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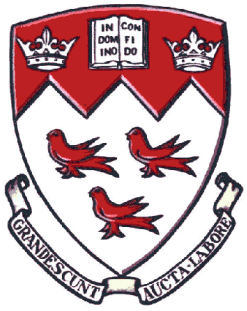

McGill University

Targeted Delivery of Single Walled Carbon Nanotubes as Drug Delivery Systems

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master's of Biomedical Engineering

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Abstract

Carbon nanotubes (CNTs) have become some of the most promising drug delivery systems due to their unique properties, especially their high surface area and their ease to penetrate cells. The aim of this thesis was to render single walled carbon nanotubes (SWNTs) more biocompatible, and to test their ability to selectively deliver suitable dosages of anti-cancer drugs to $\alpha_v\beta_3$ integrins and epidermal growth factor receptor (EGFR) expressing cancer cells used as delivery targets. Those two targets are important to consider, as they are highly present on the cell membrane of several cancer cells, such as colon, breast, leukemic, and lung cancer. Results reveal that the combination of covalent and noncovalent surface modification of SWNTs increased SWNTs biocompatibility towards RAW 264.7 and Caco-2 cells by 17.4% and 20.8%, respectively, compared to covalently modified SWNTs. Results also show that the delivery of the widely used anti-cancer drug, doxorubicin (DOX), was higher when targeted by the SWNTs. In fact, the concentration of targeted DOX was 1.4 (± 0.3) folds higher and 2 (± 0.7) folds higher than that of free DOX in Caco-2 and RAW 264.7 cells, respectively. Similarly, the cytotoxicity of the SWNT-targeted DOX on RAW 264.7 cells at 48h post exposure was 3.6 folds higher than that of free DOX. Thus, the study reveals that SWNTs are capable to enhance drug effect on cancer cell lines. Further *in vivo* studies are recommended to evaluate the full potentials.

Resumé

Grace à leurs propriétés uniques tel que leur grande surface de contact et leur aisance de pénètre les cellules humaines, les nanotubes de carbone sont désormais de prometteurs systèmes de livraison des médicaments. Le but de cette thèse est de rendre les nanotubes de carbone plus biocompatible, et de tester leur habileté de cibler la chimiothérapie vers les cellules cancéreuses qui possèdent les intégrines $\alpha_v\beta_3$ et les récepteurs EGF. Ces deux récepteurs sont spécifiquement ciblés car ils sont présents en grandes concentrations sur la surface des cellules cancéreuses telles que les cellules colorectales et les cellules du cancer du sein. Les résultats montrent que la modification covalente et non covalente de la surface des nanotubes de carbone augmente leur compatibilité envers les cellules RAW 264.7 et Caco-2 de 17% et 20.8%, respectivement, comparèrent aux nanotubes modifies de façon covalente seulement. Les résultats montrent aussi que la concentration de la chimiothérapie doxorubicine (DOX) était plus grande lorsqu'elle est délivrée par les nanotubes. En effet, la concentration de DOX délivrée par les nanotubes était 1.4 (± 0.3) et 2 (±0.4) fois plus élevée dans les cellules Caco-2 et RAW 264.7, respectivement, que celle de DOX délivrée sans système de livraison. De même, la cyto-toxicité de DOX délivré par les nanotubes de carbone aux cellules RAW 264.7 était 3.6 (± 0.7) fois plus élevée que celle de DOX délivrée sans système de livraison à 48 h post exposition. L'étude montre que les nanotubes de carbone sont capables d'augmenter la concentration du médicament dans les cellules cancéreuses. Pour étudier tout le potentiel des nanotubes de carbone, d'autres études *in vivo* seront nécessaire.

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Preface

In accordance with the McGill University thesis preparation and submission guidelines, as stated in section I-C, 1 have taken the option of writing the experimental section of this thesis as a compilation of original papers suitable for publications. The papers are presented in chapters 3, 4 and 5 and are subdivided into sections including abstract, introduction, materials and methods, results and discussion, and conclusion. A common abstract, general introduction, literature review, summary of results, overall conclusions and references are included in the thesis as required by the guidelines.

List of Abbreviations

CNT	Carbon Nanotubes
DDS	Drug Delivery Systems
DMAP	4-Dimethylaminopyridine
DMEM	Dolbecco's Medium Essential Eagle Medium
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FITR	Fourier Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffer Solution
PEG	Poly(ethylene) Glycol
RGD	Arg-Gly-Asp Peptide
SWNTs	Single Walled Carbon Nanotubes
TDDS	Targeted Drug Delivery Systems
UV-VIS	Ultraviolet-Visible spectroscopy
WST-1	water soluble Tetrazolium salts

Units:

cm^{-1}	Per centimeter
mL	Milliliters
mg	Milligrams
nm	Nanometers
µg	Micrograms
µL	Microliters
µM	Micromolar
ppm	Parts per million

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1. General Introduction

Cancer is one of the leading causes of death in developed countries. It is estimated that 177,800 new cases of cancer and 75,000 deaths occurred in Canada in 2011 [8]. Cancer cases are increasing world wide as a result of population aging, and unhealthy lifestyle choices such as smoking, lack of physical activity, and poor diets. Cancer, a malignant neoplasm, is a broad group a various diseases that are difficult to treat and that involve uncontrolled cell growth. This growth invades nearby parts of the body, consuming all the nutrients that surrounding healthy tissues are supposed to consume, Cancerous cells are able to leave the primary tumor and migrate to different parts of the body to cause multiple tumors, a process known as metastasis. Once malignant tumor cells metastasize, it gets even more difficult to treat the disease, as different kinds of toxic chemotherapy are required. Additionally, traditional chemotherapeutic agents lack cell-specificity, hence, they need to be administered at very high doses to have an effect on tumors. As a result, a lot of damage is caused to healthy tissues, leading to wide range of side effects such as depression of the immune system, fatigue, weight and hair loss, and dehydration [9]. Advanced drug delivery systems (ADDS) hold the key in improving cancer outcomes, and cancer prevention [10]. Current ADDS offer many advantages when compared to traditional treatments since they are capable of transporting large amounts of therapeutic agents to tumors. ADDS have the ability to overcome various biological barriers and to localize into the target tissue.

Carbon nanotubes (CNTs) have become some of the most promising ADDS due to their unique properties such as high surface area and ease to get internalized by cells. Conjugation of targeting molecules to SWNTs is a technique that has been used to deliver

therapeutic agents exclusively inside cancerous cells. A successful targeting molecule is the Arg-Gly-Asp (RGD) peptide that has been used by many groups to deliver drugs and nanoparticles to integrin positive cell lines [1, 2]. In fact, cyclic RGD is a highly specific probe for $\alpha_v\beta_3$ integrins [3]. Those integrins are common tumor markers expressed at high levels on the membranes of endothelial and epithelial cancer cells [4]. They have the ability to interact with compounds containing the peptide arginine-glycine-aspartic (RGD) [5]. In previous studies, RGD was used as a targeting peptide for various drug delivery systems [6]. Once at the surface of the cell membrane, the RGD peptide moiety of the CNT-RGD complex binds at the interface between the α and β subunits of the $\alpha_v\beta_3$ integrin, facilitating the delivery of the complex [5, 7].

Another major membrane receptor to target, along with the $\alpha_v\beta_3$ integrin, is the epidermal growth factor receptor (EGFR) as it is highly expressed on several kinds cancer cells [11, 12]. It plays important roles in cell growth and decreases cell apoptosis. Originally, EGFR was targeted by the epidermal growth factors (EGF), which is its natural ligand. EGF-conjugated CNTs have proved to be selectively delivered to cancer cells [13]. However, the use of EGF is decreasing since it has a strong mitogenic activity. Therefore, it as recently been replaced by GE11, a novel peptide that successfully recognizes and selectively binds to EGFR. Here, we explore the efficiency of RGD- and GE11-conjuagted SWNTs as targeted nanocarriers of therapeutic agents.

1.1 Research Hypothesis

This study hypothesizes that single walled carbon nanotubes (SWNTs) that are both covalently and non-covalenty surface modified, are less cytotoxic than covalently modified SWNTs. The study also hypothesizes that peptide-targeted and drug-loaded SWNTs are selectively delivered to the cells of interest. As SWNTs possess high surface areas, they can accept high amounts of drugs on their sidewalls. Hence, the concentration of drugs delivered by targeted SWNTs is hypothesized to be higher than that of free drugs inside the cells of interest.

The specific research objectives are:

1- To surface modify SWNTs to enhance their biocompatibility and efficiency for drug delivery applications.

2- To design and characterize ligand-conjugated SWNTs serving as drug delivery systems.

3- To measure the targeted delivery of SWNTs to $\alpha_v\beta_3$ integrins and EGFR expressing cancer cell lines.

4- To investigate the *in vitro* cytotoxicity of doxorubicin (DOX) delivered by the targeted SWNTs.

Chapter II: Literature Review

2.1 Introduction to cancer, its treatments methods and their limitations.

Cancer is one of the major causes of death in North America and worldwide. A new cancer report by the Canadian Cancer Society estimated 177,800 new cases of cancer and 75,000 cancer deaths in Canada in 2011 [8]. Despite the progress that has been made in decreasing the mortality caused by cancer, the worldwide incidence of cancer death continues to increase. Cancer includes a large group of various diseases, all of which involve unregulated cell growth. This uncontrolled cell division and growth leads to the formation of tumors, which invade nearby parts of the body. Cancer detection is hard at its early phases because it produces no symptoms. It is only as the tumor continues to grow that symptoms begin to appear. Cancerous cells are able to spread to distal parts of the initial tumor through the lymphatic system or the bloodstream, allowing a new tumor to start at a different location in the body, a process known as metastasis. Patient treatment gets very difficult once cancerous cells metastasize, and treatment options are limited to chemotherapy, radiation and surgery [14, 15]. All of those techniques, especially chemotherapy, cause severe adverse effects such as depression of the immune system, fatigue, gastrointestinal distress, nausea, hair loss, and dehydration [9]. In fact, traditional therapeutic approaches rely on the administration of unselective and harmful drugs. The medication is therefore distributed throughout the body and enters different kinds of healthy cells. This means that very high doses of harmful drugs must be administered in order to reach a good anticancer efficacy.

2.2 Targeted drug delivery as a novel strategy for cancer treatment

In order to solve the problems caused by the traditional administration of chemotherapy, it is important to both localize it only in the tumor, and to decrease its dose. Novel drug delivery systems (DDS) offer great promises in improving cancer treatment. They consist of a therapeutic agent and a guidance molecule/ligand (also known as targeting molecule/ligand, or recognizing moiety) linked to a carrier known as the delivery vehicle [16, 17]. Such carriers are on the nano scale, and include liposomes, quantum dots, dendrimers, and carbon nanotubes [18]. The main advantage of those nano-sized carriers is that they possess very high surface areas, which is highly needed for efficient drug loading and necessary for adequate cell therapy. As a result, DDS consisting of complexes of nanoparticles, drugs and targeting molecules are increasingly affecting conventional clinical practices and medical research.

The first idea for drug targeting was proposed by Paul Ehrlich in the nineteenth century [19]. He presented the idea of the "magic bullet" that has the ability to bind to specific types of cells, similarly to a key-and-lock approach. Drug targeting is therefore a selective drug delivery process that targets drugs to specific sites, such as organs, tissues or cells. As a result, the therapeutic effects of the drug would be applied only at the desired site in the body, without causing side effects in healthy tissues. Nanoparticles offer great improvements in therapeutics through site specificity, and the efficient protection and delivery of therapeutic agents. Most of the recent nano-systems are the result of extensive studies conducted in the 1960's, which include the use of liposomes, and colloidal gold particles. About three decades ago, gold nanoparticles were used as conjugates and carriers of different antibodies for specific targeting and staining [20].

This application may be considered as a precursor of recent biomedical applications of nanoparticles. The importance of nanoparticles as drug carriers lies in the concept and ability to manipulate/localize molecules, and to produce programmed and desired functions, such as controlling the release of drugs at certain temperatures and pH values [21].

Another beneficial aspect of nanoparticles as drug carriers is their ability to greatly enhance the delivery of highly hydrophobic drugs by means of encapsulation. On the other hand, numerous DDS have been developed in an attempt to minimize drug degradation and loss. As a result, they successfully increase the *in vivo* stability and the bio-distribution of drugs. In other words, they can highly improve the unfavorable pharmacokinetics of 'free' drugs. Considering all the positive attributes that DDS provide to free drugs, targeted drug delivery is being extensively researched to become a standard in cancer therapy.

2.3 Basics of active targeting

Active targeting employs modifications of drug carriers with recognizing moieties such as ligands, antibodies or peptides, that have selective affinities for certain receptors on cell membranes and tissues [22]. As a result, the drug carrier, coupled to the chosen recognizing moiety, allows transportation of thousands of drug molecules by means of the specific receptor of interest. Various receptors and antigens/antibodies have been utilized to target drugs to specific cells [23]. Those receptors are usually surface proteins that may be uniquely expressed or over-expressed on diseased cells only. Targets that have been extensively used in cancer research include receptors like folate, LDL and

peptides receptors, and membrane surface antigens/proteins as described below with more details. Some of the mostly used recognizing moieties that actively target such receptors include molecules like sugars, peptides, folic acid, RGD, and specifically engineered antibodies [24]. Once the recognizing moiety is conjugated to the drug carrier, the whole complex carrier-drug-recognizing moiety reaches the targeted cells. The cells then internalize the complex and the drug gets released at high doses. In fact, due their high surface area to volume ratio, ligand bound nanoparticles can encapsulate or be conjugated with big quantities of drug, which makes the whole delivery process much more efficient than traditional drug administration techniques. Drug delivery systems using nanocarriers for active targeting can overcome physiological barriers, and guide drugs to the desired cells. Following administration, nanoparticles can be rapidly cleared by the macrophages of the reticulo-endothelial system (RES). RES uptake of nanocarriers depends on the carrier size, surface charge, and surface hydrophobicity. Hydrophilic particles that are smaller than 100 nm undergo less clearance by the RES. They have prolonged circulation time in the blood and higher chances of interacting with the tissues of interest. Nonetheless, nanocarriers smaller than 5 nm get cleared by the kidneys.

2.3.1 Receptors used for the targeted delivery of SWNTs:

Different kinds of receptors have been used as targets by DDS for adequate therapeutic results. The uptake of the DDS by the cells is achieved via receptor-mediated endocytosis. The following receptors are some of the widely targeted receptors.

Folate Receptor

Folic acid is a vitamin essential for *de novo* nucleotide synthesis. It is taken up by cells that have membrane-associated folate receptors via receptor-mediated endocytosis. The folate receptor has two glycosyl phosphatidylinositol (GPI)-anchored isoforms, alpha and beta. FR-alpha expression is frequently amplified in epithelial cancers, whereas FR-beta expression is found in myeloid leukemia and activated macrophages associated with chronic inflammatory diseases. Conjugates of folic acid and anti-FR antibodies can be taken up by cancer cells via receptor-mediated endocytosis, thus providing a mechanism for targeted delivery to FR+ cells. Folic acid has been mainly used for tumor specific drug delivery in many cancers including breast, ovary, brain and lung malignancies [25-28].

LDL Receptors:

The low-density lipoprotein (LDL) receptor consists of five distinct domains with individualized function. Among those domains are the ligand binding domain at the N-terminus containing complement-type repeats involved in LDL binding, and the epidermal growth factor (EGF) precursor-homology repeats that contain YWTD motifs responsible for ligand dissociation. The LDL receptor is made from a variety of proteins and the cloning of its gene has broadened its knowledge to be considered a family of LDL receptors, each sharing structurally similar motifs. There are nine members of the family, which include the LDL receptor, and seven of them have been identified in mammals [29]. Each member of this receptor family undergoes the process of receptor-mediated endocytosis [29, 30]. Anionic liposomes and apolipoproteinE (apoE) enriched

liposomes were found to mimic LDL and provided site-specific delivery of antitumor agents to cancer cells via the LDL receptors.

Peptide Receptors

A large number of peptide receptors are expressed in large quantities in certain tumor cells. Receptors for peptides such as somatostatin analogs, vasoactive intestinal peptide, gastrin related peptides, cholecystokinin, and leutanising hormone releasing hormone have been localized on tumor cells [31]. Peptides/peptide analogs can be conjugated to a drug carrier system to allow tumor specific targeting of drugs or other biomolecules, following interaction with peptide receptors. Among the peptide receptors that have been extensively used in cancer research, is the epidermal growth factor receptor (EGFR). EGFR exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor (EGF) [13, 32].

2.4 Single walled carbon nanotubes as drug delivery systems

Carbon nanotubes (CNTs) are made of cylinders of graphene sheets made of pure carbon. The chemical bonding between the carbon atoms that form the sheets is sp^2 by nature, with each carbon atom joined to three neighbor atoms. A single walled nanotube structure can be achieved by wrapping the sheets of grapheme into seamless nano-cylenders. The way the sheet is wrapped is represented by two integers (n, m). Those integers enote the number of unit vector along two directions in the honeycomb crystal lattice of graphene. If m=0, the nanotubes are called “zig-zag”, if n=m, they are called “armchair” nanotubes. Otherwise, they are called chiral (fig.1).

CNTs can be classified as single walled carbon nanotubes (SWNTs) or multi walled carbon nanotubes (MWNTs), depending on the number of graphene layers that compose them. SWNTs are one-dimentional (1-D) nanoparticles with diameters range between 1 and 5 nm and lengths varying between 20 and 400 nm when used for cell therapy and cancer treatment. SWNTs have extremely high surface area (1300 m^2 /g) as all the atoms that constitute them are exposed on the surface. This property permits high loading capacities of drugs and other biomolecules on the SWNTs surface [33-35].

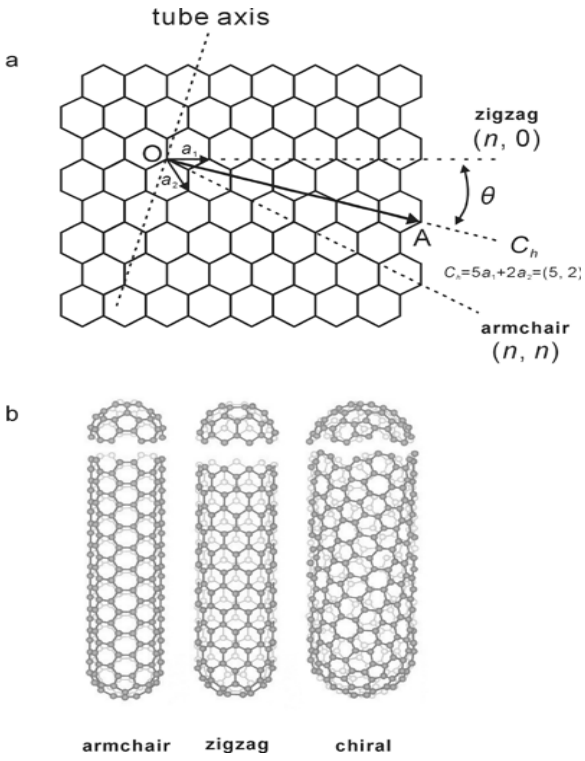

Fig. 1. Rolling graphene sheets to obtain carbon nanotubes. (a) Unrolled graphene sheet made of carbon with sp^2 hybridized orbitals. (b) The sheet is folded to from "armchair", "zig-zag", or "chiral" types of carbon nanotubes. Picture taken from Murr, L.E. et. al. [36].

2.4.2.1 Surface modification and dispersion of SWNTs for biological applications

In order to decrease the cytotoxicity caused by SWNTs, they must be exfoliated and surface modified. When in their raw nature, pristine SWNTs tend to aggregate in aqueous solutions due to their highly hydrophobic nature and the strong Van der Waals forces that hold them together. This aggregation causes them to form a film on the surface of the cells and interact with various biological compounds, inducing stress responses and decreasing cell viability. To address this issue, the SWNTs must be surface modified/functionalized. Proper surface modification renders them hydrophilic and allows them to be well dispersed in aqueous solutions. It has been shown that the SWNTs toxicity was dependent on the amount and nature of surface modification. In fact, toxicity studies have shown a decrease in cytotoxicity as surface modification increased [37]. Surface modification can be either covalent on noncovalent.

2.4.2.2 Covalent surface modification of SWNTs

Different techniques have been used to covalently modify the carbon nanotubes sidewalls, cycloaddition and oxidation being the most utilized methods. Cycloaddition is inducted by photochemical reaction of the nanotubes with azides [38, 39] or carbine generating compounds. The reactions occur on the aromatic sidewalls of the nanotubes. A very common cycloaddition reaction, the 1,3-dipolar cycloaddition, relies on the generation of azomethine-ylides that are added on the nanotubes sidewalls. The generation happens by condensing α-amino acids and aldehydes and adding them to the nanotubes, forming a pyrrolidine ring coupled to the sidewalls [40]. Oxidation is carried out with sonicating and refluxing the SWNTs in the presence of nitric acid and sulfuric acid [41]. During the process, the SWNTs get broken into smaller tubes, and carboxyl

groups are formed at the ends of the tubes and at the defect sites along the tubes. Extensive washing is then needed to remove the acids and impurities in the sample. The oxidized SWNTs, oxSWNTs, are highly soluble in water; however, they tend to aggregate in saline solutions. Therefore, it is preferred to further conjugate the carboxyl groups with hydrophilic polymers to stabilize and homogenize them in physiological environments [42, 43].

2.4.2.3 Noncovalent surface modification SWNTs

The noncovalent surface modification of carbon nanotubes relies on the use of heterobifunctional molecules. While the hydrophobic part of the molecule interacts strongly with the hydrophobic surface of the SWNTs due to π-π stacking [44], the hydrophilic part faces the environment, suspending the nantotubes in the solution. Another method to noncovalently modify SWNTs is to coat them with single stranded DNA [45]. The aromatic base units of the DNA are themselves hydrophobic and therefore interact with the nanotubes sidewalls through π-π stacking, while the sugar units of the DNA face the hydrophilic environments. This increases the biocompatibility and solubility of the SWNTs. However, it has been shown that nucleases in the serum cleave DNA-coated SWNTs, which might affect the stability of SWNTs in nuclease containing environments [45]. Noncovalent surface modification of SWNTs using phospholipid poly(ethylene)glycol (PL-PEG) was developed by Hongjie et. al. This method proved to be very efficient in terms of SWNTs solubility and versatile functionalities for cell therapy and drug delivery [1, 46]. PL-PEG is a highly biocompatible amphiphilic biomolecule of which the two hydrocarbon chains of the phospholipid strongly adhere to

the SWNTs sidewalls with the hydrophilic PEG part facing the aqueous environment. The resulting PEGylated SWNTs are highly biocompatible and stable in saline solutions and physiological environments. Another advantage that noncovalent surface modification offers is that it preserves their physical properties by keeping their chemical structure intact. Thus, it retains their imaging properties for Raman spectroscopy.

2.4.3 Potential of SWNTs in targeted delivery of drugs, proteins and genetic material

2.4.3.1 SWNTs as carriers of proteins and genetic materials

Genetic materials are known to be poorly able to cross biological membranes. It is therefore necessary to use viral or nonviral vectors to transport genes inside the cells. Viral vectors are more efficient than nonviral ones, however, they are far more toxic. Pantarotto et. al. have developed SWNT-DNA complexes and reported high DNA expression compared to naked DNA [47]. In order to bind SWNTs to DNA plasmids for gene transfection, SWNTs can be modified with positive charges that attract the negatively charged DNA [47]. For example, amine-terminated SWNTs modified by 1.3-dipolar cycloaddition were able to bind DNA sequences, and achieved reasonable transfection efficiency [2]. Small interfering RNA (siRNA) was attached to SWNTs via cleavable disulfide bonds, and the complex was delivered to cells to observe gene silencing [48].

An interesting study has shown that at 700- to 1,100-nm near-infrared (NIR) light, the strong absorbance of SWNTs can be used for optical stimulation of nanotubes inside living cells to afford various useful functions. The SWNT served as DNA delivery systems that released the DNA inside the targeted cells. The release was achieved by

applying NIR light on the treated cells, causing the complexes to heat up and to dissociate. Continuous NIR radiation can cause cell death because of excessive local heating of SWNTs *in vitro*. Selective internalization of SWNTs inside cells was achieved by labeling the SWNT's with folate receptor tumor markers. Therefore, the functionalization of SWNTs with a specific moiety allowed for the NIR-triggered cell death, without harming receptor-free normal cells. The intrinsic physical properties of SWNTs have been thus exploited to afford new types of biological transporters with many useful functionalities.

Due to their large size, anticancer proteins streptavidin, protein A, BSA, and cytochrome c do not penetrate through cells when delivered freely or without the use of delivery systems. Their conjugation with SWNTs and the delivery of the complex SWNT-protein was explored by Kam and Dai [49]. Confocal fluorescence microscopy was used to observe their translocation in mammalian cell lines, including HeLa, N1H-3T3 fibroblasts, HL60, and Jurkats. Fluorescence microscopy showed that SWNTs were able to transport the proteins cargoes inside the cells. It is believed that the translocation of the SWNTs-protein complexes was mediated by endocytosis, an energy-dependent process that engulfs large foreign particles. Once internalized within the cells, SWNT-proteins complexes were found co-localized with red endocytosis endosome marker FR 4-64 [50], suggesting the confinement of the complexes in endosomal lipid vesicles. The endosomes fused with lysosomes that, due to their acidic environment, caused the degradation of the linkages that attached the proteins to the SWNTs. This mechanism freed the proteins from the SWNTs after cell engulfment. Known to undergo cytochrome c-induced apoptosis, the HeLa and N1H-3T3 cell lines were treated with SWNT-

cytochrome c conjugates for apoptosis assay. After cell incubation with the conjugates, apoptosis was analyzed using fluorescein isocyanate (FITC) labeled Annexin, an efficient marker for apoptosis. Significantly lower amounts of cell viability were observed when incubated with SWNT-cytochrome c conjugates than with cytochrome c alone.

2.4.3.2 Techniques of drug loading onto SWNTs

Like surface modification, drug attachment to SWNTs can be achieved either covalently or noncovalently. Several methods have been used to covalently load drugs on the SWNTs sidewalls, many of which make use of linkers between the SWNTs and the drug [51]. For example, Dai et. al. delivered paclitaxel (PTX) to cancer cells by covalently attaching the drug to the PEG part of PEGylated SWNTs. Initially, PL-PEG containing NH_2 groups were used to surface-craft the SWNTs, and PTX was reacted with succinic anhydride to add COOH groups on the surface of the drug. Then, the NH_2 groups of SWNT-PEG-NH_2 were then reacted with the COOH groups of PTX in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), which served as a coupling agent. The delivery of the product SWNT-PEG-PTX showed higher efficacy in treating tumor growth then the delivery of free PTX [1]. The results showed that the tumor volume decreased by a factor of three when compared to that of the same tumor treated with free PTX. Another group covalently attached platinum (IV) to the SWNTs surface using amide-coupling reactions. First, the SWNTs were coated with PL-PEG-NH_2 through ultrasonication. The SWNT-PEG-NH_2 were then washed and conjugated covalently with platinum (IV). The formulation was successfully internalized in nasopharyngeal epidermoid carcinoma (KB), choriocarcinoma (JAR), and human testicular cancer (NTera-2) cells [52], and platinum (IV) was cleaved inside the

lysosomes of cells to be released it to an active Pt(II) species that successfully killed the cells.

Although covalent attachment of drugs on the SWNTs sidewalls is feasible, it might cause chemical changes in their structure, and thus altering their efficiency [53]. To solve this issue, noncovalent drug attachement can be used as it only involves physical conjugation of the drug to the SWNTs. As a result, the drug's chemical structure remains intact and preserves it therapeutic properties. Noncovalent conjugation can be achieved via π–π stacking, hydrophobic interactions, or electrostatic adsorption of the drug onto the SWNTs sidewalls. An example of noncovlent attachment of an anticancer drug is the attachment of doxorubicin (DOX) to SWNTs [54]. DOX was sonicated with the SWNTs for 30 minutes to allow it to adsorb on the SWNTs surface. The complex SWNT-DOX was then washed extensively to remove the unbound DOX. The interaction between the SWNTs and DOX was then studies using luminescence spectroscopy. DOX adsorbed strongly and at a high concentration on the nanotubes surface [54]. In another experiment, PEGylated SWNTs were sonicated with DOX, resulting in DOX becoming loaded onto the PEG covering the SWNTs. Because of its aromatic nature, DOX bound noncovalently to the SWNTs through π–π stacking and hydrophobic interactions [55].

2.4.3.3 Targeted delivery of SWNTs as carriers of anticancer molecules

For potential cancer treatment, it is highly important to selectively target the therapeutic agents to the tumors. Both passive targeting, which relies on the enhanced permeability and retention (EPR) effect of tumors, and active targeting guided by recognizing moieties

or targeting ligands, have been used for effective drug delivery. One of the biggest advantages of SWNTs is their ability to selectively deliver high amounts of therapeutic agents to cancerous cells [56].

Various studies showed that SWNTs are rapidly internalized into different cells and accumulate in the cytoplasm to deliver high dose of drugs [54]. Cell-specific ligands including recognizing moieties like peptides, and antibodies [52, 57, 58] have been conjugated to the SWNTs, along with the therapeutic agent, to target the SWNTs to specific cells (see fig. 2). There is still debate about the exact mechanism by which SWNTs enter the cells. It is said that the cellular uptake mechanism of SWNTs differs depending on the surface modification type and the nanotube's dimensions. Two main internalization roots have been described in the literature. These are passive diffusion through the lipid bilayer of the cell membrane, and receptor-mediated uptake of the SWNTs [34, 35, 59]. Receptor-mediated uptake relies on the interaction between ligand-conjugated SWNTs and specific receptors on the cell membrane. This interaction allows the cells to internalize the SWNTs and their cargo via endocytosis, an energy-dependent process.

Once in the cells, SWNTs are able to deliver their cargoes in the acidic environment of lysosomes, then, they exit the cells through exocytosis [60]. Double functionalization of SWNTs has been employed in order to attach different biomolecules such as fluorescent probes, and drugs on their sidewalls [52]. One of the first *in vivo* studies using SWNTs was conducted by McDevitt et. al. [58]. It was shown that SWNTs conjugated with antibodies, metal-ion chelate, and fluorescent chromophore moieties were specifically reactive with human lymphoma cancer cells. The antibodies coating the

nanotubes were able to selectively recognize and target the cells. Similarly to antibodies, folic acid has been extensively used as a recognizing moiety in cancer therapy. For example Dhar et. al. have linked folic acid to Pt(IV) prodrugs, which were then linked to SWNTs to get SWNT-Pt(IV)-FA complexes [52]. The complexes were toxic to folate receptor positive cells, but not to folate negative ones.

Another successful recognizing moiety is the Arg-Gly-Asp (RGD) peptide that has been used by many groups to deliver drugs and nanoparticles to integrin positive cell lines [1, 2]. In fact, cyclic RGD is a highly specific probe for $\alpha_v\beta_3$ integrins [3]. Those integrins are common tumor markers expressed at high levels on the membranes of endothelial and epithelial cancer cells [52]. Once at the surface of the cell membrane, RGD binds at the interface between the α and β subunits of $\alpha_v\beta_3$, facilitating the uptake of the whole system attached to the peptide [5, 7]. Cyclic RGD has been linked to PEGylated SWNTs, and the targeting capabilities of the complex SWNT-RGD were evaluated in various studies [46]. The system SWNT-RGD has been used for the targeted delivery of doxorubicin (DOX) to integrin positive cancer cells such as U87MG [53]. Results showed that brighter DOX fluorescence were observed in U87MG cells incubated with RGD-conjugated and DOX-loaded SWNTs, compared to cells treated with control formulations [53]. Additionally, RGD-conjugated and DOX-loaded SWNTs showed a high decrease in cell viability towards the U87MG compared to controls formulations effects. This is due to the specific RGD-integrin recognition and high cell uptake of the RGD-SWNTs.

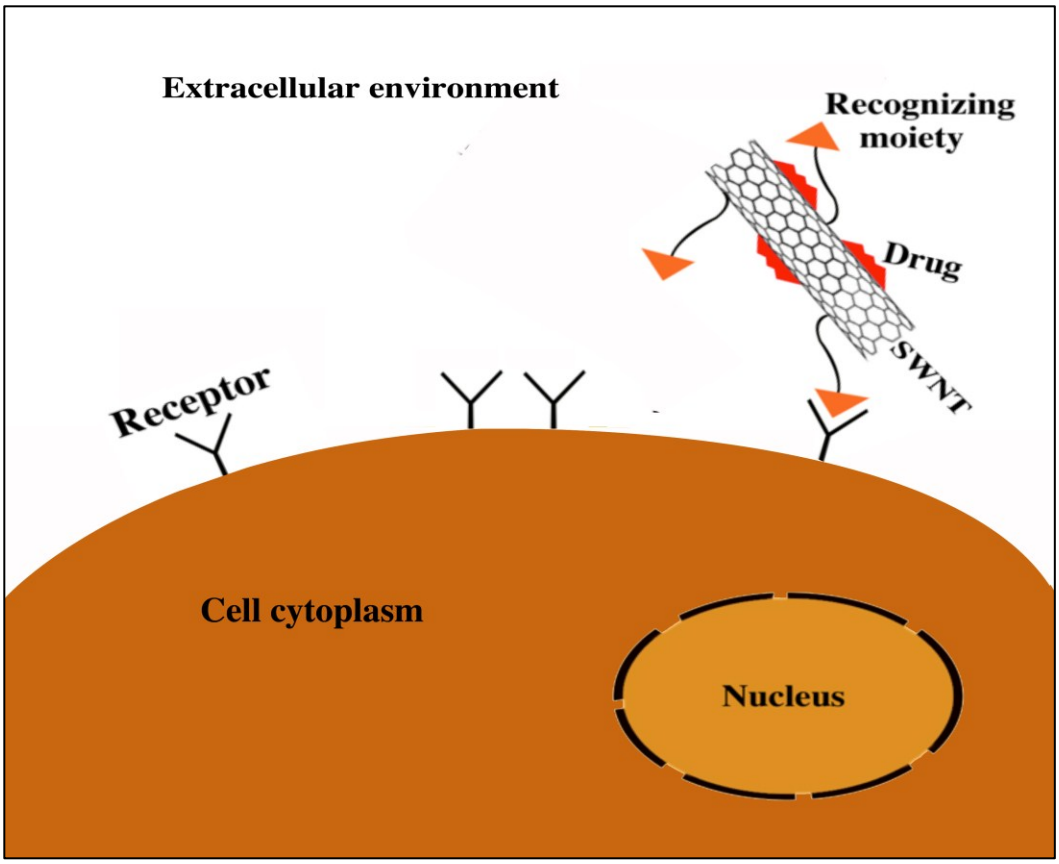

Fig. 2. Active targeting of SWNTs inducing a receptor-mediated endocytosis. As the recognizing moiety on the nanotube's surface binds to its cell receptor, the cell undergoes an endocytotic mechanism that absorbs the whole drug delivery system, resulting in high intracellular concentrations of accumulated drugs.

Dhar et. al. have developed what they called the "long delivery system", which is a complex of SWNTs carrying cisplatin as a therapeutic agent, and folic acid as a recognizing moiety [52]. The complex has been reported to be internalized by cancer cells via endocytosis, followed by the release of the drug and its interaction with the DNA. Another study showed that, after being transported by SWNTs, a similar platinum anticancer, carboplatin, has been shown to inhibit cell proliferation of urinary bladder

cancer cells *in vitro*. Paclitaxel, a poorly water-soluble anticancer drug has been conjugated to PEGylated SWNTs via a cleavable ester bond between the carboxyl groups of paclitaxel, and the amine groups of PEG [1]. The formulation showed to be more effective in suppressing tumor growth than free paclitaxel. Additionally, the presence of PEG chains on the nanotubes prolonged the formulation's circulation *in vivo* and greatly enhanced cellular uptake of the drug by cancer cells.

2.4.4 Therapeutic agents release from SWNTs

It is important to understand the mechanism by which drugs are released from the SWNTs once they are internalized in cancerous cells. Different release modes have been described, however, data describing the rate and amount of drug release from SWNTs is lacking. It has been found that, as the environmental pH becomes more acidic, higher amounts of drugs are released from the SWNTs [48]. For example, 40-50% DOX gets released from the SWNTs in 24 hours as the pH reaches 5.5. Since the microenvironment of extracellular tumor tissues is acidic, drug release in this kind of environments occurs at higher rates [61]. This release at lower pH's might be caused by weakening of hydrogen bonds between the partially negative charge of DOX and the defect sites of SWNTs. Under acidic conditions, the H^+ protons in the solution would compete with the hydrogen bond-forming groups, and weaken the hydrogen bond interactions, leading to greater release of DOX. DOX release from the SWNTs inside living cells was studies by Kang et. al. The study showed that the drug was detached from the SWNTs once the complex was engulfed in the lysosomes [62]. This is due to the low pH value inside the lysosome that loosens the π- π stacking between the drug and the SWNTs. The free DOX then

leaves the lysosomes and get into the nucleus, while the SWNTs remain in the cytoplasm and leaves the cell via exocytosis [62]. Another study showed that enzymes in the lysosomes of HeLa cells were able to cleave the disulfide bonds that covalently linked SWNTs to siRNA [63]. The free siRNA was then able to leave the lysosomal lipid vesicles to reach the cytosol of the cells, and successfully silenced the targeted gene.

Preface for Chapter 3, 4 and 5

The results from the current research have been presented in the following three chapters. Chapter 3 is an optimization of surface chemistry on single walled carbon nanotubes (SWNTs) to render them more biocompatible for *in vitro* studies. Doxorubicin (DOX) loading and release from the SWNTs is also investigated. Chapter 4 focuses on the targeted delivery of DOX to the colon cancer cell line Caco-2, using SWNTs as drug delivery systems. Chapter 5 focuses on the efficiency of a novel targeted SWNT formulation to localize DOX in epidermal growth factor receptor (EGFR) expressing cancer cell lines. The RAW 264.7 cell line was used as it is a good candidate to represent EGFR positive cancer cells. A special thanks to Meenakshi Malhotra who showed me how to perform cell culture, cell viability studies, and how to conjugate RGD to surface modified SWNTs.

Chapter 3

Development of a novel drug delivery system based on a double surface modification of single walled carbon nanotubes. Preparation, characterization and *in vitro* assessment.

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3.1 Abstract:

Single walled carbon nanotubes (SWNTs) have been extensively explored as drug delivery systems, however, their cytotoxicity is of major concern. This study attempts to reduce the toxicity of SWNTs used for *in vitro* applications. The SWNTs formulation prepared was first oxidized, then surface grafted with poly(ethylene) glycol (PEG) to obtain a new formulation; oxSWNT-PEG. The toxic effects of the SWNTs were determined on the Caco-2 and RAW 264.7 cell lines. Measurements taken at 24h, and 48h of exposure showed that formulation oxSWNT-PEG exhibited less toxicity than the already existing formulations oxSWNTs and SWNT-PEG. Viability studies of RAW 264.7 at 24h post exposure showed that the toxicity caused by oxSWNT-PEG at 50 μg/ml was 17.4% less than the toxicity caused by oxSWNT, and 3.77% less than the one caused by SWNT-PEG. Similarly, the toxicity caused by the formulation at 50 μg/ml on Caco-2 was 20.8% less than the toxicity caused by oxSWNT, and 13.3% less than the one caused by SWNT-PEG. Those results confirm the reduction of toxicity as a result of the double modification on SWNTs surface. Additionally, The formulation demonstrated a high loading capacity of anti-cancer drug doxorubicin (DOX) on the SWNTs surface, with a weight ratio of DOX:SWNT equal to 3:1. Drug release was pH- and time-dependent.

3.2 Introduction

Carbon nanotubes (CNTs) have attracted much attention in the field of cancer therapy and medical imaging [56, 64]. However, in spite of these advantages, pristine CNTs are not fit for biomedical applications, as they cause significant cytotoxic effects [65-67]. The hydrophobic surface of pristine nanotubes makes them insoluble in aqueous and physiological environments. As a result, they aggregate into large chunks that accumulate on the surface of cells and tissues, causing toxicity and poor delivery of drugs [68, 69]. In order to solve this issue, adequate surface modification of CNTs is required to solubilize and disperse them in aqueous environments. The different studies regarding the toxicity of CNTs [70] often show contradictory and inconclusive results [71][[2, 72, 73], but toxicity is always detected.

In this study, we propose an enhanced system of doubly modified single walled carbon nanotubes (SWNTs), to decrease their toxicity and increase their biocompatibility. We first enhanced the water solubility of SWNTs by using covalent surface modification on their sidewalls to obtain oxidized SWNTs (oxSWNTs). Then, we surface graphed the newly modified oxSWNTs with PEG, to obtain a new and highly biocompatible formulation: oxSWNT-PEG. WST-1 assay was performed on the Caco-2 cell line and the Raw 264.7 cell line, which proved that the formulation was less toxic than oxidized SWNTs and SWNT-PEG; two already known and widely used formulations for drug delivery.

3.3. Materials and methods:

3.3.1 Chemicals

HiPCo purified single walled carbon nanotubes (SWNTs), purchased from Unidym, (Sunnyvale, USA). Fluorescein isothiocyanate (FITC) was purchased from Aldrich Chemicals Co., (Milwaukee, USA). Dialysis cassette were obtained from Thermo Scientific (Rockford, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (PEG) was purchased from Avanti Polar Lipids (Alabama, USA). Centrifugal filters were purchased from Millipore (Billerica, USA). Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline PBS were purchased from Life Technologies (Grand Island, USA). Water Soluble Tetrazolium salts assay (WST-1 assay) was purchased from Cayman Chemical (New Orleans, USA).

3.3.2 Covalent modification of single walled carbon nanotubes using the oxidation method:

In a typical preparation, 50 mg of pristine, HiPCo purified SWNTs (diameter from 1-1.5 nm and length from 50 to 200nm, were first dispersed in 50 mL of 3:1 HNO_3:H_2SO_4 solution for 3 h using bath sonication. This step serves to both disperse, cut the nanotubes to shorter lengths, and to begin oxidizing them. After sonication, the SWNT formed a black solution of insoluble material. This mixture was then refluxed and heated at 70 °C in the acid for a period of 4 hours. The reaction conditions were chosen to minimize loss of SWNT material while ensuring adequate removal of metallic impurities. The oxidation step serves to introduce carboxylic acid groups primarily onto nanotube ends and defect sites. Oxidized metal catalyst impurities were subsequently washed away

through ultracentrifugation and filtration. The oxSWNTs were then extensively washed with deionized water until the pH of the washes reached 4.5. Finally, the ox-SWNTs in water suspension were heated resulting in a black powder with a typical recovery yield of 70% by weight. We confirmed that the SWNTs were effectively oxidized with UV-VIS-NIR spectroscopy (fig.3).

3.3.3 Conjugation of oxSWNT with FITC:

A quantity of 40 mg FITC was dissolved in 15ml of dimethylformamide (DMF), and stirred in a flask. 6mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to the mixture in order to activate the functional carboxyl groups of FITC, followed by 10mg of oxSWNT after 10 minutes. 15 μg of 4-Dimethylaminopyridine (DMAP) was added as a catalyst. The mixture was stirred overnight at room temperature in dark, as FITC is light sensitive. For purification, the mixture was transferred to a dialysis cassette with a molecular weight cut off (MWCO) of 3000. The cassette was stirred in a flask containing deionized water, protected from light. Water was replaced at every 8 hours for 2 days.

3.3.4 Surface modification of Pristine and Oxidized Single Walled Carbon Nanotubes with Poly(ethylene)glycol (PEG):

A quantity of 5 mg of each pristine and newly COOH-functionalized SWNTs were each mixed in two different 200 ml glass scintillation vial with 25 mg of PEG. 25 ml of deionized distilled water was added. PEG was completely dissolved in water by shaking. The vials were sonicated in a bath sonicator for 60 min at room temperature (~22 °C). The water in bath was changed every 15 min to avoid overheating of the

mixture. The newly surface graphed SWNTs were centrifuged for 6 hours, at 24,000*g*, room temperature [74]. The supernatant solution was collected and stored at 4°C to preserve the stability of the formulation. The formulation can be stored up to 4 weeks [74].

Before the use of the functionalized SWNT (stock solution) for *in vitro* studies, we washed them 4 times in order to completely remove the excess PEG in the SWNT solution. For that, we used centrifugal filters with a molecular weight cutoff (MWCO) of 100 kDa, in which we added 3 ml of water to each 1ml of functionalized SWNT stock solution, and the mixture was washed by spinning it 4 times at room temperature for 10 min, 4,000*g*. Finally, the washed oxSWNT-PEG in the filter was collected and fresh media was added for *in vitro* studies.

3.3.5 Doxorubicin attachment and release from oxSWNT-PEG

A quantity of 0.5 mg oxSWNT-PEG was dispersed in 5 mL sodium phosphate buffer 20mM pH 8.5, and 2.5 mg of doxorubicin hydrochloride was added. The mixture was sonicated in a bath sonicator for 10 min and incubated overnight while stirring. Unbound doxorubicin was removed by filtration and washing various times with Amicon centrifugal filters, 30 K MWCO. Absorption spectroscopy at 490 nm was used to determine the amount of unbound doxorubicin in the eluate of the filtration. For drug release studies, suspensions of DOX-loaded oxSWNT-PEG were incubated for 24h and 48h in phosphate buffer pH 5 while stirring. The solutions were washed by centrifugation to remove unbound DOX and UV-VIS-NIR spectroscopy was performed to quantify the DOX-loaded nanotubes.

3.3.6 Cell culture.

Caco-2 cells and RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). All cell lines were supplemented with 1% penicillin-streptomycin. Caco-2 cells were supplemented with 20% of fetal bovine serum (FBS), and RAW 264.7 cells were supplemented with 10% FBS. The incubations were carried out at 37°C, in 5% CO2 atmosphere, and 90% relative humidity. Media was refreshed every two or three days. Cells were trypsinized to detach them from the flasks. The cells were then harvested, plated in 96 well plates at a density of 10^4 cells per well, and incubated for 24 hours.

3.3.7 Addition of SWNTs to the cell lines for toxicity studies:

After seeding the cells and incubating them for 24 hours, the medium was removed and both cell lines were washed with PBS. They were then treated with oxSWNT, SWNT-PEG, or oxSWNT-PEG at various concentrations (200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml 6.25 μg/ml, and 3.125 μg/ml), all in triplicates repeated on the same sample (n=1). Toxicity was measured at 24h, 48h, and 72h post exposure.

3.3.8 Cell viability measurements:

Cells were washed three times with PBS to remove all SWNTs suspended outside the cells. Water Soluble Tetrazolium salts assay (WST-1 assay) [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] was used for measuring cell viability. Fresh media (100 μl) and 20 μl of the WST-1 reagent were

added to each well. The plates were then shaken vigorously for 60 seconds to thoroughly homogenize the reagent inside the wells. Finally, they were incubated for 90 minutes before performing any cell viability readings. Cell viability measurements were taken at 24 hours, 48 hours post treatment with the nanotubes. Absorbance was determined using a Perkin Elmer (Victor^3 V) multiplate reader. The readings were measured at 450 nm.

3.4 Results

3.4.1 Surface modification of SWNTs:

The hydrophobic nature of SWNTs is a major issue in drug delivery applications since the SWNTs tend to aggregate together. In order to resolve this problem, we first modified the SWNTs surface covalently by oxidizing them to obtain oxSWNTs. The covalent modification of the SWNTs sidewalls was carried out by sonication and acid reflux with nitric acid and sulfuric acid. Heating and ultrasonication of the SWNTs with nitric acid causes the formation of carboxyl groups (COOH groups) at the defect sites of the walls and at the ends of the tubes [75, 76]. UV-VIS spectroscopy confirmed the presence of COOH groups on the SWNTs sidewalls (fig. 1). The polymer PEG was used to surface-graft the oxSWNTs. The two hydrocarbon chains constituting the hydrophobic tail of PEG anchor strongly onto the walls of the SWNTs via π-π stacking [77, 78] (fig. 2). The hydrophilic head of PEG extends into the aqueous environment, imparting the water solubility and the biocompatibility of SWNTs [79, 80]. PEGylated oxSWNTs showed to be very stable in biological environments, even at high temperature up to 65

°C. The oxSWNT-PEG formulation was highly soluble in water for more than 2 weeks (fig. 3).

3.4.2 Cellular uptake of single walled carbon nanotubes inside the Caco-2 and Raw 264.7 cells lines:

We examined the cellular uptake of surface modified SWNTs using the Caco-2 and the Raw 264.7 cell lines. The three formulations oxSWNT-PEG, oxSWNTs and SWNT-PEG, tagged with FITC, penetrated the cell lines successfully (fig. 4). The mechanism for the internalization of SWNTs in the cells is not yet fully understood. Several studies showed that SWNTs that are surface-grafted with polymers, proteins or genes can be internalized into cells via endocytosis, whereas SWNTs functionalized with small molecules such as carboxylic groups and amino groups tend to act as tiny needles that can pierce through cell membranes, hence allowing for their penetration into cells. Further studies need to be performed to better understand the uptake mechanism of carbon nanotubes.

3.4.3 Cytotoxicity studies of oxSWNT-PEG:

Since safety is the top priority of any material used for biomedical applications and medicine, we aimed to study the cytotoxicity caused by the new formulation oxSWNT-PEG on two different cell lines: Caco-2 and RAW 264.7. Cell viability was measured using a WST-1 assay (fig. 5, 6, 7). WST assays are preferred to MTS and MTT assays since they are less invasive. MTT's insoluble formazan molecules accumulate inside cells and cause toxicity, leading to false cell viability results. Contrarily, the reaction between WST-1 and the cells occurs at the cell surface, making it a safer product with accurate cell viability measurements. The viability of Caco-2 cells at 24h of

exposure to the oxSWNT-PEG showed to be always high (higher than 80% compared to control unexposed cells) over a wide range of SWNTs concentration from 6.25 to 100 μg/ml (fig. 5). Viability was reduced to 63.5% at a high concentration of 200 μg/ml oxSWNT-PEG, at 24h of exposure. The viability of Raw 264.7 cells decreased to 60.1% at 24h of exposure to 100 μg/ml oxSWNT-PEG. Those results show that the Caco-2 cells are more resistant to SWNTs toxicity than the RAW 264.7 cells. We can also deduce that 100 μg/ml, and 50 μg/ml are the highest SWNTs concentrations to be used for Caco-2 cells and Raw 264.7 cells, respectively.

3.4.3 DOX loading and release from SWNTs

DOX binding and release from SWNTs can be controlled by varying the environment's pH. When in basic solutions, the high surface area of carbon nanotubes allow for efficient loading capacities of DOX through π- π stacking [81, 82]. On the basis of absorbance spectroscopy of DOX at 490 nm, we evaluated the weight ratio of DOX:SWNT to be 3:1, which demonstrates a high binding capacity of DOX on the SWNTs sidewalls. To demonstrate the detachment of DOX, the purified sample SWNT-PEG-DOX was stirred in phosphate buffer at pH 5 for 24 h. Half of the sample was then collected and washed, and the other half was further stirred at pH 5 for another 24 h. UV-VIS-NIR spectroscopy showed that the DOX peaks decreased gradually as the time of exposure of SWNT-PEG-DOX to acidic buffer increased (fig. 8). The absorbance of DOX at 490 nm dropped by ~50 and ~80% after 24 h and 48 h at pH 5. This clearly demonstrates a controlled release of DOX from the SWNTs.

3.5 Discussion

The hydrophobic nature of raw SWNTs makes them form bundles in aqueous solutions. This natural property causes a big problem for biomedical applications since cells and tissues are highly hydrophilic. The highly hydrophilic nature of carboxyl groups helped suspending the oxSWNTs in water (fig. 3), and the formulation appeared to be perfectly homogenous in water for over 5 days. It is believed that the mechanism behind the dispersion process is the formation of hydrogen bonds between the COOH groups on the sidewalls of the nanotubes and the hydroxyl (OH) groups of water molecules [83]. In addition to that, the presence of carboxyl groups on the oxSWNTs surface leads to a reduction of van der Waals interactions between the nanotubes, which highly facilitates the separation of nanotube bundles into individual tubes [77]. Those characteristics reduce the toxicity of carbon nanotubes and allow them to penetrate the cells more easily.

PEG polymers were used to surface-graft the oxSWNT to yield oxSWNT-PEG. The formulation oxSWNT-PEG was highly soluble and well dispersed in water, PBS, and cell culture media, with no clustering for over two weeks (fig. 3). In contrast, pristine SWNTs clustered instantaneously and were hard to disperse in aqueous solutions. It has been found that adsorbing PEG molecules onto the walls of the nanotubes prevents nonspecific binding of proteins and other compounds to the surface of SWNTs. This is due to the wrapping ability of the phospholipid tail of PEG, which partly covers the nanotubes walls. Furthermore, the hydrophilic chain of the molecule protects the surface of the nanotubes since it extends towards the outer environment. Another great advantage that SWNT-PEG holds in drug delivery is the ability to conjugate different

biomolecules (targeting moieties, fluorescent dyes, drugs and proteins) to the hydrophilic head of PEG. This increases their versatility as targeted drug delivery systems.

Various studies have been conducted in order to explore the cytotoxicity of carbon nanotubes. The issue of SWNTs toxicity is still controversial since the results of those studies vary drastically [84, 85], showing a large range of different and sometimes contradictory conclusions. Data has shown that the degree of cytotoxicity of carbon nanotubes might be partially dependent on the nature of chemical modification [86]. It has also been shown that as the degree of sidewall functionalization increases, the SWNTs sample becomes less cytotoxic [86]. Oxidation of nanotubes is known to decrease their cytotoxicity due to the presence of covalently linked COOH groups on their sidewalls. However, despite the fact that oxSWNTs decrease cytotoxicity compared to pristine SWNTs, they always show different levels of toxic effects, depending on the cell line and on the amount of COOH groups on the SWNTs sidewall. Similarly, studies show that the non-covalent modification of pristine SWNTs with PEG also decreases the SWNTs toxicity, both *in vitro* and *in vivo* [87]. Nevertheless, toxicity is always present.

Fig. 6 and 7 clearly show that the formulation oxSWNT-PEG exhibits less cytotoxicity than oxSWNT and SWNT-PEG. In fact, the toxicity studies at 24h of exposure on RAW 264.7 cells showed that 50 μg/ml oxSWNT-PEG were 17.4% and 3.77% less toxic than the same concentration of oxSWNT and SWNT-PEG, respectively. Likewise, 100 μg/ml oxSWNT-PEG were 16.9% and 13.3% less toxic to Caco-2 than the same concentration of oxSWNT and SWNT-PEG, respectively. Those findings represent a significant improvement for the biocompatibility of SWNTs. We believe that this

improvement was due to the presence of both COOH groups and PEG moieties on the surface of oxSWNT-PEG, which greatly helped decreasing the nanotubes toxicity.

The SWNTs were able to adsorb high quantities of DOX due to their high surface area. Contrary to neutral and basic solutions, acidic environments favor DOX detachment from SWNTs due to its increased solubility at low pH's [82, 88]. This phenomenon is for our advantage since cell lysosomes and tumors environments are acidic by nature. This acidity can facilitate the release of DOX from the nanotubes sidewalls.

3.5 Conclusion

The unique properties of CNTs have been explored for their use in drug delivery; however, their hydrophobic nature and tendency to form big chunks in physiological environments cause them to be cytotoxic. We created a new formulation of surface modified SWNTs, which proved to decrease their toxic effects when compared to existing and already used modified SWNTs. First, the SWNTs were covalently modified by the addition of COOH groups at their ends, and on their defect sites. They were then surface crafted by PEG to increase their biocompatibility. Our results show that the formulation oxSWNT-PEG was perfectly homogenous in aqueous solutions. They also show that the formulation had less toxic effects than oxSWNT and SWNT-PEG, which are two commonly used formulations. The formulation demonstrated a pH- and time-dependent controlled release of DOX. In addition to the formulation's enhanced biocompatibility and to drug loading and release capability, this double surface modification allows for the conjugation of the nanotubes with up to two other types of biomolecules: one type linked to the COOH groups on the SWNTs surface, and another

one linked to the head of PEG. Considering these positive results, oxSWNT-PEG could offer big advantages in cancer therapy.

3.6 Acknowledgements:

This work was supported by research grants form the Canadian Institute of Health Research (CIHR) to Dr. Prakash. We wish to express our sincere thanks to Catherine Tomaro-Duchesneau for assistance, and to the center for self-assembled chemical structure (CSACS) of the chemistry department, McGill University.

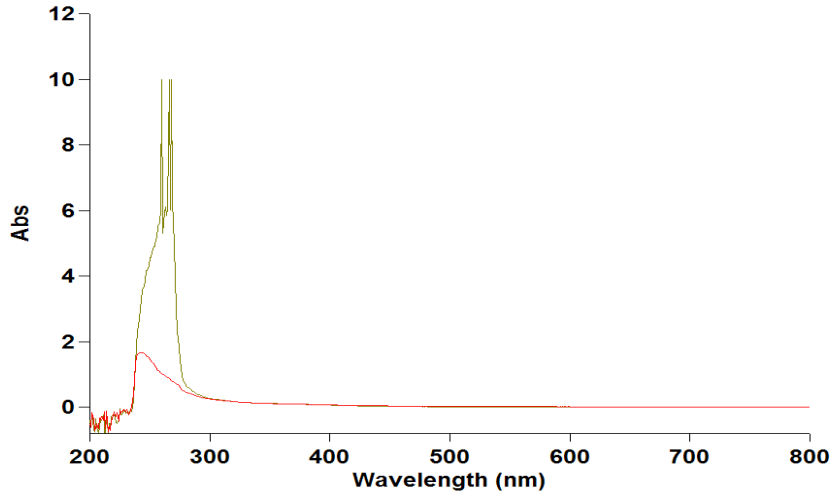

Fig. 3.1: Characterization of oxidized SWNTs using UV-VIS spectroscopy.
Detection of COOH groups on the SWNTs sidewalls following covalent modification.
Green line: Represents the light absorbance of oxSWNTs. The peak at approx. 280nm is due to the COOH groups present on the sidewalls of the oxSWNTs walls.
Red line: Represents the light absorbance of pristine SWNTs.

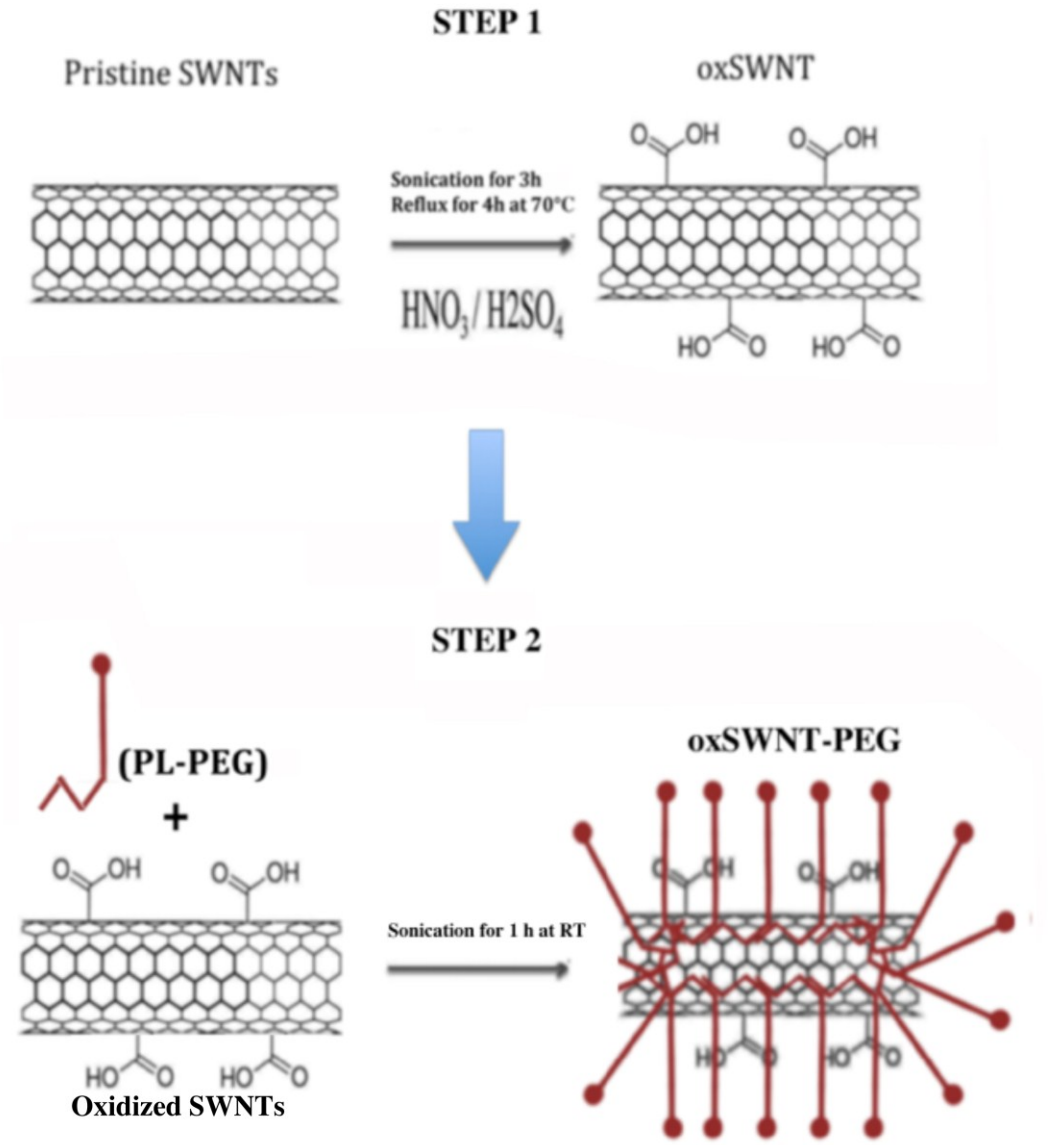

Fig. 3.1: Design of the oxSWNT-PEG formulation.
Step 1: Covalent modification of pristine SWNTs. Covalent modification of SWNTs was carried by oxidating the SWNTs with the oxidizing agents: nitric acid (HNO_3) and sulfuric acid (H_2SO_4). The SWNTs were sonicated in a bath sonicator for 3 h. The mixture was then refluxed at 70°C for 4 hours. This technique was used to break the SWNTs into smaller tubes and to add carboxyl groups (COOH groups) at the ends of the nanotubes as well as at the defect sidewalls. COOH groups decrease the cytotoxicity of SWNTs, and allow for further conjugation with different biomolecules.
Step 2: Surface grafting of the oxidized SWNTs with PEG. oxSWNT were snicated with PEG molecules containing hydrophobic phospholipid (PL) chains. The PL chains adsorbed firmly to the oxSWNT surface, creating a highly soluble and biocompatible formulation: oxSWNT-PEG

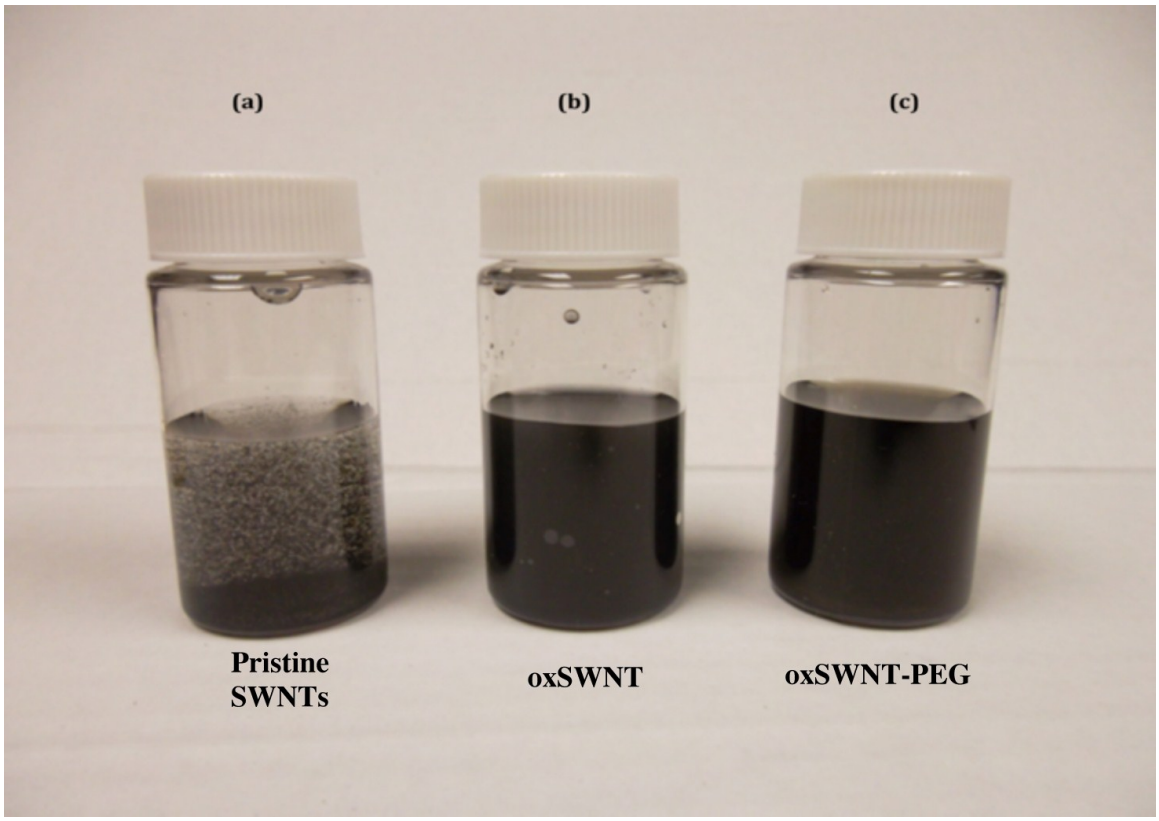

Fig. 3.3: Enhancement of SWNT dispersion in water due to surface modification (a): Pristine SWNTs forming aggregates in aqueous media (here water) due to their hydrophobic nature. (b): Covalently functionalized SWNTs with COOH groups (oxSWNT) via 3 hours of sonication with nitric and sulfuric acid, followed 4 hours of acid reflux. The oxidized walls of the SWNTs allow them to be more dispersed in aqueous media. (c): oxSWNTs surface grafted with the polymer PEG to get the new formulation oxSWNT-PEG, which showed to be very well dispersed and homogenous in many aqueous media, including water, PBS, and cell culture media. This homogenous dispersion is essential to decrease the toxicity and aggregation of carbon nanotubes in physiological environments, and to enhance the efficiency of drug delivery.

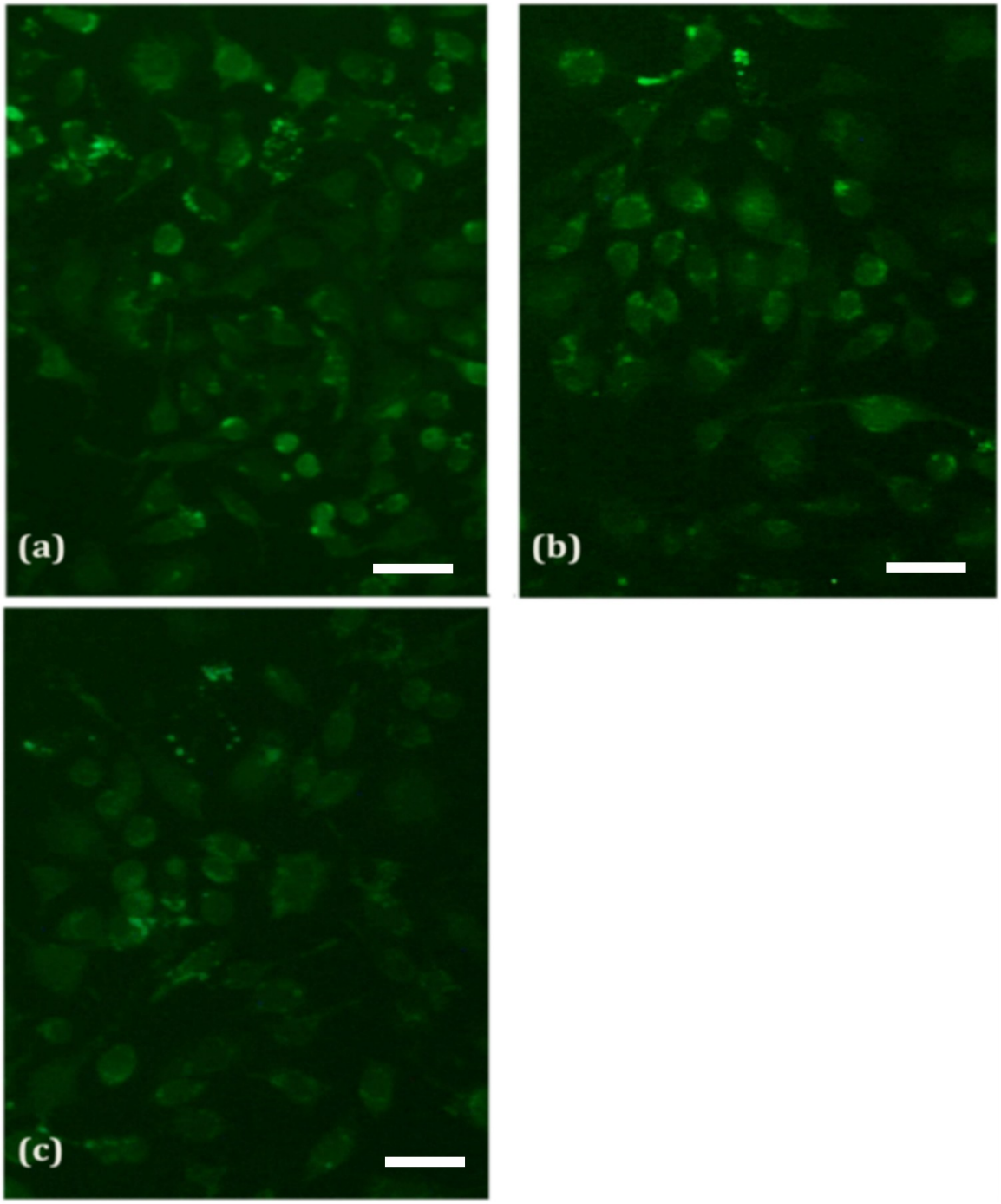

Fig. 3.4: Investigation of the uptake of surface modified and FITC tagged SWNTs by the RAW 264.7 cells. The Cells were incubated for 24h with 12.5 μg/ml of SWNTs tagged with FITC. The cells were incubated with oxSWNT-PEG (a), oxSWNTs (b), and SWNT-PEG (c). All formulations successfully penetrated the cells. Bar: 30 μm

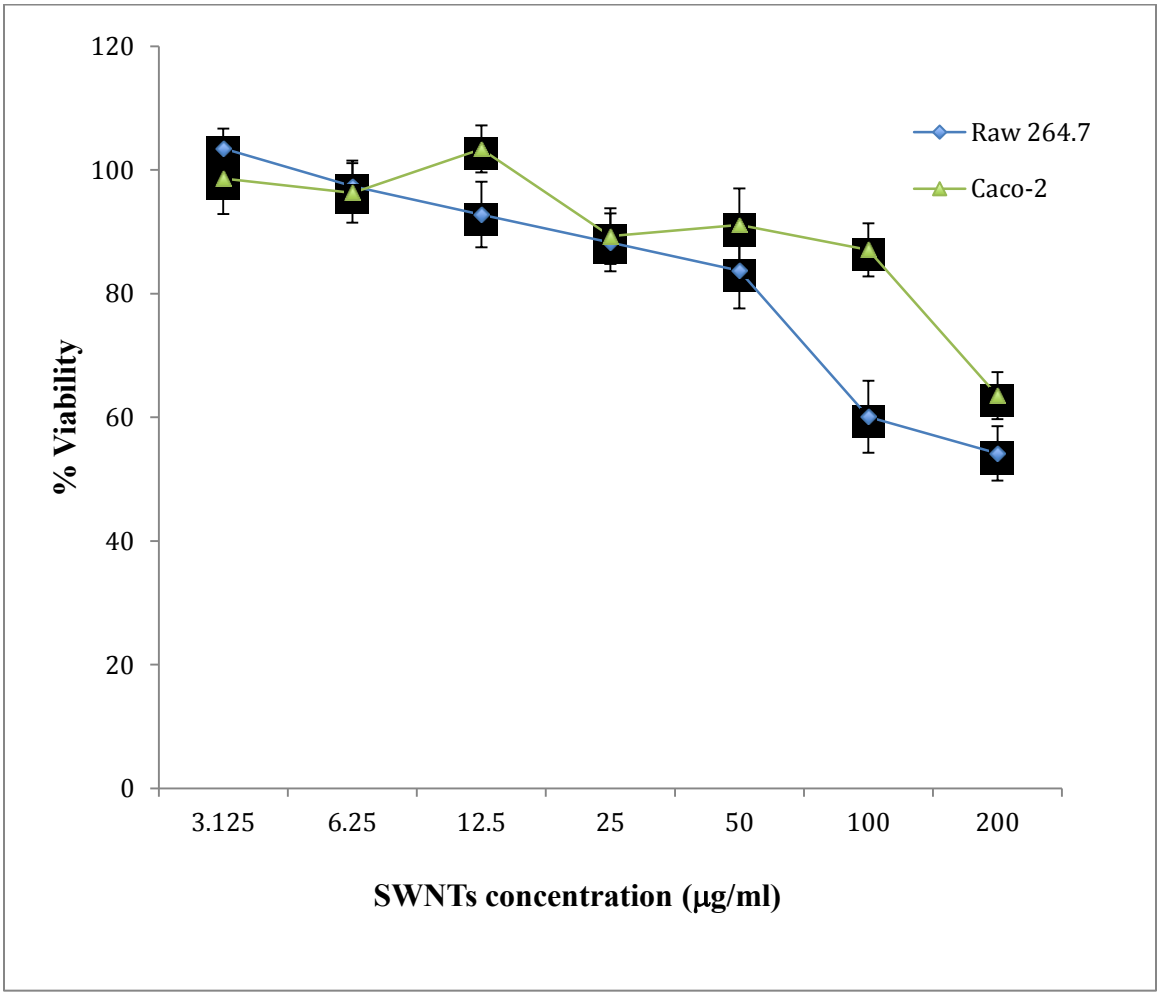

Fig. 3.5. Cell viability of RAW 264.7 and Caco-2 cells after 24h of exposure to oxSWNT-PEG. Viability was measured using a WST-1 assay. Data expressed in % of unexposed controls showed that the viability was always high (> 80%) at all SWNTs concentration up to 50 µg/ml. Exposure to 200 µg/ml oxSWNT-PEG decreased the viability of RAW 264.7 and Caco-2 to 54.2 % and 63.5%, respectively. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

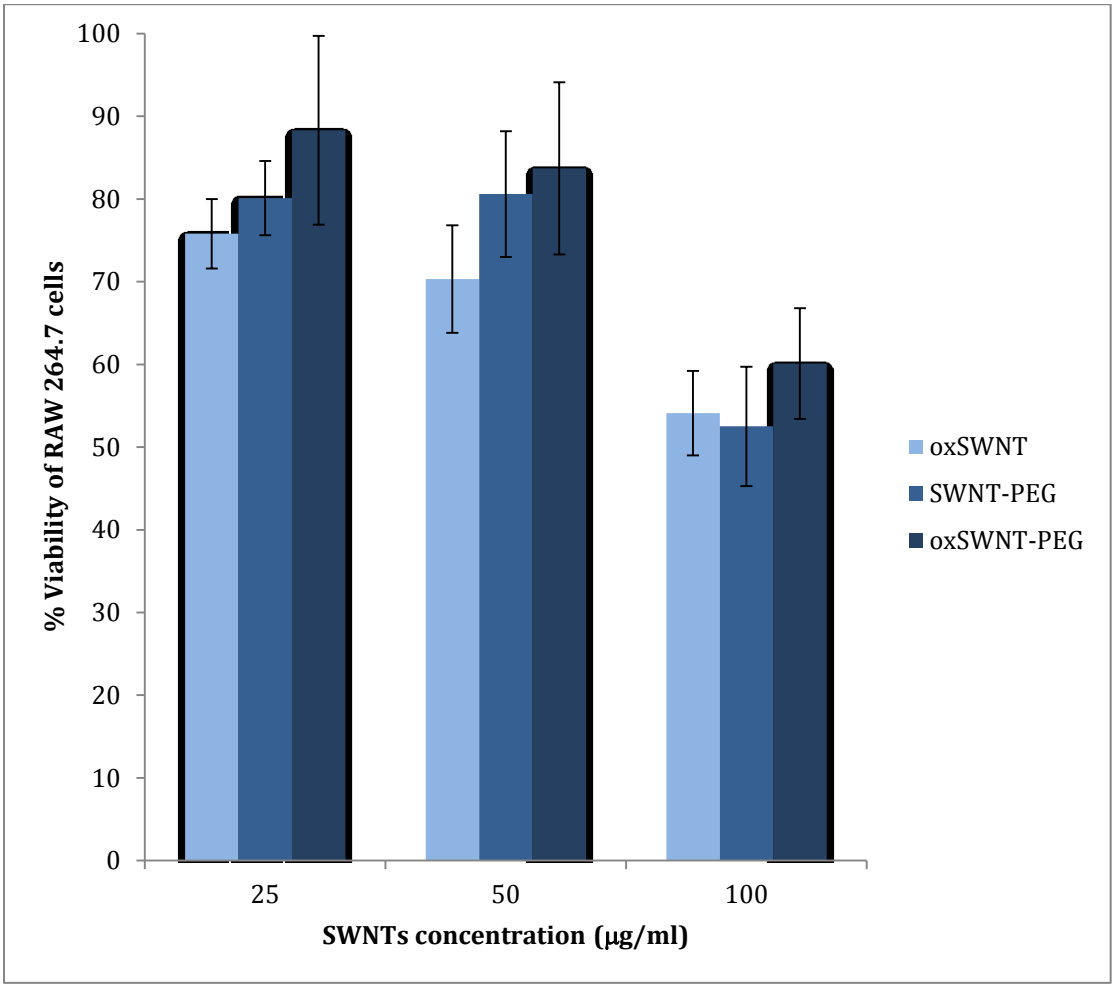

Fig. 3.6: Comparing the levels of cytotoxicity induced by oxSWNT, SWNT-PEG, and oxSWNT-PEG on RAW 264.7 cells. Cell viability was measured by a WST-1 assay at 24h of exposure. The data clearly shows that the formulation oxSWNT-PEG exhibits less cytotoxicity than oxSWNT and SWNT-PEG. In fact, 50 µg/ml oxSWNT-PEG were 17.4% and 3.77% less toxic than the same concentration of oxSWNT and SWNT-PEG on the cell line, respectively. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

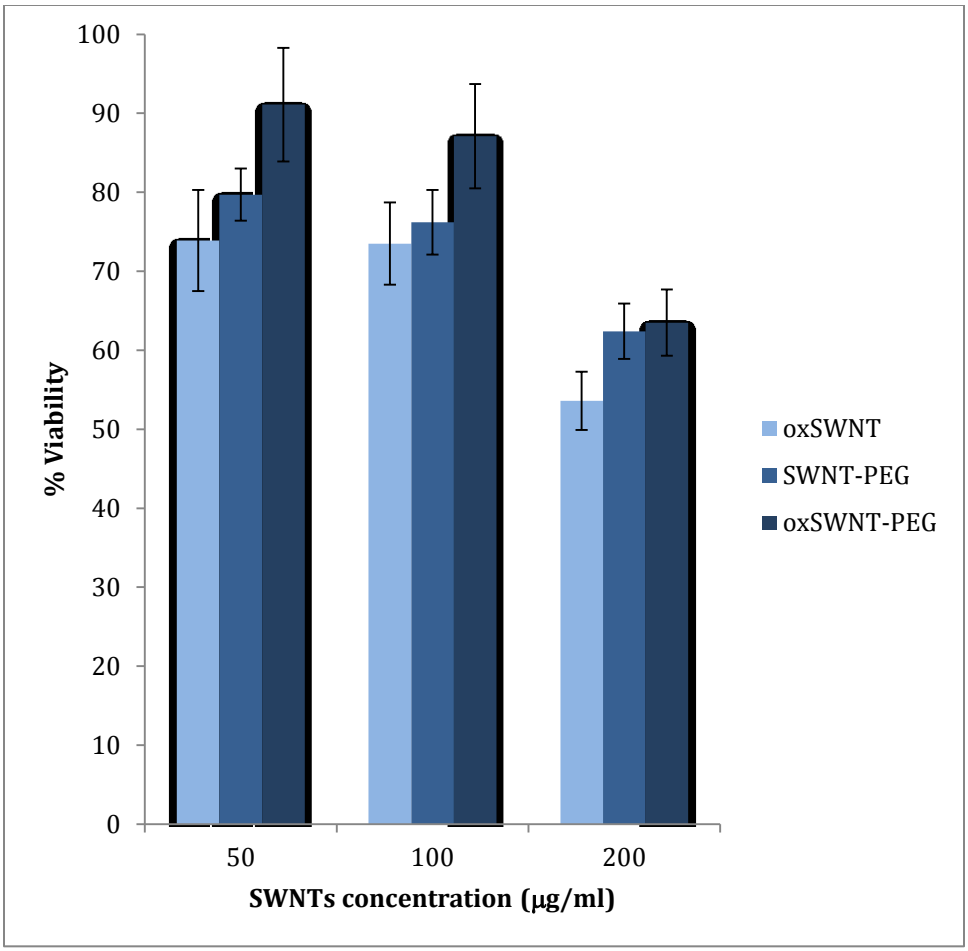

Fig. 3.7: Comparing the levels of cytotoxicity induced by oxSWNT, SWNT-PEG, and oxSWNT-PEG on Caco-2 cells. Cell viability was measured by a WST-1 assay at 24h of exposure. Data shows that the formulation oxSWNT-PEG exhibits less cytotoxicity than oxSWNT and SWNT-PEG. 50 µg/ml oxSWNT-PEG were 16.9% and 13.3% less toxic than the same concentration of oxSWNT and SWNT-PEG on the cell line, respectively. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

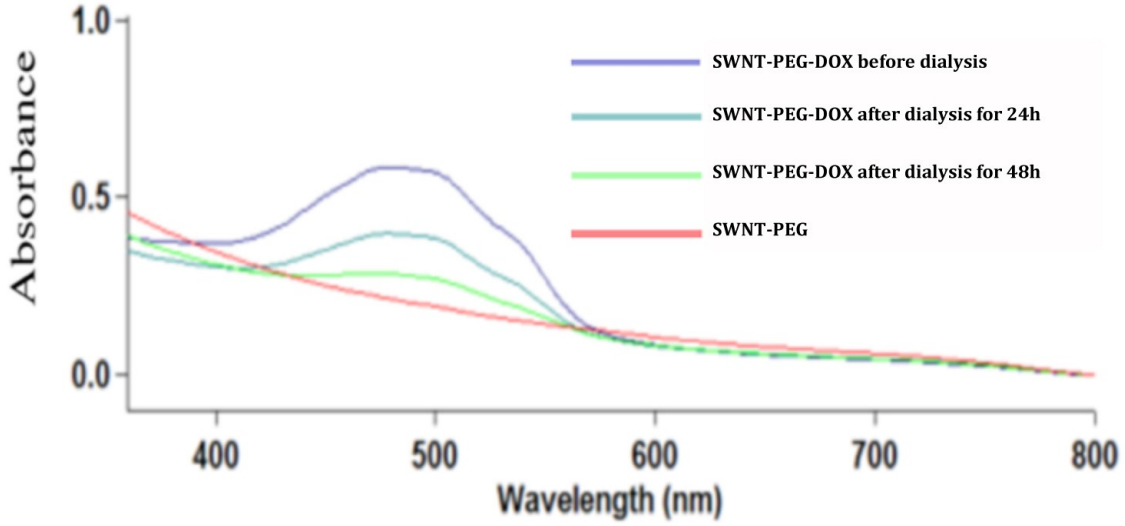

Fig. 3.8: Release kinetics of DOX from SWNT-PEG-DOX measured using UV-VIS spectroscopy. Measurements were taken before and after dialysis at pH 5 for 24h and 48h. Spectra of SWNT-PEG without DOX was taken as a reference. The spectra prove the release of DOX from the SWNTs in acidic solutions that simulate the acidic environment inside the lysosomes of cells.

Chapter 4

Targeted Delivery of Doxorubicin to the Caco-2 Cell Line Using RGD-Conjugated Single Walled Carbon Nanotubes

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4.1 Abstract

To enhance the therapeutic efficiency of drugs, we developed a targeted delivery system consisting of RGD-conjugated and doxorubicin-loaded single walled carbon nanotubes (SWNT-PEG-RGD-DOX), using SWNTs as drug carriers, RGD as the targeting moiety, and DOX as the drug. We used the Caco-2 cell line to study both the targeted delivery and the cytotoxicity of DOX carried by RGD-conjugated SWNTs (SWNT-PEG-RGD-DOX). The uptake of RGD-targeted SWNTs was 32.1% higher than that of untargeted SWNTs. Similarly; the intracellular concentration of DOX delivered by the targeted SWNTs was 1.4 folds higher than that of free DOX. SWNT-PEG-RGD-DOX induced a time-dependent cell death, and the viability of cells exposed to the formulation was around 22% lower than that of cells exposed to free DOX at 48h post exposure. The results demonstrate that our formulation had a high targeting capacity towards the cell line, in addition to having a greater anticancer effect than free DOX.

4.2 Introduction

Carbon nanotubes (CNTs) have received a lot of attention as drug delivery systems because of their excellent physical properties, such as high surface area, ability to penetrate cells, and release of molecular cargoes systems [89]. However, due to their very hydrophobic nature, the use of raw CNTs as drug carriers has been avoided. Surface modification of CNTs is necessary to enhance their solubility in aqueous solutions and to increase their biocompatibility. Several articles have described the advances of SWNTs for applications in drug delivery [89-92] and biomedical imaging [93].

Colon cancer is one of the leading causes of cancer death in the world. It is difficult to treat as it shows to be resistant to the cytotoxic effects of many therapeutic agents [94]. In fact, colon cancer cells are known to express efflux transporters and multidrug resistance related proteins (MRP) that reject drugs and reduce the permeability of the cells [95, 96]. Doxorubicin (DOX), one of the most common chemotherapies [97], has been previously studied on colon cancer cell lines. However, DOX, as many other drugs, showed to have a low therapeutic impact on colon cancers as it accumulates in very low concentrations in colon cancer cells. Drug delivery systems help greatly when such challenges occur as they provide effective concentrations of anticancer drugs at the desired sites. Those techniques generally comprise a drug delivery system, a targeting ligand, and a therapeutic agent [24].

The design of a CNT drug delivery system linked to a targeting molecule is an important factor. Targeting molecules have the ability to specifically enter the cells of interest via receptor-mediated endocytosis. Therefore, they can carry the drug delivery system to the cancerous cells without harming healthy ones. Epidermal growth factor,

(EGF), folic acid (FA), and the arg-gly-asp (RGD) peptide are all targeting molecules that successfully deliver drugs to the desired cells. The RGD peptide is known to have a high affinity to the $\alpha_v\beta_3$ integrin; a cell membrane receptor that is highly expressed on endothelial and epithelial tumor cells [98] such as Caco-2 and U-87 MG.

Studies have been conducted to investigate the toxicity of SWNTs on Caco-2 cells [99, 100], however, as far as we know, no one has yet studied the targeting efficiency and toxicity of drug-loaded SWNTs on the cell line. This study investigates the targeting capacities of RGD-conjugated and DOX-loaded SWNTs to selectively target the Caco-2 cell line, a well-established colon cancer model. Hence the targeted delivery and the cytotoxic effects of DOX delivered by our formulation on Caco-2 are presented.

4.3 Materials and methods

4.3.1Chemicals

HiPCo purified single walled carbon nanotubes (SWNTs), purchased from Unidym, (Sunnyvale, USA). Fluorescein isothiocyanate (FITC) was purchased from Aldrich Chemicals Co., (Milwaukee, USA). Dialysis cassette were obtained from Thermo Scientific (Rockford, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (PEG) was purchased from Avanti Polar Lipids (Alabama, USA). Centrifugal filters were purchased from Millipore (Billerica, USA). Cyclic RGD peptide was purchased from Peptides International (Luisville, USA). Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline PBS were

purchased from Life Technologies (Grand Island, USA). Water Soluble Tetrazolium salts assay (WST-1 assay) was purchased from Cayman Chemical (New Orleans, USA).

4.3.2 Conjugation of oxSWNT with FITC

20 mg of fluorescein isothiocyanate (FITC) were dissolved in 15ml of dimethylformamide (DMF), and stirred in a flask. 6mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were added to the mixture in order to activate the functional carboxyl groups of FITC. 10 minutes later, 5mg of oxSWNT were added to the mixture. The nanotubes were oxidized as previously described [75, 76]. 15 μg of 4-Dimethylaminopyridine (DMAP) was also added to the whole solution in order to catalyze the reaction. The mixture was protected from light and stirred overnight at room temperature to get SWNT-FITC. For purification, the mixture was transferred to a dialysis cassette with a molecular weight cut off (MWCO) of 3000. The cassette was protected from light and stirred in a flask containing deionized water. The formulation was purified for 2 days and water was replaced at every 8 hours.

4.3.3 Conjugation of RGD to PL-PEG

10 mg of PL-PEG were dissolved in a 50mM sodium phosphate buffer, pH 7.5. NHS-PEG-MAL (*N*-Hydroxysuccinimide-polyethylene glycol-maleimide) was added, and the mixture was stirred at room temperature for one hour. 5 mg of cyclic RGD (cyclo (Arg-Gly-Asp-D-Phe-Cys, PCI-3686-PI) was diluted in 1 ml dimethyl sulfoxide (DMSO), and the mixture was transferred to the buffer solution. The reaction mixture was stirred overnight at room temperature. For purification, the mixture was transferred to a dialysis cassette with a molecular weight cut off (MWCO) of 3000. The cassette was

protected from light and stirred in a flask containing deionized water. Water was replaced at every 8 hours for 2 days.

4.3.4 Addition of PEG-RGD to SWNT-FITC

1 mg of SWNT-FITC was mixed with 2.5 mg of PEG-RGD in 5 ml of deionized water. The sample sonicated in a bath sonicator for 60 min at room temperature (~22 °C) to obtain SWNT-FITC-PEG-RGD. The formulations was stored at 4°C. Before the use of the SWNTs formulation for *in vitro* studies, we washed it four times at room temperature for ten minutes at 4,000g, to remove the excess PEG-RGD in the solution. We used centrifugal filters (Amicon, Millipore) with a molecular weight cutoff (MWCO) of 100 kDa, in which we added 3 ml of water to each 1ml of SWNTs formulation. Finally, the washed formulations in the filter were collected and added to fresh media for *in vitro* studies.

4.3.5 Doxorubicin attachment to SWNT-FITC-PEG-RGD

0.5 mg SWNT-FITC-PEG-RGD was dispersed in 5 mL sodium phosphate buffer 20mM pH 8.5, and 2.5 mg of DOX was added. The mixture was sonicated in a bath sonicator for 10 min and incubated overnight while stirring. Unbound doxorubicin was removed by filtration and washing with Amicon centrifugal filters, 30 KDa MWCO. Absorption spectroscopy at 490 nm was used to determine the amount of unbound doxorubicin in the eluate of the filtration.

4.3.6 Cell Culture and Incubation with SWNTs Solutions

Caco-2 cells were cultured in DMEM. They were supplemented with 20% FBS

and1% penicillin-streptomycin. The incubations were carried out 37°C, in 5% CO_2 atmosphere, and in 90% relative humidity. Media was refreshed every two to three days. The cells were seeded in 96 well plates at 10^4 cells per well for 24 hours. SWNT-FITC-PEG-RGD was then incubated with the cells. The SWNTs concentrations used in this study were 7 μg/ml and 15 μg/ml. All experiments were done in triplicates repeated on the same sample (n=1).

4.3.8 Cell Viability Assay:

Cells were washed three times with PBS to remove all SWNTs suspended outside the cells. Water Soluble Tetrazolium salts assay (WST-1 assay) [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (purchased from Cayman Chemical) was used for measuring cell viability. Fresh media (100 μl) and 20 μl of the WST-1 reagent were added to each well. The plates were then shaken vigorously for 60 seconds to thoroughly homogenize the reagent inside the wells. Finally, they were incubated for 90 minutes before performing any cell viability readings. Cell viability measurements were taken at 12 hours, 24 hours, and 48 hours post treatment with the nanotubes. Absorbance was measured using a Perkin Elmer (Victor^3 V) multiplate reader. The readings were measured at 450 nm.

4.4 Results

4.4.1 Preparation of the conjugated SWNTs samples:

The conjugation between PEG-NH_2 and NHS-PEG-MAL was established through an ester reaction between the amine group of PEG and the NHS group of NHS-PEG-MAL to yield PEG-MAL. The thiol group of RGD was then reacted with the maleimide (MAL) group of PEG-MAL via a sulfhydryl bond. Sonication of PEG-RGD with SWNT-FITC led to the highly soluble formulations and SWNT-FITC-PEG-RGD (fig. 1). NMR studies were used to characterize the RGD-PEG conjugates (fig. 2). The multiple signals at 7.2 ppm are the aryl protons of the phenylalanine amino acid of RGD. Their presence confirms the success of the conjugation between PEG and RGD. The formulation's cytotoxicity was measured using a WST-1 assay. The results showed that it had negligible influence on cell viability (fig. 3), indicating that the cytotoxicity induced by SWNT-PEG-RGD-DOX was caused by the release of high amounts of DOX, and not by the delivery system itself.

4.4.2 Selective uptake of RGD-targeted SWNTs by Caco-2

To demonstrate the targeting ability of RGD-conjugated SWNTs, we incubated SWNT-FITC-PEG-RGD with Caco-2 for 12 hours (fig.4). Results at 12 hours post exposure to the formulations show that the fluorescence in Caco-2 cells incubated with SWNT-FITC-RGD was 32.1% more intense than the one in Caco-2 cells incubated with control SWNTs (fig.5). The SWNTs uptake by the cells appeared to be logarithmic as a function of time, plateauing at 8h post exposure. A possible explanation is that the $\alpha_v\beta_3$

integrins might have all been used within around 8 hours to internalize the RGD-conjugated nanotubes.

4.4.3 DOX uptake and cytotoxicity of SWNT-FITC-PEG-RGD-DOX on Caco-2 cells

DOX was loaded to the SWNTs via π–π stacking. On the basis of absorbance spectroscopy of DOX at 490 nm, we evaluated the weight ratio of DOX:SWNT to be 3:1, which demonstrates a high loading capacity of DOX on the SWNTs sidewalls. To measure DOX uptake, we incubated the cells for up to 12 hours with 10 μM DOX delivered by SWNT-FITC-PEG-RGD-DOX (fig. 6). Both SWNT-DOX and free DOX served as negative controls, with a DOX concentration of 10 μM. The WST-1 assay was used to measure the cytotoxicity induced by SWNT-PEG-RGD-DOX on Caco-2 cells. Results also showed that the cytotoxicity induced by the formulation was time-dependent, reaching the efficacy of the same concentration of free DOX at around 27 hours post exposure (fig. 7). At 48 h post exposure, 20 μM and 40 μM of DOX delivered by the RGD-conjugated SWNTs decreased cell viability by 23.3% and 21% when compared to equivalent concentrations of free DOX.

4.5 Discussion

Because DMF is a good dispersion media for nanotubes, oxSWNTs were initially suspended in DMF and then homogenized by quick sonication. FITC was added to the oxSWNTs sidewalls using EDC, a carbodiimide crosslinker that activates carboxyl groups for spontaneous reactions with either amino groups or carboxylic groups [101]. EDC mediated the covalent linkage between the carboxylic groups of FITC and

oxSWNTs via an acid anhydride formation. Dialysis was then performed to purify the sample. To conjugate RGD with PEG, we first linked the NH_2 groups of the hydrophilic PEG polymers to the NHS groups of NHS-PEG-MAL. We then reacted the maleimide group at the other end of the crosslinker with thiol groups of the RGD peptide.

$\alpha_v\beta_3$ integrins are common tumor markers expressed at high levels on the membranes of endothelial and epithelial cancer cells [4]. They have the ability to interact with compounds containing the peptide arginine-glycine-aspartic (RGD) [5]. In previous studies, RGD was used as a targeting peptide for various drug delivery systems [6]. Once at the surface of the cell membrane, RGD binds at the interface between the α and β subunits of $\alpha_v\beta_3$, facilitating the uptake of the whole system that contains it [5, 7]. Readings using fluorescence spectroscopy were taken at 2 hours intervals, and clearly showed that the formulation accumulated in Caco-2. Bright fluorescence signals were observed for cells incubated with the formulation (fig. 4 and 5). In contrast, we observed diminished intracellular fluorescence signals for cells treated with the RGD-free negative control SWNT-FITC-PEG**.** This difference is due to the absence of ligand-induced and receptor-mediated endocytosis of the control formulation. The results are in agreement with previous anticancer drug delivery studies, which also revealed RGD-dependent cellular uptakes [102].

Fig. 6 shows that DOX uptake was time dependent, plateauing between 8 and 10 hours post exposure. We can notice that the uptake of DOX was the same when incubated alone or delivered by the SWNTs at times prior to 4 hours post exposure. At 8 hours post exposure and above, cells exposed to SWNT-FITC-PEG-RGD-DOX showed significantly higher absorbance than cells exposed to SWNT-DOX or free DOX. At 12

hours post exposure, the ratio between targeted DOX and free DOX inside the cells was around 1.4 folds. This demonstrates that DOX can be found at higher levels inside the $\alpha_v\beta_3$ expressing Caco-2 cells when delivered by RGD-targeted SWNTs.

The WST-1 assay was used to measure the cytotoxicity induced by SWNT-PEG-RGD-DOX on Caco-2 cells. The cells were treated with different concentrations of DOX, ranging from 1 to 40 μM. SWNT-PEG-DOX, and free DOX were used as negative controls. Increasing concentrations of DOX showed a decrease in cell viability, however, viability remained at all concentrations due to the resistance of the cell line to DOX (fig. 5). Results also showed that the cytotoxicity induced by the formulation was time-dependent, reaching the efficacy of the same concentration of free DOX at around 27 hours post exposure (fig. 7). At 48 h post exposure, the highest toxicity levels were caused by SWNT-PEG-RGD-DOX at DOX concentrations higher than 10 μM. Compared to equivalent concentrations of free DOX, DOX delivered by the RGD-conjugated SWNTs induced a clear decrease in cell viability.

4.6 Conclusion:

This work presents ligand-targeted SWNTs for the active targeting and killing of Caco-2 cells. Doxorubicin is known to accumulate at low concentrations inside the cell line. To increase its concentration and enhance its therapeutic potential, we used SWNTs as drug carriers and RGD as the targeting moiety. We showed that the uptake of SWNT-FITC-RGD by Caco-2 cells was 32% higher than that of control SWNTs. This suggests a ligand-receptor interaction and a receptor-mediated endocytosis. The system had no toxic

effects on the cells in the absence of DOX. We also showed that cytotoxicity of SWNT-PEG-RGD-DOX was ~22% higher than the one induced by free DOX at 20 μg/ml and 40 μg/ml, at 48h post exposure. These results demonstrate that the delivery system had better anticancer efficacy than the free drug. However, the therapeutic effects of DOX remained limited even when delivered by the targeted-SWNTs. further optimization of drug delivery systems is required to overcome the MRP effect of cancer cells.

4.7 Acknowledgements:

This work was supported by research grants form the Canadian Institute of Health Research (CIHR) to Dr. Prakash. We wish to express our sincere thanks to Catherine Tomaro-Duchesneau for assistance, and to the center for self-assembled chemical structure (CSACS) of the chemistry department, McGill University.

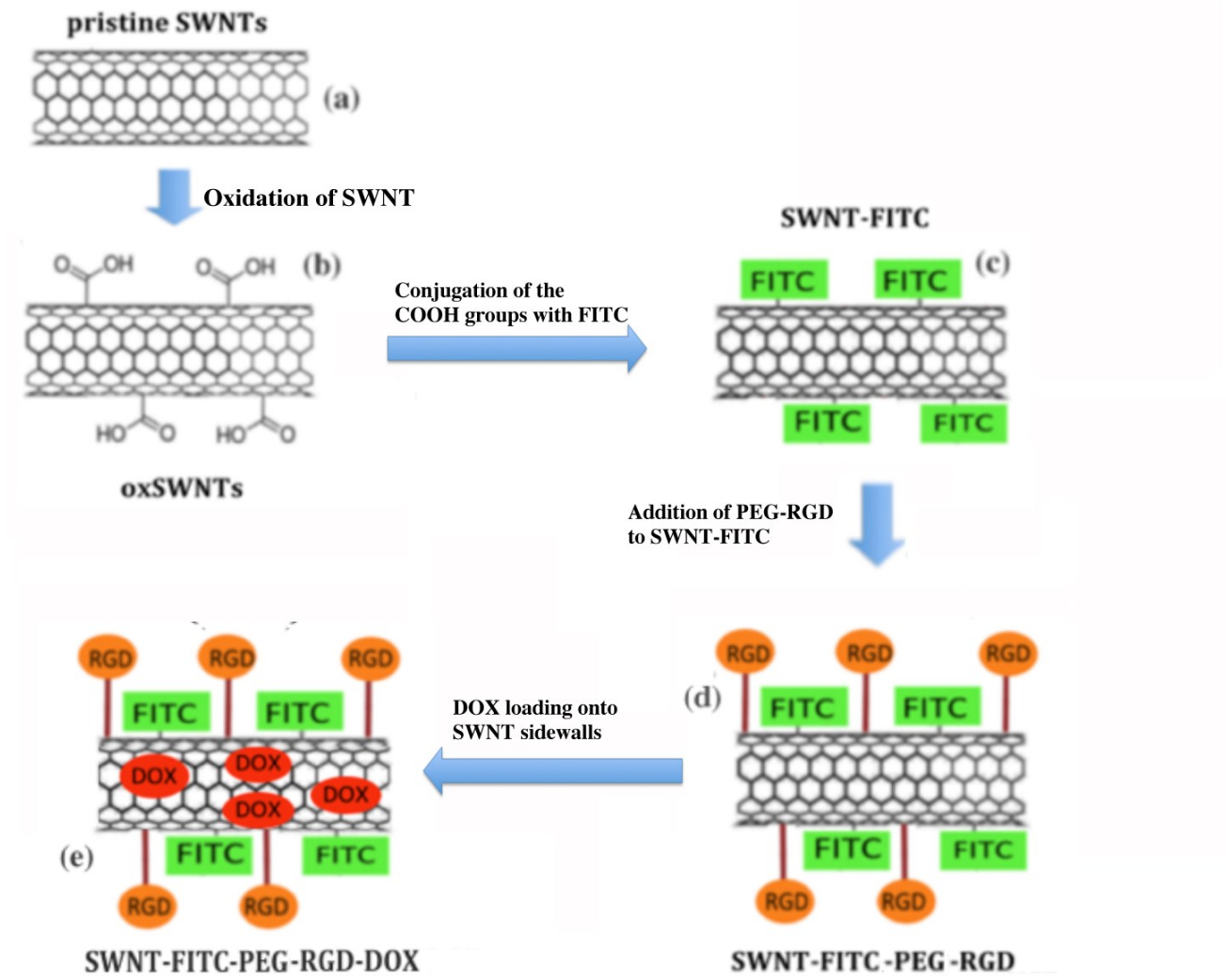

Fig. 4.1. Development of the SWNT drug delivery system using RGD as the targeting molecule, DOX as the drug and FITC as the fluorescent tag. (a) Pristine SWNT. (b) The SWNTs were oxidized with COOH groups to get oxSWNT. This covalent surface modification with nitric and sulfuric acid increases the solubility and the biocompatibility of the SWNTs, and serves as attachment sites for compounds on the SWNTs surface. (c) SWNT-FITC. The oxSWNTs were conjugated to FITC for fluorescence studies. (d) SWNT-FITC-PEG-RGD resulted by sonicating PL-PEG-RGD with SWNT-FITC. (e) Attachment od DOX onto the SWNTs sidewalls by stirring overnight in phosphate buffer at pH 8.5.

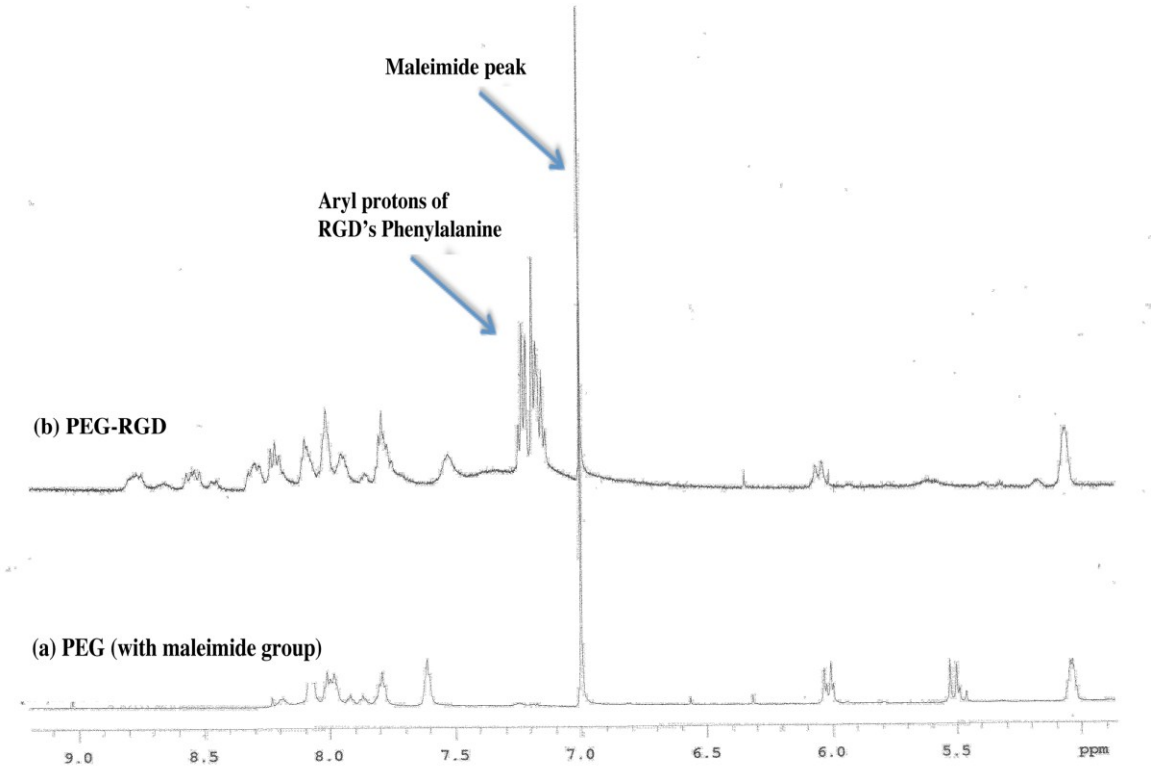

Fig. 4.2. NMR characterization of the conjugation of PEG with RGD. (a) shows the NMR signal of PEG polymers containing a maleimide group. (b) shows the NMR signal of PEG-RGD conjugates. The maleimide groups of the PEG polymers were used to link PEG with RGD via a sulfhydryl bond. The multiple signals at 7.2 ppm are the aryl protons of the phenylalanine amino acid of RGD. Their presence confirms the success of the conjugation between PEG and RGD.

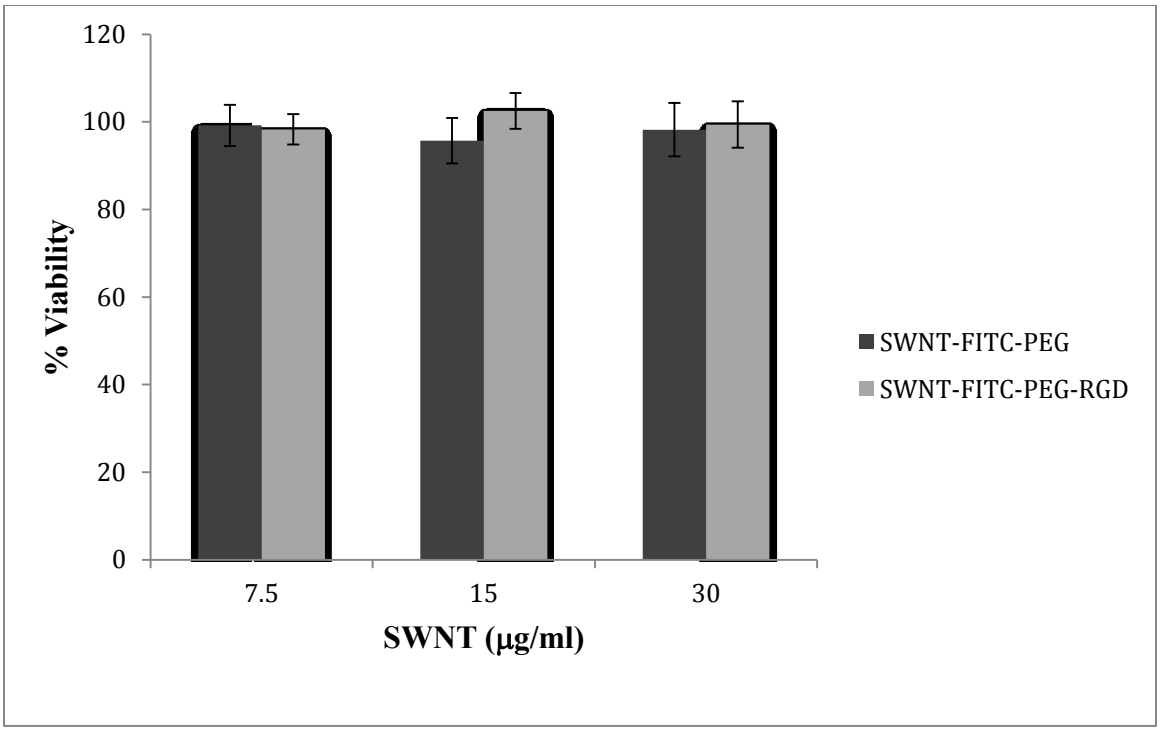

Fig. 4.3. Biocompatibility of the SWNT formulations. Cell viability was measured at 48 hours post exposure with different concentrations of SWNT-FITC-PEG-RGD and SWNT-FITC-PEG. Data expressed in % of unexposed controls showed no obvious toxicity at 7 μg/ml, 15 μg/ml, and 30 μg/ml. No obvious toxicity was detected. Toxicity studies were performed in triplicates using a WST-1 assay. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

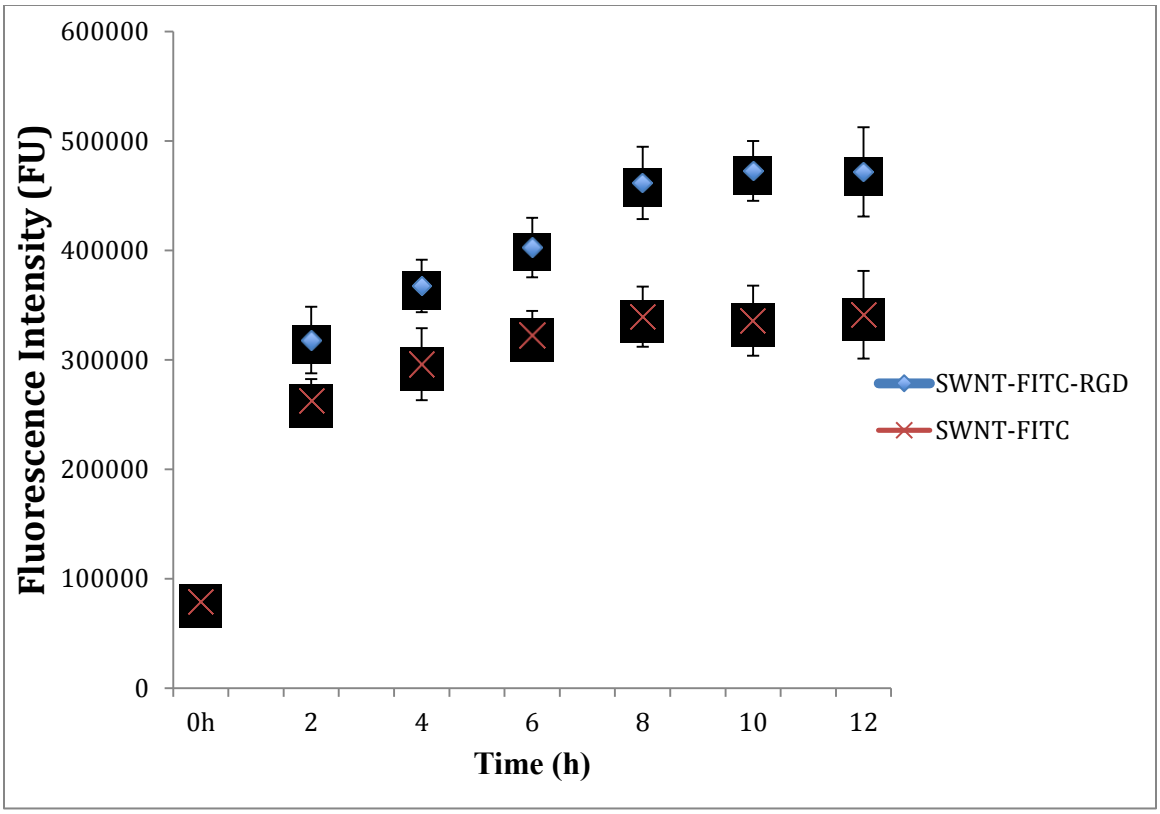

Fig. 4.4. Targeted delivery of RGD-conjugated SWNTs by Caco-2 cells via receptor-mediated endocytosis. The cells were incubated for 12 hours with 15 μg/ml of SWNT formulations. The fluorescence intensity in the cells treated with RGD-targeted SWNTs at 12h post exposure is clearly higher (32% higher) than that in the cells treated with non-targeted SWNTs (controls). Values represent the means ± SD for triplicates repeated on the same sample (n=1).

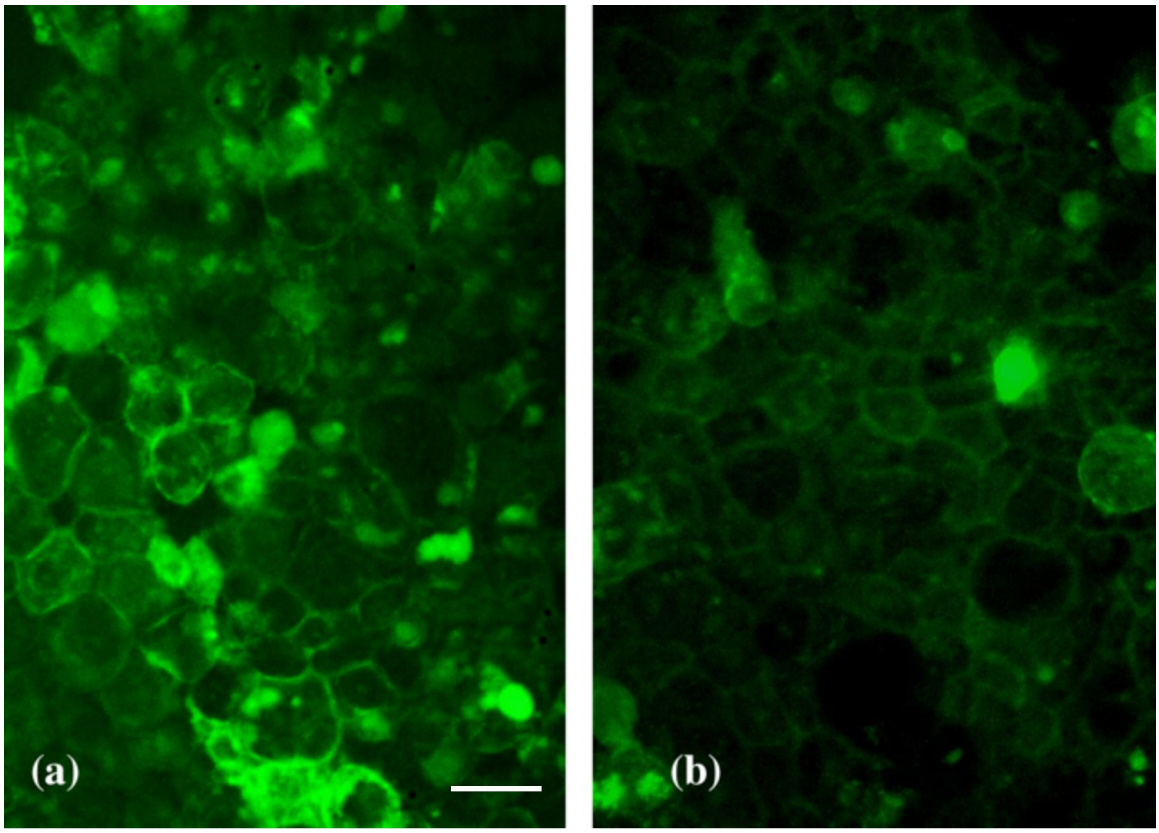

Fig. 4.5. Fluorescence microscopy of Caco-2 cells exposed to SWNTs. The cells were incubated for 12 h with 15 μg/ml of SWNTs tagged with FITC. (a) Fluorescence image of cells exposed to RGD-targeted SWNTs, showing a strong green fluorescence inside the cells. (b) Cells incubated with the untargeted SWNTs (negative control) showed a weaker fluorescence. The difference in fluorescence levels between both samples confirms that the cell uptake of SWNTs was receptor-mediated. Bar: 50 μm

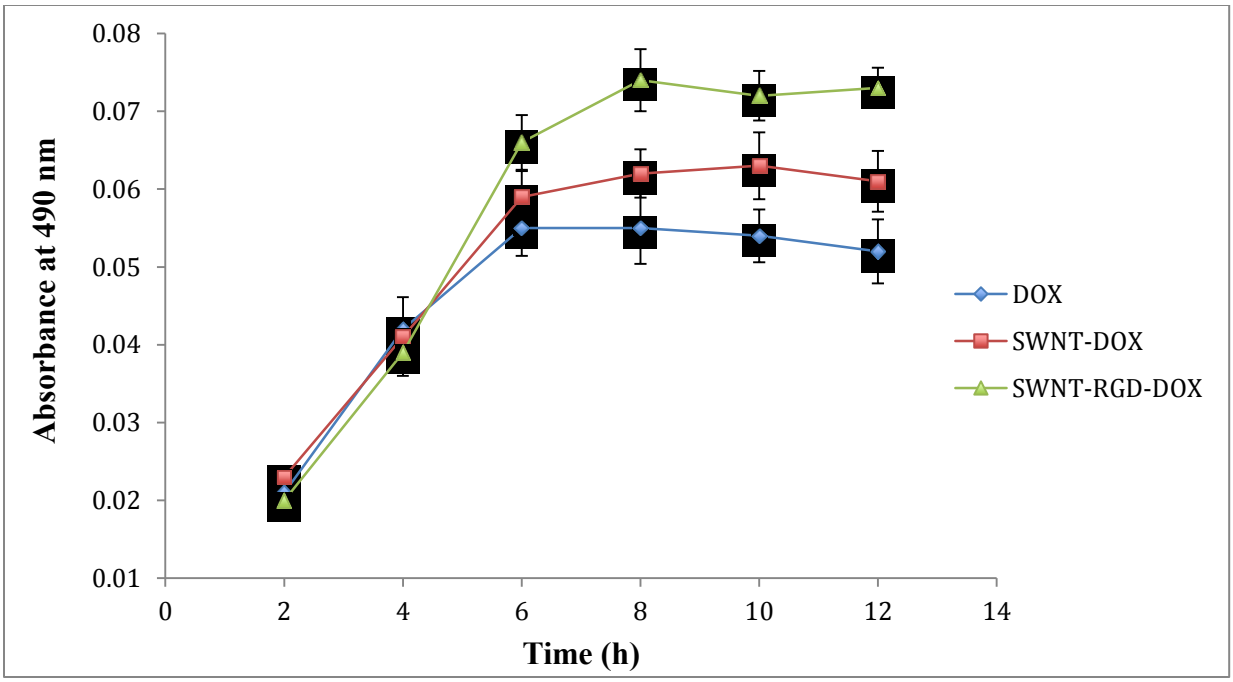

Fig. 4.6. Targeted delivery of DOX to Caco-2 cells by RGD-conjugated SWNTs. The delivery was studied by absorbance spectroscopy at 490 nm at intervals of 2 h. All cells were incubated with 5 μM DOX for up to 12 h. DOX was delivered by RGD-targeted SWNTs. SWNT-DOX and free DOX served as controls. Cells were washed 3 times with PBS before each reading. The cells exposed to SWNT-RGD-DOX showed higher optical absorbance than the cells exposed to controls as time > 4 h post exposure. At 12 h post exposure, the ratio between targeted DOX and free DOX inside the cells was around 1.4 folds. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

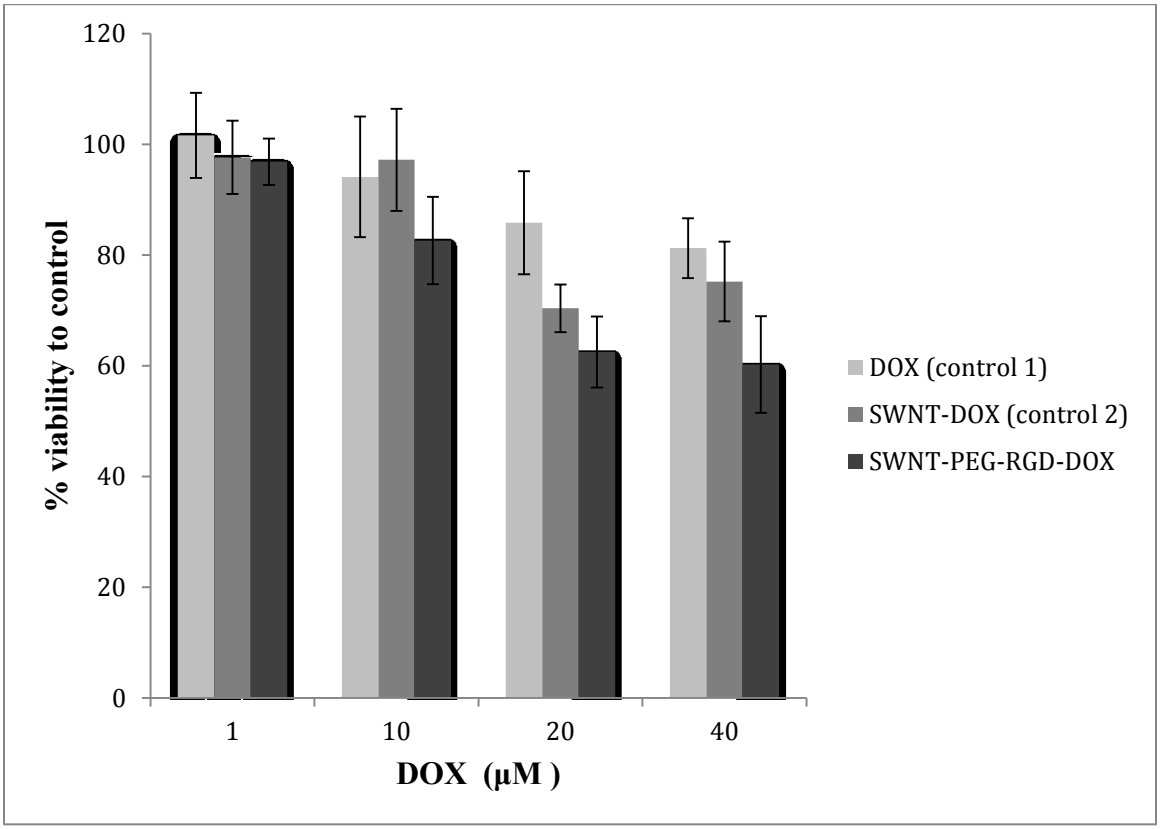

Fig. 4.7. Dose dependent cytotoxicity induced by the formulation SWNT-RGD-DOX on Caco-2. Cytotoxicity was measured at 48 h post exposure to different DOX concentrations using a WST-1 assay. SWNT-DOX, and free DOX served as controls. At higher DOX concentrations, a significant decrease in viability is noticed for cells treated with SWNT-RGD-DOX. This confirms the efficiency of ligand-conjugated SWNTs as drug delivery systems. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

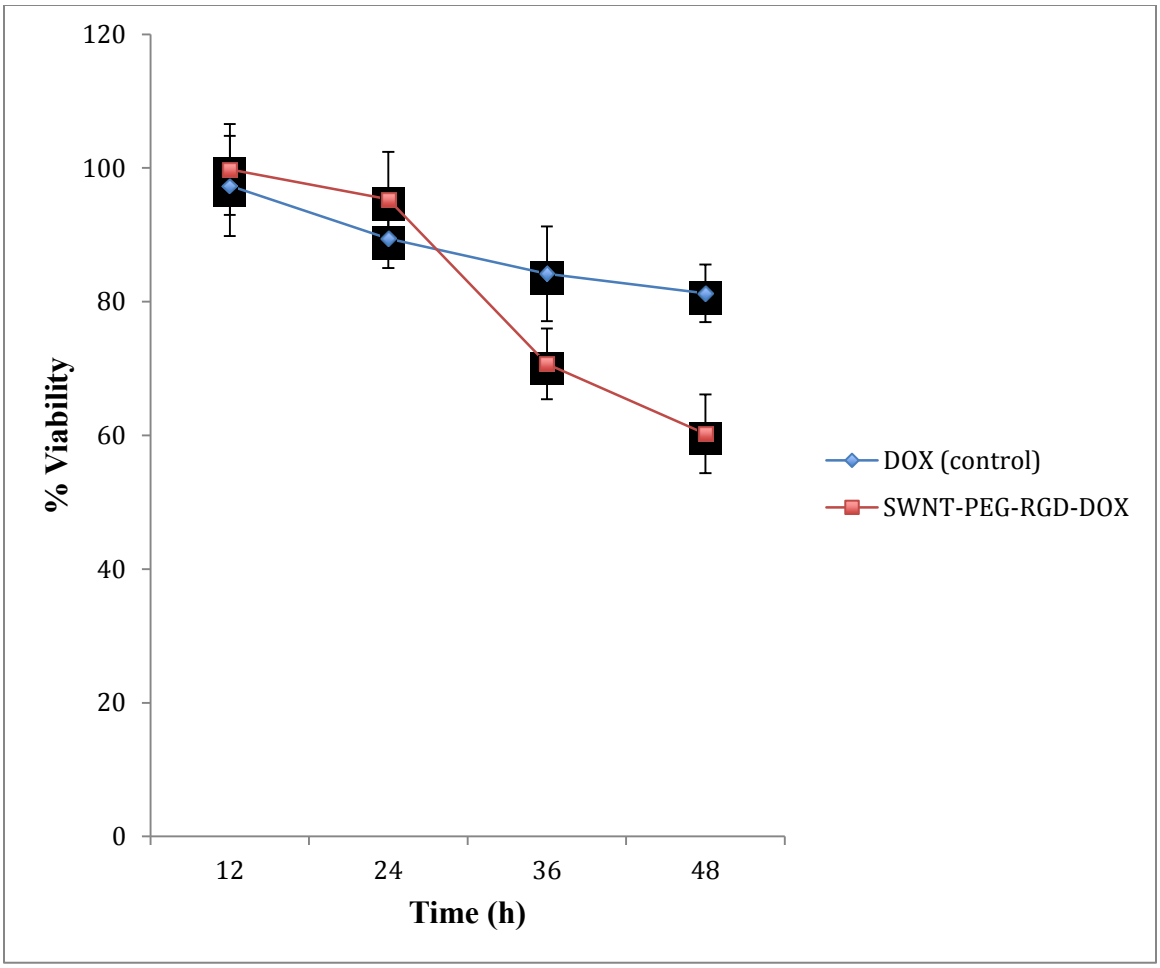

Fig. 4.8. Time-dependent cytotoxicity induced by DOX at a concentration of 40 μM on Caco-2 cells. Data is expressed in % of unexposed controls. At a concentration of 40 μM, free DOX showed to decrease cell viability more than the formulation at times lower than 30 h post exposure. At around 27 h, the effect of 40 μM DOX delivered by SWNT-PEG-RGD-DOX exhibited more cytotoxicity, decreasing cell viability to 60.23% at 48 h post exposure. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

Chapter 5

Targeted Delivery of Doxorubicin to the RAW 264.7 cancer cell line using GE11-Functionalized Single Walled Carbon Nanotubes

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5.1 Abstract

Epidermal growth factor receptor (EGFR) is a membrane receptor that is overexpressed on many types of cancerous cells. It is therefore an important target in drug delivery. Here, we developed a novel drug delivery system with single walled carbon nanotubes (SWNTs) serving as drug targeting vehicles to EGFR expressing cancer cells. SWNTs were surface graphed with poly(ethylene) glycol (PEG), loaded with anticancer-drug doxorubicin (DOX), and conjugated with GE11, a targeting peptide with a high affinity towards EGFR. The delivery system was rapidly internalized into the EGFR overexpressing RAW 264.7 cell line. At 12 hours post exposure, the uptake of FITC-tagged and GE11-targeted SWNTs was 43.3% higher than that of untargeted SWNTs. Similarly, the intracellular concentration of DOX delivered by the targeted SWNTs was almost 2 folds higher than that of free DOX. The cytotoxicity caused by 20 μM of DOX targeted by the delivery system was 3.6 higher than that of 20 μM free DOX. Incubation of the cells with the formulations at 4°C showed almost no targeted-SWNTs uptake compared to the uptake at 37°C, suggesting a temperature dependent receptor-mediated endocytosis. The findings demonstrate that our delivery system without the presence of DOX had no obvious toxicity effects on the cells. They also show that the formulation has a good potential to selectively target doxorubicin to EGFR expressing RAW 264.7 cells.

5.2 Introduction

Anticancer drugs induce various types of adverse effects due to their presence in healthy cells upon administration. Advanced drug delivery systems (ADDS) have been extensively studied in recent years as they have the ability to target drugs to the desired sites. ADDS have the ability to provide effective concentrations of drugs inside cancer cells, and to decrease the drug's toxicity towards healthy cells. An ADDS consists of a delivery vehicle loaded with anticancer drugs, and a targeting molecule that recognizes specific receptors on the cancer cell membrane. Targeting molecules have the ability to internalize the ADDS inside the cells of interest via receptor-mediated endocytosis.

The epidermal growth factor receptor (EGFR) is a glycoprotein that is overexpressed on the membrane of many human cancer cells, including breast, ovarian, leukemia, lung, head, and prostate tumor cells. It is therefore a potential target for cancer therapy. Consequently, strategies targeting EGFR have been developed as treatment options, including monoclonal antibodies, small molecule inhibitors of EGFR signal transduction, and antibody-based immunoconjugates such as immunotoxins [103]. GE11 is a new peptide known to have a high affinity towards EGFR [104]. It has been used as a recognizing moiety and conjugated to liposomes that selectively delivered therapeutic agents to cancerous cells [104]. Additionally, the peptide GE11 is more biocompatible than EGF, the natural ligand of EGFR, as it induces much lower cell mitogenic activity [105]. It is therefore being increasingly used as a targeting molecule.

Carbon nanotubes (CNTs) have shown great promise as ADDS due to their excellent physical properties as drug carriers and their ability to penetrate the cells of interest [11, 12]. However, due to their very hydrophobic nature, the use of raw CNTs as drug carriers has been avoided. Surface modification of CNTs is necessary to enhance their solubility in aqueous solutions and to increase their biocompatibility. Surface modified CNTs have become widely used for targeted drug delivery [89-92] and biomedical imaging [93]. Moieties recognizing EGFR have also been used for the targeting of drug-loaded single walled carbon nanotubes (SWNTs) [106]. However, GE11 has never been used for the targeted delivery of doxorubicin (DOX)-loaded SWNTs to EGFR positive cancerous cells. The aim of this study was to integrate several advantages of SWNTs to improve cancer treatment. The objectives were to investigate the selective uptake and toxic effects of GE11-targeted and DOX-loaded SWNTs on EGFR expressing cancerous cells. Therefore, we used the RAW 264.7, a mouse leukaemic monocyte macrophage cell line that is known to express EGFR.

5.3 Materials and Methods

5.3.1 Chemicals

HiPCo purified single walled carbon nanotubes (SWNTs), purchased from Unidym, (Sunnyvale, USA). Fluorescein isothiocyanate (FITC) was purchased from Aldrich Chemicals Co., (Milwaukee, USA). Dialysis cassette were obtained from Thermo Scientific (Rockford, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (PL-PEG) was purchased from Avanti Polar Lipids

(Alabama, USA). Centrifugal filters were purchased from Millipore (Billerica, USA). GE11 was purchased from Sheldon Biotechnology (Montreal, Canada). Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline PBS were purchased from Life Technologies (Grand Island, USA). Water Soluble Tetrazolium salts assay (WST-1 assay) was purchased from Cayman Chemical (New Orleans, USA).

5.3.2 Conjugation of FITC and GE11 to PL-PEG

For the synthesis of PEG-GE11 [105], GE11 was dissolved in PBS and mixed at 1:1.2 molar ratio with sulfosuccinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (Sulfo-LC-SPDP) dissolved in DMSO. The mixture was mixed for one hour at room temperature. It was then dried and dissolved in a solution containing tris(2-carboxyethyl) phosphine at pH 8.5 to expose the thiol groups (SH groups). The peptide was then linked to the isothiocyanate group of FITC. The complex was then purified and mixed overnight with PEG bearing NH_2 groups, in the presence of EDC. For purification, the mixture was transferred to a dialysis cassette with a molecular weight cut off (MWCO) of 3000. The cassette was stirred in a flask containing deionized water, protected from light. Water was replaced at every 8 hours for 2 days. The reaction was confirmed by fluorescence spectroscopy at 485nm/535nm.

5.3.3 Addition of PEG-FITC-GE11 to the SWNT to yield SWNT-PEG-GE11

1 mg of SWNT was mixed with 2.5 mg of PEG-GE11 in 5 ml of deionized water. The sample was sonicated in a bath sonicator for 60 min at room temperature (~22 °C) to obtain SWNT-PEG-FITC-GE11. The dispersion stability of the sample in PBS was estimated for one week at room temperature. The formulations were stored at 4°C. Before

the use of the SWNTs formulations for *in vitro* studies, we washed them four times at room temperature for ten minutes at 4,000g, to remove the excess PEG-GE11. We used centrifugal filters with a molecular weight cutoff (MWCO) of 100 kDa, in which we added 3 ml of water to each 1ml of SWNTs formulation. Finally, the washed formulations in the filter were collected and added to fresh media for *in vitro* studies.

5.3.4 Doxorubicin attachment to SWNT-PEG-GE11

0.5 mg SWNT-PEG-GE11 was dispersed in 5 mL sodium phosphate buffer 20mM pH 8.5, and 2.5 mg of DOX was added. The mixture was sonicated in a bath sonicator for 10 min and incubated overnight while stirring. Unbound doxorubicin was removed by filtration and washing with Amicon centrifugal filters, 30 KDa MWCO. Absorption spectroscopy at 490 nm was used to determine the amount of unbound doxorubicin in the elute of the filtration.

5.3.5 Cell Culture and Incubation with SWNTs Solutions

Raw 264.7 cells were cultured in DMEM. They were supplemented with 10% FBS. the cell lines were incubated with 1% penicillin-streptomycin. The incubations were carried out at 37°C, in 5% CO2 atmosphere, and in 90% relative humidity. Media was refreshed every two to three days. The cells were seeded in 96 well plates at 10^4 cells per well for 24 hours. The SWNTs concentrations were 7 μg/ml and 15 μg/ml. All experiments were done in triplicates repeated on the same sample (n=1).

5.3.7 Cell Viability Assay:

Cells were washed three times with PBS to remove all SWNTs suspended outside the cells. Water Soluble Tetrazolium salts assay (WST-1 assay) [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] was used for measuring cell viability. Fresh media (100 μl) and 20 μl of the WST-1 reagent were added to each well. The plates were then shaken vigorously for 60 seconds to thoroughly homogenize the reagent inside the wells. Finally, they were incubated for 90 minutes before performing any cell viability readings. Cell viability measurements were taken at 12 h, 24 h, and 48 h post exposure to the formulations. Absorbance was measured using a Perkin Elmer (Victor^3 V) multiplate reader. The readings were measured at 450 nm.

5.4 Results

5.4.1 Preparation of the conjugated SWNTs samples

For the conjugation of GE11 to PEG, we first linked the N-terminus of GE11 to Sulfo-LC-SPDP that has an amine reactive sulfo-NHS ester. The addition of TCEP to the complex GE11-sulfo-LC-SPDP helped cleave the disulfide bonds in the spacer arm of sulfo-LC-SPDP to obtain thiol groups (SH groups). We then purified the sample with a dialysis cassette of 2000 MWCO. FITC was added to the sample and the reaction between the isothiocyanate group of FITC and the thiol groups linked to GE11 led to the complex GE11-FITC. The reaction was confirmed by fluorescence spectroscopy at 485nm/535nm. Finally, we conjugated the COOH groups of FITC (now bound to GE11) to the NH_2 groups of PEG using EDC as a cross-linker and DMAP as a catalyst. FTIR confirmed the conjugation of FITC with PEG (fig.1). The band at 2916 cm^{-1} was the C-H

stretch of -CH_2, and the band at 2849cm^{-1} was C-H stretch of -CH-. The band at 1741 cm^{-1} was attributed to C=O stretch. Sonication of PEG-FITC-GE11 with SWNT led to the highly soluble formulations and SWNT-FITC-PEG-GE11 (fig. 2). Cytotoxicity studies using a WST-1 assay showed that the formulation SWNT-PEG-FITC-GE11 had negligible influence on cell viability (fig. 3), indicating that the cytotoxicity was caused by the release of DOX from the drug delivery system.

5.4.2 Targeted delivery of SWNT-PEG-FITC-GE11 to RAW 264.7

To study the targeted delivery of SWNT-FITC-PEG-GE11 to RAW 264.7 cells, we incubated the cells for up to 12 h. Fluorescence measurements were taken at intervals of 2 hours (fig. 4). Results at 12 hours post exposure showed that the fluorescence intensity in the RAW 264.7 cells exposed to targeted formulation was 43.3% higher than that of the cells exposed to control SWNTs. To study the trafficking mechanism of the SWNTs, we exposed the cells to the formulation and incubated them at 4°C for three hours. The results showed a very limited uptake of GE11-conugated SWNTs by RAW 267.4 cells at 4°C (fig. 5). This further suggests a temperature dependent receptor-mediated endocytosis mechanism at 37°C [107, 108].

5.4.3 DOX delivery and cytotoxicity of SWNT-PEG-FITC-GE11-DOX on RAW 264.7 cells

DOX was loaded to the SWNTs via π–π stacking. On the basis of absorbance spectroscopy of DOX at 490 nm, we evaluated the weight ratio of DOX:SWNT to be 3:1, which demonstrates a high loading capacity of DOX on the SWNTs sidewalls. To measure the uptake of DOX, we incubated the cells for up to 12 hours with 5 μM of

targeted DOX using the formulation SWNT-PEG-FITC-GE11-DOX. Both untargeted SWNT-PEG-FITC-DOX, and free DOX served as negative controls. Figure 4 shows that DOX uptake was time dependent, plateauing between 8 – 10 hours post exposure. To measure the cytotoxicity induced by targeted DOX, we treated the cells with 1 μM, 5 μM, 10 μM, and 20 μM DOX for 48h. Untargeted DOX and free DOX served as negative controls. As the concentration of DOX increased, we observed higher cytotoxicity caused by targeted DOX, compared to untargeted DOX and free DOX. In fact, the toxicity caused by 5 μM, 10 μM, and 20 μM DOX delivered by SWNT-PEG-GE11-DOX was 1.2, 2.2, and 3.6 higher than that of equivalent concentrations of free DOX, respectively.

5.5 Discussion

EGFR is a potential receptor to target as it is highly expressed on many kinds of cancer cells [11, 12]. It plays important roles in cell growth and decreases cell apoptosis. Originally, EGFR was targeted by the epidermal growth factors (EGF), which is its natural ligand, however, its use in research is decreasing since it has strong mitogenic activity. It is therefore essential to replace it by a healthier substitute. GE11 is a peptide formed by the sequence (YHWYGYTPQNVI), and has been synthesized to bind to EGFR [105]. Studies have shown that the GE11 peptide binds very specifically and efficiently to EGFR, in addition to having a much lower mitogenic activity then EGF [105]. An important part of this study was to test the targeting capability of the new

formulation SWNT-PEG-FITC-GE11 on the EGFR expressing RAW 264.7 cells. We measure the cell uptake of SWNTs by incubating the cells with 7 μg/ml GE11-targeted SWNTs. SWNT-PEG-FITC served as a negative control. After incubation with the formulations, cells were washed with cold saline to remove the excess SWNTs in the culture media, and then scanned using a spectrophotometer for their fluorescence. At all incubation time points, RAW 264.7 cells exposed to GE11-targeted SWNTs were found to have greater fluorescence than cells exposed to the negative control SWNTs. This indicates that the targeted SWNTs were internalized into the cells via receptor mediate endocytosis.

Figure 6 shows that DOX uptake was time dependent. We can notice that the uptake of free DOX was the highest at time points prior to 6 hours post exposure. At 8 hours post exposure and above, cells exposed to the targeted SWNT-DOX showed significantly higher absorbance than cells exposed to the untargeted SWNT-DOX and free DOX. At 10 and 12 hours post exposure, the ratio between targeted DOX and free DOX present inside the cells was around 2 folds. This proves the efficiency of the GE11-conjugated SWNTs as drug delivery systems.

The WST-1 assay showed that the targeted drug had a higher cytotoxic efficiency than free DOX and untargeted DOX, except for low DOX concentrations (fig. 7). As the concentration of DOX increased, we observed higher cytotoxicity caused by targeted DOX. This further suggests that SWNT-PEG-GE11-DOX could be efficiently taken up by RAW 264.7 cells with subsequent intracellular release of DOX (fig. 8). To assess the biocompatibility of the delivery system SWNT-PEG-FITC-GE11, we evaluated its cytotoxicity at three different concentrations that covered those used for cell treatment.

5.6 Conclusion

To increase the concentration of doxorubicin in the RAW 264.7 cell line, we developed a new targeted drug delivery system; SWNT-PEG-FITC-GE11-DOX, which comprised SWNTs as the carriers, GE11 as a targeting moiety, and doxorubicin as the drug. The formulation showed high stability and dispersion in aqueous solution at room temperature. The addition of GE11 to the SWNTs enhanced their selective uptake by RAW 264.7. The uptake root was proved to be receptor-mediate since less fluorescence was detected in cells treated with untargeted SWNTs. Additionally, a very limited uptake of GE11-conugated SWNTs was detected at 4°C, which further proved that endocytosis was energy-dependent.

Cell death induced by 40 μM of targeted DOX was up to 3.6 times that of free DOX at 48h post exposure. These results demonstrate that our targeted drug delivery system had high anticancer efficacy due to the active targeting of SWNTs by GE11.

5.7 Acknowledgements:

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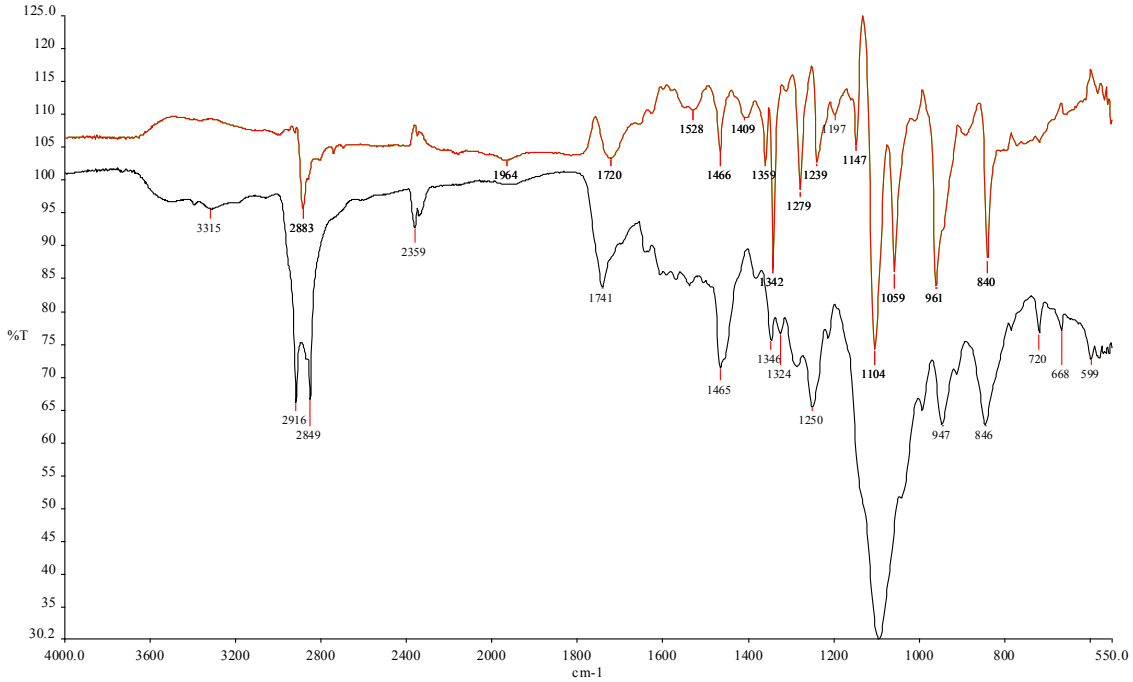

Fig 5.1. FTIR characterization of the conjugation between PEG and FITC.
The red graph and the black graph represent the transmittance of FITC and PEG-FITC, respectively. The band at 2916 cm^{-1} was the C-H stretch of -CH_2, and the band at 2849cm^{-1} was C-H stretch of -CH-. The band at 1741 cm^{-1} was attributed to C=O stretch.

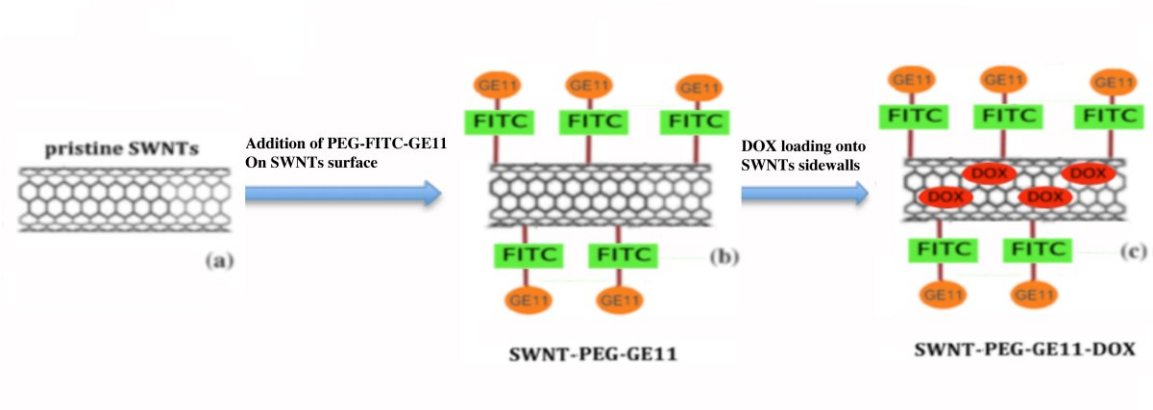

Fig. 5.2. Design strategy of the drug delivery system SWNT-PEG-FITC-GE11-DOX. (a) Pristine SWNT. (b) The SWNTs were sonicated for 1 h with PEG-FITC-GE11 to yield SWNT-PEG-FITC-GE11. The sample was then washed extensively with water to remove unbound PEG-FITC-GE11. (c) DOX was attached on the sidewalls of SWNTs by stirring it with SWNT-PEG-FITC-GE11 overnight in phosphate buffer of pH 8.5, followed by extensive washing.

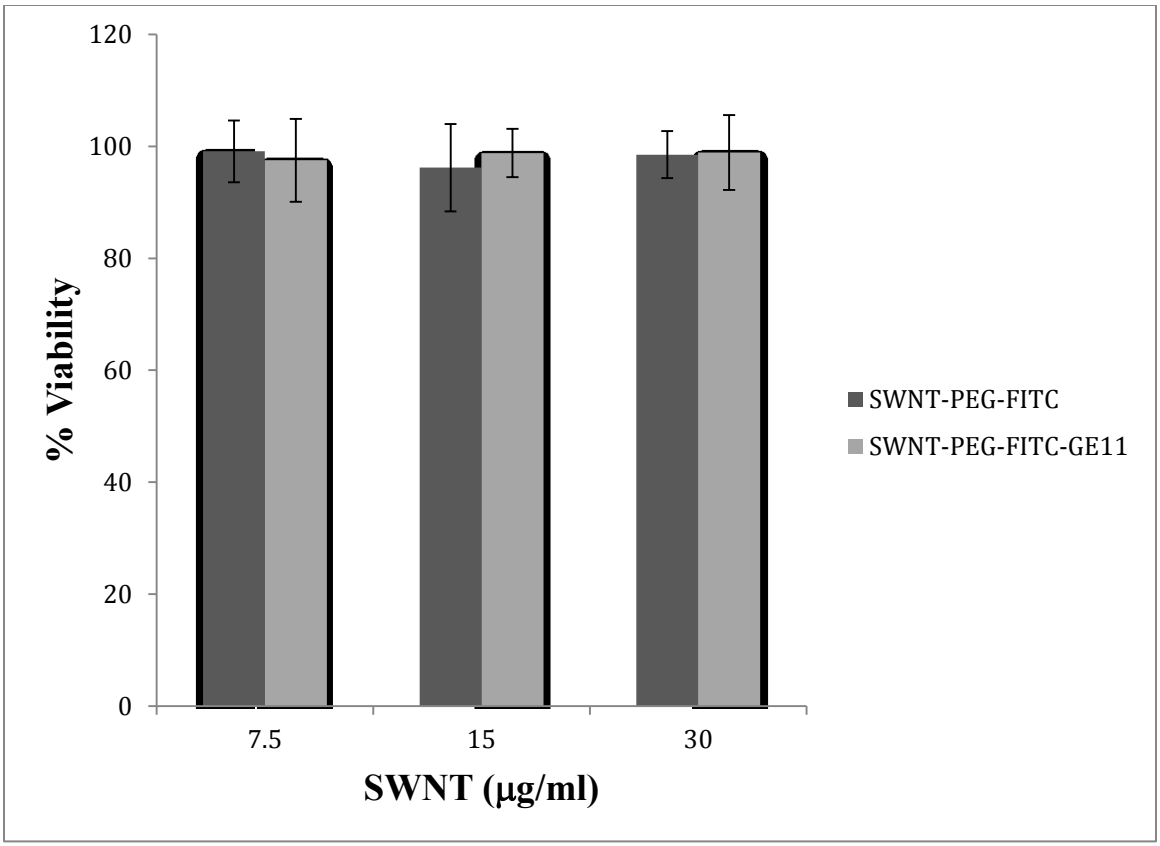

Fig. 5.3. Effect induced by the SWNT formulations after 48 hours of exposure on the viability of RAW 264.7 cells. Data expressed in % of unexposed controls showed no obvious toxicity at 7 μg/ml, 15 μg/ml, 30 μg/ml. Toxicity studies were performed in triplicates using a WST-1 assay. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

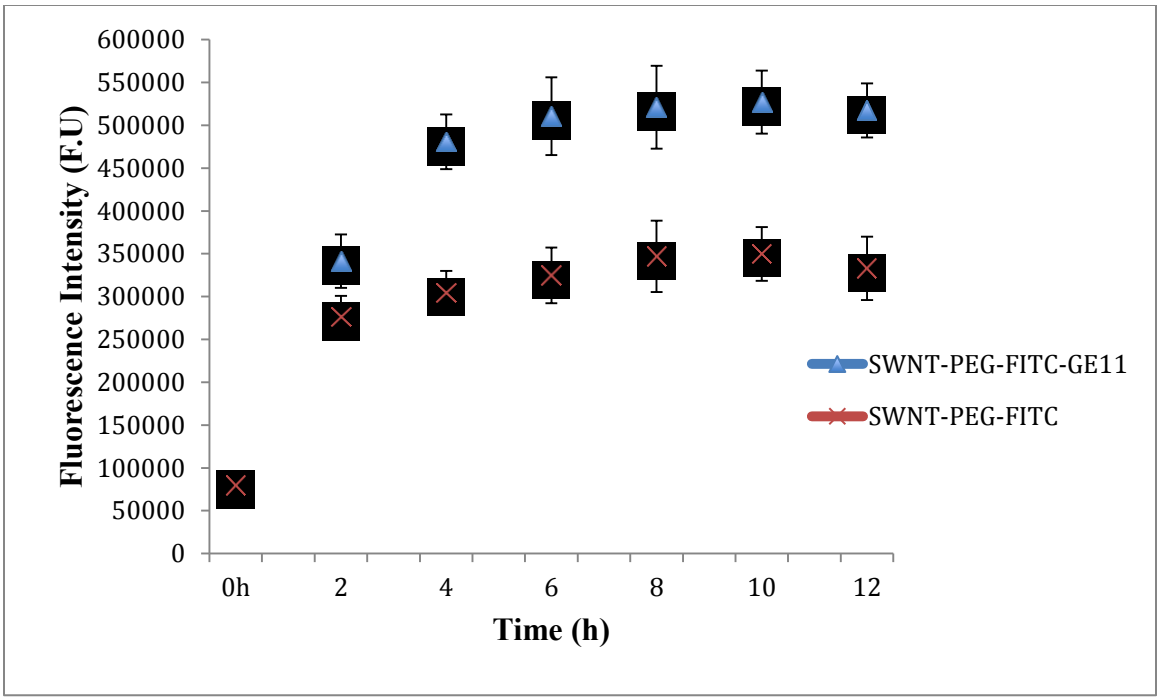

Fig. 5.4. Targeted delivery of SWNT-PEG-FITC-GE11 to RAW 264.7 cells. The targeted delivery occurs via receptor-mediated endocytosis. The cell line is known to express EGFR that are selective to GE11. The cells were incubated for 12 hours with 7.5 μg/ml of SWNT formulations. The fluorescence intensity in the cells treated with targeted SWNTs at 12h post exposure is clearly higher (32% higher) than that in the ones treated with non-targeted SWNTs (controls). Values represent the means ± SD for triplicates repeated on the same sample (n=1).

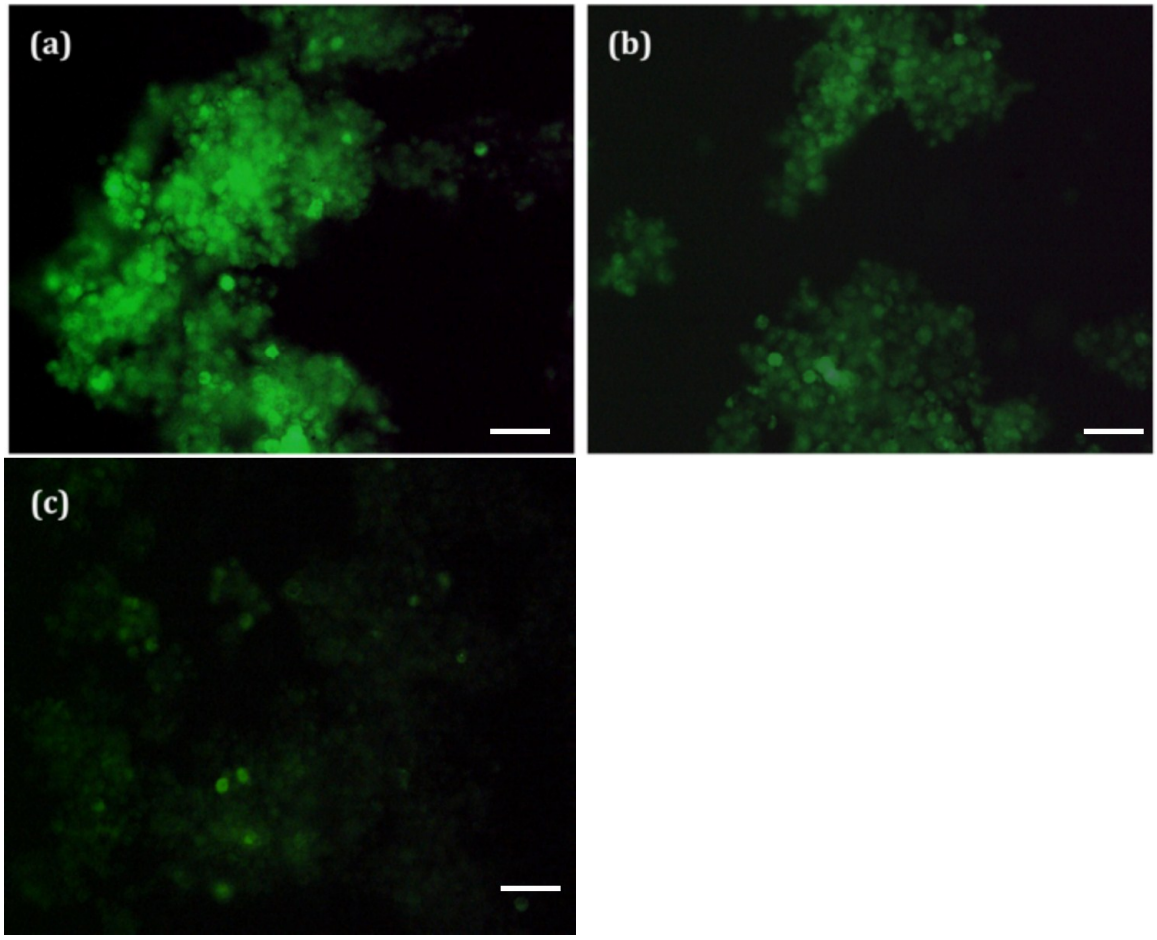

Fig. 5.5. Fluorescence microscopy of RAW 264.7 cells incubated with 15 μg/ml SWNTs for 3 h. (a) Cells incubated with SWN-PEG-FITC-GE11 at 37°C. The strong green FITC fluorescence inside the cells confirms the receptor-mediated uptake of SWNTs. (b) Cells incubated with the negative control formulation SWNT-FITC-PEG at 37°C. The fluorescence signal was significantly lower, confirming little uptake of non-targeted SWNTs. (c) Cells incubated with SWNT-FITC-PEG-GE11 at 4°C. The uptake was very minor compared to (a), which suggests a temperature dependent receptor-mediated endocytosis that further proves the selectivity of RAW 264.7 to GE11-targeted SWNTs. Bar: 100 μm

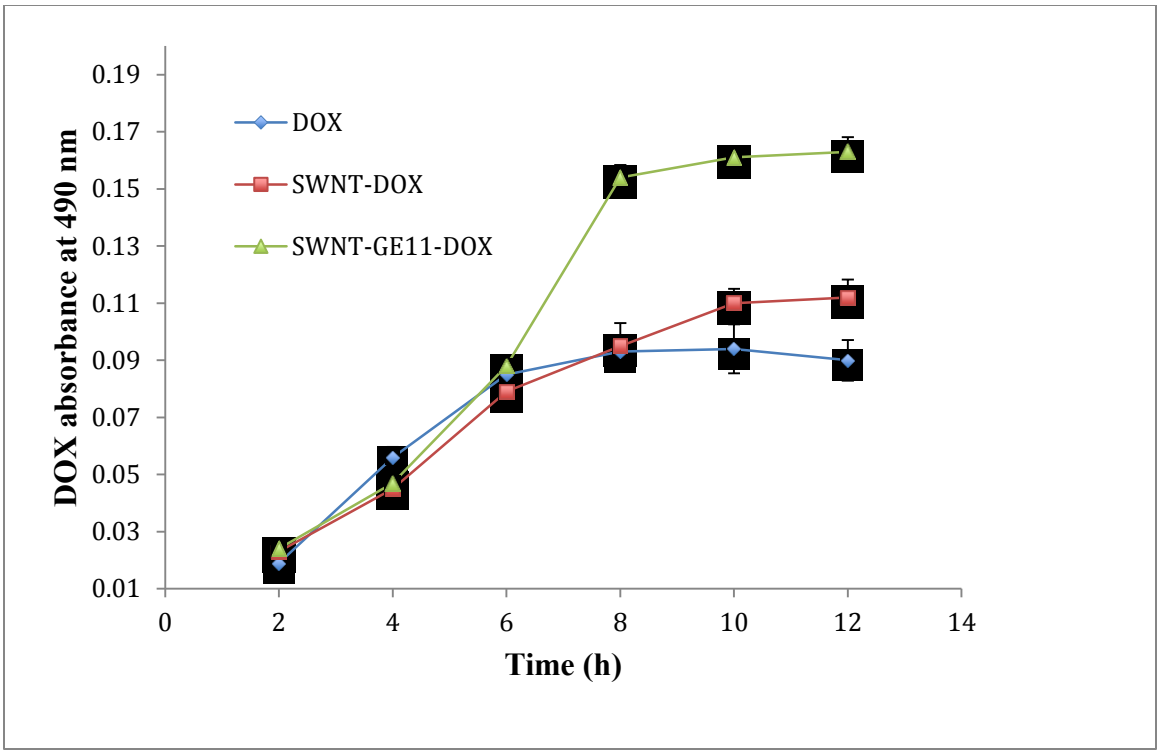

Fig. 5.6. Targeted delivery of DOX to RAW 264.7 cells. The accumulation of DOX inside RAW 264.7 was measured by absorbance spectroscopy at 490 nm. All cells were incubated with 5 µM DOX for 12 hours, and readings were taken at intervals of 2 hours. DOX was delivered by GE11-targeted SWNTs. SWNT-DOX and free DOX served as negative controls. Cells were washed with PBS before the readings. The cells exposed to SWNT-GE11-DOX showed significantly higher optical absorbance than the cells exposed to controls above 6 hours post exposure. At 10 and 12 hours post exposure, the ratio between targeted DOX and free DOX inside the cells was around 2 folds. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

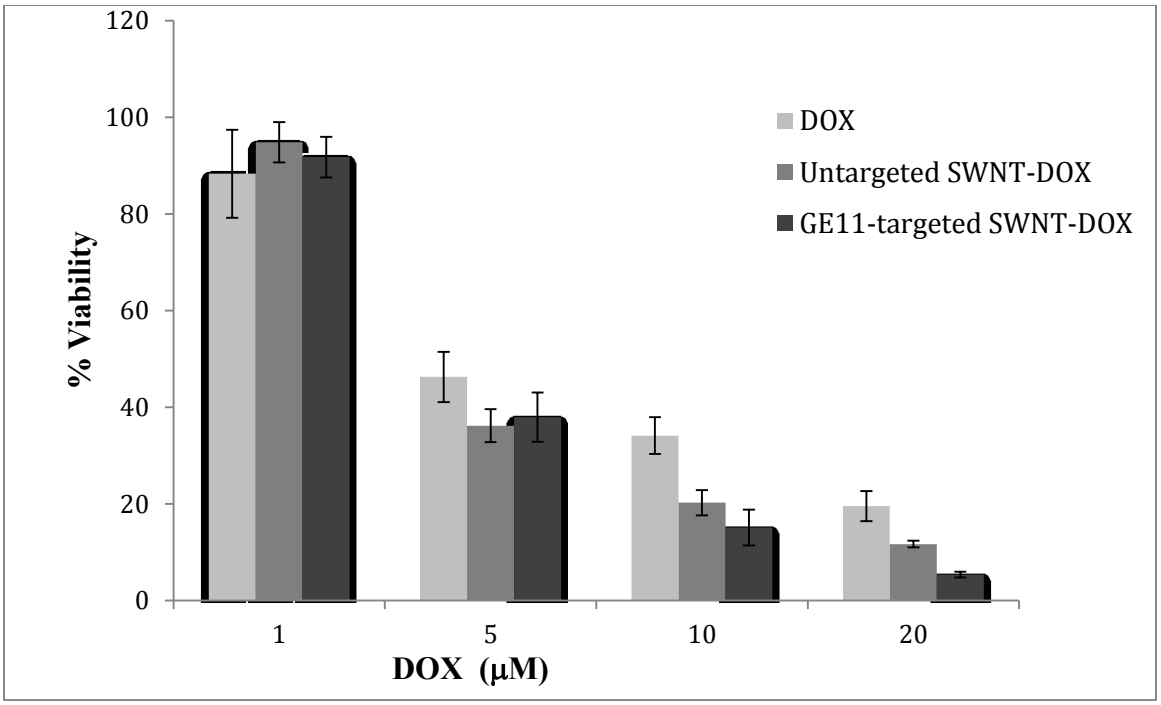

Fig. 5.7: Cytotoxicity induced by targeted DOX-SWNT formulation on the RAW 264.7 cells. DOX and untargeted SWNT-DOX served as controls. Viability was measured at 48 h post dose using a WST-1 assay. A significant decrease in viability is noticed for cells treated with DOX delivered by GE11-targeted SWNT, especially at higher DOX concentrations. The toxicity caused by 20 μM DOX delivered by SWNT-PEG-GE11-DOX was 3.6 higher than that of equivalent concentrations of free DOX. This confirms the efficiency ligand-conjugated SWNTs as drug delivery systems. Values represent the means ± SD for triplicates repeated on the same sample (n=1).

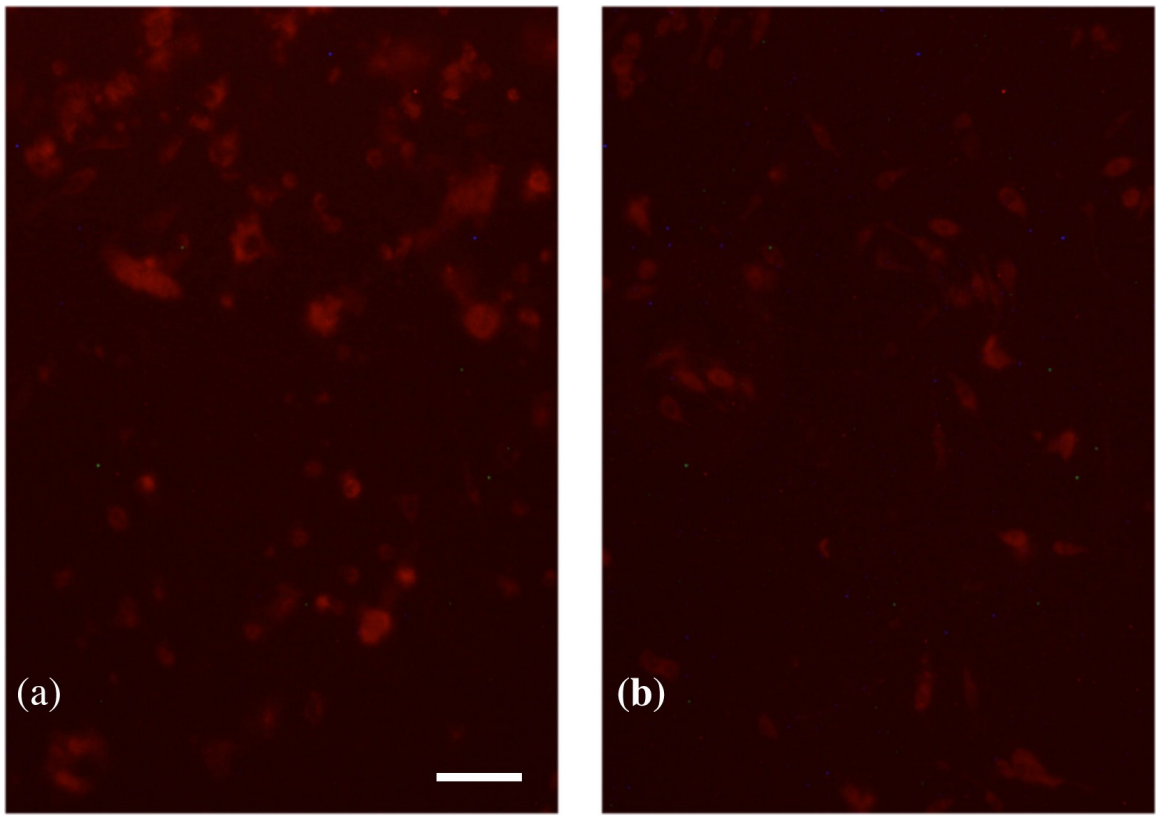

Fig. 5.8. Fluorescence microscopy of RAW 264.7 cells 24 h post exposure with SWNT-PEG-FITC-GE11-DOX.
(a) Cells treated with SWNT-PEG-FITC-GE11-DOX. (b) Cells treated with free DOX. Brighter fluorescence is detected in (a) since the drug delivery system was able to internalize higher amounts of drug inside the cells. Cells treated with the delivery system seemed to be in a worse shape than the ones treated with free DOX. This may be due to the effect of higher amounts of DOX delivered by the GE11-targeted SWNTs. Bar: 30 μm.

Chapter 6

Summary of Results

The aim of this research was to study the ability of targeted single walled carbon nanotubes (SWNTs) to selectively deliver chemotherapeutic agents for the treatment of $\alpha_v\beta_3$ integrins and epidermal growth factor receptor (EGFR) expressing cancer cells, such as colon, breast, leukemic, and lung cancer cells. The study can be summarized as the following:

1. Surface modification of SWNTs for increased biocompatibility:

Pristine SWNTs were covalently surface modified by sonication for 3 hours and reflux for 4 hours with both nitric and sulfuric acid. This process served to cut the SWNTs into smaller segments, and to add carboxylic groups (COOH-groups) at their ends and defect sites. The highly hydrophilic nature of COOH-groups increased the solubility of the oxidized SWNTs (oxSWNT) in aqueous environments. Poly(ethylene) glycol (PEG) was then used to surface craft SWNTs and oxSWNTs to further solubilize them and to increase their biocompatibility. oxSWNT, SWNT-PEG and oxSWNT-PEG showed to be very well dispersed and homogenous in various aqueous solutions, including water, PBS, and culture media for more than 7 days (fig. 3.4)

2. Cytotoxicity induced by different concentration of surface modified SWNTs on cancer cells:

Results indicate that oxSWNT, SWNT-PEG, and oxSWNT-PEG exerted different levels of toxicity. oxSWNT-PEG was the most biocompatible formulation among the 3

formulations, followed by SWNT-PEG, then by oxSWNT. The viability of Caco-2 cells exposed to 50 μg/ml of oxSWNT-PEG, SWNT-PEG, and oxSWNT was 91.1 ± 7.9%, 79.7 ± 6.8%, 73.9 ± 3.5% compared to controls at 24h post exposure, respectively. Similarly, the viability of RAW 264.7 cells exposed to 25 μg/ml of each formulation 88.3 ± 12.8%, 80.1 ± 4.5%, 75.8 ± 4.8%. Caco-2 showed to be more resistant to SWNTs at higher concentrations. At 24h post exposure, the viability of Caco-2 cells exposed to 100 μg/ml oxSWNT-PEG was 87.1 ± 5.8% while the one of RAW 264.7 was only 60.1 ± 6.5%.

3. Controlled release of DOX from the SWNTs

Upon loading DOX onto SWNT-PEG, experiments were performed to measure the release kinetics of DOX from the nanotubes sidewalls at pH 5 (lysosomal pH). UV-VIS-NIR spectroscopy was performed to measure the absorbance of DOX stacked on the nanotubes. DOX absorbance at 490 nm dropped by ~50 and ~80% after 24 h and 48 h at pH 5, showing a controlled release of DOX from SWNTs.

4. Targeted delivery of SWNTs to Caco-2 and RAW 264.7 cells:

In order to target the SWNTs to Caco-2 and RAW 264.7 cells, we conjugated them with RGD and GE11, respectively, to get the formulations SWNT-FITC-PEG-RGD and SWNT-FITC-PEG-GE11. Results showed a significant difference between the uptake of targeted and non-targeted SWNTs. Fluorescence spectroscopy at 485 nm/535 nm showed that the fluorescence in Caco-2 cells incubated with SWNT-FITC-PEG-RGD for 12 h was 32.1% more intense than the one in cells incubated with control SWNTs, i.e.

SWNT-FITC-PEG. Similarly, the fluorescence in RAW 264.7 cells incubated with SWNT-FITC-PEG-GE11 for 12h was 43.3% higher than the one in cells incubated with control SWNTs.

5. DOX uptake by Caco-2 and RAW 264.7

Spectrophotometry at 490 nm was performed to detect the difference between the uptake of DOX delivered by ligand-targeted SWNTs, and the uptake of control DOX (delivered alone). SWNTs were able to deliver higher amounts of drug inside both Caco-2 and RAW 264.7 cells. Results demonstrate that, for Caco-2 cells, the intracellular concentration of DOX delivered by SWNT-PEG-RGD-DOX was 1.4 folds higher than that of free DOX. Similar results were obtained after exposing RAW 267.4 cells with targeted DOX, as the intracellular concentration of DOX delivered by SWNT-PEG-GE11-DOX was 2 folds higher than that of free DOX.

6. Cytotoxicity induced by DOX-loaded SWNTs on the cell lines.

The cytotoxicity of SWNT-PEG-RGD-DOX and SWNT-PEG-GE11-DOX was compared to the cytotoxicity of free DOX on both Caco-2 and RAW 264.7 cells, respectively. DOX delivered by SWNT-PEG-RGD-DOX induced a time-dependent Caco-2 cell death, and its cytotoxicity towards cell line was 1.3 times higher than that of free DOX at 48h post exposure. Similarly, DOX delivered by SWNT-PEG-GE11-DOX to RAW 264.7 cells was 3.6 times more cytotoxic to the cell line than free DOX.

Chapter 7

General Discussion

Single walled carbon nanotubes (SWNTs) have evolved as efficient drug nanocarriers and have shown to be promising candidates in the field of drug delivery for cancer therapy. In the present research two SWNTs formulations were developed for the targeted delivery of therapeutic agents to cancer cells. The peptides RGD and GE11 were used as targeting moieties, and doxorubicin (DOX) was used as a drug. It was hypothesized that by using nanotubes as targeted drug delivery systems, a higher drug uptake and a higher cytotoxicity may be induced to cancer cells. The objectives of this study were to improve the biocompatibility of SWNTs, to assess the selective cell uptake of RGD- and GE11-targeted SWNTs by Caco-2 and RAW 264.7, and to compare the cytotoxicity of DOX delivered by the targeted SWNTs with the cytotoxicity of free DOX. After reviewing the results, the following general discussion can be drawn.

Double surface modification of SWNTs by oxidation and PEGylation provided a highly soluble, stable, and biocompatible oxSWNT-PEG formulation. The oxidation was carried out by sonication and acid reflux with nitric acid and sulfuric acid. Heating and ultrasonication of the SWNTs with nitric acid causes the formation of carboxyl groups (COOH groups) at the defect sites of the walls and at the ends of the tubes [75, 76]. UV-VIS spectroscopy confirmed the presence of COOH groups on the SWNTs sidewalls. The presence of carboxyl groups on the oxSWNTs surface led to a reduction of van der Waals interactions between the nanotubes, which highly facilitates the separation of nanotube bundles into individual tubes [77]. The use of PL-PEG to surface graft the oxSWNTs further stabilized the nanotubes in water and PBS. Those surface

modifications increased the homogenous dispersion of SWNTs in aqueous environments, making it easy for them to penetrate the cells, in addition to increasing their biocompatibility. In fact, the cell viability of Caco-2 and RAW 264.7 exposed to oxSWNT-PEG was higher than that of cells exposed to either oxSWNT or SWNT-PEG.

DOX binding and release from SWNTs was controlled by varying the environment's pH. When in basic solutions, the high surface area of carbon nanotubes allow for efficient loading capacities of DOX through π- π stacking [81, 82]. On the basis of absorbance spectroscopy of DOX at 490 nm, we evaluated the weight ratio of DOX:SWNT to be 3:1, which demonstrates a high binding capacity of DOX on the SWNTs sidewalls. Contrary to neutral and basic solutions, acidic environments favored DOX detachment from SWNTs due to its increased solubility at low pH's [82, 88]. UV-VIS-NIR spectroscopy was used to demonstrate the detachment of DOX. It showed that the DOX peaks decreased gradually as the time of exposure of SWNT-PEG-DOX to acidic buffer increased. The absorbance of DOX at 490 nm dropped by ~50 and ~80% after 24 h and 48 h at pH 5. This clearly demonstrates a controlled release of DOX from the SWNTs.

The targeted delivery of SWNTs to Caco-2 cells was carried on by conjugating the nanotubes with cyclic RGD; a peptide that recognizes $\alpha_v\beta_3$ integrins, which are common tumor markers expressed at high levels on the membranes of endothelial and epithelial cancer cells [4]. Once at the surface of the cell membrane, RGD binds at the interface between the α and β subunits of $\alpha_v\beta_3$, facilitating the uptake of the whole system that contains it [5, 7]. Results clearly showed that the RGD-conjuagted SWNTs accumulated at high levels in Caco-2, compared to control SWNTs. This difference is

due to the absence of ligand-induced and receptor-mediated endocytosis of the control formulation. Similarly, DOX accumulation inside the cells was time dependent, plateauing between 8 and 10 hours post exposure. At times prior to 4 hours post exposure, the amount of control DOX and targeted DOX was roughly the same when at time lower than 8 hours post exposure. Past that time, cells exposed to RGD-targeted and DOX-loaded SWNTs showed to have higher amounts of DOX than cells exposed to SWNT-DOX or free DOX. At 12 hours post exposure, the ratio between targeted DOX and free DOX inside the cells was around 1.4 folds. This demonstrated that DOX could be found at higher levels inside the $\alpha_v\beta_3$ expressing Caco-2 cells when delivered by RGD-targeted SWNTs.

Results also showed that the cytotoxicity induced by the drug delivery system SWNT-PEG-RGD-DOX was time-dependent, reaching the efficacy of the same concentration of free DOX at around 27 hours post exposure. At 48 h post exposure, the highest toxicity levels were caused by SWNT-PEG-RGD-DOX at DOX concentrations higher than 10 μM. In fact, compared to equivalent concentrations of free DOX, 20 μM and 40 μM of DOX delivered by the RGD-conjugated SWNTs decreased cell viability by 23.3% and 21%. The formulation SWNT-PEG-RGD had negligible influence on cell viability, indicating that the cytotoxicity induced by SWNT-PEG-RGD-DOX was caused by the release of high amounts of DOX, and not by the delivery system itself.

The targeting capability of GE11-targeted SWNTs was investigated on the EGFR positive RAW 264.7 cancer cell line. GE11, a peptide that is highly selective to EGFR, successfully delivered the drug delivery system SWNT-PEG-FITC-GE11 to the RAW 264.7 cell line. At all incubation time points, RAW 264.7 cells exposed to GE11-targeted

SWNTs were found to have greater fluorescence than cells exposed to the negative control SWNTs. This indicated that the targeted SWNTs were internalized into the cells via receptor mediate endocytosis. Results at 12 hours post exposure showed that the fluorescence intensity in the RAW 264.7 cells exposed to targeted formulation was significantly higher than that of the cells exposed to control SWNTs. Exposing the cells to the formulation at 4°C for three hours was necessary to study the trafficking mechanism of the SWNTs. The results showed a very limited uptake of GE11-conugated SWNTs by the cells, which suggests a temperature dependent receptor-mediated endocytosis mechanism at 37°C [107, 108]. The targeted drug had a higher cytotoxic efficiency than free DOX and untargeted DOX-loaded SWNTs. As the concentration of DOX increased, we observed higher cytotoxicity levels caused by targeted DOX, compared to untargeted DOX and free DOX. In fact, the toxicity caused by 5 μM, 10 μM, and 20 μM DOX delivered by SWNT-PEG-GE11-DOX was 1.2, 2.2, and 3.6 higher than that of equivalent concentrations of free DOX, respectively. This further suggested that SWNT-PEG-GE11-DOX could be efficiently taken up by RAW 264.7 cells with subsequent intracellular release of DOX, followed by transport of DOX into the nucleus where it takes effect.

Chapter 8

Conclusions

In the present research, the biocompatibility of surface modified SWNTs and their ability to target cancer cells were investigated. Results showed that a double surface modification, both covalent and non-covalent surface modification, of SWNTs have shown to increase their biocompatibility, compared to SWNTs that were either covalently or non-covalently surface modified. Results also showed that the formulation oxSWNT-PEG was perfectly homogenous in aqueous solutions. SWNTs loaded with doxorubicin (DOX) demonstrated a pH- and time-dependent controlled release of DOX. At neutral pH, DOX was retained on the SWNTs sidewalls. A steady release was however observed as the pH was lowered to 5 for a period of 48h.

SWNTs were successfully conjugated with the targeting ligands RGD and GE11. Conjugation was confirmed by FTIR and NMR. The drug delivery systems consisting of SWNTs and the targeting ligands were able to selectively target the Caco-2 and RAW 264.7 cancer cell lines. Their presence inside the cell was significantly higher than the presence of untargeted SWNTs. This suggests a ligand-receptor interaction and a receptor-mediated endocytosis. Similarly, compared to controls, the concentration of anti-cancer drug doxorubicin (DOX) was higher inside the cells when it was delivered by the targeted SWNTs. As a result, the cytotoxicity induced by DOX delivered by targeted SWNTs was higher than that induced by free DOX. This was due to their presence at higher concentration inside the cells exposed to the drug delivery systems. The SWNTs had no toxic effects on the cells in the absence of DOX, which shows that the cytotoxic effects were only induced by DOX.

Chapter 9

Recommendations and Future Prospects

Cancer is a complex disease that continues to kill millions of people worldwide every year. No real cure has yet been found to efficiently treat the disease, and the adverse effects of chemotherapy create a lot of additional health problems. The goal of developing drug delivery systems is to enhance the anticancer effect of drugs and to reduce their adverse effects by targeting them to tumor cells. Carbon nanotubes, especially single walled carbon nanotubes (SWNTs) have been introduced as advanced drug delivery systems as they offer several advantages as nanocarriers. However, further efforts are required to optimize their surface chemistry to further enhance their biocompatibility. With improved surface modification, further improvements in biological sensing and imaging, better tumor targeting and more prolonged blood circulation may be realized. Numerous encouraging results using SWNTs as drug delivery systems have been published in the last few years, however, more work is still needed before carbon nanotubes enter the clinic. The most important issue to be addressed is still their long-term cytotoxicity. Cytotoxicity observation periods are usually no longer than six months, which may not be sufficient to determine the long-term effects of SWNTs. Further investigations should be carried out using different *in vitro* cell lines and different animal models, with special attention paid to various doses of surface modified nanotubes.

Recently, graphene has emerged as a 2D nanomaterial with promising applications in nanomedicine. Graphene is the planar sheet of carbon that, if rolled, constitutes carbon nanotubes. Like carbon nanotubes, graphene's properties enable a

wide range of novel cancer therapies such as photothermal and photoacoustic ablation [109], which could be combined with drugs to overcome the multi-drug resistance problem in current cancer chemotherapies. Graphene has poorer optical properties than carbon nanotubes, however, the 2D shape of nano-graphene may offer interesting advantages in biomedical applications. Therefore, it is necessary to study the difference between these two closely related carbon nanomaterials structures for cancer research, and to determine which one has the greater potential.

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