APPLICATION OF LIPOPROTEIN AS DRUG DELIVERY SYSTEM FOR ANTICANCER DRUG AND DNA-BASED VACCINE

by

FARS KAED ALANAZI

(Under the Direction of D. ROBERT LU)

ABSTRACT

Various advanced drug delivery systems such as lipoprotein, red blood cell, and albumin have been investigated to overcome the limitation of the conventional systems. One of the advantages of using these advanced drug delivery systems is that they are natural plasma components. The main focus of this research is to study the application of lipoprotein as drug delivery system for cancer therapy and for DNA based vaccines.

The high requirement of LDL by malignant cells and thus the overexpression of LDL receptor can be utilized for developing a novel targeted drug delivery system. This can be achieved by targeting of the LDL particle *in vivo* and allowing the anticancer drugs to be transfered to the natural LDL inside of the body. Basically, LDL will function as a secondary carrier of anticancer molecules *in vivo* and deliver these molecules selectively toward cancerous cells via elevated LDL receptors. This approach requires the anticancer molecules to have affinity of the LDL particle endogenously and to have certain special physicochemical properties. We hypothesized that mimicking the cholesterol ester can facilitate targeting of the LDL particle and cancer cells. Therefore, we synthesized new cholesterol boron conjugates. Liposomes were prepared as the first carrier of these compounds and the factors affecting the incorporation in liposomes were studied. We also investigated the distribution of one of these compounds, BCH, in the brain after intracerebral administration using a tumor-bearing rat model. Since these compounds will face hydrolytic enzymes *in vivo* that limit their targeting activity, by liberating cholesterol from anticancer moiety, stability studies of a representative compound, BCH, in simulated biological media were carried out. Inductively Coupled Plasma (ICP), an analytical technique for determining concentration in liposomal formulation and tissue samples was evaluated. To examine the transfer of these compounds from liposomal formulation to low density lipoprotein (LDL) *in vitro,* an ultra-centrifugation method was developed and optimized.

An artificial lipoprotein system mimicking natural lipoprotein in the human body was evaluated as a novel DNA based vaccine delivery system. The artificial lipoprotein is composed of an oil-in-water emulsion, hydrophobic polyamine polymer and plasmid DNA. Characterization of the complex with DNA as well as transfection efficiency was studied.

INDEX WORDS: Drug delivery, Drug targeting, Drug design, Low-density lipoprotein, BNCT, Cholesteryl conjugate, Liposome, Cancer, Nanoemulsion, DNA vaccine, Rabies

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B.S., King Saud University, Saudi Arabia, 1997

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2003

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DEDICATION

This dissertation is dedicated with affection to my mother Turkiah, my father Kaed, my wife Hussah, my daughter Rema, my son Malik and the rest of my family, for transcendent enthusiasm, motivation, encouragement, infinite patience and intangible support.

ACKNOWLEDGEMENTS

I am very grateful to God, the one and only, for all of his countless blessings and I would like to thank God for helping and guiding me throughout my life, specifically for accomplishing my dissertation. I would like to thank God for giving me my wife, my daughter and my son. I would like to thank my wife and my kids for their endurance to bear the thousand silent hours that I spent away from them while I was conducting my research.

I would like to express my appreciation to my major advisor Dr Robert Lu. His constant encouragement and guidance are invaluable. I am very thankful to my committee members Dr Anthony C. Capomacchia, Dr H. Won Jun, Dr James C. Price, and Dr James V. Bruckner for their support and valuable suggestions. I'd like to thank Dr Hengguuang Li for his assistance. My deep gratitude to my labmates: Gina, Richard, Mohhanad, Bingqing, Ranajoy, Tiffany and others. My sincere thanks and appreciation are extended to all the faculty, staff and graduate students in the department for their friendship and help.

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CHAPTER 1

INRODUCTION AND LITERATURE REVIEW

Outline

A. Introduction

B. Function and classification of lipoprotein

C. Lipoprotein as drug delivery system for cancer therapy

    1) Cancer and targeted drug delivery for cancer treatment

    2) Low density lipoprotein pathway for targeted delivery for anticancer

    3) Our approach for targeting boron compounds via LDL pathway

    4) Liposome

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D. Lipoprotein as a drug delivery system for gene based vaccine

    1) DNA vaccination

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A) Introduction

The changes in the drug discovery and development process over the last decade have been supplemented by corresponding changes in the area of pharmaceutical research and pharmaceutical biotechnology which resulted in the improvement of the field of drug delivery. For any ideal drug delivery system, a sufficient amount of active drug must be absorbed and transported to the site of action at the right time and appropriate rate of input. Also, it implies selective distribution with minimal uptake other than at the site of action, which is important when there is only a small of margin between effective and toxic concentration. Cancer chemotherapy and DNA-based vaccine have been identified as special areas of need for improved drug delivery.

For the last few years, advanced drug delivery systems have been investigated to overcome the limitations of the conventional systems. One of the leading approaches is the utilization of the plasma components as drug delivery systems such as lipoprotein, red blood cells, and albumin. Lipoproteins as drug delivery systems are becoming an attractive area of research and they are considered excellent candidates for targeted delivery of drugs to various tissues. In this introduction the application of lipoprotein as a drug delivery system for cancer therapy and DNA-based vaccine will be discussed.

B) Classification and function of plasma lipoprotein

Lipoproteins are endogenous particles that transport lipids through the blood to various cell types, where they are recognized and taken up via specific receptors. Lipoprotein is classified into 5 classes based on densities**:** chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density

lipoproteins (LDL), high-density lipoproteins (HDL). Chylomicrons are the largest lipoproteins and they are synthesized in the intestinal tract. Their main function is transport of dietary triglycerides and cholesterol. Very Low Density Lipoproteins (VLDL) are synthesized in the liver and function to export triglyceride to peripheral tissues. LDL is the major vehicle to transport cholesteryl esters to peripheral cells. LDL contains one major apolipoprotein (i.e. apo B-100), which allows LDL to bind to the LDL receptors on the peripheral cell surfaces and to be internalized by these cells through a receptor mediated endocytosis. High-density lipoproteins (HDL) are the smallest lipoprotein due to high protein / lipid ratio. HDL works as a lipid scavenger, transporting cholesterol from various tissues back to the liver.

C) Lipoprotein as drug delivery system for cancer therapy

1) Targeted drug delivery system in cancer treatment.

Cancer occurs when cells in a part of the body begin to grow out of control. Cancer cells differ from normal cells in three main ways: they divide more rapidly, they have different surface properties (they don't stick and pack as tight as normal ones) and they do differentiate in their shape, size and etc [31]. Cancer is the second leading cause of death in the US, exceeded only by heart disease. In the US, 1 of every 4 deaths is from cancer [1]. Thus, NIH supports an estimated $5.44 billion in cancer research for year 2003 (National Institution Of Health) [2]. This number (5.44 billion dollars) for the year 2003 is higher than that of year 2001(4.37 billion dollars) by 24.5 %, and year 2002 (4.92 billion dollars) by 10.6 %, which indicates the importance and magnitude in cancer research. Surgery, radiation, chemotherapy, hormones, and immunotherapy are the modalities in treating cancer. For chemotherapy, success relies on the improvement of targeted drug delivery systems for therapeutic agents.

The application of anti-cancer agents is currently associated with significant toxicity and lack of tumor specificity. The development of novel drug delivery systems for cancer therapies that selectively deliver anticancer agents to tumor cells with limited toxicity to normal tissues is a challenge for oncology researchers [4]. A targeted drug delivery system offers the potential to enhance the therapeutic index of anticancer agents, either by increasing the drug concentration in tumor cells and/or by decreasing the exposure in normal host tissues [3]. The success of cancer therapy, in many cases, is dependent on the possibility of utilizing biological differences between malignant and normal cells to selectively deliver anticancer agents to tumor cells.

To deliver the anticancer agent to tumor tissue selectively, there are two ways: physical and biological targeting strategies [5]. Physical targeting is based on delivering anticancer agents directly to tumor tissue by physical implantation or injections of the agents precisely at the tumor site. The examples include intracerebral delivery of anticancer agents to brain tumor [6], implantation of anticancer agent-loaded wafers [7], and intra-arterial drug delivery to liver cancer [8]. Biological targeting can be based on the following tactics. First, an anticancer moiety can be delivered by specific carriers such as liposomes [3], polymer conjugates [17], bacterial [4] and virus vectors [16]. Second, the development of targeted drug delivery systems to cancer can be based on the difference in substrate uptake between cancer cells and normal cells. Due to the high growth rate of cancerous cells, they require more nutrient and various receptors, thus, are over expressed, such as folate receptors [12], transferrin receptors [14], growth factor receptors [15], and low-density lipoprotein receptors [18]. Drug delivery systems linked to ligands that target these receptors have been investigated [14], [32]. The high requirement for LDL by malignant cells and thus the overexpression of LDL receptors can be utilized for

developing a novel targeted drug delivery system. This strategy is attractive and promising. It will be discussed in this section and through various parts of the dissertation.

2-Low density lipoprotein for targeted delivery of anticancers

Growing cells need cholesterol to construct cell membranes. They acquire cholesterol via *de novo* synthesis and high affinity receptor-mediated uptake of low-density lipoprotein (LDL). Many types of tumor cells display higher level of receptor-mediated LDL uptakes compared to corresponding normal tissues. The increase in LDL receptor activity in cancer cells is suggested to be due to high cholesterol demand for cell growth and/or a mechanism directly linked to cell transformation [18]. LDL has therefore been proposed as a potential carrier for chemotherapeutic agents [19].

There are three main strategies to utilize LDL as a drug delivery system. First, LDL has been utilized as a targeting vector for cancer therapy through loading drugs into LDL particles and subsequent administration of the preloaded LDL. Anticancer drugs can be loaded into LDL particles by five main methods; 1) The dry stir method, in which the drug is dried as a thin film on a glass tube and the lipophilic drug will partition into the LDL particle upon incubation with LDL [20]. 2) The aqueous addition method for incorporating water-soluble compounds [21]. 3) Detergent assisted transfer by solubilizing the lipophilic drug with nonionic surfactant, followed by incubation with LDL [22]. 4) The solvent extraction method in which a lipophilic core is extracted and replaced by another oil containing the drug [23]. 5) Liposomal formulation in which higher drug loading can be achieved compared to the first, second and third methods [24]. This strategy (preloading drug into LDL) suffers from several limitations, such as being tedious, the possibility of losing LDL targeting

capability (due to modification of apo B100 in presence of such harsh conditions), and risk of cross contamination (i.e. it is a biohazard blood product). A second strategy is developing LDL resembling delivery systems, which follow a similar metabolic pathway to that of natural LDL. This strategy involved using a drug delivery system such as protein free microemulsion [33] and Apolipoprotein B built-in phospholipid-cholesterol liposomes [34]. The third strategy, which is a combination of the two previous pioneer strategies, is based on targeting the LDL particle *in vivo* and allowing the anticancer agent to be transferred to the natural LDL inside of the body. Basically, LDL will function as a secondary carrier of anticancer molecules *in vivo* and deliver these molecules selectively toward cancerous cells *via* elevated LDL receptor. This approach required anticancer molecules to have affinity to the LDL particle endogenously and to have certain special physicochemical properties. Our approach of developing a new drug delivery system for cancer therapy is similar to this strategy.

3-Our approach of targeting boron-containing compounds via the LDL pathway:

LDL is the endogenous carrier of cholesterol. The majority of cholesterol is obtained through the LDL receptor-mediated endocytosis mostly in the form of cholesterol ester. LDL particle is an oil droplet that is covered by a monolayer of phospholipid (Figure 1.1). The lipid core is made up mostly of triglycerides (20%) and cholesteryl esters (80%). Low-density lipoprotein particles are potential drug carriers, but only lipophilic drug species partition into the core of the system. Since cholesterol (in its ester form) is the native component of LDL, conjugation of an antitumor moiety with cholesterol facilitates the loading of these compounds into LDL. Synthesizing antitumor compounds that mimic native cholesteryl esters may

result in successfully transferring these compounds into LDL. As these compounds share similar chemical and physical characteristics with native cholesteryl esters, they can interact well with LDL. They may transfer effectively into LDL in the physiological environment and, thus, utilize the elevated LDL receptor expression on tumor cells for targeted drug delivery.

It has been demonstrated that chemical modification of an agent, giving it high lipophilicity, will enable it to be carried by lipoproteins after intravenous administration. For example, after intravenous administration of 14C-warfarin to mice bearing M5076 sarcoma, it was mainly concentrated in the liver and was found at only low concentrations in other tissues including the tumor. However, intravenous injection of its lipophilic derivatives (14C-warfarin hexadecyl ether [14C-WHE], and additionally a palmityl moiety to the parent compound) resulted in a high distribution of 14C-WHE to the tumor. These modifications raise the possibility of lipoproteins as endogenous targeting carriers into tumor cells, which have high LDL-receptor activity, and show the importance of lipophilicity in transferring drugs into LDL [25]. The effect of linking different fatty chains in incorporation of cytotoxic drug into LDL was studied [26]. Esters of elliptinium with stearic (C 18), palmitic (C 16) or oleic (unsaturated C 18) acids, a series of lipophilic derivatives of ellipticine, were synthesized. Among the three derivatives, the oleic derivative shows the most potent incorporation (83 μg/mg protein LDL) compared to stearic derivative (37 μg/mg protein LDL) and the palmitic derivative (58 μg /mg protein LDL). These results indicated that the length of fatty chains linked to an anticancer moiety has an effect on transferring to LDL. Also, it shows that difference in incorporation between saturated (stearic) and unsaturated (oleic) fatty chain conjugates [26].

In the light of all that, we hypothesized that mimicking the cholesterol ester could be a good tactic to target the LDL particles and cancer cells. Therefore, the design and synthesis in our laboratory have focused on the derivatives of cholesteryl esters containing an antitumor chemical unit. The antitumor moiety to be conjugated with cholesterol was carborane, used for Boron Neutron Capture Therapy (BNCT). The proposed scenario of targeting these compounds to cancer cells is presented in Figure 1.2. Following the administration of cholesterol-based compounds in liposomal formulation, these compounds will partition into LDL particles. LDL-cholesterol based conjugate complex enters the tumor cells *via* receptor mediated endocytosis. Degradation of LDL particles by endosomal enzymes will result in liberation of these molecules which act as tumorcides. It should be noted that BNCT does not require the release of the antitumor unit from cholesterol conjugate as other prodrugs require for therapeutic a effect. Initially, cholesteryl 1,12–dicarba–closo–dodecaboranel-carboxylate (BCH) was synthesized and evaluated *in vitro* and *in vivo*. New derivatives of BCH were also synthesized by linking different fatty chains which varied in length and saturation.

4-Liposome

Liposomal anticancer drug delivery systems (Figure 1.3) markedly alter the biodistribution of their associated anticancer agents by delaying drug clearance, retarding drug metabolism, decreasing the volume of distribution, and shifting the distribution in favor of cancerous tissues with increased capillary permeability. This increases the therapeutic indices of the associated drugs by increasing the drug concentration in solid tumors and regions of infection and reducing the drug concentration in normal tissues [27]. Also, liposomes were shown to play an important role in overcoming multidrug resistance in cancer therapy [28].

Stability of liposomes in biological systems is very important in the targeting of anticancer agents. A number of different factors influence liposomal integrity after introduction into the circulation such as: lipid-metabolism, exchange or transfer of liposomal lipid to plasma constituents or cellular membranes, interaction with plasma proteins, imperfect fusion of liposomes with cells involving partial release of entrapped material and release of lysosmal lipolytic enzymes during liposome-induced endocytosis [29].

It has been shown that the liposomal membrane becomes slightly modified by the incorporation of plasma lipoprotein causing an increase in permeability but leaving the gross morphology of the liposome unchanged. Kader A., et al. (1998) has shown that doxorubicin transferred from liposome to LDL *in vitro* [24]. The phospholipid layer of liposome interacted with the LDL resulting in leakage of liposome. The drug is presumably released on the surface of LDL, from where it diffuses into the core of the LDL from liposome. In our approach the release of drugs from the liposome in biological systems is advantageous because the cholesterol-based compounds favor the partitioning into the endogenous carrier of cholesterol (LDL). Thus, liposomes can be used as *in vivo* drug donors for transferring drug to LDL particles and then to cancerous tissue.

5-BNCT

Boron Neutron Capture Therapy (BNCT) is a binary technique that involves the concurrent presence of a flux of neutrons of adequate energy and a ^{10}B capture agent (Figure 1.4). Their interaction generates particles that damage tumor cells. The path length of ^{7}Li and ^{4}He (alpha particle) is approximately 5 μm and 8 μm respectively, i.e. approximately the diameter of a tumor cell (» 10 μm). The destructive effect of the capture reaction occurs mainly in those cancer cells that have selectively

accumulated boron, ^{10}B. The normal cells that have not incorporated significant amounts of ^{10}B do not suffer significant damage.

D) Lipoprotein as drug delivery system for DNA based vaccine

1-DNA vaccination

A DNA vaccine is a circular double stranded DNA molecule, referred to as a plasmid, containing genes encoding one or more proteins of a pathogen. When DNA vaccine is introduced into eukaryotic cells, the gene of interest is transcribed to mRNA and then it is translated into the corresponding antigenic protein in the cytoplasm of the host cell, which is capable of initiating the immune response [35]. DNA vaccine offers several advantages over traditional vaccines. For example, DNA vaccine is easy and inexpensive to produce. DNA vaccine is safe (non-infectious) since it uses only enough genes from the pathogens that encode the particular proteins for a protective or therapeutic immune response, without introducing unnecessary proteins. DNA vaccine is stable at ambient temperature in dry or in an aqueous solution that makes it easy to ship to less developed areas in the world [36].

2-Viral and non-viral vaccine based carrier

DNA vaccine is similar to gene therapy for the need of a carrier or vector to deliver the exogenous genes *in vivo*. Generally large doses of DNA are needed to initiate immune responses, thus requiring advanced DNA vaccine delivery systems [37]. DNA vaccine could be delivered either by organisms or DNA based carriers.

Live or attenuated organisms as DNA vaccine vectors are either bacteria or viruses. The concept of bacterial vectors is based on the extension of using an attenuated pathogenic bacterium not only to produce DNA but also to directly deliver the DNA vaccine into the host cells. One important advantage of bacterial vectors is that the viable invasive bacteria have sophisticated systems that mediate their own

uptake into most of the cells [37]. Examples of bacterial vectors for DNA vaccine delivery are *Salmonella enterica* [38], *S typhimurium* [37], and *Listeria spp* [39]. Several viral vectors for DNA vaccines have been developed and the concept relies on the generation of recombinant viruses, containing recombinant viral genomes packaged into true virus particles [40]. Poxvirus, adenovirus, Alphavirus, and poliovirus are examples of gene-based vaccine vectors [41]. One important advantage for using viruses as vaccine vectors is the efficient delivery of DNA vaccine [41]. Despite extensive research efforts, use of viruses and bacteria as delivery systems for DNA vaccine has been hampered due to risk of side effects (e.g. risk of pathogenicity in immunocompromised humans) and lack of safety in humans [42, 43, 44].

To deliver DNA molecules into cells by non-organism based carriers, some facts about DNA molecules should be realized. DNA is a large molecule, which is not internalized by eukaryotic cells [46]. Therefore, it has to be condensed to a size that can be taken into cells. Also, the negative charges of the DNA have to be masked to allow specific uptake into cells. The advantages of using non-organism vectors (DNA-based carrier) are their ease of preparation, their flexibility regarding the size to be transferred and their safety profile. The main drawback of using these vectors is their lower efficiency compared to that of viral and bacterial vectors [46]. DNA-based carriers are mostly polymer-based carriers (bearing cationic polar head groups) that electrostatically interact with negatively charged phosphate backbones of DNA [47], which are capable of binding and condensing DNA. A summary of the polymer-based DNA delivery systems is presented in Table 1.1.

3-Lipoprotein as DNA carrier

Lipoproteins are naturally occurring biological oil-in-water emulsions and serve as carriers for cholesterol and other lipids in the systemic circulation. The first attempt to use lipoprotein as nonviral gene vector was by Hara, et al. (1997). Reconstituted chylomicron remnants (RCR) were used to deliver DNA to the liver since it is a favorable target organ for this kind of carriers [66]. Cationic lipids containing a quaternary amine head group were first complexed with negatively charged DNA. The resulting hydrophobic complex was incorporated into RCR. Injection of RCR into the portal vein of mice resulted in levels of transgene expression that was 100 fold higher than that of naked DNA. This finding suggested that usefulness of lipoprotein as DDS for gene therapy and in this case in treating liver diseases such as hepatoma, viral hepatitis, and other liver disorders.

In the same year (1997), Kim's group (Department of Pharmaceutical & Pharmaceutical Chemistry, University of Utah) published their first paper about the use of low-density lipoprotein (LDL) as a novel gene vector [68]. Their vector system was called "Terplex" and composed of LDL, stearyl-poly-L-lysine and DNA. Stearyl-PLL was incorporated into LDL particles and the positive charge on the surface of the polymer was able to condense DNA. An *in vitro* transfection study showed promising results [64] and *in vivo* studies demonstrated that the Terplex DNA system remained in the systemic circulation longer than naked DNA. Also, liver and heart were the major organs of transgene expression after intravenous injection in rats [69].

Recently, an artificial lipoprotein system was developed in our lab as a novel gene delivery system [66]. The artificial lipoprotein system mimics the natural lipoprotein in human body. It is composed of 70 % triglyceride (triolein), 25 %

phospholipid (22.7% egg yolk phosphatidylcholine and 2.3% lysophosphatidylcholine), 3% cholesterol ester (cholesterol oleate) and 2% cholesterol. The new delivery system was capable of transfecting human a glioma cell line *in vitro* with much lower toxicity compared to Lipofectamine ™. The cell viability after four days of transfection was 75 % and 24 % for p-PLL/ nanoemulsion/ DNA and Lipofectamine ™, respectively, indicating its lower cytotoxicity. The advantages of using artificial lipoprotein as a DNA vaccine vector are the following: (1) it is easy to produce since it is formulated from commercially available natural lipids, which reduce the cost and time of production. (2) It has low toxicity compared to cationic liposomes. (3) Varying the lipidized poly lysine and nanoemulsion ratio can optimize the size and charge of the overall complex. (4) Unlike natural LDL "Terplex system", artificial lipoprotein is biosafe since it is not a plasma product. (5) Targeting moieties, such as apolipoprotein B and transferrin, necessarily for gene delivery to specific cells can be potentially incorporated into the artificial lipoprotein. (6) The oily core of our system can be used as a reservoir for hydrophobic immune adjuvant to help the initiation of systemic immunization.

4- Rabies

Rabies is a serious disease that is caused by a virus. Each year, it kills more than 50,000 people and millions of animals around the world. Rabies virus infects the central nervous system, causing encephalopathy and ultimately death. Since 1885, people have been vaccinated against rabies virus by inactivated virus vaccine. Simple vaccine (derived from brain tissue of infected sheep or goat) is still used throughout the world due to its low cost in spite of its major side effects. The costly inactivated virus vaccine developed through growing virus in cell culture is used by most of the industrial countries. DNA vaccination utilizing the naked DNA strand has promising

immunization results in rabies [45]. It has been shown that the DNA vaccine is 100 percent effective against rabies in monkeys [48]. These findings indicated that DNA vaccines might have a promising future in human rabies immunization.

E) Objectives of this research

For the last few years, advanced drug delivery systems have been investigated to overcome the limitation of the conventional systems. Cancer chemotherapy and DNA-based vaccines have been identified as special areas of need for improved drug delivery. Lipoproteins as drug delivery systems have become an attractive area of research and they are considered excellent candidates as novel drug deliver systems. The usage of a lipoprotein as drug delivery system has been investigated in this research.

The following study objectives were pursued and will be elaborated in the following sections:

1. Development of cholesterol- based conjugates for target drug delivery
2. Synthesis, preformulation and liposomal formulation of cholesteryl carborane esters with various fatty chains.
3. Distribution of a cholesterol-based anticancer conjugate in the brain and its stability in cholesterol esterase and lipoprotein lipase media.
4. Density gradient separation of carborane-containing liposomes from LDL and detection by inductively coupled plasma spectrometry
5. Poly-peptide artificial lipoprotein as a novel DNA vaccine delivery system

In chapter 2, we have reviewed most of studies that have been conducted to develop new cholesteryl drug conjugates and it is considered the first review paper in

this area. This review paper highlights these studies and their potential medical applications. In chapter 3, new cholesterol-based compounds were synthesized which were designed for boron neutron capture therapy. Various liposomal formulations as well as the preformulation characterization of these new compounds were examined. In chapter 4, brain distribution profiles of one of these cholesterol-based compounds (BCH) in tumor-bearing rats were studied. Also, examination of BCH stability in simulated biological media containing cholesterol esterase and lipoprotein lipase enzymes was carried out. In chapter 5, utilization of Inductively Coupled Plasma Spectroscopy to analyze boron compounds in a liposomal formulation and biological samples is presented. In addition, this chapter contains a detailed method to separate liposome loaded with BCH from LDL using an ultracentrifuge. In chapter 6, a novel artificial lipoprotein system has been evaluated as DNA based vaccine delivery system. Factors affecting the transfection efficiency were studied, such as amount of lipidized surface protein. In addition, comparison study with A marketed trasfecting liposome was conducted.

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Figure 1.1 LDL particle

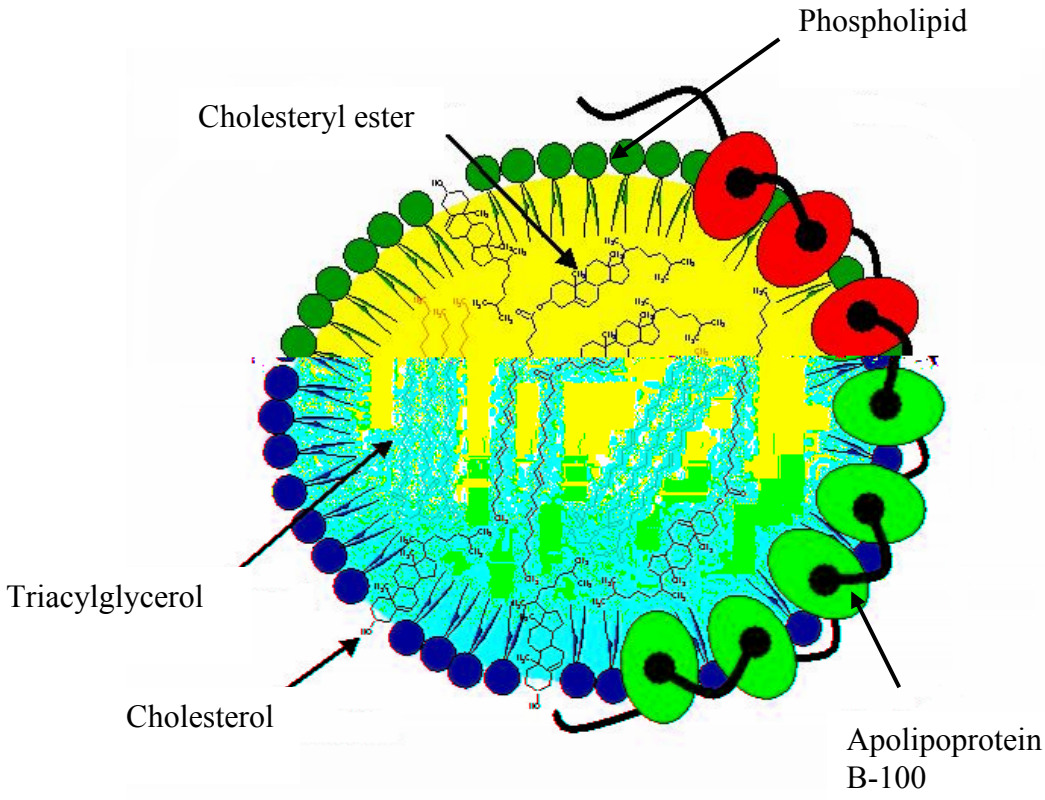
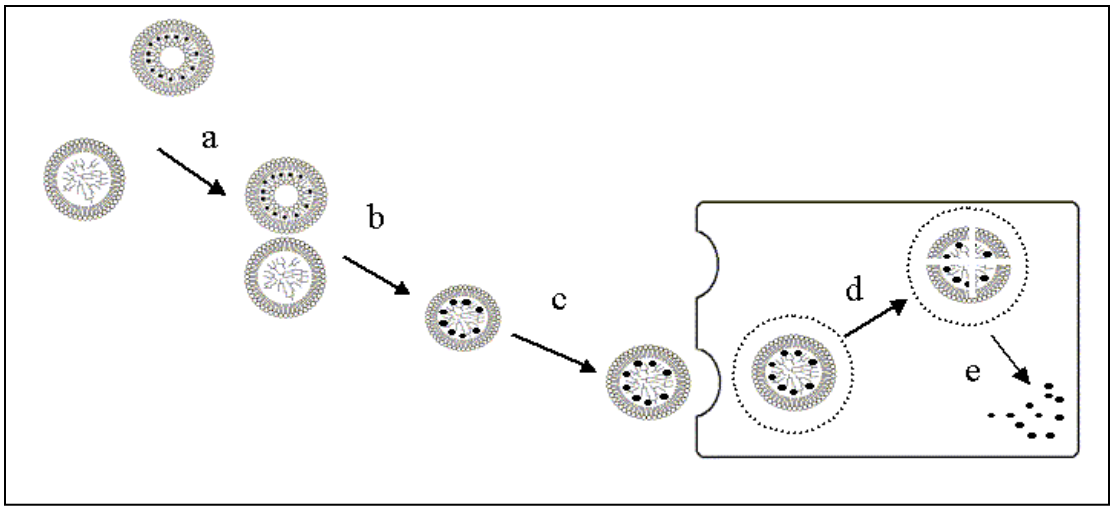


Figure 1.2 LDL pathway for anticancer-targeted delivery



a. Administration of liposomal cholesterol based compounds. Liposomes become unstable because of blood components. Drug becomes free from its vector (i.e. liposome)
b. Lipophilic drug will partitioning into LDL particle
c. LDL-cholesterol based compound complex will bind to LDL receptor and it enters tumor cell via LDL receptor mediated endocytosis.
d. Complex will transfer to endocytotic vesicles or endosomes.
e. Degradation of LDL vector and release of cholesterol based compound to act as cytotoxic molecules.

Figure 1.3 Liposome structure

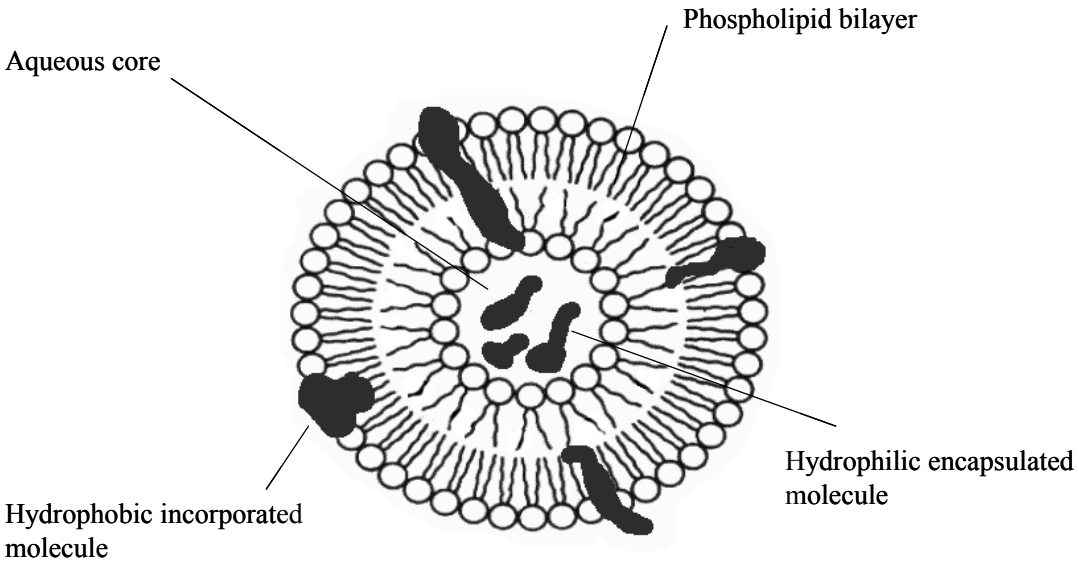


Figure 1.4 Boron Neutron Capture Therapy

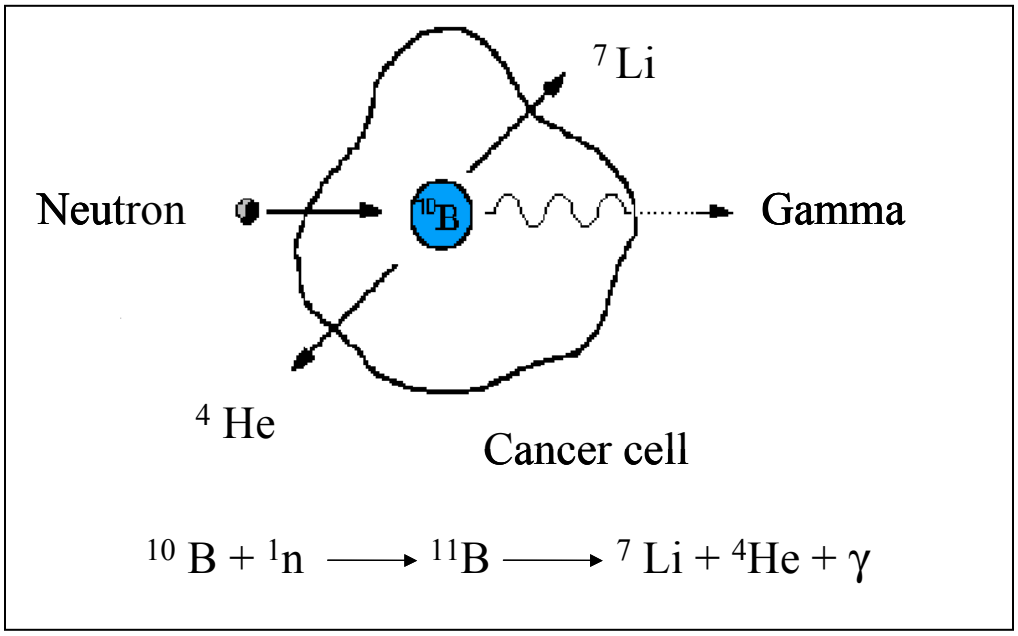


Table 1.1 Summary of Non-viral gene delivery systems.

Classification	Example	Note	Reference
Cationic polymer-based System	PLL (poly-L-lysine)	• Cationic polymer, condense DNA • Prone to aggregate at pH 7.4	49
	PLL-g-Dex	• Hydrophilic dextran increased PLL/ DNA complex solubility and thermal stability	50
	PEG-g-PLL	• Linking PEG reduced toxicity of PLL and enhanced transfection efficiency	51
	PEI (polyethyleneimine)	• Cationic polymer, condense DNA • Transfect cells *in vivo* and *in vitro* • Protect DNA from lysosomal degradation • Prone to aggregate at pH 7.4 • Toxic to cell	52
	PEGylated PEI	• Increase water solubility of DNA complex • Reduce the surface charge	53
	Chitosan	• Cationic polymer, condense DNA • Molecular weight of the polymer effect DNA interaction • Good candidate for transfecting gastrointestinal epithelia	54
	Dendrimers	• Example PAMAM, pDMAEM • Cationic polymer, condense DNA	55

Classification	Example	Note	Reference
Noncondensing polymer- based System	PVP (polyvinylpyrrolidone)	• Amphiphilic Molecule • Hydrophilic portion interacts with DNA via H-bond, van der waals and/ or ionic interaction. • Uptake by cells via hydrophobic interaction with cell membrane	56
Biodegradable polymer- based System	PAGA (poly (D, L-lactide-co-glycolide))	• A biodegradable analogue of PLL • Soluble in water. Less toxic and has higher transfection efficiency • Unstable due to hydrolysis of ester linkage in PAGA	57
	PHP ester (poly (4-hydroxy-1-proline ester)	• Biodegradable cationic polymer • Have lower cytoxicity	58
Liposome	Cationic liposome	• Cationic lipids such as DOTMA, DC-Chol and DOSPA • Form large size complex • DNA is not well condense	59
	Neutral liposome	• Entrapped DNA in the aqueous compartment of liposome	60
	Lipopolyplex-based system	• PLL used to precondense DNA and to form less complex size with DNA • Protamine and lipopoly-Llysine are also used with liposome	61

Classification	Example	Note	Reference
Emulsion based gene delivery system		• Cationic lipid emulsions • Concentration of non-ionic surfactant effects size and transfection efficiency • Oil component in o/w emulsion is effecting emulsion as gene delivery systems	62,63
Lipoprotein based gene delivery system	Terplex system	• Composed of LDL, stearyl-poly-L-lysine and DNA • Tested *in vitro* and *in vivo*	64
	chylomicron remnant particles	• Cationic lipid / DNA hydrophobic complex was incorporated into the oily core of RCR	65
	Artificial lipoprotein	• Composed of palmitoyl-PLL, nanoemulsion, DNA • Less toxicity than cationic liposome	66
Microspheres	PLGA and chitosan microspheres	• Aiming to sustained and high protein production	67

CHAPTER 2

DEVELOPMENT OF CHOLESTEROL-BASED CONJUGATES FOR TARGETED DRUG DELIVERY [1]

[1] Alanazi, F., Halpern, D. S., and Lu, D. R. 2003 STP Pharmaceutical Sciences, 13, 27-35. Reprinted here with permission of publisher

Abstract

Cholesterol-based drug conjugates have a good potential for targeted delivery of therapeutic agents to various disease sites. Many studies have been conducted to develop new cholesteryl drug conjugates for the purpose of diagnosis or treatment of diseases related to liver, adrenals, ovaries, atherosclerotic lesion, viral infections and tumors. This review paper highlights these studies and their potential medical applications. The chemical structures of the new cholesteryl drug conjugates are also reviewed to provide useful information for the further development in this area.

Keywords: Drug targeting, drug design, diagnosis, virus, tumor

I. Introduction

The development of new therapeutic compounds that are the derivatives of naturally occurring biomolecules has become one of principal approaches in pharmaceutical chemistry. An extremely successful example is the development of new antiviral compounds, such as Zidovudine (AZT) and Didanosine (DDI), based on the structure of nucleoside. Many studies have also been conducted to search new wonder drugs based on the structure of other biomolecules; one of these biomolecules is cholesterol. Cholesterol is an important substance that is widely distributed throughout the body, either in its free form or in its fatty acid ester forms. The conjugation of cholesterol with various medicinal agents can provide several advantages to enhance disease targeting capability and therapeutic efficacy. The aim of this review paper is to highlight the research work carried out by many different laboratories in developing cholesterol-based drug conjugates for targeted delivery.

II. Cholesterol and its biological properties

Cholesterol is a hydrophobic compound found in the tissues of all vertebrates but seldom in significant amounts in higher plants. It is a well-known member of the sterols, a biologically important group of lipid alcohols. The chemical structure of cholesterol can be seen in Figure 2.1-A, which consists of aliphatic side chain attached to a steroid nucleus with a hydroxyl group at position 3. In blood circulation, it is transported mainly as cholesteryl esters (Figure 2.1-B). Cholesterol is insoluble in water and has been widely used in pharmaceutical formulations as emollient agents, emulsifying agents and excipients for incorporation of drugs in oily formulations [1].

Cholesterol serves many biological functions. It is a major structural component of cell membranes [2]. The cholesterol content of cell membranes varies depending on cell types and specific membrane functions [3]. The ratio of cholesterol to phospholipid affects the stability, permeability, and protein mobility of cell membranes [4]. Membranes of intracellular organelles, such as mitochondria, have a low cholesterol ratio and are thus fluidic and permeable [3]. Cell membranes with a high cholesterol ratio, such as those in epidermis layer of the skin, have high stability and relatively low permeability that reflect their major functionality as a protective barrier [3, 5]. The outer membranes of most cells have an intermediate cholesterol/phospholipid ratio and possess both protective and metabolite-transport functions.

Distribution of cholesterol in human organs and tissues varies (Figure 2.2). Cholesterol is stocked up in the adrenals, testes, and ovaries as fatty acid esters that are used as chemical precursors to synthesize steroid hormones including sex hormones and adrenal corticoid hormones [6]. In the liver, cholesterol serves as the precursor for bile acids that are steroid carboxylic acids for emulsification of dietary lipids. Cholesterol is also linked with the amino acids, glycine or taurine, to form the bile salts [7].

The total amount of cholesterol bound to lipoproteins in the blood circulation is normally about 150-200 mg per 100 ml of serum. The body obtains cholesterol from two routes, either from dietary sources or from *de novo* biosynthesis. Under normal conditions, the total amount of cholesterol from these two sources remains constant because the rate of cholesterol synthesis in the liver is under a feedback control. When the dietary intake of cholesterol is high, its synthesis in liver remains low but the synthesis increases when the dietary intake is reduced.

The primary carriers for cholesterol transport in blood circulation are various lipoproteins. Dietary cholesterol is transported from the intestine to the liver within large lipoprotein particles (Chylomicrons). The liver secretes very low density lipoprotein (VLDL) containing cholesterol and cholesterol esters into the blood circulation. VLDL is partially converted to low density lipoprotein (LDL) through the action of endothelial cell associated lipoprotein lipase. LDL then serves as the major transport lipoprotein for cholesterol, supplying both free and esterified cholesterol to body tissues. High-density lipoprotein (HDL) acts as a cholesterol scavenger to transport cholesterol from various tissues back to the liver.

A simplified presentation of the cholesterol distribution pathway can be seen in Figure 2.3. Based on its wide distribution in the body, many laboratories have investigated the probability of using various cholesterol-based conjugates for targeted drug delivery. The work includes several targeting sites as indicated in Figure 2.3: liver, adrenals, ovaries, atherosclerotic lesions, viral infections and tumor cells.

III. Cholesteryl conjugates for diagnostic purpose

Early diagnosis of disease state can improve the results of treatment and decrease the risk of associated complications. Targeted delivery of imaging molecules to specific organs and tissues is essential for sensitive and accurate diagnosis. Cholesterol has been conjugated with imaging molecules for the detection of manifestations associated with adrenal glands, liver disease and atherosclerosis.

III.1. Adrenal glands

Adrenal scintigraphy generates useful information relating to the disease state of the adrenal cortex. It also permits the non-invasive diagnosis of steroid-processing related

diseases such as Cushing's syndrome and adrenal cortical lesions such as adrenal adenomas. Cholesterol is the chemical precursor from which all adrenal cortical steroid hormones are synthesized. Therefore, conjugation of cholesterol with an imaging agent provides a potential approach for adrenal visualization and examination of hormonal abnormalities. The imaging cholesteryl conjugates were designed to be administered into blood circulation and transported by lipoproteins similar to the native cholesterol molecules. They were internalized to adrenocortical cells via the binding to low density lipoprotein receptors.

The first cholesteryl conjugate for human adrenal imaging was the gamma emitting agent ^{131}I-19-iodocholesterol (NM-145, Figure 2.4-A) [8]. This compound has been used to visualize abnormal adrenal functions [9,10,11]. 6β-^{131}I iodomethyl-19-norcholest-5 (10)-en-3 β-ol (NP-59, Figure 2.4-B) was also reported as the imaging agent designed to target the steroid secreting adrenal cortex [12,13,14]. In medical application, NM-145 and NP-59 may be use for distinguishing between adrenal producing adenoma (APA) and bilateral adrenal hyperplsia (BHA). The differentiation provides an important indicator for selection of various treatment options. After the agents are taken up by adrenal gland, "hot" areas can be visualized indicating the increased uptake. The visualization may be unilateral or bilateral for APA and BAH, respectively [15].

A cholesteryl conjugate with radioactive selenium was also developed. The compound, ^{37}Se-selenomethyl-norcholesterol (Scintadren, Figure 2.4-C), was reported to examine adrenal disorders [16,17,18]. Selenium, as the imaging moiety conjugated to cholesterol, had an advantage over iodine since the thyroid uptake was not a serious concern [19]. Cholesteryl-p-[^{18}F] fluorobenzoate ([^{18}F] CFB, Figure 2.4-D) was also

shown promise as an imaging agent for adrenal disorders [20]. The considerations for designing cholesteryl conjugates as adrenal imaging agents involve several factors. First, the conjugates need to be selectively accumulated in adrenal cortex compared to other organs. Second, the accumulation has to be rapid for patient convenience and should have lower toxicity. Third, these compounds should be chemically stable with respect to resistance to *in vivo* hydrolysis since majority of them are the cholesteryl conjugates attached to imaging moiety through an ester bond. Linking a bulky group to the carbonyl group of ester bond was found to make the conjugate more stable in biological fluids [21].

III.2. Liver

It has been shown that lack of hydrophobicity and lack of hepatic tissue targeting capability are the main limitations for effectively using various contrast agents for the detection of liver lesions by computed tomography (CT) [22]. Liver plays an important role in lipid metabolism and linking an imaging agent to cholesterol may facilitate the radioimaging agent to be taken up by the liver [23]. Cholesteryl iopanoate (Figure 2.4-E) was evaluated as a potential imaging agent in hepatic CT [22,24]. After intravenous injection of cholesteryl iopanoate to rabbits with hepatic adenocarcinoma, CT imaging of tumors was examined and the result showed the successful detection of tumors as small as 2 mm in diameter [25].

III.3. Atherosclerosis

Atherosclerosis affects the coronary arteries and may lead to heart attack, congestive heart failure and arrhythmias [26,27]. Its early treatment with drugs and controlled diet is generally successful, while in the late stage, surgical intervention is

required [28]. Development of non-invasive detection methods for atherosclerosis at its early stage thus becomes critical.

An atherosclerotic lesion begins when the endothelium is injured which leads to various responses by the endothelium. One of the responses involves the releasing of a variety of cytokines, which are chemotactic for monocytes [29]. Cytokines stimulate monocytes to concentrate around the lesion area and to ingest modified LDL (oxidized LDL). The cholesterol-rich monocytes and macrophages are called foam cells [30]. The monocyte can ingest a significant amount of LDL resulting in rupture of the cell membrane and release of free cholesterol, cytokines, and procoagulants into the surrounding tissue. This process leads to the atherosclerotic plaque, which consists of a mass of cholesterol-rich monocytes covered by a fibrous cap. They are pushed out into the vessel lumen by smooth muscle cells [31]. The atherosclerotic plaque may grow slowly and may produce a severe stenosis or total arterial occlusion. Significant accumulation of LDL in arterial wall is a sign of atherosclerosis [32]. Many attempts have been made in various laboratories to utilize this fact for atherosclerosis diagnosis.

Radiolabeled LDL has been examined for detection of atherosclerosis [33,34,35,36,37,38]. Lupattelli et al. (1999) have evaluated the use of ^{131}I-labeled low-density lipoprotein in ten male patients for early detection of atherosclerosis. The result indicated that seven out of the ten patients showed hot spots in the areas with early atherosclerosis [39]. The utilization of radiolabeled LDL, however, has several drawbacks including false positive results [40] and rapid degradation in the targeted cells [41]. Since cholesterol (in its ester forms) is the native component of LDL, conjugation of radioimaging agents with cholesterol facilitates the loading of imaging agents into LDL.

Xiao et al. (1999) have examined cholesteryl iopanoate (^{125}I-CI) as an imaging agent and found that it was efficiently loaded into acetylated low density lipoprotein (AcLDL) *in vitro* (41). Intravenous injection of the cholesteryl iopanoate/AcLDL preparation in rabbits that developed early atherosclerosis showed accumulation of ^{125}I-CI in the atherosclerotic lesion (Aorta). The radioactivity in the atherosclerotic lesion was eight times higher than in the normal regions, indicating the selectivity of the uptake and the potential in early detection of atherosclerosis using a radiolabeled cholesteryl conjugate.

These studies have shown the possibility of using various cholesteryl conjugates for diagnosis of ailments in adrenal glands, liver and atherosclerosis. These conjugate compounds are shown in Figure 2.4. Chemically, the imaging units are generally linked to cholesterol through an ester bond, either at position 3, 6 or 19 (Figure 2.4).

IV. Cholesteryl conjugates as antiviral compounds

One of the major limitations with some antiviral agents is low antiviral efficacy due to poor penetrability through cell membranes and difficulty in reaching viruses in various cells [42]. For example, the antisense activity of oligonucleotides as potential antiviral agents often suffers from two obstacles: poor penetration into the living cells and chemical instability in biological fluids [43]. Therefore, development of new derivatives of the existing antiviral agents may overcome some of their weakness and enhance their efficacy for antiviral therapy.

Conjugation of antiviral agents to cholesterol, for their increased antiviral activity, has been reported by many laboratories. 3′-deoxyadenosine (cordycepine, Figure 2.5-A) is an extract from *cordyceps militaris* and its d3 trimer core (A2′ P5′ A2′ P5′ A) shows inhibitory activity for reverse transcriptase [44]. The 2′-O- and 5′-O-cholesterol

conjugates of (2′-5′) d3 (A-A-A) exhibited a 1000 fold increase in its anti-HIV-1 activity. The increase in antiviral activity appeared attributed to the increase in cellular uptake of the cholesteryl derivatives via membrane fluidization or receptor-mediated endocytosis [45]. Letsinger et al. (1989) has shown that conjugation of cholesterol at the 3′-terminal of oligonucleotide enhanced the antiviral activity significantly. Linking another cholesteryl moiety at the other end of the oligonucleotide, however, did not enhance the activity [46,47]. Mohan et al. (1994) has shown that 4-amino-5-hydroxy-2, 7-naphthalenedisulfonic acid was not active in inhibiting HIV–cytopathogenesis and reverse transcriptase (RT), while its cholesteryl conjugate (Figure 2.5-B) exhibited significant inhibitory activity. It has been suggested that the cholesteryl group interacts with the hydrophobic core of the enzyme and the naphthalenesulfonic acid is responsible for chelating with the metal ions that are essential for the catalytic activity of the RT enzyme [48]. Galabov et al. (1998) has shown that a cholesteryl ester of cinnamic acid (Figure 2.5-C) had promising antiviral activity [49]. Sergheraert et al. (1993) has shown that the cholesteryl D4T phosphate derivative (Figure 2.5-D) not only inhibited the replication of HIV in a lymphatic cell line (CEM-CI13) more effectively than the parent D4T compound but also had less toxicity [50]. Conjugation of cholesterol with 3′-azido-3′-deoxythymidine (AZT) (Figure 2.5-E) was achieved to specifically target macrophages infected with HIV via LDL or scavenger receptors. Also, It has been shown that cholesteryl AZT was successfully incorporated into LDL particle reaching about 200 cholesteryl AZT molecules per LDL particle [51].

The chemical structures of these cholesteryl conjugates used as antiviral agents can be seen in Figure 2.5. The cholesteryl conjugation in general occurs at position 3 of

cholesterol. The cholesteryl moiety plays a significant role in increasing the antiviral activity in part because the original compounds possess charged functional group and thus have difficulty in penetrating the cell membrane. The cholesteryl group provides the required lipiophilicty for cellular interaction and membrane penetration.

V. Cholesteryl conjugates for targeted drug delivery to cancer cells

Cholesterol is very important to mammalian cells since it is the major component for cell membrane construction. Cell proliferation and cholesterol demand are correlated [52]. Due to the high proliferation rate, cancer cells require and take up more cholesterol than the normal cells in order to build the cell membranes [53]. More than 90% of cholesterol is obtained through LDL receptor-mediated endocytosis [54]. LDL is the major carrier to transport cholesterol and cholesteryl esters to cells. It contains a significant amount of cholesterol either in the free form (25%) or in the ester form (75%). Approximately, there are 500 cholesterol molecules and 1500 cholesterol ester molecules per LDL particle. It is shown that the cholesterol level in many types of cancer in patients is low compare to normal patients [55,56] and the level declines in a first-order kinetics with the progression of disease [57]. Many cancer cells have been found to have elevated LDL receptor expression indicating the high demand of cholesterol for these types of cancer cells.

Ho et al. (1978) has observed that LDL receptor activity in leukemic cells was higher than that of mononuclear blood cells from healthy subjects [58]. Many other types of cancer cells including lung, brain, colon, and kidney show the elevation in LDL receptor expression and, thus, higher consumption of LDL [59, 60, 61, 62, 63]. The researchers at UCSF (Maletinska et al., 2000) have studied 9 human brain glioma cells

and found all of them have high expression of LDL receptor ranging from 128,000 to 950,000 per cell [62].

Fibroblasts cells were killed when exposed to LDL loaded with 25-hydroxycholesteryl oleate, a potent suppressor of HMG-CoA reductase [64]. Many studies have also been conducted to use drug-loaded LDL for targeted drug delivery to cancer cells [54, 65, 66, 67, 68, 69, 70, 71]. These approaches, however, require the pre-loading of LDL with anticancer drugs and the subsequent intravenous injection of the LDL/drug complex. It is also not practical to administer the complex for a long period of time because of the associated high cost and time-consuming preparation. Since cholesterol is the native substrate for LDL, it becomes attractive to develop cholesteryl drug conjugates that can be transferred into LDL after the administration into blood circulation. Such a approach allows the multiple dosing of cholesteryl drug conjugates with conventional pharmaceutical formulations and also allows a larger amount of drug administration.

Firestone et al. (1984) linked nitrogen mustards to cholesterol (Figure 2.6-A) and showed that the conjugation could be used to successfully deliver anticancer drugs through LDL pathway [72]. Halbert et al. (1984) coupled methotrexate to cholesterol as a methotrexate-α-benzyl-γ-cholesteryl diester (Figure 2.6-B) and the conjugate showed the cytotoxicity after loading in a microemulsion *in vitro* [73]. Versluis et al. (1998) synthesized a daunorubicin cholesteryl derivative (Figure 2.6-C) [74] and showed that the liver uptake of this conjugate was 5 fold more in rats with upregulated hepatic LDL receptor expression than that of normal ones [75]. Chlorambucil was conjugated to cholesterol and was examined for its distribution from micelles to LDL [76].

Other efforts have also been made to increase the activity of anticancer compounds by conjugation with the cholesterol moiety for enhancing the lipophilicity. Salomone et al (2001) demonstrated that cholesteryl butyrate (Figure 2.6-D), as a lipid matrix of solid lipid nanospheres, had more anti-proliferation activity than Na-butyrate on melanoma cells [77]. Cyano-containing 5,6-*seco*-cholesteryl derivatives (Figure 2.6-E) were synthesized as novel Cdc25A protein phosphatase inhibitors [78] and the presence of a cholesteryl group significantly contributes to the associated anticancer activity [79,80].

In our laboratory, cholesteryl carborane conjugates were synthesized to mimic native cholesterol esters as novel anticancer compounds [81, 82]. As these compounds share similar chemical and physical characteristics with the native cholesteryl esters, they may be effectively transferred into LDL in biological fluids and, thus, follow the LDL pathway for targeted delivery of tumor-destroying capacity utilizing the elevated LDL receptor expression on tumor cells. Correspondingly, design and synthesis in our laboratory have focused on derivatives of cholesteryl esters containing an antitumor chemical unit. Carborane becomes a good candidate as the antitumor unit because its potential use for boron neutron capture therapy (BNCT) of tumors. Unlike the utilization of other prodrugs, BNCT does not require release of the antitumor unit in the cells, allowing us to concentrate on the study of targeting capability.

Cholesteryl carborane ester compounds that have been synthesized in our laboratory are shown in Figure 2.7. Among these compounds, we have extensively evaluated the cholesteryl 1, 12 –dicarba–closo–dodecaboranelcarboxylate (BCH) for its interaction with lipoproteins and its cellular uptake in two human brain glioma cell lines

(SF-763 and SF-767) and one human normal neuron cell line (HCN-1a) [83,84]. Because it is also essential for the compound to remain in the cells for a sufficient time period to allow the subsequent neutron radiation in BNCT, we further determined whether the compound was retained in the cells after the uptake.

The results from our experiments indicated that the cholesteryl carborane ester, BCH, carried by liposomes, resulted in more than adequate cellular uptake of BCH. The boron uptake was significantly higher than that required for successful BNCT. Specifically, for the SF-763 cells, the boron uptake reached 283.3 μg boron/g cells, about 11 times higher than the required boron level (≥ 20-25 μg boron per g cells). For SF-767 cells, the boron uptake reached 264.0 μg boron/g cells, about 10 times higher than the required boron level. The amount of BCH uptake by the two tumor cell lines was much more (up to 14 times more) than that by the normal neuron cell line ($p<0.05$). The cellular uptake of BCH by the SF-767 tumor cell line was also greatly affected by the absence of lipoprotein. In the presence of lipoprotein in culture medium, BCH uptake was 4 times as much as that in the absence of lipoprotein ($p<0.05$). These results indicated that the cellular uptake was associated with the rapidly growing rate of the tumor cells and the uptake process appeared related to the lipoprotein presence in the culture medium [83,84].

It has been reported that liposomes can interact with LDL in aqueous medium leading to the transfer of drug from liposomal formulation to LDL [85]. We have conducted an in-vitro transfer study to examine whether BCH was transferred from the liposomal formulation to LDL and whether there was an effect in the presence of other serum components. The results indicated that the presence of other serum components

significantly assisted the transfer of BCH from the liposomal formulation to LDL. The simple incubation of the liposomal formulation with LDL in phosphate-buffered saline, however, did not result in any significant transfer of BCH to LDL.

In addition to the requirement for the cells to obtain adequate amount of BCH, it is also essential for the boron compound to remain in the cells for a sufficient time period so that neutron radiation may be applied. The results from our retention experiment indicated that a significant amount of the BCH taken up in the human glioma cells was retained in the cells after the subsequent 24-hour incubation without the presence of BCH [83,84].

VI. Future prospective:

Conjugation of the cholesterol moiety to active medicinal compounds for either disease diagnosis or disease treatment is an attractive approach for targeted drug delivery. There are many additional areas in which this targeting tactic could be applied to improve the therapeutic efficacy and to reduce the toxicity to normal cells. For example, brain drug delivery presents a challenge due to the presence of blood brain barrier (BBB). Kyotorphin [86], niflumic acid [87], thyrotropin-releasing hormone (TRH) [88,89] and leucine-enkephalin [90] were victorised by cholesterol for brain delivery and the experimental results appeared promising. It is well known that LDL receptors are present on the BBB capillary endothelial cells and, therefore, they could be utilized for transporting cholesteryl-based or other compounds to the brain [91,92]. Furthermore, cholesterol accumulates in ovarian tissue and is used to synthesize sex hormones. Therefore, cholesteryl drug conjugates could also be used for drug targeting to ovary. Ades et al. (2001) showed that injection of labeled cholesteryl oleate in patients bearing

ovarian tumors resulted in eight-fold uptake by the malignant tumors compared to the contralateral normal ones [93].

Although many cholesteryl drug conjugates have been studied and promising results have been shown as indicated in this review article, further experiments are needed to illustrate the mechanism of targeted delivery. Since most of the cholesteryl drug conjugates are very hydrophobic, suitable pharmaceutical formulations are also required to enhance the solubility of these conjugates in blood circulation and to improve their interactions with lipoproteins and other functional proteins. Risks from the reaching of cholesteryl conjugates to non-target tissues and cells need to be investigated to fully assess the risk/benefit ratio. Since the liver most likely takes up significant amount of these cholesteryl conjugates, the associated pharmacological and toxic effects should be specifically examined. Literature data have indicated that local excess of cholesterol, the metabolic product of cholesteryl conjugates, can be cytotoxic. Kellner-Weibel et al. (1999) has shown that free cholesterol produced by hydrolysis of cytoplasmic cholesteryl ester was transported through acidic vesicles to the plasma membrane, and the build-up of free cholesterol in this pool caused cell death by necrosis and apoptosis [94].

The information related to biodistribution and pharmacokinetics of various cholesteryl drug conjugates is also essential for their practical use. When active drug molecules are conjugated to cholesterol, their properties, such as hydrophilicity/lipophilicity and molecular weight, are largely altered and consequently their biodistribution, pharmacokinetics as well as efficacy are significantly affected. De Smidt et al.(1991) has shown that conjugation of A 16-mer oligonucleotide (ODN) to cholesterol yielded a change in its in vivo distribution behaviors. The plasma half-life of

chol-ODN was 9-11 times greater than that of control ODN. The chol-ODN was found to bind quantitatively to both high density lipoproteins (HDL) and LDL, but not to albumin. On the contrary, control ODN did not have any affinity for plasma lipoproteins [95]. Therefore, the delivery mechanism of these new cholesteryl conjugates at cellular and molecular levels involving their interactions with proteins, cells, receptors and membranes becomes important and requires extensive investigation. Nevertheless, utilization of cholesteryl drug conjugates for targeted delivery provides a novel approach which has the potential to enhance their therapeutic efficacy and benefit the medical treatment of various human diseases.

IV. References

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Figures legends:

Figure 2.1 Structure of cholesterol (cholest-5-en-3β-ol, $C_{27}H_{46}O$) and cholesterol ester

Figure 2.2 Distribution of cholesterol in human organs and tissues

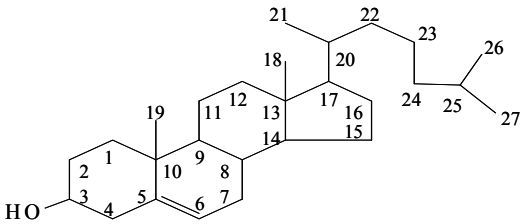
Figure 2.3 Cholesterol distribution pathway and the potential sites for drug targeting

Figure 2.4 Chemical structures of cholesteryl conjugates used for diagnosis. Structures A, B, C, D and E are referred as NM-145, NP-59, Scintadren, [^{18}F] CFB and cholesteryl iopanoate, respectively.

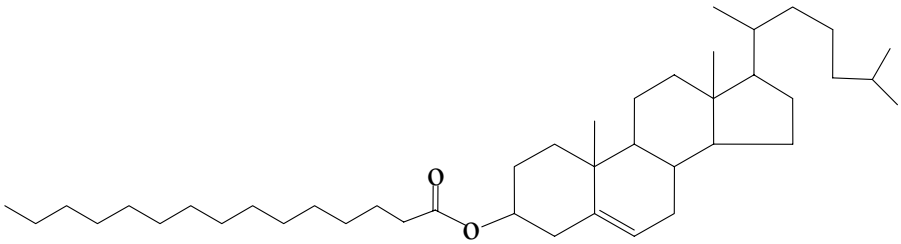
Figure 2.5 Chemical structures of cholesteryl conjugates used as antiviral compounds. Structures A, B, C, D and E are referred as cordycepine-cholesterol conjugate, cholesteryl naphthalenesulfonic acid derivative, cholesteryl ester of cinammic acid, cholesteryl D4T phosphate diester, and AZT-cholesterol derivative, respectively.

Figure 2.6 Chemical structures of cholesteryl conjugates used as anticancer compounds. Structures A, B, C, D, and E are referred as nitrogen mustards-cholesteryl conjugate, methotrexate-α-benzyl-γ-cholesteryl diester, daunorubicin cholesterol conjugate, cholesteryl butyrate, and K-o-cholestanyl xanthate (Cdc25A protein phosphatase inhibitor), respectively.

Figure 2.7 Chemical structures of cholesteryl carborane conjugates used as boron-containing anticancer compounds. Structures A, B, C, and D are referred as cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH), Cholesteryl-3β-12-octyl-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate, Cholesteryl-3β-12-(1-octenyl)-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate, Cholesteryl-3β-12-octadecyl-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate, respectively.



A



B

Figure 2.1

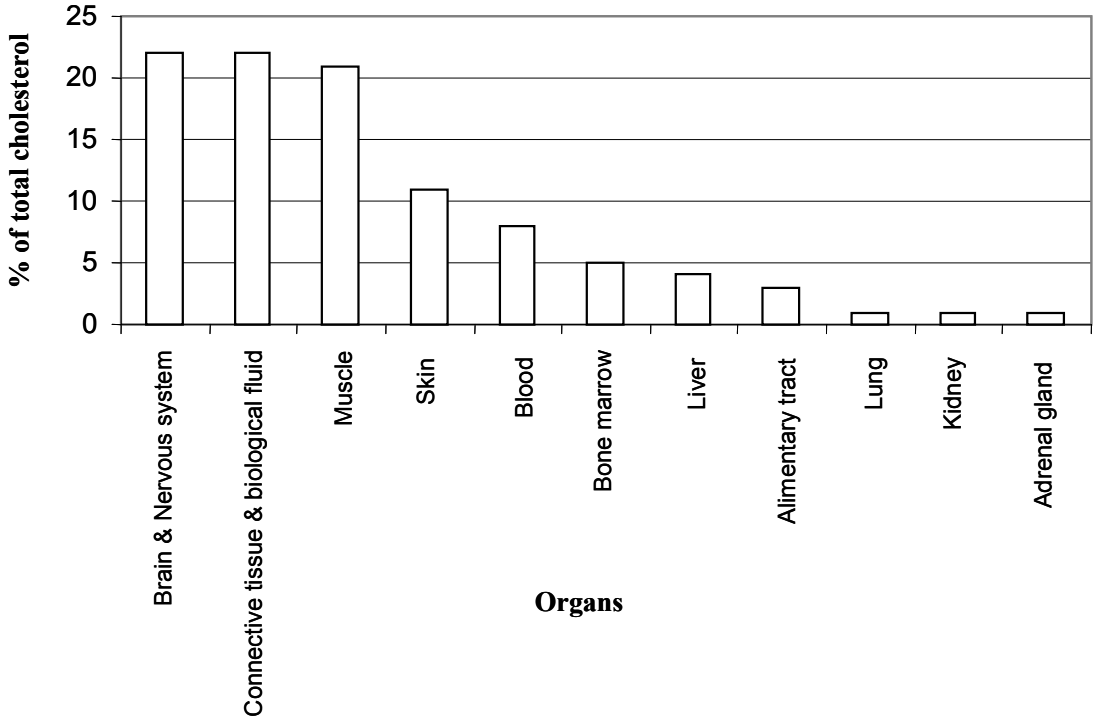

% of total cholesterol
25
20
15
10
5
0
Brain & Nervous system
Connective tissue & biological fluid
Muscle
Skin
Blood
Bone marrow
Liver
Alimentary tract
Lung
Kidney
Adrenal gland
Organs

Figure 2.2

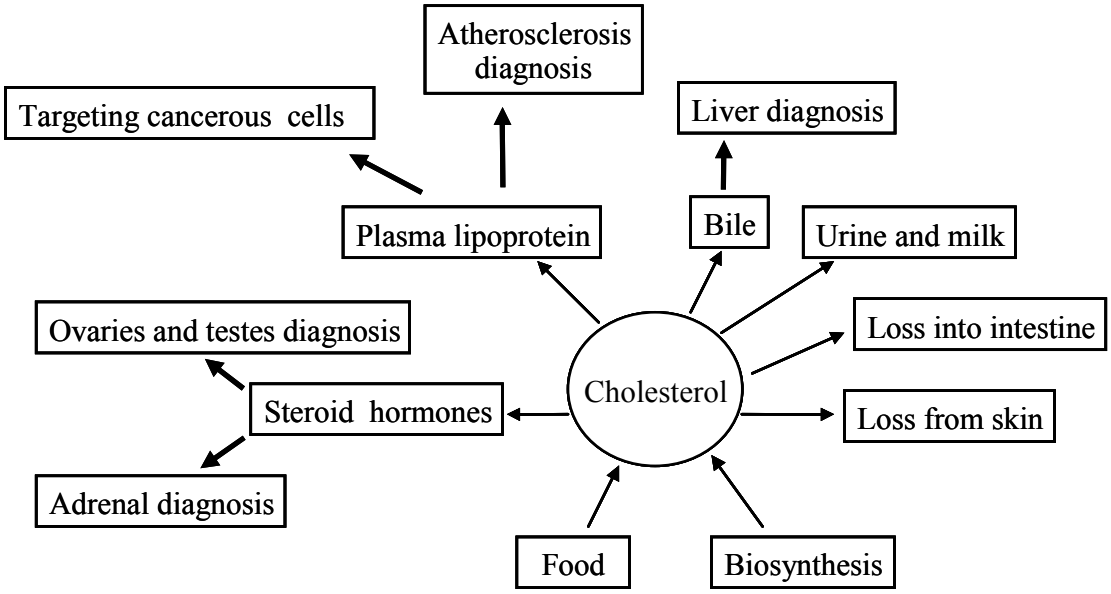
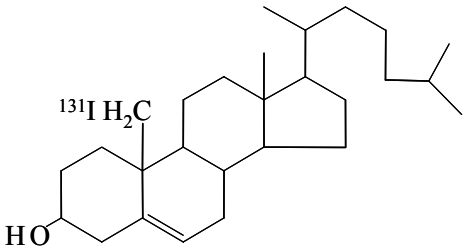
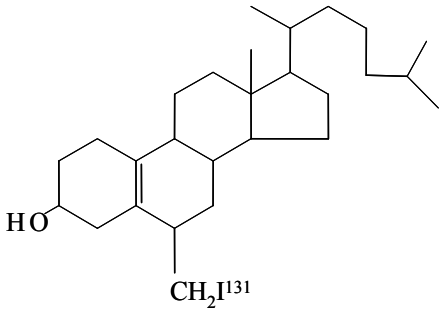

Atherosclerosis diagnosis
Targeting cancerous cells
Liver diagnosis
Plasma lipoprotein
Bile
Urine and milk
Ovaries and testes diagnosis
Loss into intestine
Cholesterol
Steroid hormones
Loss from skin
Adrenal diagnosis
Food
Biosynthesis

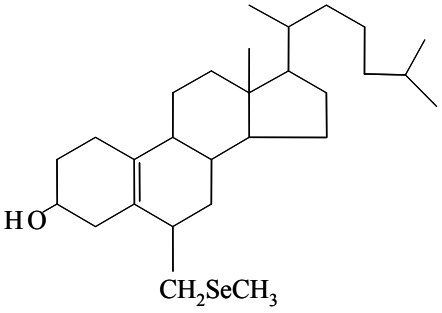
Figure 2.3



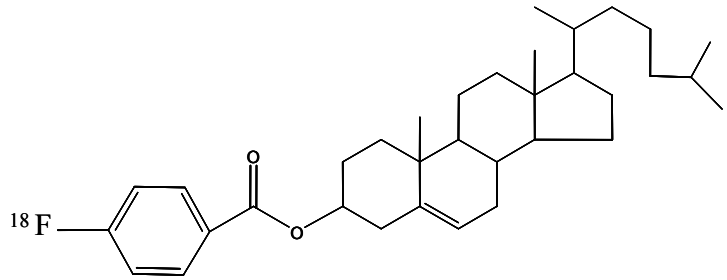
A



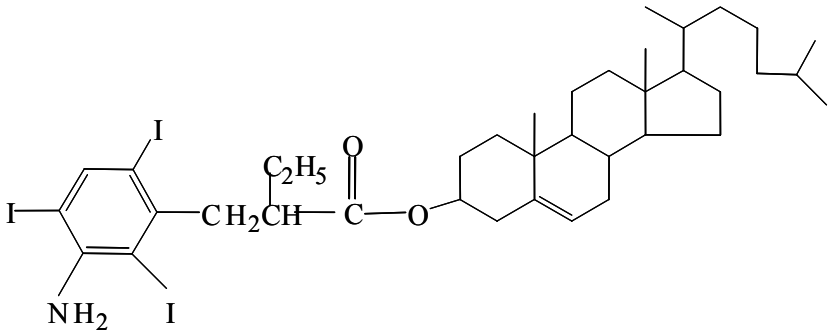
B



C

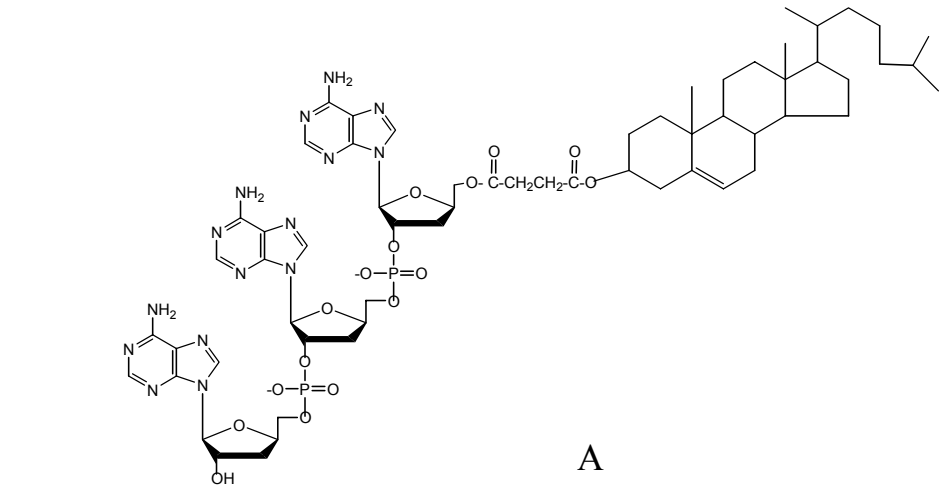


D

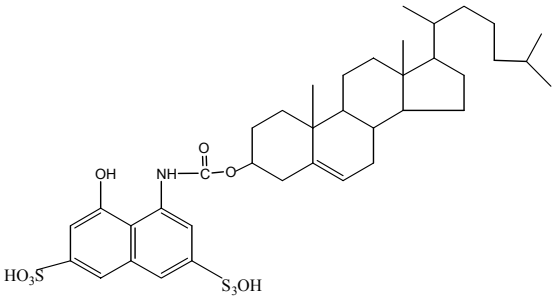


E

Figure 2.4



A



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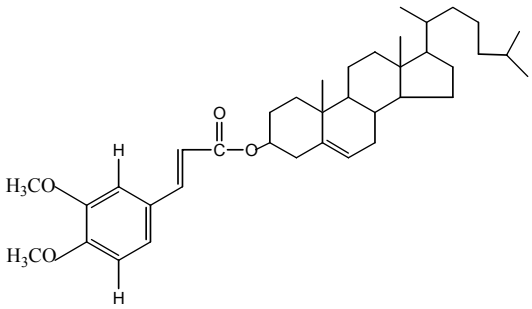
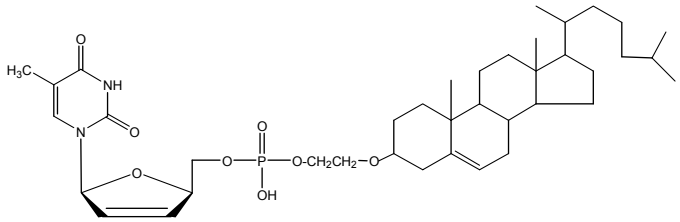


C

D



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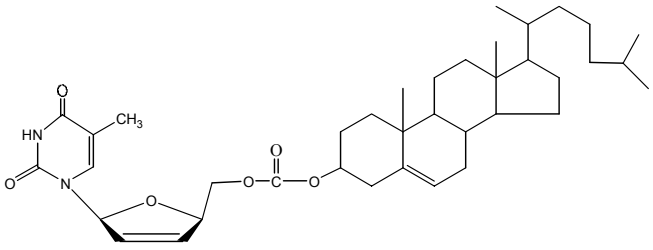

Figure 2.5

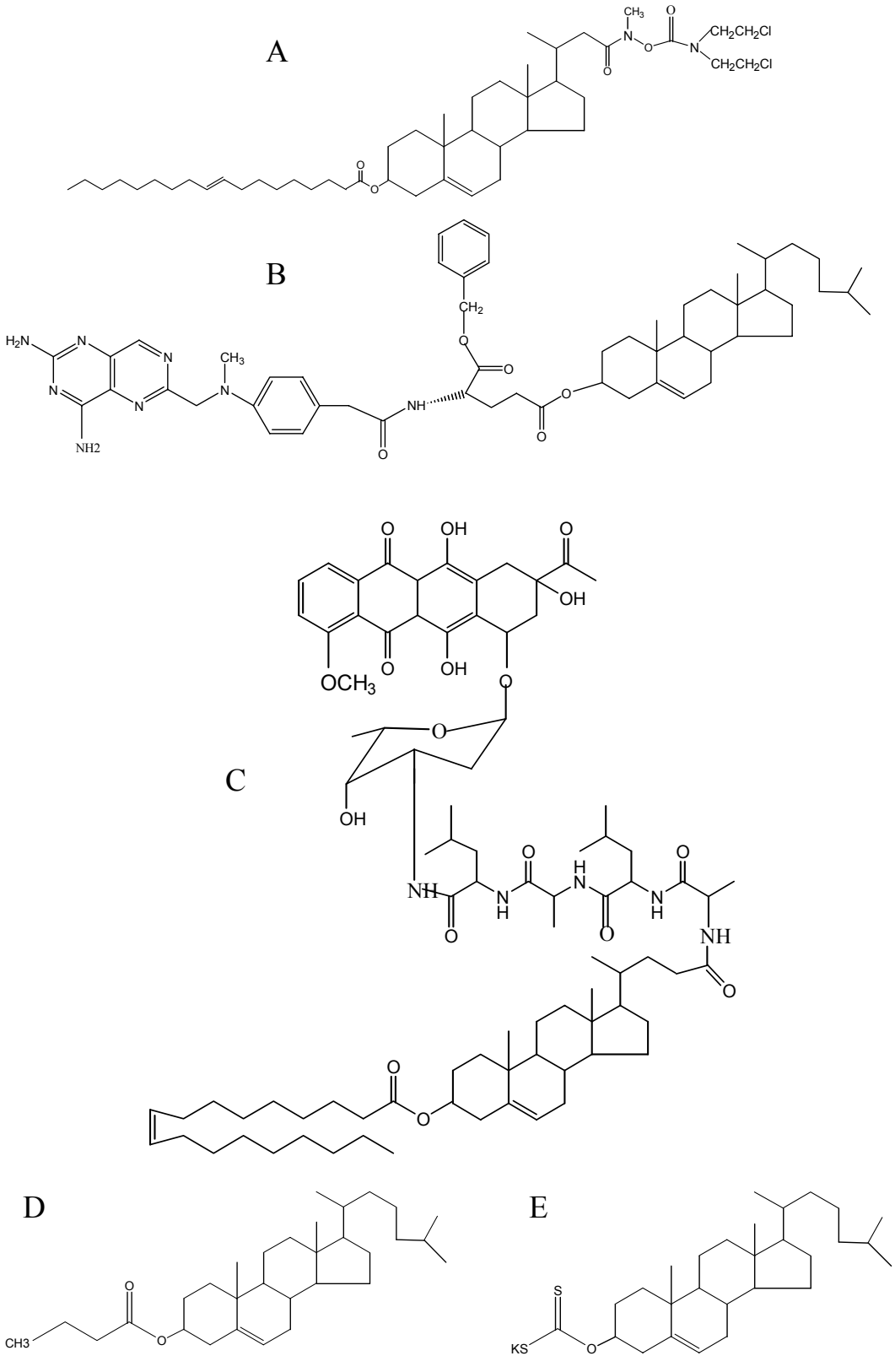

Figure 2.6

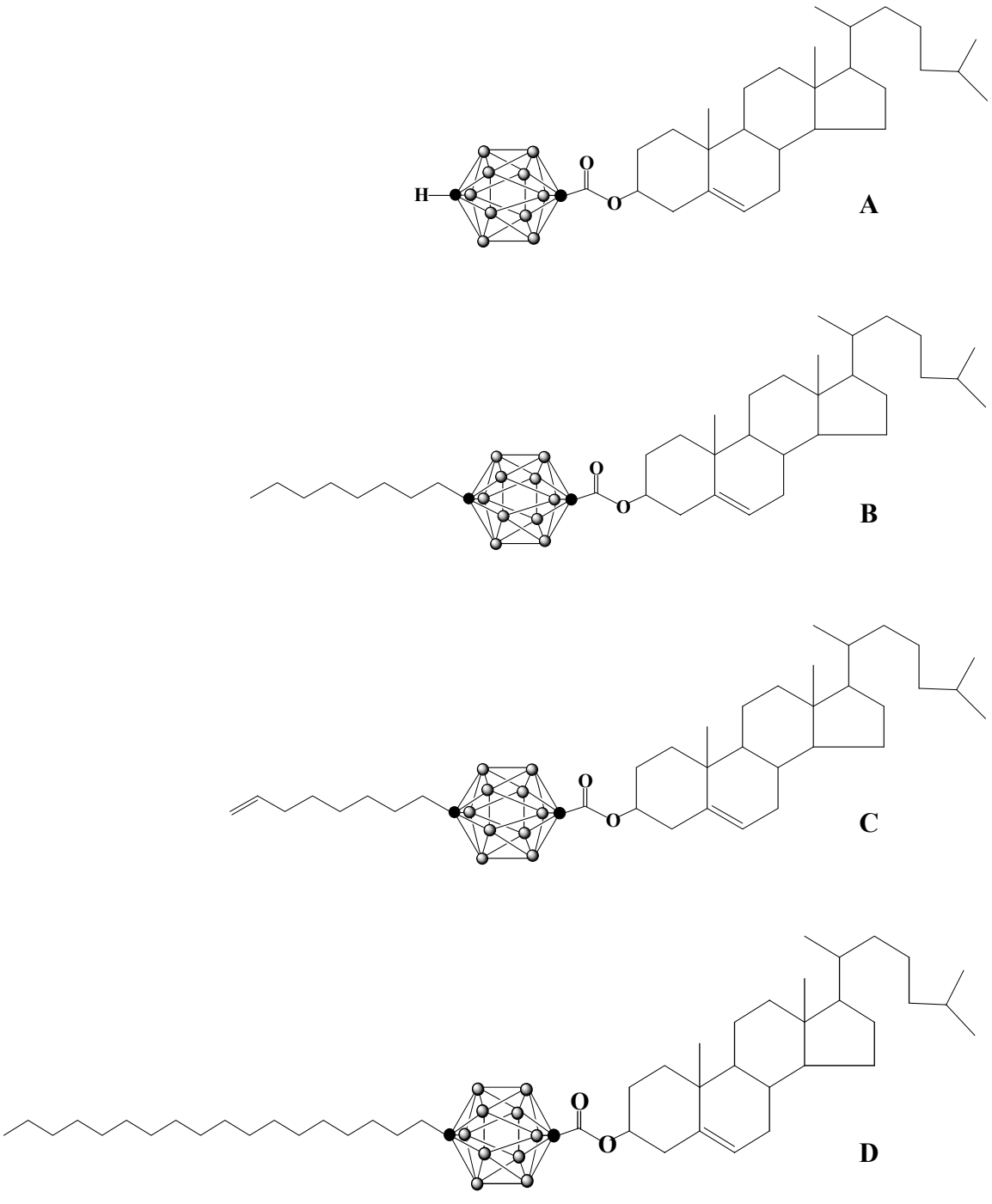

Figure 2.7

CHAPTER 3

SYNTHESIS, PREFORMULATION AND LIPOSOMAL FORMULATION OF CHOLESTERYL CARBORANE ESTERS WITH VARIOUS FATTY CHAINS [1]

[1] Alanazi, F., Li, H., Halpern, D.S., Øie, S. and Lu, D. R. 2003, International J. Pharmaceutics, 255, 189-197. Reprinted here with permission of publisher.

Abstract:

The elevated expression of LDL receptor on tumor cells provides one attractive approach for targeted drug delivery to tumor cells. Suitable antitumor compounds, however, need to be synthesized and developed which mimic the native cholesteryl esters (as major constituent of LDL) in chemical structure for targeted delivery to tumor cells through the over-expressed LDL receptors. In the present study, new antitumor compounds were designed containing cholesterol, fatty chain and carborane which is used as the antitumor unit. Three new compounds were synthesized with a three-step reaction scheme. Similar to the native cholesteryl esters, these compounds are extremely hydrophobic and, before any further biological studies, suitable liposomal formulations for these new compounds are required. Various liposomal formulations as well as the preformulation characterization of these new compounds were thus examined. The incorporation efficiency of the compounds in liposomes was found to vary significantly depending on the type of fatty chain attached and the ratio of cholesterol: phospholipid used as the excipients of liposomal formulation.

Keywords: Drug design; Drug targeting; Low density lipoprotein; Liposome; Cholesterol ester; BNCT

1. **Introduction:**

Targeted drug delivery to cancer cells has a great potential to enhance the chemotherapeutic efficacy for the treatment of human malignant cancers. One of the targeting strategies is based on the fact that rapidly dividing cancer cells have high cholesterol demand (Vitols, S., 1991; Polo et al., 2002; Sarkar et al., 2002). Cancer cells usually grow very aggressively and take up significant amounts of exogenous cholesterol (in ester form) for their proliferation and cell membrane construction. Many types of cancer cells show an elevation in LDL (low density lipoprotein) receptor expression and, thus, higher consumption of LDL (Gueddari et al., 1993; Firstone et al, 1994; Yen et al., 1995; Maletineska et al., 2000; Chen et al., 2001). LDL contains about 1500 molecules of cholesterol esters per LDL particle and functions as the main carrier of cholesteryl esters in blood circulation (Kostner et al., 1989). Therefore, the development of new anti-cancer compounds mimicking the native cholesterol esters presents a potentially effective approach for targeted drug delivery to cancer cells via the elevated LDL receptors (Versluis et al, 1998; Feakes et al, 1999; Ji and Lu, 2001; Sarkar et al., 2002).

The new compounds can be designed as derivatives of cholesterol esters containing an antitumor chemical unit. Carborane becomes a good candidate as the antitumor unit because its potential use for boron neutron capture therapy (BNCT) of tumors (Gutman, 2000; Ujvary et al., 2001; Tietze et al., 2002). Unlike the utilization of other prodrugs, BNCT does not require the release of the antitumor unit in cells. Figure 3.1. shows the general designing strategy for the new compounds which contain cholesterol, carborane and saturated or unsaturated fatty chains with various chain length. The chemical structures mimic native cholesterol esters in human (such as cholesteryl

esters of stearate and oleate) and the fatty chains are varied to compensate the addition of the antitumor carborane unit. As the new compounds share similar chemical and physical characteristics with native cholesteryl esters, they may interact well with LDL and, thus, utilize the elevated LDL receptor expression on cancer cells for targeted delivery.

Recently, we have synthesized a carborane cholesteryl ester. The compound, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH, see Figure 3.1, C), is extremely hydrophobic and thus was formulated in liposomes for cell culture studies. The cell culture results indicated that BCH, carried by liposomes, resulted in more than adequate cellular uptake. The boron uptake was about 10-11 times higher than the required boron level for successful BNCT (Peacock and Lu, 2001). In the present study, three new carborane-containing cholesterol ester mimics were synthesized. In chemical structure, these compounds are similar to BCH but include various fatty chains (octyl, octenyl and octadecayl) on the second carbon atom of the carborane cage. Para-carborane was used because it is boron-rich (containing 10 boron atoms) and it imposes less steric hindrance to further modification on the second carbon atom comparing to the meta or ortho isomers. Because these compounds are extremely hydrophobic, suitable liposomal formulations for these new compounds are required before any further biological studies. Therefore, we studied preformulation characteristics and liposomal formulations for these new compounds. The effect of different fatty chains (conjugated with the carborane cage) on the drug loading in liposomal formulation was evaluated. Since these new compounds are cholesterol derivatives, the amount of cholesterol used in the liposomal formulation was also varied to maximize the drug loading.

2. Material and methods:

2.1. *Reagents and methods*

P-carborane, butylithium, 1-bromooctane, 8-bromo-1-octene, 1-bromooctadecane, phosphomolobdic hydrate, 4-dimethylaminopyridine, chloroform-d and tetrahdrofuran anhydrous were purchased from Aldrich Chem. Co. (Milwaknkee, WI). Cholesterol, Dipalmitoyl DL-α-phosphotidylcholine (DPPC), and N, N-Dicyclohexylcarbodimide were purchased from Sigma Chemical Co. (St.Louis, MO).

All reactions used anhydrous solvents and were carried out under dry nitrogen atmosphere. Carbon dioxide (from CO_2 gas cylinder) was dried by bubbling through concentrated sulfuric acid. Chromatographic separations were performed using silica gel (60-200 Mesh) (J.T.Baker). Thin layer chromatography was performed on silica gel 60 F_{254} (E. M. Industries, Inc., Germany). Elemental analysis was provided by Atlantic Microlab (Norcross, GA). Mass spectrometry (ESI) was provided by Mass spectrometry facility in the University of Georgia (Athens, GA). NMR data were recorded on a Brucker-400 AMX spectrometer and the chemical shifts are reported in ppm (δ). Coupling constant were reported in hertz. The abbreviations used are as follows: s: singlet, d: doublet, t: triplet, m: multiplet, dd: double doublet, brs: broad singlet.

2.2. Synthesis procedure

2.2.1. *Syntheses of compound* Ia, Ib and Ic

An appropriate amount of p-carborane dissolved in dry tetrahydrofuran (THF, 30 ml) was treated with 1.6 M n-BuLi (equal mmol to p-carborane) at –78 ^{o}C and under positive flow of dry nitrogen. The mixture was stirred for one hour, warmed to room temperature, and stirred for additional 20 minutes. The mixture was cooled to –78 ^{o}C and

stirred for 15 minutes. 1-Bromooctane (for Ia), 8-Bromo-1-octene (for Ib) or 1-Bromooctadecane (for Ic) was added in equal mmol to p-carborane over period of 15 minutes to the reaction mixture. It was stirred for one hour, warmed up to room temperature, and stirred for additional two hours. The reaction mixture was diluted with ethyl acetate (25 ml), and quenched with 1 N HCl solution (25 ml). The aqueous layer was extracted with ethyl acetate (2 x 15 ml). The organic extracts were combined, washed with saturated Nacl solution, dried over Na_2SO_4, filtered and concentrated by rotor evaporator. The residues were purified on silica gel column chromatograph eluted with (0-20%) dichloromethane in petroleum ether to afford compounds Ia, Ib, Ic, containing octyl, octenyl and octadecayl group, respectively.

2.2.2. *Syntheses of compound* IIa, IIb, and IIc

Compound I (in appropriate amount) was dissolved in dry THF (25 ml). The solution was cooled to –78 ^oC and n-BuLi (1.6 M in hexane, equal mmol to compounds I) was added in dropwise. The mixture was stirred for one hour, warmed up to room temperature, and stirred for additional one hour. It was, then, cooled to –78 ^oC and dry carbon dioxide was bubbled through the mixture for 2 hours. The mixture was warmed up to room temperature and stirred overnight while keeping CO_2 bubbling through it. The reaction mixture was diluted with ethyl acetate (25 ml) and quenched with 1 N HCl (15 ml). The aqueous layer was extracted with ethyl acetate (2 x 20 ml). The organic extracts were combined, washed with saturated NaCl, dried over Na_2SO_4, filtered and concentrated by rotor evaporator. The residues were purified on silica gel column chromatography eluted with petroleum ether to afford compounds IIa, IIb, IIc, containing octyl, octenyl and octadecayl group, respectively.

2.2.3. *Syntheses of compound* IIIa, IIIb, and IIIc

Compound II (an appropriate amount) and cholesterol (equal mmol to compound II) were dissolved in Dichloromethane (35 ml). 4-DMAP (5-10% of compound II in weight) was added. The reaction mixture was stirred for one hour, added with N, N-Dicyclohexylcarbodimide (equal mmol to compound II), and stirred for 12 hours at room temperature. The reaction mixture was concentrated, filtered, and purified on silica gel column chromatography eluted with petroleum ether to afford compounds IIIa, IIIb, and IIIc, containing octyl, octenyl and octadecayl group, respectively. The products were recrystalized using acetone and dichloromethane.

2.3. Preformulation characterization of the new cholesteryl carborane esters

Melting Point was measured using differential scanning calorimeter (Perkin Elmer Corporation, Norwalk, CT). Optical and polarizing microscopes were used to observe the crystalline status of the new compounds. Solubility of the new compounds in deionized water was conducted at 25 ^{o}C and inductively coupled plasma mass spectrometry was used for analysis.

2.4. Incorporation of cholesteryl carborane esters in liposomal formulations

Multilamellar liposomes (MLV's) were prepared by thin film hydration method. Cholesterol, phospholipid and the new compounds were dissolved in a 2:1 mixture of chloroform and methanol in a round bottom flask. The new compounds to lipid ratio was 1:50 (w/w) and total lipid (cholesterol and phospholipid) to water ratio was 1:38 (w/w). Cholesterol and phospholipid were used in three different molar ratios (0:1, 0.33:1, 1:1). The solvent mixture was evaporated under reduced pressure and a thin film of lipid was formed on the bottom of the flask. The film was hydrated at a temperature above the

phase transition temperature of the lipid using deionized water and glass beads (5 mm diameter) were added to the dry lipid film to ease the dissolution. The hydration process was performed for several hours using a shaking water bath. Liposomes were separated from the glass beads by filtration through a Buchner funnel. The liposomal suspension was centrifuged at 30,000x g for 45 min at 4 ^{o}C and the amount of the boron content in liposomal suspension and supernatant was measured by ICP-AES (Inductively coupled plasma atomic emission spectroscopy). Phosphorus content was also obtained by ICP-AES and phospholipid content of the liposomal formulations was calculated accordingly. The percentage of the new compounds incorporated in liposomes (the incorporation efficiency or I.E.) was calculated based on boron concentration and phospholipid content.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was performed using Statgraphics plus 2 software to compare the mean values of % incorporation efficiency for the three different levels of ratio (0:1, 0.33:1, 1:1). Multiple Range Test (Fisher's least significant difference procedure, LSD) was used to determine which means are significantly different from others.

3. Results:

Three new carborane conjugates (IIIa, IIIb, IIIc) have been synthesized with a three-step procedure, which is depicted in scheme 3.1. In the first step, mono-substitution reactions were achieved by coupling p-carborane with the different fatty chains. The overall yield of the reactions was about 80%. Verification of the proposed structures was performed using thin layer chromatography (TLC) and proton nuclear magnetic spectroscopy (NMR). The results were as following: **1-octyl-1, 12-dicarbo-*closo*-**

dodecaorane (Ia): ^{1}H NMR ($CDCL_3$, 400Hz) δ 2.75 (s, 1H, B-CH), 2.61-1.38 (m, 10H, B-H), 1.35-1.1 (m, 14H, CH_2), 0.86 (t, 3H,J=7.31Hz).

1-(7-octenyl)- 1,12-dicarbo-*closo*-dodecaorane (Ib): ^{1}H NMR (CDCL3, 400Hz) δ 5.76 (m, 1H, CH_2= $\underline{CH}_{1)}$, 4.96 (dd, 1H, $\underline{CH_2}$= $CH_{1,}$ J=1.46 Hz, J=14.17 Hz), 4.91 (dd, 1H, $\underline{CH_2}$= $CH_{1,}$ J=1.05 Hz, J=9.12 Hz), 2.74 (s, 1H, B- CH_1), 2.65-1.4 (m, 10H,B-H), 1.40-1.01 (m, 12H, CH_2).

1-octadecyl-1, 12-dicarbo-*closo*-dodecaorane (Ic): ^{1}H NMR (CDCL3, 400Hz) δ 2.75 (s, 1H, B-CH), 2.65-1.52 (m, 10H, B-H), 1.43- 1.03 (m, 34H, CH_2), 0.88 (t, 3H, J=6.62 Hz).

In the second step, compounds I were carboxylated with CO_2 to afford the carboxylic acid derivatives, the intermediate of the target compounds. The overall yield was about 50%. Verification of the proposed structures was performed using thin layer chromatography, proton nuclear magnetic spectroscopy and mass spectroscopy. The results were as following: **12-octyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylic acid (IIa):** ^{1}H NMR (CDCL3, 400Hz) δ 9.01 (brs, 1H, COOH), 3.1-1.4 (m, 10 H, B-H), 1.3-0.9 (m, 14H, CH_2), 0.86 (t, 3H, J=7.3 1Hz). MS (ESI) m/z calcd for $C_{11}H_{28}$ B_{10} O_2 [M^+]: 300, found [M^+]: 300; [M^+ -1-CO_2]: 255; [$2M^+$]: 600.

12-(1-octenyl)- 1,12-dicarbo-*closo*-dodecaorane-carboxylic acid (IIb): ^{1}H NMR (CDCL3, 400Hz) δ 8.5 (brs, 1H, COOH), 5.78 (m, 1H, CH_2= $\underline{CH}_1$), 4.97 (dd, 1H, $\underline{CH_2}$= $CH_{1,}$ J=1.69 Hz, J=15.48 Hz), 4.92 (dd, 1H, $\underline{CH_2}$= $CH_{1,}$ J=0.84 Hz, J=9.33 Hz), 3.1-1.5 (m, 10 H, B-H), 1.4-0.75 (m, 12H, CH_2). MS (ESI) m/z calcd for $C_{11}H_{26}$ B_{10} O_2 [M^+]: 298, found [M^+]: 298, [$2M^+$]= 596.

12-octadecyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylic acid (IIc): ^{1}H NMR (CDCL3, 400Hz) δ 3.2-2.9 (brs, 1H, COOH), 2.8-1.5 (m, 10H, B-H), 1.4-1.05 (m, 34H, CH_2), 0.88 (t, 3H, J= 6.56 Hz). MS (ESI) m/z calcd for $C_{21}H_{48}$ B_{10} O_2 $[M^+]$: 440, found $[M^+]$: 440, $[2M^+]$=880, $[3M^+ +1]$=1321.

In the third step, coupling the carboxylic acid derivatives (compounds II) with cholesterol was achieved using 4-dimethylaminopyridine as catalyst and dicyclohexylcarbodiimide as dehydrating reagent. The overall yield was about 30%. Structural verification of the final target compounds was performed using thin layer chromatography, proton and ^{13}C nuclear magnetic spectroscopy, differential scanning calorimeter and elemental analysis. The results were as following: **Cholesteryl-3β-12-octyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylate (IIIa):** ^{1}H NMR (CDCL3, 400Hz) δ 5.45 (s, 1H, Chol- 6), 4.48 (s, 1H, Chol-3) 2.85-1.48 (m), 1.45-1.05 (m), 0.98 (s, 3H,C-$\underline{CH_3}$), 0.90 (d, 3H, CH_2-$\underline{CH_3}$, J=6.48 Hz), 0.87 (d, 3H, CH- $\underline{CH_3}$, J=1.76 Hz), 0.85 (d, 6H, CH- $\underline{CH_3}$, J=1.72 Hz), 0.65 (s, 3H, C-$\underline{CH_3}$). ^{13}C NMR ($CDCL_3$) δ 23.5, 57.35, 56.5, 50.3, 42.68, 40.07, 39.9, 37.75, 36.87, 32.19, 29.7, 29.47, 28.6, 28.4, 27.52, 24.21, 23.21, 22.95, 21.4,19.66, 19.10, 14.46, 12.23.

Cholesteryl-3β-12-(1-octenyl)-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate (IIIb): ^{1}H NMR (CDCL3, 400Hz) δ 5.79 (m, 1H, CH_2= $\underline{CH_1}$), 5.3 (m, 1H, Chol-6), 4.97 (dd, 1H,J=1.65, J=15.52), 4.93 (dd, 1H, J=3.99), 4.42 (m, 1H, Chol-3) 3.2-1.6 (m), 1.6-1.1 (m), 0.98 (s, 3H, 3H, C-CH_3), 0.9 (d, 3H, CH_1- $\underline{CH_3}$, J=6.48Hz), 0.86 (d, 6H, CH_1-$\underline{CH_3}$, J= 4.93 Hz), 0.66 (s, 3H, C- $\underline{CH_3}$); ^{13}C NMR ($CDCL_3$) δ 139.39, 139.33, 123.52, 114.73, 57.05, 56.52, 50.32, 42.70, 39.92, 377, 36.58, 36.19, 34.05, 32.28, 29.30, 29.08, 29.03, 28.63, 28.43, 27.54, 23.24, 22.98, 21.42, 19.69, 19.12, 12.253.

Cholesteryl-3β-12-octadecyl-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate (IIIc): ^{1}H NMR (CDCL3, 400Hz) δ 5.31 (s, 1H, Chol-6), 4.43 (s, 1H, Chol-3), 2.2-1.4 (m), 1.3-1.1 (m), 0.99 (s, 3H, C-$\underline{CH_3}$), 0.89 (d, 3 H, CH_2- $\underline{CH_3}$, J=6.2 Hz), 0.87 (d, 3H, CH_1- $\underline{CH_3}$, 1.75 Hz), 0.85 (d, 6H, CH_1- $\underline{CH_3}$, J=1.74 Hz), 0.66 (s, 3H, C-$\underline{CH_3}$); ^{13}C NMR ($CDCL_3$) δ 138.95, 123.09, 56.63, 56.09, 49.89, 42.28, 39.5, 37.35, 31.93, 31.85, 29.69, 29.66, 29.58, 29.53, 29.41, 29.36, 29.14, 28.21, 28.01, 22.82, 22.70, 22.55, 19.26, 11.83.

The elemental analysis results of these new compounds are shown in Table 3.1 and the results indicate that the actual percentage of C and H found in the final products is almost exact to that calculated theoretically. The results from the preformulation characterization of the new compounds are shown in Tables 3.2. These compound are extremely hydrophobic and poorly soluble in water. Conjugating the side chain (IIIa, C_8) to BCH resulted in increased melting point from 235 ^{o}C to 251 ^{o}C. Also, changing from saturated (IIIa, C_8) to unsaturated side chain (IIIb, C_8) led to an increase in melting point by 10 ^{o}C. Further increase in the number of carbon of the saturated fatty chain from 8 to 18 (IIIc) decreased the melting point from 251 to 210 ^{o}C. Under optical and polarizing microscopy, all the compounds were found to be in crystalline form. The size and shape of these crystalline forms were not uniform.

The incorporation efficiency of BCH and the three new compounds in liposomal formulation was evaluated based on the drug:lipid ratio of 1:50 (w/w). The ratio of total lipid (cholesterol and phospholipid) to water was kept constant at value of 2.6%. The ratio of cholesterol and phospholipid, however, were used in three different molar values (0:1, 0.33:1, 1:1). The experimental results are shown in Fig 3.2. The incorporation of BCH in liposomal formulations was increased (statistically significant difference at 95%

confidence level) with an increase in cholesterol/phospholipid ratio from 0:1 to 0.33:1 and further increase of this ratio to 1:1 led to a decrease in I.E (statistically insignificant). For compound IIIa, an increase in the cholesterol/phospholipid ratio from 0:1 to 0.33:1 was accompanied with a decrease in I.E (statistically insignificant) and the increment in the ratio from 0.33:1 to 1:1 led to increase in I.E (statistically insignificant). For compound IIIb, I.E. was increased (statistically significant) from 0:1 to 0.33:1 and I.E. was decreased (statistically significant) from 0.33:1 to 1:1. For compound IIIc, the increase in the cholesterol/phospholipid ratio resulted in a statistically significant decrease in the I.E.

4. Discussion:

Boron neutron capture therapy of cancer is based on the irradiation of boron (^{10}B) compound with low-energy thermal neutrons to produce cell-destroying alpha particles (i.e. Helium nucleus) (Barth et al., 1996; Chen et al, 1997). However, to minimize the destruction of the neighboring normal cells and maintain the BNCT effectiveness, it is very important to selectively deliver sufficient amount (20-25 μg of boron per gram of cell) of boron to tumor cells. Recently, attempts have been made in our laboratory and others to synthesize boron-containing cholesteryl esters and other hydrophobic boron compounds to facilitate the interactions to LDL for targeted drug delivery. Ji and Lu (2001) synthesized a cholesteryl ester linked to a hydrophobic p-carborane cage (BCH). Feakes et al. (1999) constructed a series of boron compounds by linking o-carborane to cholesterol with various fatty spacers between them. Laster et al. (1991) synthesized carborane carboxylic acid esters of fatty alcohols to replace the cholesterol ester in the core of LDL. Cell cultures studies indicated that some of these compounds, including

BCH, were taken up by cancer cells to reach the cellular boron concentration up to 280 μg boron/g cells (more than 10 times higher than that required for successful BNCT) (Laster et al. 1991; Peacock and Lu, 2001). Because of their highly hydrophobic nature, liposomal formulations or other type of lipid carriers are generally required to solubilize these types of boron compounds.

In this paper, three new boron compounds were synthesized. An attempt was made initially to synthesize these new compounds directly from BCH by substituting the hydrogen atom (linking to the second carbon on carborane cage) with various fatty chains. However, the reaction was not successful because BCH (specifically, the ester bond between carborane cage and cholesterol) was not stable in the reaction condition with extremely strong base (n-butylthium). The reaction step was redesign to first link p-carborane to various fatty chains and then to couple them with cholesterol. In the first step, monosubstitution (Ia, Ib and Ic) of fatty chains on one side of carborane was achieved by reducing the temperature to -78 ^{o} C, using the n-BuLi and fatty chains in equivocal molar to p-carborane and adding the reagents slowly. Cholesterol was coupled with the carboxylic acid derivatives, IIa, IIb and IIc, using dicyclohexylcarbodiimide and 4-dimethylaminopyridine.

Similar to BCH, these new compounds are extremely hydrophobic and poorly soluble in aqueous solution. Melting points of these compounds follow the similar pattern as those of native cholesterol esters: the short chain esters have higher crystalline melting temperatures and extension of the fatty chain shows a general trend to lower crystalline melting temperatures (Ginsburg et al., 1984). With the increase in the number of carbons, the crystal becomes less stable and less ordered leading to a decrease in the

melting point. Presence of double bond in the fatty chain of compound IIIc resulted in an increase in the melting point, apparently due to the stronger intermolecular interactions.

Owing to the poor water solubility, liposomal formulation was used to solublize BCH for biological studies and our previous study showed that liposomal formulation was a suitable vehicle for this type of cholesteryl carborane ester compounds. However, the incorporation of new compounds in liposomes needs to be investigated before further biological studies. The present study used three liposomal formulations containing different cholesterol:phospholipid ratios and examined the incorporation of four cholesteryl carborane ester compounds.

The type of fatty chains attached to carborane had a significant effect on the incorporaton of these new compounds in liposomal formulation. For BCH and compound IIIb, the highest I.Es (90.65 ± 6.33 % and 35.35 ± 2.64 %, respectively) were achieved at 0.33:1 cholesterol:phospholipid ratio. For compound IIIa, the highest I.E. (45.39 ± 3.15 %) was achieved at 1:1 cholesterol:phospholipid ratio. For compound IIIc, the highest I.E (80.73 ± 5.64 %) was achieved at 0:1 cholesterol:phospholipid ratio. The results are in agreement with the literature data that the incorporation of hydrophobic drugs in a liposomal formulation depends on the size and spatial structure of the drug molecule and the presence of certain function groups (Samedro et al., 1993). Preez and Prieb (1990) have shown that presence of iodine in position 2 or demethoxylation in position 4 of anthracyclines significantly enhanced the entrapment of the compounds in multilamellar liposomes. Although the addition of various fatty chains to carborane unit changed the incorporation efficiency in our study, no clear trend can be found directly related to the size of the fatty chains.

Changes in cholesterol:phospholipid ratio as the excipients of liposomal formulation also affected the incorporation efficiencies in our experiments. Cholesterol and these hydrophobic compounds are located in the phospholipid bilayer of liposomes. It is known that the incorporation of cholesterol in the phospholipid membrane should not exceed 50 % in molar fraction (Lia et al., 1985). New, R.R.C. (1990) showed that the increase of cholesterol beyond a certain concentration reduced the incorporation efficiency due to the disruption of linear structure in liposomal membrane and resulting in the reduction in intramolecular interaction. Lai et al. (1985) concluded that the formation of a cholesterol-phospholipid complex did not require the presence of 3β-OH and the cholesteryl hemisuccinate had similar effect as cholesterol on the thermotropic properties of dipalmitoyl phosphotidylcholine. These observations suggest that the amount of cholesterol affects the physical stability of phospholipid bilayer and thus the co-existence of cholesterol and cholesteryl esters in the liposomal membrane. Our experiment confirmed that the incorporation efficiencies for various cholesteryl carborane esters depended on the amount of cholesterol used in liposomal formulation. Based on the studies, selection can be made to use different liposomal formulations in the future biological studies involving these new compounds.

In conclusion, the addition of various fatty chains to the cholesteryl carborane ester can be achieved through a three-step chemical reaction. Similar to BCH, these new compounds are extremely hydrophobic and present in crystalline forms. The melting points are ranged from 210 to 260 ^{o}C among these compounds. The incorporation efficiencies of these compounds in liposomes vary significantly depending on the type of

fatty chain attached and the ratio of cholesterol:phospholipid used as the excipients of liposomal formulation.

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Figures and tables legends:

Figure 3.1: Designing strategy of new cholesteryl carborane ester compounds. Structure A, B, C , D, E and F are referred as cholesteryl stearate, cholesteryl oleate, BCH, IIIa, IIIb, and IIIc , respectively.

Figure 3.2: Incorporation efficiency of BCH and the three new compounds in various liposomal formulations. (*) Indicates statistical significant difference at 95% level of confidence.

Scheme 3.1: A three-step reaction for the synthesis of cholesterol carborane ester compounds.

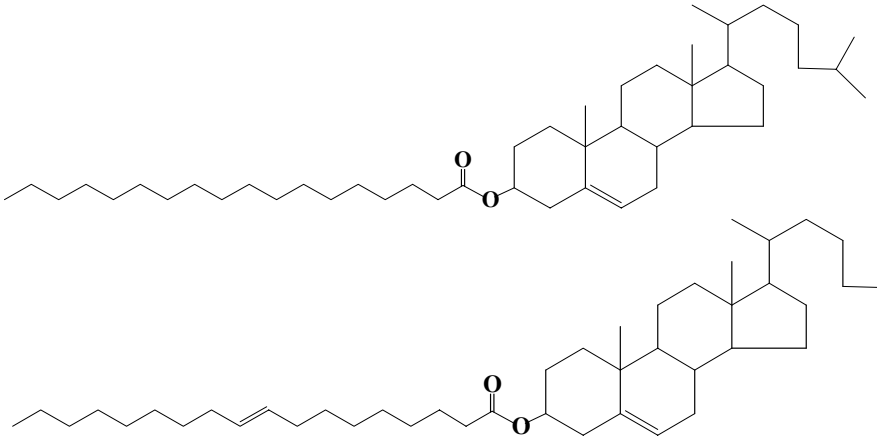
Reagent and solvent:

(1) n-BuLi in hexanes, anhydrous THF, dry N_2 and bromoalkane or bromoalkene at $-78\ ^{o}$ C.

(2) n-BuLi in hexanes, anhydrous THF, dry CO_2 at $-78\ ^{o}$ C.

(3) Dichloromethane, Cholesterol, 4-DMAP and N, N- Dicyclohexyl Carbomidine

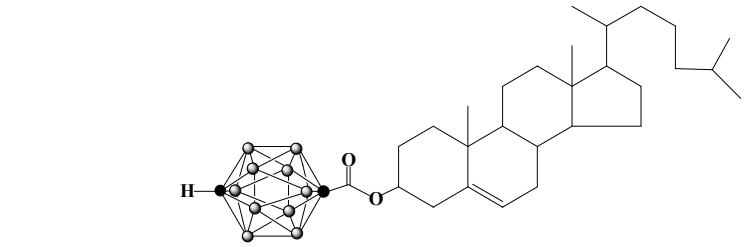
Native cholesteryl esters in human



A

B

New cholesteryl carborane esters



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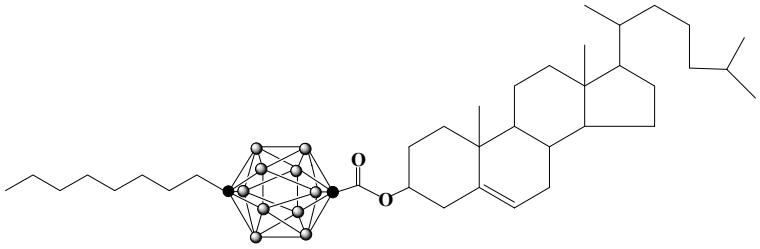
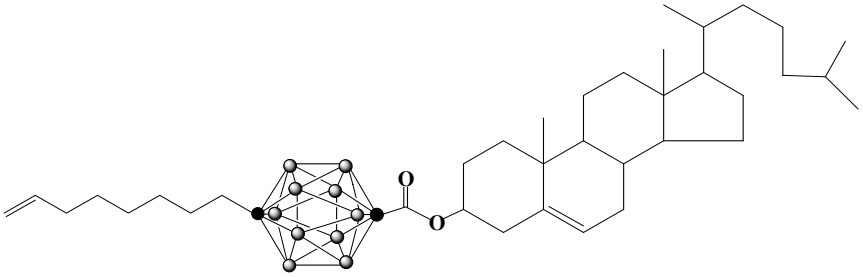
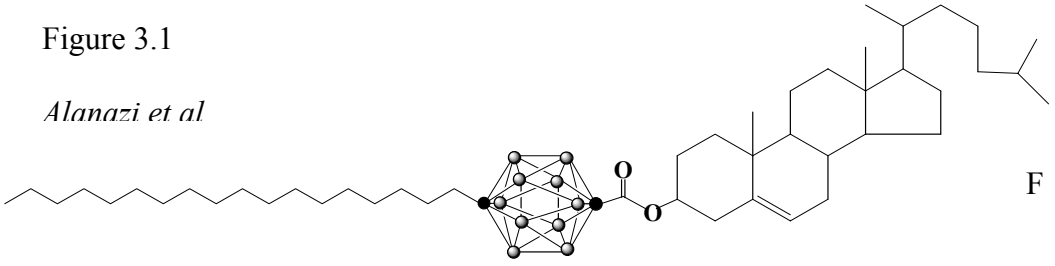


D



E



F

Figure 3.1

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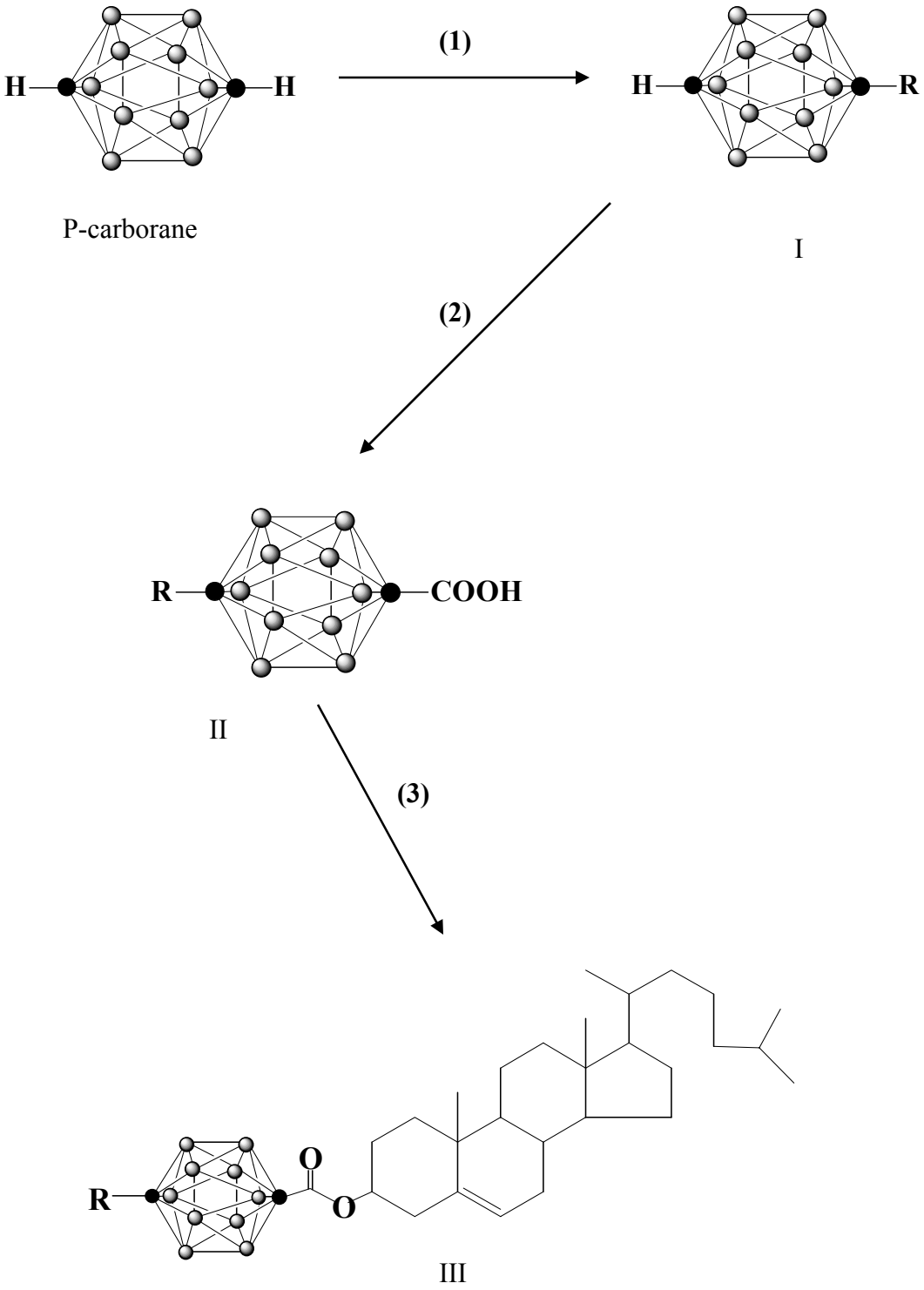

Scheme 3.1
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Table 3.1 Elemental analysis of the final cholesteryl carborane ester compounds.

Compound	% C		% H	
	Theoretical	Found	Theoretical	Found
IIIa	68.152	68.25	10.76	10.92
IIIb	68.42	68.59	10.58	10.69
IIIc	71.23	71.31	11.45	11.61

Table 3.2 Preformulation properties of cholesteryl carborane ester compounds.

Compound	R	Molecular weight	Mp ^{o}C	Internal structural	Aqueous solubility [a]
BCH	H	556.93	235	Crystalline	--
IIIa	$-(CH_2)_7CH_3$	669.085	251	Crystalline	--
IIIb	$-(CH_2)_6CH=CH_2$	667.07	260	Crystalline	--
IIIc	$-(CH_2)_{17}CH_3$	809.354	210	Crystalline	--

[a] Aqueous solubility at 25^{o}C is less than 50 ng/ 1ml of water.

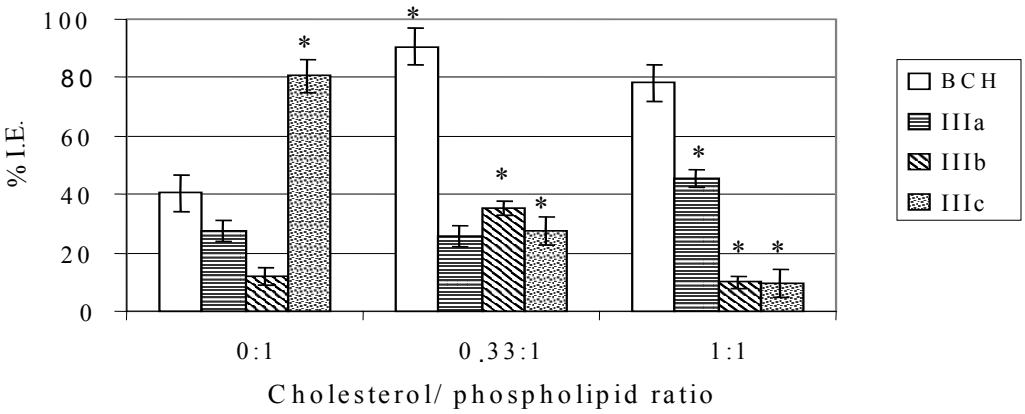

Figure 3.2

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CHAPTER 4

DISTRIBUTION OF A NEW CHOLESTEROL-BASED ANTICANCER CONJUGATE IN BRAIN AND ITS STABILITY IN CHOLESTEROL ESTERASE AND LIPOPROTEIN LIPASE [1]

[1] Fars K Alanazi and D.Robert Lu. To be submitted to Journal of Applied Research

Abstract

Purpose: To study the brain distribution profile of a new cholesterol-based boron anticancer conjugate (cholesteryl 1,12-dicarba-*closo*-dodecaborane 1- carboxylate, BCH) in tumor-bearing rats, to evaluate the radiation window of BCH after it is administrated intracerebrally in a brain tumor, to investigate the *in vivo* affinity of BCH to the tumor and normal tissues in an animal model, and to examine the stability of BCH in two hydrolytic media (cholesterol esterase and lipoprotein lipase). **Methods:** Intracerebral implantation of rat 9L glioma cells was performed to produce a tumor-bearing rat model. BCH liposomes were prepared as a solubilization formulation due to the extremely hydrophobic nature of BCH. Ten μl of BCH liposomes containing 150 μg BCH/ml was administrated to tumor-bearing rats intracerebrally. BCH liposomal formulation (0.673 μmole) was added to an enzymatic medium and incubated at 37 ^{o} C. Inductive coupled plasma mass spectrometry (ICP-MS) and HPLC were used for analysis. **Result:** The BCH concentration in the tumors at 2 hrs and 6 hrs after the administration were 27.70 and 12.51 μg BCH/gm of tumor tissue, respectively. The ratio of BCH in tumor to that in normal tissues at 2 hrs, 6 hrs, 8hrs and 14 hrs were 5.46, 1.56, 1.12 and 1.01, respectively. The data of the affinity study showed that the average BCH concentration in the tumor was 4.45 μg/gm of tissue and in the normal tissues was 3.91 μg /gm of tissue, when the BCH formulation was injected 2 mm away from tumor site. Results indicated good stability of BCH in presence of CE and LPL **Conclusion:** the decline of BCH concentration, greater than 54% within 4 hours, indicated that BCH in liposomal

formulation distributed from the site of injection quickly. The radiation window appeared to be less than 2 hrs. BCH is stable in presence of CE and LPL.

Keywords: brain tumor, drug targeting, liposome, cholesterol esterase, lipoprotein lipase

Introduction:

Each year more than 17,000 people in the United States have been diagnosed with brain tumor [1]. The prognosis for brain tumor is poor due to the lack of effective treatment options. For the chemotherapeutic option, it is critical to deliver a sufficient amount of therapeutic agents to the brain tumor site. However, delivery of therapeutic agents to brain is technically challenging due to presence of blood brain barrier (BBB) [2]. Many attempts of special drug delivery have been made to overcome this obstacle. One of the most direct approaches of bypassing the BBB is to intracerebrally administer the agents within brain parenchyma through local delivery to tumor tissue [3]. The therapeutic molecules then diffuse from the site of administration to surrounding cancer cells. This process offers the considerable advantage of high drug concentrations at the tumor site with limited exposure to normal tissues and organs in the rest of the body. Recently, our laboratory has developed a cholesterol-based anticancer agent containing carborane as the anticancer unit for boron neutron capture therapy (BNCT). The new compound, cholesteryl 1,12-dicarba-*closo*-dodecaborane 1-carboxylate (BCH) mimics the natural cholesteryl ester in structure and was found to be effectively taken up by brain glioma cells in cell culture studies [4,5]. The intracerebral administration of BCH in animal models, however, was not conducted.

Therefore, the purpose of the present study is to investigate the biodistribution of BCH in the brain after intracerebral administration of BCH using a tumor-bearing rat model. The ratio of BCH concentrations in tumor versus surrounding normal tissue at different time points is an important factor for determination of the radiation window (when the ratio reaches a maximum) and it was investigated after intracerebral BCH administration.

The uptake of BCH by normal cell line (HCN-1a) and tumor cell lines (SF-763 and SF-767) was investigated and the amount of BCH uptake by the two different tumor cell lines was up to 14 times higher than normal cell line [4]. This study was performed *in vitro* comparing BCH uptake in the cell lines in tissue culture. To truly test the drug affinity in normal and cancer tissue, it is important that the affinity study be performed *in vivo* (using a suitable animal model instead of tissue culture). Therefore, in the present study an *in vivo* method was developed and used to compare selectivity differences in BCH uptake by normal and cancer tissues in tumor bearing rats.

BCH stability in biological media (brain) is very important since it faces hydrolytic enzymes *in vivo* that limit its targeting activity [6]. BCH is composed of a cholesterol unit linked to the p-carborane moiety *via* an ester bond. It is challenged with presence of enzymes that act as ester bound cleavers. Examples are cholesterol esterase (CE) and lipoprotein lipase enzymes (LPL) in the brain. Cholesterol esterase (CE) functions in the brain to break down the ester bond of cholesterol esters resulting in cholesterol and fatty acid [7]. LPL functions in the brain to hydrolyze triglycerides and to transport membrane compounds such as lipid, cholesterol and vitamin E to neurons [8]. Therefore, BCH was incorporated in vesicles prepared from DL-α-phosphotidylcholine,

Dipalmitoyl (DPPC) and utilized as a substrate for an *in vitro* stability study in the presence of cholesterol esterase (CE) and lipoprotein lipase (LPL).

Experimental Section

Materials

BCH, (cholesteryl 1,12-dicarba-*closo*-dodecaborane 1-carboxylate) was synthesized and purified according to a method published early (Ji and Lu, 2002) [9]. Cholesterol and Dipalmitoyl DL-α-phosphotidylcholine (DPPC) were purchased from Sigma Chemical Co. (St.Louis, MO). CE (cholesterol esterase) and LPL (lipoprotein lipase) were purchased from Sigma. All other chemicals were analytical grade.

Preparation of liposomes

BCH was formulated in liposome formulations to enhance its solubility due to its extreme hydrophobicity. The BCH liposomal formulation was prepared by thin film hydration method as described (Alanazi et al 2003) [10]. Briefly, cholesterol, phospholipid and BCH were dissolved in a 2:1 mixture of chloroform and methanol in a round bottom flask. BCH to lipid ratio was 1:50 (w/w) and total lipid (cholesterol and phospholipid) to water ratio was 1:38 (w/w). Cholesterol and phospholipid ratio was 0.33:1 molar ratio [10]. The solvents were evaporated under reduced pressure and a thin film of lipid was formed on the bottom of the flask. The film was hydrated at a temperature above the phase transition temperature of the lipids with deionized water and glass beads (5 mm diameter) were added to ease the dissolution. The hydration process was performed for several hours using a shaking water bath. Liposomes were separated from the glass beads by filtration through a Buchner funnel. The size of the liposomes was reduced using an

Emulsiflex B3 (Avestin, Ottawa, Canada) and measured at 25^{o}C using a Nicomp Submicron Particle Sizer (Model 380, Nicomp, CA).

Tumor-Bearing Rat Model

Male Fisher rats weighing about 250 gm were used throughout the experiment. Rats were kept in standard cages (2 rats per cage) without restriction to water and food during the experiment. Rat 9L glioma cell line was used to introduce brain tumor in the animal. The cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM, Fisher, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 100μg/ ml streptomycin, and 100U/ ml of penicillin (Sigma, St. Louis, MO) at 37^{o}C and 5% CO_2 in 150 mm^2 flasks (Corning, Corning, NY). When the cells reach confluence, they were washed with DMEM, harvested using trypsin, centrifuged, re-suspended with phosphate buffer, counted, and kept in an ice bath for immediate use. The animals were anesthetized intramuscularly with ketamine (40 mg/kg) and xylazine (2.7mg/kg). The rat scalps were shaved and sterilized with 70% ethyl alcohol. The rats were placed on a stereotaxic apparatus (Stoelting, Wood Date, IL) and a middle incision was performed. The skull was exposed and a small burr hole was made 1mm anterior to Lambdoid suture and 3mm lateral (right) to Sagittal suture [11]. Four μl of the rat 9L glioma cell suspension (4 x 10^4 cells) was injected intracerebrally 5 mm deep from the surface of the skull using a Hamilton syringe. The injection was performed over a period of 2 minutes and the needle was withdrawn slowly to minimize the flow back of cells in the needle track. The hole was filled with bone wax and the wound was closed with running silk sutures. The animals were watched over until they recovered from anesthesia and then returned to their cages.

Intracerebral administration of BCH liposomal formulation

The rats were ready for further BCH distribution studies when the tumor reached a relatively large size (about 14-16 days after implantation of tumor cells). The animals were injected intracerebrally with BCH liposomal formulation at the tumor site using the same procedure as described above for tumor implantation. Ten μl of BCH liposomal formulation (150 μg BCH/ml) was administrated for each rat. To study BCH distribution from the tumor site, four groups of rats (n = 3 for each time point) were sacrificed at 2, 6, 8 and 14 hrs post-administration. Tumor tissue was carefully separated and surrounding normal tissue samples were also prepared. To study BCH affinity to normal and cancerous tissue *in vivo (*affinity study), BCH in the liposomal formulation was injected intracerebrally 2 mm away from the tumor site [Figure 4.7]. Animals (n =3) were sacrificed after 18 hrs of post-administration. Normal tissue samples and tumor tissue samples were taken at the opposite direction and both sampling sites were the same distance from the BCH injection site. All tissue samples were homogenized, digested in a Teflon-lined acid bomb, and analyzed for boron content using ICP-MS (Inductively coupled plasma-mass spectrometry). Based on the HPLC analysis, the result from the chemical stability of BCH in brain simulating media containing enzymes acting as ester bond cleavers indicated the stability of BCH in the brain (see below). Therefore, it is reasonable to use ICP-MS for boron analysis since it offers a higher analytical sensitivity for BCH (5 ng/1ml) than HPLC (100 ng/ ml) method.

Histological Examination

To validate the tumor-bearing model, brains of control animals after 14 days of post-tumor implantation were obtained, fixed immediately with 10 % buffered formalin

and sliced into coronal sections (3-5 mm in thickness). Tissue slides were placed in a paraffin tank, consolidated into a single unit using a Shandon HistoCenter 2 [TM] and cut into 4 μm sections using a Finesse Rotary Microtome (Thermo Shandon Inc, Pittsburgh, PA). Micrometer sections were placed on a glass slide and subjected to washing with 70-100% of ethanol and 100 % xylene to dehydrate sample and to remove the paraffin layer. Staining of the sample with Hematoxylin and Eosin was performed using a robotic staining system (Leica Autostainer XL, Lecia Microsystems Inc, Bannockburn, IL). After staining, the samples were examined under light microscopy.

Chemical stability of BCH in CE and LPL

Ten mg of CE (6.83 unit is enough to theoretically hydrolyze 5.9 μmole of BCH; 8.7 times higher than the BCH concentration used) and 4 mg LPL (424,000 unit which is enough to theoretically hydrolyze 106 μmole of BCH; 157 times higher than BCH concentration used) were dissolved in 7.5 ml of phosphate buffered saline (pH 7.4), respectively. BCH liposomal formulation (0.673 μmole of BCH) was added and the mixture incubated at 37^{o}C. At appropriate time intervals, 200 μl samples were withdrawn and immediately added to 600 μl acetonitrile kept in an ice bath to precipitate the protein (enzyme). The samples were vacuum dried (Speed Vac [®] plus, Savant, NY) at low temperature, reconstituted with HPLC mobile phase (methanol: isopropanol 50: 50), vortexed, and centrifuged at 3000 RPM for 10 minutes. The supernatant was analyzed with a reverse phase HPLC system based on an existing method [12] (Table 4.1). A liposomal formulation of cholesterol oleate was prepared as a control and underwent the same procedure as described above.

Results

Liposomes containing BCH were prepared by a thin film hydration method and the size distribution of the resulting liposomes was examined by photon correlation spectroscopy (number weighted mean). A bimodal distribution was observed. Approximately 91% of the vesicles had a mean diameter of 49 ± 6.3 nm and the remaining 9% of the vesicles had a mean diameter of 170.1 ± 21.3 nm. The average BCH content in the liposomal formulation was about 150 μg/ml.

After 14 days post-implantation of tumor cells in the rat brains, the tumor was readily visualized (Figure 4.1). Histological examination of the brain tissue indicated the presence of large pleomorphic nuclei and abnormal mitotic figures (Figure 4.2), further confirming the presence of tumor. Physical changes were also observed in the tumor-bearing rats, including weight loss and changing the hair to white-brownish, due to abnormal nasal excretion indicating presence of brain cancer. Therefore, the tumor-bearing model was considered to be suitable for subsequent BCH distribution studies. The concentration of BCH in tumor tissues after intracerebral administration at the tumor site was 27.70 ± 5.5 μg/gm tissue and 12.51 ± 2.5 μg/gm tissue at 2 hrs and 6 hrs, respectively. The concentration of BCH in the surrounding normal tissues was 5.07 ± 1.0 μg/gm tissue and 7.99 ± 1.6 μg/gm tissue at 2 hrs and 6 hrs, respectively (Figure 4.3). Ratios of BCH concentration in tumor tissue and in surrounding normal tissue were 5.46, 1.56, 1.12 and 1.01 at 2, 6, 8 and 14 hrs, respectively (Figure 4.4). The BCH concentrations in the tumor tissue and in the normal tissue, both sampled at 2 mm from the intracerebral BCH injection site at opposite directions, were 4.45 ± 0.89 μg/gm tissue and 3.91 ± 0.78 μg/gm tissue, respectively (Figure 4.5).

The chemical stability of BCH in the presence of cholesterol esterase and lipoprotein lipase was further examined and the results presented in Figure 4.8. BCH was formulated in liposomes to increase its aqueous solubility and incubated with cholesterol esterase and lipoprotein lipase, while cholesterol oleate liposomal formulation was used as control. After 24 hours, 90% and 100 % of BCH remained in cholesterol esterase and lipoprotein lipase media, respectively. On the other hand, 78 % and 47 % of cholesterol oleate remained in CE and LPL media, respectively.

Discussion

Recently, BCH was synthesized in our laboratory as a new boron neutron capture therapy (BNCT) targeting agent [Figure 4.6]. BCH has been developed based on the fact that rapidly dividing cells like malignant tumor cells require more cholesterol to construct cell membranes. In the human body, cholesterol is carried as cholesteryl esters by low-density lipoprotein (LDL) and other lipoproteins. To acquire more cholesterol, many types of tumor cells possess elevated LDL receptor activities than the corresponding normal cells [13-16]. A recent study at UCSF reported that seven human glioblastoma multiforme cell lines had very high numbers of low-density lipoprotein (LDL) receptors per cell, indicating that the LDL pathway may provide a method for selectively targeting compounds to glioblastoma multiforme cells [17]. Correspondingly, the design and synthesis of anticancer compounds in our laboratory have focused on derivatives of cholesteryl esters, resulting in the development of BCH. In our cell culture studies, the amount of BCH taken by the glioma cells was much more (up to 14 times) than that by the normal brain neuron cells. The cellular uptake of BCH by glioma cells (SF-763 and SF-767) after 16 hours of incubation was 283.3 ± 38.9 μg boron/g cells and 264.0 ± 36.5

μg boron/g cells, respectively, about 10 times higher than the required boron level for effective BNCT [5]. A mechanistic study indicated an inhibition of BCH uptake in the presence of the anti-LDL receptor antibody and a positive correlation of BCH uptake with the amount of LDL in the culture medium, strongly suggesting the involvement of LDL receptor-mediated pathway [18].

Following positive results from the cell culture studies, the present study focused on the distribution of BCH in rat brain after intracerebral administration using a tumor-bearing rat model. Many previous studies have indicated that the intracerebral delivery of anticancer compounds can significantly enhance their efficacy [11, 19, 20, 21].

In this paper, distribution of BCH liposomal formulation in the brain after intracerebral injection was studied. Tumor-bearing rats obtained by implanting 9L brain glioma cells were used as animal modesl to evaluate the radiation window of BCH and to test BCH affinity to normal and cancer cells *in vivo*. BCH concentration in tumor tissue (site of injection) decreased from 27.70 ± 5.54 μg/gm tissue to 12.51 ± 2.5 μg/gm tissue at 2 hr and 6 hr, respectively. The decline of BCH concentration, greater than 54% within 4 hours, indicates that BCH distributed from the site of injection quickly. In addition, concentration of BCH in normal tissues increased from 5.07 ± 1.014 μg/gm tissue to 7.99 ± 1.598 μg/gm tissue at 2 hr and 6 hr, respectively. The result shows out that BCH diffused from the injection site to the neighboring normal tissues. The result is in agreement with the brain distribution pattern of liposomal Boroncaptate in tumor bearing rats after intracerebral injection [11]. Finding a suitable irradiation window between intracerebral injection and neutron irradiation is important for successful BNCT therapy. An adequate radiation window appeared to be less than 2 hours due to rapid BCH

distribution. An earlier time for irradiation may be used after intracerebral injection of BCH. For examining the affinity of BCH to tumor and normal cells *in vivo*, the concentration of BCH in tumor and normal tissues after intracerebral injection of BCH liposomal formulation 2 mm away from the tumor site was 4.45 ± 0.89 μg/gm tissue and 3.91 ± 0.78 μg/gm tissue, correspondingly. The result indicated statistically insignificant difference between the BCH concentration in tumor and normal tissues at 95% confidence level (student's *t* test).

Since BCH is composed of a cholesterol unit linked to p-carborane moiety *via* ester bond, it faces challenge in the presence of enzymes that act as ester bound cleavers existing in the brain, such as CE and LPL [7, 8, 22,23]. The hydrolysis of the ester bond liberates cholesterol from anticancer moiety resulting in demolishing the proposed targeting activity. Therefore, in the present study, BCH stability was investigated. To simulate the brain *in vivo* environment, we used buffered solutions of cholesterol esterase and lipoprotein lipase. The applicability of cholesteryl ester-containing phospholipid vesicles as a useful substrate for studying cholesteryl ester hydrolysis in vitro has been demonstrated [24, 25]. BCH in the liposomal formulation was relatively stable when incubated in two hydrolytic media, with 90% and 100 % of BCH remaining during the incubation with CE and LPL, respectively. In contrast, the control cholesterol oleate showed 78 % and 47 % degradation for CE and LPL, respectively under the same conditions [Figure 4.8]. The presence of a bulky group near to the ester bond provides protection from hydrolysis by steric hindrance [26, 27]. Thus, BCH stability in the presence of CE and LPL could be attributed due to existence of the bulky p-carborane group that slows down these enzymes to act on the site. Absence of a bulky group in cholesterol

oleate led to its instability. The difference in BCH degradation in presence of CE or LPL is about 10 %. Alternatively, LPL has a higher hydrolysis effect on cholesterol oleate (47% remaining) than CE (78% remaining). It could be because LPL has a greater hydrolytic effect on the phospholipid since it also has phospholipase activity [28] and more free cholesterol oleate was exposed to hydrolytic medium as a result.

In conclusion, a new BNCT targeting compound (BCH) was tested for brain distribution and its stability in *in vivo* environment was challenged. The result indicated that BCH in a liposomal formulation distributed from the site of injection quickly. The adequate radiation window was less than 2 hrs. The result also pointed out that BCH diffused from the injection site to the neighboring normal tissues. The result indicated a statistically insignificant difference between the BCH concentration in tumor and normal tissues. BCH was stable in presence of cholesterol esterase and lipoprotein lipase as a simulated *in vivo* environment.

Acknowledgment:

This study was supported in part by NIH Research Grant CA 67352(DRL). Authors would like to thank Isotron Inc. for their support. Also, authors would like to thank Dr Barry G. Harmon (Department of Veterinary Pathology, College of Veterinary Medicine, UGA) for his assistant with the histopathology examination.

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Figures and tables legends:

Figure 4.1: Brain picture of a rat with a developed brain tumor after implantation of 9L glioma cells (16 days after implantation); picture taken by digital camera, dark color is the tumor tissue located on the upper part of the right hemisphere.

Figure 4.2: Histological examination indicated presence of tumor in rats' brain. (A) Brain section through cerebrum. Neoplastic mass (arrows) with hemorrhage of one margin (arrowheads). (B) Neoplastic mass, note the large pleomorphic nuclei (a) and abnormal mitotic figures (b). Hematoxylin-and-eosin staining (H&E).

Figure 4.3: The distribution of BCH in the brain after it was injected in the tumor site directly

Figure 4.4: Result of the radiation window study

Figure 4.5: Result of the affinity study. Three rats were sacrificed in each group after 18 hr post injection. There was a statistically insignificant difference between the average concentration of boron in the tumor and the normal tissue.

Figure 4.6: Cholesteryl 1,12-dicarba-closo-dodecarborane 1-carboxylate (BCH) (Lu and Ji, U.S. Patent Application No. 09/609, 957)

Figure 4.7: An *in vivo* affinity comparison study

Figure 4.8: Hydrolysis of BCH in two hydrolytic media (CE and LPL). CE, LPL, BCH, and OL are stated for cholesterol esterase, lipoprotein lipase, a new-targeted boron compound, and cholesterol oleate.

Table 4.1: Chromatographic condition

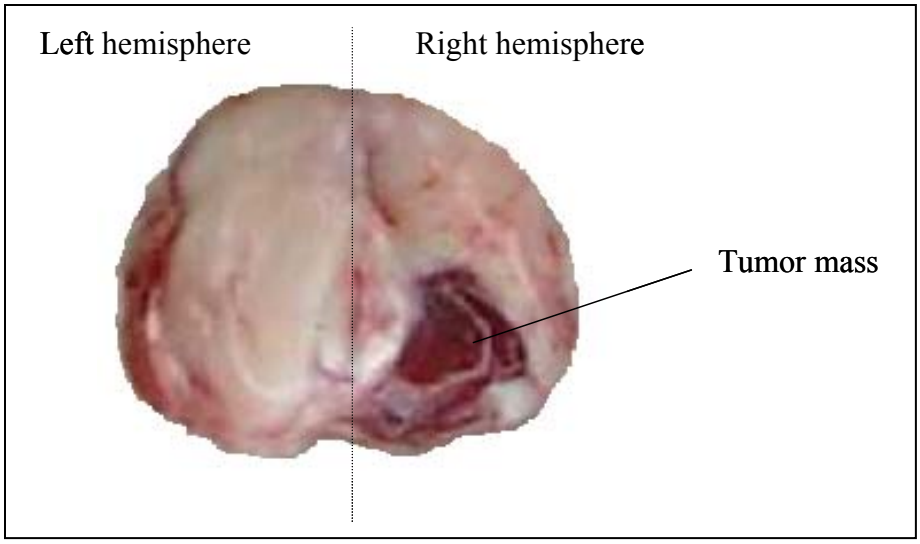

Figure 4.1

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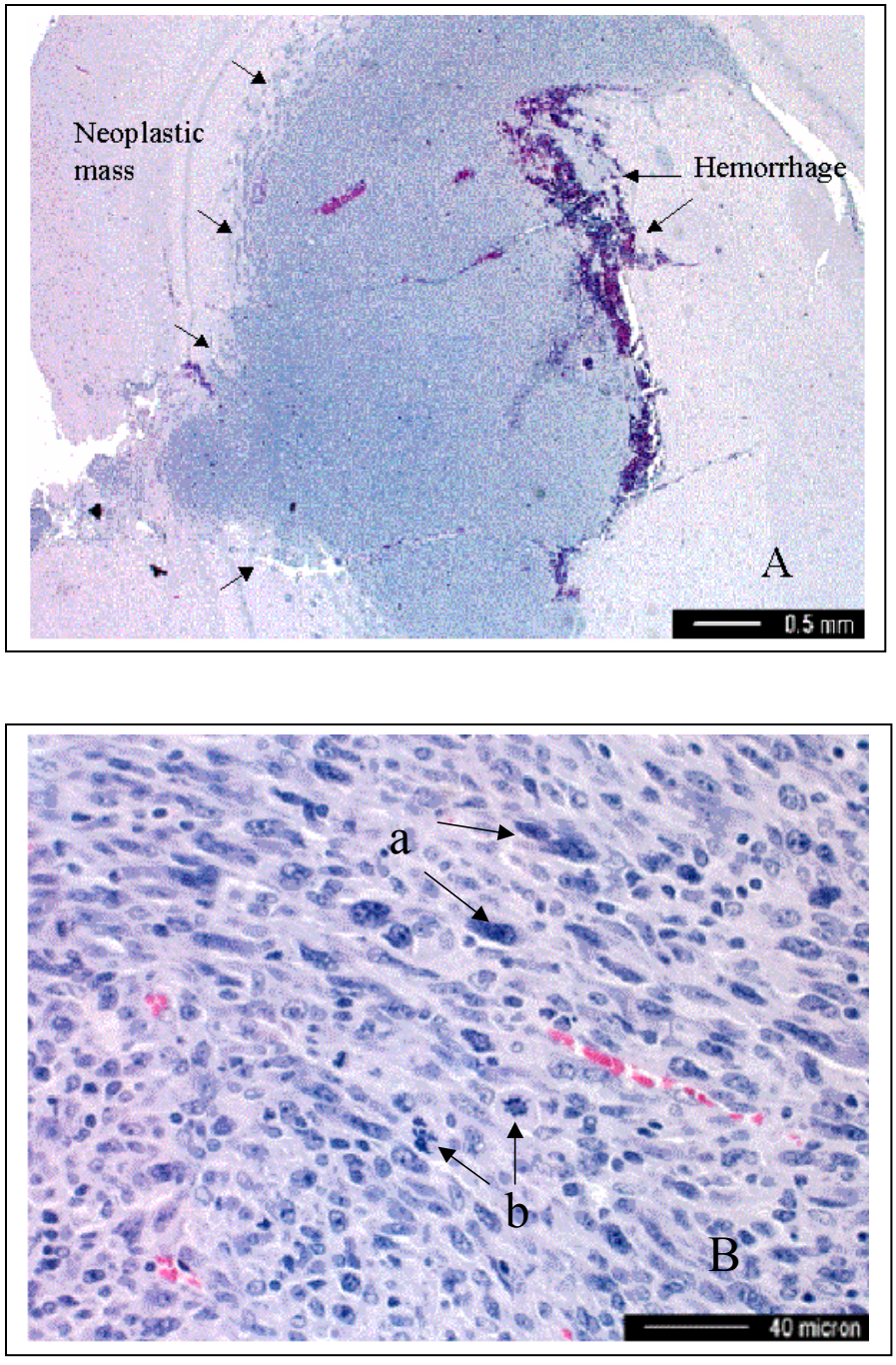

Figure 4.2

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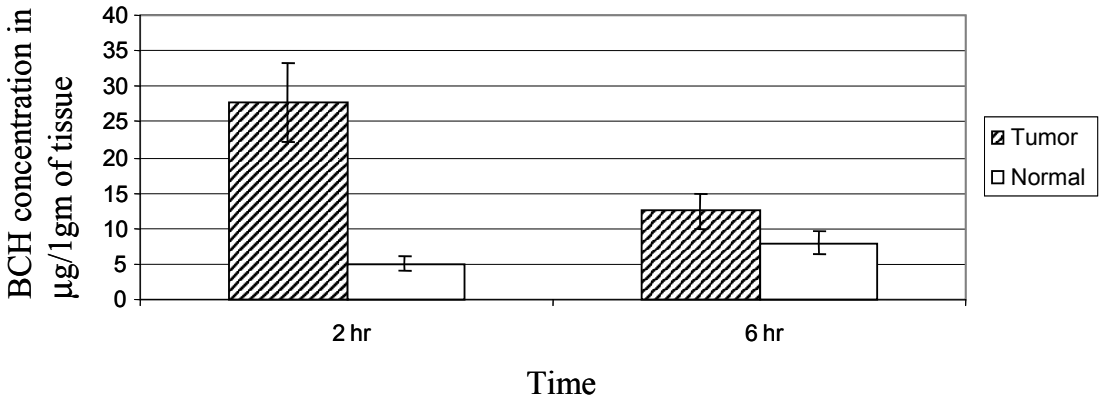

Figure 4.3

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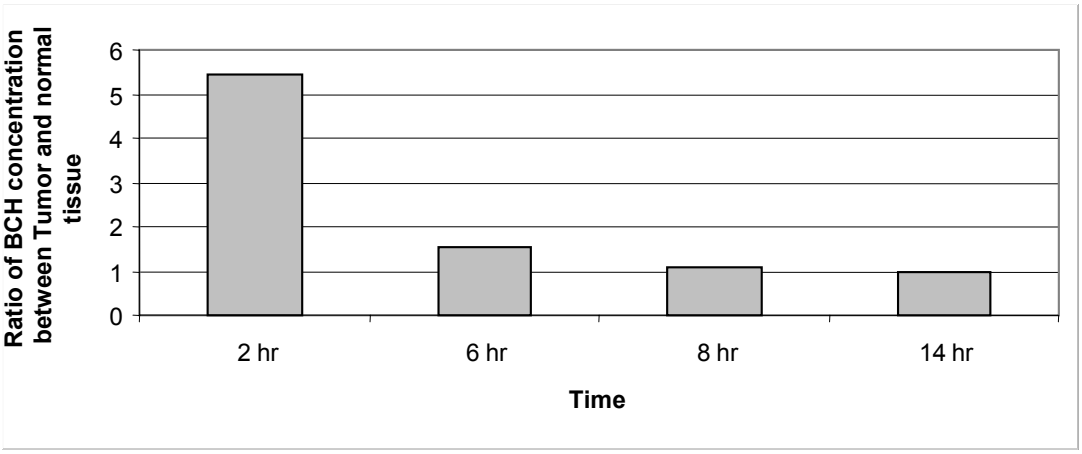

Figure 4.4

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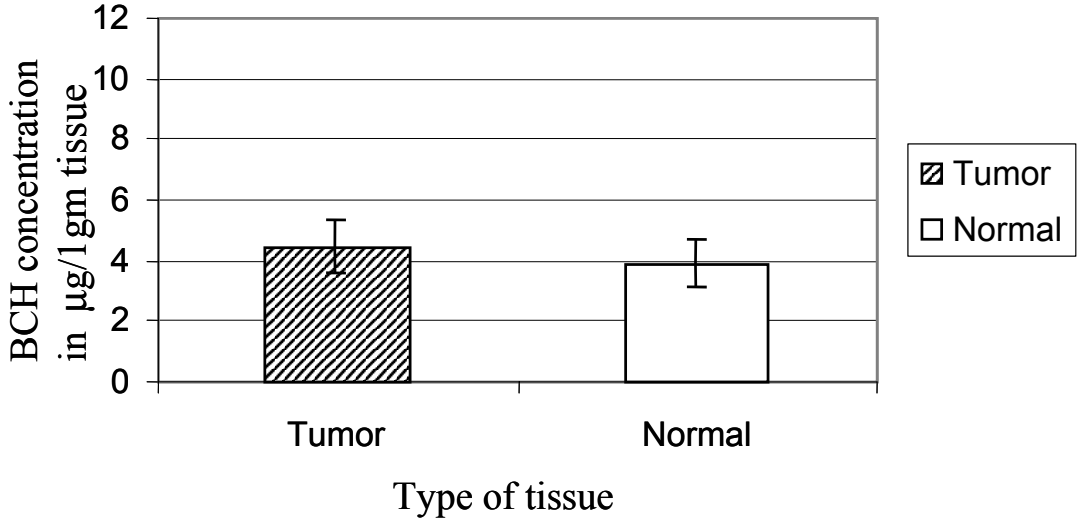

Figure 4.5

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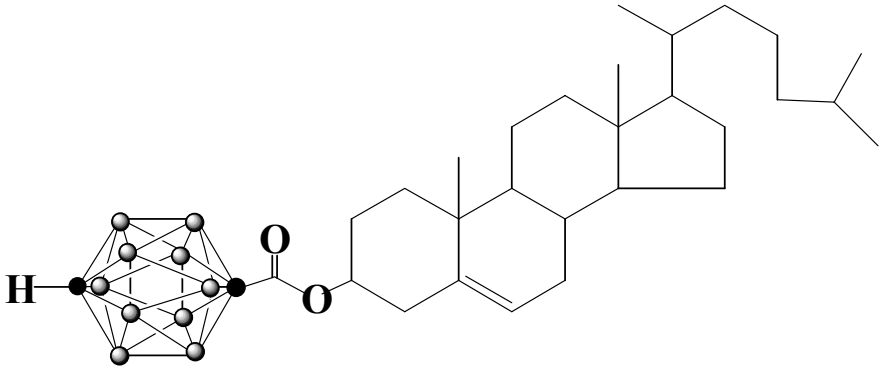

Figure 4.6

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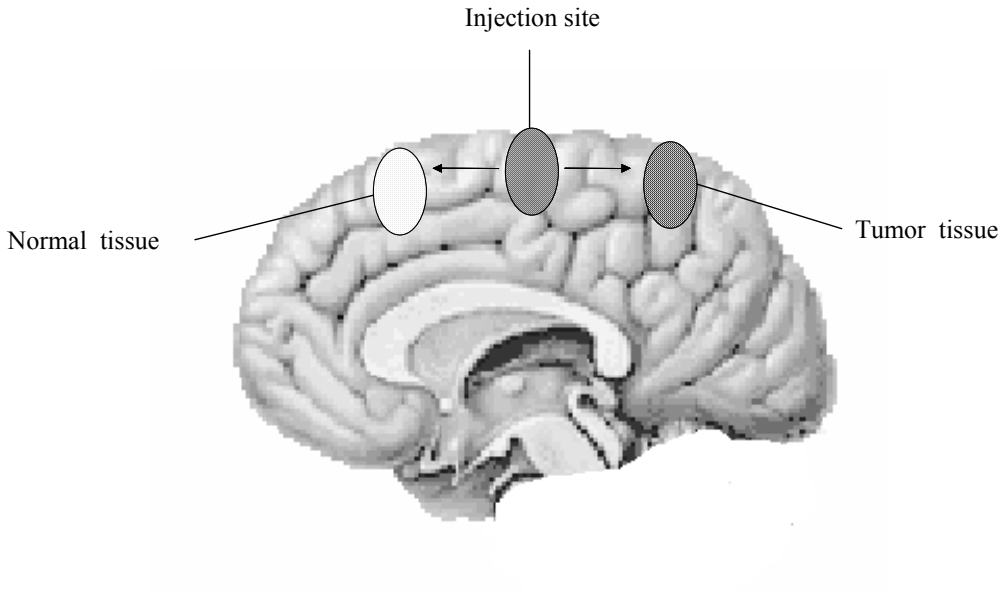

Figure 4.7

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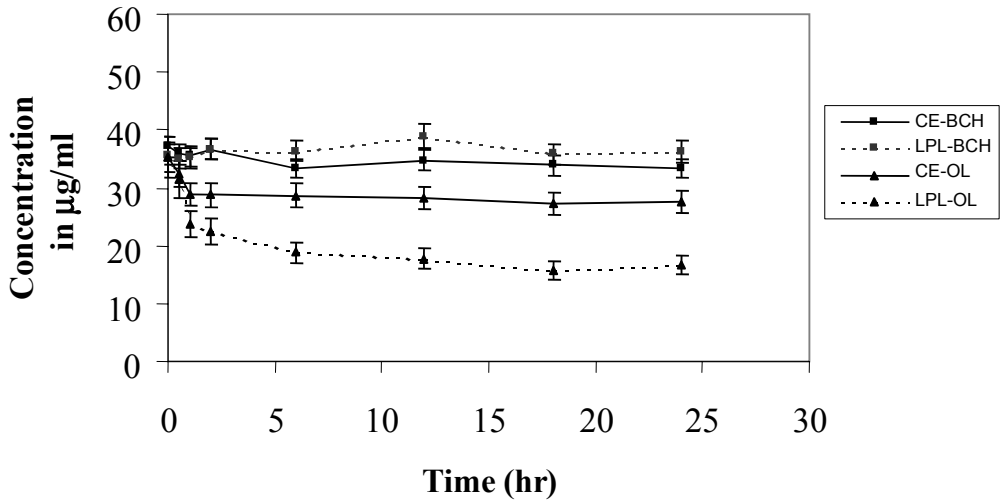

Figure 4.8

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System	Water model 2690
Column	Hewlett-Packard ZORBAX SB C-18, 3.5 µm
Column length	150 mm
Internal diameter	3 mm
Precolumn	Hewlett-Packard ZORBAX SB C-18, 12.5 x 4.6 mm, 5 µm
Mobile phase	Methanol: 2-propanol (1:1) (v/v)
Flow rate	0.5 ml /minute
Detector	Photodiode array detector (λ= 202 nm)
Temperature	40 ^{o} C
Internal stander	Cholesteryl Heptadecanoate (Rt: 11 mint)
Retention time of BCH	5.25 minutes
Retention time of CO	10 minutes

Table 4.1

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CHAPTER 5

DENSITY GRADIENT SEPARATION OF CARBORANE-CONTAINING LIPOSOME FROM LDL AND DETECTION BY INDUCTIVELY COUPLED PLASMA SPECTROMETRY [1]

[1] Alanazi F and D Robert Lu. To be submitted to Drug Delivery

Abstract

Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) was used for analyzing the new cholesterol-based compounds (BCH, BCH-Da, BCH-Db, and BCH-Dc) in liposomal formulations. Not only the boron compounds but also the phospholipid composition of the liposome formulation were quantitatively analyzed. This combination provides a feasible method for analyzing element-based compounds encapsulated in phospholipid vesicles. ICP-MS was, also, utilized for analyzing BCH in a brain distribution study. In addition, an ultracentrifugation method was developed to separate liposomes from low-density lipoprotein (LDL). Factors such as density gradient and size of liposomes were adjusted to optimize separation. Details of how to separate liposomes from LDL by ultracentrifugation technique are presented.

Keywords: Liposomes, low-density lipoprotein, ultracentrifugation, Inductively Coupled Plasma, boron compound.

1. Introduction

New cholesterol-based carborane compounds have been synthesized in our laboratory as potential anticancer targeting molecules [Alanazi F. et al. 2003 and B. Ji et al 2002]. These compounds are designed to mimic the native cholesterol esters and to be delivered through the elevated LDL receptor for cancer treatment. Due to the extremely hydrophobicity, liposomal formulations were prepared to solublize these compounds. Additionally, liposomes have been chosen to facilitate the delivery of targeted molecules to an endogenous cholesterol carrier (LDL), and to tumor tissue accordingly [Kader A. et al, 1998].

Finding a good analytical method for analyzing these compounds in liposomal formulation and in biological samples is essential. Inductively Coupled Plasma Spectrometry (ICP) can be used in the quantitative analysis in the following areas: natural materials (such as rocks, minerals, soil, sediment air, water, and plant) and animal tissue. Furthermore, it has been used widely for quantitative analysis of compound-containing elements, for example, boron compounds [Svantesson E et al. 2002, Hiratsuka Jet al 2000], selenium compounds [Li F, et al., 1999, Zheng J et al. 2002], and platinum compounds [Liu J et al., 2002].

An advantage of using ICP as a bio-analytical technique includes its ability to identify and quantify most of the elements [Thompson M., et al, 1989]. Since many wavelengths of varied sensitivity are available for determination of any one element, ICP is suitable for all concentrations from ultra-trace levels to major components [Dean J.R, 1997]. Detection limits are generally low for most elements with a typical range of 1μg/ L (lower limit) and 1g/ L (upper limit). Positively, the main advantage of employing an

ICP when performing quantitative analysis is the fact that multi-elemental analysis can be accomplished rapidly. A complete multi-element analysis can be undertaken in a period as short as 30 seconds, consuming less than 0.5 ml of sample solution.

In the present study ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) was used for analyzing the new cholesterol-based compounds (BCH, BCH-Da, BCH-Db, BCH-Dc) in liposomal formulations. These compounds are boron derivatives. Each drug molecule contains 10 boron atoms. Detection of the boron in the samples was used as indirect method for analyzing the whole drug molecule. Multi-element analysis as provided by ICP-AES allows measurement boron concentration as well as phosphorus concentration at the same time. Consequently, phosphorus concentration was used to calculate phospholipid concentration to determine incorporation efficiency. Additionally, Inductively coupled plasma-mass spectrometry (ICP-MS) was used for analyzing BCH in brain tissue samples in a brain distribution study.

One of the objectives of the study after synthesizing the cholesterol-based compounds and incorporating them in a suitable drug delivery system (liposomes) is to examine the transfer of these compounds from the liposomal formulation to low density lipoprotein (LDL) *in vitro* with different incubation media. To conduct this study it was imperative in our laboratory to develop a method to separate the liposomal formulation from LDL after finishing the incubation period. Ultra-centrifugation was used for separation based on the fact that at solvent density higher than the liposomes or lipoproteins, they float at rates based on their densities. In the present study, details of how to separate liposomes from LDL by ultra-centrifugation are presented.

2. Experimental section

2.1 Material

BCH, (cholesteryl 1,12-dicarba-*closo*-dodecaborane 1- carboxylate), BCH-Da (Cholesteryl-3β-12-octyl-1, 12-dicarbo-*closo*-dodecaorane-1-carboxylate), BCH-Db (Cholesteryl-3β-12-(1-octenyl)-1,12-dicarbo-*closo*-dodecaorane-1-carboxylate), BCH-Dc(Cholesteryl-3β-12-octadecyl-1,12-dicarbo-*closo*-dodecaorane-1-Carboxylate) have been synthesized and purified according to a method published by [Alanazi F. et al. 2003 and B. Ji et al 2002]. Cholesterol, Nile Red dye and Dipalmitoyl DL-α-phosphotidylcholine (DPPC) were purchased from Sigma Chemical Co. (St.Louis, MO). Liposomal formulations of these compounds have been prepared as described earlier [Alanazi F. et al. 2002]. Low-density lipoprotein from human plasma (5-7 mg/mL protein solution in 0.15 M NaCl; 0.01% EDTA; pH 7.4) was obtained from Sigma Chemical Co. (St.Louis, MO). All other chemicals were analytical grade. ICP instruments are VG Inductively Coupled Plasma Mass Spectrometer (ICP-MS) and The Thermo Jarrell-Ash 965 Inductively Coupled Argon Plasma (ICP-AES). Densities of the prepared solution were measured with a specific gravity balance (Henery Troemner, Philadephia,USA).

2.2 Analysis of boron compounds in liposomes using ICP-AES

The concentration of boron compounds in the liposomal formulations was measured by ICP-AES. About 0.5-1 ml samples were diluted to sufficient volume (about 2 ml). Deinonized water was used to formulate liposomes and for dilutions. The steps involved in the analysis of compounds in liposomal samples by ICP-AES are: the liposomal formulation was introduced to the system by using an electronic controlled pump. In the second step, the nebulization step, the sample was converted from liquid to

aerosol via nebuliser. In the third step, the desolvation /volatization step, water was driven off and remaining solid and liquid portions were converted to gases. In the fourth step, the atomization step, gas phase bonds were broken, and only atoms were present. In the fifth step, excitation/ emission step, atoms gain energy from collisions and emit light of a characteristic wavelength (Table 5.1). In the sixth step, the separation/ detection step, a grating disperses light that was quantitively measured. Two control steps were used to justify the analysis: Running a standard matrix containing most of the elements between the unknown samples to exclude any fluctuation of the data due to instrumental changes during the analysis and running boric acid (Boron reference solution, Fisher chemicals, New jersey, USA) as external standard for boron analysis.

2.3 Analysis of tissue samples in ICP-MS

Brain tissue samples were collected from normal tissue and tumor originated tissue. The concentration of boron compounds was measured using ICP-MS. Tissue samples were weighed and placed in a Teflon tube. Five hundred µl of nitric acid was added. The Teflon tube was capped and sited in the metallic jacket (Teflon bomb). It was, then, placed in oven at about 140 ^{o}C for more than 60 minutes. After leaving it to cool, digested solution was transferred to a plastic tube. The remaining traces of digested solution in the Teflon tube was washed with 500 µl of deionized water and transferred to the plastic tube to complete the sample (to be analyzed) to 1 ml. The steps involved in the analysis of BCH in tissue samples by ICP-MS were the same of ICP-AES, except the analyte was analyzed with a mass spectrometer. Because ICP-MS was used for single element analysis (i.e. boron), only boric acid as an external control was used.

2.4 Ultra-centrifugation for separating LDL from liposomal formulation

Liposomal formulation of BCH as a representative model of the other compounds was used in this study. Preparation of BCH liposomal formulation was described previously [Alanazi F. et al. 2003]. Size of the liposome was reduced using extrusion methods (LipoFast extrusion device, Avestin, Ottawa, Canada). Nicomp Submicron Particle Sizer was used to measure particle size (Model 380, Nicomp, CA). For determining the density of BCH liposomes, 200 µl of BCH liposomal formulation was added to 1.8 ml of phosphate buffer saline (PBS) (tube A). About fifty µl of Nile Red dye solution (100 µg/ml Nile Red dye in acetone) was placed in a glass tube (tube B) and dried using nitrogen gas. Tube A was added to tube B and the dye resuspended through the mixture *via* shaking. The density of the mixture was then adjusted to 1.21 g/ml with KBr. The mixtures were placed in centrifuge tubes (13 ml in size, n = 6). The rest of the tubes were filled with additional KBr solutions (densities; 1.03 g/ml, 1.04 g/ml, 1.05 g/ml, 1.063 g/ml, 1.07 g/ml and 1.08 g/ml for tube 6, 5, 4, 3, 2,and 1, respectively) (table 5.2). The tubes were centrifuged at 40,000 rpm at 10 ^{o} C for 1 hr, and the rotor was allowed to stop without using the brake (SW 40 rotor). Also, the effect of liposome size on the floating behavior was studied. Large liposomes were prepared with the same procedure as small liposomes except their size was not reduced by the extrusion method. Two hundred µl of large liposomes (MLV) and small liposomes went through the same conditions described for tube 3 (the bottom density was 1.21 g/ml and the top density was 1.063 g/ml) (Table 5.3). In addition, large liposomes and LDL were compared in terms of floating behavior when they were treated similarly. Two hundred µl of BCH liposomal formulation and 100 µl of LDL were centrifuged at similar conditions to that of tube 3

(Table 5.4). All tubes are exposed to UV light to make the visualization of the Nile Red dye complex with either liposomes or LDL possible.

3. Results and Discussion

Cholesteryl carborane conjugates were synthesized in our laboratory to mimic native cholesterol esters as novel anticancer compounds [Alanazi F. et al. 2002 and B. Ji et al 2002]. As these compounds share similar chemical and physical characteristics with native cholesteryl esters, they may be effectively transferred into LDL in biological fluids and thus, follow the LDL pathway for targeted delivery utilizing the elevated LDL receptor expression on tumor cells [Alanazi F. et al. 2003].

Finding a good analytical method for analyzing these compounds in liposomal formulations and in biological samples was essential to examine their potential targeting efficiency. Thus, Inductively Coupled Plasma Spectrometry (ICP) was used to analyze these compounds based on indirect boron analysis. In the present study, the usefulness of ICP as analytical techniques for such compounds in liposomal formulation and brain tissue is presented.

Elements are often measured by ICP-AES in units of ppm metal (part-per-million) based on mass:

$$\text{ppm metal} = \frac{\text{g metal}}{10^{6}\ \text{g sample}} = \frac{\mu\text{g metal}}{\text{gm of sample}}$$

An ICP requires that the elements, which are to be analyzed, be in solution. The samples are mostly aqueous samples (water as solvent or suspending media) with density of 1 gm / ml. Therefore, it could be presented as follows:

$$\text{ppm metal} = \frac{\mu\text{g metal}}{\text{gm of sample}} = \frac{\mu\text{g metal}}{\text{ml of sample}}$$

Elements are often measured by ICP-MS in units of ppp metal (part-per-pillion):

$$\text{ppp metal} = \frac{\mu\text{g metal}}{\text{kg of sample}} = \frac{\mu\text{g metal}}{\text{Liter of sample}}$$

Results from analyzing boron compounds in liposomal formulation are presented in Figure 5.1. When ICP-AES was used to measure boron compound concentrations in liposomal formulation, the method was simple and easy since it doesn't require drug extraction from liposomes (i.e. no pre-analysis treatment). In addition, It was very fast and it had a reasonable limit of detection for boron (0.5 µg / ml) and phosphorous (0.09 µg / ml). The method was linear in the range of 50-0.5 µg of B / ml and the linearity correlation coefficient was 0.9994 (Fig 5.5). The determination of phospholipids in liposome suspension is necessary to estimate the loss or dilution of phospholipids. Many methods, such as the spectrophotometric method based on digestion of phospholipid molecules with acid, have been developed for the determination of phospholipids [New R.R.C, 1990]. However, these methods are laborious and time-consuming. Notably, only two reports on the determination of phospholipids in liposomal suspension by ICP-AES were found [Kamidate T et al., 1996 and Suita T et al., 1999]. It has been shown that the sensitivity and precision of phospholipid analysis by ICP-AES are equivalent to those in the spectrophotometric methods. In the present study ICP-AES, therefore, was used to obtain boron compound concentrations as well as phospholipid concentration at the same

time and on the same run. This finding provides a feasible method for analyzing element-based compounds encapsulated in phospholipid vesicles. Results of the determination of boron compound concentrations in brain tissues using ICP-MS are presented in Table 5.5. The detection limit of boron analysis by ICP-MS is at least three orders of magnitude lower than of that of ICP-AES (1 ng B/ ml). The method was linear in range of 500-0 ng B/ ml and the linearity correlation coefficient was 1 (Fig 5.6). While the methods has the same advantage of ICP-AES, it needs the tissue samples to be digested which increases time of analysis (sample preparation). Using ICP for quantitative analysis is limited by contamination of samples with exogenous boron and phosphorus. Using glassware resulted in increased boron concentration by two to three fold when samples were measured with ICP-MS (data is not presented). Using non-deionized water, also, had similar effect. Therefore, in all studies plastic tubes and deionized water were used to overcome these limitations. Dilution of the samples is required since the viscous liquid may result in clogging of the instrumentation.

Overexpression of low-density lipoprotein (LDL) receptors occurs in several cancer cell lines and offers a unique strategy for drug targeting [Graziani SR et al.,2002]. Liposome has been shown to increase drug loading into LDL [Kader A ET AL., 1998]. One of the objectives of our study after synthesizing the cholesterol-based compounds and incorporate them in a suitable drug delivery system (liposomes) is to examine the transfer of these compounds from liposomal formulation to low density lipoprotein (LDL) *in vitro* with various incubation media. To conduct this study it was necessary in our laboratory to developed a method to separate liposomal formulation from LDL after the incubation period.

To separate LDL from liposomes, it is sensible to look at the existing methods to separate lipoproteins into their various classes and applying one of these methods to separate LDL from liposome. The technique most commonly used in clinical research laboratories is ultracentrifugation. This technique has been used to separate liposomes from lipoproteins [Kader A et al., 1998, Wasen et al., 1997and Wasen et al., 1993].

Liposomal Nystatin (particle size 321±192 nm) was recovered from solution at density values range of 1.21 to 1.25 g/ml (on the bottom) and LDL was recovered at a density 1.063 g/ml (on the top)[Wasen et al., 1997]. Also, it has been reported (A.Kader et al, 1998) that doxorubicin liposomal preparation floated on the top of solution with density 1.006 g/ml and LDL remained at the bottom. The size of the liposomes was not clarified (≈200 nm). Not only did liposomes float differently according to these reports, but also the processes were time consuming (20 hrs). In the present study, a description of how to separate liposomes from LDL by an ultra-centrifugation technique is presented.

The result of determining the density of BCH liposomes (small liposome) is shown in Figure 5.2. Nile Red complex with liposomes gave a fluorescent pink color under UV light. Liposomes were recovered on the top of tube 1 and 2. This indicated that the liposomes had a density less than 1.07 g/ml; but in tubes 4, 5 and 6, liposomes were retained at the interface between the two densities (the top densities: 1.05-1.03 g/ml and the bottom density: 1.21 g/ml). This indicated that the liposomes had a density greater than 1.05 g/ml. Results from tube 4 indicated that liposomes were distributed throughout the 1.063 layer. This showed that the liposomes had a density of 1.063 g/ml. In general, LDL has a density of 1.019-1.063 g/ml. Therefore; the same experimental conditions of tube 3 (top density 1.063 and bottom density is 1.21) were used to find the density of

LDL. The results showed that LDL was retained at the interface between the two densities. Thus, separation of LDL from liposomes isn't complete because they were overlapped on the bottom fraction of the 1.063 layer. To separate them efficiently liposomes should float on the top of the 1.063 density solution and LDL should be retained between the two solutions. The only factor that can be modified to adjust the floatation is the size of liposome. Also, no report has been found in the literature on the effect of liposome size on improving the LDL–liposome separation. The effect of liposome size on floating behaviors was studied. The result is shown in Figure 5.3 and Table 5.3. Large liposomes (tube a, 1.63 μm) were recovered on the top of the 1.063 layer. On the other hand, small liposomes (tube b, 36 nm) were distributed throughout the 1.063 layer. This can be explained based on isopycnic theory (also termed density gradient centrifugation and sedimentation equilibrium centrifugation). The separation is based on particle density. When samples were underlaid at the bottom of the tube, liposomal particles floated to their corrected densities during centrifugation (i.e molecules move to the position in the gradient where their density is the same as the gradient material). Small liposomes had a grater phospholipid to water ratio (large volume of liposomal phospholipid) than large liposomes [Versulis et al, 1999]. Consequently, the small liposomes are denser than the large liposomes. Mohannad et al.,(2002) reported similar phenomena with submicron emulsions (SE); the largest particle size SE were recovered first and then the smallest ones. To confirm the success of this method to separate LDL from liposomes, large liposomes and LDL were ultracentrifuged in separate tube under the same conditions (Table 5.4). Liposomes were

recovered on the top of the 1.063 layer and LDL was retained at the interface between the two density layers (Figure 5.4).

In conclusion: Inductively Coupled Plasma Spectrometry (ICP) was used to analyze these compounds indirectly based on boron analysis. ICP-AES was used to obtain boron compound concentrations as well as phospholipid concentration at the same time and on the same run. This finding provides a feasible method for analyzing element-based compounds encapsulated in phospholipid vesicles. ICP-MS was used for analyzing BCH in brain tissue samples in brain distribution study. Details of how to separate liposomes from LDL by ultra-centrifugation technique are presented. Not only time, speed, and density gradient but also the size of the liposome have effects on the separation using centrifugation method. This method gave a good separation between LDL and liposome and will be used in future studies.

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5. Figures and table legends:

Table 5.1 Properties and ICP-AES limit of detections of boron and phosphorus

Table 5.2 Density gradient used for determination density of BCH liposomal pellets

Table 5.3 Density gradient used for comparison between large and small liposome

Table 5.4 Density gradient used for comparison between LDL and large liposome

Table 5.5 Results from analyzing BCH in brain tissue sample by ICP-MS

Figure 5.1 Results from analyzing boron compounds in liposomal formulation by ICP-AES

Figure 5.2 Results of determining density of BCH liposomal pellets

Figure 5.3 Results of comparison between large liposome (a) and small liposome (b)

Figure 5.4 Results of comparison between LDL (a) and large liposome (b)

Figure 5.5 Linearity plot of ICP-AES

Figure 5.6 Linearity plot of ICP-MS

Table 5.1

Element	Mole. wt	Detection limits (ppm)	Wavelength (nm)
Boron (B)	10.81	0.50-600	249.68
Phosphorus (P)	30.97	0.09-900	178.29

Table 5.2

Tube #	Top ρ (gm/ ml)	Bottom ρ (gm/ ml)	Liposome ρ (gm/ ml)
1	1.08	1.21	< 1.08
2	1.07	1.21	< 1.07
3	1.063	1.21	= 1.063
4	1.05	1.21	> 1.05
5	1.04	1.21	> 1.04
6	1.03	1.21	> 1.03

Table 5.3

Tube #	Type of liposome	Particle size	Density (g / ml)
a	Large	1.6 μm	> 1.063
b	Small	0.034 μ m	=1.063

Table 5.4

Tube #	Component	Density (g /ml)
a	LDL	1.063-1.2
b	Large liposome	<1.063

Table 5.5

Tissue type	Time [b]	
	2 hr	6 hr
Tumor [a]	27.7 ± 5.54	12.5 ±2.5
Normal [a]	5.07 ± 1.014	7.99 ± 1.598

(a)- concentrations are in µg /gm of tissue

(b)- time after injection of BCH intracerebrally

Figure 5.1

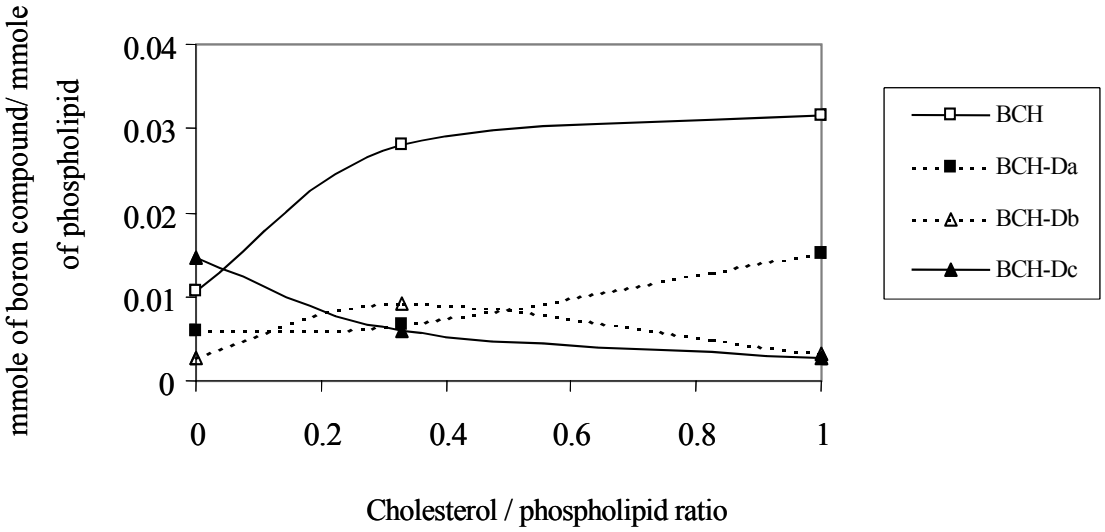

mmole of boron compound/ mmole of phospholipid
0.04
0.03
0.02
0.01
0
0
0.2
0.4
0.6
0.8
1
Cholesterol / phospholipid ratio
BCH
BCH-Da
BCH-Db
BCH-Dc

Figure 5.2

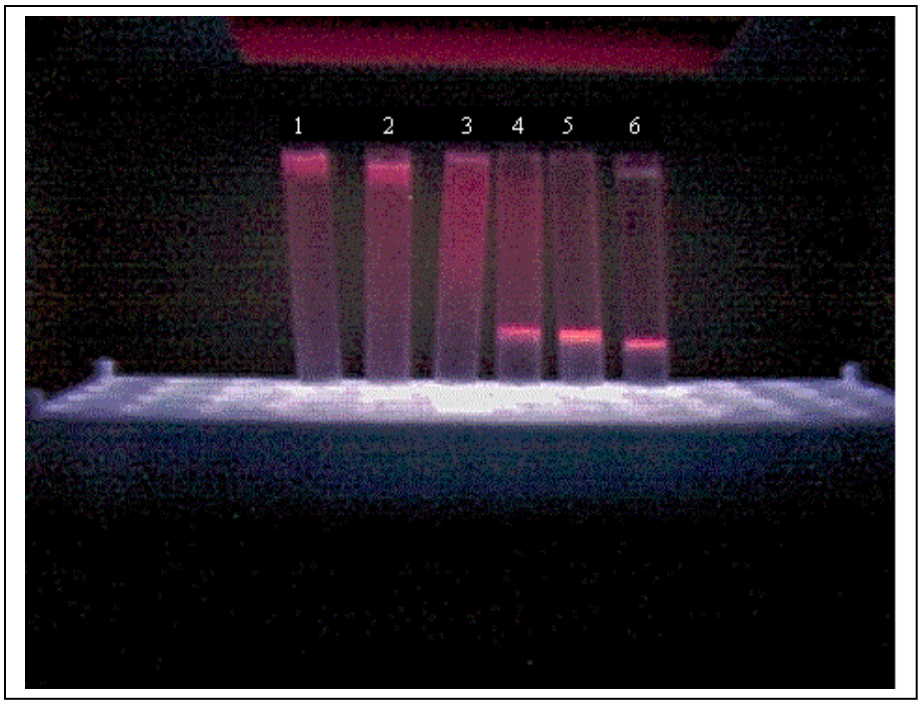


Figure 5.3

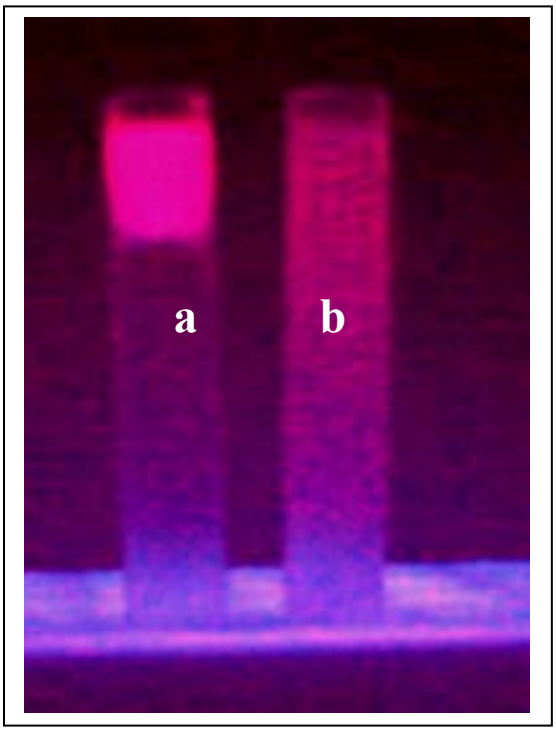


Figure 5.4

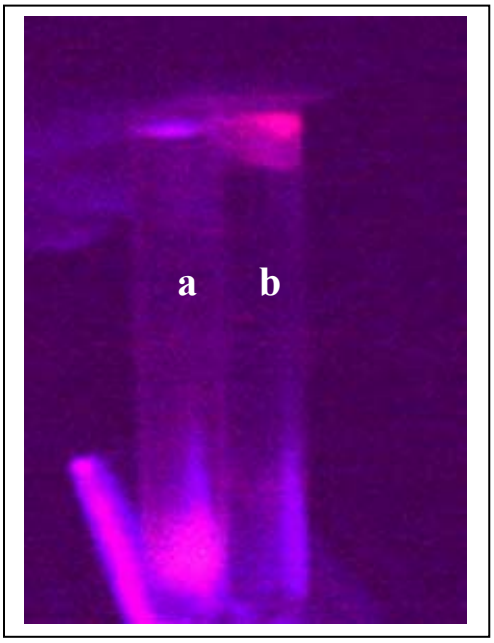


Figure 5.5

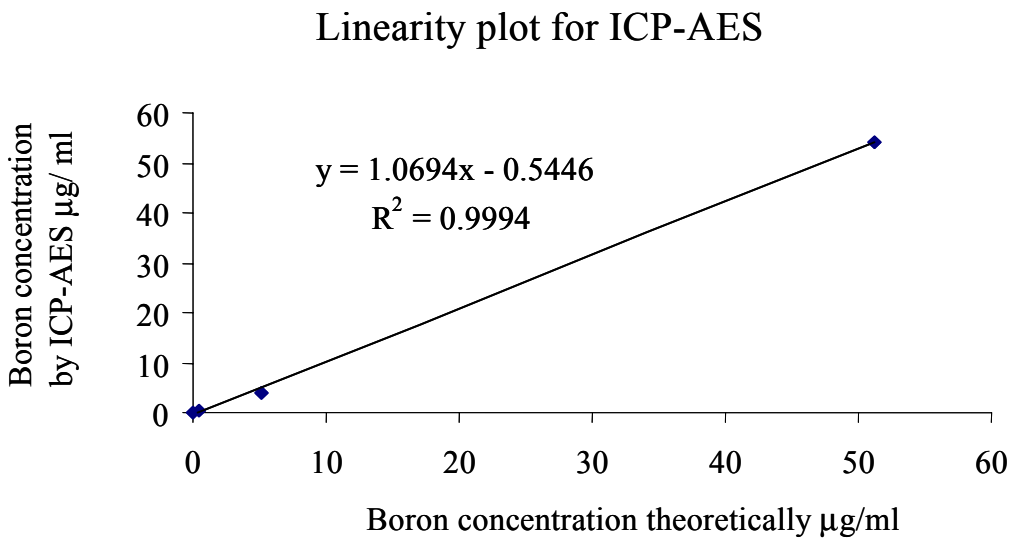
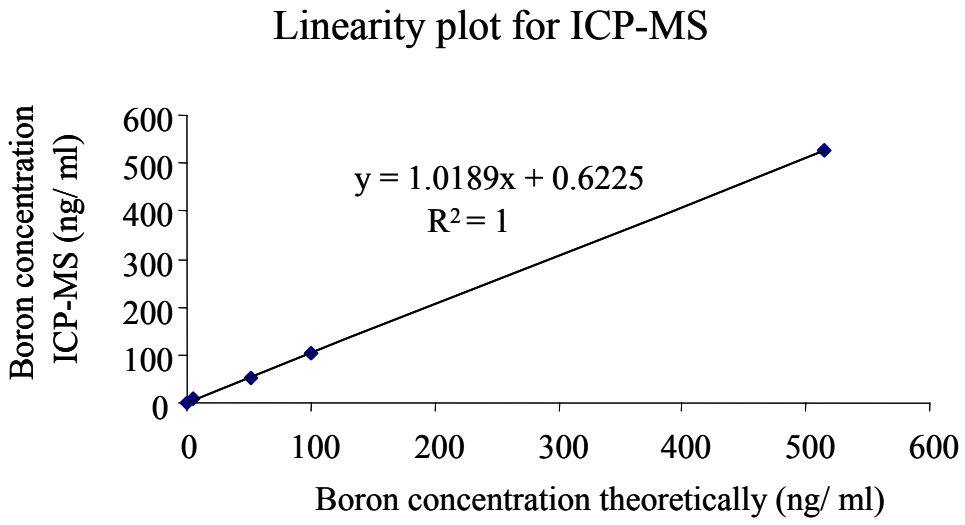

Linearity plot for ICP-AES
Boron concentration by ICP-AES µg/ ml
y = 1.0694x - 0.5446
R2 = 0.9994
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Boron concentration theoretically µg/ml

Figure 5.6


Linearity plot for ICP-MS
Boron concentration ICP-MS (ng/ ml)
600
500
400
300
200
100
0
y = 1.0189x + 0.6225
R² = 1
0
100
200
300
400
500
600
Boron concentration theoretically (ng/ ml)

CHAPTER 6

POLY-PEPTIDE ARTIFICIAL LIPOPROTEIN AS A NOVEL DNA VACCINE DELIVERY SYSTEM

[1] Alanazi Fars and D.Robert Lu. To be submitted to Journal of Pharmaceutical Research

Abstract:

An artificial lipoprotein nanoemulsion, which consisting of palmitoyl-polylysine, trioline, phospholipids and cholesterol, was characterized as a novel DNA vaccine carrier for rabies glycoprotein **Methods:** Phospholipid nanoemulsions resembling the chemical composition of human lipoprotein were prepared by a sonication method. N-alkylation was used to conjugate the palmitoyl side chain to poly-L-lysine. The artificial lipoprotein was constructed by assembling palmitoyl-poly-L-lysine (p-PLL) onto phospholipid nanoemulsion particles. The fatty chains on the poly-peptide serve as an anchor to interact with the phospholipid chains. Rabies plasmid DNA vaccine (7 kb) and plasmid DNA (without rabies gene as a control) were incorporated onto the artificial lipoprotein through electrostatic interactions. Agarose gel electrophoresis (0.4 %) and zeta potential measurements were conducted to examine the charge balance on the particle surface at different experimental stages. Transfection efficiency was measured by flow cytometry **Results:** The phospholipid nanoemulsion was prepared with a multiple-step procedure, producing a particle sizes of 54.3 ± 24 nm. Attachment of p-PLL onto the nanoemulsion formed stable complexes and electrophoretical measurement showed that upon addition of p-PLL, the complex reverses direction compared to the original nanoemulsion. Zeta Potential values of the artificial lipoprotein/plasmid DNA complex were -43.67, -18.35, 12.51, 16.86, 21.29 mV and its mobility was -3.41, -1.43, 0.98, 1.32, 1.66 (μ/s)(V/cm) for 0:1, 0.125:1, 0.25:1, 0.5:1, 1:1 p-PLL: triolein ratio, respectively (the amount of DNA was kept constant at 2 μg/50μl of nanoemulsion). These results indicated that the surface net charge can be balanced based on p-PLL quantity. Results from an *in vitro* cell culture study using the artificial lipoprotein/plasmid DNA encoding rabies glycoprotein showed

sufficient transfection in human glioma cell line SF-767 and comparable transfection with commercial Lipofectamine™ system. **Conclusion:** Artificial lipoprotein can be constructed by integrating phospholipid nanoemulsion and palmitoyl-poly-L-lysine. These artificial lipoprotein particles efficiently carried rabies plasmid DNA vaccine resulting in sufficient transfection in tumor cells.

Keywords: DNA, vaccine delivery, nanoemulsion, transfection efficiency, palmitoyl poly-L-lysine, rabies, lipoprotein

Introduction:

DNA vaccine is a simple ring of DNA containing a gene encoding an antigenic protein. DNA vaccine uses enough genes from the virus that encode the particular proteins against which a protective or therapeutic immune response is desired, without introducing unnecessary toxic proteins or viral components. Vaccination with DNA came from the discovery by Wolff et al. (1990) when they found that injection of naked DNA into muscle of mice resulted in transfection and expression of the reporter gene (1). Accordingly, researchers have explored DNA encoded antigens as a novel vaccination to cover a large number of pathogens (2). DNA vaccines have many advantages over inactivated virus vaccines because the production of DNA is much easier and less expensive. DNA vaccine, also, can initiate both humoral and cellular immune responses without being pathogenic like the attenuated organisms (3).

DNA vaccine is similar to gene therapy requiring a carrier or vector to deliver the exogenous genes *in vivo*. Naked DNA could be delivered either by organisms or DNA

based carriers. Use of live or attenuated organisms like bacteria (4,5) or viruses (6) as DNA vectors are considered too risky due to their poor safety (2). DNA based carriers are basically capable of binding and condensing DNA. They are mostly cationic lipids or liposomes (bearing cationic polar head groups) that electrostatically interact with negatively charged phosphate backbone of DNA (7). The main draw back of these systems is their significant cellular toxicity (8-11).

Concerns about viral-induced immune responses, the risk associated with replication-competent viruses, and toxicity of cationic lipids have stimulated efforts toward the development of alternative gene delivery systems such as lipoprotein (11-14) Recently, we have developed an artificial lipoprotein delivery system as a novel gene carrier (11). Our vector carried plasmid DNA and the reporter gene was expressed in glioma cells. Since one of the main requirements of the new gene delivery system is its ability to carry genetic material into the cells with low cytotoxicity, cellular toxicity of the new artificial lipoprotein gene vector was examined and compared to that of Lipofectamine ^{TM} 2000 (cationic liposome). The artificial lipoprotein showed 50 % less cellular toxicity compared to Lipofectamine.

In this paper, based on the encouraging results of being a less cytotoxic gene carrier, the novel artificial lipoprotein, consisting of palmitoyl poly L lysine (p-PLL), trioline, phospholipids and cholesterol, was characterized as a novel DNA vaccine carrier. The rabies glycoprotein genome was used as a model of the naked DNA vaccine. The surface charge parameters (zeta potential and mobility) of artificial lipoprotein / plasimd DNA encoding rabies glycoprotein was determined. The effect of varying the ratio of lipophilic cationic poly-L-lysine to the nanoemulsion on transfection efficiency

(expressing the rabies virus glycoprotein in human glioma cell line SF767) was studied. In addition, a comparison study of the capability of transfecting a human glioma SF-767 cell line between the artificial lipoprotein and Lipofectamine^{TM} 2000 (a commercial liposomal formulation for transfection) was conducted.

Material and method:

Material:

Egg yolk phosphatidylcholine (99%), cholesterol (99%), triolein, cholesterol oleate (98%), L-α -lysophosphatidylcholine (99%), poly-L-lysine hydrobromide (MW 48100 Dalton based on viscosity), and Palmitoyl chloride (98%) were purchased from Sigma (St. Louis, MO USA). Electrophoresis grade agrose was purchased from Bio-Rad (Hercules, CA, USA). Lipofectamine^{TM} 2000 was purchased from Invetrogen Corporation (Carlsbad, CA, USA). All others chemical were analytical grade obtained from Sigma or J.T. Baker (Philipsburg, NJ, USA).

Preparation of emulsion:

The nanoemulsion (NE) was prepared as prescribed by (Shawer et al 2002) (15). In brief, the lipid components of oily phase [triolein (70 %), egg yolk phosphatidylcholine (22.7%), lysophosphatidylcholine (2.3%), cholesterol oleate (3%) and cholesterol (2%)] were dissolved separately in chloroform and mixed thoroughly. Organic solvent was removed by a stream of nitrogen gas. NaCl solution (2.4 M) was added to dried lipids in ratio 1:10 (ml of 2.4 M NaCl to total weight of lipids in mg). The mixture was sonicated under nitrogen flow for 30 min using a Model 450 Sonifier (Branson Ultrasonics Corporation, Danbury, CT) with a duty cycle dial setting of 90% of the output of 40% watts. The temperature was maintained at 55^{o} C during sonication. The

prepared emulsion was then passed through an emulsifex B3 device (Avestin, Ontario, Canada) at pressure of 70 psi for 10 times to reduce the particle size of the nanoemulsion. The NE was dialyzed against phosphate buffered saline (PBS, pH 7.4) using Spectra/ pro 2 molecular porous membrane tubing with molecular weight cut-off 6000-8000 dalton (Spectrum Medical Industries, Inc. Houston, Texas). Submicron particle Sizer Model 370 (Nicomp Paticle Sizing System, San Barbra, CA) was used to measure particle size distribution. The sample was diluted automatically by the instrument and the measurements were obtained at 25^{o} C.

Lipidization of poly-L-lysine

Lipidization of Poly-L-Lysine (PLL) was performed as described by (Kim et al., 1997) (16). In brief, 17.5 mg palmitoyl chloride was dissolved in 2 ml of dioxane. PLL (50 mg) was dissolved in solution of 2 ml dimethyl sulfoxide (DMSO) and 200 µl NaOH (1N). The first solution was added slowly to the latter. The mixture was allowed to react for 24 hr at room temperature. The reaction was stopped and the mixture was filtered (Whatman filter papers) and added to excess diethyl ether, which resulted in precipitation of palmitoyl Poly-L-Lysine (p-PLL). Precipitated polymer was redissolved in DMSO and reprecipitated in diethyl ether. The product was purified by dialysis against distilled water overnight at temperature 4 ^{o} C, followed by lyophilization. The resulting polymer was verified by H-NMR (d-DMSO) and it was characterized by agarose gel electrophoresis using Nile Red as the fluorescent dye. Agarose gel (0.4 %) was prepared in TAE buffer (40 Mm Tris-acetic acid, 1 mM EDTA, pH 8.0). Twenty µl of PLL solution (1 mg/ ml) and 20 µl of p-PLL solution (1mg/ ml) were added to dried Nile Red Dye and incubated for 30 min. Six µl of glycerin was added to increase the density of the sample and then

the samples were loaded into the well of the agarose gel. Horizontal mini-gel system (CBS Scientific Company Inc, CA, USA) was used to run the electrophoresis and it was carried out for one hour at 90 volts, 20 mA at room temperature. The mobility of the samples in the electric field was visualized by ChemiImager ™ System (Alpha Innotech Corporation, San Leandro, CA).

Incorporation of P-PLL into nanoemulsion

Fifty µl of NE was diluted with 0.2 ml PBS solution (in 1.5 ml microcentrifuge tube, n=5). Various amounts of palmitoyl poly-L-lysine (p-PLL) were incubated for one hour at 37 ^{o} C with diluted NE based on the weight ratio of p-PLL to triolein (the major component of NE). The weight ratios were 0:1, 0.125:1, 0.25:1, 0.5:1, 1:1 and 2:1 of p-PLL to triolein. After the incubation, samples' electrophoretic mobility was examined using agarose gel (0.4 %) as described above. Zeta potential and mobility of the p-PLL/ NE particles were measured by Submicron Particle Size Analyzer 90Plus (Brookhaven Instrument Corporation, Holtsville, NY, USA). All samples were subjected to dilution with HPLC water before the measurement. HPLC water was used as baseline control. Results were recorded with built-in PC computer system.

Assembly of the complex of nanoemulsion, p-PLL and rabies plasmid DNA

Rabies DNA glycoprotein (PC DNA_3 RG) was generously given by Dr Fu's lab (Pathology Department, School of Veterinary Medicinal, UGA). NE (50 µl) was diluted with 0.2 ml PBS solution (in 1.5 ml microcentrifuge tube, n= 5). Various amounts of palmitoyl poly-L-lysine (p-PLL) was incubated for one hour at 37 ^{o} C with diluted NE based on the weight ratio of p-PLL to triolein. The weight ratios were 0:1, 0.125:1, 0.25:1, 0.5:1, 1:1 of p-PLL to triolein. After the incubation, 2 µg of $PCDNA_3$ RG

(plasmid DNA) was added and incubated for 15 minutes at room temperature. Twenty µl of samples were loaded onto 0.4 % agarose gel and electrophoresis was carried out as described above. Zeta potential and mobility of the p-PLL/ NE particles were measured by Submicron Particle Size Analyzer 90Plus (Brookhaven Instrument Corporation, Holtsville, NY, USA).

Transfection of Rabies plasmid DNA

Transfection study was carried out using human glioma cell line SF-767 obtained from the tissue bank of brain tumor research center (University of California-San Francisco, CA, USA). The cells were grown in 75 cm^2 plastic cell culture flasks containing 13 ml of Eagle's Minimum Essential Media (EMEM) (A CAMMBREX Com., Walkersville, Maryland) supplemented with 10% Fetal bovine serum (FBS), 100 unit/ ml penicillin, and 100 µg/ ml streptomycin (Sigma Chemicals, St. Louis, MO). Cells were allowed to grow for 10 days to maintain the exponential growth. Transfection was performed in 6-well culture plates (35 mm in diameter) and 3 x 10^5 cells were seeded in each well. Before cells were transfected, they were allowed to grow for 24 hr. For transfection, NE (50 µl) was diluted with 0.2 ml PBS solution and incubated with palmitoyl poly-L-lysine (p-PLL) for one hour at 37 ^{o} C. After the incubation, 2 µg of $PCDNA_3$ RG (plasmid DNA) was added and incubated for 15 minutes at room temperature. NE /p-PLL/DNA complex was incubated with cells for 12 hr at 37 ^{o} C, in a 5 % CO_2 atmosphere after 1 ml of EMEM (without FBS) was added. One ml of EMEM containing 20% FBS was added to the transfection media and incubated for 36 hr.

The transfected cells were rinsed three times with phosphate buffered saline (PBS) and then fixed in a acetone solution (80% v/v, acetone: water) for 10 minute in –20

^{o} C freezer. Fixed cells were air-dried and stored in refrigerator pending indirect immunofluorescence (IFA) detection. For IFA, cells were rinsed three times with PBS and a 1:50 dilution of a rabies gG-specific mouse monoclonal antibody (Accurate Chemical and Scientific Corporation, Westbury, NY) was added. Cells were incubated for 1 hr at 37 ^{o} C and then rinsed three times with PBS (3-4 minutes each). A 1:100 dilution of a secondary goat anti-mouse fluorescein (FITC)-conjugated antibody (Accurate Chemical and Scientific Corporation, Westbury, NY) was added and cells were incubated for 1 hr at 37 ^{o} C. Cells were rinsed three times with PBS, air-dried, and examined using an Olympus fluorescence microscopy (Olympus American Inc, Melville, NY). Lipofectamine was used as positive control and p-PLL/ NE carrying PC DNA_3 (plasmid only without rabies gene) was incubated with cells and it was a used as negative control.

Evaluation of transfection efficiency

Transient expression of rabies glycoprotein was determined using flow cytometric evaluation as described above. Binding of rabies gG-specific mouse monoclonal antibody was revealed by secondary staining using secondary goat anti-mouse fluorescein (FITC)-conjugated antibody. Since flow cytometry runs cells in suspension, expressed cells were washed and suspended in phosphate buffered saline (PBS). Suspended Cells were incubated with the first antibody (1:50 dilution) for 1 hr. Cells were centrifuged, media was discarded, and cells washed with PBS. Cells were incubated with the secondary antibody (1:100) for 1 hr, centrifuged, washed with PBS, and resuspended in PBS. Twenty thousand cells were examined in FACS Calibur flow cytometry (Becton Dickimson, San Jose, CA) device using FLOWJO software (Tree Star Inc, San Carlos,

CA). Clumps and debris were excluded using forward and side-scatter windows. Transfection efficiency was calculated based on the percentage of the positive cells that expressed rabies glycoproteins in total number of cells. Lipofectamine was used as comparable trasfecting agent. DNA to Lipofectamine ratios (w/v) were 1:0.5, 1:2, 1:3 and 1:5 according to the manufacture protocol for optimization of the transfection. p-PLL/ NE carrying PC DNA_3 (plasmid only without rabies gene) was incubated with cells and it was used as negative control. In addition, p-PLL / PC DNA_3 RG (without nanoemulsion) was used as positive control and the quantity of p-PLL added was similar to that of ratio 1:1 and 0.5:1 (p-PLL: triolein)

Result:

Preparation of emulsion:

Milky transparent emulsion was prepared using a sonication method. Temperature was kept at 55^{o} C, above the phase transition temperature of lipids to ease emulsification. Sonication was conducted under a nitrogen environment to reduce lipid oxidation during emulsification. The size of the emulsion was reduced using Emulsifex B3 device (Avestin, Ontario, Canada). The mean particle size (measured as number weighted) was reduced to 54.3 ± 24.8 nm (Fig 6.1). Zeta potential of nanoemulsion was measured at – 43.67 ± 1.38 mV and its mobility, –3.4 ± 0.11 (μ / s)(V/cm). The negative surface charge of NE particle made it an unstable vector for DNA due to the negative charge of DNA resulting in electrostatic repulsion.

Lipidization of poly-L-lysine

Characterization of the palmitoyl poly-L-lysine polymer was performed by ^{1} H-NMR using a Brucker-400 AMX spectrometer and result was ^{1} H-NMR (400Hz, d-

DMSO) δ 0.82 (d, 3H, CH_2-$\underline{CH_3}$, J = 5.6 Hz), δ 2.14 (CH_2-$\underline{CH_2}$ –CO_2-). Zeta potential of synthesized polymer (p-PLL, Fig 6.2B) and poly-L-lysine (PLL, Fig 6.2A) were 40.64 ± 3.51 and 37.65 ± 3.43 respectively. The chemical modification of poly-L-lysine didn't change the surface charge of this polymer indicating it is suitable in carrying DNA. Since p-PLL and PLL are positively charged, they would move toward cathode (-ve) (Fig 6.3). The difference between p-PLL and PLL can be clearly seen in the picture of electrophoresis. Hydrophobic poly-L-lysine had fluorescence emission while unmodified poly–L-lysine didn't emit any fluorescence. Thin layer chromatography (TLC, polar phase) was used to identify the product using Ninhydrin, a colorimetric reagent to detect amino acids. However, both p-PLL and PLL didn't moved from starting point due to their high molecular weight and high polarity even though different mobile phase systems were used.

Incorporation of p-PLL into nanoemulsion particles

NE has a negatively charged surface and its zeta potential was – 43.67 ± 1.38 mV. Results from agarose gel electrophoresis (Fig 6.4) showed that it was moved to the anode (Lane 1). Incorporation of p-PLL into the nanoemulsion particle resulted in changing the surface charge of the NE. Results from gel electrophoresis showed that the incorporation neutralized the surface charge which resulted in retardation of the electrophoretic mobility of NE / p-PLL complex in the electrical filed (Lane 2 to Lane 6). The result indicated that p-PLL could be successfully incorporated into the NE.

Assembly of the complex of nanoemulsion, p-PLL and rabies plasmid DNA

Since the net charge on DNA is negative, there will be electrostatic repulsion between DNA and NE. Therefore, incorporation of p-PLL into the NE to make the

surface charge positive would make p-PLL/ NE electrostatically attractive with DNA. It can be seen in Fig 6.7 that plasmid DNA (Lane 1) migrated toward the anode due to its negative charge. In Lane 2 plasmid DNA was incubated with p-PLL and no DNA molecules were observed because p-PLL condensed DNA molecules. In Lane 3 plamid DNA was incubated with NE only and DNA molecules migrated toward the anode indicating unsuccessful interaction between DNA and NE due to the electrostatic repulsion. Lanes 4 to 7 were complexes of NE with different amount of p-PLL and 2 μg DNA (the ratio of p-PLL to triolein was 0.125:1, 0.25:1, 0.5: 1, and 1:1, respectively). These lanes did not show any fluorescence emission indicating that DNA molecules were condensed by p-PLL/ NE vector (i.e. DNA was tightly held by the complex).

Because it is important in cellular transfection, the surface charge of the p-PLL/ NE / DNA complex was further determined. Zeta Potential of the artificial lipoprotein/ plasmid DNA complex was -43.67, -18.35, 12.51, 16.86, 21.29 (Fig 6.5) and its mobility (Fig 6.6) was -3.41, -1.43, 0.98, 1.32, 1.66 for 0:1, 0.125:1, 0.25:1, 0.5:1, 1:1 p-PLL: triolein ratio, respectively (the amount of DNA was kept constant at 2 μg/50μl of nanoemulsion).

Transfection of Rabies plasmid DNA

The expression of rabies glycoprotein was visualized under fluorescence microscopy (Fig 6.10). Transfection efficiency of the artificial lipoprotein and Lipofectamine^{TM} 2000 (a commercial cationic lipid formulation) in human glioma SF-767 cell line is presented in Figure 6.9. The transfection efficiency of the negative control (p-PLL/ NE / plain plasmid DNA) is 0.30 ± 0.23. The transfection efficiency of the positive controls (p-PLL/ DNA) was 8.67 ± 0.51(L1) and 6.86 ± 0.1 (L0.5) when the

quantity of p-PLL added was similar to that of ratio 1:1 and 0.5:1 (p-PLL: triolein), respectively. The transfection efficiency for the p-PLL/ NE / plasmid DNA complex (carrying rabies gene) was 6.76 ± 0.32, 6.25 ± 0.21, 36.33 ± 8.08 and 96.30 ± 0.14 for p-PLL: triolein ratio of 0.125:1, 0.25:1, 0.5:1, 1:1, respectively. The transfection efficiency for Lipofectamine^{TM} 2000 was 4.46 ± 0.23, 5.39 ± 0.43, 23 ± 3.85 and 3.5 ± 0.59 for DNA / lipofectamine ratio of 1:0.5, 1:2, 1:3 and 1:5, respectively.

Discussion:

DNA based vaccine is composed of nucleic acid material encoding an antigenic protein, which can be synthesized *in vivo* for induction of antigen-specific immune responses. Experiments in animal models have shown the possibility of producing effective prophylactic DNA vaccines against various viruses, bacteria and parasites (17). However, successful DNA vaccine immunization is largely dependent on development of delivery systems that can efficiently and safely introduce the exogenous genetic material into the target cells.

Owing to the poor safety of using viruses and bacteria as a gene vaccine carrier (2) and the cytotoxoicity of DNA/cationic liposome complexes (8-11), many attempts were conducted to find an efficient and safe DNA vaccine carrier with less cellular toxicity. Lipoproteins have drawn attention for being drug delivery systems for many years (15, 18). Kim et al. (1997) developed a terplex system, stearyl-poly-L-lysine/ low-density lipoprotein, as novel gene delivery vector, which showed promising results (16). Hara et al. (1997) utilized the hydrophobic core of the chylomicron remnant to solubilize DNA /cationic lipids complex as a novel gene targeted delivery system to liver. Recently, artificial lipoprotein system was developed in our lab as a novel gene delivery system

(11). The artificial lipoprotein system mimics the natural lipoprotein in human body, which is composed of 70 % triglyceride (triolein), 25 % phospholipid (22.7% egg yolk phosphatidylcholine and 2.3% lysophosphatidylcholine), 3% cholesterol ester (cholesterol oleate) and 2% cholesterol. The new delivery system was capable of transfecting human glioma cell line *in vitro* with much lower toxicity compared to Lipofectamine ^{TM}. Cell viability after four days of transfection was 75 % and 24 % for p-PLL/ nanoemulsion/ DNA and Lipofectamine ^{TM}, respectively. Based on the encouraging results in the cytotoxicity study, evaluation of the new artificial lipoprotein gene carrier for DNA based vaccine was accomplished in this paper.

Our system is an oil-in-water nanoemulsion (NE) stabilized by an interfacial film of natural phospholipids. Theoretically, presence of a hydrophilic polar head group (negatively charged phosphate group) on the phospholipid at the oil-water interface makes the NE carry a negative charge at neutral pH. Results from gel electrophoresis (Fig 6.4, Lane 1, the movement toward anode) and measurement of zeta potential (–43.67 ± 1.38 mV) and mobility (–3.4 ± 0.11 [μ / s][V/cm]) confirmed that the net charge of the NE was negative. DNA is a double helix polymer of nucleotides and it carries negative charge too. Thus, the NE is considered an unsuitable carrier for DNA due to electrostatic repulsion (Fig 6.7, Lane 1, DNA was not held by nanoemulsion). Polymers carrying polyamine groups are usually used to assist in carrying and condensing DNA molecules. Therefore, poly–L-lysine (PLL) was used to neutralize the negative charge of the NE, which would lead to reduction of the electrostatic repulsion between DNA and NE, and to condense the DNA molecules. Lipidization of PLL was required for a stable nanoemulsion-poly–L-lysine complex that was achieved by linking

palmitoyl fatty chain to the ε–methylene of PLL. ^{1}H-NMR results confirmed successful conjugation of the fatty chain to the PLL. In addition, electrophoresis results showed that the lipidized PLL interacted with the hydrophobic Nile Red dye resulting in a red fluorescent band, while the unmodified poly-L- lysine lacked this fluorescent (Fig 6.3). This result can be explained on the fact that Nile Red dye is fluorescent in hydrophobic environment since the dye is very soluble in lipids (19). Incorporation of p-PLL into NE was successfully achieved and the addition of p-PLL in ratio of 0.125-2 to triolein was capable of retaining the electrophoretic mobility of the NE indicating the neutralization of the surface charge of the NE by the incorporated p-PLL (Fig 6.4).

Assembly of the artificial lipoprotein carrier with DNA can be seen in the Fig 6.8. The schematic illustration showed that free cationic polyamine group of p-PLL would interact with negatively charged DNA. Once polyamine binds to DNA, the electrostatic repulsion between the nucleic acid phosphates will be neutralized leading to DNA collapse in to a compact state, reducing its volume four to six orders of magnitude less than that of uncondensed DNA (20). Condensed DNA was shown to not give fluorescence with ethidium bromide because the condensation prevents ethidium bromide from intercalating into the DNA molecules (11). Results from gel electrophoresis (Fig 6.7) showed that DNA was condensed by p-PLL (Lane 2) as well as p-PLL/ nanoemulsion complex (Lane 4-7) but not with NE alone (Lane 3). DNA molecules were held by p-PLL/ NE complex when a sufficient amount of p-PLL was used.

The negative charge of the DNA-carrier complex should be masked to allow the cellular uptake since cell membrane has a negative charged surface. Therefore, the surface charge of the p-PLL/ NE / DNA complex was determined. The zeta potential and

mobility of the artificial lipoprotein / DNA carrier is shown in (Fig 6.5 and 6.6). Increasing the ratio of p-PLL to triolein while fixing the DNA amount at 2 μg led to reversing the zeta potential and mobility from negative toward positive value.

Transient expression of rabies glycoprotein was visualized under fluorescence microscopy (Fig 6.10). Binding of FITC antibody with glycoprotein on the cell membrane of the transfected cells made the cells appear as light green fluorescent cells. The non-transfected cells, however, lacked of antibody specific affinity (i.e. absence of rabies glycoprotein) and exhibited a black background. The percentage transfection efficiency (% TE) was obtained by flow cytometric assay. The transfection efficiency results are shown in Fig 6.9. The highest transfection efficiency was achieved when the cells were incubated with the artificial lipoprotein complex composing of p-PLL/ NE /DNA at (1:1) p-PLL: triolein weight ratio. The %TE of the negative control is very low (0.30 ±0.23) indicating the sensitivity of the detection method. Increasing the ratio of p-PLL to triolein resulted in an increase in the %TE of the artificial lipoprotein. This phenomenon could be attributed to the increase in the amount of p-PLL which resulted in an increase in the positivity of the surface net charge of the lipoprotein / DNA complex. Accordingly, the DNA complex had a greater affinity to interact with the negatively charged cell membrane (23). In addition, it has been shown that PLL can activate phospholipases (21) and, thus, may affect membrane permeability (22). Therefore, the amount of p-PLL incorporated into NE could increase the artificial lipoprotein DNA system cellular internalization. The artificial lipoprotein/ DNA complex showed up to 11 fold and 5.3 fold increase in the %TE compared to p-PLL/ DNA for ratio 1:1 and 0.5:1, respectively. These results indicated the importance of the artificial lipoprotein to

facilitate DNA uptake by the giloma cell line. The highest %TE with Lipofectamine™ 2000 was 23 ± 3.85 and it was achieved with 1:3 DNA: lipofectamine ratio. The artificial lipoprotein DNA system compared to lipofectamine showed about 4.2 times increase in %TE based on the highest %TE. This result showed the capability of the artificial lipoprotein to achieve high transfection efficiency.

In conclusion, a novel artificial lipoprotein system has been developed as DNA based vaccine delivery system. The system mimics natural lipoprotein in composition and it contains surface protein (lipidized poly-L-lysine) as a DNA interacting and condensing molecule. The amount of p-PLL incorporated into nanoemulsion had an effect on transfection efficiency and 1:1 ratio of p-PLL: triolein showed the highest transfection efficiency. Presence of artificial lipoprotein was very important for DNA- cellular internalization. *In vitro* transfection of human glioma cells using the artificial lipoprotein DNA system was found to be more efficient than a commercial trasfecting liposomal formulation.

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Figure and table legends:

Figure 6.1 Size distribution of nanoemulsion by Photon Correlation Spectroscopy
The number weighted mean particle size was 54.3 ± 24.8 nm .

Figure 6.2 Structure of poly-L-lysine (PLL, 2A) and palmitoyl poly-L-lysine (p-PLL, 2B)

Figure 6.3 Agrose gel electrophoresis of PLL and p-PLL stained with Nile Red dye

Figure 6.4 Agrose gel electrophoresis of the complex of nanoemulsion and p-PLL stained with Nile Red dye (Lane 1: nanoemulsion; Lane 2 to Lane 6 were nanoemulsion / p-PLL complex with ratio of P-PLL to triolein to be 0.125:1, 0.25:1, 0.5:1, 1:1 and 2:1 , respectively)

Figure 6.5 Zeta potential of the nanoemulsion particles and their complexes with different amount of p-PLL (DNA was kept constant at 2 μg)

Figure 6.6 The mobility of the nanoemulsion particles and their complexes with different amount of p-PLL (DNA was kept constant at 2 μg)

Figure 6.7 Agrose gel electrophoresis of the complex of nanoemulsion and p-PLL with Rabies plasmid DNA vaccine stained with ethidium bromide (Lane 1 and 8 : pure DNA; Lane2: DNA / p-PLL; Lane 3 : DNA / nanoemulsion; Lane 4 to 7 were complex of nanoemulsion with different amount of p-PLL and DNA. The ratio of p-PLL to triolein was 0.125:1, 0.25:1,0.5:1, and 1:1, respectively)

Figure 6.8 Construction of p-PLL/ nanoemulsion/ DNA complex

Figure 6.9 Percentage transfection efficiency in human glioma SF-767 cell line with the artificial lipoprotein and lipofectamine. Negative control is (p-PLL/

nanoemulsion/ plasmid DNA not carrying rabies gene). L1 and L 0.5 are positive controls and they are refereed to DNA/ p-PLL (the quantity of p-PLL added is similar to ratio 1:1 and 0.5:1, respectively). NE 1, NE 0.5, NE 0.25, and NE 0.125 is stated for p-PLL/ nanoemulsion/ DNA (the ratio of p-PLL to triolein was 1:1, 0.5:1, 0.25:1, and 0.125:1, respectively) and DNA quantity was kept constantan at 2 μg. Lipo 0.5, Lipo 2, Lipo 3, Lipo 5 is stated for DNA/ lipofectamine ratio 1:0.5, 1:2, 1:3 and 1:5, respectively (DNA quantity was kept constant at 2 μg).

Figure 6.10 Picture of transfected cells with p-PLL/ nanoemulsion/ DNA under fluorescence microscopy. Figure 10-A is for ratio 1:1 and Figure 10-B is for ratio 0.5:1

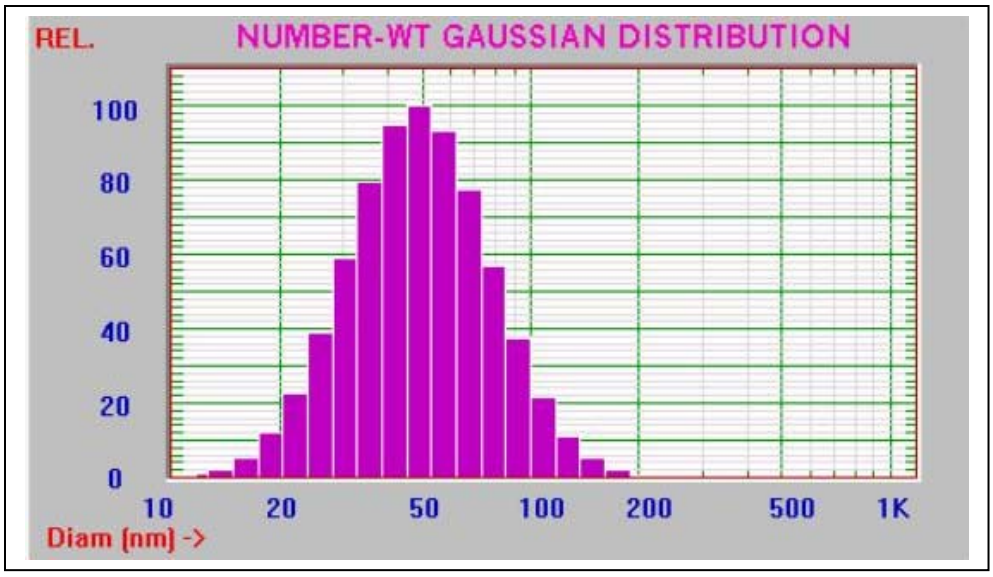
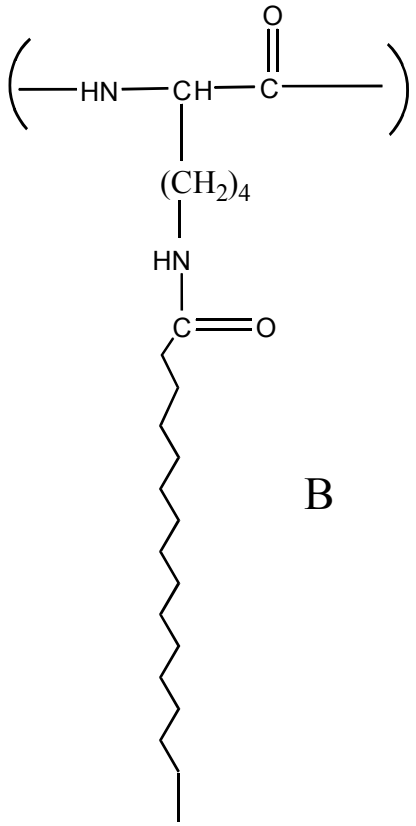
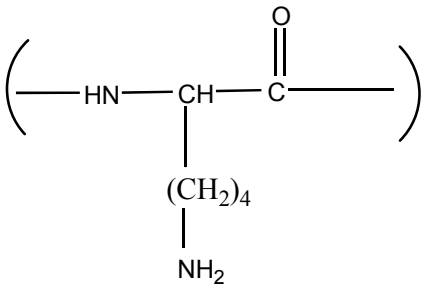

REL.
NUMBER-WT GAUSSIAN DISTRIBUTION
100
80
60
40
20
0
10
20
50
100
200
500
1K
Diam (nm) ->

Figure 6.1



B



A

Figure 6.2

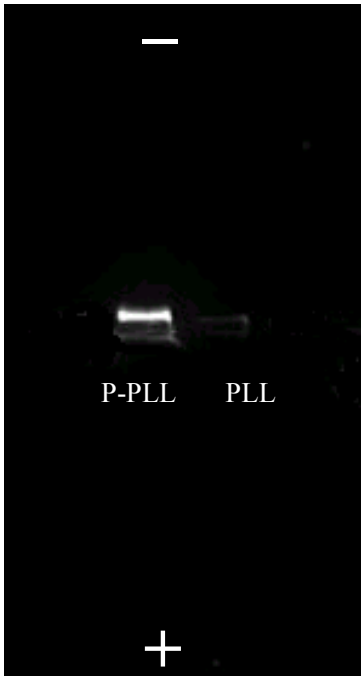


Figure 6.3

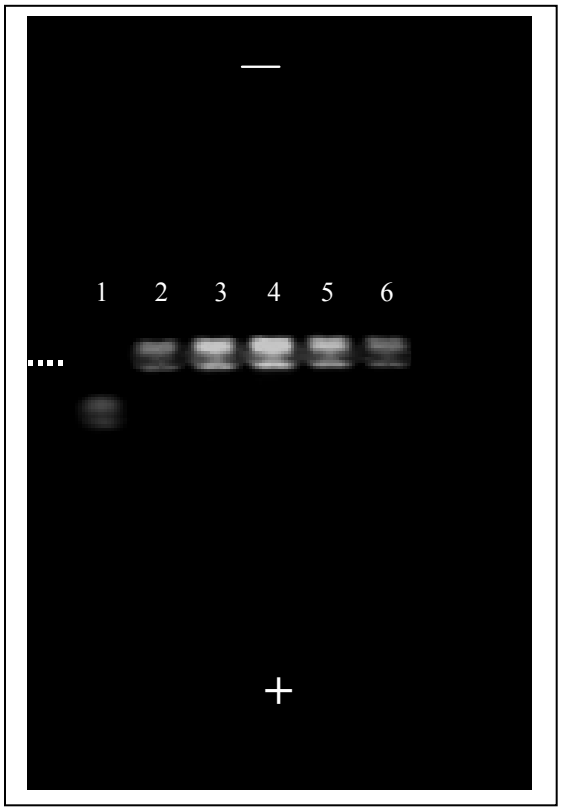


Figure 6.4

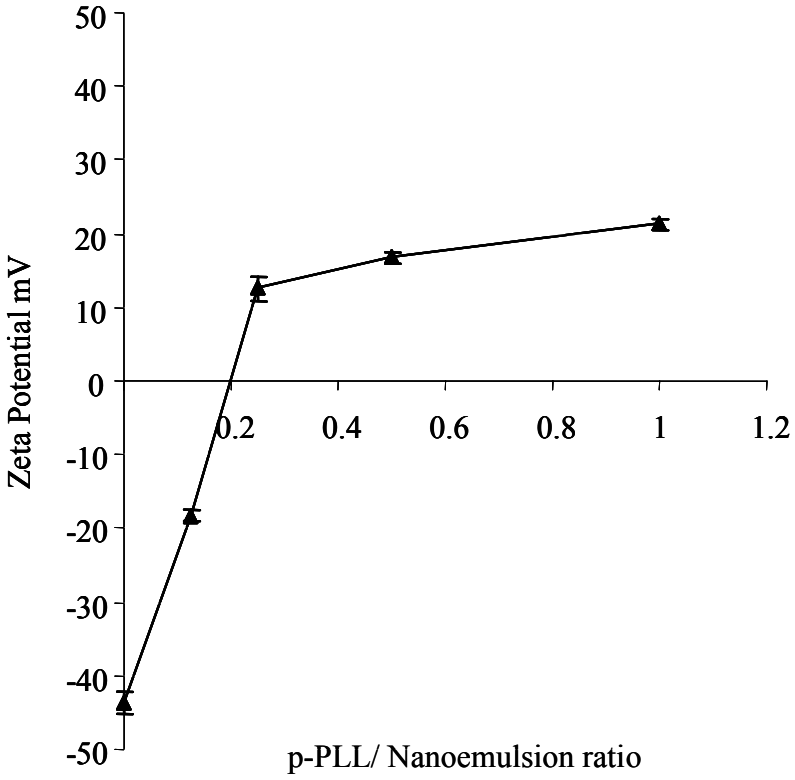

50
40
30
20
10
0
-10
-20
-30
-40
-50
Zeta Potential mV
0.2
0.4
0.6
0.8
1
1.2
p-PLL/ Nanoemulsion ratio

Figure 6.5

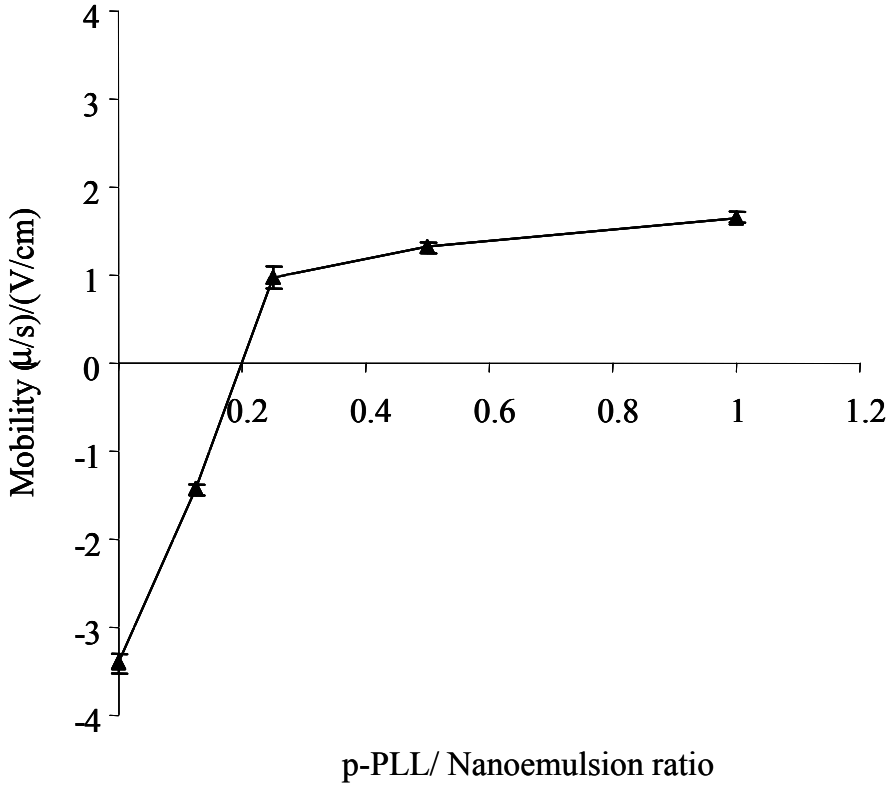

4
3
2
1
0
-1
-2
-3
-4
0.2
0.4
0.6
0.8
1
1.2
Mobility (μ/s)/(V/cm)
p-PLL/ Nanoemulsion ratio

Figure 6.6

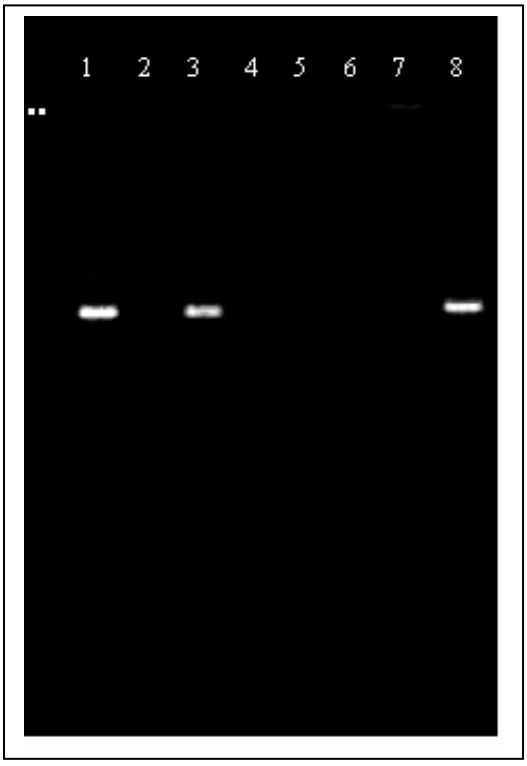


Figure 6.7

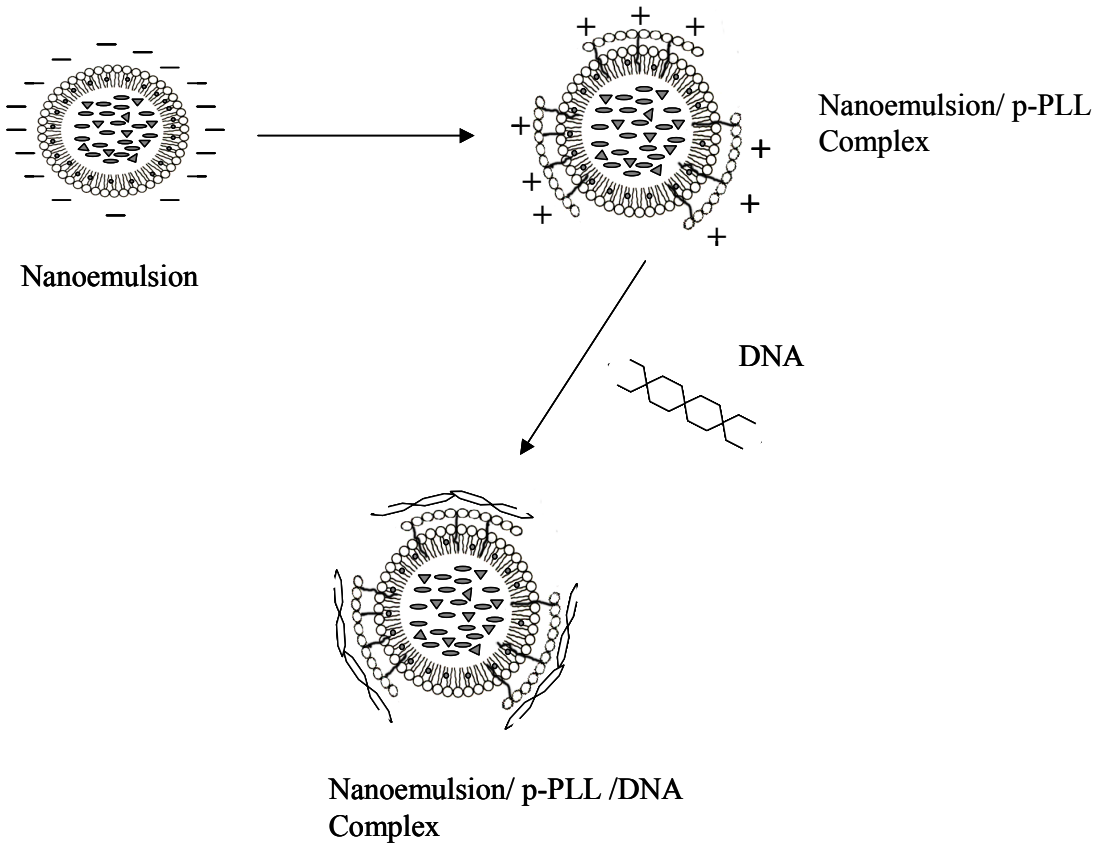

Nanoemulsion
Nanoemulsion/ p-PLL
Complex
DNA
Nanoemulsion/ p-PLL /DNA
Complex

Figure 6.8

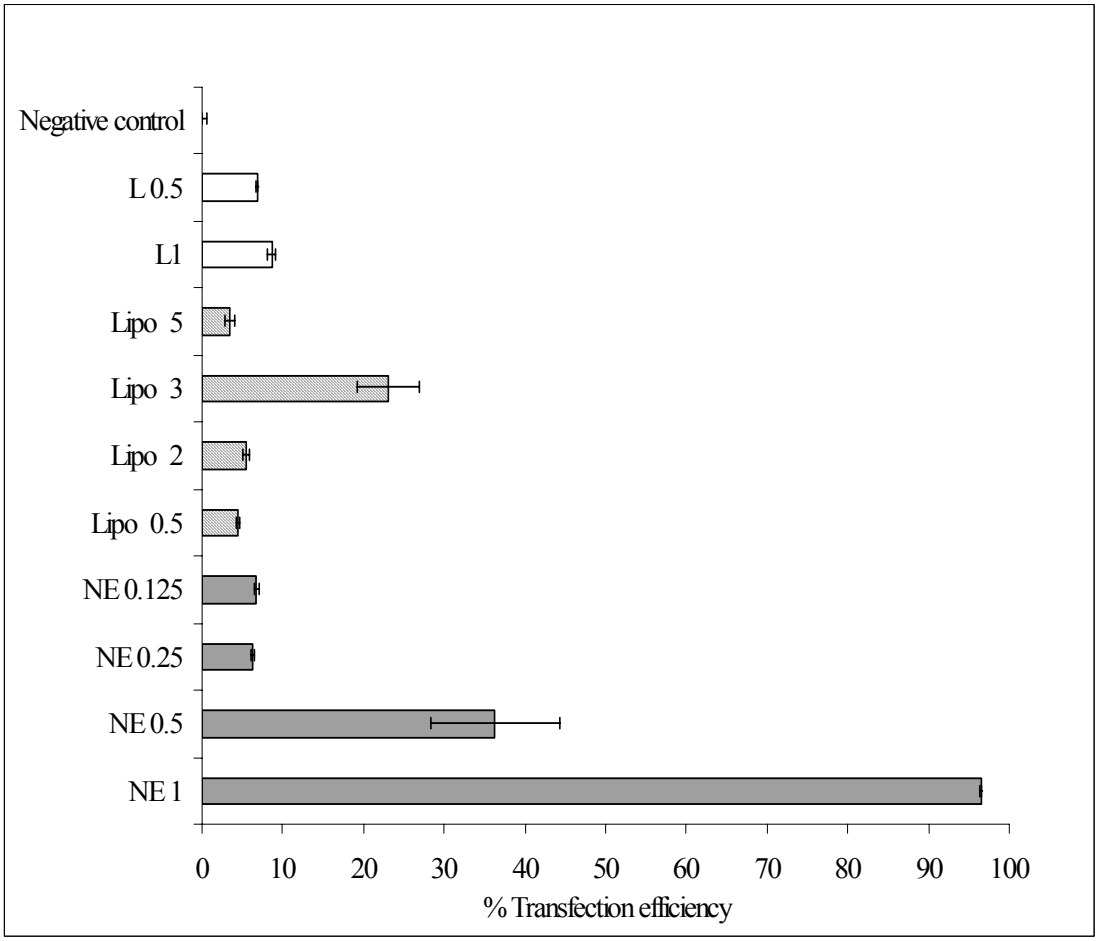

Negative control
L 0.5
L1
Lipo 5
Lipo 3
Lipo 2
Lipo 0.5
NE 0.125
NE 0.25
NE 0.5
NE 1
0
10
20
30
40
50
60
70
80
90
100
% Transfection efficiency

Figure 6.9

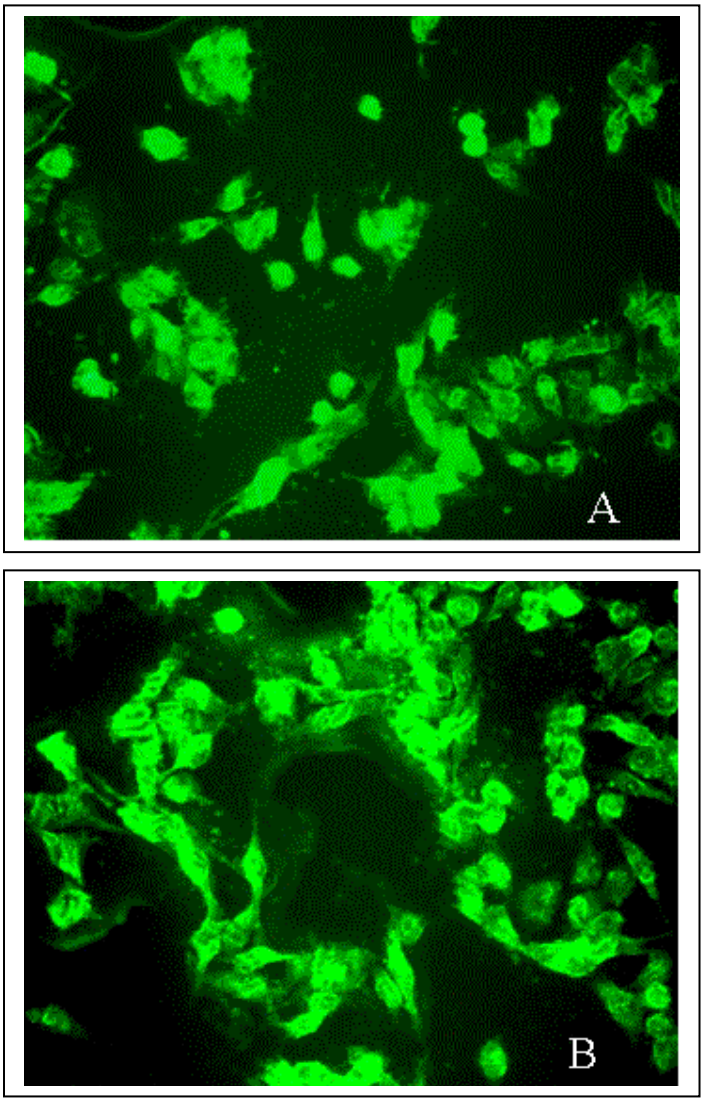


Figure 6.10

CHAPTER 7

CONCLUSIONS

In this research we have studied the application of using lipoprotein as drug delivery system for anticancer agents and DNA based vaccine. The conclusions are listed according to the order in which they appeared in the dissertation.

In chapter 2, the review paper highlights the potential medical applications of cholesterol-based drug conjugates. Many studies have been conducted to develop new cholesteryl drug conjugates for the purpose of diagnosis or treatment of diseases related to liver, adrenals, ovaries, atherosclerotic lesion, viral infections and tumors.

In chapter 3, the addition of various fatty chains to the cholesteryl carborane ester was achieved through a three-step chemical reaction. Similar to BCH, these new compounds were extremely hydrophobic and presented in crystalline forms. The melting points ranged from 210 to 260 ^{o} C among these compounds. The incorporation efficiencies of these compounds in liposomes vary significantly depending on the type of fatty chain attached and the ratio of cholesterol: phospholipid used as the excipients of the liposomal formulation.

In chapter 4, a new BNCT targeting compound (BCH) was evaluated for brain distribution and its stability in simulating *in vivo* environment was challenged. The result indicated that BCH in liposomal formulation distributed from the site of injection quickly. The adequate radiation window appeared to be less than 2 hrs. The result, also, pointed out that BCH diffused from injection site to the neighboring normal tissues. The

result showed statistically insignificant difference between the BCH concentration in tumor and normal tissues. BCH was stable in the presence of cholesterol esterase and lipoprotein lipase.

In chapter 5, Inductively Coupled Plasma Spectrometry (ICP) was used to analyze these compounds indirectly based on boron analysis. ICP-AES was used to obtain boron compound concentrations as well as phospholipid concentration at the same time and on the same run. This finding provides a feasible method for analyzing element-based compounds encapsulated in phospholipid vesicles. ICP-MS was used for analyzing BCH in brain tissue samples during the brain distribution study. Also, separation of liposomes from LDL by ultra-centrifugation technique is presented. Not only the time, speed, and density gradient but also the size of the liposome has an effect on the separation. This method gave us a good separation between LDL and liposome and it will be used in future studies.

In chapter 6, a novel artificial lipoprotein system has been evaluated as DNA based vaccine delivery system. The system mimics the natural lipoprotein in composition and it contains surface poly peptide (lipidized poly-L-lysine) as a DNA interacting and condensing molecule. The amount of p-PLL incorporated into nanoemulsion had an effect on the transfection efficiency and the ratio 1:1 of p-PLL: triolein showed the highest transfection efficiency. The presence of artificial lipoprotein was very important for DNA- cellular internalization. In vitro transfection of human glioma cells using the artificial lipoprotein DNA system was found to be more efficient than a commercial liposomal formulation.