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# MECHANISMS UNDERLYING EFFECTS OF CHITOSAN AND NANOMATERIALS IN TISSUE ENGINEERING MONOTYPIC AND CO-CULTURE CELL MODELS

by

Wenjuan Gao

A dissertation

submitted in partial fulfillment

of the requirements for the degree of

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## Committee Approval

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# List of Abbreviations

| | |
|---|---|
| U87 | Human brain glioblastoma cells |
| MTT | Thiazolyl blue tetrazolium bromide |
| DMSO | Dimethyl sulfoxide |
| FBS | Fetal bovine serum |
| AKT | Protein kinase B |
| p-AKT | Phosphorylated protein kinase B |
| ERK | Extracellular-signal-regulated kinases |
| p-ERK | Phosphorylated extracellular-signal-regulated kinases |
| MEM | Modified Eagle's medium |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| PVDF | Polyvinylidene difluoride |
| TBS | Tris-buffered saline |
| TBS-T | Tween-20 in Tris-buffered saline |
| ANOVA | Analysis of variance |
| SEM | Standard error of mean |
| CNTs | Carbon nanotubes |
| SMWCNTs | Short multi-walled carbon nanotubes |
| DRG | Dorsal root ganglion |
| PNS | Peripheral nervous system |
| LDH | Lactate dehydrogenase |
| DMEM | Dulbecco's modified Eagle's medium |

| | |
|---|---|
| 'DiO' | 3,3'-Dioctadecyloxacarbocyanine perchlorate |
| SD | Standard deviation |
| GFAP | Glial fibrillary acidic protein |
| PBS | Phosphate buffered saline |
| BCA | Bicinchoninic acid |
| ECL | Enhanced Chemiluminescence |
| ROS | Reactive oxygen species |

## Abstract

Chitosan has been used for a wide range of biomedical applications because of its satisfactory biocompatibility. Experimental results demonstrated that chitosan exhibited anti-microbial activities through its interaction(s) with microbial cell surface. We hypothesized that the properties of chitosan can be exploited to inhibit cancer cell growth. The commercial use of nanomaterials such as carbon nanotubes (CNTs) and nanoparticles (e.g., silver and gold nanoparticles) for novel applications is increasing exponentially. However, the impact of these nanomaterials on human and environmental health remains unclear. In this study, we investigated the effects of chitosan, chitosan in combination with nanoparticles, and chitosan in combinations with nanoparticles and/or three therapeutic drugs (i.e., Adriamycin, Methotrexate, and Cisplatin) on human brain glioblastoma U87 cells. We also investigated the effects of functionalized (i.e., carboxylated and hydroxylated), non-functionalized short multi-walled carbon nanotubes (SMWCNTs), chitosan, and chitosan in combination with SMWCNTs on dorsal root ganglion (DRG) neurons which constitute an excellent model *in vitro* of neurons derived from the peripheral nervous system (PNS). The close interactions between DRG neurons and Schwann cells stimulated us to develop a co-culture model consisting of DRG neurons and Schwann cells to investigate our hypothesis that co-culturing DRG neurons with Schwann cells imparts protection on them against cytotoxicity induced by silver or gold nanoparticles. The results of this project contribute significantly to the applications of chitosan and nanoparticles in tissue engineering, cancer therapy, and improve our understanding of how exposure to CNTs and silver and gold nanoparticles impacts the PNS and mechanisms underlying DRG neurons-Schwann cells interactions.

## Executive summary

Chitosan and nanomaterials (e.g., metal nanoparticles and CNTs) have been widely used in many fields, especially in biomedical applications. Before a new material can be employed for tissue engineering or other biomedical applications, it is necessary to test its biocompatibility and/or putative toxicity. Cell culture models (e.g., monotypic cell models and co-culture models) *in vitro* constitute convenient systems for investigating the putative toxicity of nanomaterials (e.g., metal nanoparticles and CNTs). There are three goals in my dissertation research. The first goal is to investigate the effects of chitosan, chitosan in combination with nanoparticles (i.e., gold and silver nanoparticles), and chitosan in combinations with nanoparticles and/or three chemotherapeutic drugs (i.e., Adriamycin, Methotrexate, and Cisplatin) on U87 cells (human brain glioblastoma cell line) (chapter II). The second goal is to evaluate the putative cytotoxicity of CNTs, chitosan, and chitosan in combination with CNTs on DRG neurons (chapter III). The third goal is to develop a co-culture model *in vitro* employing immortalized DRG neurons and Schwann cells and employ it to investigate our hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against neurotoxicity induced by silver or gold nanoparticles (chapter IV). These three goals are different applications of tissue engineering cell culture models. Chapter II is tissue engineering cell culture models for drug discovery/pharmacological studies. Chapter III and chapter IV are tissue engineering cell culture models for nanotoxicity studies.

# Chapter I

# Functional enhancement of chitosan and nanoparticles in cell culture, tissue engineering, and pharmaceutical applications[*]

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**Abstract:** As a biomaterial, chitosan has been widely used in tissue engineering, wound healing, drug delivery, and other biomedical applications. It can be formulated in a variety of forms, such as powder, film, sphere, gel, and fiber. These features make chitosan an almost ideal biomaterial in cell culture applications, and cell cultures arguably constitute the most practical way to evaluate biocompatibility and biotoxicity. The advantages of cell cultures are that they can be performed under totally controlled

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environments, allow high throughput functional screening, and are less costly, as compared to other assessment methods. Chitosan can also be modified into multilayer composite by combining with other polymers and moieties to alter the properties of chitosan for particular biomedical applications. This review briefly depicts and discusses applications of chitosan and nanoparticles in cell culture, in particular, the effects of chitosan and nanoparticles on cell adhesion, cell survival, and the underlying molecular mechanisms: both stimulatory and inhibitory influences are discussed. Our aim is to update the current status of how nanoparticles can be utilized to modify the properties of chitosan to advance the art of tissue engineering by using cell cultures.

**Keywords: chitosan, nanoparticles, tissue engineering, biocompatibility, nanotoxicity.**

## INTRODUCTION

Chitosan, produced by deacetylation of chitin, is a presumed non-toxic and hydrophilic polysaccharide (Tomihata and Ikada, 1997; Fukuda et al., 2006). Commercially, chitin and chitosan are obtained from shellfish sources such as crabs and shrimps. Chitin was first discovered by the French scientist Henri Braconnot in 1811 and “modified chitin” was renamed “chitosan” by Hoppe-Seiler in 1894 (Winterowd and Sandford, 1995). Research on chitin and chitosan accelerated in the 1900s and recently hundreds of articles have been published on chitosan. Bioapplications of chitosan were probably more popularized in the last 25 years; chitosan is currently better known to be a dietary supplement to the public than its other biomedical applications. Because of its low cost, large-scale availability, anti-microbial activity, as well as biodegradation and

biocompatibility (Khor and Lim, 2003), chitosan has been widely used by researchers as an important and promising biomaterial in tissue engineering (Dorj et al., 2012; Hu et al., 2012), wound healing (Charernsriwilaiwat et al., 2012; Wang et al., 2012), drug delivery (Chen et al., 2012a; Liang et al., 2012), and other biomedical applications (Amarnath et al., 2012; Kumar et al., 2012). Other important properties of chitosan include its compatibility to natural glycosaminoglycan (GAG) (Muzzarelli et al., 2005; Bhardwaj and Kundu, 2012) and that it can be molded into a variety of forms, such as powder, film, sphere, gel, and fiber. These compelling features are important qualities in cell culture. Many researchers have chosen chitosan or the combinations of chitosan with other materials to culture different cell types (Khor and Lim, 2003; Charernsriwilaiwat et al., 2012).

Recently adding nanoparticles in cell cultures for treatments and therapeutic uses is among the newest developments other than probing and imaging in nanotechnology and biotechnology. Nanotechnology, first appeared in the twentieth century, is an area of science devoted to the manipulation of atoms and molecules of materials in the nanometer range. Nanoparticles include all particles that possess at least one dimension that is less than 100 nm: the material origins of the particles can be organic, inorganic, metals, polymers, etc. Because of the wide-range of potential applications, nanotechnology has recently emerged as one of the most commercially viable technologies. Nanoparticles possess unique properties, more importantly, a large surface-to-volume ratio; thus, many of these particles possess high surface reactivity. These favorable properties are being exploited in many directions in science and technology, more so recently in biomedical applications.

Nanoparticles can enter the body through the lung and skin by absorption or through the gastrointestinal track via intake of food, drink, and medication. Due to the ubiquitous existence of nanoparticles and other nanomaterials, human exposure to these nanoparticles is inevitable. Nanoparticles can enter and affect different organs and tissues such as brain, liver, kidney, heart, blood, etc., and induce cytotoxic effects (Lai et al., 2008b). These particles may alter and inhibit cell growth leading to various pathophysiological states in humans and animals. Consequently, nanotoxicology research is now gaining much more attention and its importance is gradually being recognized.

Cell culture can be used to investigate cytotoxicity of nanoparticles including their effects on cell adhesion, cell survival, etc. Cell cultures in vitro can be performed under controlled environments with predictable and reproducible results, and are relatively inexpensive. Hence, research studies using nanoparticles in cell cultures have drawn considerable interest recently.

Combinations of material science in biological field proved to be fruitful and promise to hold great potential in biomedical developments: In 2004, Gu et al. (2004) induced proliferation of hepatocytes by immobilizing these cells on 24-nm gold colloids; they also constructed a silver nanocrystalline chitosan for wound dressing (Lu et al., 2008). In 2009, they found that the gold colloid/chitosan scaffold could promote adhesion and proliferation of keratinocytes (Zhang et al., 2009; Lu et al., 2010). Commercialization of these new technologies is sprouting almost as fast as they are developed, such as some of the wound dressing materials associated with nanoparticles (Rustogi et al., 2005; Ulkur et al., 2005).

In the biological and biomedical fields, studies involving chitosan, nanoparticles, or cell cultures alone are numerous in the literature. As the science of nanotechnology continues to advance, researchers are exploring different ways to modify chitosan for various biomedical applications. Moderate amount of work thus far has been performed to evaluate the biocompatibility of chitosan and modified chitosan in cell cultures, especially cultures of fibroblasts.

Applications of chitosan and its modified forms to tissue engineering is a fast developing field. This review highlights applications of chitosan and nanoparticles in cell cultures, in particular the effects of chitosan and nanoparticles on cell adhesion and cell survival: both stimulatory and inhibitory influences are discussed. Our aim is to update the current status of how nanoparticles can be utilized to modify the properties of chitosan to advance the art of tissue engineering (Lai et al., 2011) by using cell cultures.

## USES OF CHITOSAN IN CELL CULTURES

### DEACETYLATION OF CHITOSAN

Many studies showed that the degree of deacetylation and variation in molecular weights are the two factors that determined the effects of chitosan on cell growth. Generally, chitosan dissolves in dilute acidic solutions such as HCl, acetic acid, and some other organic acids (Sashiwa et al., 2000). Howling et al. (2001) examined the effects of chitin and chitosan solution at various deacetylation levels (37%, 58%, and 89%), molecular weights (12,000–263,800 Da), as well as different concentrations (2.5–500 μg/mL) on the proliferation of human dermal fibroblasts and immortalized human keratinocytes (HaCaT) in vitro. Their study showed that at high degrees of deacetylation, chitosan stimulated

fibroblast proliferation better than chitosan with lower levels of deacetylation; but the effects on keratinocytes were different. At high levels of deacetylation (89% deacetylated), chitosan inhibited HaCaT proliferation up to about 26%, while at lower degrees of deacetylation (37% deacetylated), chitosan had no effect on HaCaT proliferation at the reported concentrations. These findings indicated that the deacetylation level of chitosan is a key factor in regulating the mitogenic activity of fibroblasts and keratinocytes, but the cell responses with molecular weight differential was not clearly delineated in the report.

## MODIFICATION OF CHITOSAN AND ITS PROPERTIES

Modification of chitosan either by substituting the surface functional groups or cross-linking chitosan with different layers of polymers can alter the mechanical and biological properties of chitosan. Chen et al. (2002) investigated the effects of carboxymethyl (CM)-chitosan at different concentrations (50–500 μg/mL) and different molecular weights (3,200 Da, 15,000 Da and 35,000 Da) on normal human skin fibroblast and keloid fibroblast. They showed that CM-chitosan promoted proliferation of normal skin fibroblast significantly but inhibited proliferation of keloid fibroblast, because the CM-chitosan could decrease the ratio of type I/III collagen in keloid fibroblast by suppressing the secretion of type I collagen; but CM-chitosan had no effect on the secretion of types I and III collagen in the normal skin fibroblast. While both high and low concentrations of the CM-chitosan promoted initial proliferation, they noted that at the high concentration (500 μg/mL), CM-chitosan exerted a more positive effect on initial cellular proliferation. On the other hand, at the lower concentration (100 μg/mL), CM-chitosan exerted a lower

positive effect, compared to that of the higher concentration on initial cellular proliferation. Nevertheless, the duration of the effect exerted at the lower CM-chitosan concentration was longer than that achieved at the higher CM-chitosan concentration. In addition, molecular weight made no difference in growth stimulation for the skin fibroblast, the lower molecular weight (3,200 Da) CM-chitosan exerted almost the same effect on the growth stimulation of normal skin fibroblast as that achieved by the higher molecular weight (35,000 Da) CM-chitosan. However, the growth inhibition of keloid fibroblast increased slightly with the decrease of CM-chitosan molecular weight although that difference was not significant (~2%).

## CELL CULTURES ON CHITOSAN FILM

### CELL GROWTH ON CHITOSAN FILM

Chitosan solution can be employed to produce chitosan film/membrane using solution-casting technique (Tomihata and Ikada, 1997). This film can serve as a scaffold for cell cultures. Fakhry et al. (2004) employed two kinds of commercially available chitosan of different deacetylation and molecular weights, Chitosan-H (molecular weight: 140,000 Da; degree of deacetylation: 80%) and Protasan CL212 (molecular weight: 270,000 Da; degree of deacetylation: 70%), to prepare chitosan film/membrane. They used these films to culture mouse osteoblasts and fibroblasts and found that the two chitosan films could enhance the initial attachment and proliferation of osteoblasts, but the films were not as effective with fibroblasts. Their observation indicated that manipulation of deacetylation and molecular size of chitosan could modulate the in vitro level of cell attachment and spreading. Indeed, chitosan film inhibited the proliferation and differentiation of

embryonic rat cerebral cortical single stem cells and neurosphere in serum-free medium; but when serum was included, it induced the neurosphere-forming cells into an extensive cellular substratum of "protoplasmic cells" on which process-bearing cells spread (Hung et al., 2006). Zheng et al. (2003) studied the cytocompatibility of chitosan and CM-chitosan in human skin fibroblasts. They found that chitosan film inhibited cell growth and eventually the cells detached from the film. By contrast, the skin fibroblasts adhered to and differentiated well on CM-chitosan films. These findings demonstrated that cytocompatibility of CM-chitosan films in fibroblasts was better than that of plain chitosan films; these results were good examples that cell proliferation and differentiation were controlled by complex environmental conditions (stresses) that involve more than just manipulation of cell types alone.

Lahiji et al. (2000) hypothesized that chitosan promotes the survival and function of human osteoblasts and chondrocytes. Human osteoblasts propagating on chitosan films continued to express Type I collagen whereas chondrocytes expressed Type II collagen. Their results demonstrated the biocompatibility of chitosan as a substrate for the growth and continued function of human osteoblasts and chondrocytes. Consequently, chitosan shows some potential as a tissue engineering tool for the repair of osseous and chondral defects.

The effects of hexanoyl chitosan (H-chitosan) on cytotoxicity, attachment, proliferation, and spreading of L929 (mouse fibroblast) cells were reported (Neamnark et al., 2007). The attachment of the cells on H-chitosan film was better than that on the chitosan counterpart for a short time (<5 h) after seeding, while the proliferation of the

cells on H-chitosan film was better than that on the chitosan counterpart after 2 and 3 days in culture.

To simulate a membrane layer that is closer to the natural membrane that can be used as a structure scaffold and function as a barrier with selectivity, a single layer material for such is nearly impossible but a bilayer membrane is more realistic. A bilayer structure of chitosan film and sponge as a scaffold can support growth and proliferation of human neofetal dermal fibroblasts (Ma et al., 2001). In fact, blending materials with different chemical and physical properties (biocomposites) are finding numerous applications in biomedical technology.

## BLENDING POLYMERS WITH CHITOSAN AS CELL SCAFFOLD

To improve the biocompatibility and other properties (e.g., permeability) critical for a wide-range of biomedical applications, blending chitosan with other polymers has been widely investigated. Silica-chitosan complex membrane (SiCM) was developed by Suzuki et al. (1999). The composition of the SiCM was stepwisely controlled by adjustments of mixing ratios between silica and chitosan from 0% to 50%. Their study showed that 50% SiCM was especially effective in increasing adhesion and growth of human embryonic kidney cells and human lung diploid cells. Bettini et al. (2008) used sugar and phosphate to modify the chitosan film: phosphate and sugar were added into the chitosan film-forming solutions. The resulting chitosan film exerted a beneficial effect on affinity with human diploid fibroblasts (HDF, cell strain WI-38). A novel absorbable scaffold with an asymmetric structure composed of chitosan and gelatin was fabricated by a freezing and lyophilizing method as a bilayer skin substitute (Mao et al.,

2003). Data from that study suggested that scaffold constructed by this artificial bilayer was flexible, had good mechanical properties and induced no contraction in the cell cultures tested. Using the suspension of chitosan hydrogel mixed with gelatin, novel chitosan/gelatin membranes were prepared (Nagahama et al., 2009); human MG-63 osteoblast-like cells were used to examine the effect of chitosan/gelatin membranes on cell adhesion. The cells incubated with chitosan/gelatin membranes for 24 h exhibited good adhesion to the chitosan/gelatin substratum, suggesting that these modified chitosan membranes are useful for biomedical applications. Huang et al. (2005) investigated the degradation of chitosan-gelatin scaffold and its effect on mouse fibroblast activity and adhesion. The degradation rate and material loss of chitosan-gelatin scaffold were faster than those of chitosan alone even though both chitosan-gelatin and chitosan alone supported fibroblast viability equally well. A similar study was also performed by Chupa et al. (2000): they evaluated the potential applications of GAG-chitosan and dextran sulfate (DS, a semi-synthetic GAG analog)-chitosan complex membrane materials for controlling the proliferation of human vascular endothelial (EC) and smooth muscle cells (SMC). Their results showed that while chitosan alone supported cell attachment and growth, GAG-chitosan materials inhibited spreading and proliferation of ECs and SMCs in vitro. In contrast to the GAG-chitosan, DS-chitosan supported proliferation of both cell types, although it is a semi-synthetic GAG analog. Their results indicated that GAG-chitosan could also be used to modulate the proliferation of EC cells. A series of chitosan/poly-l-lysine composite films were produced from chitosan/poly-l-lysine blended solutions by Zheng et al. (2009) The effects of the composite films on the behavior of MC3T3-E1 osteoblast-like cells (from mouse) were assessed. Those

researchers found that the increase in poly-l-lysine weight fraction in the blended solutions could result in different nanoscaled surface topographic features, and MC3T3-E1 cells strongly responded to those nanotopographic features. Consequently, their novel observation demonstrated nanotopography of chitosan and chitosan-derived materials could exert remarkable influences on behavior of cells cultured in vitro. Thus, topographic modification of chitosan-derived substratum on a nanoscale may be exploited in regulating behavior of cells in culture, and as such can be productively exploited in diverse applications in tissue engineering, as well as in engineering an environment to foster bone regeneration.

Elastin and poly-l-lysine were used to modify a hybrid bulk scaffold which contained polyethylene oxide, chitin, and chitosan (Kuo and Chung, 2012). Bovine knee chondrocytes were seeded in the scaffolds and cultured in a spinner-flask bioreactor over 4 weeks. Results of this study showed that elastin- and poly-l-lysine-grafted polyethylene oxide/chitin/chitosan scaffolds were effective in producing cartilaginous components. It appears that modified chitosans have more and better control of biomedical applications than just chitosan alone due to better physicochemical properties.

## COMPLEX COMPOSITE MODIFICATIONS OF CHITOSAN FILM

Once chitosan is viewed as a building component of composite materials for biomedical applications, the possibilities of creating new biocomposites with chitosan are almost unlimited. With such, majority of the new applications thus far are associated with improving structural properties of scaffolding and tissue connection. The following are some of the major applications of chitosan biocomposites.

***Applications with bone/cartilage***

This constitutes another strategy to develop novel tissue engineering applications. For example, Chen et al. (2006) utilized fractional factorial design methodology to co-immobilized four different GAGs (chondroitin-4-sulfate, chondroitin-6-sulfate dermatan sulfate and heparin) and prepared eight different combinations of GAG/chitosan membranes. They evaluated the effects of these GAG/chitosan membranes on several properties of chondrocytes, including adhesion, morphology, and proliferation. This was their attempt to provide a rational method to predict and evaluate the proper formulation of GAG/chitosan membranes for various applications in tissue engineering. Recently, chitosan/chondroitin sulfate/nano-$SiO_2$ composite scaffold was fabricated by lyophilization (Kavya et al., 2012). Biocompatibility and cell attachment-proliferation studies performed using MG-63 cells (human osteosarcoma cell line) suggested this novel nanocomposite scaffold could be a suitable candidate for bone tissue engineering.

The polypeptide Arg-Gly-Asp-Ser (RGDS) sequence of fibronectin was used to modify chitosan membrane using the photochemical immobilization technique (Karakecili et al., 2007): this substrate could enhance the attachment and proliferation of L929 (mouse fibroblast) cells. Schneider et al. (2007) cross-linked chitosan with hyaluronan (a polysaccharide) (CHI/HA) using a water-soluble carbodiimide: the film had a much improved elastic modulus and was more resistant to enzymatic degradation by hyaluronidase but was not overly thick. Consequently, the increased film stiffness improved the adhesion and spreading of chondrosarcoma cells. Thus, the CHI/HA cross-linked films could be used for various biomedical applications due to their more favorable mechanical and adhesive properties, and are a more robust matrix material due

to their higher tolerance to enzymatic degradation. Higher resistance to enzymatic degradation allows the engineered tissue to develop into more mature structure before the supportive scaffold collapsed. A series of nano-hydroxyapatite/chitosan cross-linking composite membranes (nano-hydroxyapatite; 0-30% by weight) were successfully developed by a simple casting/solvent evaporation method by Li et al. (2012). The nano-hydroxyapatite content greatly affected the morphology as well as the tensile property of composite membrane. In vitro cytotoxicity testing suggested that the developed nano-hydroxyapatite/chitosan cross-linking composite membrane was non-cytotoxic to L929 cells after 24 h of incubation. Therefore, the nano-hydroxyapatite/chitosan cross-linking composite membrane with favorable cytocompatibility, water adsorption (wettability) and tensile strength might serve as the vehicle for bone tissue engineering.

Chitosan has also been employed to reinforce calcium phosphate cement (CPC). Moreau and Xu (2009) examined the proliferation and osteogenic differentiation of mesenchymal stem cells (MSCs) cultured on high-strength CPC-chitosan scaffold. On the CPC-chitosan scaffold, MSCs differentiated into the osteogenic lineage and expressed high levels of bone marker alkaline phosphatase (ALP). The results of Moreau and Xu (2009) suggested the stronger CPC-chitosan scaffold may be useful for tissue engineering research in stem cell-based bone regeneration.

Alginate-chitosan semi-interpenetrating polymer network (IPN) scaffolds were prepared by a freeze-drying process (Tigli and Gumusderelioglu, 2009). The attachment and proliferation abilities of ATDC5 murine chondrogenic cells on alginate, 70:30% (v/v) alginate:chitosan and 50:50% (v/v) alginate:chitosan scaffolds, were assessed. The results of this study indicated that alginate:chitosan semi-IPN scaffolds could promote

chondrocyte proliferation and that 50:50% (v/v) alginate:chitosan scaffolds showed some promise as an ideal material for applications in cartilage tissue engineering in vitro, especially from the prospective of structural analysis and cell-based functional screening.

Venkatesan et al. (2012) constructed chitosan-carbon nanotube scaffolds by a freeze-drying method. Carbon nanotube was uniformly dispersed in chitosan matrix. Cytotoxic effects and cell proliferation of scaffold were investigated employing the MTT assay using MG-63 cells (human osteosarcoma cell line). The cell proliferation, protein content, ALP and mineralization of the cells cultured on composite scaffolds were higher than those on the chitosan scaffold due to the addition of carbon nanotube suggesting that chitosan/f-MWCNT scaffolds may be promising biomaterials for bone tissue engineering.

***Applications with fibroblasts and other cell types***

Chitosan can also be used to functionalize with other polymerizable molecules to improve the biocompatibility and mechanical properties of the resulting copolymer, such as poly-L-lactic acid, poly(L-lactide-co-epsilon-caprolactone) and poly (ε-caprolactone) (Ding et al., 2004; Mei et al., 2005; Jiao et al., 2007; Yang et al., 2012; Zhang and Cui, 2012). Immobilization of chitosan onto poly-L-lactic acid (PLLA) film surface by plasma graft polymerization has been reported. Two cell lines, L929 (mouse fibroblast) and L102 (human hepatocytes), were cultured on the modified PLLA surface. Results indicated that cell spreading on this film was minimal and the colonies tended to become rounded. Thus, the film was demonstrated to be a poor adhering substrate. Nonetheless, cells grown on this substrate proliferated at almost the same speed as those cultured on a glass surface.

These results suggested that the new substrate could be used to control the morphology of cells.

Another method to construct a novel cytocompatible graft co-polymer of chitosan and L-lactic acid was reported by Yao et al. (2003). They dissolved chitosan powder in an aqueous solution of L-lactic acid and then poured the solution into a frame mold, and maintained at a proper temperature for film formation. With this type of co-polymer films, they were able to ascertain that the growth rate of human fibroblast on the co-polymer films, with various L-lactic acids to chitosan ratios, was higher than that on chitosan alone, but the growth of those cells decreased as the ratio of L-lactic acid to chitosan increased. All the copolymer and chitosan films enhanced initial fibroblast proliferation, but the proliferation of the cells on both types of films eventually became slower than that of the controlled cells cultured after 9 days. Evidently, despite their results of slower cell growth at prolonged time, the co-polymer films provided a better scaffold than chitosan alone with improved mechanical properties for cell growth and thus could be useful in applications in designing grafting in tissue engineering.

Keratin-chitosan film is another useful composite film fabricated (Tanabe et al., 2002). This film was fabricated by mixing solutions of keratin and chitosan casting into the polypropylene mold, and then followed by drying at 50°C overnight. With this procedure, films with average 0.01–0.02 mm thickness were obtained. The role of chitosan was to reinforce the mechanical properties of keratin film. Results of that study (Tanabe et al., 2002) showed the keratin-chitosan film was a good substrate that supported the attachment and proliferation of L929 mouse fibroblast cells; this finding

also suggested that keratin-chitosan film could be a good supporting substrate for other mammalian cells in culture.

Poly (vinyl alcohol) (PVA)/chitosan blend has also been fabricated (Chuang et al., 1999). Human skin fibroblasts seeded and cultured on the PVA/chitosan blended membrane showed better attachment and spreading compared to that of fibroblasts seeded and cultured on the pure PVA membrane alone (Chuang et al., 1999). This observation suggested that chitosan enhanced the biocompatibility of the PVA and that the PVA/chitosan blended membrane could be employed as another useful biofilm for cell culture applications. In 2009, Costa et al. (2009) and Mansur et al. (2009) synthesized chitosan/PVA blends with different chitosan/PVA mass ratios and chemically cross-linked these blends with glutaraldehyde. VERO cells (isolated from kidney epithelial cells from an African green monkey) were used to assess the biocompatibility and cytotoxicity of these chitosan/PVA blends. Their studies demonstrated that changing the mass ratio of chitosan to PVA could alter the "swelling behavior" of the blended materials. Swelling control is an important property for tissue engineering that the physical performance of the materials must be predictable. The results from cell biocompatibility assays have demonstrated that all these blends were non-toxic and biotolerant and potentially suitable for prospective use in skin tissue engineering and drug delivery.

## THREE-DIMENSIONAL CELL CULTURE MATRICES INVOLVING CHITOSAN GEL AND CHITOSAN NANOPARTICLES/NANOFIBER COMPLEXES

## CELL GROWTH IN 3-DIMENSIONAL CHITOSAN STRUCTURES

Under physiological conditions, cells normally proliferate and grow in various organs and tissues *in vivo* in a truly three-dimensional (3-D) matrix surrounded by one or more other cell types (Lai and Leung, 2005; Zhang et al., 2005). Thus, a 3-D in vitro cell culture environment more closely resembles that of the in vivo physiological environment. Methods or materials fare well in the 2-D setting may not fare well in the 3-D situation; one of the major obstacles of 3-D culture is the delivery or availability of nutrients that are needed for the cells to survive/proliferate. Passive diffusion does not provide enough supports for the cells in deep layers: this situation may not be as critical in 2-D culture. Indeed, the use of chitosan has been recently exploited to create such an environment in vitro. For example, chitosan can be made into a gel and/or nanoparticle/nanofiber complex that can provide a 3-D structure. Poly (vinyl alcohol) (PVA)/chitosan blended hydrogel has been fabricated for that purpose (Koyano et al., 1998; Minoura et al., 1998). The hydrogel with 40% (weight) chitosan content was superior to collagen for the attachment and growth of L929 mouse fibroblasts. Karp et al. (2006) created micropatterns in the chitosan gel using a photolithographic method which was simple and rapid. Cardiac fibroblasts, cardiomyocytes and osteoblasts could form arrays on these chitosan-patterned surfaces and remained stable for up to 18 days. Zheng et al. (2012) recently presented a new method for primary neonatal rat cardiomyocytes cultured in vitro using alginate/collagen/chitosan hydrogel. The results showed a significant increase in cardiac myocyte numbers, and the expression levels of CACNL1A1 and Connexin 43 were up-regulated significantly, as compared with those in 2-D cultures. By using an electrospinning technique, PCL (poly (ε-caprolactone))/chitosan/PCL scaffolds were

prepared layer by layer (Sasmazel, 2011). The hybrid scaffolds exhibited nanofiber structures and such layered scaffolds have provided improved substrate delivery to facilitate the growth and differentiation of SaOs-2 osteosarcoma cells cultured in vitro. A biomimetic poly (propylene carbonate) (PPC) porous scaffold with nanofibrous chitosan network (PPC/CSNFs, chitosan nanofibers) for bone tissue engineering was fabricated by a dual solid-liquid phase separation technique (Zhao et al., 2012). The in vitro culture of bone MSCs showed that PPC/CSNFs scaffold exhibited a better cell viability than PPC scaffold. Bhardwaj and Kundu (2012) fabricated polyelectrolyte complex silk fibroin/chitosan blended porous scaffolds and examined its ability to support in vitro chondrogenesis of MSCs. These results suggested that silk fibroin/chitosan blended 3D scaffolds were suitable scaffold for mesenchymal stem cell-based cartilage repair. A similar study showed that silk fibroin/chitosan composite nanofibers could support the growth and osteogenic differentiation of human fetal osteoblastic cells, indicating that these nanofibers would be potentially suitable for bone tissue engineering applications (Chen et al., 2012b).

Fukuda et al. (2006) advanced the use of chitosan hydrogel in the co-culture paradigm for cells from different animal species. They synthesized a photo-cross-linkable chitosan using the protocol of Ono et al. (2000). Fukuda and co-workers demonstrated human hepatocellular carcinoma (HepG2) cells and NIH-3T3 mouse fibroblasts could be co-cultured in the chitosan hydrogel where the two cell types were spatially separated. They also noted that the NIH-3T3 fibroblasts attached evenly to the chitosan surface surrounding the HepG2 spheroids and proliferated over time to cover the entire surface of the hydrogel.

Manipulating neural cells in culture has consistently provided challenges for tissue engineering researchers, not least of all because of the paucity of existing appropriate neural cell models in vitro (Lai and Leung, 2005; Zhang et al., 2005). On the other hand, model systems in vivo have revealed significant challenges that differ from those of in vitro models (Lai and Leung, 2005; Zhang et al., 2005). Nevertheless, some advances have been evident. Zahir et al. (2008) investigated the survival and differentiation of neural stem/progenitor (NSPCs) cells cultured on chitosan matrices in vivo in a complete transection model of spinal cord injury. Firstly, they isolated NSPCs from the subependyma of lateral ventricles of adult green fluorescent protein (GFP)-transgenic rat forebrains, and then seeded the GFP-positive neurospheres onto the inner lumen of chitosan tubes to generate multicellular sheets ex vivo. Finally, they implanted the bioengineered neurosphere tubes into a completely transected spinal cord for 5 weeks and then assessed for cell survival and differentiation. They found that the implanted NSPCs showed excellent survival; moreover, the implanted cells differentiated into astrocytes and oligodendrocytes. These results demonstrated that the chitosan tubes exhibited excellent potential for use in stem cell delivery and neural regeneration. For neural tissue engineering, hydrogel-based scaffolds can provide appropriate physicochemical and mechanical properties to support neurite extension. Valmikinathan et al. (2012) developed a novel chitosan-based photocrosslinkable hydrogel system with tunable mechanical properties and degradation rates. When human MSCs were cultured in photocrosslinkable chitosan hydrogels, negligible cytotoxicity was observed. Photocrosslinkable chitosan hydrogels facilitated neural differentiation from primary cortical neurons and enhanced neurite extension from dorsal root ganglia as compared to

agarose based hydrogels. These data demonstrated the potential of photocrosslinked chitosan hydrogels designed for neural tissue engineering. Electrospun polyvinyl alcohol (PVA)/chitosan nanofibrous scaffolds have been synthesized (Alhosseini et al., 2012). The scaffolds were used for in vitro cell culture in contact with PC12 cells which are neurons-like, they were found to exhibit the most balanced physicochemical and biological properties to meet the basic required specifications for nerve cells. Thus, it could be concluded that addition of chitosan to the PVA scaffolds enhanced viability and proliferation of nerve cells, thereby confirming the biocompatibility of the scaffolds. Therefore, PVA/chitosan nanofibrous scaffolds have the potential to be used in nervous tissue engineering and repair.

The thermosensitive chitosan-gelatin-glycerol phosphate hydrogels were synthesized so that they can be employed as a cell carrier for nucleus pulposus (NP) cell regeneration (Cheng et al., 2010). NP cells cultured in the hydrogels displayed normal GAG production, mRNA production and gene expression. This finding suggested that this hydrogel may be suitable for intervertebral disc replacement in tissue engineering. Ma et al. (2010) prepared injectable hydrogels from chitosan derivative, methacryloyloxy ethyl carboxyethyl chitosan (EGAMA-CS)/polyethylene glycol dimethacrylate (PEGDA)/N, N-dimethylacrylamide (DMMA) by photopolymerization. Their cell culture studies demonstrated that these hydrogels exhibited the desirable properties in promoting attachment and proliferation of human bone sarcoma (SW1353) cells. Thus, their results suggested that these hydrogels may well be the ideal matrix material for bone tissue engineering. More recently, porous chitosan-gelatin/hydroxyapatite composite scaffolds were developed (Isikli et al., 2012). These scaffolds promoted Saos-2 cells attachment

and proliferation indicating that scaffolds prepared from chitosan, gelatin and hydroxyapatite were good cell carriers for bone tissue engineering.

An important issue to tissue engineering is the influence of mechanical characteristics and matrix architecture of substrates used in cell culture. Iyer et al. (2012) examined the influence of porous structures, hydrogels (chitosan-gelatin), and membranes of chitosan-based material on the growth of normal human fibroblasts and their matrix production in a serum-free system. They used chitosan alone and in combination with gelatin. They found that increased viability of fibroblasts on chitosan gelatin porous scaffold with decreased proliferation relative to tissue culture plastic surface. The total protein, collagen and tropoelastin contents were higher in the spent media derived from cells cultured with chitosan gelatin porous scaffolds compared with corresponding levels from cells treated with chitosan membrane or hydrogel alone. An increase in collagen content was also observed in the matrix, suggesting increased matrix deposition. Therefore, matrix production is influenced by the form of chitosan structures, which significantly affects the regenerative process.

## EFFECTS OF CHITOSAN NANOPARTICLES ON CELLS IN CULTURE

Chitosan nanoparticles have been synthesized using either the batch processing methods or the spinning disc processor (Bodna et al., 2005; Loh et al., 2008). Evidence has shown that chitosan nanoparticles may exert differential bactericidal and pharmacological effects on prokaryotic and eukaryotic cells in culture (Shi et al., 2006; Grenha et al., 2007). Shi et al. showed that chitosan nanoparticles had significant bactericidal effects on bacteria such as Staphylococcus aureus (S. aureus) and Staphylococcus epidermidis (S.

epidermidis), but had no cytotoxic effect on mouse fibroblast cells (Shi et al., 2006). Grenha et al. also demonstrated that chitosan nanoparticles are biocompatible with human respiratory epithelial cells in vitro (Grenha et al., 2007). Nevertheless, chitosan nanoparticles cannot be expected to be biocompatible to all mammalian cell types. Materials that are non-toxic at macroscale can become toxic at nanoscale (Rustogi et al., 2005). Nafee et al. (2009) investigated the effects of the chitosan/PLGA [poly (D, L-lactide-co-glycolide)] nanoparticles on three cell lines (i.e., African green monkey kidney COS-1 cells, human alveolar cancer A549 cells and human bronchial epithelial Calu-3 cells). These chitosan nanoparticles were cytotoxic to COS-1 cells in a dose-related manner; however, they were not cytotoxic to A549 cells in the dose range investigated. Similarly, these chitosan nanoparticles were nearly non-cytotoxic to Calu-3 cells compared to COS-1 cells. Similar results were obtained by employing different cytotoxic assays (e.g., MTT, LDH release) by the investigators.

**EFFECTS OF COLLAGEN-CHITOSAN NANOFIBERS**

A few studies have focused on elucidating the effects of another novel chitosan nanofiber, namely collagen-chitosan complex nanofibers, which have been fabricated employing an eletrospinning technique (Chen et al., 2007). Tangsadthakun et al. (2007) investigated the influence of molecular weight of chitosan on the physical and biological properties of collagen/chitosan scaffolds. They found that low-molecular-weight chitosan within the collagen-chitosan nanofibers rendered the nanofibers more effective in promoting and accelerating the proliferation of mouse L929 fibroblasts. Duan et al. (2006) constructed a nanofibrous composite membrane of poly (lactide-co-glycolide) (PLGA) and

chitosan/poly (vinyl alcohol) (PVA) and tested their effects on attachment and proliferation of rabbit dermal fibroblasts. They concluded that the nanofibrous composite membrane they fabricated show a good tendency toward promoting the attachment and proliferation of rabbit dermal fibroblasts and suggested that the composite membrane was a good candidate for application in skin tissue engineering/reconstruction. Feng et al. (2009) developed a novel natural nanofibrous galactosylated chitosan (GC) scaffold and investigated its effect on primary cultures of rat hepatocytes. They observed that hepatocytes cultured on GC nanofibrous scaffold formed stably immobilized 3-D flat aggregates and exhibited superior bioactivity with higher levels of liver-specific functions compared to the 3-D hepatocyte spheroid aggregates formed on GC films alone. Consequently, their findings pointed to the utility of the GC-based nanofibrous scaffolds in the constructs of bioartificial liver-support devices and the versatility of these scaffolds were also suitable as substrates for primary cultures of hepatocytes in tissue engineering applications, such as liver regeneration and related translational research.

## NANOPARTICLES AND CELL SURVIVAL

### TOXICITY OF NANOPARTICLES AND RELATED NANOMATERIALS

Despite the important and accelerating advances of nanoscience and nanotechnology, there has been increasing concern that some of the nanomaterials are not as harmless as people have assumed. Those concerns have spurred a new field of research, namely, “nanotoxicology” (Jandhyam et al., 2008; Lai et al., 2008c).

Cell cultures of a variety of human and other mammalian cell types constitute versatile model systems in vitro for high throughput screening of putative toxicity of

nanomaterials, including nanoparticles, and for elucidating any underlying molecular mechanisms (Rustogi et al., 2005; Jandhyam et al., 2008; Lai et al., 2008c). The putative nanotoxicity of various nanomaterials has been examined: such nanomaterials included, but were not limited to, carbon nanotubes (Agharkar et al., 2008), carbon nanopipes (Whitby et al., 2008), silicon dioxide (Rustogi et al., 2005) and many metal oxides.

In 2005, Limbach et al. (2005) evaluated the uptake and transport of industrially important cerium oxide nanoparticles into human lung fibroblasts in vitro by exposing them to suspensions of these nanoparticles, with concentrations ranging from 100 ppb to 100 ppm. At such low but pathophysiologically relevant concentrations, the size of the nanoparticles was a dominant factor in determining the rate of uptake, while total particle surface area was of secondary importance. In 2006, the same research group employed a human mesothelioma cell line and a rodent fibroblast cell line to test the putative cytotoxicity in vitro of seven industrially important soluble and insoluble nanoparticles, namely $SiO_2$, $Ca_3$ $(PO4)_2$, $Fe_2O_3$, ZnO, $CeO_2$, $TiO_2$, and $ZrO_2$. They found that solubility was the critical factor in determining the cytotoxicity of the nanoparticles (Brunner et al., 2006). In 2011, Shavandi et al. (2011) assessed the toxicity of silver nanoparticles in murine peritoneal macrophages. A significant decrease in cell viability was observed at concentration of silver nanoparticles from 1 ppm to 25 ppm when the results were compared to that in the control group after 24 h of exposure of the cells to the nanoparticles in culture.

Lai and co-workers were the first to develop cell models in vitro for high throughput screening of putative cytotoxicity of nanomaterials, including nanoparticles, in neural cells (Rustogi et al., 2005; Lai et al., 2007, 2008a,c, 2009, 2010; Agharkar et al., 2008;

Jandhyam et al., 2008; Jaiswal et al., 2010, 2011; Jain et al., 2011; Lu et al., 2011). Their comprehensive and systematic studies have revealed that nanoparticles of metallic and non-metallic oxides exerted differential cytotoxic effects on neural cells derived from the central nervous system as cell models in vitro (Rustogi et al., 2005; Lai et al., 2007, 2008a,c, 2009, 2010; Jandhyam et al., 2008). More recently, they have also developed non-tumor neural cells derived from the peripheral nervous system for high throughput screening of putative cytotoxicity of nanomaterials, including nanoparticles, on peripheral neural cells (Jaiswal et al., 2010, 2011; Jain et al., 2011; Lu et al., 2011). Results of their previous and ongoing studies have also indicated that nanoparticles of metallic and non-metallic oxides exerted differential cytotoxic effects on neural cells derived from the peripheral nervous system (Jaiswal et al., 2010, 2011; Jain et al., 2011; Lu et al., 2011). Their results (Rustogi et al., 2005; Lai et al., 2007, 2008a, c, 2009, 2010; Jain et al., 2011; Jandhyam et al., 2008; Jaiswal et al., 2010, 2011; Lu et al., 2011) have highlighted the importance and need for screening and elucidating the putative cytotoxicity of nanoparticles in neural cells if the nanoparticles are to be employed in biomedical and other applications that exert impact on the nervous system. Indeed, one emerging area where this nanotoxicity concern needs to be adequately addressed is the area of targeted drug delivery to the central nervous system and other peripheral organs.

To enhance targeted drug delivery, nanoparticle-loaded polymer capsules are often employed to enable selective drug delivery to the desired target organ(s). In accord with the considerations discussed above, prior to—at least along with—developing the applications of nanoparticles for drug delivery, the putative nanotoxicity of such particles needs to be assessed. Some examples of this kind of investigations are available in the

literature. Kirchner et al. (2005a) demonstrated that polymer capsules containing coating of CdTe nanoparticles exhibited higher toxic effects than those of non-coated capsules. Nevertheless, by comparison, the silica-coated CdSe/ZnS nanoparticles were far less cytotoxic (Kirchner et al., 2005b). On the other hand, not all nanoparticles containing Si are benign and non-toxic. Di Pasqua et al. (2008) assessed the cytotoxicity of MCM-41, a mesoporous silica nanomaterial, in human neuroblastoma SK-N-SH cells. They found that MCM-41 was more toxic than spherical silica (i.e., $SiO_2$) nanoparticles. As we have alluded to above, $SiO_2$ nanoparticles are known to be cytotoxic to neural and non-neural cells (Rustogi et al., 2005; Lai et al., 2007, 2008a, 2010; Jandhyam et al., 2008; Jain et al., 2011; Jaiswal et al., 2011).

## APPLICATIONS OF NANOPARTICLES

Cyclodextrins (CDs) are cyclic oligosaccharides widely used in pharmaceutical industries to control and/or improve on the solubility, release and absorption of drugs so as to ascertain the proper delivery of such drugs. Thus, injectable CDs are useful in drug delivery applications. Their utility notwithstanding, one disadvantage of using CDs is that the CDs can induce hemolysis and nephrotoxicity. Consequently, there is a need to obviate such adverse effects induced by the CDs: in this area, research is still continuing. For example, concentration-related cytotoxicity of β-CDC6, an amphiphilic β-cyclodextrin derivatized nanoparticles, has been demonstrated by using mouse fibroblasts and human polymorphonuclear cells in culture (Memisoglu-Bilensoy et al., 2006).

Among the metal nanoparticles, nano-gold particles possess favorable biocompatibility and stability (Gu et al., 2001, 2002, 2003, 2006). The utility of nano-

gold particles was first highlighted in the immune-gold staining procedures in electron microscopic applications in the 1970s. But studies on the effects of the nano-gold particles on proliferation of mammalian cells only began to emerge in recent years (Lai et al., 2008b). In 2005, Mukherjee et al. (2005) noted that a novel mechanism by which nano-gold particles inhibited the proliferation of human umbilical EC cells in vitro was via binding with heparin-binding proteins. However, the toxicity of the nano-gold particles could be decreased by functionalizing with peptide of the sequence Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) (Fuente et al., 2006).

Ramis-Castelltort et al. (2008) designed a nanostructured material composed of a gold nanoparticle core functionalized with hyaluronan (HA) molecules on its surface. They tested this conjugate based on Gold-HA nanoparticles for cosmetic applications with the intent to improve features including stability, skin-penetration and water absorption/retaining effect, but their results remained to be confirmed. In order to improve the biocompatibility of polyethylene terephthalate (PET) mesh, scaffolds composed of PET-gold nanoparticles (PET-AuNP) were developed through conjugating various concentrations of AuNP to the PET surface (Whelove et al., 2011). Their results demonstrated that exposure of L929 murine fibroblasts to the PET-AuNP scaffolds improved their cell integrity, decreased their production of reactive oxygen species and lowered bacterial adhesion to the PET; thus, their findings suggested AuNPs could enhance the biocompatibility of the PET mesh. Kumari and Singh (2012) synthesized gold nanoflowers (flower-like, three dimensional branched gold nanoparticles) and constructed glycolic acid-g-chitosan-gold nanoflower nanocomposite scaffold. The nanohybrid scaffold was stable at the medium pH and it was biocompatible through cell

(SP2/0 mouse myeloma cell line) viability study. These Au nanoflowers released the carried drug (cyclophosphamide) at rates depending on the depth (location) of the nanoparticles in buffer solution (pH 7.4). Therefore, these gold nanoflowers might be a viable additive for drug delivery for the glycolic acid grafted chitosan-based system, but the drug released mechanism in vivo was not confirmed.

Nano-gold particles can also be used for assay development. A nanoparticle-based antimicrobial susceptibility assay, utilizing the concanavalin A-induced clustering of dextran-coated gold nanoparticles, was developed (Perez et al., 2008). This gold nanoparticles-based assay reportedly provided reliable and faster results within 3 h, as compared to conventional methods that took 24–48 h. Liu et al. (2008) reported a one-step biomolecular detection method using gold nanoparticle bioconjugates. This detection method not only permitted the analysis of cancer protein biomarkers but also potentially allowed the development of bioassays for fast detection and quantitative analysis of DNAs, therapeutic drugs and other biological targets.

Silver nanoparticles have been widely used in cosmetic and medical applications. For example, silver nanoparticles have been used as an additive to burn-dressing. Depending on the methods by which they are produced, the physical and chemical properties of the nanoparticles may significantly differ. Ji et al. (2008) investigated the effects of two types of silver nanoparticles, those of colloidal silver and plasma silver, in human periodontal ligament cells. Consistent with the hypothesis stated above was their finding that the colloidal silver nanoparticles showed higher toxicity than plasma-generated silver nanoparticles, especially in inhibiting proliferation of the ligament cells. Another biomedical application of silver nanoparticles relates to their use in ameliorating

joint inflammation. Use of the ointment containing silver nanoparticles led to faster recovery from temporomandibular joint arthritis (Lee et al., 2008); this beneficial effect was attributed to the anti-inflammatory action of the silver nanoparticles.

In summary, many nanoparticles appear to show some toxicity in various cell types. Consequently, in considering the use of nanoparticles in pharmaceutical and other biomedical applications, one should eliminate the putative cytotoxicity of such particles, which very often exert as a complicating and undesirable characteristic. On the other hand, one can productively exploit the toxicity of nanoparticles in conjunction with existing anti-cancer drugs to arrive at improved drug formulations of combination therapies to enhance their potential and efficacy for treating different kinds of cancers. All in all, the literature suggests that the strategic use of combinations of nanomaterials with other materials may lead to the development of more biocompatible materials with the desired characteristics for different pharmaceutical and other biomedical applications.

## APPLICATIONS OF CHITOSAN AND NANOPARTICLES IN BIOENGINEERING

A recent advance in nanotechnology is the development of a functional nanosystem (such as carbon nanotubes, quantum dots, polymeric micelles, metallic nanoparticles and liposomes), by incorporation, adsorption, or covalent coupling of polymers, carbohydrates, nucleic acids or polysaccharides to the surface of nanoparticles (Nahar et al., 2006). Various types of functional nanosystems are being extensively explored for diverse applications in the biomedical field. A major problem in tissue engineering is high tissue accumulation of non-biodegradable nanoparticles that may hinder mobility

and cause inflammatory/toxicity problems, thus rendering them as “not-so-popular and not-so-desirable” therapeutic and diagnostic systems. This pathophysiological condition is presumed to be related to the immune response elicited by the non-biodegradable nanoparticles and evidently needs further research.

In bioengineering, the layer-by-layer technique used to build thin polyelectrolyte multilayer films is a new promising approach to modify biomaterial surfaces (Karakecili et al., 2007). Adopting this approach, one can combine chitosan with nanoparticles in current and future applications. For instance, chitosan can be used to modify nanoparticles; on the other hand, it can also be functionalized with nanoparticles. The composites of chitosan bonded with nanoparticles may have advantages over chitosan and the bonding materials alone. Chitosan-gold hybrid nanospheres and gold nanoparticles encapsulated with chitosan have been fabricated (Tan and Zhang, 2005; Guo et al., 2008). Because these hybrid composites are relatively new materials, their putative toxicity has yet to be determined. Similarly, biomedical applications of these materials remain to be developed: presumably cell culture systems in vitro may be suitably employed for assisting such developments.

## PHARMACEUTICAL APPLICATIONS OF CHITOSAN AND NANOPARTICLES

Because of good biocompatibility, chitosan has excellent potential in numerous pharmaceutical applications; consequently, it has captured the attention of researchers in pharmaceutical sciences (Dodane and Vilivalam, 1998). The fact that chitosan is highly biocompatible, biodegradable, and shows the ability to open intercellular tight junctions

renders it an almost ideal candidate for formulating drugs for oral delivery (Berradaa et al., 2005). For example, a chitosan-dibasic orthophosphate hydrogel has been formulated and synthesized (Ta et al., 2009). The potential of employing this gel as a prolonged drug delivery vehicle was demonstrated using fluorescein isothiocyanate-dextran, β-lactoglobulin and bovine serum albumin. Moreover, this hydrogel was not toxic to SaOS-2 (human epithelial-like osteosarcoma) cells.

Nanoparticulate delivery system has the potential to improve drug stability, increase the duration of the therapeutic effect and permit administration through non-parental routes. Sarmento et al. (2007a, b) prepared alginate/chitosan nanoparticles and dextran sulfate/chitosan nanoparticles for use in oral insulin delivery. They found that the nanoparticles significantly lowered serum glucose levels in streptozotocin-induced diabetic rats at insulin doses of 50 and 100 IU/kg. The hypoglycemic effect and insulinemia levels were considerably better than those obtained from oral insulin administration alone. Their results indicated that alginate/chitosan nanoparticles and dextran sulfate/chitosan nanoparticles showed favorable characteristics as agents to be employed for formulating oral delivery system for insulin and potentially for other therapeutical proteins. Rekha and Sharma (2009) synthesized and evaluated lauryl succinyl chitosan particles for the applications in oral insulin delivery and absorption. Their results demonstrated that the chitosan particles modified with both hydrophilic (i.e., succinyl) and hydrophobic (i.e., lauryl) moieties had improved the release characteristics, mucoadhesiveness as well as the permeability of insulin compared to those of the native chitosan particles. Thus, their findings suggested that these novel chitosan derivatives may be promising candidates for oral peptide delivery.

Chitosan has also been employed to enhance drug efficacy, especially in formulating anti-cancer drugs. Kim et al. (2008) developed self-assembled glycol chitosan (HGC) nanoparticles for the sustained and prolonged delivery of anti-angiogenic small peptide (containing the anti-angiogenic Arg-Gly-Asp (RGD) peptide) drugs in cancer therapy. They observed that intravenous and intratumoral administration of RGD-HGC nanoparticles into B16F10 tumor-bearing mice resulted in significant decreases in tumor growth and microvessel density compared with corresponding parameters in the tumor-bearing mice injected with native RGD peptide. Thus, their findings suggested the HGC nanoparticles may be valuable in anti-angiogenic therapy and for local and regional tumor therapy. HGC nanoparticles loaded with the anticancer drug docetaxel (DTX) were investigated by Hwang et al. (2008). The DTX-HGC nanoparticles exhibited higher anti-tumor efficacy as indicated by reduced tumor volume and increased survival rate in mice bearing A549 lung cancer cells. Furthermore, the formulation of DTX-HGC nanoparticles resulted in decreased toxicity of DTX in mice compared to toxicity of free DTX in tumor-bearing mice. Consequently, their findings suggested that this type of nano-sized drug carriers may be ideal for formulating drugs for cancer treatment.

A new type of drug delivery system (DDS) involved chitosan-modified single walled carbon nanotubes (SWNTs) for controllable loading/release of an anti-cancer drug, doxorubicin (DOX) (Ji et al., 2011). The DDS containing DOX could effectively kill the HepG2 SMMC-7721 cells and depress the growth of liver cancer in tumor-bearing nude mice. Thus, this finding demonstrated that the efficacy of the chitosan-containing DDS with DOX was superior to that of free DOX and suggested that the chitosan-containing

DDS holds promise for delivering anti-cancer drugs because of its improved efficacy and low side effects.

## CONCLUSIONS

As a biocompatible material, chitosan has found many and diverse biomedical applications. Nevertheless, there is lots of room for improvement in mechanical properties and biocompatibility of chitosan before it becomes an ideal biomaterial for biomedical and tissue engineering applications. For instance, blending chitosan with other polymers can improve its properties and vice versa. Chitosan can also be used to modify other materials to enhance their biocompatibility. On the other hand, chitosan can induce both stimulatory and inhibitory effects on fibroblasts and several other cell types. These contrasting data could be attributed, at least in part, to dissimilar properties and chemical compositions of the biopolymers employed, thus rending it difficult to derive molecular mechanisms underlying such dissimilar effects of chitosan. By the same token, blending chitosan with other biopolymers can very well complement what chitosan cannot achieve alone biocompatibility wise. Consequently, there is a definite need for a systematic approach to categorically classify all the aforementioned parameters.

Due to the ubiquitous existence of nanomaterials, human exposure to nanoparticles is inevitable. Nanoparticles can enter the body through lungs, skin, or via the gastrointestinal track with intake of food, drinks, and medications, or simply by direct exposure to adverse environment such as working in a dusty tunnel. These particles can affect brain, liver, kidney, heart, blood, and other organs and tissues. They are known to induce many cytotoxic effects on neural and non-neural cell types. They may distort and

inhibit cell growth leading to various pathophysiological states in humans and animals. Consequently, nanotoxicology research is now becoming an important endeavor and is gaining and receiving much more attention in the biomedical field.

Before a new material can be employed for tissue engineering or other biomedical applications, it is necessary to test its biocompatibility and/or putative toxicity. Cell cultural models in vitro constitute convenient systems for high throughput screening of the putative toxicity of nanoparticles and other nanomaterials (Rajaraman et al., 1974; Lai et al., 2007, 2008a, 2009, 2010; Jaiswal et al., 2010, 2011; Jain et al., 2011; Lu et al., 2011). Use of these cell models is relatively inexpensive as compared to the use of animal models. The knowledge obtained from the research can lead to prevention of human exposure to nanoparticles and/or nanomaterials that are proven hazardous. Thus far, many nanoparticles show some toxicity to some cell types. Consequently, one needs to pay more attention to the cytotoxicity of nanoparticles and other nanomaterials. On the other hand, one can exploit the inhibitory effects of nanoparticles on cell survival in cancer chemotherapy and drug treatment. Nevertheless, evaluation of nanotoxicity with standard protocols is an urgent issue as more nanomaterials are being used in industry: that includes exposure to workers in confined quarters as well as to the public in an open environment. Whilst cell models are viewed to be the most viable means to pursuit nanotoxicity, the traditional discussion of the pros and cons of cell model versus animal model still persist and is beyond the scope of this review.

Biopolymer films, either based on chitosan itself or chitosan binding with other polymers, have been demonstrated to work well with various types of cells in culture: this encouraging characteristic of chitosan holds promise for a variety of applications in

current and future developments of tissue engineering. However, it would be unrealistic to expect that chitosan and its derivatives would fulfill all the needs in tissue and bioengineering. Most of our organs and tissues are multifunctional; there are receptors and organelles on or beneath the surface of the tissues and cells that function interactively in networks to either promote or suppress biological activities so that our bodily functions can be properly controlled. In fact, these push-pull properties from the multifaceted observations of chitosan and its aforementioned modified forms mimic certain characteristics of tissues and organs. This biomimicry provides a foundation that researchers can build upon. From a tissue engineering point of view, scientific advances have been made in great strides in the last few decades, but the overall state of the art is still in the developmental stage as compared to the development of the electronic industry. One should expect that the development of bioengineering be at a slower rate due to the complexity and variability of the biosystems. To say the least, research needs and opportunities in tissue and bioengineering are abundant now and in the foreseeable future.

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## More information for Chapter I

### TOXICITY OF CARBON NANOTUBES

Carbon nanotubes (CNTs) have distinctive structural, mechanical, electrical, and optical properties due to their small size and mass, their strong mechanical potency, and their high electrical and thermal conductivity (He et al., 2013). The combination of these properties bestows them potentially useful for applications in many fields, including, but not limited to, fabrication of sensors, electrodes, catalysts, actuators, transistors, and capacitors. CNTs have been widely used in biomedical applications such as cancer therapy (Zhang et al., 2011), tissue engineering (MacDonald et al., 2005), and biosensors (Usui et al., 2012).

The promise of CNTs for so many different biomedical applications has led to a strong interest in studying their potential toxicity to human and environmental health. The toxicity of CNTs is attributed to their physicochemical properties, including structure, length and aspect ratio, surface area, degree of aggregation, surface topology, bound functional group(s), manufacturing method, concentration, and dose offered to cells or organisms (Vardharajula et al., 2012). There have been numerous reports on effects of CNTs in various cellular models. However, toxicity data are still very varied and significantly controversial. Some investigations have reported toxic effects following the exposure of several cell types to CNTs, while others demonstrate that very low or no decrease of cell viability.

Due to the morphological similarity of CNTs to asbestos fibers, many studies in pulmonary toxicity of CNTs have been carried out. Numerous in vitro and in vivo studies have shown that CNTs may induce prominent pulmonary inflammation and induction of

cytotoxic effects on lungs (Kayat et al., 2011). Liu and colleagues elucidated that functionalized CNTs (COOH-CNT) induced autophagic cell death in human lung adenocarcinoma A549 cells through the AKT-TSC2-mTOR pathway and caused acute lung injury in vivo (Liu et al., 2011).

Macrophages in the lungs play an important role in the induction of acute and chronic pulmonary inflammation, since they work as phagocytes that ingests foreign substances including CNTs. Some studies investigated the toxicity and mechanism of uptake of CNTs by macrophages. Cheng et al. found that a decrease in human macrophage cell viability was correlated with uptake of multi-walled CNTs (MWNTs) due to mainly necrosis (Cheng et al., 2009). Similarly, it has also been shown that a high concentration (100 μg/ml) of MWCNTs was cytotoxic to macrophage and MWCNTs directly damaged liposomal membranes (Shimizu et al., 2013).

Since CNTs have been reported to be able to cross the blood brain barrier (BBB), there is great interest in investigating potential neurotoxicity (Roldo et al., 2013). Belyanskaya et al. reported that single-walled CNTs (SWCNTs) induced acute toxic effects in primary cultures from both, the central and peripheral nervous system of chicken embryos. They also found that the level of toxicity is at least partially dependent on the agglomeration state of the tubes (Belyanskaya et al., 2009). Furthermore, MWNTs were also able to inhibit regenerative axon growth in a dose-dependent manner (Wu et al., 2012). It has also been shown that the functional group significantly affects cellular toxicity. Mattson et al. reported that embryonic rat-brain neurons could attach to and grow across the surfaces of MWNTs. On unmodified MWNTs, neurons extend only one or two neurites while neurons grown on MWNTs coated with the bioactive molecule 4-

hydroxynonenal elaborated multiple neurites (Mattson et al., 2000). In a similar study, MWNTs modified by amino groups showed stimulation of neuronal neurite outgrowth by activation of the ERK signaling pathway (Matsumoto et al., 2010).

Thus, with the progress in the field of toxicity of CNTs research, it can be asserted that the biocompatibility of CNTs towards cells relies on various properties, including the concentration, degree of aggregation, and surface modifications of CNTs. Functionalized CNTs are generally biocompatible and low toxic for the biomedical purposes. More toxicity evaluations are encouraged to confirm these findings and clarify the toxicological mechanism and determine the safe dosage for each type of CNTs intended for biomedical applications.

## GOALS AND GENERAL HYPOTHESES OF MY DISSERTATION RESEARCH THEME

From my literature review, it is evident that chitosan and nanomaterials (e.g., metal nanoparticles and CNTs) have been widely used in many fields, especially in biomedical applications. Before a new material can be employed for tissue engineering or other biomedical applications, it is necessary to test its biocompatibility and/or putative toxicity. Cell cultural models (e.g., monotypic cell models and co-culture models) *in vitro* constitute convenient systems for investigating the putative toxicity of nanomaterials (e.g., metal nanoparticles and carbon nanotubes). Consequently, the first goal of my dissertation research is to investigate the effects of chitosan, chitosan in combination with nanoparticles (i.e., gold and silver nanoparticles), and chitosan in combinations with nanoparticles and/or three chemotherapeutic drugs (i.e., Adriamycin, Methotrexate, and

Cisplatin) on U87 cells (human brain glioblastoma cell line). I employed a monotypic cell model *in vitro* to investigate hypothesis 1 that the properties of chitosan can be exploited to inhibit U87 cells proliferation and growth and this inhibitory effect of chitosan is greater if I combine it with nanoparticles and/or chemotherapeutic drugs.

The second goal of my dissertation research is to evaluate the putative cytotoxicity of CNTs, chitosan, and chitosan in combination with CNTs on dorsal root ganglion (DRG) neurons, which constitute an excellent model *in vitro* of neurons derived from the peripheral nervous system (PNS), because the effects of CNTs and chitosan on neural cells have not been reportedly studied. Hypothesis 2 is that treatment with CNTs induces a dose-related decrease in the survival/proliferation of DRG neurons, functionalization of CNTs modulates their cytotoxicity, and chitosan has little or no effect on the survival/proliferation of DRG neurons.

Among *in vitro* cell models, co-culture models gained attention with accumulating evidence showing their capacity for mechanistic investigation in tissue engineering research. The close interactions between DRG neurons and Schwann cells may remedy the cytotoxicity induced by silver or gold nanoparticles. The third goal of my dissertation research is to develop a co-culture model *in vitro* employing immortalized DRG neurons and Schwann cells and employ it to investigate our hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against cytotoxicity induced by silver or gold nanoparticles.

To address my goals and hypotheses, I employed some monotypic cell models *in vitro* to investigate the effects of chitosan, chitosan in combination with nanoparticles, and chitosan in combination with nanoparticles and/or chemotherapeutic drugs on U87

cells (chapter II) and the effects of CNTs, chitosan, and chitosan in combination with CNTs on DRG neurons (chapter III). I also developed a co-culture cell model consisting of immortalized DRG neurons and Schwann cells for neurotoxicity and DRG neurons-Schwann cells interactions studies (chapter IV).

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## Chapter II

# Effects of chitosan and nanoparticles on human glioblastoma cells are enhanced in combination treatments with the chemotherapeutic drug Adriamycin*

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**ABSTRACT**

Chitosan has increasingly gained popularity in biomedical applications. Experimental results demonstrated that chitosan exhibited anti-microbial activities through its interaction(s) with microbial cell surface. We hypothesized that the properties of chitosan can be exploited to inhibit

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cancer cell proliferation and growth. In this study, we investigated the effects of chitosan, chitosan in combination with nanoparticles (namely, nanosilver and nanogold particles), and chitosan in combinations with nanoparticles and/or three chemotherapeutic drugs (namely, Adriamycin, Methotrexate, and Cisplatin) on human brain glioblastoma U87 cells. We found that chitosan, chitosan in combination with nanoparticles, and the three chemotherapeutic drugs exerted different inhibitory effects on the survival/proliferation of U87 cells. The inhibitory effects of the drugs individually on the survival/proliferation of U87 cells were greater when employed in combination with chitosan and nanoparticles. We further noted that all these treatments for 72 hours did not induce any increases in lactate dehydrogenase (LDH) release from U87 cells suggesting that necrosis may not be a major cell death mechanism underlying the effects of these treatments. However, these treatments induced marked increases in the cellular production of reactive oxygen species (ROS). Western blot analysis revealed that these treatments also exerted differential effects on the expression of p-AKT and p-ERK (important cell survival/proliferation signals) in U87 cells. Thus, our findings suggested that alterations in ROS generation and in AKT and ERK signaling were involved in the treatment-induced lowering of the survival/proliferation of U87 cells. Taken together, these results suggested that chitosan and nanoparticles may have chemotherapeutic potential in the design of new and/or improved treatments for glioblastoma.

*Keywords*: chitosan, nanoparticles, chemotherapeutic drugs, glioblastoma.

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## 1. Introduction

A putatively biocompatible material, chitosan is the deacetylated product of chitin and is a linear polysaccharide composed of randomly distributed β-(1, 4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan

has been widely employed by researchers as an important and promising biomaterial in tissue engineering [1,2], wound healing [3,4], and drug delivery [5,6] because of its low cost, large-scale availability, anti-microbial activity, as well as its biodegradability and biocompatibility [7].

Amongst the most recent developments and advances in applications of nanoparticles in nanotechnology and biotechnology is the deployment of nanoparticles in cell cultures in drug discovery and drug delivery studies: these advances are considered ground-breaking in addition to the more established applications of nanoparticles as probes and in imaging [8]. Nanoparticles include all particles that possess at least one dimension that is less than 100 nm. The material origins of the particles can be organic, inorganic, metals, polymers, etc. Nanoparticles possess unique properties, more importantly, a large surface-to-volume ratio which accounts for the high surface reactivity for many of these particles. These favorable properties are being exploited in multiple applications, especially in recent biomedical ones [8]. Among the metal nanoparticles, nanogold and nanosilver particles have gained particular interest in biomedical applications because nanogold particles possess favorable biocompatibility and stability [9] and nanosilver particles possess antimicrobial properties [10]. Thus, it is not surprising nanoparticles such as nanogold and nanosilver may have utility in cancer nanotechnology.

Glioblastoma is the most common primary brain tumors in adults. It is the highest grade (grade IV as defined by the World Health Organization) astrocytoma and is characterized by increased proliferation and invasion into the surrounding normal tissues [11-14]. Despite some recent improvement in the treatment of this malignancy, the

prognosis of patients with glioblastoma remains extremely poor [12,13]. Over the past 30 years, the median survival time of patients with malignant glioblastomas has only been improved from 6 months to 16 months [13,15,16]. Thus, there is an urgent need to find new and/or improved treatments for glioblastoma [12,13,16].

Adriamycin, Methotrexate and Cisplatin are three widely used drugs in cancer chemotherapy. Adriamycin is one of the most frequently used anticancer agents for the treatment of a variety of cancers including, but not limited to, small cell lung cancer, breast cancer, sarcoma, lymphoma, and acute leukemia [17,18]. Methotrexate is an antimetabolite (antifolate) drug used in treatment of cancers and autoimmune diseases [19,20]. Cisplatin has been used to treat various types of cancers including sarcomas, some carcinomas, glioblastoma, lymphomas, and germ cell tumors [13,20,21]. Even though the three drugs have been widely used in cancer chemotherapy, over the years they have been demonstrated to be much less efficacious as monotherapies in treating different cancers because upon being treated with these drugs, the cancers invariably develop resistance to one or more of these drugs [13]. Nevertheless, they may still be efficacious in combination chemotherapy [13].

Combining the knowledge of material science with that of biopharmaceutical sciences proved to be fruitful and promising in devising novel experimental strategies in discovering new and/or improved combination therapies for treating deadly cancers such as glioblastoma [8,13]. These [8,13] and other [22,23] considerations have prompted us to initiate the current series of studies to determine the feasibility of combining chitosan and nanoparticles with well-known chemotherapeutic agents such as Adriamycin, Methotrexate and Cisplatin in the design of new combination chemotherapies for treating

glioblastoma. For example, previous studies demonstrated chitosan film and chitosan in combination with nanoparticles could promote wound healing [24] and proliferation of keratinocytes [25]. Therefore, we hypothesized that chitosan film can inhibit cancer cell growth because chitosan exhibits anti-microbial activities through its interaction(s) with microbial cell surface to alter cell permeability [26]. We also hypothesized that this inhibitory effect of chitosan is greater if we combine it with nanoparticles and/or chemotherapeutic drugs. This study, therefore, aims to investigate these hypotheses by examining the effects of chitosan, chitosan in combination with nanoparticles (namely, nanogold and nanosilver particles), and chitosan in combinations with nanoparticles and/or three chemotherapeutic drugs (namely, Adriamycin, Methotrexate, and Cisplatin) on human brain glioblastoma U87 cells.

## 2. Materials and methods

### *2.1 Chemical reagents and antibodies*

Chitosan (from shrimp shells, minimum 75% deacetylated, and molecular weight 190-375 kDa), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Adriamycin, Methotrexate, and Cisplatin were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Tetrachloroauric (III) acid ($HAuCl_4 \bullet 3H_2O$), trisodium citrate ($C_6H_5Na_3O_7 \bullet 2H_2O$) and silver nitrate ($AgNO_3$) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The monoclonal antibody against AKT, phospho-AKT (Ser473), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2) and β-actin were obtained

from Cell Signaling Technology (Beverly, MA, USA). Goat polyclonal to rabbit IgG and rabbit polyclonal to mouse IgG were purchased from Abcam Inc. (Cambridge, MA, USA). All chemicals were of analytical grade unless otherwise stated.

*2.2 Preparation and dilution of stock solution of drugs*

Stock solution of 10 mM Adriamycin or Methotrexate was prepared with DMSO and further dilutions (0.001, 0.01 and 0.1 μM) were prepared with culture medium. Cisplatin stock solution of 13 μM was prepared with DMSO and further diluted solutions (0.013, 0.13 and 1.3 μM) were prepared with culture medium. The stock solutions were sterilized using a 0.22 μm microfilter in a laminar flow hood and stored at -20$^{o}$C. All dilutions were prepared fresh before being added to the cells.

*2.3 Cell culture*

Human astrocytoma U87 cells, obtained from ATCC (Manassas, VA, USA), were cultured in modified Eagle's medium (MEM) supplemented with 10% (v/v) FBS and incubated at 37$^{o}$C and 5% $CO_2$.

*2.4 Preparation of chitosan films*

The preparation of chitosan films was as described previously [24]. Briefly, a certain amount of chitosan was weighed and dissolved in 100 ml 1% (v/v) acetic acid solution at room temperature overnight. The solution was filtered to remove insoluble particles in the chitosan solution, poured onto a plastic plate, and then oven-dried at a constant temperature of 40$^{o}$C for 24 hours to form a solid film. The dry transparent film was

carefully peeled off from the plastic plate, washed with 5% (w/v) NaOH aqueous solution until the pH reached about neutral and then repeatedly washed with distilled water. After that, the chitosan film was punched out in the form of circles with ~15 mm in diameter. Subsequently, the circular membranes were sterilized in 70% (v/v) ethanol overnight and then exposed to ultraviolet light for 40 minutes on each side. Finally each circular piece of chitosan film was rinsed extensively with sterile phosphate-buffered saline (PBS) and then placed into a 24-well culture plate.

*2.5 Preparation of nanosilver and nanogold particles*

To prepare nanosilver particles, $AgNO_3$ and $C_6H_5Na_3O_7 \cdot 2H_2O$ solutions were filtered through a 0.22 μm microporous membrane filter prior to being used for preparing nanosilver particles. Nanosilver particles were prepared according to the literature [27] by adding $C_6H_5Na_3O_7 \cdot 2H_2O$ solution to boiling $AgNO_3$ aqueous solution. The prepared concentration of nanosilver particles was about 108 μg/ml and their size was about 60 nm as characterized by scanning electron microscopy [24].

To prepare nanogold particles, $HAuCl_4 \cdot 3H_2O$ and $C_6H_5Na_3O_7 \cdot 2H_2O$ solutions also were filtered through a 0.22 μm microporous membrane filter prior to being used for preparing nanogold particles. Nanogold particles were prepared according to the literature [28] by adding $C_6H_5Na_3O_7 \cdot 2H_2O$ solution to boiling $HAuCl_4 \cdot 3H_2O$ aqueous solution. The prepared concentration of nanogold particles was about 49 μg/ml and their size was about 34 nm as characterized by transmission electron microscopy [29].

*2.6 Preparation of nanosilver/chitosan and nanogold/chitsan scaffolds*

A specified amount of nanosilver or nanogold solution was added into each well of the 24-well culture plate in which a sterile chitosan film had already been placed. After 12 hours, nanosilver or nanogold solution was aspirated with residual nanoparticles attached on the film, and sterile PBS was added twice into each well to wash the film.

*2.7 Cell survival/proliferation assay*

Cell survival/proliferation was determined by using the modified MTT assay [30-32]. U87 cells were seeded with equal density in each well of the 24-well plates with or without specified concentrations of chitosan film, nanosilver/chitosan scaffolds or nanogold/chitsan scaffolds on the bottom of each well and cultured as described above. After 1 hour (allowing cells attached to the bottom of each well), cells were treated with or without (ie, the control) specified concentrations of the drugs investigated (namely, Adriamycin, Methotrexate or Cisplatin). At the end of the incubation period, 100 μL MTT dye (0.5% (w/v) in PBS) was added into each well and the plate was incubated for an additional 4 hours at 37°C. The purple-colored insoluble formazan crystals in viable cells were dissolved using DMSO and the subsequent absorbance (designated as X) of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA).

The chitosan, nanosilver or nanogold particles by themselves had absorbance. Thus, their absorbance (ie, the control sets of wells) had to be subtracted from the absorbance of live cells with different treatments as depicted in the preceding paragraph [32]. The control sets of wells were set up alongside those sets of wells in the plates as detailed in the preceding paragraph except that the control sets of wells did not contain any seeded

cells. At the end of the specified culture period, 100 μL of MTT dye (0.5% (w/v) in PBS) was added into each well and the plates were incubated for an additional 4 hours at 37°C. The subsequent absorbance (designated as Y) of the content of each well was measured at 570 nm as described above. (X-Y) was taken as the absorbance attributed to viable cells in each well [32].

*2.8 Lactate dehydrogenase (LDH) release*

Necrotic cell damage and cell death was determined by measuring the activity of LDH released from the cells into the culture medium. As described previously, U87 cells were treated with or without chitosan film or chitosan in combinations with nanosilver particles and/or Adriamycin and cultured in MEM in petri dishes at 37°C for 72 hours. Subsequently, the culture medium from each petri dish was removed and kept at -80°C until they were used for assaying LDH activity therein. LDH activity released by cells into the culture medium was assayed by the procedure of Clark and Lai [33].

*2.9 Western blot analysis*

Western blot analysis was carried out as described previously [23]. Briefly, cell lysates were prepared from cells treated with or without chitosan film or chitosan in combinations with nanosilver particles and/or Adriamycin. Equal amounts of cell lysate proteins (20 μg) were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 70 minutes. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane at 100 V for 1 hour.

The membrane was blocked with 5% (w/v) non-fat dry milk in TBS-T (0.05% (v/v) Tween-20 in Tris-buffered saline) at 4˚C for 4 hours and washed with TBS-T 5 times for 5 minutes each. Subsequently, the membrane was incubated at 4˚C overnight with primary antibodies at the following concentrations: rabbit anti-AKT [1:1000], rabbit anti-phospho-AKT [1:1000], rabbit anti-ERK [1:1000], rabbit anti-phospho-ERK [1:1250] or mouse anti-β-actin [1:1000]. Then the membrane was incubated with a goat polyclonal antibody to rabbit IgG for 45 minutes after being washed with TBS-T 5 times for 5 minutes each. To verify equal loading of samples, the membrane was subsequently incubated with monoclonal antibodies to β-actin, followed by a rabbit polyclonal antibody to mouse IgG. The expression of the protein of interest was determined using enhanced chemiluminescence (ECL) detection.

*2.10 Reactive oxygen species (ROS) assay*

The intracellular accumulation of ROS was determined by using the fluorescent probe $H_2$DCFDA (2', 7'-dihydrodichlorofluorescein diacetate), which is an indicator of ROS [34]. U87 cells were seeded with equal density in each well (with or without chitosan film or chitosan in combinations with nanosilver particles and/or Adriamycin) of the 24-well plates and cultured in an incubator for 72 hours. After that, 200 µL 10 µM of $H_2$DCFDA dye (dissolved in DMSO) was added into each well and incubated for 45 minutes and then the medium was replaced with 200 µL sterile PBS. The fluorescence formed from the oxidized dye was measured at excitation of 492 nm and emission of 521 nm employing a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA).

*2.11 Protein determinations*

ROS activity was normalized to the amount of protein present in the homogenates. Protein content of the homogenates was determined using the bicinchoninic acid (BCA) assay in a 96-well plate reader with the wavelength set at 562 nm [35].

*2.12 Statistical analysis*

All measurements were repeated with 3 sets at a minimum of 6 samples for each set, and all data were recorded as the mean ± standard error of the mean (shown in Figures). Data analysis was carried out by one-way analysis of variance (ANOVA), followed by post-hoc Student–Newman–Keuls test for multiple comparisons using the software KaleidaGraph version 4 (Synergy Software, Reading, PA, USA). Significance level was set at $p<0.05$.

## 3. Results

*3.1 Effects of chitosan, chitosan in combination with different concentrations of chemotherapeutic drugs on survival of U87 cells*

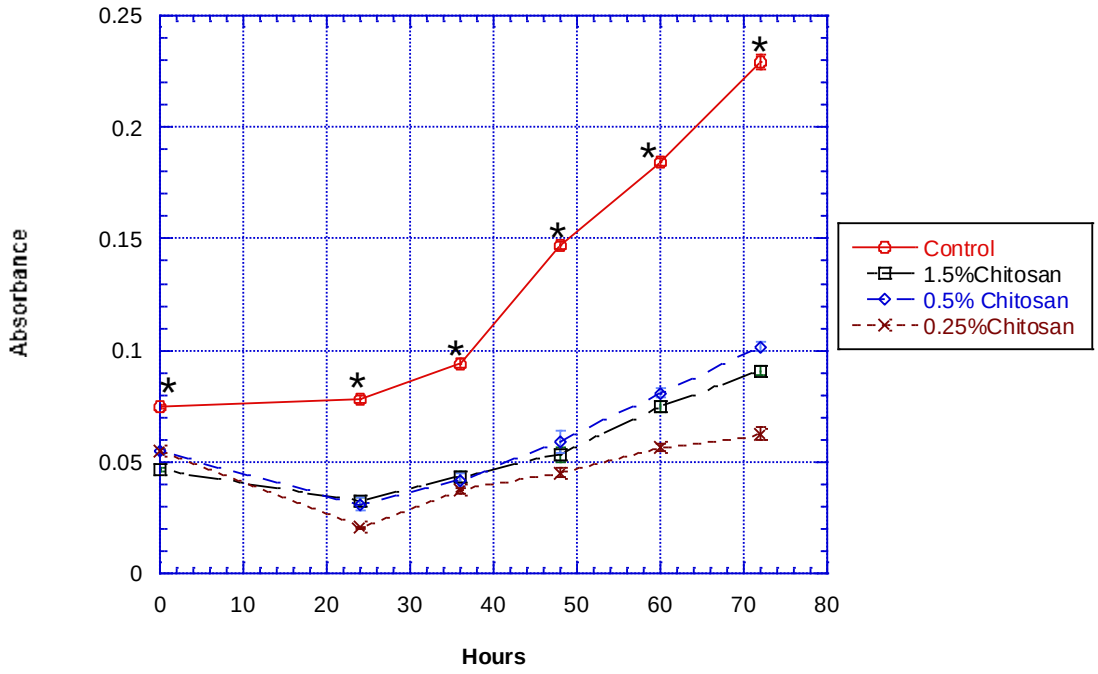


**Fig. 1A.** Effects of different concentrations of chitosan on survival of human glioblastoma U87 cells.

**Notes:** U87 cells were treated with different concentrations of chitosan for various times up to 72 hours. Afterwards, their survival/proliferation was determined using the MTT assay. Values are mean ± SEM of 6 determinations. Untreated U87 cells are marked with red circles. U87 cells treated with 1.5% chitosan are marked with black squares, those treated with 0.5% chitosan are marked with blue diamonds, and those treated with 0.25% chitosan are marked with brown crosses. Values marked with * are significantly different ($p<0.05$, by ANOVA and post-hoc Student–Newman–Keuls test) from corresponding mean value in treated cells.

**Abbreviations:** ANOVA, analysis of variance; SEM, standard error of the mean.

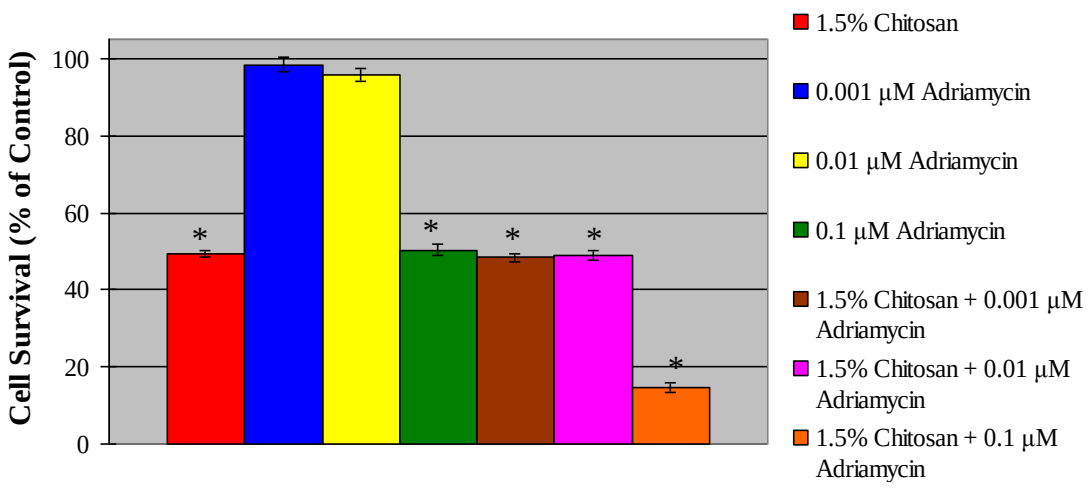


**Fig. 1B.** Effects of 1.5% chitosan, different concentrations of Adriamycin, and 1.5% chitosan in combination with different concentrations of Adriamycin on survival of human glioblastoma U87 cells.

**Notes:** U87 cells were treated with 1.5% chitosan alone, different concentrations of Adriamycin alone, or 1.5% chitosan in combination with different concentrations of Adriamycin for 72 hours. Afterwards, their survival/proliferation was determined using the MTT assay. Values are mean ± SEM of 6 determinations. U87 cells treated with 1.5% chitosan are marked with red column, those treated with 0.001 μM Adriamycin are marked with blue column, those treated with 0.01 μM Adriamycin are marked with yellow column, those treated with 0.1 μM Adriamycin are marked with green column, those treated with 1.5% chitosan in combination with 0.001 μM Adriamycin are marked with brown column, those treated with 1.5% chitosan in combination with 0.01 μM Adriamycin are marked with pink column, and those treated with 1.5% chitosan in combination with 0.1 μM Adriamycin are marked with orange column. Values marked with * are significantly different ($p<0.05$, by ANOVA and post-hoc Student–Newman–Keuls test) from corresponding mean value in control (ie, untreated) cells.

**Abbreviations:** ANOVA, analysis of variance; SEM, standard error of the mean.

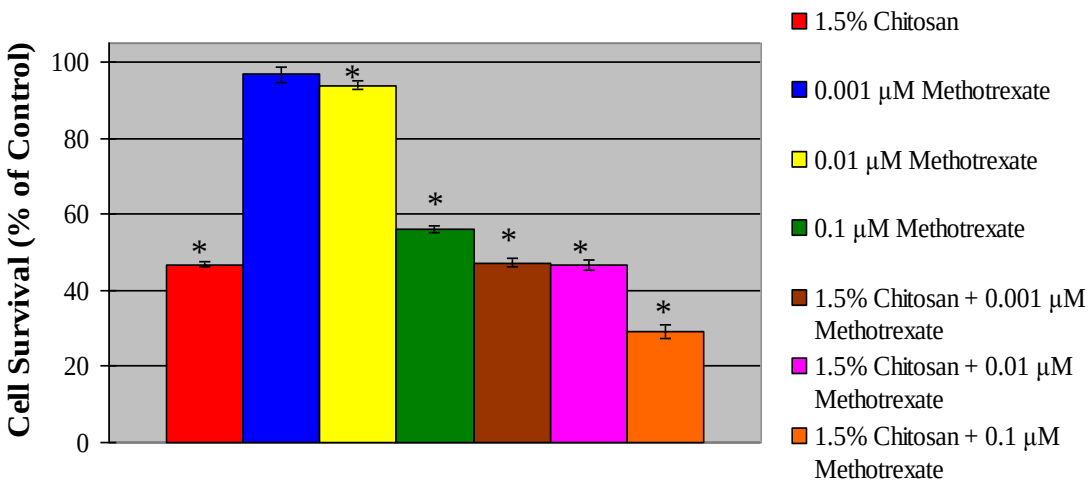


**Fig. 1C.** Effects of 1.5% chitosan, different concentrations of Methotrexate, and 1.5% chitosan in combination with different concentrations of Methotrexate on survival of human glioblastoma U87 cells.

**Notes:** U87 cells were treated with 1.5% chitosan alone, different concentrations of Methotrexate alone, or 1.5% chitosan in combination with different concentrations of Methotrexate for 72 hours. Afterwards, their survival/proliferation was determined using the MTT assay. Values are mean ± SEM of 6 determinations. U87 cells treated with 1.5% chitosan are marked with red column, those treated with 0.001 µM Methotrexate are marked with blue column, those treated with 0.01 µM Methotrexate are marked with yellow column, those treated with 0.1 µM Methotrexate are marked with green column, those treated with 1.5% chitosan in combination with 0.001 µM Methotrexate are marked with brown column, those treated with 1.5% chitosan in combination with 0.01 µM Methotrexate are marked with pink column, and those treated with 1.5% chitosan in combination with 0.1 µM Methotrexate are marked with orange column. Values marked

with * are significantly different ($p<0.05$, by ANOVA and post-hoc Student–Newman–Keuls test) from corresponding mean value in control (ie, untreated) cells.

**Abbreviations:** ANOVA, analysis of variance; SEM, standard error of the mean.

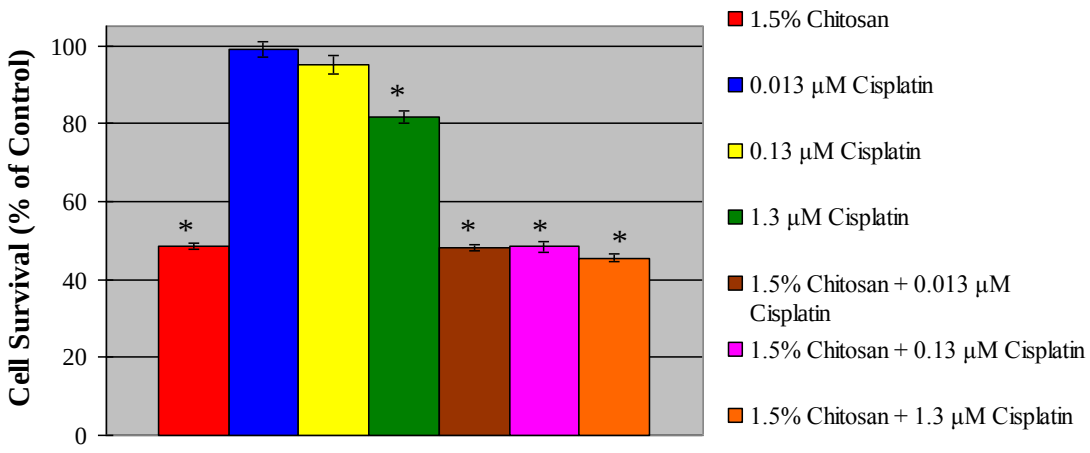


**Fig. 1D.** Effects of 1.5% chitosan, different concentrations of Cisplatin, and 1.5% chitosan in combination with different concentrations of Cisplatin on survival of human glioblastoma U87 cells.

**Notes:** U87 cells were treated with 1.5% chitosan alone, different concentrations of Cisplatin alone, or 1.5% chitosan in combination with different concentrations of Cisplatin for 72 hours. Afterwards, their survival/proliferation was determined using the MTT assay. Values are mean ± SEM of 6 determinations. U87 cells treated with 1.5% chitosan are marked with red column, those treated with 0.013 µM Cisplatin are marked with blue column, those treated with 0.13 µM Cisplatin are marked with yellow column, those treated with 1.3 µM Cisplatin are marked with green column, those treated with 1.5% chitosan in combination with 0.013 µM Cisplatin are marked with brown column, those treated with 1.5% chitosan in combination with 0.13 µM Cisplatin are marked with pink column, and those treated with 1.5% chitosan in combination with 1.3 µM Cisplatin

are marked with orange column. Values marked with * are significantly different ($p<0.05$, by ANOVA and post-hoc Student–Newman–Keuls test) from corresponding mean value in control (ie, untreated) cells.

**Abbreviations:** ANOVA, analysis of variance; SEM, standard error of the mean.

We first determined the effect of several concentrations of chitosan, in the form of a film, on survival/proliferation of human glioblastoma U87 cells. As shown in Fig. 1A, within 72 hours, untreated U87 cells began to proliferate 36 hours after plating and were in the log phase of growth from then till 72 hours. All three concentrations chitosan (namely, 0.25, 0.5 and 1.5%) tested significantly ($p<0.05$) decreased the rate of proliferation of U87 cells (Fig. 1A). We therefore decided to employ 1.5% chitosan in all subsequent studies to investigate the effects of chitosan in combination with nanoparticles with or without the addition of a chemotherapeutic drug.

As shown in Fig. 1B, C and D, chitosan induced approximately 50% decrease in survival/proliferation of U87 cells. Exposure of U87 cells for 72 hours to either Adriamycin or Methotrexate alone at concentrations below 0.1 μM did not affect their survival/proliferation as determined by the MTT assay (Fig. 1B and C). On the other hand, at the concentration of 0.1 μM, both Adriamycin and Methotrexate alone induced a nearly 50% decrease in survival/proliferation of U87 cells (Fig. 1B and C). Compared to Adriamycin and Methotrexate, Cisplatin showed less inhibitory effect on U87 cells (compare Fig. 1B, C and D). At the highest concentration (1.3 μM) employed, Cisplatin only induced less than 20% decrease in survival/proliferation of U87 cells (Fig. 1D). The rank order of the effects of the three drugs in lowering survival/proliferation of U87 cells

was: Adriamycin > Methotrexate >> Cisplatin.

All three drugs enhanced its inhibitory effect in lowering survival/proliferation of U87 cells when used in combination with chitosan; Adriamycin in combination with chitosan was the most effective among the three drugs examined (compare Fig. 1B, C and D). Thus, in all subsequent experiments, we employed 1.5% chitosan with or without 0.1 μM Adriamycin to determine the combination treatment effects of chitosan with nanoparticles in the absence or presence of Adriamycin.

*3.2 Effects of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of U87 cells*

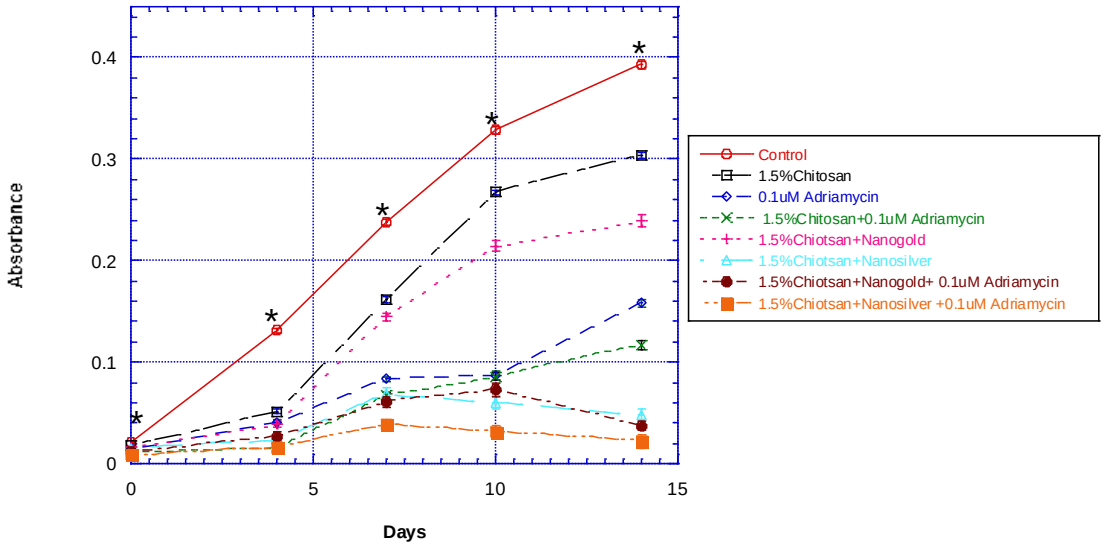


**Fig. 2.** Effects of 1.5% chitosan and 1.5% chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival of human glioblastoma U87 cells.

**Notes:** U87 cells were treated with 1.5% chitosan alone, 0.1 μM Adriamycin alone, 1.5% chitosan in combination with nanoparticles, or 1.5% chitosan in combination with nanoparticles plus 0.1 μM Adriamycin for various times up to 14 days. Afterwards, their

survival/proliferation was determined using the MTT assay. Values are mean ± SEM of 6 determinations. Untreated U87 cells are marked with red circles. U87 cells treated with 1.5% chitosan are marked with black squares, those treated with 0.1 μM Adriamycin are marked with blue diamonds, those treated with 1.5% chitosan in combination with 0.1 μM Adriamycin are marked with green crosses, those treated with 1.5% chitosan in combination with nanogold particles are marked with pink pluses, those treated with 1.5% chitosan in combination with nanosilver particles are marked with light blue triangles, those treated with 1.5% chitosan in combination with nanogold particles plus 0.1 μM Adriamycin are marked with brown circles, and those treated with 1.5% chitosan in combination with nanosilver particles plus 0.1 μM Adriamycin are marked with orange squares. Values marked with * are significantly different ($p<0.05$, by ANOVA and post-hoc Student–Newman–Keuls test) from corresponding mean value in treated cells.

**Abbreviations:** ANOVA, analysis of variance; SEM, standard error of the mean.

We examined the effects of chitosan, chitosan in combination with nanoparticles and/or 0.1 μM Adriamycin on survival/proliferation of U87 cells in culture for up to 14 days. As shown in Fig. 2, all the treatments induced time-related decreases in the survival/proliferation of U87 cells. There were apparent differences in the effects exerted by different treatments. Over the 14 days' treatment, chitosan with nanosilver plus 0.1 μm Adriamycin was the most effective combination treatment, chitosan with nanogold plus 0.1 μm Adriamycin was the second most effective combination treatment, and chitosan alone was the least effective treatment in lowering the survival/proliferation of U87 cells (Fig. 2). These results suggested that a combination of chitosan, nanoparticles, and

Adriamycin was more effective than chitosan or Adriamycin alone, or chitosan with nanoparticles. Chitosan with nanosilver particles showed greater effect than chitosan with nanogold particles indicating that nanosilver particles were more cytotoxic to U87 cells than nanogold particles (Fig. 2). Because chitosan with nanosilver plus 0.1 μm Adriamycin was the most effective combination treatment in lowering the survival/proliferation of U87 cells (Fig. 2), we chose it for further study in combination treatments.

*3.3 Effects of chitosan, chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin on LDH release from U87 cells into the medium*

To further elucidate the effects of the combination treatments on U87 cells, we determined the effects of these treatments on inducing LDH release from U87 cells into the medium because LDH release from cells is a marker of necrotic cell damage and cell death [22]. Treatment of U87 cells with these treatments for 72 hours did not induce any increases in LDH from these cells, suggesting that necrosis may not be a major cell death mechanism underlying the effects of chitosan, chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin in U87 cells (Fig. 3).

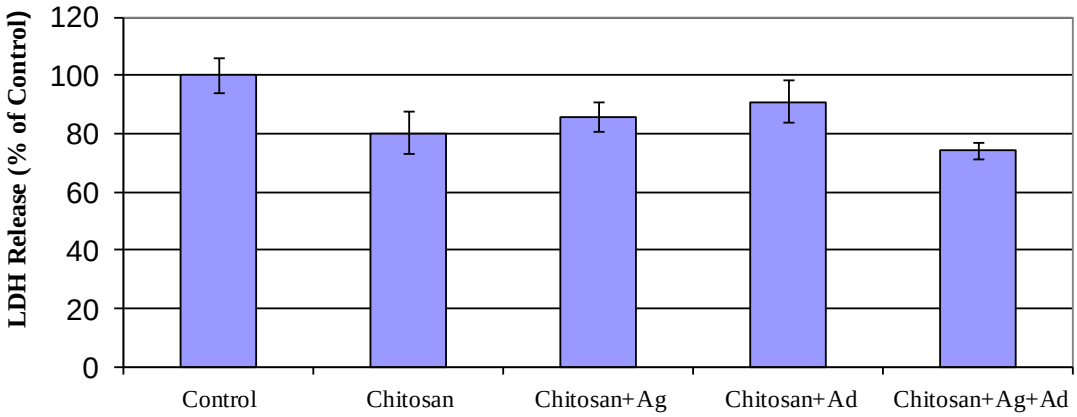


**Fig. 3.** Effects of 1.5% chitosan, 1.5% chitosan in combination with nanosilver particles and/or 0.1 μM Adriamycin on LDH release from human glioblastoma U87 cells into the medium.

**Notes:** U87 cells were treated with 1.5% chitosan alone, 1.5% chitosan in combination with nanosilver particles, 1.5% chitosan in combination with 0.1 μM Adriamycin, or 1.5% chitosan in combination with nanosilver particles and 0.1 μM Adriamycin for 72 hours. Then the culture medium were collected as described in Materials and methods. LDH activity released by cells into the culture medium was assayed by LDH release assay.

**Abbreviations:** LDH, lactate dehydrogenase; Ag, nanosilver particles; Ad, Adriamycin.

*3.4 Effects of chitosan, chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin on the AKT, p-AKT, ERK, and p-ERK protein expression in U87 cells*

To further elucidate the molecular mechanisms underlying the effects of the combination treatments on U87 cells, we examined the expression of cell survival

signaling pathways after the cells had been exposed to the treatments for 72 hours. Since PI3K/AKT signaling is overly active in most of the glioblastomas [12,36], we investigated the effects of the treatments on expression of the AKT signaling pathway by Western blot analysis (Fig. 4). Our results indicated that the treatments did not induce any changes in total AKT expression in U87 cells (Fig. 4A). However, the effects of the treatments on their phosphorylated AKT (p-AKT) expression were different from those on total AKT (Fig. 4A and B). Treatment with chitosan in combination with nanosilver particles and treatment with chitosan in combination with nanosilver particles and 0.1 μM Adriamycin induced increases in their expression of p-AKT (Fig. 4B). However, treatment of U87 cells for 72 hours with chitosan alone or with chitosan in combination with 0.1 μM Adriamycin did not affect their expression of p-AKT (Fig. 4B).

Activation of the ERK signaling pathway resulting in phosphorylation of ERK is one of the regulatory pathways implicated in controlling cell proliferation/growth [12,23,37]. Since ERK plays an important role in cancer cell proliferation [12,23], we also determined the effects of the combination treatments on the protein expression of this signaling pathway in U87 cells (Fig. 4C and D). Our results showed a pattern different from the corresponding effects of combination treatments on the AKT signaling pathway (Fig. 4). The total ERK expression in U87 cells remained almost unchanged after all treatments administered (Fig. 4C). Treatment of U87 cells for 72 hours with chitosan alone resulted in a decrease in their phosphorylated ERK (p-ERK) expression (Fig. 4D). Treatment of U87 cells with chitosan in combination with nanosilver particles and treatment with chitosan in combination with nanosilver particles and 0.1 μM Adriamycin led to a significant increase in their expression of p-ERK (Fig. 4D). However, treatment

of U87 cells for 72 hours with chitosan combined with 0.1 μM Adriamycin did not affect their expression of p-ERK (Fig. 4D).

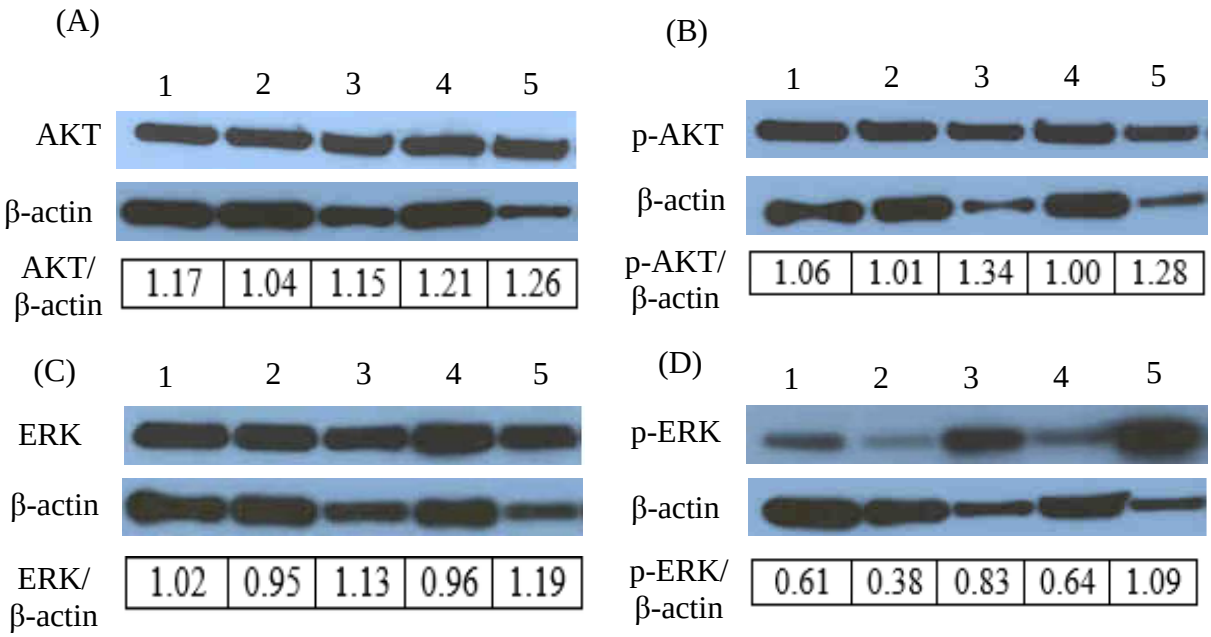


**Fig. 4.** Effects of 1.5% chitosan, 1.5% chitosan in combination with nanosilver particles and/or 0.1 μM Adriamycin on the AKT, p-AKT, ERK, and p-ERK protein expression in human glioblastoma U87 cells.

**Notes:** U87 cells were treated with 1.5% chitosan alone, 1.5% chitosan in combination with nanosilver particles, 1.5% chitosan in combination with 0.1 μM Adriamycin, or 1.5% chitosan in combination with nanosilver particles and 0.1 μM Adriamycin for 72 hours. Then cell lysates of treated and untreated (ie, control) U87 cells were prepared as described in Materials and methods. The expression of AKT, p-AKT, ERK, p-ERK was determined by Western blot analysis using β-actin as the loading control: Lane 1, lysate of control (ie, untreated) U87 cells; lane 2, lysate of U87 cells treated with 1.5% chitosan; lane 3, lysate of U87 cells treated with 1.5% chitosan in combination with nanosilver particles; lane 4, lysate of U87 cells treated with 1.5% chitosan in combination with 0.1

μM Adriamycin; lane 5, lysate of U87 cells treated with 1.5% chitosan in combination with nanosilver particles and 0.1 μM Adriamycin. The blots were from a typical experiment. Two other experiments yielded essentially the same trend of results.

**Abbreviations:** AKT, protein kinase B; p-AKT, phosphorylated protein kinase B; ERK, extracellular-signal-regulated kinases; p-ERK, phosphorylated extracellular-signal-regulated kinases.

*3.5 Effects of chitosan, chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin on ROS generation from U87 cells*

To examine the effects of chitosan, chitosan in combination with nanosilver particles and/or 0.1 μM Adriamycin on the generation of ROS, we used the $H_2$DCFDA assay. As shown in Fig. 5, treatment of the U87 cells with all treatments for 72 hours resulted in marked increases of the intracellular ROS levels. Treatment with chitosan in combination with nanosilver particles and/or 0.1 μM Adriamycin induced higher levels of ROS in these cells than treatment with chitosan alone and chitosan in combination with 0.1 μM Adriamycin.

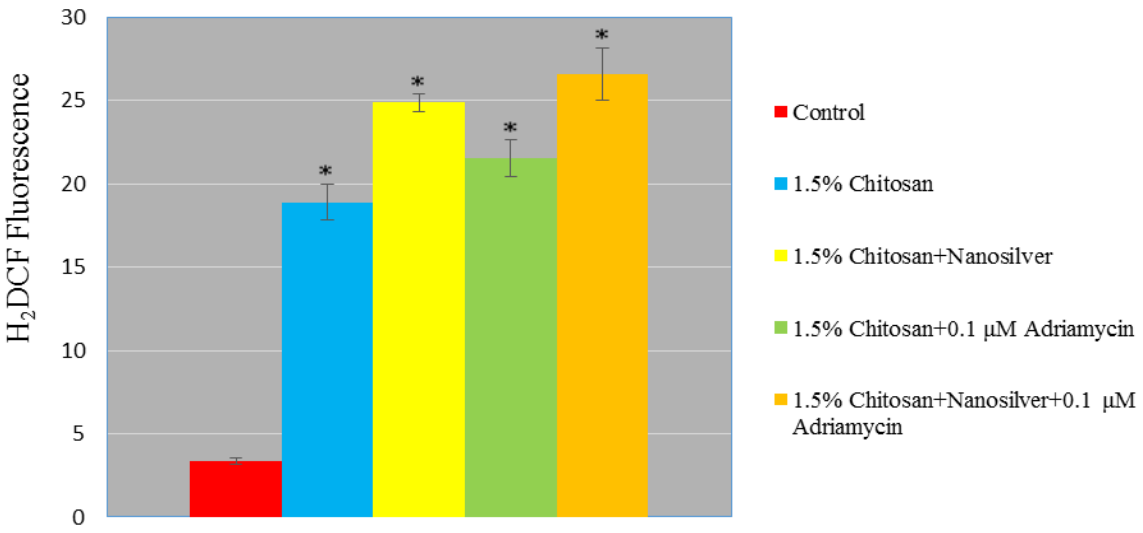


**Fig. 5.** Effects of 1.5% chitosan, 1.5% chitosan in combination with nanosilver particles and/or 0.1 µM Adriamycin on the ROS generation from human glioblastoma U87 cells.

**Notes:** U87 cells were treated with 1.5% chitosan alone, 1.5% chitosan in combination with nanosilver particles, 1.5% chitosan in combination with 0.1 µM Adriamycin, or 1.5% chitosan in combination with nanosilver particles and 0.1 µM Adriamycin for 72 hours. Then treated and untreated (ie, control) U87 cells were prepared as described in Materials and methods. Untreated U87 cells are marked with red column, those treated with 1.5% chitosan are marked with blue column, those treated with 1.5% chitosan in combination with nanosilver particles are marked yellow column, those treated with 1.5% chitosan in combination with 0.01 µM Adriamycin are marked with green column, those treated with 1.5% chitosan in combination with nanosilver particles plus 0.1 µM Adriamycin are marked with orange column. Values marked with * are significantly different ($p<0.05$, by ANOVA and post-hoc Student–Newman–Keuls test) from corresponding mean value in control (ie, untreated) cells.

**Abbreviation:** ROS, reactive oxygen species.

## 4. Discussion

To our knowledge this study is the first to report on the effects of chitosan and nanosilver and nanogold particles, with and without the combination treatment with chemotherapeutic drugs (namely, Adriamycin, Methotrexate, and Cisplatin) on human glioblastoma U87 cells. In particular, this study investigated our hypothesis that the anti-cancer property of chitosan is enhanced if it is employed in combination treatment with nanogold or nanosilver particles and/or anti-cancer drugs. In accord with our hypothesis, we found chitosan and the nanoparticles exerted some anti-survival/proliferative effects on U87 cells and the presence of the chemotherapeutic drugs tested markedly enhanced the anti-survival/proliferative effects of chitosan and the nanoparticles on U87 cells (Fig. 1 and 2).

Treatment with chitosan alone lowered the survival/proliferation of glioblastoma U87 cells (Fig. 1 and 2). While treatment with Adriamycin, Methotrexate or Cisplatin alone at concentrations tested below 0.1 μM did not significantly affect the survival/proliferation of U87 cells (Fig. 1B, C, and D), at treatment concentration of 0.1 μM or higher, all three chemotherapeutic drugs, when used alone, induced significant decreases in the survival/proliferation of U87 cells, with the rank order of Adriamycin > Methotrexate >> Cisplatin (Fig. 1B, C, and D). Moreover, 1.5% chitosan in treatment combination enhanced this inhibitory effect of the three drugs (especially that of Adriamycin) in U87 cells (Fig. 1B, C, and D). Additionally, in combination treatments with chitosan, both nanogold and naosilver particles further enhanced the inhibitory effect of Adriamycin on the survival/proliferation of U87 cells (Fig. 2). We also noted that chitosan in combination with nanosilver particles showed greater inhibitory effects

than chitosan in combination with nanogold particles on the survival/proliferation of the U87 cells (Fig. 2), suggesting that nanogold particles are more biocompatible than nanosilver particles.

Our observation that the combination inhibitory effects of chitosan, Adriamycin and nanosilver particles on the survival/proliferation of U87 cells were marked (Fig. 2) prompted us to investigate some of the cell survival/proliferation signaling mechanisms underlying the effects of chitosan, chitosan in combination with nanosilver particles, and chitosan in combination with nanosilver particles and Adriamycin on U87 cells. Since AKT and ERK signaling pathways play a major role in survival and proliferation of many types of cells including cancer cells [12,23,38], we determined the effects of these treatments on the protein expression of the two signaling pathways in human glioblastoma U87 cells by Western blot analysis.

We found that treatment with chitosan, chitosan in combination with nanosilver particles, and chitosan in combination with nanosilver particles and Adriamycin exerted differential effects on the expression of p-AKT and p-ERK proteins in U87 cells (Fig. 4). Treatment of U87 cells for 72 hours with chitosan alone resulted in a marked and significant decrease in p-ERK expression (Fig. 4D). This finding suggested that the anti-proliferative and/or anti-survival effect of chitosan alone may be mediated by the marked decrease in p-ERK level in U87 cells [12,23,38]. However, chitosan in combination with nanosilver particles and chitosan in combination with nanosilver particles and 0.1 μM Adriamycin led to increases in the expression of the p-AKT and p-ERK (Fig. 4B and D).

Although activation (ie, via phosphorylation) of AKT and ERK pathways is usually associated with the delivery of a survival/proliferation signal, recent studies have linked

the activation of AKT and ERK with induction of apoptosis. For example, activation of ERK by fluoxetine and imatinib [39] in U87 cells has been reportedly associated with induction of apoptosis. Thus, it is conceivable that our observation that chitosan in combination with nanosilver particles and chitosan in combination with nanosilver particles and 0.1 μM Adriamycin led to increases in the expression of the p-AKT and p-ERK (Fig. 4B, and D) may be associated with the activation of apoptosis rather than the lowering of survival/proliferation signaling in U87 cells.

ROS mediate thymoquinone-induced colon cancer cells apoptosis and activate ERK signaling has also been reported [40]. To test this possibility, we investigated the effects of these treatments on ROS generation from U87 cells. Our results showed that all treatments elevated the level of intracellular ROS of U87 cells, especially treatment with chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin (Fig. 5). Thus, our proposed mechanism of chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin anti-survival/proliferative effects can be summarized as follows. Chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin treatment induced ROS generation, which increased AKT and ERK in an attempt to bypass the stress injury. However, AKT and ERK fail to confer a survival role, and the cells undergo apoptosis. Clearly, further studies are needed to clarify this mechanistic issue.

## 5. Conclusions

Our studies demonstrated that exposure of human brain glioblastoma U87 cells to chitosan and nanogold or nanosilver particles induced decreases in their survival/proliferation. The three anti-cancer drugs (namely, Adriamycin, Methotrexate, and

Cisplatin) exerted differential inhibitory effects on the survival/proliferation of U87 cells. The inhibitory effects of the drugs individually on the survival/proliferation of U87 cells were greater when employed in combination with chitosan and nanoparticles. These treatments induced marked increases in the cellular production of ROS. Western blot analysis showed that these treatments also exerted differential effects on the expression of p-AKT and p-ERK in U87 cells. Thus, our findings suggested alterations in ROS generation and in AKT and ERK signaling were involved in the treatment-induced lowering of the survival/proliferation of U87 cells. Taken together, these results suggest that combination treatments containing chitosan, nanoparticles, and the more conventional chemotherapeutic agents such as Adriamycin may have chemotherapeutic potential in the design of new and/or improved treatments for glioblastoma.

## 6. Conflicts of interest

The authors report no conflicts of interest in this work.

## 7. Acknowledgments

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# Chapter III

## Effects of short multi-walled carbon nanotubes and chitosan in dorsal root ganglion (DRG) neurons*

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**Abstract:** Although carbon nanotubes (CNTs) have found their way into diverse industrial and biomedical applications, the impact of CNTs on human and environmental health has not been elucidated: this is especially the case regarding the putative toxic effects of CNTs on cells of the nervous system. Chitosan is generally regarded as a biocompatible material showing potential in nerve regeneration research and related applications. Nevertheless, the effects of chitosan on neural cells are virtually unknown.

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In this study we have investigated the effects of functionalized (namely, carboxylated and hydroxylated), non-functionalized short multi-walled carbon nanotubes (SMWCNTs), chitosan, and chitosan in combination with SMWCNTs on dorsal root ganglion (DRG) neurons which constitute an excellent model *in vitro* of neurons derived from the peripheral nervous system (PNS). We found SMWCNTs induced concentration-related decreases in the growth of DRG neurons, and the non-functionalized SMWCNTs were more cytotoxic than the functionalized ones. SMWCNTs induced necrotic damage in the neurons and decreased their expression of phospho-Akt. Chitosan alone definitely induced a lowering of the proliferation/growth of DRG neurons but had virtually no effect in inducing lactate dehydrogenase (LDH) release from the neurons. Our findings from confocal microscopy strongly suggested that exposure of DRG neurons to SMWCNTs resulted in the entry of the SMWCNTs into the cytoplasm of the neurons. Taken together, our results also suggested that the cytotoxic effects of SMWCNTs in DRG neurons could be attributed, at least in part, to their penetration into the cytoplasm of the neurons. Thus, our results may have pathophysiological implications in how exposure to SMWCNTs impacts the structure and function of the PNS.

**Key Words:** carbon nanotubes, chitosan, cytotoxicity of carbon nanotubes, DRG neurons, nanotoxicity.

## INTRODUCTION

Carbon nanotubes (CNTs) are the graphite sheets rolled into cylindrical tubes of nanoscale diameter and length in nano- or micro-meter ranges. Based on the number of concentric cylinders of graphite sheets, CNTs are categorized into single- or multi-

walled.[1] They have unique chemical, electrical, and mechanical properties, which render them potentially useful for applications in many fields, including, but not limited to, fabrication of sensors, electrodes, catalysts, actuators, transistors, and capacitors.[2-4] CNTs have gained increasing popularity in biological and/or biomedical applications at the molecular and cellular levels, not least of all because they are electrically conductive, have diameters ranging between ~1 nm (single-walled carbon nanotubes) and 10–100 nm (multi-walled carbon nanotubes), and have aspect ratios close to that of nerve fibers.[5] Some studies have shown that CNTs can be modified so that they can become more biomimetic and can be adapted to facilitate molecular mechanisms such as biomolecular recognition and transport.[6-8] Thus, further advances in these and related areas of biomedical research and development will undoubtedly lead to the fabrication of novel, more biocompatible biomimetic devices and sensors.[6–8] Their ever-increasing uses in industrial and biomedical applications render humans more and more exposed to CNTs, especially in the workplace and other arenas where they are found.[2-4,9] However, the human and environmental health hazard of exposure to CNTs has not been assessed even though recent animal studies have clearly demonstrated various types of CNTs, upon entering the body, can induce toxicity to several organs and tissues, including the liver.[3,9,10] Nonetheless, the effects of CNTs on the nervous system and on neural cells are virtually unknown.[4]

We have initiated a series of studies to systematically investigate the putative cytotoxicity of a variety of nanomaterials, including CNTs, in several mammalian cell types.[4,11-21] Because the putative neurotoxicity of CNTs has not been reportedly

studied,[4,9] we have developed several neural cell models *in vitro* to facilitate such cytotoxicity studies.[11-21]

Chitosan is the fully or partially deacetylated form of chitin.[22] Due to its presumed biocompatibility, biodegradation, and other similarly favorable biological properties, chitosan has been tested for its suitability as a scaffolding material in nerve regeneration studies.[23] Two such studies ─ one employing 9L gliosarcoma cells and primary cultures of mouse cerebrocortical neurons[24] and the other primary chick dorsal root ganglion neurons[25] ─ reported chitosan and chitosan-derived materials were apparently biocompatible and did not exert any significant cytotoxic effects on the neural cell types investigated *in vitro*. However, because primary cultures of neurons are known to have very limited life span, they are thus unsuitable for investigating mechanisms underlying neuronal degeneration and regeneration, especially those mediating long-term effects. Consequently, to overcome such limitations, we have employed immortalized dorsal root ganglion (DRG) neurons (50B11)[26] to develop peripheral nervous system (PNS) neural cell models *in vitro* suitable for investigating effects of chitosan on neurons and elucidating mechanisms underlying nerve degeneration and regeneration.[26, 27]

Here we report on our investigation of the putative cytotoxic effects of both functionalized and non-functionalized short multi-walled carbon nanotubes (SMWCNTs), chitosan, and chitosan in combination with SMWCNTs on DRG neurons, which constitute an excellent model *in vitro* of neurons derived from the PNS.[19-21,26,27] The aim of this study is to investigate the hypotheses that treatment with SMWCNTs induces a dose-related decrease in viability of DRG neurons, functionalization of SMWCNTs modulates their cytotoxicity, and chitosan has little or no effect on the growth of DRG

neurons. In addition, we also investigate the possibility that important signaling pathways that regulate cell survival/proliferation (namely, extracellular signal-regulated kinase (ERK) signaling pathway and Akt signaling pathway) are mediating such effects on DRG neurons.

## MATERIALS AND METHODS

### Chemical reagents and antibodies

SMWCNTs were purchased from Cheap Tubes Inc. (Brattleboro, VT, USA). Chitosan, thiazolyl blue tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). The monoclonal antibody against Akt, phospho-Akt (Ser473), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2) and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Goat polyclonal to rabbit IgG and rabbit polyclonal to mouse IgG were purchased from Abcam Inc. (Cambridge, MA, USA). 3, 3'-Dioctadecyloxacarbocyanine perchlorate ('DiO') and red protein gel stain concentrate in DMSO were purchased from Invitrogen (Grand Island, NY, USA). All chemicals were of analytical grade and unless otherwise stated were obtained from Sigma-Aldrich (St Louis, MO, USA).

### Cell culture

DRG (50B11) neurons were kind gifts from Dr Höke's Laboratory at Johns Hopkins School of Medicine. DRG neurons were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 10% (v/v) FBS.

**Cell viability assay**

Cellular viability was determined by using the modified MTT assay.[14,28,29] Cells were seeded into the wells of 24-well plates at a density of $1.2\times10^4$ cells/well in the presence of 0 (control), 1, 10, 50, or 100 μg/mL SMWCNTs with or without the 1.5% chitosan film on the bottom of each well[30] and cultured as described above (The 1.5% chitosan film was prepared as described previously.[30]). At the end of the specified culture period, MTT dye (0.5% (w/v) in PBS) was added to each well and the plates were incubated for an additional 4 hours at 37°C. The purple-colored insoluble formazan crystals in viable cells were dissolved using 200 μL DMSO and the subsequent absorbance (designated as X) of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA) as described previously.[29]

The SMWCNTs by themselves had absorbance: thus, their absorbance (i.e., the control sets of wells) had to be subtracted from the absorbance of live cells with different treatments as depicted in the preceding paragraph.[14] The control sets of wells were set up alongside those sets of well in the plates as detailed in the preceding paragraph except that the control sets of wells did not contain any seeded DRG neurons. At the end of the specified culture period, 100 μL of MTT dye (0.5% (w/v) in PBS) was added to each well and the plates were incubated for an additional 4 hours at 37°C. The subsequent absorbance (designated as Y) of the content of each well was measured at 570 nm as described above. (X-Y) was taken as the absorbance attributed to viable cells in each well.[14]

**Lactate dehydrogenase (LDH) release**

Necrotic cell damage and cell death was determined by measuring the activity of LDH released from the cells into the culture medium. LDH release from cells is a marker of necrotic cell damage and cell death.[15,18] DRG neurons were cultured in DMEM in 75 $cm^2$ flasks until they were ~50% confluent and then treated with different concentrations of SMWCNTs for 0, 1, 3, and 5 days at 37 °C. Subsequently, the culture medium from each flask were removed and kept at −80 °C until they were used for assaying LDH activity therein. LDH released by cells into the culture medium was assayed by the procedure of Clark and Lai.[31]

**Western blot analysis**

Expression of proteins of interests was determined by Western blot analysis essentially as described previously. [15,18] Cell lysates were prepared from cells treated with or without 100 μg/mL SMWCNTs. Equal amounts of cell lysate proteins were electrophoresed by SDS-PAGE for 85 minutes at 120 V. The separated proteins were transferred electrophoretically to PVDF membrane for 1 hour at 100 V.

The membranes were blocked with 5% (w/v) non-fat dry milk in TBS-T (0.05% (v/v) Tween-20 in Tris-buffered saline) for 4 hours at 4˚C and washed with TBS-T: 5×5 minutes. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies at the following concentrations: rabbit anti-Akt [1:1000], rabbit anti-phospho-Akt [1:1000], rabbit anti-ERK [1:1000], rabbit anti-phospho-ERK [1:1000] and mouse anti-β-actin [1:1000]. Then the membranes were incubated with a goat polyclonal to rabbit IgG for 45 minutes after being washed with TBS-T: 5×5 minutes. To verify equal loading of samples, the membranes were subsequently incubated with monoclonal

antibody to β-actin, followed by a rabbit polyclonal to mouse IgG. The expression of the protein of interest was determined using ECL (enhanced chemiluminescence) detection. Average pixel intensities of protein bands were quantified using NIH software UN-SCAN IT.

**Confocal laser scanning microscopy**

DRG neurons were cultured on coverslips in 6-well plates in the presence of non-functionalized SMWCNTs at 10 μg/mL for 3 days. Then the neurons were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde at room temperature for 15 minutes. Subsequently, the fixed neurons were washed with PBS to remove the remaining paraformaldehyde and the neurons were stained with 'DiO' and red protein gel stain according to the manufacturer's instructions. Briefly, 100 μL of 12.5 μg/mL 'DiO' was added to the fixed neurons on each coverslip and the latter was incubated for 20 minutes at 37°C. Then the neurons on the coverslips were washed with PBS three times and 200 μL diluted (dilution 1:20000) red protein gel stain was added to each coverslip and the latter was incubated at room temperature for 20 minutes. After the staining steps, the neurons on the coverslips were washed three times with PBS and were examined and their images captured using an Olympus (Center Valley, PA, USA) FV1000 confocal laser scanning microscope.

**Statistical analysis of data**

Experiments were performed at least three times with a minimum of 6 replicates for each set, and all data were recorded as mean ± standard deviation (shown in figures). The

results from different treatment groups were analyzed using one-way ANOVA with post-hoc Tukey test for multiple comparisons between groups. The level of significance was set at $p<0.05$.

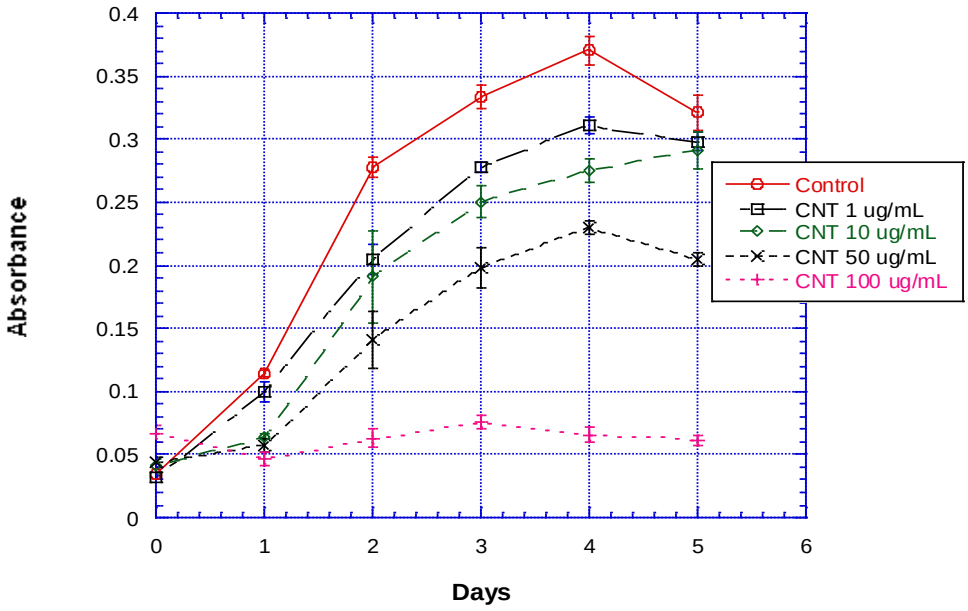
## RESULTS

### Effects of SMWCNTs on survival of DRG neurons

Employing the modified MTT assay, we systematically compared the effects of non-functionalized SMWCNTs with those of two functionalized (namely, carboxylated and hydroxylated) SMWCNTs on DRG neurons (Fig. 1). As shown in Figure 1A, exposure of DRG neurons to non-functionalized SMWCNTs induced a concentration- and time-related decrease in survival, proliferation, and/or growth of the neurons.

Exposure of DRG neurons to carboxylated (Fig. 1B) or hydroxylated (Fig. 1C) SMWCNTs also induced concentration- and time-related decreases in survival, proliferation, and/or growth of the neurons. However, while the effects of the functionalized SMWCNTs were somewhat similar, the effects of the non-functionalized SMWCNTs were generally more pronounced than those of the functionalized SMWCNTs, especially at the higher treatment concentrations of SMWCNTs (compare Fig. 1A with Fig. 1B and Fig. 1C). Taken together, these results suggest that non-functionalized SMWCNTs are generally more cytotoxic to DRG neurons compared to the functionalized SMWCNTs.

**(A)**


Absorbance
Days
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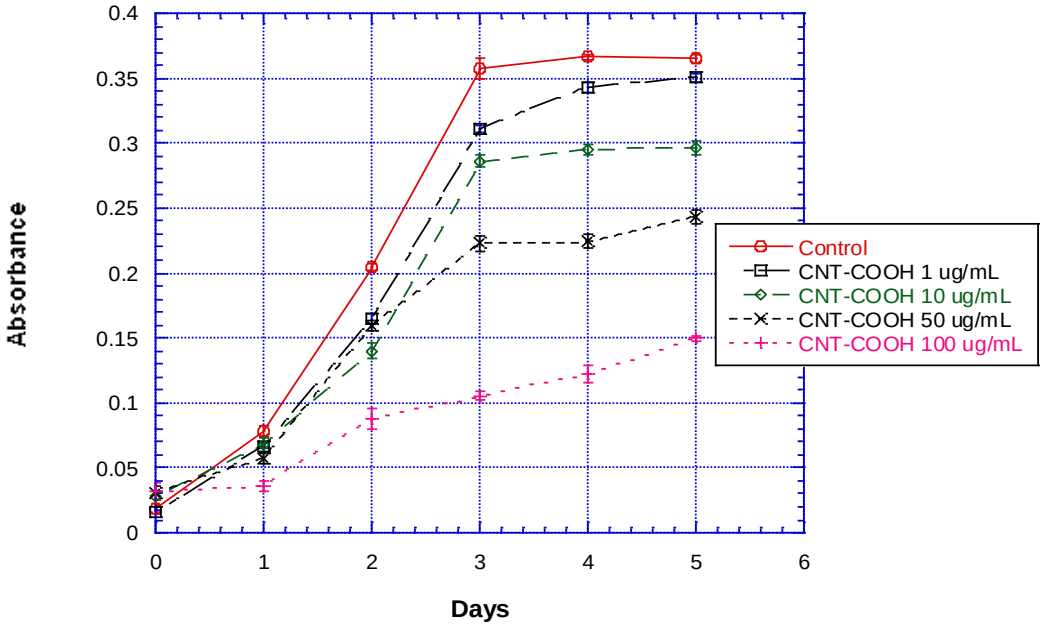
CNT 50 ug/mL
CNT 100 ug/mL

**(B)**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CNT-COOH 50 ug/mL
CNT-COOH 100 ug/mL

**(C)**

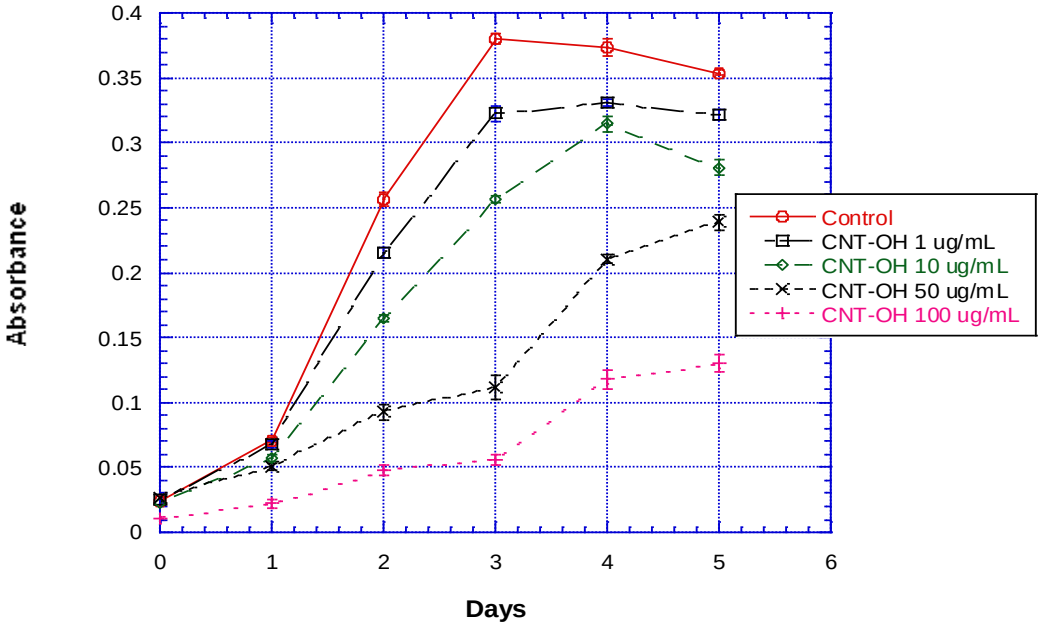



**FIGURE 1.** Effect of different concentrations of SMWCNTs on the growth of DRG neurons for 5 days. DRG neurons were treated with A) non-functionalized SMWCNTs, B) carboxylated SMWCNTs, and C) hydroxylated SMWCNTs for 5 days.

**Effects of 1.5% chitosan and SMWCNTs on survival of DRG neurons**

Chitosan has been considered as having the potential to be a candidate material suitable for application in nerve regeneration. However, the effects of chitosan on the neural cells (e.g., DRG neurons) are virtually unknown. We therefore examined the effects of chitosan and chitosan in combination with non-functionalized SMWCNTs on survival and growth of DRG neurons. As shown in Figure 2, exposure to chitosan alone appeared to definitely induce a lowering of the proliferation/growth of DRG neurons. Exposure of DRG neurons to chitosan in combination with non-functionalized SMWCNTs also induced concentration- and time-related decreases in survival, proliferation, and/or growth of the neurons. Moreover, the presence of 1.5% chitosan accentuated the

concentration- and time-related decreases in survival and/or growth of DRG neurons induced by non-functionalized SMWCNTs (compare Fig. 2 with Fig. 1A).

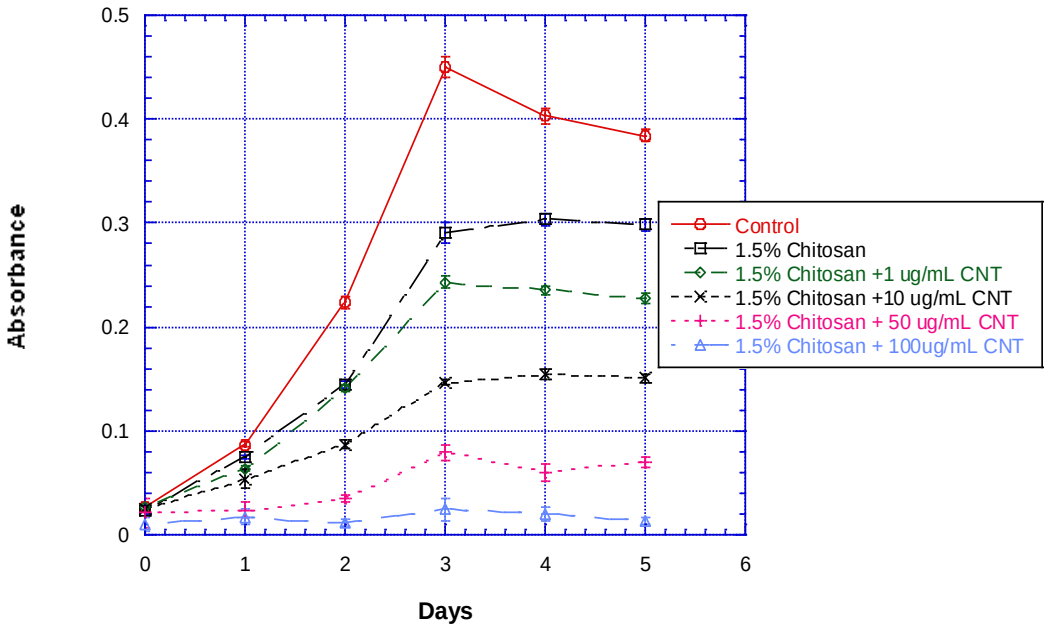



**FIGURE 2.** Effects of 1.5% chitosan and different concentrations of non-functionalized SMWCNTs on growth of DRG neurons for 5 days. DRG neurons were treated with 1.5% chitosan and 1, 10, 50 or 100 µg/mL SMWCNT for 5 days.

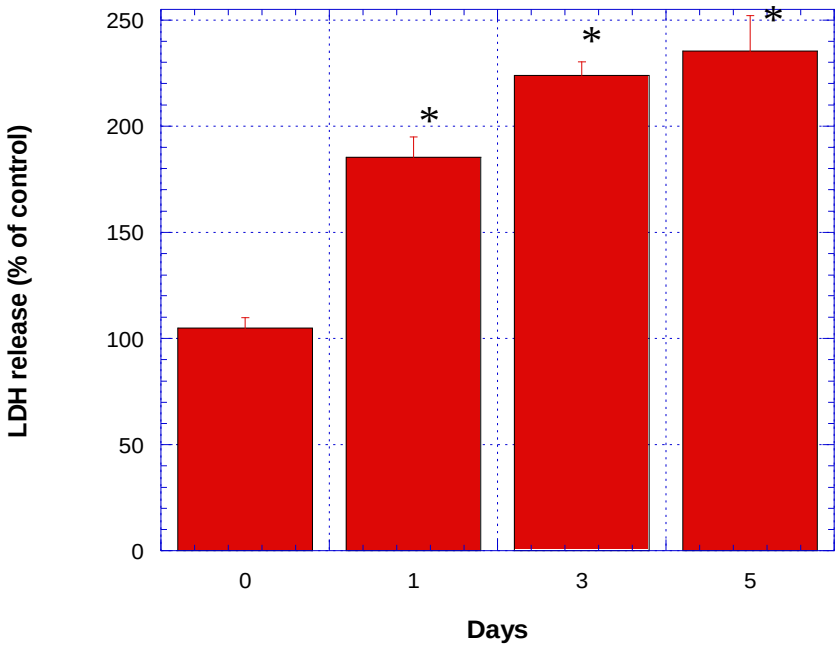
**Effects of non-functionalized SMWCNTs and carboxylated SMWCNTs on LDH release from DRG neurons into the medium**

Because non-functionalized and functionalized SMWCNTs exerted concentration- and time-related decreases in survival/growth of DRG neurons (Fig. 1), we investigated that possibility that necrosis is one mechanism mediating the effects of SMWCNTs in DRG neurons. We therefore compared the effects of exposing DRG neurons to 100 µg/mL (a concentration in which the exposed DRG neurons showed substantial decreases in survival/growth) of non-functionalized (Fig. 3A) or carboxylated (Fig. 3B) SMWCNTs

on their release of LDH into the medium over the 5-day period. When LDH levels in the culture medium of DRG neurons exposed to 100 μg/mL non-functionalized SMWCNTs were compared to those of the controls (namely, untreated DRG neurons), LDH releases from the DRG neurons treated with non-functionalized SMWCNTs showed significant time-related increases ($p<0.0001$; Fig. 3A), indicating that the non-functionalized SMWCNTs induced time-related increases in necrotic damage and cell death in the treated DRG neurons. Thus, these findings (Fig. 3A) strongly suggest necrosis may at least be one cell death mechanism underlying the cytotoxicity of the non-functionalized SMWCNTs in DRG neurons.

As shown in Figure 3B, exposure of DRG neurons to carboxylated SMWCNTs also induced increases in LDH release from DRG neurons. However, these increases were not time-related. Treatment of DRG neurons with carboxylated SMWCNTs elicited the highest increases (~ 40%) in LDH release from neurons treated for 1 day, elicited ~ 20% increases in LDH release from neurons treated for 3 days, and only ~ 5% increases in LDH release from neurons treated for 5 days. A comparison of the data shown in Figures 3A and 3B allows us to conclude that under identical exposure conditions, non-functionalized SMWCNTs induced more increases in LDH release from DRG neurons than carboxylated SMWCNTs. Thus, these results (Fig. 3A and B) are compatible with the notion that, consistent with findings based on the MTT assay (Fig. 1), non-functionalized SMWCNTs are generally more cytotoxic to DRG neurons compared to the carboxylated SMWCNTs.

**(A)**




**(B)**

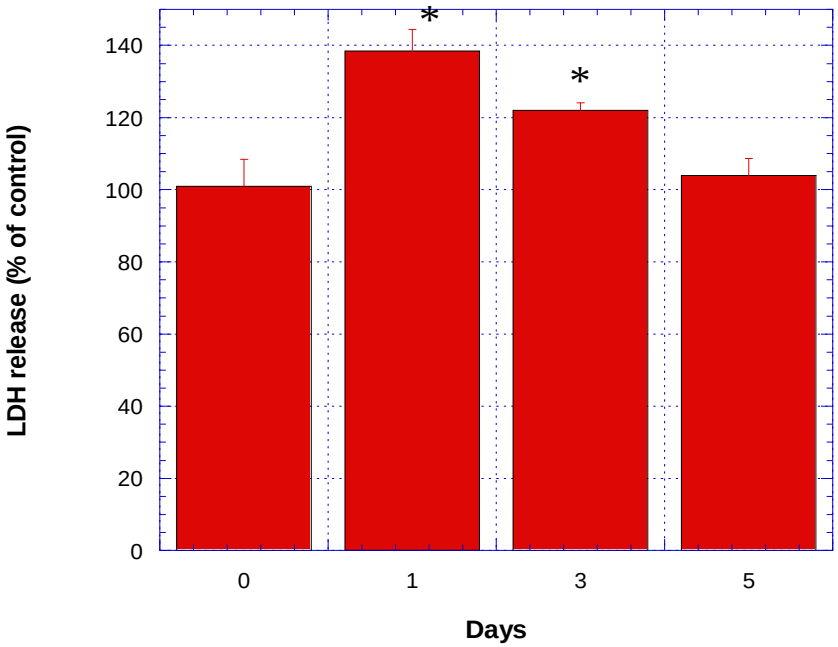



**FIGURE 3.** Effects of 100 μg/mL SMWCNTs on LDH release from DRG neurons into the medium. DRG neurons were treated with A) non-functionalized SMWCNTs and B) carboxylated SMWCNTs; * $p<0.0001$ versus control.

**Effects of 1.5% chitosan and non-functionalized SMWCNTs on LDH release from DRG neurons into the media**

Because we observed that not only non-functionalized SMWCNTs exerted concentration- and time-related effects on survival/proliferation of DRG neurons (Fig. 1A) but also 1.5% chitosan appeared to modulate such effects (compare Fig. 1A with Fig. 2), we investigated the possibility that 1.5% chitosan might similarly modulate on the effect of non-functionalized SMWCNTs on LDH released by DRG neurons into the medium. We found that, in the presence of 1.5% chitosan, treatment of DRG neurons with 100 μg/mL non-functionalized SMWCNTs induced them to release LDH into the medium in a time-related manner ($p<0.0001$; Fig. 4). Because the data in Fig. 4 closely paralleled those in Fig. 3A, we concluded the presence of 1.5% chitosan did not markedly influence the effects of non-functionalized SMWCNTs in inducing DRG neurons to release LDH into the medium. In other words, 1.5% chitosan did not significantly modulate the necrotic effect of non-functionalized SMWCNTs (employed at 100 μg/mL) on DRG neurons.

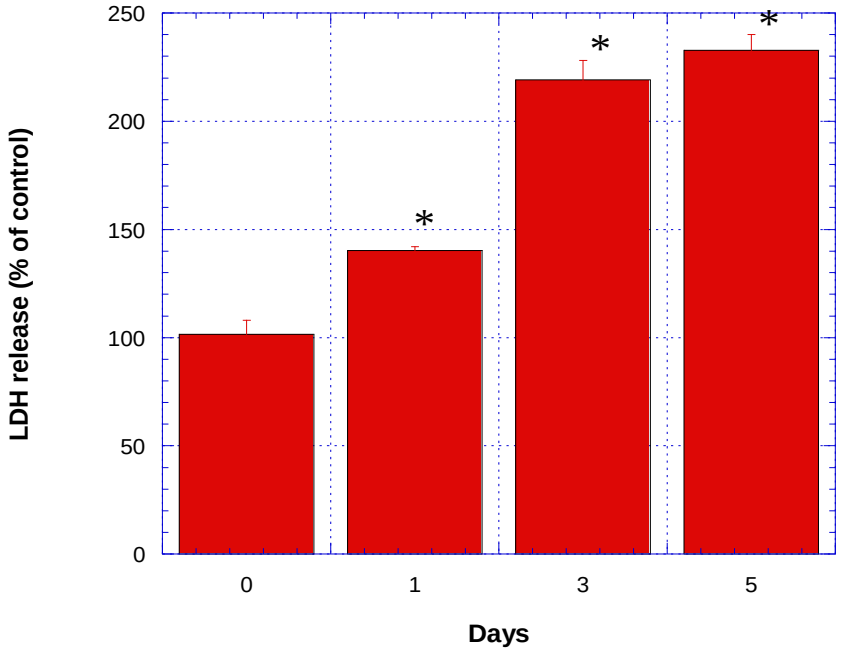


**FIGURE 4.** Effects of 1.5% chitosan and non-functionalized SMWCNTs on LDH release from DRG neurons into the medium. DRG neurons were treated with 1.5% chitosan with or without 100 μg/mL non-functionalized SMWCNTs; * $p<0.0001$ versus control.

**Effects of non-functionalized SMWCNTs, carboxylated SMWCNTs, and 1.5% chitosan on the Akt, p-Akt, ERK, and p-ERK protein expressions in DRG neurons**

Even though we found that necrosis is likely the one cell-death type underlying the cytotoxic effects of non-functionalized SMWCNTs in lowering the survival of DRG neurons (Fig. 3A), there remained the possibility that other mechanisms (e.g., cell survival/proliferation signaling) may also contribute to their cytotoxic effects. We therefore investigated this hypothesis by examining the effects of non-functionalized SMWCNTs on expression of key cell survival/proliferation signaling pathways (namely, Akt and ERK) in DRG neurons (Fig. 5). Our results showed that exposure of DRG neurons to 100 μg/mL non-functionalized SMWCNTs induced time-related decreases in the expression of phospho-Akt (Fig. 5B). However, the same treatment only induced small changes in expression of Akt, ERK, and phospho-ERK in the treated DRG neurons (Fig. 5). Because phospho-Akt is an important signaling protein involved in cell survival and/or proliferation, our observation suggested that another mechanism whereby the non-functionalized SMWCNTs could lower the survival and/or proliferation of DRG neurons was through decreasing signaling pathways such as those involving phospho-Akt.

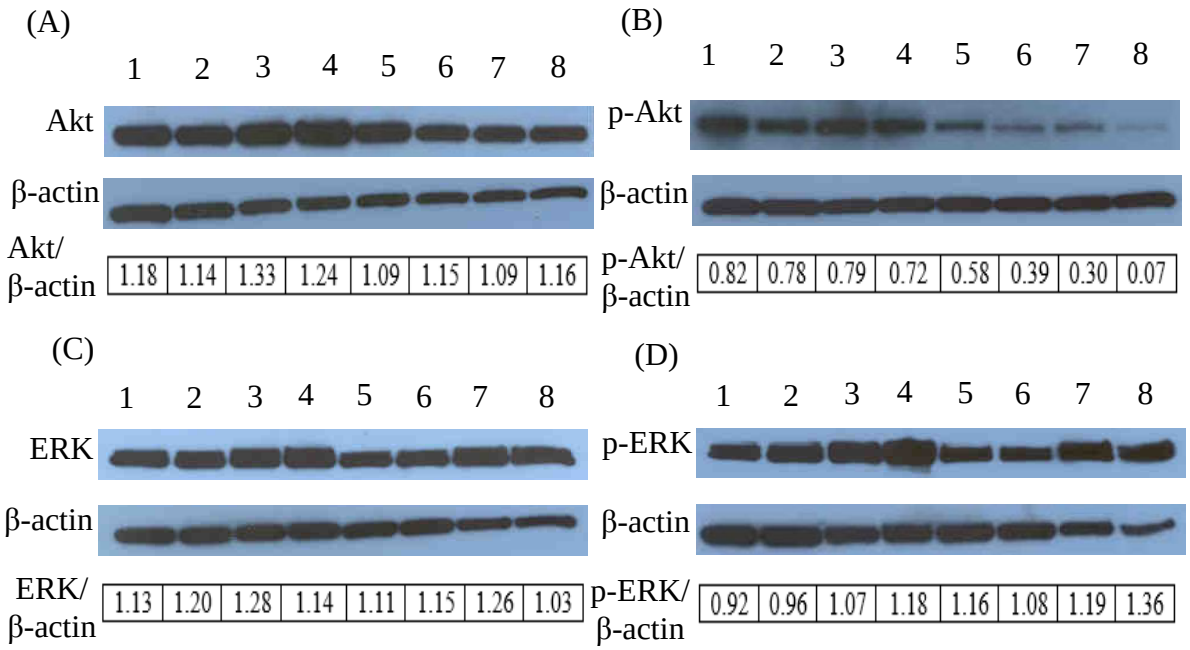



**FIGURE 5.** Effects of non-functionalized SMWCNTs at 100 μg/mL on expression of cell survival/proliferation signaling proteins: (A) Akt; (B) p-Akt; (C) ERK; (D) p-ERK. Lanes 1-4 denoted, respectively, control day 0, control 1 day, control 3 days, and control 5 days; Lanes 5-8 denoted, respectively, non-functionalized SMWCNTs 0 day, non-functionalized SMWCNTs 1 day, non-functionalized SMWCNTs 3 days, and non-functionalized SMWCNTs 5 days.

To investigate the possibility that, similar to non-functionalized SMWCNTs, functionalized SMWCNTs may influence the Akt and ERK cell survival/proliferation signaling pathways in DRG neurons, we also examined the effects of carboxylated SMWCNTs in these cells. As shown in Figure 6B, the expression phospho-Akt was also decreased in DGR neurons treated with 100 μg/mL of carboxylated SMWCNTs, but such decreases did not follow a time-related pattern. Nevertheless, the effects of carboxylated

SMWCNTs and non-functionalized SMWCNTs on expression of Akt, ERK, and phospho-ERK in the treated DRG neurons were similar (compare Figs. 5 and 6). Consequently, our results also suggested the Akt signaling pathway may also likely be involved in mediating the effects of carboxylated SMWCNTs in lowering the survival and/or proliferation of the treated DRG neurons.

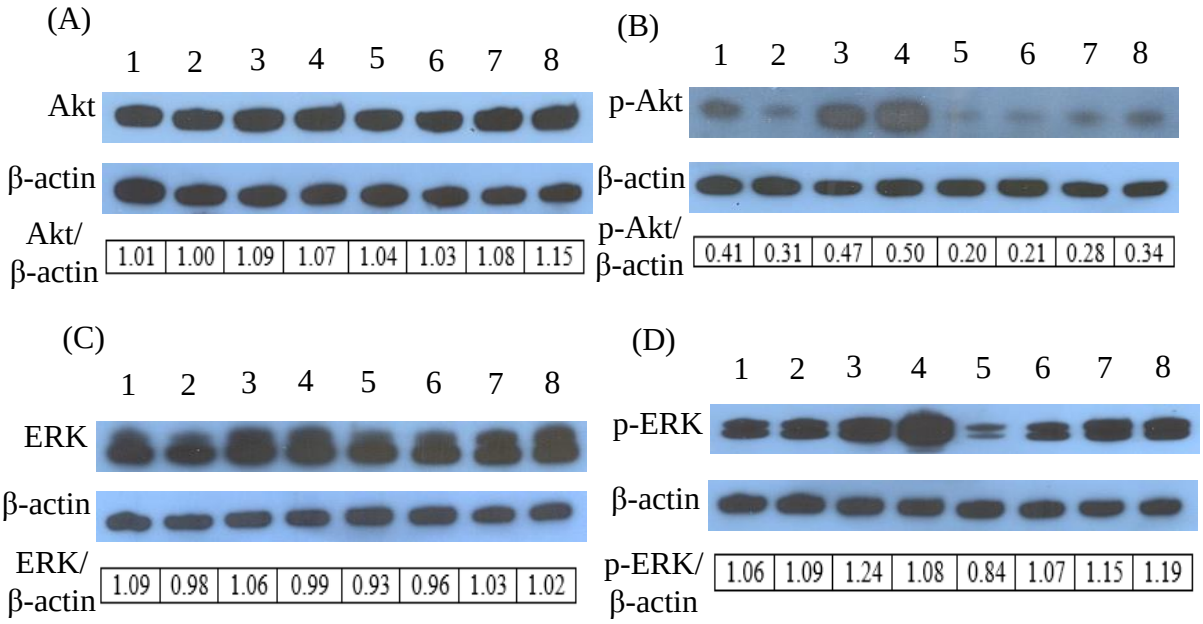



**FIGURE 6.** Effects of carboxylated SMWCNTs at 100 μg/mL on expression of cell survival/proliferation signaling proteins: (A) Akt; (B) p-Akt; (C) ERK; (D) p-ERK. Lanes 1-4 denoted, respectively, control 0 day, control 1 day, control 3 days, and control 5 days; Lanes 5-8 denoted, respectively, carboxylated SMWCNTs day 0, carboxylated SMWCNTs 1 day, carboxylated SMWCNTs 3 days, and carboxylated SMWCNTs 5 days.

Because the presence of 1.5% chitosan accentuated the concentration- and time-related decreases in survival and/or growth of DRG neurons induced by non-functionalized

SMWCNTs (compare Fig. 2 with Fig. 1A), we investigated the possibility that chitosan may modulate the effects of non-functionalized SMWCNTs on the expression of cell survival/proliferation signaling pathways (namely, Akt and ERK) in DRG neurons (Fig. 7). Our results demonstrated that, similar to treatment with non-functionalized SMWCNTs alone at 100 µg/mL, treatment of DRG neurons with 1.5% chitosan together with 100 µg/mL of non-functionalized SMWCNTs also induced time-related decreases in their expression of phospho-Akt (Fig. 7B). Similarly, the expression of Akt, ERK, and phospho-ERK remained largely unaltered in DRG neurons given the combination treatment (Fig. 7). Thus, these findings suggested that the modulatory effect exerted by 1.5% chitosan on altering the survival/proliferation of DRG neurons induced by 100 µg/mL of non-functionalized SMWCNTs could not be attributed to the modulation by chitosan of the Akt and ERK signaling pathways in DRG neurons (compare Fig. 7 with Fig. 5).

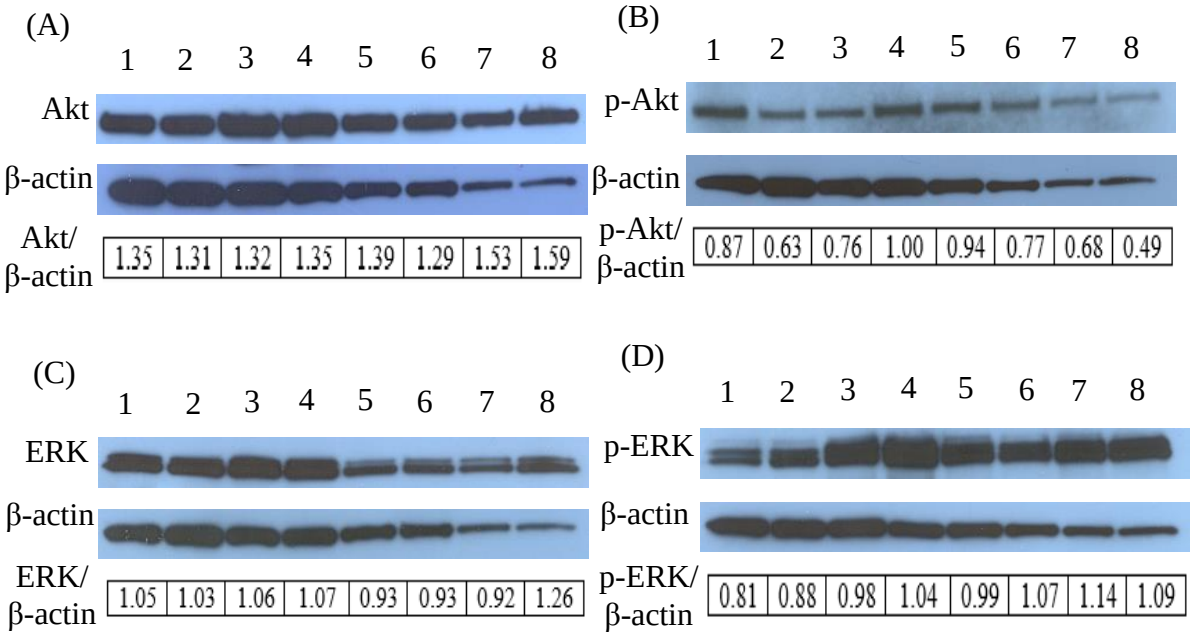


**FIGURE 7.** Effects of 1.5% chitosan and non-functionalized SMWCNTs at 100 μg/mL on expression of cell survival/proliferation signaling proteins: (A) Akt; (B) p-Akt; (C) ERK; (D) p-ERK. Lanes 1-4 denoted, respectively, control 0 day, control 1 day, control 3 days, and control 5 days; Lanes 5-8 denoted, respectively, 1.5% chitosan + non-functionalized SMWCNTs day 0, 1.5% chitosan + non-functionalized SMWCNTs 1 day, 1.5% chitosan + non-functionalized SMWCNTs 3 days, and 1.5% chitosan + non-functionalized SMWCNTs 5 days.

**Entry of SMWCNTs into DRG neurons induced decreases in cell survival and cell death**

The results of this study clearly demonstrated that both functionalized and non-functionalized SMWCNTs induced decreases in cell survival in DRG neurons (Figs 1 and 2), at least in part through inducing necrotic damage and/or necrotic cell death (Figs 3 and 4). Furthermore, both types of SMWCNTs appeared to exert effects on cell survival/proliferation signaling (especially the Akt pathway) (Figs 5 and 6). While it is conceivable and likely that SMWCNTs could exert some of the above-mentioned effects simply when they were in physical contact with DRG neurons, it is more likely that some SMWCNTs penetrated through the cell membranes of the DRG neurons and exerted their effects directly in the cytoplasm of those cells. Consequently, we designed one series of experiments employing confocal microscopy to test the possibility that some SMWCNTs — especially ones that were not clustered together — had gained entry into DRG neurons under the conditions of this study, particularly as we had tentative light microscopic evidence that they could penetrate into blood cells.[4] To facilitate the more precise

localization of the SMWCNTs in DRG neurons, we employed the 'DiO' stain to stain the plasma membrane of DRG neurons green and red protein gel stain to stain all proteins — particularly proteins in the cytoplasm — red, after the neurons were treated with non-functionalized SMWCNTs at a low concentration (namely, at 10 μg/mL) for 3 days (Fig. 8).

Figure 8 is a representative image of the many images we had captured employing the confocal microscope. The confocal images showed that some of the non-functionalized SMWCNTs — especially the smaller-sized particles or particle-clusters — were inside the cytoplasm of the neurons and several particle groups — especially the larger-sized ones — that were not inside of the neurons. As shown in Figure 8, the side panel (on the right edge of the image) represented a cross-section scan by the confocal microscope along the vertical yellow line through the entire object (DRG neurons). The panel below the image represented a cross-section scan by the confocal microscope along the horizontal yellow line through the image. The “cross-hairs” showed that the particle(s) in relation to the red stain which strongly suggested that the particle(s) was inside the cytoplasm of the neuron (Fig. 8). By this approach, we were able to observe and confirm the localization of multiple smaller clusters of non-functionalized SMWCNTs in the cytoplasm of the treated DRG neurons.

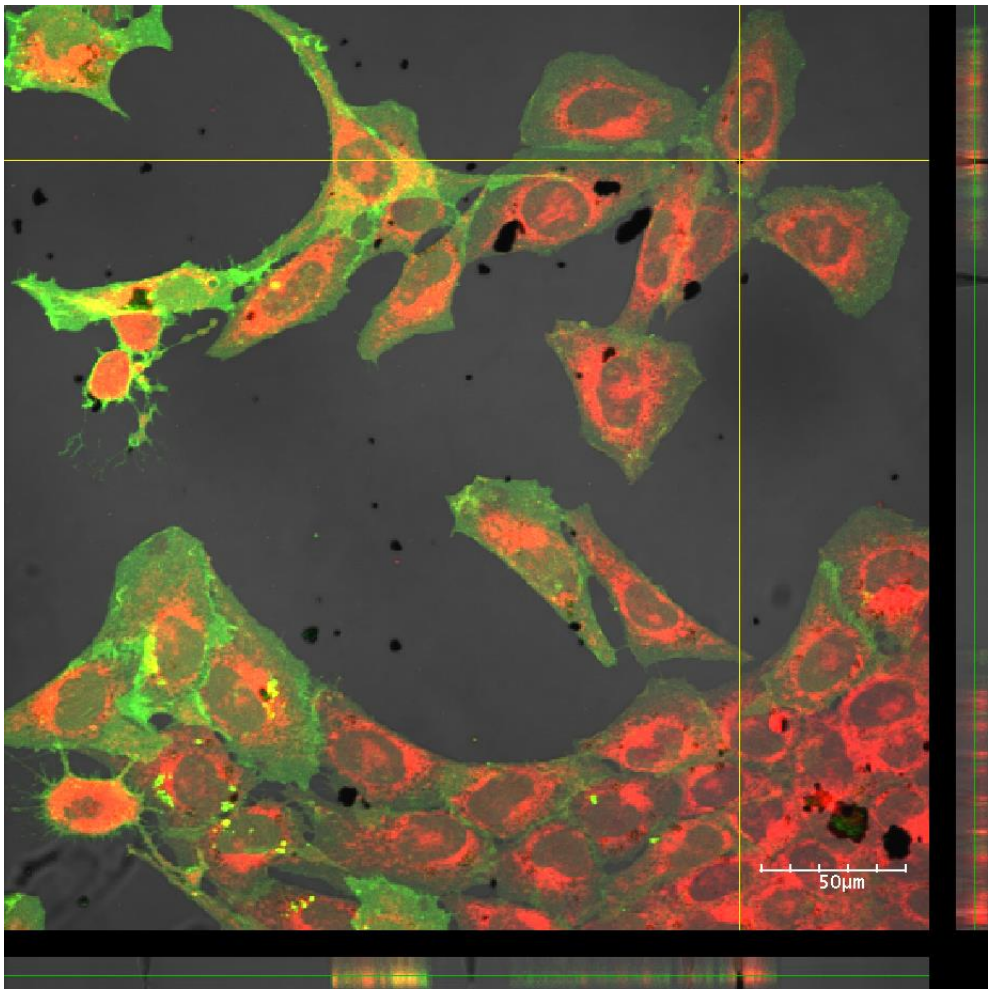



**FIGURE 8.** Entry of SMWCNTs into DRG neurons. DRG neurons were cultured in the presence of non-functionalized SMWCNTs at 10 µg/mL for 3 days. Then the neurons were stained with 'DiO' (a green fluorescent stain that selectively stains the plasma membrane) and red protein gel stain. The stained neurons were examined with an Olympus FV1000 confocal laser scanning microscope.

## DISCUSSION

To our knowledge, this paper is the first to report on the putative cytotoxic effects of functionalized (namely, carboxylated and hydroxylated) and non-functionalized short

multi-walled carbon nanotubes (SMWCNTs) in the presence or absence of chitosan in cultured dorsal root ganglion (DRG) neurons. We investigated the effects of both functionalized and non-functionalized SMWCNTs because we had previously noted that functionalization altered the cytotoxic properties of CNTs.[4] Furthermore, we also elucidated some of the molecular and signaling mechanisms underlying the cytotoxic effects of SMWCNTs, with and without added chitosan, in DRG neurons.

Although treatment with both functionalized (namely, carboxylated and hydroxylated) and non-functionalized SMWCNTs induced time- and dose-related decreases in survival, proliferation and/or growth of DRG neurons, their effects at the lowest treatment dose of 1 μg/mL were minimal (Fig. 1). Our findings also indicated that especially in the highest dose (100 μg/mL) used, the carboxylated or hydroxylated SMWCNTs were less cytotoxic than the non-functionalized SMWCNTs to cultured DRG neurons (Fig. 1). Moreover, the presence of 1.5% chitosan definitely attenuated the time- and dose-related decreases in survival and/or growth of DRG neurons induced by non-functionalized SMWCNTs (Figs. 1A and 2). Thus, our results with DRG neurons (Fig. 1) confirmed our earlier findings that the carboxylated or hydroxylated SMWCNTs were less cytotoxic than the non-functionalized SMWCNTs to Adriamycin-resistant murine sarcoma S180A10 cells.[4]

There is a paucity of literature on the putative cytotoxic effects of carbon nanotubes in neural cells. Our findings show some similarities as well as differences to those reported in the few published papers on effects of multi-walled carbon nanotubes (MWCNTs) on neural cells.[5,32,33] Wu et al.[32] employed a dissociated dorsal root ganglia neural cell culture model derived from DRG collected after the conditioning lesion of

sciatic nerve five days prior to the ganglia collection to determine the effect of overnight exposure to 0.1, 1, 5, or 10 μg/mL of non-functionalized MWCNTs (width of 25 nm and length of 10-20 μm), dispersed in 10% surfactant in sterile saline with 4-minute sonication. They used the regenerative response of such conditioned DRG neurons to assess the effect of exposure to their range of concentrations of MWCNTs. They found that while they did not observe any concomitant apoptosis induced by the MWCNTs even at their highest dose employed (i.e., 10 μg/mL), they did detect dose-related compromise of regenerative axon growth both in length and extent of branching at doses of 1 μg/mL and higher.[32] Similar to the observations of Wu et al.,[32] Mattson et al.[5] also found that primary cultures of hippocampal neurons derived from embryonic rats when cultured on a substratum of unmodified multi-walled carbon nanotubes (diameter of 20 nm and length of 20-100 μm) for 3 days exhibited neurite growth but limited neurite branching although they did not reportedly monitor if exposure to the unmodified multi-walled carbon nanotubes they employed induced any changes to the survival of the cultured hippocampal neurons. Thus, our findings (Fig. 1), together with those of Wu et al.[32] and Mattson et al.[5] strongly suggest that multi-walled carbon nanotubes exert some cytotoxic effects on neurons.

There are some indications that the cytotoxicity of carbon nanotubes is strongly influenced by their surface modifications.[5,33,34] However, how their surface modifications influence their cytotoxicity in neural cells has only been studied to a very limited extent.[5,33] For example, Mattson et al.[5] observed that when primary cultures of hippocampal neurons derived from embryonic rats were cultured on a substratum of multi-walled carbon nanotubes coated with the bioactive molecule 4-hydroxynonenal

exhibited neurite growth and marked neurite branching, not detected in the same type of neurons cultured on unmodified multi-walled carbon nanotubes. Similarly, Matsumoto et al.[33]reported that when cultured with low concentrations (0.11–1.7 μg/mL) of functionalized SMWCNTs, modified by amino groups, and in the presence of added nerve growth factor, the neuronal neurite outgrowth from cultured chick embryonic DRG neurons and from rat PC12h cells was significantly promoted. In accord with the findings of Mattson et al.[5] and Matsumoto et al.,[33] our results (Fig. 1) also demonstrated that functionalized (namely, hydroxylated and carboxylated) SMWCNTs are far less cytotoxic than non-functionalized ones to cultured DRG neurons. Clearly this is an important area that deserves further investigation.

To further determine some of the putative mechanisms underlying the cytotoxic effects of non-functionalized and functionalized SMWCNTs on DRG neurons, we monitored the effects of the nanotubes on LDH release (a marker of necrotic damage and/or necrotic cell death) by DRG neurons into the medium when the neurons were treated with the non-functionalized or the carboxylated SMWCNTs. We found that, compared with control, untreated DRG neurons, those treated with SMWCNTs released significantly more LDH into the medium (Fig. 3), indicating that SMWCNTs induced necrotic damage to DRG neurons. Similar to the findings of this study, a recent report also demonstrated that unmodified multi-walled carbon nanotubes (MWCNTs; average diameter of 150 nm and length of 8 μm, suspended in phosphate-buffered saline after sonication) also induced dose-related decreases in the survival of murine macrophage RAW264 cells: such effects were significant at MWCNT concentrations of 10 and 100 μg/mL.[35] Shimizu et al.[35] further noted that at 100 μg/mL, the MWCNTs they had

employed induced necrotic damage to the RAW264 cells as indicated by the increase in LDH release induced by the MWCNTs. To test their hypothesis that the necrotic effect induced by the MWCNTs is exerted via interaction of the MWCNTs with the lipid bilayer of the plasma membrane of RAW264 cells, they monitored the calcein release from calcein-encapsulated liposomes upon exposing such liposomes to MWCNTs at 100 μg/mL.[36] They found that exposure of the calcein-loaded liposomes to the MWCNTs did indeed induce them to release the calcein into the exterior: thus, this observation lends some credence to their hypothesis.[35]

In addition to necrosis being one mechanism that could account for the decreases in survival of DRG neurons induced by SMWCNTs as indicated by LDH release from SMWCNT-treated DRG neurons (Figs. 1 and 3), we hypothesized that another mechanism whereby SMWCNTs can exert their effects is through the alterations of the cell survival/proliferation signaling pathways of DRG neurons. Because Akt and ERK signaling pathways play a major role in survival and proliferation of many cell types including neurons,[18,20,33] we investigated this hypothesis by assessing the effects of non-functionalized and carboxylated SMWCNTs on the protein expressions of these signaling pathways in DRG neurons by Western blot analysis. Treatment of DRG neurons with 100 μg/mL of non-functionalized SMWCNTs resulted in time-related decreases in the expression of phospho-Akt (Fig. 5B). By contrast, the expression phospho-Akt in DRG neurons treated with 100 μg/mL of carboxylated SMWCNTs showed multi-phasic decreases and increases related to the duration of exposure to the carboxylated SMWCNTs (Fig. 6B). On the other hand, treatment of DRG neurons with 100 μg/mL of non-functionalized and carboxylated SMWCNTs only induced small changes in their expression of Akt, ERK,

and phospho-ERK (Figs. 5 and 6). Thus, our findings suggested that the anti-proliferative and/or anti-survival effects induced by SMWCNTs may be largely mediated by the marked decreases in phospho-Akt level in DRG neurons. It is important and relevant to point out that, to our knowledge, the effects of SMWCNTs on these signaling pathways in neurons other than DRG neurons have not been reportedly studied. In this context, our findings of changes in phospho-Akt expression in DRG neurons (Fig. 6) are somewhat reminiscent of those reported by Liu and colleagues who had shown that treatment of human lung adenocarcinoma A549 cells with carboxylated single-walled CNTs at 1 mg/mL for 24 hours induced decreases in their expression of phospho-Akt.[36]

Treatment with 1.5% chitosan alone definitely induced a lowering of the proliferation/growth of DRG neurons. That the presence of 1.5% chitosan accentuated the concentration- and time-related decreases in survival and/or growth of DRG neurons induced by non-functionalized SMWCNTs (compare Fig. 2 with Fig. 1A) prompted us to investigate the possibility that chitosan may modulate the effects of non-functionalized SMWCNTs on the expression of Akt and ERK in DRG neurons (Fig. 7). We found that the expression patterns of phospho-Akt, Akt, phospho-ERK and ERK in DRG neurons induced by treatment with non-functionalized SMWCNTs at 100 μg/mL in the presence or absence of 1.5% chitosan did not significantly differ, suggesting that the presence of 1.5% chitosan did not modulate the effects of non-functionalized SMWCNTs on their expression of these signaling pathways. Thus, these signaling pathways are unlikely to be involved in the modulation by chitosan on the effects exerted by non-functionalized SMWCNTs on the survival and/or growth of DRG neurons.

When cells are exposed to carbon nanotubes, such nanotubes may penetrate into the interior of the cells. Consequently, the penetration of the nanotubes into cellular cytoplasm may influence their putative cytotoxicity to the cells they have penetrated. Cheng et al.[37] noted that treatment of matured human monocyte-derived macrophage cells with multi-wall carbon nanotubes (with average diameter of 68 nm and lengths between 2 to 164 μm) for 4 days at 37ºC induced dose-related decreases in their survival at concentrations of 2.5 to 20 μg/mL. Based on their observations employing 3-D dark-field scanning transmission electron microscopy as well as confocal and scanning electron microscopy, they concluded that the multi-walled carbon nanotubes they had employed entered the macrophages both actively and passively frequently inserting through the plasma membrane into the cytoplasm and the nucleus. They further suggested that such carbon nanotubes might cause incomplete phagocytosis or mechanically pierce through the plasma membrane of the macrophages leading to oxidative stress and the death of the macrophages.[37] The results of this study employing confocal microscopy (Fig. 8) also allow us to conclude that treatment of DRG neurons with non-functionalized SMWCNTs under the conditions we had employed led to the accumulation of multiple small clusters of those SMWCNTs in their cytoplasm and their accumulation in the DRG neurons were associated with various degrees of cytotoxicity as discussed above. Thus, our findings are quite similar to those of Cheng et al.[37] although with one exception: under the conditions of this study, we observed that the SMWCNTs accumulated in the cytoplasm of the DRG neurons did not penetrate into their nuclei (Fig. 8). This apparent difference between our observations and those of Cheng et al.[37] could be attributed to the dissimilar dimensions of the carbon nanotubes and/or experimental conditions employed.

## CONCLUSIONS

The results of this study have clearly demonstrated that SMWCNTs induced time- and concentration-related decreases in survival, proliferation and/or growth of DRG neurons, and the non-functionalized SMWCNTs were more cytotoxic than the functionalized ones, especially at the higher treatment concentrations. Treatment of DRG neurons with non-functionalized SMWCNTs induced necrotic damage and/or cell death in the neurons as indicated by the enhanced LDH release by the treated DRG neurons into their surrounding medium. Furthermore, our results suggested that DRG neurons treated with a high concentration of non-functionalized SMWCNTs exhibited decreased expression of phospho-Akt. Our findings with confocal microscopy revealed that when DRG neurons were treated with non-functionalized SMWCNTs, there were multiple small clusters of such nanotubes in their cytoplasm, strongly suggesting that the cytotoxic effects of the SMWCNTs were, at least in part, associated with the presence of the SMWCNTs in the cytoplasm of the treated DRG neurons. Thus, our results may have pathophysiological implications in how exposure to SMWCNTs impacts the structure and function of the PNS. Clearly, this is an important area that merits further investigation.

## ACKNOWLEDGMENTS

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# Chapter IV

## Co-culturing dorsal root ganglion neurons with Schwann cells protects them against the cytotoxic effects of silver and gold nanoparticles*

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**Abstract** Previous studies using monotypic nerve cell cultures have shown that nanoparticles induced neurotoxic effects on nerve cells. Interactions between neurons and Schwann cells may protect against the neurotoxicity of nanoparticles. In this study, we developed a co-culture model consisting of immortalized dorsal root ganglion (DRG) neurons and Schwann cells and employed it to investigate our hypothesis that co-culturing DRG neurons with Schwann cells imparts protection on them against neurotoxicity induced by silver or gold nanoparticles. Our results indicated that cells survived better in co-cultures when they were exposed to these nanoparticles at the higher

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concentrations employed compare to when they were exposed to these nanoparticles at the same concentrations in monotypic cultures. Synapsin expression was increased in DRG neurons when they were co-cultured with Schwann cells and treated with or without nanoparticles. Glial fibrillary acidic protein (GFAP) expression was increased in Schwann cells when they were co-cultured with DRG neurons and treated with nanoparticles. The expression of ERK and p-ERK was altered in treated DRG neurons, Schwann cells, and DRG neurons co-cultured with Schwann cells. Furthermore, we found co-cultured with Schwann cells stimulated neurofilaments polymerization in DRG neurons and produced the morphological differentiation. Silver nanoparticles induced morphologically disorganized in monotypic cultures (i.e., Schwann cells or DRG neurons alone). However, there were more 'normal' morphological cells in co-cultures than in monotypic cultures. All these results suggested co-culturing DRG neurons with Schwann cells imparts some protection on them against neurotoxicity induced by silver or gold nanoparticles and altering the expression of neurofilaments, synapsin, and GFAP could account for the phenomenon of "protection" in co-cultures.

**Keywords** neurotoxicity, co-culture, dorsal root ganglion (DRG) neurons, Schwann cells, silver nanoparticles, gold nanoparticles.

## Introduction

Because of their unique physical and chemical properties, such as the electrical, optical, and chemical properties, nanoparticles have attracted significant attention in numerous applications in diverse industries over recent decades [1]. Among the various kinds of available nanoparticles, metallic nanoparticles have attracted more scientific and

technological interest due to their potential applications in novel technologies [2]. Silver and gold nanoparticles have become particularly popular in many applications because of their presumed inertness [3]. For example, silver nanoparticles, which have been found to be very effective as an antibacterial agent, are increasingly used in many types of products and are thus produced on a large and industrial scale [4, 5]. Similarly, gold nanoparticles, which widely used as photothermal therapy agents and as imaging agents, have attracted enormous scientific and technological interest owing to their ease of synthesis, chemical stability, and unique optical properties [6]. However, the impacts of these nanoparticles on human and environmental health have not been elucidated [5-8]: this is especially the case regarding the putative neurotoxic effects of these nanoparticles on cells of the nervous system. Systematic toxicological studies are still needed to fully understand the health hazard potentials of silver and gold nanoparticles [9].

Various *in vivo* and *in vitro* models have been devised to investigate the toxicity of nanomaterials. *In vivo* models most often require animal sacrifice, such as rats [10] and zebra fish [11]. These models have provided some insights, but they are highly complex, include multiple interrelated and/or interdependent parameters, have potentially low reproducibility, and are time-consuming and labor-intensive to generate. More importantly, they do not readily facilitate the elucidation of the underlying cellular and molecular mechanisms. By contrast, *in vitro* cell-based cytotoxicity studies including two-dimensional (2D) and three-dimensional (3D) cell culture models are more attractive and can support nanotoxicity assessment because this approach allows researchers to rapidly obtain more reliable mechanistic information in nanotoxicology [1, 8]. To date, the majority of cell-based cytotoxicity studies use traditional two-dimensional (2D)

monolayer cells. 3D cell culture models are better models than the traditional 2D monolayer culture because cell responses in 3D cultures are more similar to *in vivo* behavior compared to 2D culture. However, there are still many hurdles such as the maturity of the technology and the cost that must be overcome before these systems can be widely accepted [12]. For over a decade, we have been developing a variety of neural and non-neural cell types as 2D *in vitro* models for systematic investigation of putative cytotoxicity of various nanomaterials, including metallic and non-metallic nanoparticles [13-18]. More recently, we have developed two non-tumor neural cell models *in vitro* for systematic investigation of putative neurotoxicity of various nanomaterials including nanoparticles employing dorsal root ganglion (DRG) neurons and Schwann cells, which are physiologically important neural cell types of the peripheral nervous system (PNS) [19-22].

Our previous studies using monotypic nerve cell cultures have shown that several metallic and non-metallic nanoparticles including silver and gold nanoparticles exerted neurotoxic effects on DRG neurons and Schwann cells [19-21]. The known mutually beneficial interactions between DRG neurons and Schwann cells prompted us to further investigate the neurotoxicity of silver and gold nanoparticles in DRG neurons and Schwann cells using a co-culture model instead of the more commonly employed monotypic models. Cross-talk between DRG neurons and Schwann cells may protect them against the neurotoxicity induced by silver or gold nanoparticles. Therefore, we hypothesized that co-culturing DRG neurons with Schwann cells imparts some protection on them against the neurotoxicity of silver or gold nanoparticles. To investigate our hypothesis, we developed a co-culture model consisting of immortalized DRG neurons

and Schwann cells and employed it to compare the neurotoxic effects of silver and gold nanoparticles on monotypic cultures (i.e., Schwann cells or DRG neurons alone) and on DRG neurons co-cultured with Schwann cells employing established cytotoxicity testing approaches [13-22]. Furthermore, we further elucidate some of the neurochemical mechanisms underlying the phenomenon of "protection" in co-cultures. We examined the expression of some cellular biomarkers, namely synapsin and glial fibrillary acidic protein (GFAP), the cell survival signaling pathway protein extracellular signal-regulated kinase (ERK), and phosphorylated ERK (p-ERK) in DRG neurons and Schwann cells either cultured singly in monotypic cultures or in co-cultures in the presence or absence of silver or gold nanoparticles by Western blot analysis. By immunofluorescence staining, we also monitored their morphological changes in monotypic cultures (i.e., Schwann cells or DRG neurons alone) and in DRG neurons co-cultured with Schwann cells with or without treatment with nanoparticles.

## Materials and methods

### Chemical reagents and antibodies

Thiazolyl blue tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Tetrachloroauric (III) acid ($HAuCl_4 \bullet 3H_2O$), trisodium citrate ($C_6H_5Na_3O_7 \bullet 2H_2O$) and silver nitrate ($AgNO_3$) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The monoclonal antibodies against GFAP (GA5), neurofilament-L (C28E10 and DA2), ERK, and p-ERK were obtained from Cell Signaling Technology (Beverly, MA, USA). The monoclonal

antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The polyclonal antibody against synapsin, monoclonal antibody against GFAP (2A5), goat anti-rabbit IgG secondary antibody, goat anti-mouse IgG secondary antibody, goat anti-mouse IgG H&L (Alexa Fluor® 647) secondary antibody, and goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody were purchased from Abcam Inc. (Cambridge, MA, USA). All chemicals were of analytical grade and were usually obtained from Sigma-Aldrich.

**Preparation of silver and gold nanoparticles**

Silver and gold nanoparticles were prepared as described previously [18, 20]. To prepare nanosilver particles, $AgNO_3$ and $C_6H_5Na_3O_7 \cdot 2H_2O$ solutions were filtered through a 0.22 μm microporous membrane filter prior to being used for preparing nanosilver particles. Nanosilver particles were prepared by adding $C_6H_5Na_3O_7 \cdot 2H_2O$ solution to boiling $AgNO_3$ aqueous solution. The prepared concentration of silver nanoparticles was about 108 μg/mL and their size was about 60 nm as characterized by scanning electron microscopy [23]. To prepare nanogold particles, $HAuCl_4 \cdot 3H_2O$ and $C_6H_5Na_3O_7 \cdot 2H_2O$ solutions also were filtered through a 0.22 μm microporous membrane filter prior to being used for preparing nanogold particles. Nanogold particles were prepared by adding $C_6H_5Na_3O_7 \cdot 2H_2O$ solution to boiling $HAuCl_4 \cdot 3H_2O$ aqueous solution. The prepared concentration of gold nanoparticles was about 49 μg/mL and their size was about 34 nm as characterized by transmission electron microscopy [24].

**Cell culture**

S16 Schwann cells, obtained from ATCC (Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 10% (v/v) FBS and 25 mM glucose at 37˚C and with 5% (v/v) $CO_2$. DRG (50B11) neurons were kind gifts from Dr Höke's Laboratory at Johns Hopkins University School of Medicine. DRG neurons were cultured in DMEM supplemented with 25 mM glucose and 10% (v/v) FBS at 37˚C and with 5% (v/v) $CO_2$.

**Cell survival/proliferation assay**

Cell survival/proliferation was determined by using the modified MTT assay [15-18]. Schwann cells were seeded (3000 cells/well) into a 24-well plate and allowed to attach and grow for an hour. Then the same number of DRG neurons were seeded onto the substratum layer of Schwann cells and cultured as described above. After an hour, cells were treated with or without (i.e., the control) specified concentrations of silver or gold nanoparticles. Monotypic cultures (i.e., Schwann cells or DRG neurons alone) were also set up similarly. The plates so prepared were incubated for 1, 2, 3, 4, or 5 days at 37˚C. At the end of the incubation period, 100 μL of 5 mg/mL MTT dye in phosphate buffered saline (PBS) was added into each well and the plates were incubated for an additional 4 hours at 37 ˚C. The purple-colored insoluble formazan crystals in viable cells were dissolved using 200 μL DMSO and the subsequent absorbance (designated as X) of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA) [25].

The medium, silver or gold nanoparticles by themselves had absorbance: thus, their absorbance (i.e., the control sets of wells) had to be subtracted from the absorbance of live cells with or without different concentrations of nanoparticles treatments. The control sets of wells

were set up alongside those sets of wells in the plates as detailed in the preceding paragraph except that the control sets of wells did not contain any seeded cells. At the end of the specified culture period, 100 μL of 5 mg/mL MTT dye in PBS was added into each well and the plates were incubated for an additional 4 hours at 37˚C. The subsequent absorbance (designated as Y) of the content of each well was measured at 570 nm as described above. (X-Y) was taken as the absorbance attributed to viable cells in each well. The absorbance of co-cultures of DRG neurons and Schwann cells was compared with the sum of absorbance of monotypic cultures (i.e., Schwann cells or DRG neurons alone).

**Western blot analysis**

Expression of synapsin, GFAP, ERK and p-ERK was determined by Western blot analysis. Cells treated with or without silver or gold nanoparticles were collected and homogenized. Protein content of the homogenates dissolved by 10 M NaOH for 3 days was then determined using the bicinchoninic acid (BCA) assay as described previously [17, 26]. Equal amounts of protein from the samples were loaded onto the lanes of the gels, and the proteins were separated by polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. Monoclonal or polyclonal antibodies against the respective proteins were then used to probe the proteins of interest. The PVDF membrane containing the target protein was then developed using the enhanced chemiluminescence (ECL) technique on an X-ray film to assess the extent of expression of respective proteins [17, 26].

**Immunofluorescent staining**

Schwann cells, DRG neurons, or DRG neurons co-cultured with Schwann cells were cultured on 18 mm coverslips in 6-well plates in the absence or presence of specified concentrations of silver nanoparticles for 3 days. Then the cells were fixed for 15 minutes in 4% (w/v) paraformaldehyde, permeabilized and blocked with 0.3 % Triton X-100, 10% goat serum in PBS for 60 minutes at room temperature. Then, the cells were incubated with different primary antibodies: anti-GFAP (2A5) (1:100, Abcam) and anti-neurofilament-L (C28E10) (1:100, Cell Signaling), or anti-synapsin (1:200, Abcam) and anti-neurofilament-L (DA2) (1:100, Cell Signaling) antibodies at 4˚C overnight with shaking. The next day, decant the solution and wash the cells three times in PBS 5 times for 5 minutes each. Then the cells were incubated with goat anti-mouse IgG H&L (Alexa Fluor® 647) (1:1000, Abcam) and goat anti-rabbit IgG H&L (Alexa Fluor® 488) (1:1000, Abcam) for 2 hours with shaking at room temperature in the dark. Coverslips were mounted onto slides with Prolong Gold Antifade Reagent (Cell Signaling) and imaged using an Olympus (Center Valley, PA, USA) FV1000 confocal laser scanning microscope.

## Statistical analysis of data

Experiments were performed at least three times with a minimum of 6 replicates for each set, and all data were recorded as mean ± standard deviation (SD). Data analysis was carried out by one-way analysis of variance (ANOVA), followed by post-hoc Student–Newman–Keuls test for multiple comparisons using the software KaleidaGraph version 4 (Synergy Software, Reading, PA, USA). Significance level was set at $p<0.05$.

## Results

### Effects of different concentrations of gold nanoparticles on survival/proliferation of Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells

Employing the modified MTT assay, we studied the effects of different concentrations of gold nanoparticles on survival/proliferation of Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells. At lower treatment concentrations, from 25 to 125 μL, gold nanoparticles did not affect viability of the cells (Figs. 1A, 1B, and 1C). At treatment concentration of 250 μL, gold nanoparticles induced time-related decreases in survival of DRG neurons or Schwann cells in monotypic cultures. However, the cells survived better in co-cultures when they were exposed to 250 μL of gold nanoparticles compare to when they were exposed to gold nanoparticles in monotypic cultures (compare the orange line with the brown line in Fig. 1D). All these results suggest that gold nanoparticles at higher concentration (i.e., 250 μL) are cytotoxic to DRG neurons and Schwann cells and co-culturing DRG neurons with Schwann cells imparts some protection on them against the neurotoxicity induced by gold nanoparticles. Thus, we employed the concentration of 250 μL gold nanoparticles to do all subsequent experiments. Interestingly, we found that the survival/proliferation of untreated cells (i.e., control) in co-cultures was better than that of untreated cells (i.e., control) in monotypic cultures after 4 days' culture (compare the dark line with the green line in Figs. 1A, 1B, 1C, and 1D).

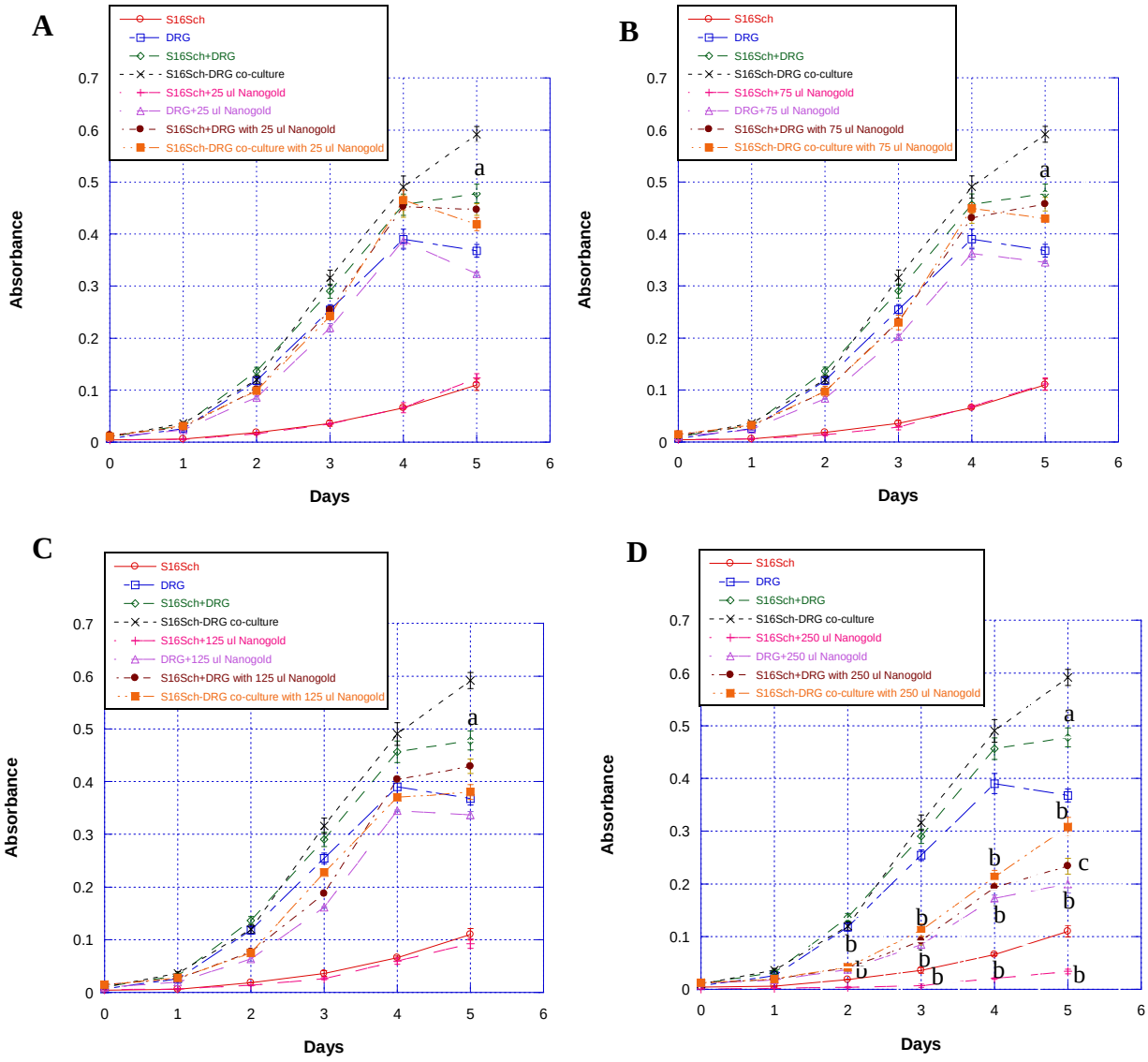


**Fig. 1** Effect of treatment with different concentrations of gold nanoparticles on survival/proliferation of Schwann cells alone, DRG neurons alone, or DRG neurons co-cultured with Schwann cells.

**Notes:** Schwann cells alone, DRG neurons alone, or DRG neurons co-cultured with Schwann cells were treated with or without (i.e., control) different concentrations of gold

nanoparticles for various times up to 5 days. Afterwards, their survival/proliferation was determined using the MTT assay. Values are mean ± SD of 6 determinations. The concentration of gold nanoparticles is 49 μg/mL. (**A**) Cells were treated with 25 μL gold nanoparticles. (**B**) Cells were treated with 75 μL gold nanoparticles. (**C**) Cells were treated with 125 μL gold nanoparticles. (**D**) Cells were treated with 250 μL gold nanoparticles. Untreated Schwann cells alone are marked with red circles. Untreated DRG neurons alone are marked with blue squares. The absorbance of untreated DRG neurons alone plus untreated Schwann cells alone is marked with green diamonds. Untreated DRG neurons co-cultured with Schwann cells are marked with black crosses. Schwann cells alone treated with gold nanoparticles are marked with pink pluses. DRG neurons alone treated with gold nanoparticles are marked with purple triangles. The absorbance of DRG neurons alone plus Schwann cells alone treated with gold nanoparticles is marked with brown circles. DRG neurons co-cultured with Schwann cells treated with gold nanoparticles are marked with orange squares. Values marked with a are significantly different ($p<0.05$, by ANOVA and post-hoc Student-Newman-Keuls test) from corresponding mean value in control (i.e., untreated) co-culture cells; Values marked with b are significantly different ($p<0.05$, by ANOVA and post-hoc Student-Newman-Keuls test) from corresponding mean value in control (i.e., untreated) cells; Values marked with c are significantly different ($p<0.05$, by ANOVA and post-hoc Student-Newman-Keuls test) from corresponding mean value in treated co-culture cells.

**Abbreviations:** DRG, dorsal root ganglion; MTT, thiazolyl blue tetrazolium bromide; SD, standard deviation.

**Effects of different concentrations of silver nanoparticles on the survival/proliferation of Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells**

To assess if silver nanoparticles may exert neurotoxic effects similar to those of gold nanoparticles, we also examined the effects of different concentrations of silver nanoparticles on survival/proliferation of Schwann cells alone, DRG neurons alone, and on DRG neurons co-cultured with Schwann cells. We found that at the concentration range employed, silver nanoparticles were more neurotoxic to these cells than gold nanoparticles (compare Fig. 2 with Fig. 1). At treatment concentrations of 25 μL and higher, silver nanoparticles induced concentration- and time-related decreases in survival of Schwann cells or DRG neurons in monotypic cultures (Figs. 2A, 2B, 2C, and 2D). Similarly, we found the "protection" phenomenon when DRG neurons co-cultured with Schwann cells treated with silver nanoparticles at higher concentrations. As shown in Figs 2B and 2C, cells survived better in co-cultures when they were exposed to silver nanoparticles compare to when they were exposed to the silver nanoparticles in monotypic cultures (compare the orange line with the brown line in Figs. 2B and 2C), especially at concentration of 125 μL (compare Fig. 2B with 2C). There were almost no live cells remaining after these cells were treated with 250 μL of silver nanoparticles (Fig. 2D). Since at concentration of 125 μL, cells survived much better in co-cultures than in monotypic cultures, we employed the concentration of 125 μL silver nanoparticles to do all subsequent experiments.

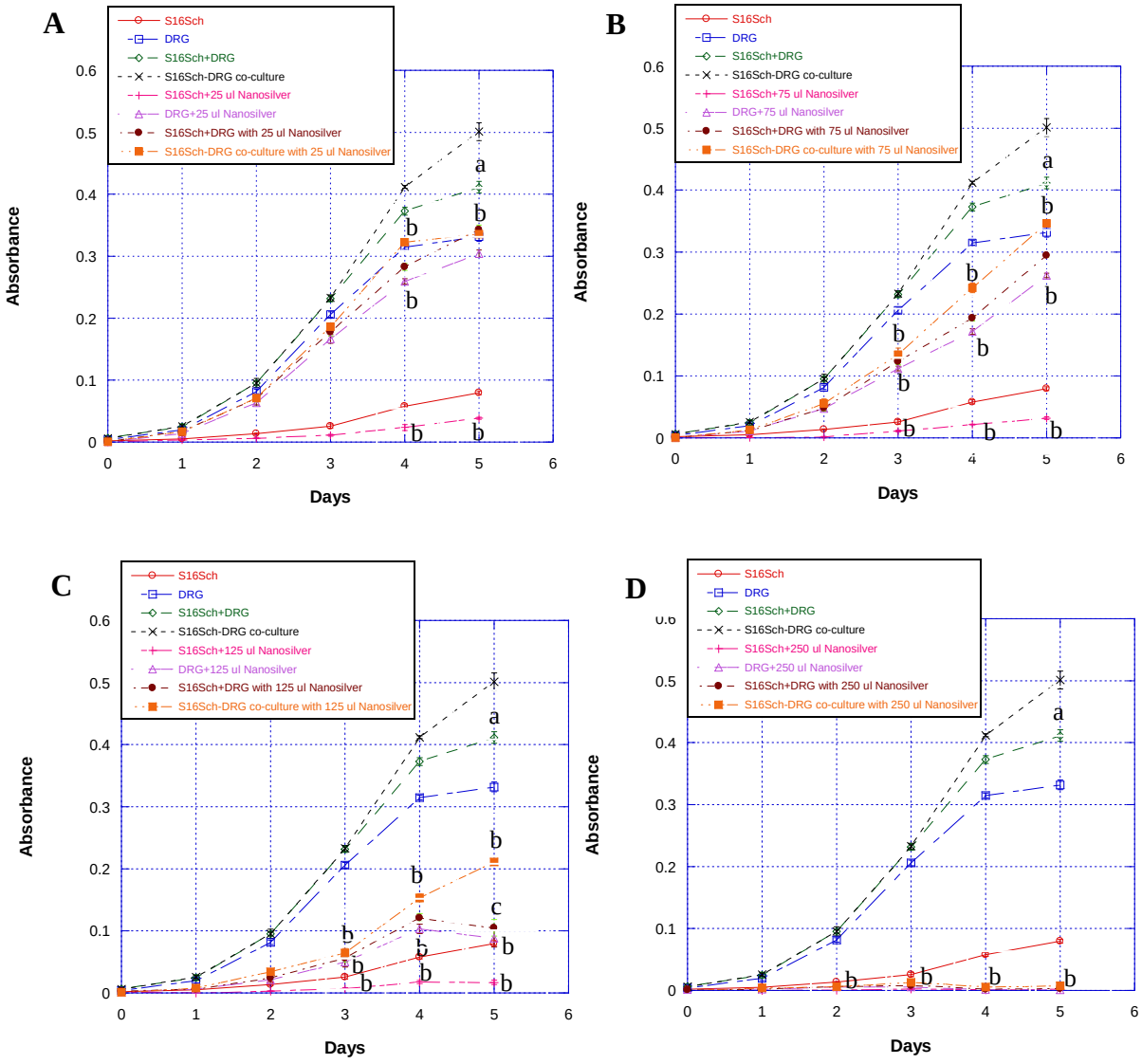


**Fig. 2** Effect of treatment with different concentrations of silver nanoparticles on survival/proliferation of Schwann cells alone, DRG neurons alone, or DRG neurons co-cultured with Schwann cells.

**Notes:** Schwann cells, DRG neurons, and DRG neurons co-cultured with Schwann cells cultured for various times up to 5 days. Afterwards, their survival/proliferation was determined using the MTT assay. Values are mean ± SD of 6 determinations. The concentration of silver nanoparticles is 108 µg/mL. (A) Cells were treated with 25 µL

silver nanoparticles. (B) Cells were treated with 75 μL silver nanoparticles. (C) Cells were treated with 125 μL silver nanoparticles. (D) Cells were treated with 250 μL silver nanoparticles. Schwann cells alone are marked with red circles. DRG neurons alone are marked with blue squares. The absorbance of DRG neurons alone plus Schwann cells alone is marked with green diamonds. DRG neurons co-cultured with Schwann cells are marked with black crosses. Schwann cells alone treated with silver nanoparticles are marked with pink pluses. DRG neurons alone treated with silver nanoparticles are marked with purple triangles. The absorbance of DRG neurons alone plus Schwann cells alone treated with silver nanoparticles is marked with brown circles. DRG neurons co-cultured with Schwann cells treated with silver nanoparticles are marked with orange squares. Values marked with a are significantly different ($p<0.05$, by ANOVA and post-hoc Student-Newman-Keuls test) from corresponding mean value in control (i.e., untreated) co-culture cells; Values marked with b are significantly different ($p<0.05$, by ANOVA and post-hoc Student-Newman-Keuls test) from corresponding mean value in control (i.e., untreated) cells; Values marked with c are significantly different ($p<0.05$, by ANOVA and post-hoc Student-Newman-Keuls test) from corresponding mean value in treated co-culture cells.

**Abbreviations:** DRG, dorsal root ganglion; MTT, thiazolyl blue tetrazolium bromide; SD, standard deviation.

**Effects of silver or gold nanoparticles on synapsin and GFAP expression**

To further elucidate some of the cellular and molecular mechanisms underlying this phenomenon of “protection” in co-cultures, we examined the expression of two cellular

biomarkers, namely synapsin (a marker of neuronal and synaptic function [27]) and GFAP (a glial marker [28]), in DRG neurons and Schwann cells either singly in monotypic cultures or in co-cultures in the presence or absence of 125 μL of silver nanoparticles or 250 μL of gold nanoparticles by Western blot analysis. As expected of a neuronal biomarker, DRG neurons, but not Schwann cells, expressed synapsin abundantly. Similarly, co-cultures of DRG neurons and Schwann cells also expressed synapsin abundantly (Fig. 3A). Our results also showed that treatment of DRG neurons alone for 5 days with either silver or gold nanoparticles resulted in a decrease in their synapsin expression (compare lanes 2, 5, and 8 in Fig. 3A). By contrast, synapsin expression was increased in DRG neurons when they were co-cultured with Schwann cells and treated with silver nanoparticles (compare lanes 5 and 6 in Fig. 3A), or gold nanoparticles (compare lanes 8 and 9 in Fig. 3A), indicating that co-culturing with Schwann cells protect them from the cytotoxicity of silver or gold nanoparticles. This conclusion is consist with cell survival/proliferation assay results (Figs. 1 and 2). We also found that expression of synapsin in untreated DRG neurons was increased after co-culturing with Schwann cells (compare lanes 2 and 3 in Fig. 3A), suggesting that Schwann cells can promote the synthesis of synapsin in DRG neurons which is consistent with previous studies [29, 30] showing that Schwann cells can promote synaptogenesis.

As expected of a glial marker, Schwann cells, but not DRG neurons, expressed GFAP abundantly (Fig. 3B). Similarly, co-cultures of DRG neurons and Schwann cells also expressed GFAP abundantly (Fig. 3B). We also found that treatment of Schwann cells alone for 5 days with gold nanoparticles resulted in a decrease in their GFAP expression (compare lanes 1 and 7 in Fig. 3B). Similarly, treatment of Schwann cells

alone with silver nanoparticles led to a substantive decrease in their expression of GFAP (compare lanes 1 and 4 in Fig. 3B). Thus, our GFAP expression findings are consistent with the notion that silver nanoparticles are more cytotoxic than gold nanoparticles to Schwann cells. On the other hand, GFAP expression was increased in Schwann cells when they were co-cultured with DRG neurons and treated with silver nanoparticles (compare lanes 4 and 6 in Fig. 3B) or gold nanoparticles (compare lanes 7 and 9 in Fig. 3B), indicating that co-culturing with DRG neurons appeared to render the Schwann cells less susceptible to the cytotoxicity of the nanoparticles. However, expression of GFAP in untreated Schwann cells appeared to remain unchanged after co-culturing with DRG neurons (ratio of GFAP/β-actin of co-culture is half of that Schwann cells in monotypic culture because there was only half lysate of Schwann cells in co-culture). These results indicated that without treatment co-culturing with DRG neurons had no effect on the expression of GFAP in Schwann cells.

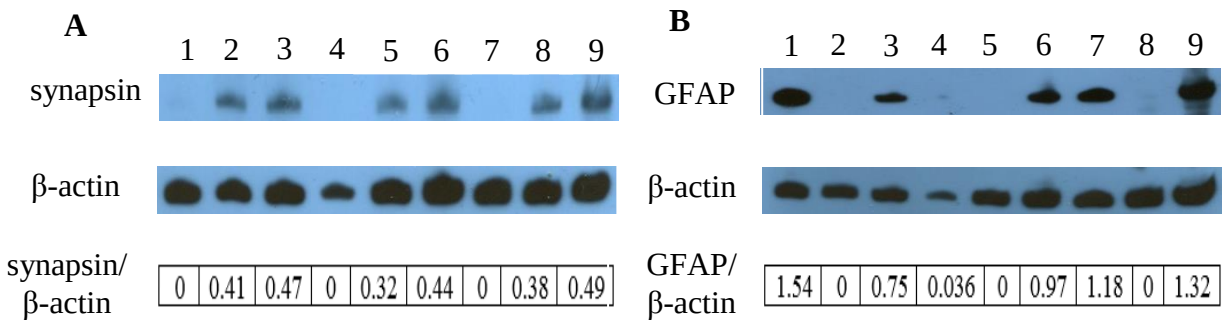


**Fig. 3** Effect of silver and gold nanoparticles on expression of synapsin and GFAP in Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells.

**Notes:** Schwann cells alone, DRG neurons alone, or DRG neurons co-cultured with Schwann cells were treated with 125 μL of silver nanoparticles or 250 μL of gold nanoparticles for 5 days. Then cell lysates of treated and untreated cells (i.e., control) were prepared as described in Materials and methods. The expression of synapsin (A) and GFAP (GA5) (B) was determined by Western blot analysis using β-actin as the loading control: lane 1, lysate of untreated Schwann cells alone; lane 2, lysate of untreated DRG neurons alone; lane 3, lysate of untreated DRG neurons co-cultured with Schwann cells; lane 4, lysate of Schwann cells alone treated with 125 μL of silver nanoparticles; lane 5, lysate of DRG neurons alone treated with 125 μL of silver nanoparticles; lane 6, lysate of DRG neurons co-cultured with Schwann cells and treated with 125 μL of silver nanoparticles. lane 7, lysate of Schwann cells alone treated with 250 μL of gold nanoparticles; lane 8, lysate of DRG neurons alone treated with 250 μL of gold nanoparticles; lane 9, lysate of DRG neurons co-cultured with Schwann cells and treated with 250 μL of gold nanoparticles. The blots were from a typical experiment. Ratio of band intensities was calculated using ImageJ software. Two other experiments yielded essentially the same patterns of results.

**Abbreviations:** DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein.

**Effects of silver or gold nanoparticles on cell signaling protein expression**

Because we found that silver and gold nanoparticles (at the higher concentration) exerted neurotoxic effect on Schwann cells, DRG neurons, and DRG neurons co-cultured with Schwann cells (Figs. 1 and 2), we investigated the possibility that alteration of cell signaling pathway(s) is one mechanism mediating the effects of silver or gold

nanoparticles in these cells. We therefore examined the effects of these nanoparticles on expression of ERK and p-ERK proteins. Our results showed that treatment of Schwann cells alone (compare lanes 1, 4 and 7 in Fig. 4A), DRG neurons alone (compare lanes 2, 5, and 8 in Fig. 4A), and DRG neurons co-cultured with Schwann cells (compare lanes 3, 6, and 9 in Fig. 4A) with the nanoparticles decreased the total ERK expression. The effects of the nanoparticles on their p-ERK expression were different from those on total ERK expression (compare Fig. 4A with 4B). Treatment of Schwann cells alone with silver nanoparticles for 5 days led to a significant increase in their expression of p-ERK (compare lanes 1 and 4 in Fig. 4B). However, treatment of Schwann cells alone for 5 days with gold nanoparticles resulted in a significant decrease in their p-ERK expression (compare lanes 1 and 7 in Fig. 4B). The effects of these nanoparticles on the protein expression of p-ERK in DRG neurons were different from their effects in Schwann cells. Phosphorylated ERK protein expression was decreased when DRG neurons alone (compare lanes 2 and 5 in Fig. 4B) or co-culture with Schwann cells (compare lanes 3 and 6 in Fig. 4B) were treated with silver nanoparticles. However, treatment of DRG neurons alone (compare lanes 2 and 8 in Fig. 4B) or co-culture with Schwann cells (compare lanes 3 and 9 in Fig. 4B) for 5 days with gold nanoparticles did not affect their expression of p-ERK. These findings suggested that silver and gold nanoparticles exerted different effects on p-ERK protein expression in DRG neurons, Schwann cells, and DRG neurons co-cultured with Schwann cells and alteration of this cell survival/proliferation signaling was involved in the treatment-induced lowering of the survival/proliferation of Schwann cells, DRG neurons, and DRG neurons co-cultured with Schwann cells.

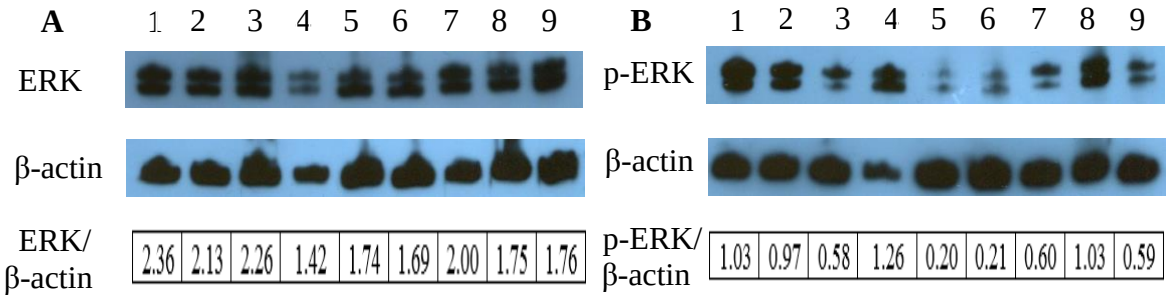


**Fig. 4** Effect of silver and gold nanoparticles on expression of ERK and p-ERK in Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells.

**Notes:** Schwann cells, DRG neurons, or DRG neurons co-cultured with Schwann cells were treated with 125 μL of silver nanoparticles or 250 μL of gold nanoparticles for 5 days. Then cell lysates of treated and untreated cells were prepared as described in Materials and methods. The expression of ERK (A) and p-ERK (B) was determined by Western blot analysis using β-actin as the loading control: lane 1, lysate of Schwann cells alone; lane 2, lysate of DRG neurons alone; lane 3, lysate of DRG neurons co-cultured with Schwann cells; lane 4, lysate of Schwann cells treated with 125 μL of silver nanoparticles; lane 5, lysate of DRG neurons treated with 125 μL of silver nanoparticles; lane 6, lysate of DRG neurons co-cultured with Schwann cells and treated with 125 μL of silver nanoparticles. lane 7, lysate of Schwann cells treated with 250 μL of gold nanoparticles; lane 8, lysate of DRG neurons treated with 250 μL of gold nanoparticles; lane 9, lysate of DRG neurons co-cultured with Schwann cells and treated with 250 μL of gold nanoparticles. The blots were from a typical experiment. Ratio of band intensities was calculated using ImageJ software. Two other experiments yielded essentially the same patterns of results.

**Abbreviations:** DRG, dorsal root ganglion; ERK, extracellular signal-related kinase; p-ERK, phosphorylated extracellular signal-related kinase.

**Effects of silver nanoparticles on morphological changes in Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells employing immunofluorescence staining**

To evaluate morphological changes after DRG neurons co-cultured with Schwann cells and treated with or without nanoparticles, we used immunofluorescence staining to compare expression of two cytoskeletal proteins (neurofilament, a neuronal marker and GFAP, a glial marker) in Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells after these cells were treated with or without silver nanoparticles because silver nanoparticles are more cytotoxic than gold nanoparticles in these cells via confocal microscopy (all settings were same when took all the images in order to compare). As was observed using confocal microscopy (Fig. 5), DRG neurons were stained with neurofilament (shown in green) and Schwann cells were stained with GFAP (shown in red). Fig. 5 clearly revealed obviously strong and filamentous staining in DRG neurons co-cultured with Schwann cells both in control culture and in silver nanoparticles treatment culture (compare Fig. 5 with Fig. 6). After DRG neurons co-cultured with Schwann cells, DRG neurons had developed a radial differentiated-like morphology with processes departing from the cell body (Fig. 5C, white arrows). These results indicated that co-cultured with Schwann cells stimulated neurofilament polymerization in DRG neurons and produced the morphological differentiation. Upon treatment with silver nanoparticles, the fine processes of DRG neurons disappeared with

an elongated morphology and strong neurofilament staining around nucleus was observed (Figs. 5B and 5D, white arrows). As shown in Fig. 6, DRG neurons were only stained with neurofilament (shown in green). In the presence of silver nanoparticles, changes in the cell shape were visible (compare Fig. 6A with Fig. 6B). Some DRG neurons displayed shrinkage (Fig. 6D, white arrows), that is indicative of a loss of cell viability. As shown in Fig. 7, Schwann cells were only stained with GFAP (shown in red). Compare Fig. 5 with Fig. 7, stronger red stain was observed in Fig. 5 indicating that Schwann cells expressed more GFAP when they were co-cultured with DRG neurons than they were cultured alone. After Schwann cells were treated with silver nanoparticles, some Schwann cells displayed swelling (Fig. 7D, white arrow). Also treatment of silver nanoparticles showed a decrease in number of cells both in co-culture and in monotypic cultures (compare Fig. 5A with 5B, Fig. 6A with 6B, Fig. 7A with 7B) which is inconsistent with cell survival/proliferation assay results (Fig. 2). All these results indicated that silver nanoparticles were toxic to these cells and are consistent with our hypothesis that co-culturing DRG neurons with Schwann cells protects them against the cytotoxic effects of silver nanoparticles.

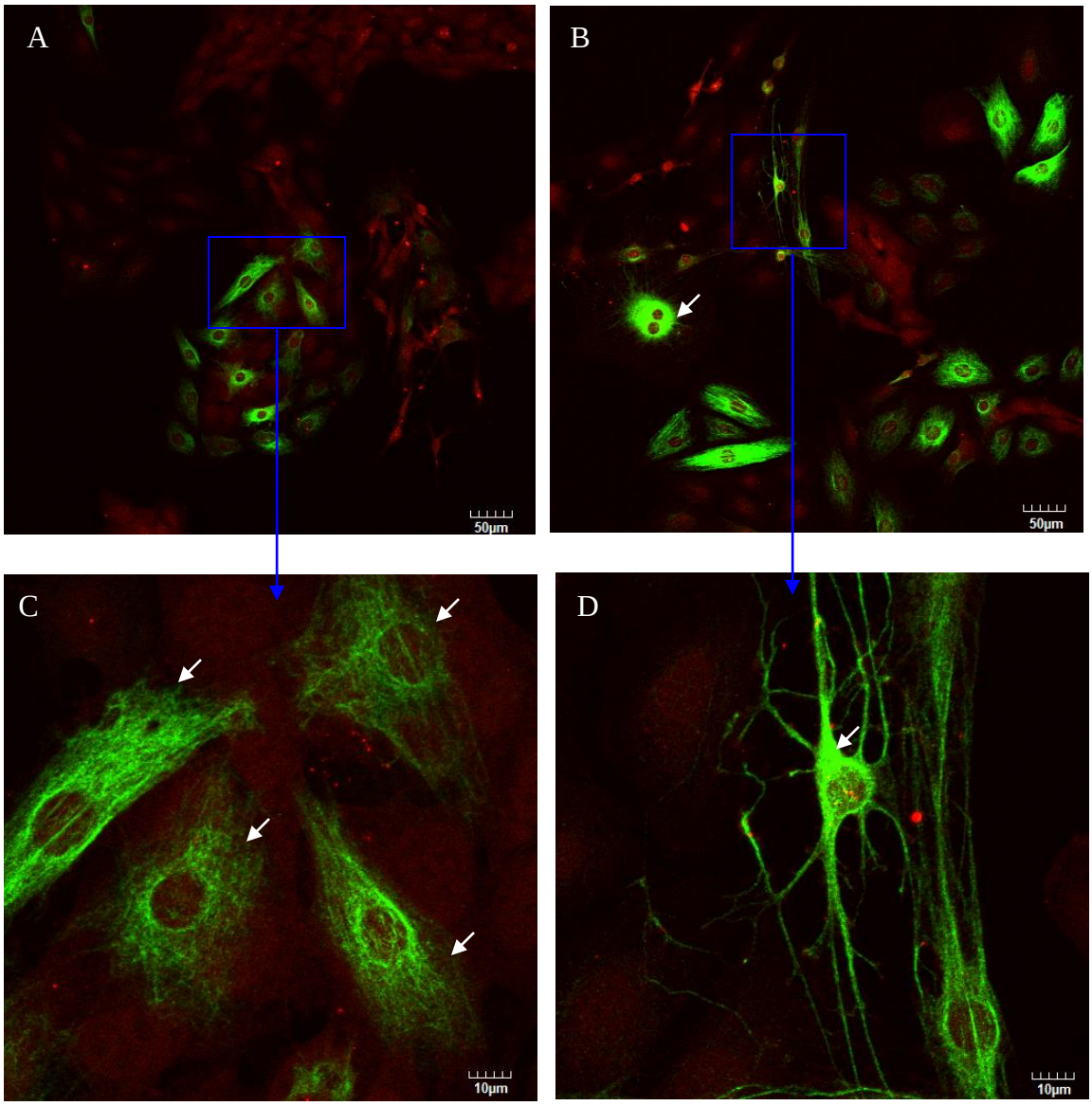


**Fig. 5** Confocal images of DRG neurons co-cultured with Schwann cells in the absence (A, C) or presence (B, D) of silver nanoparticles at two magnifications (upper and lower rows).

**Notes:** DRG neurons co-cultured with Schwann cell (at the same ratio) cultured in the absence or presence of silver nanoparticles for 3 days. Then cells were incubated with anti-GFAP (2A5) (red) and anti-neurofilament-L (C28E10) (green) primary antibodies as

described in Materials and methods. White arrows in Fig. 5C point fine processes departing from the DRG neurons cell body. White arrows in Figs. 5B and 5D point strong neurofilament staining around DRG neurons nucleus. Bars represent indicated size.

**Abbreviations:** DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein.

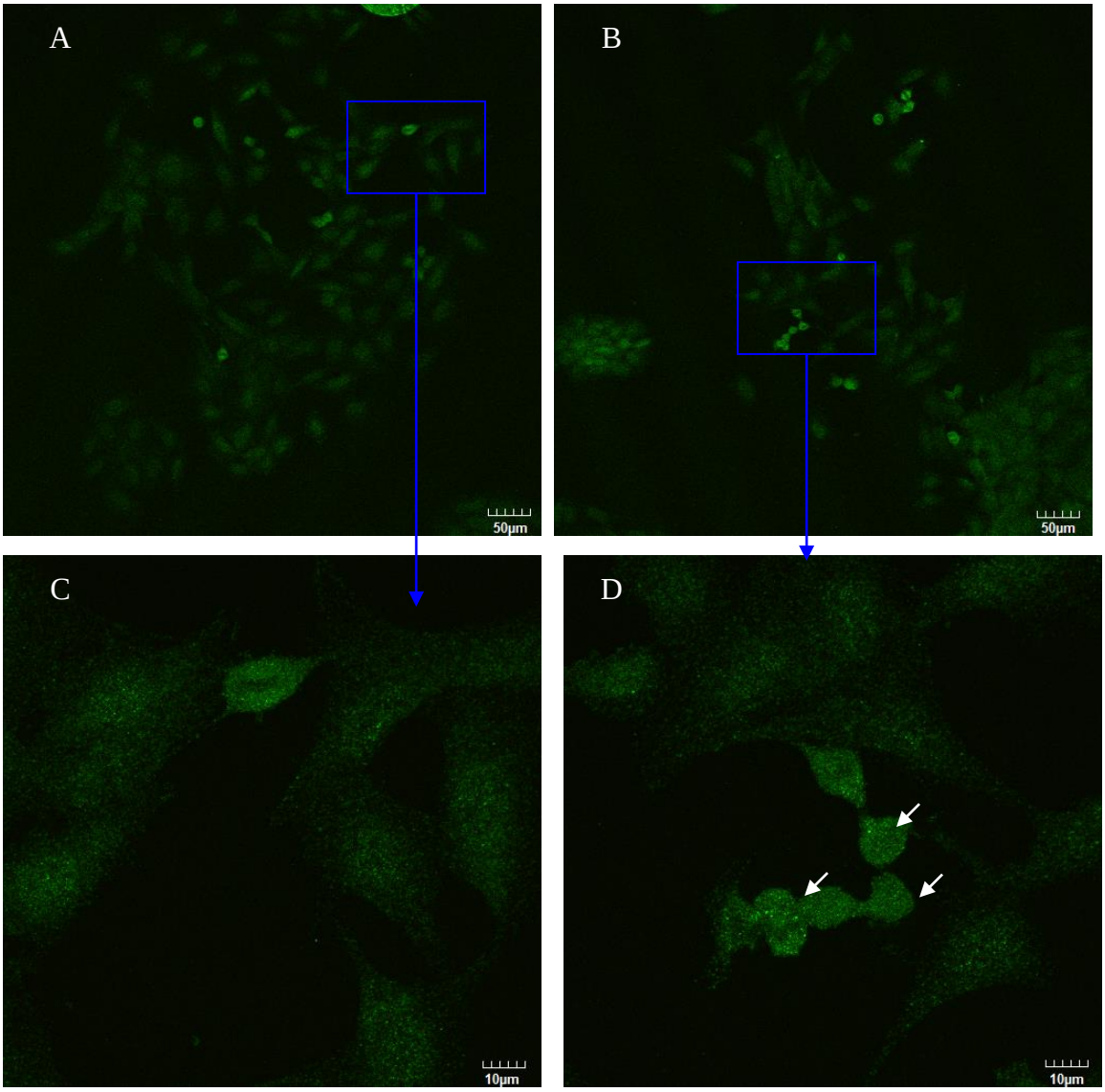


**Fig. 6** Confocal images of DRG neurons in the absence (A, C) or presence (B, D) of silver nanoparticles at two magnifications (upper and lower rows).

**Notes:** DRG neurons in the absence or presence of silver nanoparticles for 3 days. Then cells were incubated with anti-GFAP (2A5) (red) and anti-neurofilament-L (C28E10) (green) primary antibodies as described in Materials and methods. White arrows point at shrinkage DRG neurons. Bars represent indicated size.

**Abbreviations:** DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein.

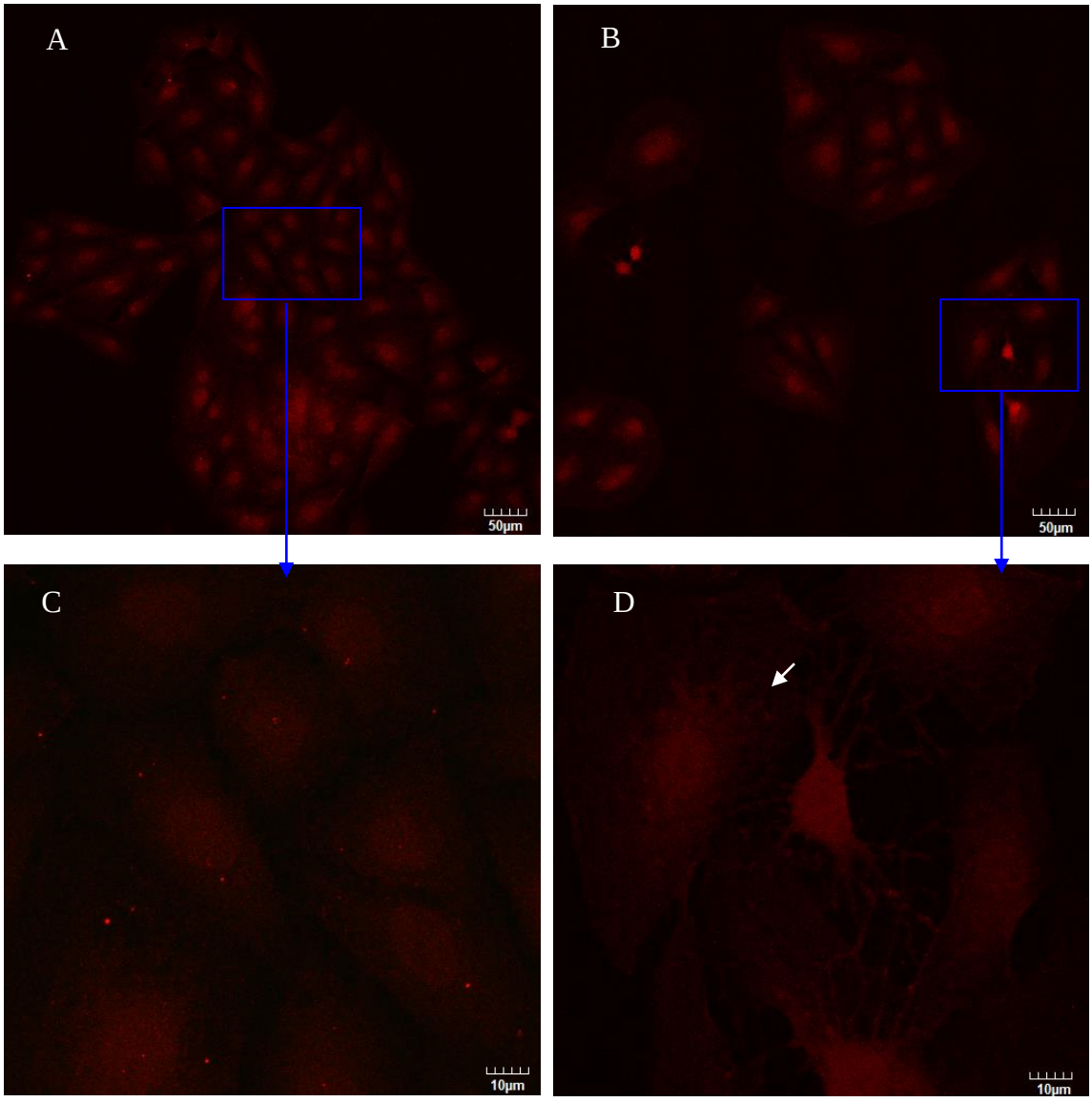


**Fig. 7** Confocal images of Schwann cells in the absence (A, C) or presence (B, D) of silver nanoparticles at two magnifications (upper and lower rows).

**Notes:** Schwann cell in the absence or presence of silver nanoparticles for 3 days. Then cells were incubated with anti-GFAP (GA5) (red) and anti-neurofilament-L (C28E10) (green) primary antibodies as described in Materials and methods. White arrow points at swelling Schwann cells. Bars represent indicated size.

**Abbreviation:** GFAP, glial fibrillary acidic protein.

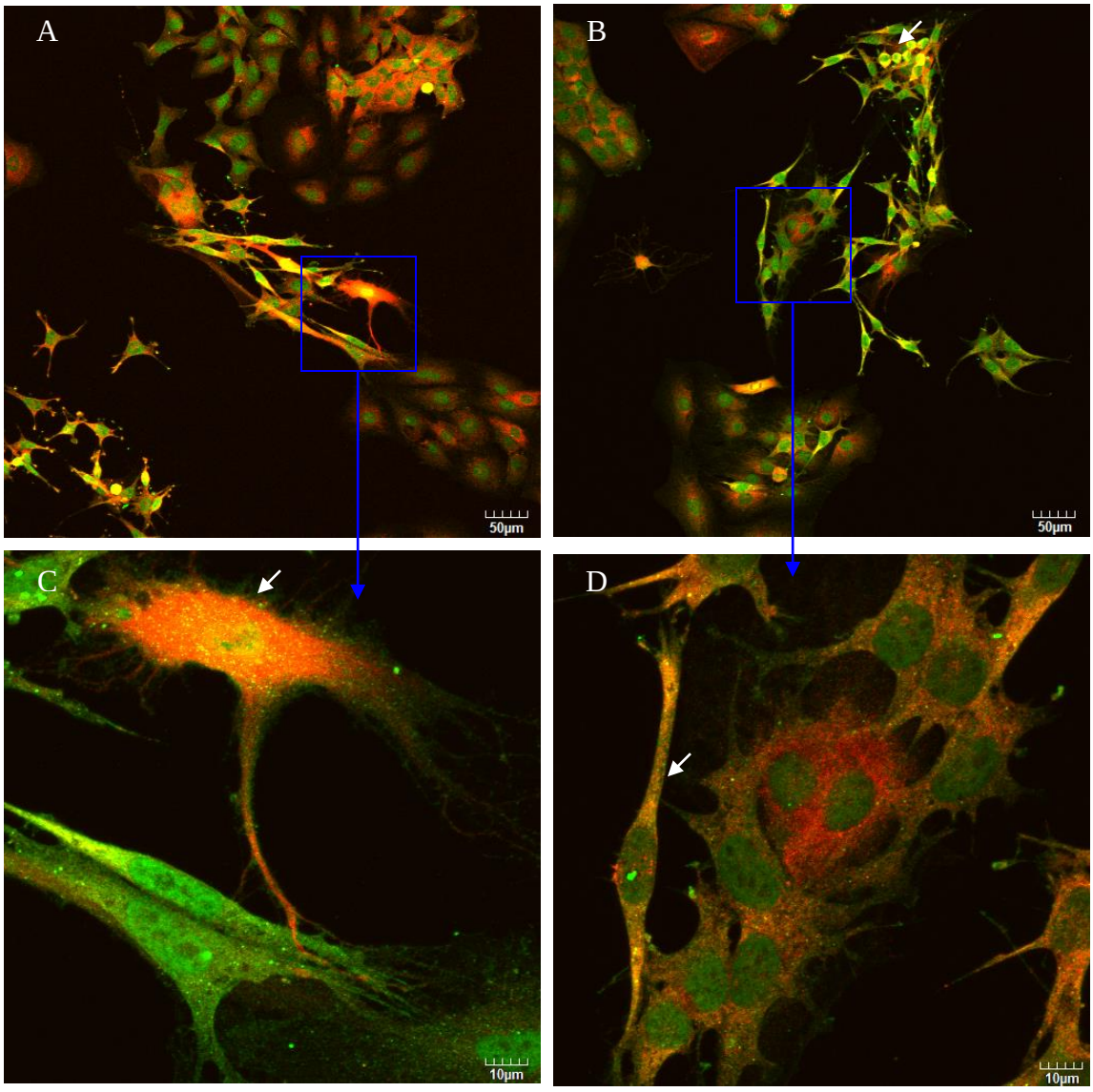


**Fig. 8** Confocal images of DRG neurons co-cultured with Schwann cells in the absence (A, C) or presence (B, D) of silver nanoparticles at two magnifications (upper and lower rows).

**Notes:** DRG neurons co-cultured with Schwann cells (at the same ratio) were cultured in the absence or presence of silver nanoparticles for 3 days. Then cells were incubated with anti-synapsin (green) and anti-neurofilament-L (DA2) (red) primary antibodies as described in Materials and methods. White arrow in Fig. 8C points at fine processes. White arrows in Figs. 8B and 8D point at shrinkage DRG neurons. Bars represent indicated size.

**Abbreviation:** DRG, dorsal root ganglion.

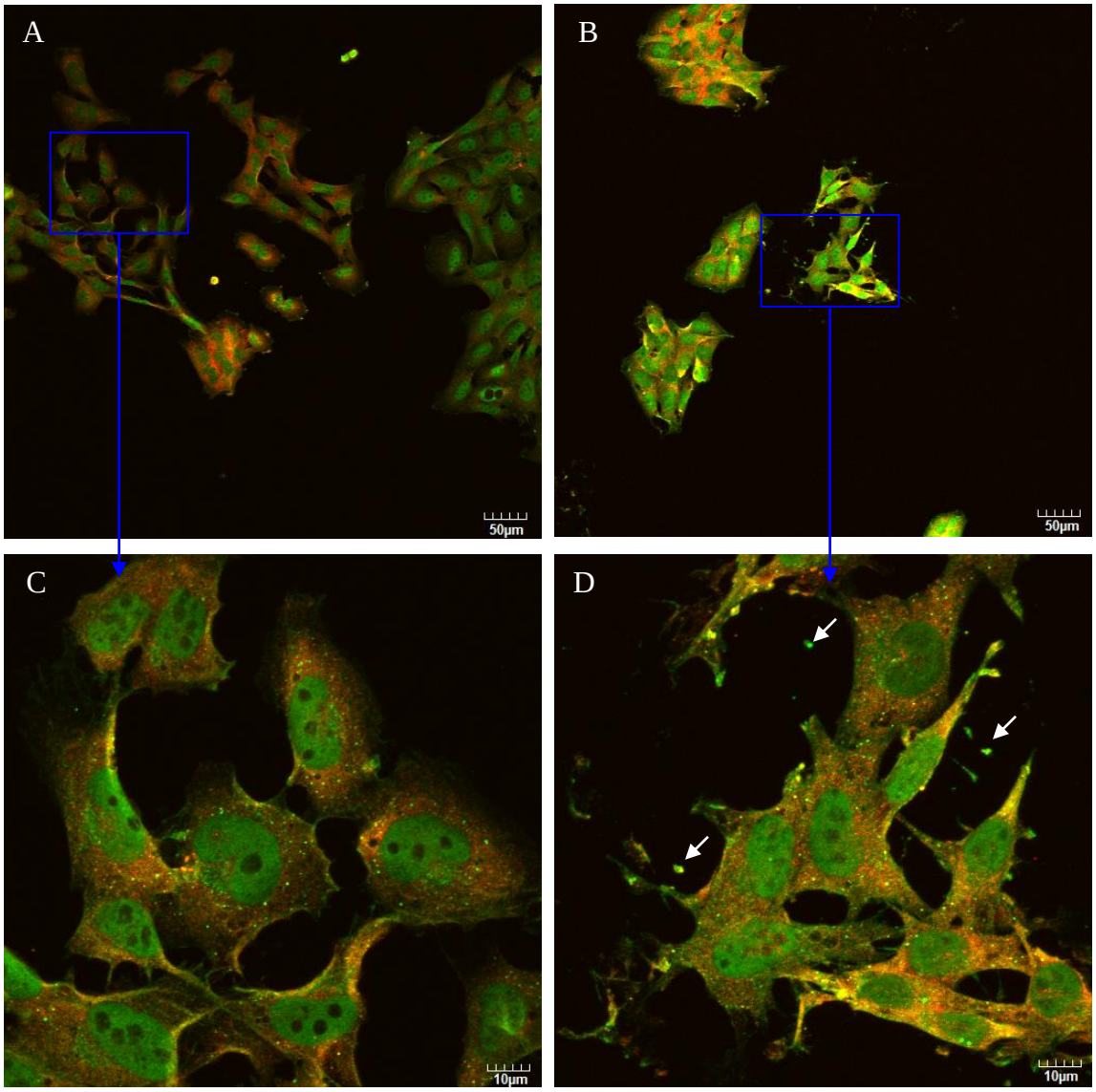

**Fig. 9** Confocal images of DRG neurons in the absence (A, C) or presence (B, D) of silver nanoparticles at two magnifications (upper and lower rows).

**Notes:** DRG neurons were cultured in the absence or presence of silver nanoparticles for 3 days. Then cells were incubated with anti-synapsin (green) and anti-neurofilament-L (DA2) (red) primary antibodies as described in Materials and methods. White arrows point at disrupted staining for sysnapsin. Bars represent indicated size.

**Abbreviation:** DRG, dorsal root ganglion.

To gain further insight into the cellular and molecular mechanisms underlying this "protection" phenomenon in co-culture, we compared expression and localization of synapsin and neurofilament. Schwann cells did not stain with synapsin and neurofilament (data not shown). As shown in Fig. 8 and Fig. 9, DRG neurons are stained with both neurofilament (shown in red) and synapsin (shown in green). However, their expression and localization are different. Synapsin was distributed throughout the cell body associated with punctuate structures. Also it showed membrane localization. Neurofilaments were mostly located in the perinuclear region. Co-culturing with Schwann cells altered neurofilament and synapsin expression. After DRG neurons co-cultured with Schwann cells, expression of neurofilament and synapsin increased (compare Fig. 8A with Fig. 9A). This observation is consistent with previous results (Figs. 3, 5A, and 5C). When DRG neurons contacted one another, expression of neurofilament and synapsin also increased. On the other hand, monotypic cultured DRG neurons were characterized by a rounded morphology, with few processes (Figs. 9A and 9C). When DRG neurons co-cultured with Schwann cells, some DRG neurons showed differentiated

morphology with many fine processes (Fig. 8C, white arrow). After these cells treated with silver nanoparticles, these fine processes became less (compare Fig. 8C with 8D) and some DRG neurons displayed shrinkage (Figs. 8B and 8D, white arrows) with an elongated morphology. The membrane pattern of synapsin immunostaining was clearly observed in untreated DRG neurons (Fig. 9C). However, the pattern was disrupted in DRG neurons after they were treated with silver nanoparticles. A disrupted staining for sysnapsin was observed (Fig. 9D, white arrows), that is indicative of a decrease of synapsin expression. This observation is consistent with Western blot analysis (Fig. 3A).

## Discussion

Although many studies have shown that nanoparticles exerted neurotoxic effects on nerve cells [19-21, 31], most of the studies performed so far have been done in pure monotypic cell cultures [19-21, 31]. Those monotypic cultures are able to unravel the cellular and molecular mechanisms underlying the neurotoxicity of nanoparticles and other nanomaterials in neural cells. However, they lack the interplay between different cell types, as it occurs *in vivo*. By contrast, co-cultures, where neurons are cultivated on the top of glial cells (i.e., Schwann cells), closely mimic the physiological conditions and provide the means to directly evaluate the interactions between neurons and glial cells [32]. Schwann cells always surround DRG neurons *in vivo* and protect them from pathophysiological assaults, therefore, we developed a co-culture model consisting of immortalized DRG neurons and Schwann cells and employed it to investigate some neurochemical mechanisms underlying interactions between DRG neurons and Schwann cells with or without silver and gold nanoparticles treatment.

First, we studied the effects of silver or gold nanoparticles on DRG neurons and Schwann cells either singly in monotypic cultures or in co-cultures by MTT assay. We compared the absorbance of co-cultures of DRG neurons and Schwann cells with the sum of absorbance of monotypic cultures (i.e., Schwann cells or DRG neurons alone). Our results showed that cells survived better in co-cultures when they were exposed to nanoparticles compare to when they were exposed to nanoparticles in monotypic cultures: these findings support our hypothesis that co-culturing DRG neurons with Schwann cells imparted some protection on them against neurotoxicity induced by silver or gold nanoparticles.

We examined the expression of two cellular biomarkers (synapsin and GFAP) in DRG neurons and Schwann cells either singly in monotypic cultures or in co-cultures in the presence or absence of 125 μL of silver nanoparticles or 250 μL of gold nanoparticles (concentrations in which the co-cultured cells survived much better than monotypic cultures) by Western blot analysis. Recent studies have illustrated that glial cells promote synapse formation, maintenance [33, 34] and Schwann cells promote synaptogenesis via transforming growth factor-β1 [35]. Consistent with these observations is our finding in this study that synapsin expression in DRG neurons was increased in co-cultures after they were treated with or without silver or gold nanoparticles (Fig. 3A). Furthermore, it was interesting to find that expression of GFAP in untreated Schwann cells remained almost unchanged after co-culturing with DRG neurons (Fig. 3B). However, GFAP expression was increased in Schwann cells when they were co-cultured with DRG neurons and treated with silver nanoparticles or gold nanoparticles. Thus, this observation demonstrated that without treatment co-culturing with DRG neurons had no effect on the

expression of GFAP in Schwann cells. However, with treatment co-culturing with DRG neurons altered GFAP expression and promoted the survival of Schwann cells. Thus, the Western blot analysis results (Fig. 3) are consistent with cell survival data (Fig. 1D and Fig. 2C), suggesting that there is a reciprocal control of cell survival between DRG neurons and Schwann cells [29] and the altered expression of synapsin and GFAP is consistent with our hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against the neurotoxicity induced by silver or gold nanoparticles.

Consistent with previous reports [36, 37], we found that silver nanoparticles were more cytotoxic to these cells than gold nanoparticles (compare Fig. 2 with Fig. 1). Haase and colleagues [38] also found that at all concentrations (10, 20, 30, 50, 100 μg/mL) tested silver nanoparticles were much more toxic on primary culture of astrocytes and neurons than gold nanoparticles. One neurotoxic effect of nanoparticles, such as silicon dioxide nanoparticles, is via a lowering of cell survival/proliferation signaling molecule expression, leading ultimately to death of neural cells [17]. Consistent with this observation is our finding in this study that treatment of Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells with nanoparticles decreased the total ERK expression (Fig. 4A). Moreover, we also found that p-ERK protein expression was decreased when DRG neurons alone or co-cultured with Schwann cells (Fig. 4B) were treated with silver nanoparticles. These findings suggested that the anti-proliferative and/or anti-survival effect of silver nanoparticles may be mediated by the marked decrease in p-ERK level in DRG neurons. However, gold nanoparticles did not affect their expression of p-ERK (Fig. 4B). Silver nanoparticles led to increases in the

expression of the p-ERK in Schwann cells (Fig. 4B). Similarly, Rinna et al. [39] found that treatment with silver nanoparticles increased expression of p-ERK in human epithelial embryonic cells and this activation was associated with ROS generation and DNA damage. Thus, our findings that silver nanoparticles activated ERK may be associated with ROS generation and DNA damage rather than the lowering of survival/proliferation signaling in Schwann cells. Clearly, further studies are needed to clarify this mechanistic issue.

Since we found that co-culturing with Schwann cells altered synapsin expression in DRG neurons and co-culturing with DRG neurons altered GFAP expression in Schwann cells after Schwann cells were treated with silver or gold nanoparticles, we monitored the effects of silver nanoparticles on morphological changes in Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells employing immunofluorescence staining. As expected of a neuronal biomarker, DRG neurons, but not Schwann cells, were stained with neurofilament (Figs. 5, 6 and 7). DRG neurons showed only a faint immunostaining with neurofilament in monotypic culture (Fig. 6). However, when DRG neurons were co-cultured with Schwann cells, strong and filamentous staining was observed (Fig. 5). Also DRG neurons showed morphological alterations in co-culture, such as some of the DRG neurons changed to a triangular shape with many fine processes (Fig. 5C). All these results indicated that Schwann cells may have the property of promoting neurofilament polymerization and producing the morphological differentiation in DRG neurons in co-culture. Clearly, these are novel but more systematic studies are required to fully elucidate the molecular mechanisms underlying the interactions between Schwann cells and DRG neurons. On the other hand,

silver nanoparticles disrupted the normal cell morphology (compare Figs. 5, 6, and 7). Some DRG neurons displayed shrinkage (Figs. 6B and 6D) and some Schwann cells displayed swelling (Figs. 7B and 7D). Xu and colleagues [40] studied neurotoxicity of silver nanoparticles in rat brain after intragastric exposure. They also found that silver nanoparticles could induce neuron shrinkage and astrocyte swelling. Our results and Xu's results suggested that silver nanoparticles had similar neurotoxicity effects in peripheral nervous system and in central nervous system.

Synapsin is associated with cytoskeletal elements including neurofilaments, microtubules, and postsynaptic densities [41]. Even through, we found that co-culturing with Schwann cells increased expression of synapsin in DRG neurons by Western blot analysis, in order to determine the expression and distribution of synapsin within individual DRG neurons and gain further insight into the cellular and molecular mechanisms underlying interactions between DRG neurons and Schwann cells in co-culture, we compared expression and distribution of synapsin and neurofilament in DRG neurons alone and in DRG neurons co-cultured with Schwann cells by immunofluorescent staining. Fletcher et al. [42] found that synapsin was concentrated in brightly fluorescent puncta around cell bodies of hippocampal neurons. Even though, the neurons we studied are from peripheral nervous system, we also found similar synapsin distribution with central nervous system neurons (Figs. 8 and 9). To our knowledge, this is the first study showing that puncta structure in peripheral nervous system neurons. We further found that synapsin in DRG neurons showed membrane localization, indicating that synapsin may be an important link between cytoskeleton and membrane [41]. Different with synapsin, neurofilaments were mostly located in the perinuclear region,

which is similar with the localization of neurofilaments in neuroblastoma cells [43]. We also found when DRG neurons contacted one another, expression of neurofilament and synapsin also increased suggesting that the increased expression of synapsin and neurofilament was triggered by contact between neurons (Figs. 8 and 9) [42].

Many studies revealed that glia cells not only support neurons in trophic aspect but also have more active functions in neurons. For example, a number of recent observations have provided evidence that glia cells in the central nervous system can promote synaptogenesis, modulate synaptic activity, influence the electrical activity of neurons, and regulate neuronal migration and process outgrowth [29]. Falcão et al. [32, 44] demonstrated that astrocytes, in an indirect neuron-astrocyte co-culture model, had neuroprotective properties when they communicate with neurons and interact with toxic stimuli as unconjugated bilirubin. Similarly, in one our previous study, U87 cells in non-contact co-culture can protect SK-N-SH cells against glutathione depletion induced by ethacrynic acid treatment [45]. In this study, we found that glia cells (i.e., Schwann cells) in the peripheral nervous system also had the property of promoting synaptogenesis. Furthermore, we found that Schwann cells had the property of promoting neurofilament polymerization, producing the morphological differentiation in DRG neurons and DRG neurons can protect Schwann cells from pathophysiological assaults. As far as we are aware, ours is the first study to demonstrate that co-culturing DRG neurons with Schwann cells imparts some protection on them against neurotoxicity induced by silver or gold nanoparticles. Thus, recent reports [32, 44, 45], as well as the findings of this study, are in accord in emphasizing that glial cells (i.e., Schwann cells, astrocytes) can protect neurons from pathophysiological assaults. However, more extensive and in-depth

examinations should be considered carefully in future studies to better understand the neurochemical mechanisms underlying interactions between DRG neurons and Schwann cells.

## Conclusions

To the best of our knowledge this is the first study to report the neurochemical mechanisms underlying interactions between DRG neurons and Schwann cells using a co-culture cell model consisting of immortalized DRG neurons and Schwann cells *in vitro* with or without silver or gold nanoparticles treatment. Our studies are the first to demonstrate that co-culturing DRG neurons with Schwann cells imparts some protection on them against neurotoxicity induced by silver and gold nanoparticles and altering the expression of neurofilament, synapsin and GFAP could, at least in part, account for the phenomenon of "protection" in co-cultures. We further found that silver nanoparticles were more cytotoxic than gold nanoparticles and alteration of ERK cell survival/proliferation signaling was involved in the neurotoxic effects of silver or gold nanoparticles. Taken together, our results are consistent with our hypothesis and may have pathophysiological implications in the biocompatibility and health hazard of silver and gold nanoparticles. Moreover, this *in vitro* co-culture model presented here is excellent for studying interactions between DRG neurons and Schwan cells and for initial testing of neurotoxicity of candidate nanoparticles, since it overcomes the limitations and disadvantages of the monotypic neural culture models as well as those of the primary cultures of DRG neurons co-cultured with Schwann cells or other glial cell types.

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## Disclosure

The authors report no conflicts of interest in this work.

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# Chapter V

## General discussion and conclusions

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The use of nanomaterials has risen exponentially over the last decade. Applications are manifold and include, but not limited to drug delivery, medical imaging, diagnostics, and cancer therapy.[1,2] However, concerns have been expressed about the health risks and environmental impact of such materials and whether they can cause adverse effects.[3] Cell cultures *in vitro* can be performed under controlled environments with predictable and reproducible results, and are relatively inexpensive. Hence, cell culture models have been widely used to investigate cytotoxicity of nanomaterials.[3] Our studies utilizing monotypic cell models and co-culture cell models investigated applications of chitosan and nanoparticles (ie, silver and gold nanoparticles) in cancer chemotherapy (Chapter II) and the putative toxic effects of CNTs, silver and gold nanoparticles on neural cells (ie, DRG neurons and Schwann cells) of the PNS (Chapter III and Chapter IV).

Our studies are the first to demonstrate the anti-cancer property of chitosan is enhanced after it is employed in combination treatment with nanogold or nanosilver particles and/or anti-cancer drugs (Chapter II). We also found that the anti-survival/proliferative effect of chitosan, chitosan in combination with nanosilver particles and/or 0.1 μM Adriamycin may be mediated by the ROS overproduction involving AKT and ERK signaling pathways. Our proposed mechanism is that chitosan in combination with nanosilver particles and/or 0.1μM Adriamycin treatment induced ROS generation, which increased AKT and ERK in an attempt to bypass the stress injury. However, AKT and ERK fail to

confer a survival role, and the cells undergo cell death. Clearly, further studies are needed to clarify this mechanistic issue. These results were already published and presented in international and local conferences.[4-7]

The main disadvantages of primary cell cultures include (1) a limited life span, (2) increased genetic variability between model systems and cultures, (3) mixture of different neuronal populations in each preparation, as well as (4) high resource requirements.[8] The availability of an immortalized cell line of DRG neurons[9] allows us to address this limitations and develop a model *in vitro* employing immortalized DRG neurons to evaluate the putative cytotoxicity of carbon nanotubes (CNTs), chitosan, and chitosan in combination with CNTs on the PNS (Chapter III). Our studies showed that SMWCNTs induced concentration-related decreases in the growth of DRG neurons, and the non-functionalized SMWCNTs were more cytotoxic than the functionalized ones. Chitosan exerted a small negative impact on the growth of DRG neurons. We also found that SMWCNTs induced necrotic damage in the neurons and decreased their expression of phospho-Akt. More importantly, we observed that some of the non-functionalized SMWCNTs were inside the cytoplasm of the neurons. Thus, the results of our studies may have toxicological and other pathophysiological implications in exposure of PNS to SMWCNTs. These results were also published and presented in international and local conferences.[10,11]

Employing immortalized DRG neurons and Schwann cells, we developed a co-culture cell model and investigated the neurotoxicity of silver and gold nanoparticles on peripheral nerve cells. We found that Schwann cells in co-culture exerted beneficial effects on DRG neurons and protected DRG neurons against neurotoxicity of silver or gold nanoparticles by altering the expression of synapsin and neurofilament. We also

found that GFAP expression in Schwann cells was changed after co-cultured with DRG neurons and treated with silver or gold nanoparticles. All these results suggested that Schwann cells had the property of promoting synaptogenesis, promoting neurofilament polymerization, producing the morphological differentiation in DRG neurons and DRG neurons can protect Schwann cells from pathophysiological assaults. These conclusions are consistent with our hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against the neurotoxicity induced by silver or gold nanoparticles. Some of these results were published in NSTI Nanotechnology Conference & Expo, Nanotech 2015. Biotech, Biomaterials and Biomedical, Chapter 1.[12]

In conclusion, our studies showed that chitosan may have the potential in the design of new and/or improved treatments for glioblastoma. CNTs, silver, and gold nanoparticles have neurotoxic effects on the PNS. Our studies have yielded excellent monotypic cell models and co-culture cell model for elucidating the cellular and molecular mechanisms underlying the cytotoxicity of nanomaterials.

## Future work

Our studies have developed a co-culture PNS cell model. To fully understand the mechanisms underlying the protections of Schwann cells on DRG neurons and the interactions between DRG neurons and Schwann cells, there are needs for additional studies to characterize this co-culture cell model. In future, we intend to do the cell cycle analysis and monitor the morphological changes at different time. Clearly, this is an interesting area that merits further investigation.

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