining moieties.[16]

We hypothesized that augmenting the properties of naturally derived polymers (collagen, in this study) through incorporation of MWCNTs might enhance *in vitro* osteogenic differentiation and mineralization of MSCs.

In our study, MWCNTs were distributed in collagen matrix, and strongly entrapped in collagen (1 mg/ml) at the concentrations below 100 ppm. At these concentrations (0 - 100ppm), MWCNT-collagen scaffolds showed excellent transparency, which is appropriate for cell culture and microscopic observation (Figure 3.1). MSCs were used to mimic the subsequent stages of osteoblastic differentiation *in vitro*. In the initial phases of differentiation process, the undifferentiated MSCs became committed and started to proliferate to osteoprogenitors and pre-osteoblasts subsequently. After we changed with the differentiating media after 4 days culture in 48well plate on those scaffolds, pre-osteoblasts were matured into osteoblasts. During this process, matrix was matured, AP (Figures 3.1 and 3.3) and collagen I were released. Subsequently, cells started to mature the extracellular matrix and mineralized matrix nodules appeared (Figure 3.4). Higher level of AP was released from MSCs on MWCNT-collagen scaffolds and collagen than plastic surface (Figure 3.1). The levels of AP were higher on all different types of MWCNT-collagen scaffolds than collagen and plastic surface (Figure 3.3). In addition, MSCs on both MWCNT-collagens (short) and MWCNT-OH-collagen (short) scaffolds induced significant increase in mineralization of matrix nodules (Figure 3.4).

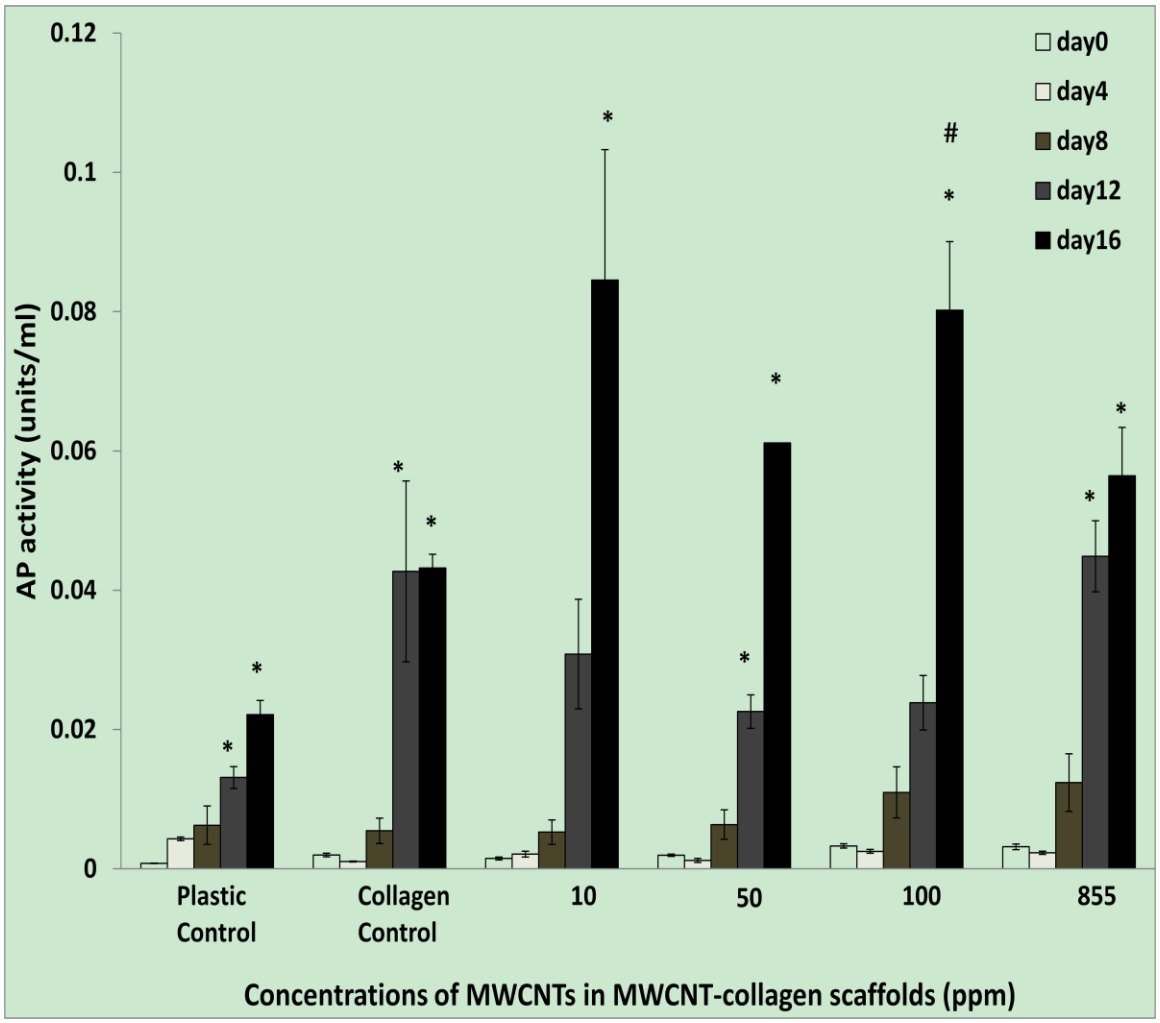


Figure 3.1 Effect of concentration of MWCNTs on MSCs' differentiation in MWCNT-collagen scaffolds. Different concentrations of MWCNTs were added to collagen scaffolds. MSCs were seeded onto the MWCNT-collagen scaffolds. When MSCs reach confluence, the cell culture media was replaced with differentiating media. AP activity was measured at four day interval. * denotes a significantly higher difference than the control (0 hour) ($p<0.05$). # denotes significantly higher difference than both plastic and collagen control at the same time point ($p<0.05$).

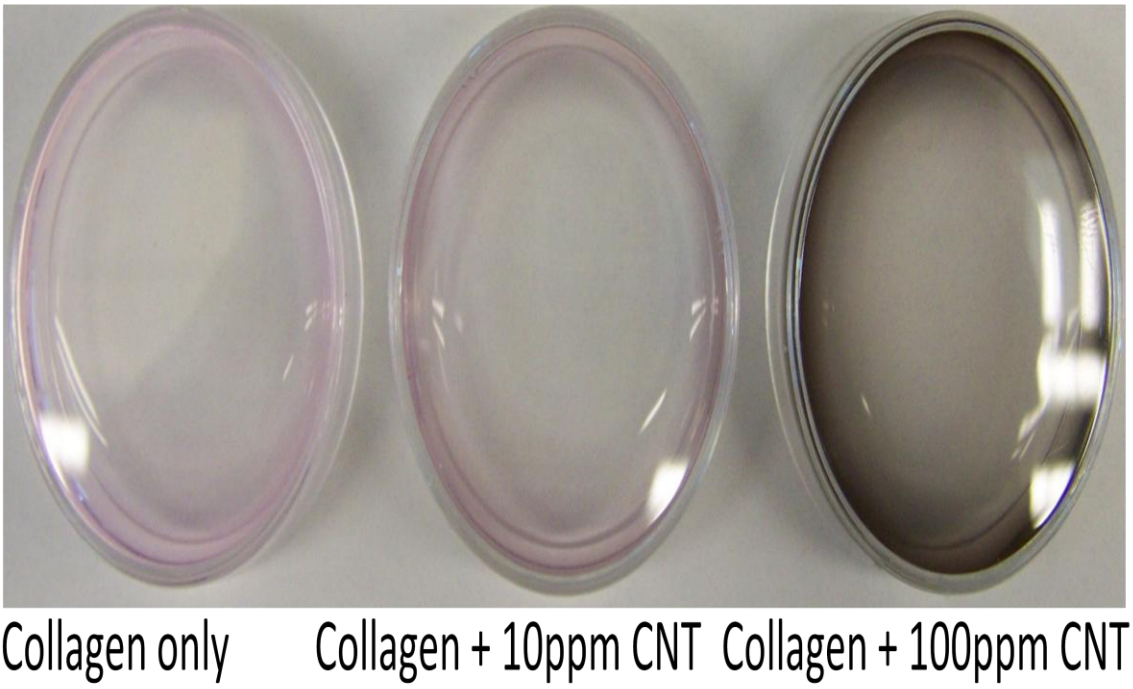


Figure 3.2 Transparency of collagen, collagen0 MWCNT composite materials coated dishes. This shows a comparison of the color and the transparency of the collagen-coated dish and the MWCNT-collagen coated dish. The MWCNT-collagen coated dish looked slightly gray but had good transparency.

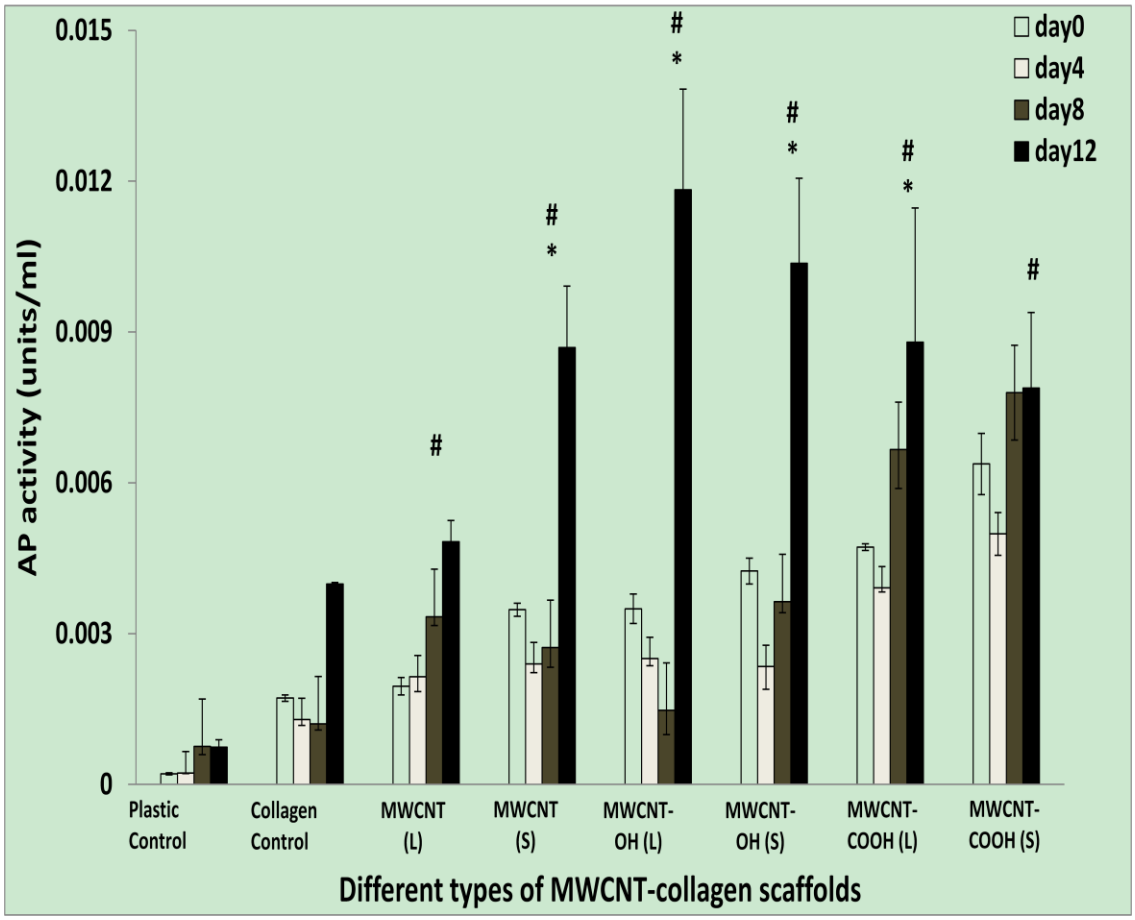
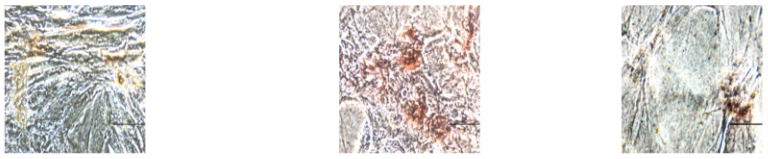


Figure 3.3 AP activity of MSC cultured on the different types of MWCNT-collagen scaffolds at 0, 4, 8, and 12 days. * denotes a significantly higher difference than control (day 0) ($p<0.05$). # denotes a significantly higher difference than the plastic and collagen control at the same time point ($p<0.05$). MWCNT (L): MWCNT-collagen scaffold (long). MWCNT (S): MWCNT -collagen scaffold (short). All types of MWCNT concentration in scaffolds were 10ppm.



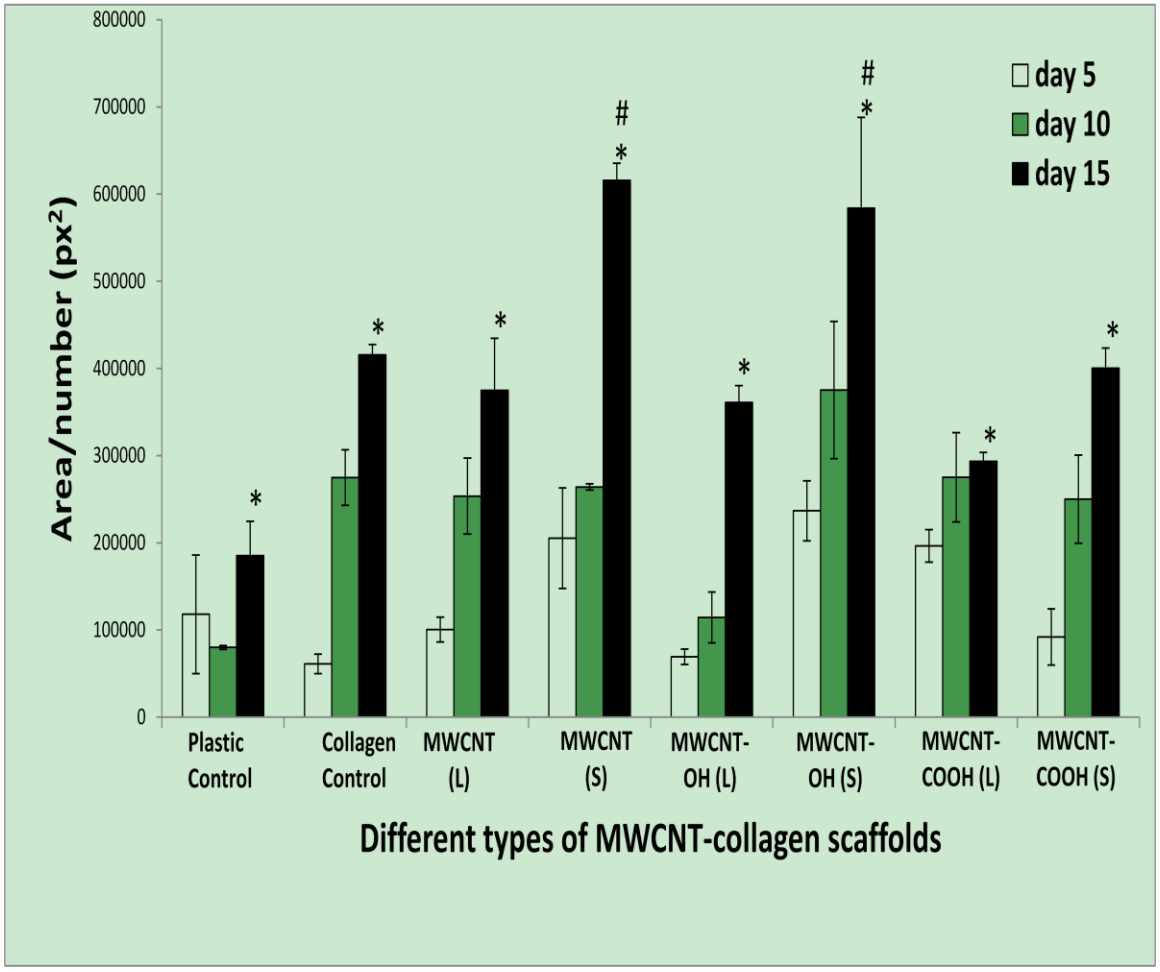
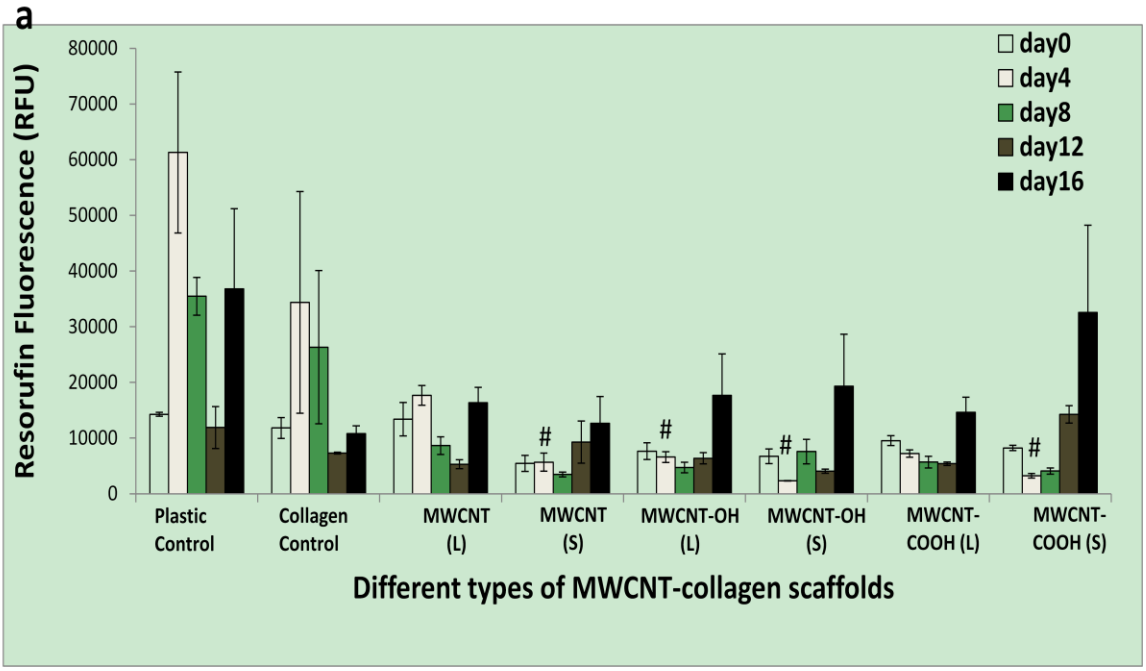


Figure 3.4 Von Kossa staining of MSC cultured on different cell culture matrixes. Quantification of nodular von Kossa staining using image analysis in the same experiment. * denotes a significantly higher difference than day 5 control ($p<0.05$). # denotes a significantly higher difference than the same time point plastic scaffolds control ($p<0.05$). All types of MWCNT concentration in scaffolds were 10ppm.



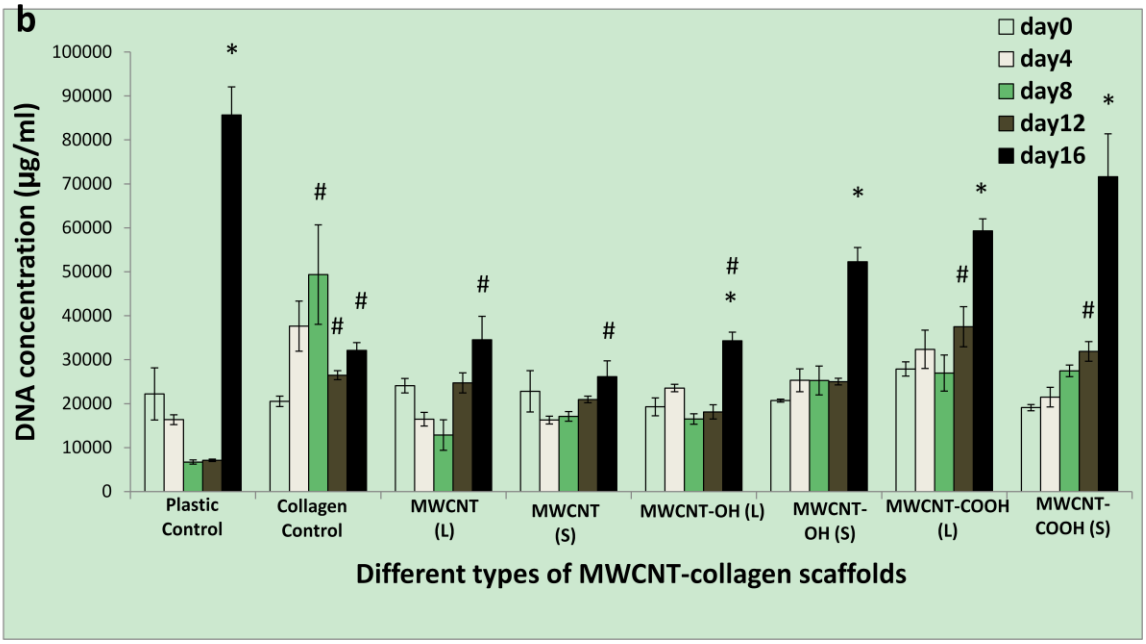


Figure 3.5 Effects of MWCNTs on cell viability and cell proliferation on MWCNT-collagen scaffolds. Three different types (MWCNT, MWCNT-OH,

Figure 3.5 (continued)

MWCNT-COOH) of MWCNTs (20 - 30 nm in diameter) with two different length (long; 10-20 nm and short; 0.5 - 2.0 nm) were added to collagen scaffolds at 10 ppm of MWCNTs. When MSCs reach confluence, the cell culture media was replaced with differentiating media. Cell viability and cell proliferation were measured after replacement of differentiating media. * denotes a significantly higher difference than the control (day 0) ($p < 0.05$). # denotes a significantly higher difference than the plastic control at the same time point ($p<0.05$).

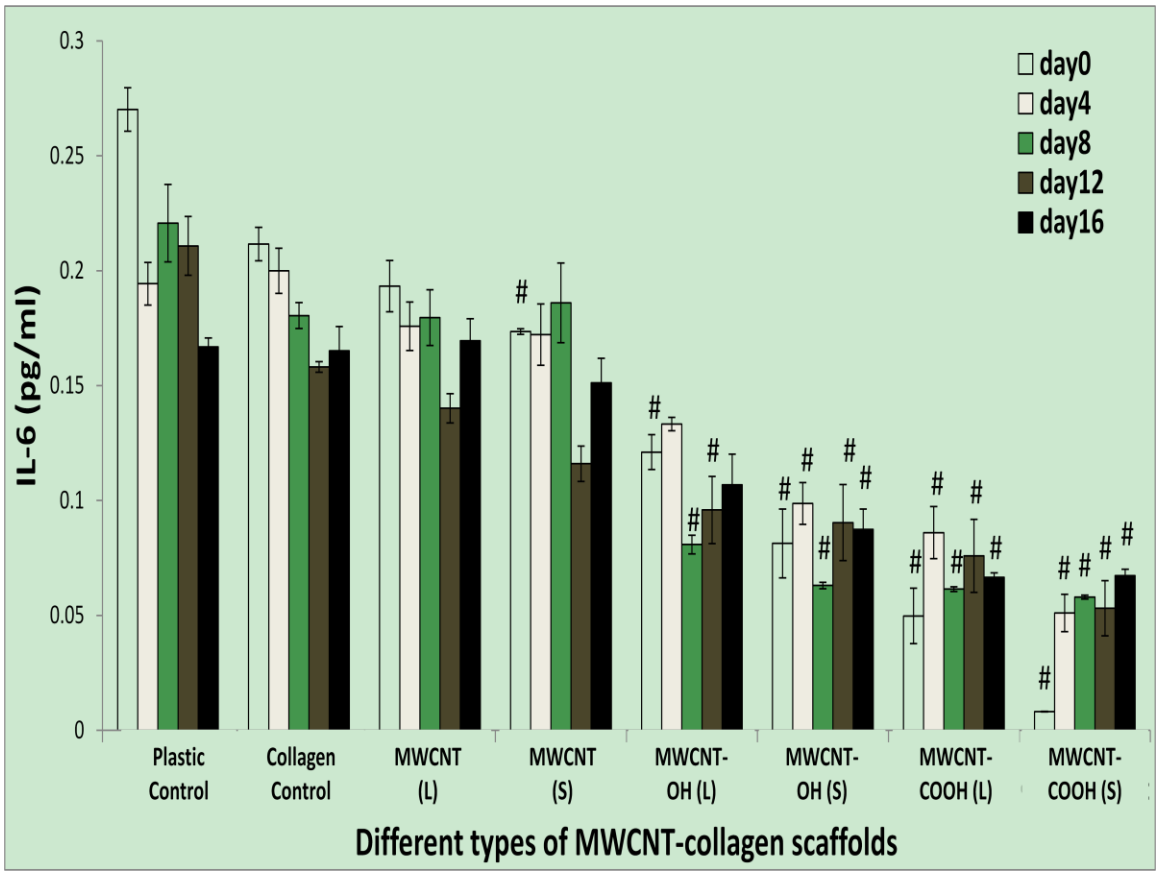


Figure 3.6 IL-6 expressions from MSC on different types of MWCNT-collagen scaffolds. Effects of MWCNT-collagen scaffolds on inflammatory response. Three different types (MWCNT, MWCNT-OH, MWCNT-COOH) of MWCNTs (20 - 30 nm in diameter) with two different length (long; 10-20 nm and short; 0.5 - 2.0 nm) were added to collagen scaffolds at 10 ppm of MWCNTs. When MSCs reach confluence, the cell culture media was replaced with differentiating media. IL-6 expresion was measured at four day interval after replacement of differentiating media. # denotes a significantly lower difference than the plastic control at the same time point ($p<0.05$).

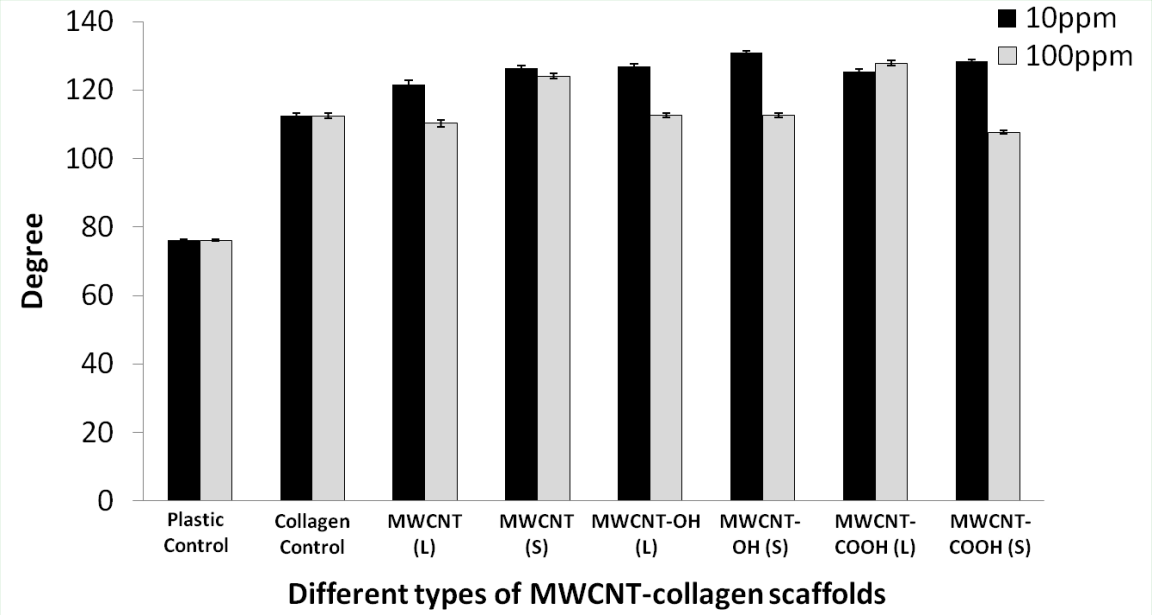


Figure 3.7 Contact angle measurements on various MWCNT-collagen scaffolds. The water ability of the surface was investigated by water contact angle (WCA) measurement. The testing was measured by using a 2 μL water droplet on the surfaces under ambient conditions with an optical contact angle meter. The image was captured at 6 s after the water droplet was dripped on the surface.

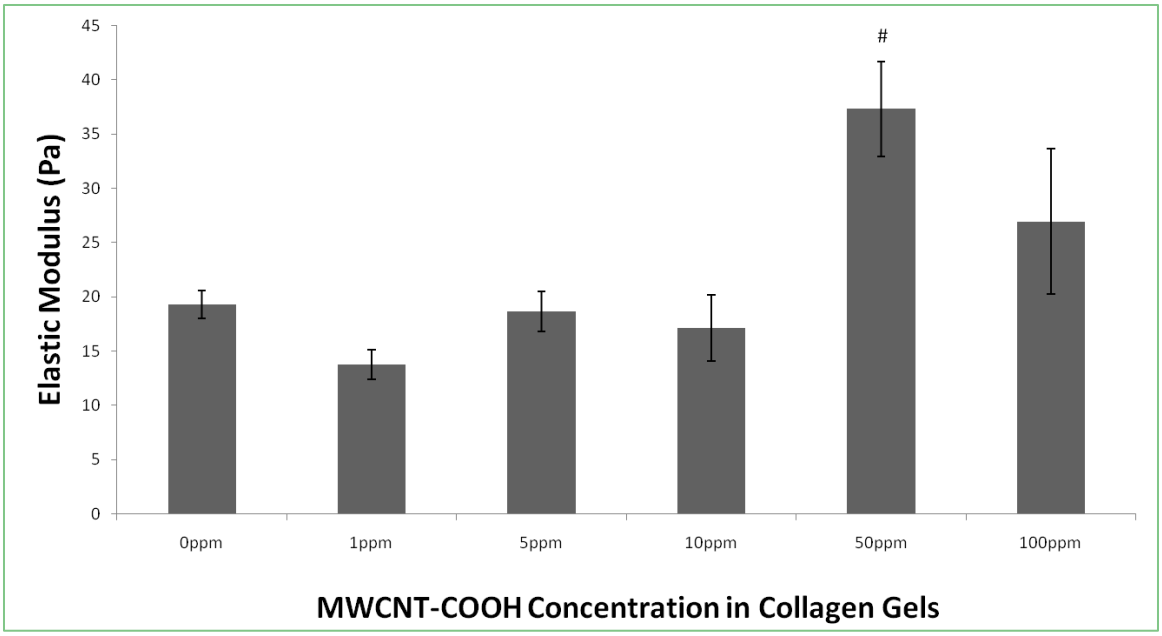


Figure 3.8 Measurements of elastic modulus in collagen gels with varying MWCNT-COOH concentration. Compressive testing was performed on MWCNT-collagen scaffolds (191mm^2) at 10 mm/min until failure was reached, using an Instron 5542 apparatus (Instron, Norwood, MA). Yong modulus was determined from the elastic regime slope of stress–strain curves (<40% strain). # denotes a significant difference in elastic modulus from the pure collagen gel.

Structurally, collagen provides tensile strength to tissues via its hierarchical assembly of collagen subunits. However, in addition to biologic signaling and macroscopic mechanical properties, collagen also possesses nano-scaled features that are mediators of cell activity. Previous studies have shown that changes made to certain features, such as structural curvature of the collagen fibrils, can regulate the activities of adherent cells. Nano-structure and nano-scale materials play important roles in tissue

engineering applications. CNTs have been used to modulate cell behavior through their electrical conductivity, and mechanically to reinforce or tailor the structural properties of tissue engineered scaffolds. In addition, CNTs have been used to increase the surface roughness and surface area of scaffolds for cell adhesion.[1] In this study, collagen itself enhanced MSCs differentiation and mineralization due to its nano-scaled feature. MWCNTs added to collagen scaffolds induced an additional increase in MSCs differentiation and mineralization. Additional increase in MSC differentiation and mineralization may be due to the increased stiffness and tensile strength of MWCNT-collagen scaffolds (Figure 3.8). However, it is still not clear that surface properties of MWCNT-collagen scaffolds affect MSC differentiation and mineralization in this study. Collagen scaffolds with functionalized MWCNTs (-COOH and -OH) induced significantly lower level of IL-6 than other scaffolds and plastic surface. Additional surface analysis and different methods for incorporating nanomaterials will be required to verify whether surface properties of nanocomposite scaffolds increase or decrease inflammatory response as well as cell differentiation. Future strategies could involve incorporating nanoscale materials inside or on the outer surfaces of 3D scaffolds to investigate mechanical property-dependent changes in cellular processes. A more complete and fundamental understanding of cellular micro-biomechanics and its relationship to MSC proliferation and differentiation will be a critical element for the development of functional tissues *in vitro*.

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CHAPTER 4

EFFECT OF EXPOSURE CONDITIONS ON SWCNT-INDUCED INFLAMMATORY RESPONSE IN HUMAN ALVEOLAR EPITHELIAL CELLS

4.1 Abstract

There has been an increasing interest in the development and applications of carbon nanotubes (CNTs) due to their huge potential in industrial and medical applications, but the toxicological properties of these materials have not been well characterized, especially the effects of nanoparticle exposure under different conditions on cellular responses. Nano-structured particles are potentially hazardous when they deposit in the respiratory system. In this study, we characterized the effects of single walled CNT (SWCNT) exposure on interleukin-8 (IL-8) expression in human alveolar epithelial cells (A549) under various exposure conditions. We measured the level of IL-8 expression in the presence and absence of serum following exposure of SWCNTs. The results demonstrated that IL-8 expression was enhanced in the presence of serum. When A549 cells were exposed to low concentrations of SWCNTs, IL-8 expression kept increasing, even after removal of SWCNTs from the media. In addition, SWCNT exposure under dynamic cell growth condition induced changes in IL-8 expression.

4.2 Introduction

Nanotechnology has the tremendous potential to improve the effectiveness of a number of industrial products and could have a substantial impact on the development of new products in all sectors, ranging from disease diagnosis and treatments to environmental remediation [1,2]. Nanotechnology is anticipated to become the strategic and dominating science and engineering field of the 21st century. Based on this expectation,

the U.S. President and the National Science and Technology Council to promote nanotechnology and to predict that it would lead the United States to the next industrial revolution.[3] Since the discovery of the carbon nanotubes (CNTs) in 1991,[4] there has been an increasing interest in these materials because of their huge potential in industrial and medical applications. CNTs have been widely used in polymer nanocomposites,[5] biomedical applications,[6-9] and electronics[10] due to their physical, chemical, thermal and optical properties.[11] Consequently, the size of the global market for CNTs has dramatically increased.[12] However, any potentially adverse effects of CNTs have not been well characterized, especially in the lungs and skin, which are regarded as the most likely exposure sites during manufacture and material handling.[13-19] Possible exposure conditions of CNTs and their adverse effects will vary among application types and environments.

Animal studies with engineered nanoparticles have shown some adverse effects on the lung (e.g., pulmonary inflammation and progressive fibrosis)[3,20,21] and cardiovascular systems (e.g., inflammation, blood platelet activation, plaque formation, and thrombosis).[22-24] Other studies have suggested that discrete nanoparticles may enter the bloodstream from the lungs and translocate to other organs such as the brain, through the olfactory nerve.[16,25,26] *In vitro* studies demonstrated that highly purified single walled carbon nanotubes (SWCNTs) induced the release of inflammatory makers such as nitric oxide and Interleukin-8 (IL-8).[21,27-34] Casey and co-workers also demonstrated the possible effect of media components on SECNT-induced cellular responses.[35] The interactions among SWCNTs, media components, and cells may affect cell viability, cellular inflammatory responses, and other toxicological responses. However, the

mechanisms of these interactions have not been well characterized. In addition, most cases of *in vivo* and *in vitro* studies involved high concentrations of SWCNTs (50~ 800 μg/ml) and extended exposure times (>24 hours).[31,33,36,37]

This study was spurred by the following fundamental questions: 1) What other environmental factors may affect the nanoparticle-related inflammatory responses or lead to cellular toxicity? 2) Are these additional factors related to the translocation of nanoparticles? 3) Is the nanoparticle-induced inflammatory response alleviated by removing nanoparticles following exposure? In this study, we monitored the SWCNT-induced IL-8 expression as an inflammatory markers under different exposure conditions, including concentrations, exposure times, and the presence of serum. We also measured the SWCNT-induced IL-8 expression in static and dynamic cell growth conditions. SWCNT-induced IL-8 expression was enhanced in the presence of serum and kept increasing after removal of SWCNTs, especially following exposure to low concentrations of SWCNTs. In addition, cellular exposure to SWCNTs in a dynamic cell growth condition changed IL-8 expression and cell proliferation.

4.3 Materials and Methods

4.3.1 Materials and Reagents

HiPco derived SWCNTs were purchased from Cheap Tubes Inc. (Brattleboro, VT). The Pierce bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Fisher Scientific (Rockford, IL). The Human IL-8 CytoSetTM was obtained from Invitrogen Corporation (Carlsbad, CA).

4.3.2 Cell Culture

A549 (human alveolar epithelial cells), a human lung adenocarcinoma with the alveolar type II phenotype, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in T-75 culture flasks in a humidified incubator at 37^{o}C with 5% CO_2 and 95% air, and grown to confluence for 2-3 days in F-12K medium (Thermo Fisher Scientific, Rockford, IL). Media was supplemented with penicillin/streptomycin (Invitrogen, CA) and 10% of fetal bovine serum (Hyclone, UT). Cells were sub-cultured using trypsin-EDTA (Invitrogen, CA) upon reaching 80-90% confluence.

4.3.3 Experimental Design

In order to investigate the effect of serum on SWCNT-induced IL-8 expression and cell proliferation, A549 cells were cultured in 24-well plates at a density of 4×10^{4} cells/well. Cells were incubated until they reached more than 90% confluence and were then exposed SWCNTs. SWCNTs stock suspensions were made in both the serum free and serum containing media. Cells were exposed to two different concentrations (4 μg/ml and 8 μg/ml) of SWCNTs for 0, 1, 3, 6 and 24 hours (Figure 4.1). Cell and media samples were collected and immediately stored at -80^{o}C until analysis.

In order to study the effects of exposure time on SWCNT-induced IL-8 expression and cell proliferation, A549 cells were prepared and incubated in the same manner described above, but were exposed to six different concentrations (2, 4, 8, 20, 40, and 80 μg/ml) of SWCNTs in the serum containing media for 0, 1, 3, 6 and 24 hours (Figure 4.2). Cell and media samples were collected and immediately stored at -80^{o}C until analysis.

To study the effects of exposure and recovery time on SWCNT-induced IL-8 expression and cell proliferation, A549 cells were prepared and incubated in the same manner described above. Cells were exposed to two different concentrations (4 and 40μg/ml of SWCNTs in serum containing media for 6 (Figure 4.3) and 24 hours (Figure 4.4). Following exposure to SWCNTs, the culture media containing SWCNTs was replaced with the fresh SWCNT-free media. Cells grew in the fresh media up to 96 hours. Cell and media samples were collected and immediately stored at -80^{o}C until analysis.

For experiments implementing a dynamic cell growth environment during SWCNT exposure, A549 cells were seeded at 3×10^{5} cells (per well) into six well BioFlex plates (Flexcell International, PA) containing 2 ml of complete F-12k medium. After cells reached confluence, they were exposed to SWCNT**s** at different concentrations (5, 10, and 20 μg/ml) under static or dynamic conditions for 24 hours. The dynamic cell growth condition was implemented using Flexcell® Tension Plus™ 4000T system, which used vacuum pressure to apply cyclic strain to cells cultured on BioFlex plates. During the course of dynamic cell growth, cyclic stretching was applied to silastic well-bottoms of BioFlex plates to attain 5% surface elongation at the frequency of 0.2 Hz, which corresponds to 45% of the total lung capacity, similar to the normal breathing conditions in the lung (Tschumperlin and Margulies, 1998). Directly after 24 hour exposure, both media and cell lysate samples were collected and stored at -80 ℃ until analysis.

4.3.4 Preparation of SWCNTs

The residual metal content of the SWCNTs used in this study was 3% - 12% by weight, and the individual size ranges of SWCNTs were 0.8-1.2nm in diameter and 100-1000nm in length. The SWCNTs were added to distilled water and sonicated for 10

minutes with 10 second cooling time per 1 minute sonication cycle using Sonicator 3000 manufactured by Misonix (Farmingdale, NY). After sonication, a nontoxic surfactant polyvinylpyrrolidone (PVP) (Fisher Scientific, PA) was added to the SWCNT solution to inhibit the aggregation of SWCNTs in the solution. Stock suspensions of SWCNTs were added to cell culture media (F-12K) with or without 10% of fetal bovine serum.

4.3.5 Cell Proliferation Assay

Cells grown in each well were lysed using 100 μL of triton X-100 following each exposure condition and stored at -80 ℃ until analysis. Total protein concentrations from each sample were measured using a BCA total protein assay (Pierce, IL) to evaluate cell proliferation.

4.3.6 Interleukin 8 Expression

Levels of IL-8 were determined from the supernatant of A549 cells by specific sandwich ELISAs, according to the manufacturer's instructions. IL-8 human antibody pair and buffer kit were purchased from Invitrogen (Carlsbad, CA).

4.3.7 Statistical Analysis

Statistically significant differences were set $p \leq 0.05$. Statistical analyses were carried out using one-way analyses of variance (ANOVA) followed by Dunnett's multiple comparison tests.

4.4 Results

4.4.1 Effects of SWCNTs on Cell Proliferation and IL-8 Expression in Serum-containing and Serum-free media

As the exposure time increased, IL-8 expression dramatically increased following cellular exposure to SWCNTs in serum-containing media. The level of IL-8 expression in serum-containing media was significantly higher than the control (1 hour exposure) after 24 hours of exposure at both concentrations (4 μg/ml and 8 μg/ml) (Figure 4.1A). Cell proliferation was not significantly diffrent as exposure time increased at both concentrations (4 μg/ml and 8 μg/ml). However, cell proliferation was higher in serum-containing media than in serum-free media at 4 μg/ml of SWCNTs (Figure 4.1B).

4.4.2 Effects of Exposure Time on Cell Proliferation and IL-8 Expression at Different Concentrations of SWCNTs

Cells were exposed to six different concentrations ((2, 4, 8, 20, 40, 80 μg/ml)) of SWCNTs. IL-8 expression significantly increased (Figure 4.2A) as the exposure time increased over 24 hours at all concentrations of SWCNTs. However, no significant changes in cell proliferation were observed at most concentrations of SWCNTs during 24 hour exposure.

4.4.3 Effects of Recovery Time on SWCNT-induced IL-8 Expression and Cell Proliferation

Following exposure to SWCNTs at a lower concentration (4 μg/ml) for 6 hours, the media was replaced with fresh SWCNT-free media. 72 and 96 hours after removing the SWCNTs, L-8 levels were significantly higher than the levels seen in the control grown for the same period of time (Figure 4.3A). Protein concentrations were significantly higher than the 0 hour control, 48 and 96 hour after removing SWCNTs

(Figure 4.3B). However, following exposure to SWCNTs at a higher concentration (40 μg/ml) for 6 hours, IL-8 expression stopped increasing after removal of SWCNTs (Figure 4.3A). No significant change in cell proliferation was observed in this case (Figure 4.3B). At a longer exposure time (24 hours), the levels of IL-8 and cell proliferation were different. Following exposure to SWCNTs at both concentrations (4 and 40 μg/ml) for 24 hours, IL-8 expression significantly increased after removal of SWCNTs (Figure 4.4A), but cell proliferation significantly decreased at both concentrations (4 and 40 μg/ml) even after removal of SWCNTs (Figure 4.4B).

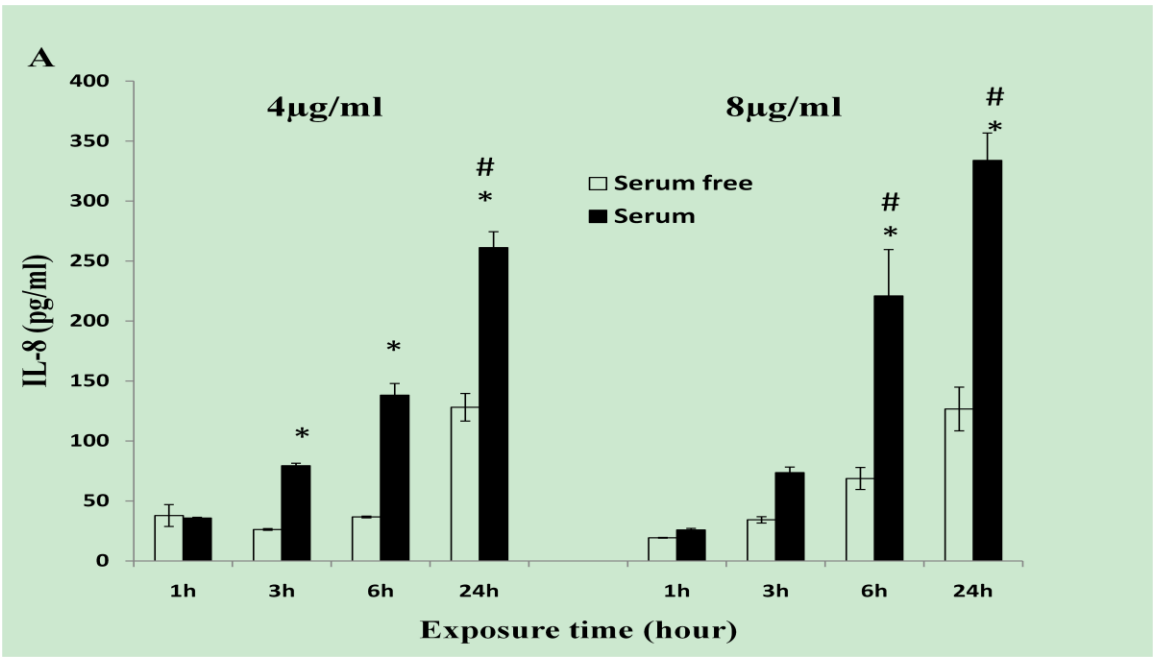
4.4.4 Effects of Dynamic Environment on SWCNT-induced IL-8 Expression and Cell Proliferation

To demonstrate the effects of dynamic cell growth conditions on the SWCNT-induced IL-8 expression and cell proliferation, cells were grown in a dynamic cell growth condition while being exposed to SWCNT at 5, 10, and 20 μg/ml for 24 hours (Figure 4.5). Following exposure to SWCNTs for 24 hours, A549 cells grown in the dynamic cell growth condition showed a significant increase in IL-8 level at all exposure concentrations as compared to cells grown in the static condition. Under the dynamic cell growth condition, the IL-8 levels at all SWCNT concentrations (5, 10, and 20 μg/ml) were significantly higher than the control (0 μg/ml after 24 hour cyclic stretching). Under static cell growth condition, IL-8 level was significantly higher at 20 μg/ml of SWCNTs than the control (0 μg/ml under static condition) (Figure 4.5A). Dynamic cell growth conditions also altered cell proliferation during the exposure of cells to SWCNTs. After 24 hours of cyclic stretching in the dynamic growth environment, cells demonstrated increased proliferation regardless of whether SWCNT was present in their media, however, cells grown under static conditions did not demonstrate the same increase.

Following cyclic stretching for 24 hours, A549 cell proliferation was enhanced either in the absence (0 μg/ml) or presence (20 μg/ml) of SWCNTs (Figure 4.5B). However, cell proliferation was not significantly changed following exposure to SWCNTs under static cell growth condition. There was no significant difference in cell proliferation between static and dynamic cell growth conditions at SWCNTs concentrations of 5 and 10 μg/ml of. Cell proliferation at SWCNT concentration of 10 μg/ml of was significantly lower than the control (0 μg/ml after 24 hour cyclic stretching) under dynamic cell growth conditions (Figure 4.5B).

4.5 Discussion

In this study, we evaluated the pro-inflammatory effect of SWCNTs on A549 cells under several different exposure conditions. SWCNT-induced IL-8 expression was enhanced in the presence of serum. IL-8 expression was induced by SWCNTs and kept increasing even after removing SWCNTs; especially following exposure to lower concentrations of SWCNTs. SWCNT exposure under dynamic cell growth conditions induced changes in IL-8 expression and cell proliferation. However, we did not observe any significant change in IL-8 expression with increasing concentration, although we observed significant change in the levels of another inflammatory marker (i.e. nitric oxide (NO) in both A549 and primary normal human bronchial epithelial (NHBE) cells in a previous publication [33]. Interestingly, Herzog and co-workers showed that SWCNT suppressed inflammatory responses (IL-8 and IL-6 expression) in A549 cells [31]. In the case of nanomaterials, the uncertainties are great because the characteristics of nanoparticles may be different from those of larger particles with the same chemical composition.



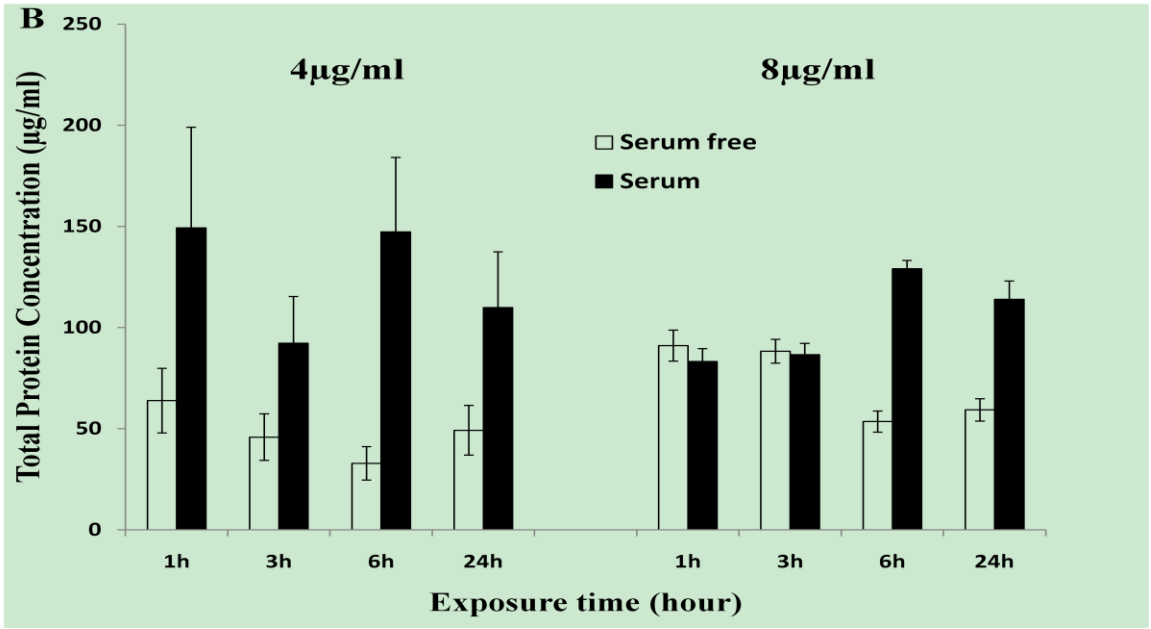


Figure 4.1 Effects of SWCNTs on cell proliferation and IL-8 expression in serum-containing and serum-free media. IL-8 was measured by ELISA (Figure

Figure 4.1 (continued)

4.1A) and total protein concentration was determined by the BCA protein assay (Figure 4.1B), following exposure of SWCNTs at 4 μg/ml and 8 μg/ml either in the presence (10% of serum in the media) or absence of serum. * denotes a significant difference between data in the serum containing and serum free media ($p<0.05$). # denotes a significantly higher difference than the 1 hour exposure control ($p<0.05$).

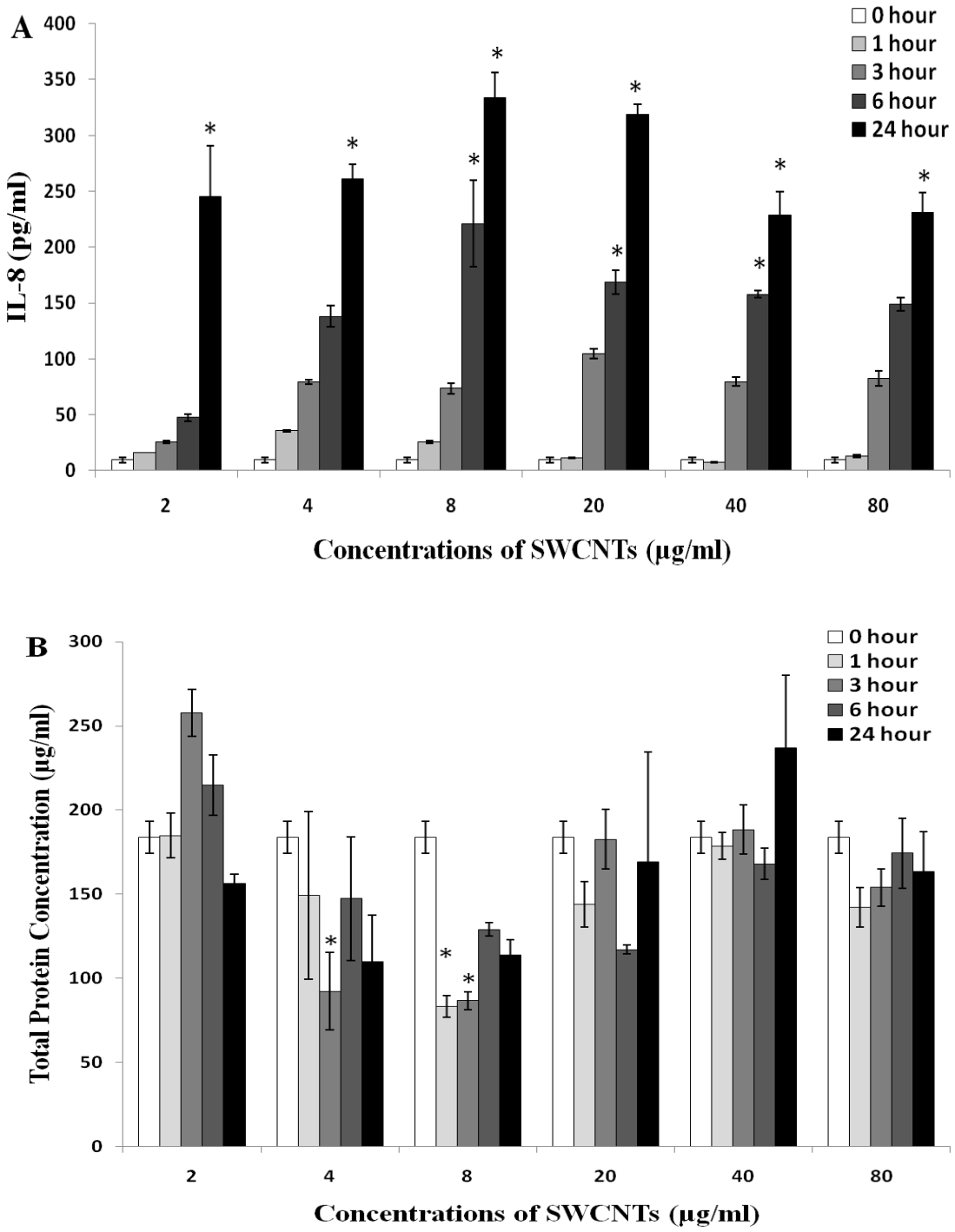


Figure 4.2 Effects of exposure time on cell proliferation and IL-8 expression at different concentrations of SWCNTs. A549 cells were exposed to SWCNTs

Figure 4.2 (continued)

at six different concentrations, IL-8 and cell proliferation were measured as exposure time increased. IL-8 was measured by ELISA (Figure 4.2A) and total protein concentration was determined by the BCA protein assay (Figure 4.2B). 0 hour were used as control. * denotes a significantly higher (or lower) difference than the control (0 hour) ($p < 0.05$).

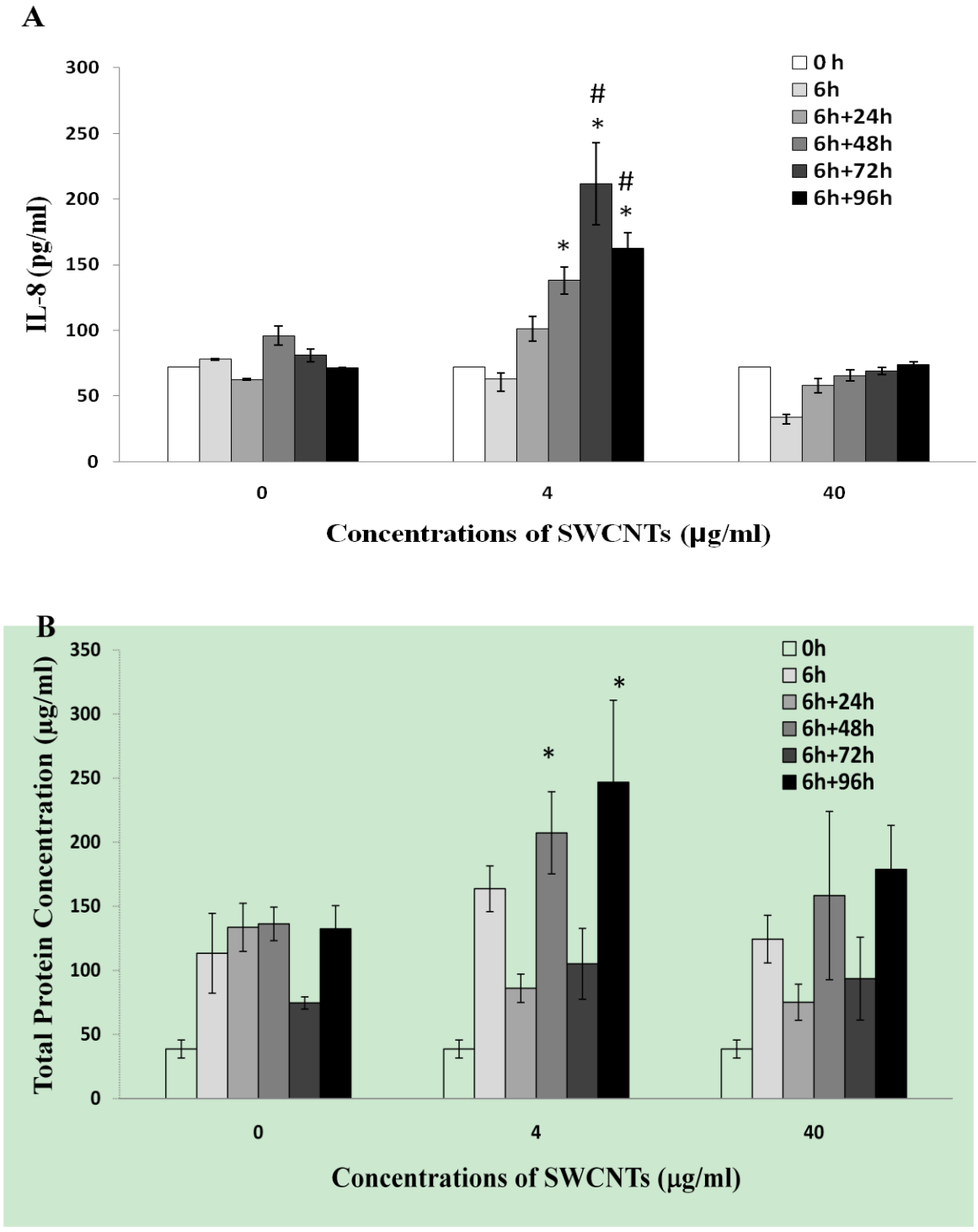


Figure 4.3 Effects of recovery time on SWCNT-induced IL-8 expression. A549 cells were exposed to SWCNTs at low and high concentrations for 6 hours,

Figure 4.3 (continued)

media was then replaced with SWCNT-free media. IL-8 and cell proliferation were consistently measured at 0, 24, 48, 96 hour after removal of SWCNTs. IL-8 was measured by ELISA (Figure 4.3A) and total protein concentration was determined by the BCA protein assay (Figure 4.3B). Untreated samples and 0 hour were used as controls. * denotes a significantly higher difference than the control (0 hour) ($p < 0.05$). # denotes a significantly higher difference than the same time point control at 0 μg/ml of SWCNTs ($p < 0.05$).

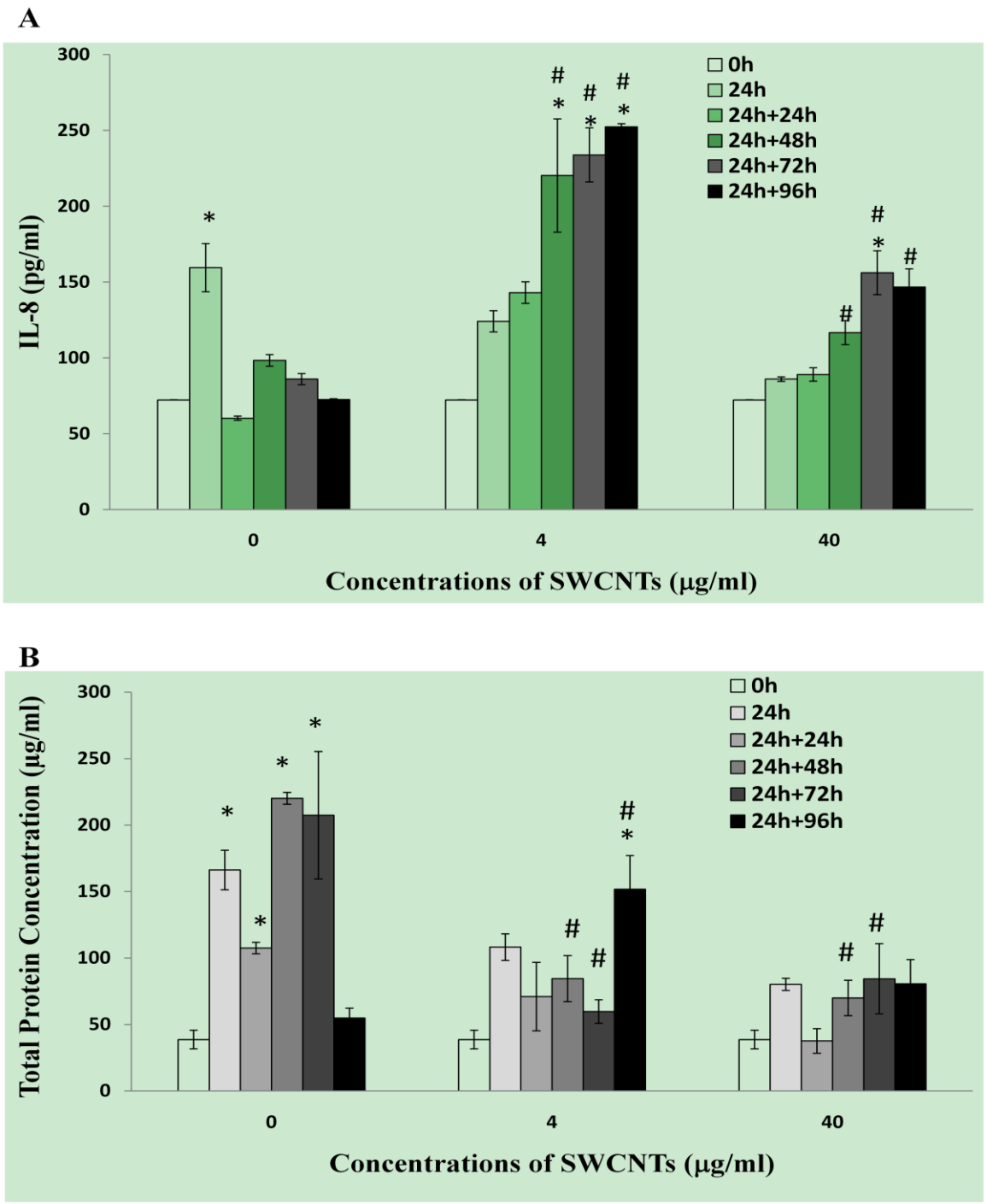


Figure 4.4 Effects of recovery time on SWCNT-induced IL-8 expression. A549 cells were exposed to SWCNTs at low and high concentrations for 24 hours, media was then replaced with SWCNT-free media.

Figure 4.4 (continued)

IL-8 and cell proliferation were consistently measured at 0, 24, 48, 96 hour after removal of SWCNTs. IL-8 was measured by ELISA (Figure 4.4A) and total protein concentration was determined by the BCA protein assay (Figure 4.4B). Untreated (0 μg/ml of SWCNTs) and 0 hour samples were used as controls. *denotes a significantly higher difference than the control (0 hour) ($p < 0.05$). # denotes a significantly higher difference than the same time point control at 0 μg/ml of SWCNTs ($p < 0.05$).

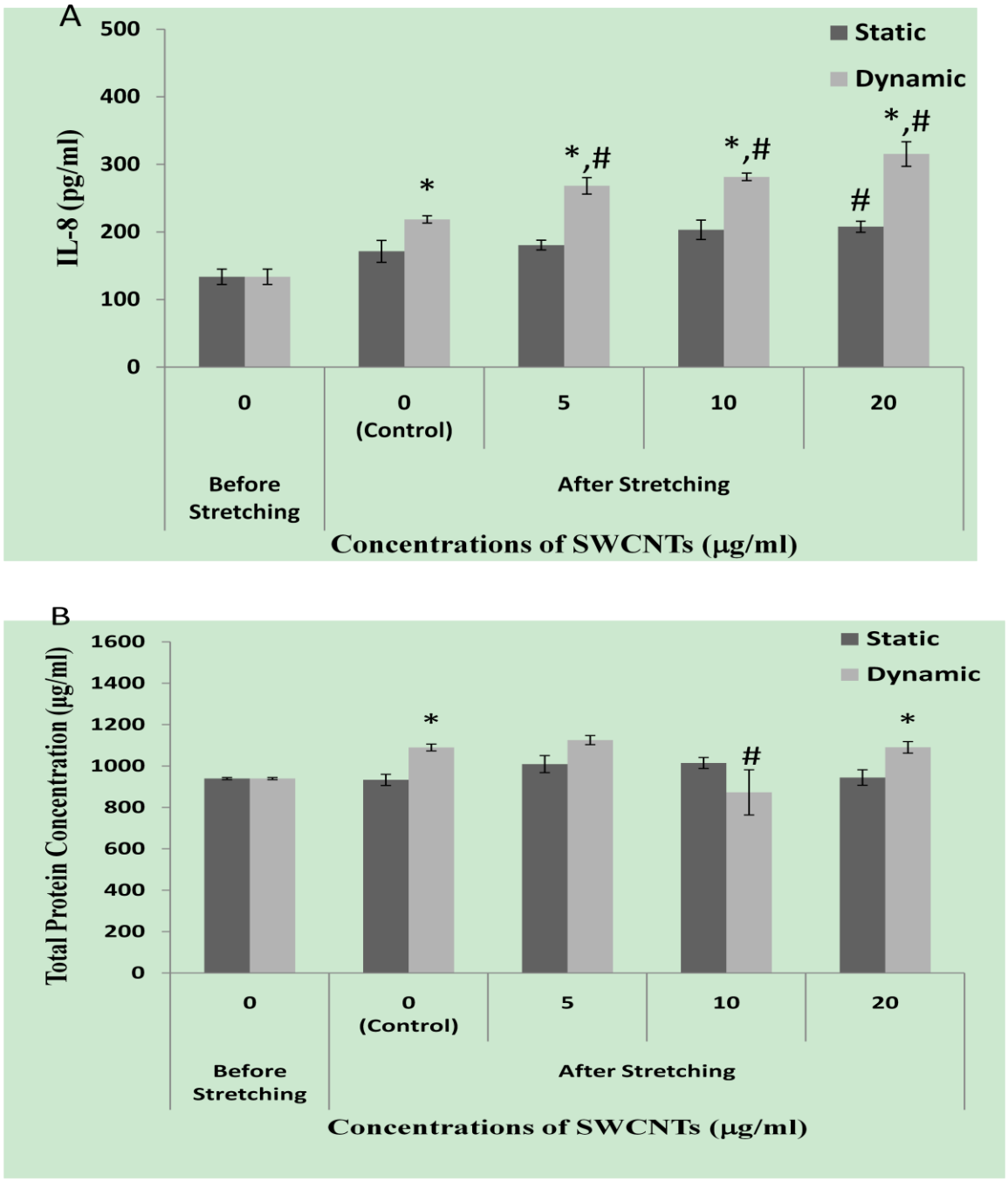


Figure 4.5 Effects of the dynamic environment on SWCNT-induced IL-8 expression and A549 cell proliferation. A549 cells were exposed to SWCNTs at 5, 10, and 20 μg/ml. Cells were grown under static and dynamic cell growth conditions

Figure 4.5 (continued)

for 24 hours. IL-8 was measured from culture media to characterize the inflammatory response of A549 cells exposed to different concentrations of SWCNTs under static and dynamic cell growth conditions (Figure 4.5A). Total amount of protein was measured from cell lysate of each sample (Figure 4.5B). * denotes a significantly higher difference than the static condition at each concentration of SWCNT ($p<0.03$ in Figure 5A and $p<0.05$ in Figure 5B). # denote a significantly higher (or lower) difference than the control for dynamic cell growth condition ($p<0.05$).

In this study, the changes in cell proliferation were not time-dependent or insignificant following exposure to SWCNTs. The SWCNT exposure started following confluence of cells. Some fluctuation in cell proliferation can be explained by likely damage to cellular components, while cells continued to proliferate. This was especially evident in the dynamic cell growth condition where part of the cell layers may have repeatedly detached and been recovered.

Previous studies showed that SWCNTs strongly interact with a large range of molecular species.[38-43] Higher toxicity was previously observed in samples that were exposure to SWCNTs at high concentrations (400- 800μg/ml) when grown in serum-free media.[44] However, at the lower concentrations (4 and 8 μg/ml) of SWCNTs from this study, higher inflammatory responses were observed in the presence of serum (Figure 4.1A) while no significant decrease in cell proliferations was observed (Figure 4.1B). Increased toxicity might be due to nutrient deficiencies and higher concentration of SWCNTs.[44-46] In this study, we observed that more SWNCTs penetrated into the cell in

the presence of serum (data not shown). Casey and co-workers suggested that SWCNTs may interact with media components by physical adsorption such as van der Waals force. They also demonstrated that serum affected the dispersion of the nanotubes.[35] Facilitated translocation of SWCNTs into the cells in the presence of serum might affect the enhanced IL-8 expression. However, the mechanism of the interaction between SWCNTs and unknown components of serum is not yet clear.

SWCNTs at high concentration (40 μg/ml) were more agglomerated. When we replaced the SWCNTs-containing media with fresh culture media following a 6 hour exposure, the aggregated forms of SWCNTs were more efficiently removed. In this case, IL-8 expression did not increase after removing SWCNTs following a shorter exposure (6 hour exposure) (Figure 3A). However, IL-8 expression kept increasing even after removal of SWCNTs following a relatively longer exposure (24 hours) (Figure 4.4A). Cell proliferation was also reduced after longer exposure times to higher concentrations of SWCNTs. Although their experimental systems were different from ours, other studies demonstrated that higher apoptotic cell death was observed at lower SWCNT exposure concentrations than higher concentrations in mouse model [27,47]. The effects of SWCNTs on cell proliferation and inflammatory responses were different between experimental systems (i.e. *in vitro* and animal model, static and dynamic systems) [48]**.** In our study, the SWCNT-induced IL-8 expression was significantly higher in the dynamic condition than in the static condition. An increased level of IL-8 under dynamic cell growth conditions could be due to the initial increase in IL-8 caused by the implementation of a dynamic cell growth condition [49,50]. Elevated levels of pro-inflammatory cytokines during SWCNT exposure under a dynamic cell growth condition may result from the altered

interaction between SWCNTs and A549 cell monolayers. Moreover, due to this altered interaction, cell proliferation was dramatically changed under dynamic cell growth conditions. Our results showed that the levels of inflammatory response could be altered under different exposure conditions. To identify the mechanisms of nanoparticle-induced inflammatory response under different exposure conditions, other inflammatory markers and various candidate biological markers (cellular component, macromolecules, other metabolic parameters, and cellular function) need to be monitored. In addition, comparative studies with normal cell lines and novel experimental set-ups for multiplexed screening of biological markers will be required to unravel the uncertainty of nanoparticle-induced inflammatory responses [51]. Continued *in vivo* and *in vitro* toxicological research is needed to identify potential health endpoints related to occupational exposure to engineered nanoparticles. Epidemiological studies of exposed workers will be needed to establish associations between exposures to engineered nanoparticles and adverse health effects and to assess other potential exposure-response relationships.

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CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Summary and Conclusions

Carbon nanotubes have been widely used in biomedical engineering and materials science, especially since researchers suggest CNTs might be able to be used to bone tissue engineering application. At the same time, the urgent need for toxicological studies on carbon nanotubes has arisen. The objectives of this research were to estimate the CNTs-collagen scaffolds in tissue engineering application, and to find out the effect of exposure conditions on SWCNT-induced inflammatory response in human alveolar epithelial cells.

5.1.1 Applications of CNTs-collagen scaffolds in tissue engineering

In Chapter 3, we designed CNTs –collagen composite scaffolds using different types of MWCNT (functionalized and non-functionalized) with good optically transparent. Then the mesenchymal stem cell's viability, differentiation, mineralization and inflammatory response on those scaffolds were studied. The aim of this study was to test whether those type of scaffolds can improve mesenchymal stem cell differentiation and mineralization without changing too much of the cell viability and inflammatory marker release. The cell differentiation and mineralization levels were higher in the MWCNTs and collagen mixture scaffolds than the plastic-only and collagen-only were. The cell viability and inflammatory maker IL-6 level didn't change too much when comparing different types of MWCNT-collagen mixture scaffolds. Those results suggested that MWCNT and collagen mixture scaffolds might be able to provide a good

nanostructure, mechanical binding and stiffness for MSC differentiation and mineralization.

5.1.2 Toxicity assessment of SWCNT

In Chapter 4, inflammatory marker (IL-8) expression in human alveolar epithelial cells under several different exposure conditions of SWCNT was measured. In this study we found IL-8 expression was enhanced in the presence of serum, and at low concentrations IL-8 expression kept increasing even after removal of SWCNTs from the media. We therefore surmise that serum was a defensive response of these lung cells to SWCNT exposure. Also the less aggregation of low concentrations of SWCNT might be the reason why IL-8 expression kept increasing even after removing SWCNTs, SWCNTs without aggregation were easily getting into cell and breaking down some mechanism of cell.

5.2 Recommendations

There has been a growth of interest in carbon nanotube materials because of their huge potential in industrial and research applications. Several studies of the application of carbon nanotubes have been carried for the substrate of cell culture, drug delivery systems and medical implant materials. However, there are not many researchers using CNT and collagen to test MSC`s viability and proliferation, especially no studies using CNT and collagen composite for estimating MSC`s differentation and mineralization. Our study does show the MWCNT-collagen composite increased in the cell differentiation and mineralization level, than the plastic and collagen-only surface. But the mechanism of the interaction between MWCNT, collagen and cell were not clear yet. Therefore, efforts should be made to force on mechanical study of MWCNT and collagen.

When considering the possible reason why cell differentiation and mineralization were changed, it seems important for future studies to address the possible study of the interaction between the MWCNT and collagen fiber, the surface properties of those composite scaffolds, and the interaction between the scaffold and cell. In addition, several studies on the effect of SWCNT on the lung tissue have been reported and there appears to be some inconsistency between the research findings. The conclusions we got during our research do show SWCNTs toxicity change at different exposure conditions, however, the reason behind that is not conclusive yet. Efforts should also be devoted to identifying the determinate of SWCNTs toxicity. In this respect, it can be recommended that more *in vivo* and *in vitro* toxicological research is needed to identify potential health endpoints related to occupational exposure to engineered nanoparticles.