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Lipoprotein-resembling submicron emulsion for drug and gene delivery
(Under the direction of D. ROBERT LU)

Several anticancer compounds that have shown excellent efficacy are known to have high incidence of toxicity. The main reason of such toxicity can be due to lack of targeting ability of the drug in the body where in doesn't discriminate between normal and cancer cells. The main focus of this research is to create a new-targeted drug/gene delivery system.

Low-density lipoprotein, LDL is known to carry cholesterol in the body and deliver it to cells via receptor-mediated endocytosis. Elevated LDL-receptor activity was reported in several cancer cells. Consequently, LDL has been identified as a potential drug carrier system for anticancer drug targeting to benefit from preferential delivery into cancer cells.

Due to the practical problems associated with loading drugs into LDL, we investigated the possibility of creating a surrogate system that resembles the native lipoproteins in its chemical and physical properties. We incorporated a cholesterol-based compound, BCH, in lipoprotein-resembling submicron emulsion and studied the physical properties of the formulation as well the drug uptake into cancer cells (9 L rat glioma, and SF-767 human glioblastoma multiforme).

The second component of the artificial lipoprotein system is a hydrophobized protein/polymer that will associate at the surface of the submicron emulsion. We investigated the ability of hydrophobized polylysine to associate with the emulsion particles and the ability of this polymer/lipid system to carry DNA.

Different targeting molecules can be associated with these emulsion particles to target different tissues depending on the hydrophobized protein/polymer used. We described a detailed method to carry protein hydrophobization with minimal denaturation of the protein.

In conclusion were we able to formulate a lipoprotein-resembling submicron emulsion. We've shown the ability of this system to solubilize water insoluble compound and to deliver the drug to cancer cells. We've also shown the potential application of the artificial lipoprotein system in gene delivery.

INDEX WORDS: Drug targeting, low-density lipoprotein, submicron emulsion, cholesteryl derivatives, BNCT, cholesteryl ester transfer protein, Gene delivery, polylysine, protein lipidization.

LIPOPROTEIN-RESEMBLING SUBMICRON EMULSION FOR DRUG AND GENE DELIVERY

by

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To my beloved family Mahmoud, Suad,

Rana, Ruba, and Mahdi

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Lipoproteins are macromolecular complexes composed of lipids and proteins. Lipoproteins function as a transport system for different water insoluble lipid. Structurally, they are composed of a monolayer of phospholipids, cholesterol, and proteins called apolipoproteins surrounding the core components containing cholesteryl esters, and triglycerides, Figure 1.1. The apolipoproteins are partly exposed at the surface, enabling them to bind to specific enzymes or receptor proteins on cell membranes thus directing the lipoprotein to its site of metabolism.

Classification and function of plasma lipoproteins

Plasma lipoproteins are divided into 5 major classes according to the densities at which they are isolated upon salt gradient ultracentrifugation, Table 1.1.[1] Lipoprotein particles undergo continuous metabolic processing so that they have somewhat variable properties and differ in chemical composition, apolipoprotein content, hydrated density and other physicochemical properties. Chylomicrons are triglyceride-rich emulsion particles having the largest size of all the lipoproteins (80-1,000 nm in diameter). They are synthesized by the mucosal cells of the small intestine under conditions of high dietary triglycerides intake. They contain the highest lipid/protein ratio (lowest density). Once these particles enter the lymph and plasma they acquire apolipoprotein E and C and taken up by the liver. Very low-density lipoprotein, VLDL, is secreted from the liver, where most of the dietary lipids are processed. It is known that in the biological system, lipoprotein lipase interacts with VLDL and this results in the liberation of fatty acids from the triglyceride core. Consequently the VLDL remnant particle is either taken up by

the LDL receptor in the liver or mostly converted to LDL in the vascular space via intermediate density lipoprotein (IDL), as the case in humans.[2-4]

Low-density lipoproteins, LDL, is formed after the metabolism of VLDL in plasma and composed mainly of cholesteryl esters. The main role of LDL is to provide cells with the cholesterol they need for cell membrane synthesis and repair. Cells have also the ability to generate their own cholesterol via *de novo* synthesis. The latter constitute only 10% of the cellular needs of cholesterol.[5] High-density lipoprotein, HDL, are the smallest lipoproteins with the highest protein/lipid ratio. HDL is secreted by the liver and intestine as nascent HDL. The function of HDL is quite the opposite that of LDL. HDL scavenges excess cholesterol from the cells throughout the body and delivers it to cells that need it, or to the liver, where it can be converted to bile salts and excreted.

LDL pathway for targeted drug delivery

Low-density lipoprotein is the main plasma component for cholesterol transport in the body. It is composed of submicron emulsion component and a protein, apolipoprotein B100. The submicron emulsion is composed of a monolayer of phospholipid and cholesterol containing a core of cholesteryl esters and triglycerides. Apolipoprotein B100 is believed to interact with LDL-receptors present at the surface of the cells and initiate receptor-mediated endocytosis of the whole particle, Figure 1.2.[1] Cells process LDL particles in the lysosomal compartment where cholesteryl esters are hydrolyzed to cholesterol and fatty acids for use in the synthesis of new cell membranes. Due to the increased need of cholesterol in rapidly dividing cancer cells, LDL receptor activity has been reported to increase in certain types of cancer. Goldstein *et al.* reported that leukemic cells isolated from patients with acute myeloid leukemia had 3-100 times higher LDL receptor activity than normal white blood cells.[6] Elevated LDL receptors have been also reported in colon, kidney, and liver tumors.[7] Increased cholesterol consumption in certain intracranial tumors was also reported by Collins *et al*.[8] In a recent study, Forte *et al.* measured a relatively high number of LDL receptors in seven human

glioma cell lines, suggesting the potential of using the LDL pathway for drug targeting.[9] Several researchers showed the ability of LDL to carry anticancer compounds for targeted delivery.[10, 11] Riedel et al. proposed utilizing the LDL pathway to target antiviral compounds to mononuclear cells for HIV treatment.[12, 13]

Utilizing human lipoproteins, especially LDL, as a drug carrier has several advantages: 1) biocompatibility, 2) the oil core can be a suitable media to solubilize hydrophobic compounds, 3) anticancer compounds can be targeted via the increased LDL receptor in some cancer cells to benefit from the preferential delivery into cancer cells, 4) ability to modify the pharmacokinetic profile of incorporated compounds in these particles. Another advantage of using LDL as drug carrier is through the ability of targeting other cell receptors by carrying chemical modifications on these particles. For example, acetylated LDL is rapidly taken up by macrophages, while lactosylation of LDL is catabolized by hepatocytes.[14, 15] Conjugating LDL with monoclonal antibodies has also been investigated to evaluate the ability of targeting these drug containing particle through antibody-cell surface antigen instead of the LDL pathway.

Drug loading into LDL

Four main methods for loading drugs into LDL has been described in the literature; 1) The dry stir method, in which the drug is dried as a thin film on a glass tube and then incubated with LDL for 2hrs at 37°C. The lipophilic drug will partition into the LDL particle.[16] 2) The aqueous addition method is used for incorporating water-soluble compounds. Unbound drug after incubation with LDL is removed by gel filtration.[17] 3) Detergent assisted transfer is achieved by solubilizing the lipophilic drug with nonionic surfactant, followed with incubation with LDL.[18] 4) The solvent extraction method is the only method that can achieve efficient loading into LDL. In this method LDL is first mixed with starch and freeze dried, then the lipophilic core is extracted with heptane, then the drug is solubilized in another oil to replace the extracted core.[19, 20] Kader et al. compared different methods for drug loading into LDL.[21] Doxorubicin-

containing multi-lamellar large vesicles (leaky liposomes) achieved the highest drug loading into LDL in comparison to the dry film method, detergent assisted and direct addition of the drug.

Practical problems of using LDL as drug carrier

Insufficient drug loading into LDL is the main obstacle of using this system for drug delivery. The solvent extraction method was found to incorporate sufficient amounts of drug into LDL, Unfortunately, the harsh conditions of extracting the core of LDL render apolipoprotein B-100 inactive. Consequently, the LDL loses its targeting capability. Another limitation arises from the fact that LDL separation and subsequent loading of drugs is a very tedious procedure and time consuming. Finally, using human LDL to be used in another human may require rigorous viral and microbial screening to prevent the possibility of causing different kinds of infections to the recipient patient.

Lipoprotein resembling systems for targeted drug delivery

Due to the limitation of using the natural lipoproteins for drug delivery, researchers attempted to use lipoprotein surrogate systems to deliver anticancer compounds via the lipoproteins pathway. The first attempts to create such surrogate systems was carried by biochemists for the purpose of characterizing the metabolic pathways of different lipoproteins containing no drugs as they weren't designed for drug delivery purposes. Ginsburg and his group were the first to formulate a protein-free microemulsion resembling LDL.[22] Maranhao et al. studied the kinetic behavior of these LDL-resembling microemulsion, and found out that they have similar kinetic parameters as the native LDL.[23, 24] Maranhoa have also formulated chylomicrons resembling particles and studied the metabolic process of these particles.[25] All of these experiments were carried out using radio-labeled components and contained no drug molecules. In 1995, Van Berkel reported the incorporation of a derivative of the nucleoside analogue iododeoxyuridine into recombinant chylomicrons for treatment of hepatitis B virus.[26] Their results showed that these particles were able to acquire apolipoprotein E and

deliver its content via the LDL pathway in vivo. Apolipoprotein E is known to interact with the LDL receptor with higher affinity than Apolipoprotein B-100 that is present on the surface of LDL.[27] The same group attempted to utilize the HDL pathway for targeted delivery using recombinant HDL particles.[28-30]

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

Boron neutron capture therapy (BNCT) is a binary cancer therapy that is composed of boron, ^{10}B, containing compound and a beam of low energy neutrons. For successful BNCT, boron must first localize preferentially in tumor cells, then irradiated by a beam of low energy neutrons to yield high energy and short-range α particles and ^{7}Li nuclei.[31] Kahl and Callaway were the first to exploit the LDL pathway for targeted delivery intended for BNCT. They reconstituted human LDL with long-chain unsaturated fatty alcohol esters of carborane carboxylic acid.[32] Their findings indicated superior boron uptake, retention, and efficacy than with other boron compounds previously studied in vitro.[33]

Due to the limitation and practical problems of using human LDL for drug delivery, we have suggested a surrogate system with the ability to interact with human lipoproteins for targeted delivery of newly synthesized boronated cholesterol, BCH (cholesteryl 1,12- dicarba-closo-dodecaborane-1-carboxylate) for BNCT, Figure 1.3.[34] This compound mimics the native cholesteryl esters present in the core of LDL for drug targeting and can potentially be used for boron neutron capture therapy (BNCT) of cancers. BCH was designed as the cholesterol-based compound because 1) for BNCT, it is not necessary for this conjugate to release the therapeutically active unit (the parent drug) and 2) the conjugate contains one carborane cage (i.e., 10 boron atoms per molecule), maximizing the amount of boron delivered to cancer cells. It is an important requirement to have about 20 μg of ^{10}B per gram of cells to achieve successful BNCT.[35, 36] Recently, Feakes *et al.* reported the synthesis of different types of boronated cholesterol derivatives and it's incorporation in liposomes for BNCT.[37]

Our strategy, as described in chapter 2, is to incorporate the cholesteryl ester of carborane, BCH, into phospholipid submicron emulsion resembling in lipid composition and structure the human very low-density lipoprotein (VLDL). VLDL core is mainly composed of triglycerides (which can be a good solvent for many hydrophobic drugs) to achieve sufficient incorporation. The ability to formulate this system from commercially available lipids with efficient drug loading overcomes the practical problems associated with the use of native lipoproteins as drug carriers.[38] It is known that in the biological system, lipoprotein lipase interacts with VLDL and this results in the liberation of fatty acids from the triglyceride core. Consequently the VLDL remnant particle is either taken up by the LDL receptor in the liver or mostly converted to LDL in the vascular space via intermediate density lipoprotein (IDL), as the case in humans. [2-4]

Another unique feature of this system is its similarity to native lipoproteins that allows its interaction with the native lipoproteins. Lipid-exchange between different classes of lipoproteins has been established by others to be dependent on the presence of certain exchange proteins like cholesteryl ester transfer protein (CETP) and phospholipid transfer protein.[39] CETP was described as less selective to its substrate as it transfers not only cholesteryl esters (CE) but also triglycerides.[40] This can be advantageous since drug molecules of similar structure of CE may also be a substrate for this enzyme and be transferred to body's lipoproteins when incorporated in this submicron emulsion due to the unique resemblance between both particles, as presented in chapter 3. Drug transfer to LDL can be another attribute of this formulation for selective delivery via the LDL pathway.

Development of artificial lipoprotein for pharmaceutical use

Most of the lipoprotein-resembling systems described in the literature focused on the lipid component of the native lipoprotein. The VLDL-resembling submicron emulsion we used for BNCT is also a protein free system. Native lipoproteins are composed of both lipid and protein components. For example, apolipoprotein B-100

constitutes about 22% of the LDL weight. A unique characteristic of apolipoproteins is their ability to associate with the lipid component of the lipoprotein spontaneously. Such a characteristic is due to the presence of hydrophobic regions in the amino acid sequence of these proteins. These regions arrange into the lipidic core of the lipoproteins while the hydrophilic portions of the apolipoproteins protrude to the surface of the lipoprotein serving as ligand for receptor mediated metabolism of the lipoproteins. We described the development of artificial lipoprotein as a drug/gene carrier system (Shawer and Lu, 2001, US Patent Application (# 60/234,141)). The artificial lipoprotein we developed is composed of two components, first, a submicron emulsion resembling in composition that of native lipoprotein. Different drug molecules (especially water-insoluble drugs) can be formulated into the core of the submicron emulsion component for delivery and transport. The second component of the system is a chemically lipidized protein created by the attachment of fatty acid chains to the protein. The protein component is to be associated with the surface of the submicron emulsion through the interactions between the lipid chains introduced into the proteins and the submicron emulsion. Protein lipidization procedure can be conducted for different types of proteins or polymers. This property will allow the utilization of different surface ligands on the submicron emulsion for advanced drug targeting. The inclusion of the modified protein or polymer may also enhance the stability of the carrier system.

Avidin, for example, is a glycoprotein that can be lipidized (using the method described) and associated with the submicron emulsion system, which can then function as a universal station for the attachment of various biotinylated proteins (enzymes, toxins, antibodies, etc.), polymers or drugs. Lipidized avidin can also be associated with native LDL and therefore allow the binding to biotinylated polymers, protein, and drugs to benefit from the LDL pathway for delivery. The properties of avidin-biotin interaction have been utilized in the preparation of immunoliposomes.[41] The advantages of using this system with a biotinylated antibody include the intact functionality of the antibody.

The antibody will be functional since the active site has not been modified or affected, Figure 1.4. We also overcome the problem of non-selective modification and disorientation of the antibody in the submicron emulsion arising from direct modification on the antibody (i.e the antibody binding site buried inside the submicron emulsion).

Different approaches have been reported in the literature describing chemical conjugation of proteins (monoclonal antibodies) in order to associate with liposomes. Niedermann et al. developed a hydrophobic anchor-coupling agent to carry the association of proteins with liposomes.[42] Others carried the conjugation reaction using reactive phospholipids.[43, 44] In chapter 5 we describe another method for chemical lipidization of proteins using lysozyme as model protein. The main difference between the artificial lipoprotein system and immunoliposomes is the lipid structure. Liposomes have bilayer system of phospholipids, while the submicron emulsion is a monolayer system of phospholipids in which the core contains a hydrophobic core in contrast to the liposome, which has an aqueous core.

Artificial lipoprotein for gene delivery

Gene therapy has great potential for treating different types of diseases. With the completion of the human genome project, we will understand more about the functions of these genes and thus significantly increase the possibility for curing wide range of diseases. However, one of the main obstacles for gene therapy is to effectively deliver these genes into target cells and tissues. Many research groups have investigated different techniques for delivering genetic material into cells. Two main strategies have been adopted for gene delivery system involving either viral vectors or nonviral carrier systems. Viral vectors are very efficient, but their use is narrowed by the potential side effects of the virus itself, such as over-expression, immunogenicity and activation of oncogenes in the human body.[45] On the other hand, the nonviral systems in general are less efficient but have a better safety profile, if properly formulated, than the virus-based

systems. Nonviral carrier systems, contrary to viral vectors, are not limited by size and amount of DNA material that can be incorporated.

The main approaches for nonviral gene delivery involve cationic liposomes,[46-53] and cationic polymers.[54-57] Both systems depend on the ionic interaction between the negatively charged DNA and the positively charged lipid or polymer. Such interaction was shown to have a condensing effect on the DNA molecules. Combination of cationic lipid and polymer has been studied in an attempt to increase the efficiency of these nonviral systems. Huang et al. reported potentiation of these lipoplexes (liposome/DNA complex) transfection efficiency when they were combined with polycationic polymers to produce virus-like particles.[58] Kim et al. was the first group to combine low-density lipoprotein (LDL) with chemically hydrophobized polylysine to examine the ability of natural lipoprotein, LDL, to deliver genetic material via the LDL pathway. [59-61] This novel system, called the terplex system, is based on a balanced hydrophobicity and net surface charge among LDL, hydrophobized polylysine and DNA. Delivery via receptor mediated endocytosis, biocompatibility, and effectiveness are among the advantages shown for the terplex system. Huang et al. 1997, described the use of reconstituted chylomicrons remnants (RCR) as nonviral vector. [62] DNA was complexed with cationic lipid and solubilized in the core of these RCR particles. Both of these lipoprotein-based systems appeared to offer resistance against serum effect, which is critical for in vivo systemic application.

The artificial lipoprotein nonviral gene delivery system we developed, chapter 4, is composed of palmitoyl polylysine associated with lipoprotein-resembling submicron emulsion prepared from commercially available lipids, Figure 1.5.[63] The interaction between the chemically modified polymer and the submicron emulsion is through hydrophobic interactions, and between the polymer and DNA through charge interaction. This artificial lipoprotein system allows us to control the particle size or charge, and to solubilize different compounds in the core of the submicron emulsion (hydrophobic

drugs or transfection enhancers). Other advantages include: 1) the feasibility of using this artificial lipoprotein for *in vitro* or *in vivo* transfection, 2) easiness of preparation of this system, and 3) the consistency in the production of our surrogate system, all pf these attributes enhance the efficacy of this gene delivery system.

In summary, my research objectives are:

1) To develop lipoprotein-resembling submicron emulsion capable of carrying extremely water insoluble compound, BCH, as described in chapter 2.
2) To study the in vitro distribution and cellular uptake of BCH formulated in lipoprotein-resembling submicron emulsion, as described in chapter 3.
3) To investigate the ability of polymer associated submicron emulsion to carry DNA as a potential system for gene delivery, chapter 4.
4) To establish an effective chemical conjugation method for protein lipidization with minimal denaturation for the purpose of association with submicron emulsion particles, chapter 5.

Table 1.1: Chemical composition of human lipoproteins

Complex	Source	Density (g/ml)	%Protein	%TG[a]	%PL[b]	%CE[c]	%C[d]	%FFA[e]
Chylomicron	Intestine	<0.95	1-2	85-88	8	3	1	0
VLDL	Liver	0.95-1.006	7-10	50-55	18-20	12-15	8-10	1
IDL	VLDL	1.006-1.019	10-12	25-30	25-27	32-35	8-10	1
LDL	VLDL	1.019-1.063	20-22	10-15	20-28	37-48	8-10	1
*HDL_2	Intestine, liver (chylomicrons and VLDLs)	1.063-1.125	33-35	5-15	32-43	20-30	5-10	0
*HDL_3	Intestine, liver (chylomicrons and VLDLs)	1.125-1.21	55-57	3-13	26-46	15-30	2-6	6

[a]Triacylglycerols, [b]Phospholipids, [c]Cholesteryl esters, [d]Free cholesterol, [e]Free fatty acids

*HDL2 and HDL3 derived from nascent HDL as a result of the acquisition of cholesteryl esters

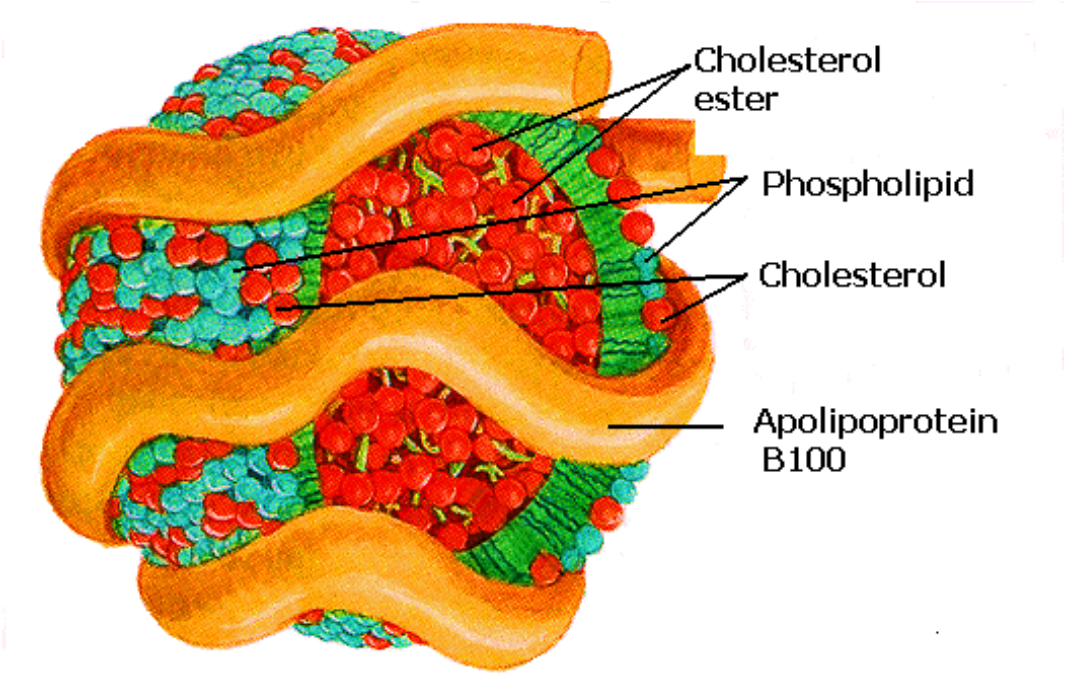

Figure 1.1: Low-density lipoprotein structure.

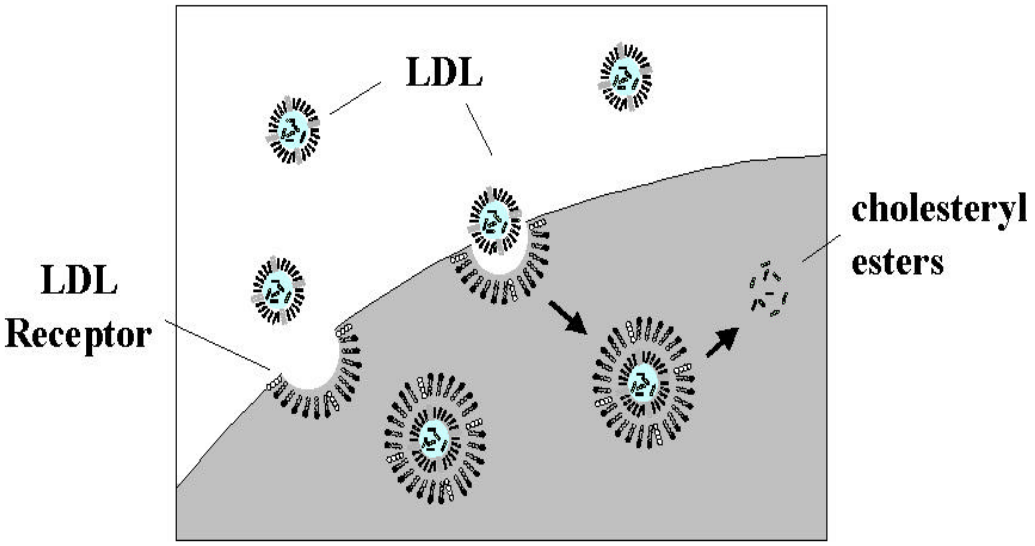

Figure 1.2: Receptor-mediated endocytosis of LDL particles via interaction with LDL receptor at the cell membrane and subsequent processing of the LDL particle in the lysosomal compartment.

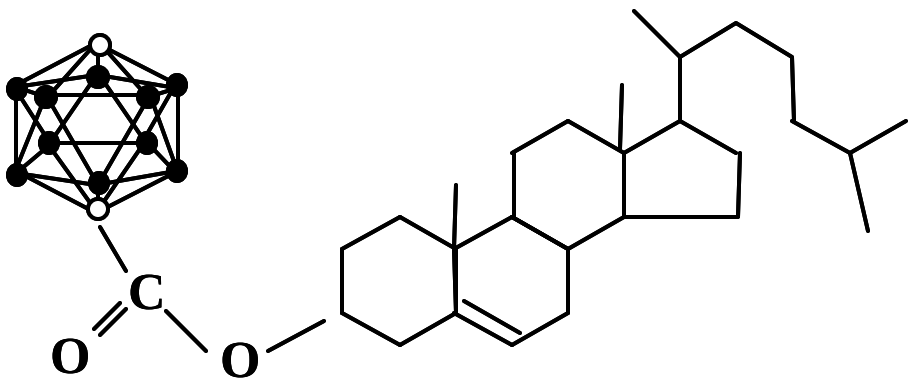

Figure 1.3: Cholesteryl –Carborane (BCH), cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate for BNCT

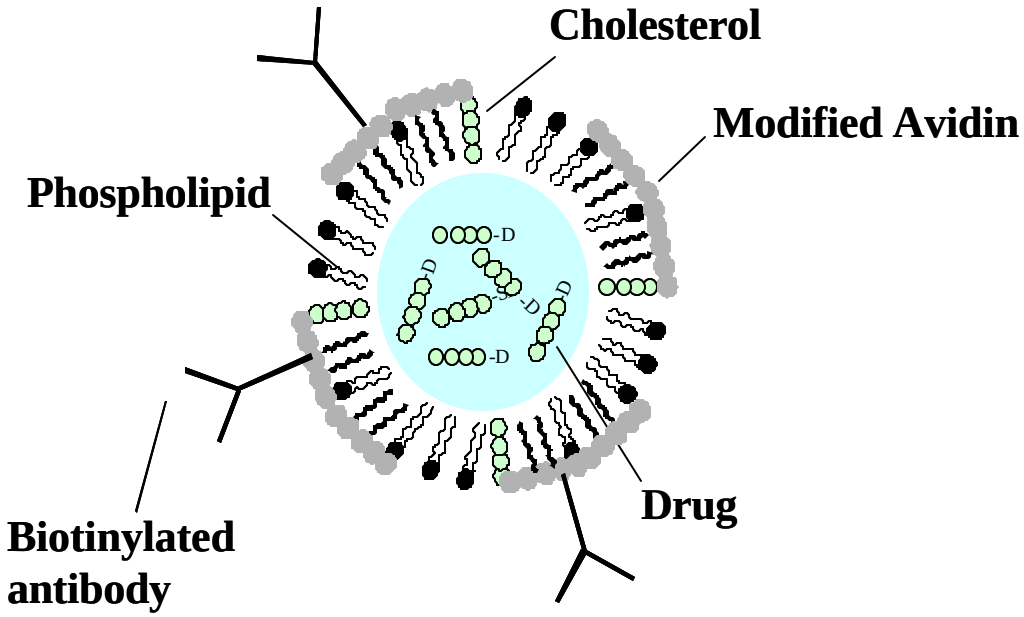

Figure 1.4: Schematic representation of artificial lipoproteins for bio-targeting of different compounds via tissue specific monoclonal antibodies.

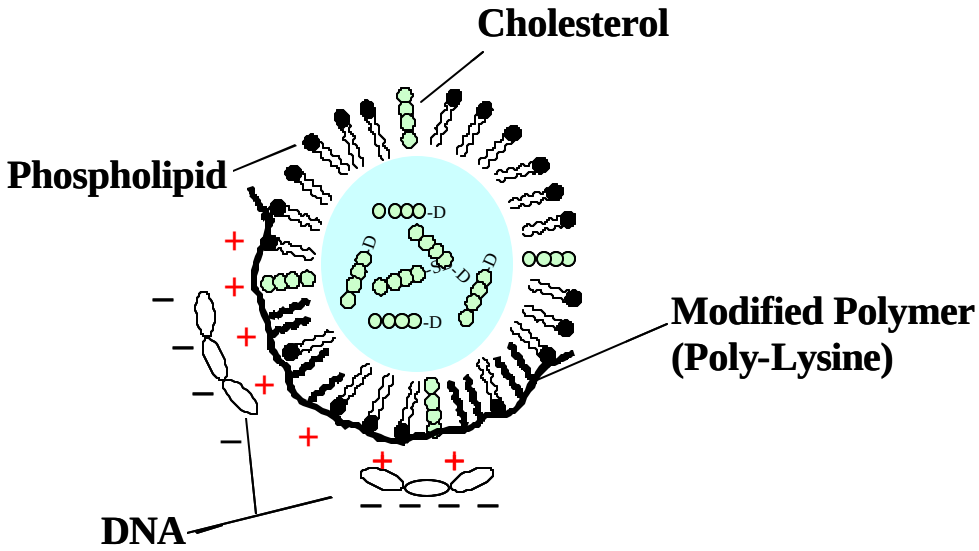

Figure 1.5: Schematic representation of artificial lipoprotein for gene delivery.

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CHAPTER 2

VLDL-RESEMBLING PHOSPHOLIPID-SUBMICRON EMULSION FOR CHOLESTEROL-BASED DRUG TARGETING[1]

[1] Shawer, M., P. Greenspan, S. Øie, R. Lu. Accepted by *Journal of Pharmaceuitcal Sciences*. 2001. Reprinted here with permission of publisher, 3/02

Abstract

The objective of the current study was to develop and evaluate VLDL-resembling phospholipid-submicron emulsion (PSME) as a carrier system for new cholesterol-based compounds for targeted delivery to cancer cells. BCH, a boronated cholesterol compound, was originally developed in our laboratory to mimic the cholesterol esters present in the LDL and to follow a similar pathway of cholesterol transport into the rapidly dividing cancer cells. The VLDL-resembling system was then designed to solubilize BCH, facilitate the interaction with LDL and thus assist the BCH delivery to cancer cells. BCH-containing PSME was prepared by sonication. Chemical compositions and particle sizes of different PSME fractions were determined. The lipid structure of PSME and location of BCH in the formulation were assessed based on experimental results. Density gradient ultracentrifugation fractionated the emulsion into 3 particle size populations with structures and compositions resembling native VLDL. *In vitro* interaction between PSME and LDL was evident by agarose electrophoresis as both formed a single band with an intermediate mobility. The transfer of BCH from PSME to LDL was also observed in the presence of other serum components including serum proteins. Cell culture data showed sufficient uptake of BCH in rat 9L glioma cells (>50μg boron/g cells). In conclusion, this system has the capability to incorporate the cholesterol-based compound, interact with native LDL and assist the delivery of this compound into cancer cells in vitro.

KEY WORDS: Drug targeting, low-density lipoprotein, submicron emulsion, cholesteryl derivatives, BNCT.

Introduction

Low-density lipoprotein (LDL), and high-density lipoproteins (HDL) are the natural carriers of cholesteryl esters in the body. Certain human and animal tumor types have been shown to have elevated LDL-receptor activity primarily because the rapidly dividing cancer cells require higher amounts of cholesterol to build new cell membranes. Goldstein *et al.* reported that leukemic cells isolated from patients with acute myeloid leukemia had 3-100 times higher LDL receptor activity than normal white blood cells.[1] Elevated LDL receptors has been also reported in colon, kidney, liver tumors.[2] Increased cholesterol consumption in certain intracranial tumors was also reported by Rudling *et al.*[3] In a recent study, Forte *et al.* measured a relatively high number of LDL receptors in seven human glioma cell lines, suggesting the potential of using the LDL pathway for drug targeting.[4]

To utilize the LDL as a drug carrier, and the LDL pathway for preferential drug delivery to cancer cells, our laboratory has recently synthesized a cholesteryl ester of carborane, cholesteryl 1,12- dicarba-closo-dodecaborane-1-carboxylate (BCH, Figure 2.1). This compound mimics the native cholesteryl esters present in the core of LDL for drug targeting and can potentially be used for boron neutron capture therapy (BNCT) of cancers. BCH was designed as the cholesterol-based compound because 1) for BNCT, it is not necessary for this conjugate to release the therapeutically active unit (the parent drug) and 2) the conjugate contains one carborane cage (i.e., 10 boron atoms per molecule), maximizing the amount of boron delivered to cancer cells. It is an important requirement to have about 20 μg of ^{10}B per gram of cells to achieve successful BNCT.[5, 6] Recently, Feakes *et al.* reported the synthesis of different types of boronated cholesterol derivatives and it's incorporation in liposomes for BNCT.[7]

To effectively deliver the compound into cancer cells through the LDL receptors and overcome its extremely hydrophobic characteristics, BCH must be loaded into LDL

in vitro or incorporated into an LDL-surrogate system. Previous methods for loading LDL with drugs in vitro were unsuccessful possibly because apolipoprotein B-100 became denatured and inactivated by the process.[8] Another limitation of using the *in vitro* LDL loading approach is that sufficient incorporation of drug may not be achieved. Kahl *et al.* attempted to reconstitute human LDL with long-chain unsaturated fatty alcohol esters of carborane carboxylic acid.[9] Their findings indicated superior boron uptake, retention, and efficacy than other boron compounds previously studied in vitro. In a later report they demonstrated that some of these compounds tended to aggregate and disrupt the LDL particles, which could result in uptake by the reticuloendothelial system (RES) rather than by their intended targets.[10] Kwon *et al.* reported the use of polymeric micelles to solubilize hydrophobic drugs and mimic native lipoproteins.[11]

In the present study, we prepared and evaluated VLDL-resembling phospholipid submicron emulsion (PSME), an artificial carrier resembling the lipid composition of very low-density lipoprotein (VLDL), into which BCH was incorporated. The potential of using this carrier system to deliver the cholesteryl ester of carborane (BCH) to rat brain glioma cells has also been examined.

Materials and Methods

Materials

Triolein (99%), egg yolk phosphatidylcholine (99%), cholesterol (99%), and human low-density lipoprotein (LDL, 5.2 mg protein/ml) were purchased from Sigma (St. Louis, MO). L-α-Lysophosphatidylcholine (99%) was from Avanti (Alabaster, AL). Cholesterol oleate (99%) was obtained from Acros (NJ, USA). Rat 9L glioma cells were obtained from Dr. Jeffrey Olsen at Emory University. Dulbecco's Modified Eagle's Media (DMEM), Dulbecco's phosphate-buffered saline (PBS) and trypsin-EDTA were obtained from VWR Scientific Products, Inc. (Suwanee, GA). Fetal bovine serum (FBS) and lipoprotein deficient serum (LPDS) were obtained from BioCell Laboratories, Inc.

(Rancho Dominguez, CA). Analytical grade chloroform, methanol and other chemicals were obtained from J.T. Baker (Phillipsburg, NJ). BCH (cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate) was prepared in our laboratory according to the method described by Ji and Lu.[12]

Preparation of VLDL-resembling phospholipid submicron emulsion containing BCH

Stock solutions of individual lipid and BCH were prepared in chloroform. Various lipids and BCH were mixed and the mixture was composed of the following ratio (w/w): Triolein: egg phosphatidylecholine: lysophosphatidylcholine: cholesterol oleate: cholesterol: BCH, 70: 22.7: 2.3: 3.0: 2.0: 2.0, respectively.[13] All components were combined and chloroform was evaporated under a stream of nitrogen. The preparation was then desiccated overnight at 4°C to remove residual solvent. Following addition of 10 ml of 2.4 M NaCl for 102 mg of lipid and BCH mixture, the preparation was sonicated under nitrogen for 30 min using a probe sonicator (Branson Sonifier 450) at output 5, while the temperature was maintained at 55°C. Inductively coupled plasma (ICP) and thin layer chromatography (TLC) were used to monitor possible degradation of BCH with the sonication condition.

Particle size and chemical composition

Different particle-size populations of BCH-containing PSME were separated by density gradient ultracentrifugation as described by Marranhao *et al*.[14] Particle size of each fraction was measured by photon correlation spectroscopy (PCS, NICOMP particle sizing systems, Santa Barbara, CA). Non-aqueous reverse phase HPLC chromatography was used to determine the chemical composition (BCH, cholesterol, cholesterol oleate and triolein) of each fraction with different particle sizes. Separation of different chemical components was achieved by C-18 HPLC column using isocratic elution with 50:50 (v:v) methanol:2-propanol, and was detected by UV absorption at 202 nm.[15] The phospholipid content in each fraction was measured as phosphorous concentration by inductively coupled plasma (ICP).

PSME structure and location of BCH

To assess the structure of the BCH-containing PSME, theoretical particle size was calculated based on the following assumption: first, the outer shell of sphere-shaped particles is composed of phospholipids and surface cholesterol whereas the other components' effects are negligible. Second, the volume is contributed by the triolein, cholesteryl oleate and cholesterol at the core. Particle surface area and volume can be calculated using the molecular dimensions of these components as follows:

Surface area (nm^2)= $4 \pi r^2 = (f. N_{PL}) + (0.37) N_{Surface\ Ch.}$, and

Volume (nm^3)= $(4/3) \pi r^3 = (1.68 N_T) + (1.13 N_{CO}) + (0.62 N_{Core\ Ch})$

Where N represents the number of moles of phospholipid (PL), triolein (T), cholesteryl oleate (CO), and cholesterol (Ch) at the core or surface. The amount of Ch in the core for each fraction was calculated from the solubility of cholesterol in the triolein and cholesteryl oleate core according to Miller and Small.[16]

Owing to the condensing effect of cholesterol on phosphatidylcholine, the surface area of each phosphatidylcholine molecule must be adjusted according to the amount of cholesterol present at the surface.[17, 18] f represents the surface area of one molecule of phosphatidylcholine at a given PL:Ch molar ratio according to the data presented by Lecuyer and Dervichian.[17] The surface area of one cholesterol molecule is 0.37 nm^2 according to the later reference and is not affected by the presence of PL. Partial molecular volume of T, CO, and Ch (1.68, 1.13, 0.62 nm^3, respectively) were calculated from the densities; ρ_{Ch}=1.45g/ml, ρ_{CO}=0.96g/ml, and ρ_T=0.913g/ml, and the molecular weight of each.[16] The radius r =3 V/S, can be calculated after measuring the composition of these particles.[19] The location of BCH in the PSME particles was determined by comparing the different behaviors of all lipid components, including BCH, with regard to changes in volume and surface area among different particle sizes.

Interaction of BCH-containing submicron emulsion with LDL

The fractions of different sizes of the phospholipid submicron emulsion containing BCH were dialyzed (Spectra/Por® membrane; MWCO 12-14 kDa) against PBS and then incubated individually (3 mg total lipid in each fraction) with 50 ìl human LDL (5.2 mg protein/ml) for 2 hrs in 2 ml PBS at 37°C with gentle shaking. Agarose gel electrophoresis was performed according to the method described by Greenspan *et al.* using Nile Red as the fluorescent dye to determine the electrophoretic mobility of the BCH-containing submicron emulsion and to examine its interactions with human LDL.[20] In brief, 0.6% agarose gel was prepared in 50 mM barbital buffer, pH 8.6. Five ìl of Nile Red in acetone (100 ìg/ml) was dried out in test tube. The incubation sample, LDL or submicron emulsion was then added to the tube individually (50 ìl sample to each tube) and mixed until Nile Red was in solution. Five ìl of sucrose solution (30%, w/v) was added. Each electrophoretic well was loaded with 11 ìl of sample preparation. Electrophoresis was conducted for 1 hr at 56 V at room temperature. Different electrophoretic bands on the gel were visualized under UV lamp.

Transfer of BCH from PSME to LDL in PBS and LPDS

LDL (100 µl) were incubated with 200 µl of the second fraction of BCH-containing PSME in 2 ml of either phosphate buffer saline (PBS) or lipoprotein deficient serum (LPDS) for 1 hr at 37°C. After the incubation, the density of each tube was adjusted to 1.08 g/ml with solid KBr. All samples were placed into centrifuge tubes and the remainder of the tube was filled with KBr solution of 1.006 g/ml density. Samples were subjected to 285,000 g for 1 hr at 11°C and centrifugation was allowed to stop without use of break. Additional samples were added with dried Nile Red before centrifugation and subsequently detected under UV lamp to identify the volume fractions separated for PSME and LDL. The top 1.5 ml and the bottom 2 ml, where PSME and LDL were present, respectively, based on the Nile Red method, were collected from each tube. Quantitative analysis of BCH was conducted by HPLC as described before. Control

samples were also analyzed, which had the same procedure described above except no LDL was involved.

Cellular uptake of BCH formulated in phospholipid submicron emulsion

Rat 9L glioma cells were grown in 150 cm^2 plastic cell culture flasks containing 26 ml of DMEM with 10% FBS and 1% antibiotics, penicillin-streptomycin (5,000 I.U./ml, and 5000 µg/ml), (referred to as the growth medium). In subsequent experiments, flasks containing 26 ml of growth medium were seeded with approximately 5×10^6 cells and placed in a humidified, 5% CO_2 –95% air incubator at 37°C. After 24 hours, when cell culture flasks were about 60% confluent, growth medium was removed and replaced with 26 ml of pre-warmed fresh growth medium containing the prepared VLDL-resembling submicron emulsion (unfractioned) with two different concentrations of BCH. A control study using BCH-free submicron emulsion was also performed.

After 18 hours of incubation at 37°C, the media were removed; the cells were rinsed 3 times with PBS, harvested with trypsin-EDTA and counted under microscope with hemocytometer. The cells were then pelleted by centrifugation at 3500 rpm for 10 minutes and assayed for boron. The BCH concentration (as boron concentration) in cell culture samples was determined by inductively coupled plasma-mass spectroscopy (ICP-MS). Concentrated nitric acid (0.5 ml) was added to the cell culture sample in a Teflon-lined acid digestion bomb. The bomb was closed and placed into a preheated oven at 150°C for 3 hrs. Samples were then diluted and analyzed by ICP-MS. Student t-test (one tailed) was used to compare the uptake at two different concentrations of BCH in the media.

Results

Preparation of BCH-containing phospholipid submicron emulsion

BCH was incorporated in sufficient quantities into phospholipid submicron emulsion resembling VLDL in lipid composition. The mean particle size of the

formulation before fractionation was 155 ± 5 nm as measured by PCS. Density gradient ultracentrifugation was used to fractionate the emulsion into three particle size populations of the same density, and the mean particle sizes were 161 ± 2, 76 ± 1 and 40 ± 3 nm, respectively. A fourth fraction, apparently consisting of small unilamellar liposomes, was also detected that didn't move to the top even after 5 hrs of centrifugation at 40,000 rpm and was recovered at the interface formed between the lower two density layers (1.065 and 1.02 g/ml).[21] The particle size of the fourth fraction, however, was not detected by photon correlation spectroscopy indicating the size was very small. The chemical composition of each fraction with different particle size, separated by density gradient ultracentrifugation, was examined by HPLC and is shown in Table 2.1.

To further examine the relationship between particle size and its effect on chemical composition of other components and to determine the location of BCH in the phospholipid submicron emulsion particles, the molecules per volume and molecules per surface area of BCH, triolein, cholesteryl oleate, cholesterol and phospholipids were calculated and are shown in Figures 2.3 and 2.4, respectively. The figures indicated that the number of molecules of BCH, triolein, and cholesteryl oleate per volume was constant, while that per surface area increased by decreasing the surface (i.e., by increasing the particle size). On the other hand, the number of phospholipids was the same per surface area, whereas that per volume decreased as the particle size increased.

The theoretical particle size of each of these fractions was also calculated from the chemical composition of each size fraction as measured by HPLC. The calculated particle size was in a good agreement with the measured diameter, Table 2.1, indicating that the particle structure of the PSME is well represented by the assumptions described above.

Stability of BCH under sonication conditions

When a suspension of the same amount of BCH was sonicated in water under sonication conditions similar to those used to prepare the submicron emulsion containing

BCH, no boron was detected by ICP after filtration. This indicates that the sonication of BCH affected neither its solubility (BCH is extremely water-insoluble) nor its stability (the cholesteryl ester of carborane remained intact). If the ester bond in BCH had been degraded, carborane acid, which is fairly water soluble, would have been formed and detected. TLC examination of BCH-containing phospholipid submicron emulsion samples also showed that BCH was stable to the sonication conditions involved during preparation.

Agarose electrophoresis and interaction study

The electrophoretic mobility of the different particle-size fractions of the phospholipid submicron emulsion was toward the positive electrode, indicating that these particles had a slightly negative charge. The mobility was increased as the particle size decreased. Incubating human LDL with the different size fractions demonstrated an interaction between the BCH-containing PSME and LDL to form one band on the gel with an intermediate electrophoretic mobility that came between the native LDL and the phospholipid submicron emulsion alone, Figure 2.2. Direct addition of LDL and phospholipid submicron emulsion mixture to the electrophoresis gel without preincubation showed two separate bands for each (picture not shown).

Transfer of BCH from PSME to LDL in PBS and LPDS

After the submicron emulsion particles were separated from LDL, analysis of the LDL fraction revealed that 20±4% of BCH originally in the submicron emulsion was transferred to the human LDL after 1hr incubation at 37°C in LPDS media. This transfer was not observed when the incubation was carried in PBS (no BCH was detected at the LDL fraction after centrifugation).

Cellular uptake of BCH formulated in phospholipid submicron emulsion

To evaluate the cellular uptake of BCH in PSME particles in vitro, rat 9L glioma cell line was used. The results, shown in Table 2.2, indicates sufficient uptake by the rat tumor cells that was above the required value for successful BNCT. Both concentrations

(8.25 and 16.5 μg B/ml) achieved high uptake by the cells. The uptake at these two different concentrations was statistically different ($p<0.005$).

Discussion

Lipoproteins are small biological particles carrying triglycerides and cholesteryl esters. They are composed of phospholipid submicron emulsion and certain types of apolipoproteins. The phospholipid submicron emulsion represents the main structure of the lipoproteins, which have a hydrophobic core that solubilize cholesteryl esters. Depending on the type of lipoproteins, the composition of the submicron emulsion varies. The VLDL is particularly interesting since it contains a relatively small amount of proteins (about 5-10 % protein) and large amount of triglycerides (about 50-65 %), which are used to sufficiently solubilize various hydrophobic cholesteryl esters. VLDL also contains about 15-20 % phospholipid, 5-10 % free cholesterol and 10-15 % cholesteryl esters. VLDL can either be metabolized by the liver or transformed into intermediate density lipoprotein, IDL, and eventually to LDL.[22]

Our laboratory has recently designed and synthesized a cholesteryl ester of carborane. The compound mimics native cholesteryl esters and is intended for use as an anticancer BNCT compound, in which it is not required for this conjugate to release the parent boron unit to be active. The tumor-targeting capability of this compound is based on the fact that many cancer cells have elevated LDL-receptor activity owing to increased cholesterol need for membrane construction. However, because of its hydrophobic nature and the proposed mechanism involving LDL interactions, the compound must be solubilized in a suitable pharmaceutical formulation to facilitate the interaction with LDL and to be taken up by cancer cells through LDL receptors.

In this study, we incorporated the cholesteryl ester of carborane into a phospholipid submicron emulsion that resembled VLDL in lipid composition. The

formulation was intended to solubilize a highly hydrophobic compound and facilitate its interactions with LDL and consequently with the LDL receptors.

A sonication method was used to prepare the phospholipid submicron emulsion containing BCH. No degradation of BCH was observed during preparation indicating BCH is relatively stable and can tolerate the sonication condition well. Sufficient amount of BCH can be incorporated into the phospholipid submicron emulsion as indicated by Table 2.1.

Theoretical calculations of particle size were in good agreement with the results obtained from photon correlation spectroscopy. The calculation was dependent on the lipid composition of each fraction with reasonable assumptions (stated above). These results indicate that the phospholipid submicron emulsion particles are likely to have the same lipid organization in a sphere structure as native lipoproteins.

In native lipoproteins, cholesteryl esters and triglycerides are present in the core, whereas phospholipids are located at the surface of the particles. To determine the location of BCH in phospholipid submicron emulsion, we have examined the molecular surface area and molecular volume for each component of the emulsion in respect to the particle size. Figures 2.3 and 2.4 show the number of molecules per volume and surface area for BCH, cholesterol, cholesteryl oleate, phospholipids and triolein, respectively, when particle size varied. From Figures 2.3 and 2.4, it can be seen that BCH behaves like triolein and cholesteryl oleate and not like phospholipid. When particle size increased, the number of molecules of BCH, triolein, and cholesteryl oleate per volume was constant while the number of molecules of phospholipid per volume decreased. Correspondingly, when particle size increased, the number of molecules of BCH, triolein, and cholesteryl oleate per surface area increased while the number of molecules of phospholipid per surface area kept constant. The results indicated that phospholipid is predominately located at the surface, as the decreased surface area due to the size increase did not affect the number of molecules of phospholipid per surface area. BCH,

triolein, and cholesteryl oleate, on the other hand, are mainly located in the core of the phospholipid submicron emulsion. In these figures, cholesterol had an intermediate behavior. This can be explained by the presence of cholesterol in the core, due to the complex solubility pattern of cholesterol in the core (which is affected by the amount of cholesterol oleate present), and its presence at the surface simultaneously. As indicated above, the theoretical size calculation also points toward the similarity between the PSME and native lipoproteins. Therefore, the structure of the phospholipid submicron emulsion containing BCH can be graphically represented in Figure 2.5, which is very similar to the lipid structure of native VLDL.

Bijsterbosch *et al*. demonstrated that a daunorubicin conjugate with oleoyl cholanic acid (LAD) was associated at the surface rather than the core of similar lipid particles.[21] This difference between BCH and LAD behavior can be explained by the extremely low water solubility of BCH, rendering it inaccessible to water. This is advantageous since firm incorporation of BCH in the core of the phospholipid submicron emulsion can be achieved, and the drug would not leak out as long as the particle is intact.

Rensen *et al*. studied the association of apolipoprotein-E with lipid particles, similar to our BCH-containing PSME.[13, 24] He reported that apolipoprotein E bound to these lipid particles and they were taken up through the LDL receptors. In this paper we also demonstrate another possible interaction between these BCH-containing PSME particles and LDL as physical association between the two particles (as evident in agarose electrophoresis). With incubation of the BCH-containing PSME and human LDL, the particles appeared to associate but not precipitate with LDL (see Figure 2.2). The results also indicate that the association detected under agarose electrophoresis was of physical nature since the particles separated again under ultracentrifugation. Lipid-exchange between different classes of lipoproteins was demonstrated by others to be dependent on the presence of certain exchange proteins like cholesteryl ester transfer

protein (CETP) and phospholipid exchange protein. In our study, BCH was transferred from the submicron emulsion particles to human LDL in the presence of other serum component including serum proteins. No transfer was detected when the same experiment was carried out in PBS. The transfer thus appeared an active process and not merely due to physical contact between these particles. Certain component(s) in the serum, probably serum proteins, appeared responsible for the interaction and the transfer of BCH to the human LDL.

The use of phospholipid submicron emulsion as a suitable formulation for BCH has also been demonstrated in our cell culture study. When the formulation containing BCH was incubated with 9L rat glioma cells, an effective cellular uptake has been observed. Although the initial concentration of the boronated cholesterol compound was very low in the media (8.25 μg B/ml), the cellular uptake results are promising and above the requirement to achieve successful BNCT (> 20 μg of B/g of cells). Earlier studies with another boron compound, sodium borocaptate (BSH), required much higher concentration in the media to achieve the needed concentration of boron in these cells.[25] At this stage, it is difficult to distinguish between intracellular BCH and membrane bound BCH. The exact mechanism of BCH uptake from these PSME particles is not clear.

VLDL is a triglyceride-rich lipoprotein with the biological function as the carrier of natural fat. Excluding the protein content (10 % of VLDL, dry weight), triglyceride and phospholipid constitute 56-72 % and 17-22 %, respectively, of VLDL, similar to the percentages used in our PSME formulation. It is known that in the biological system, lipoprotein lipase interacts with VLDL and this results in the liberation of fatty acids from the triglyceride core. Consequently the VLDL remnant particle is either taken up by the LDL receptor in the liver or mostly converted to LDL in the vascular space via intermediate density lipoprotein (IDL), as the case in humans.[26-28] We have prepared the VLDL-resembling phospholipid submicron emulsion to function as a carrier solubilizing

the hydrophobic BCH compound and to facilitate the interactions with LDL leading to the drug transfer to LDL. It is expected that with proper composition and formulation of VLDL-resembling PSME and appropriate cholesterol-based compounds, [7, 23, 29] we may be able to mimic the VLDL-LDL biological process to target delivery of anticancer drugs to cancer cells through the increased number of LDL receptors on these cells.

Conclusions

The VLDL-resembling PSME appears to be a sufficient carrier system that can incorporate the cholesterol-based compound, interact with native LDL and assist the delivery of this compound into cancer cells in vitro. The sonication method used in the preparation did not affect the compound or the lipids during the formulation. Our study suggests that these PSME particles have similar lipid composition and structural organization as native VLDL. The PSME has also the ability to interact and associate with LDL *in vitro.* The further development of VLDL-resembling formulation may allow us to mimic the VLDL-LDL biological process for targeted drug delivery to cancer cells.

Table 2.1: Composition and particle size of different fractions of BCH-containing PSME

PSME Fraction	First	Second	Third	Fourth
Particle Size (nm, Measured)	161±2	76±1	40±3	NA
Particle Size (nm, Calculated)	158	81	31	NA
BCH (%w/w)	2.4±0.1	2.2±0.1	1.8±0.1	0.5±0.1
Cholesterol (%w/w)	1.9±0.1	2.3±0.1	3.1±0.2	3.7±0.2
Cholesteryl oleate (%w/w)	3.0±0.2	2.8±0.1	2.5±0.1	0.3±0.1
Phospholipids (%w/w)	6.5±0.6	12.6±0.4	27.4±0.4	89.3±0.8
Triolein (%w/w)	86.3±0.5	80.1±0.2	65.3±0.9	6.1±0.1

[a] All value are means ± SEM of three experiments.

Table 2.2: BCH uptake in 9L glioma cell culture

Experiment	Initial concentration of B in the media (μg/ml)	Concentration of B in the cells after 18 hrs incubation (μg/g cells)
Control	0	0
Low concentration [a]	8.25	49.3±4.3
High concentration [a]	16.5	61.3±4.1

[a] All values are means ± SEM of four experiments.

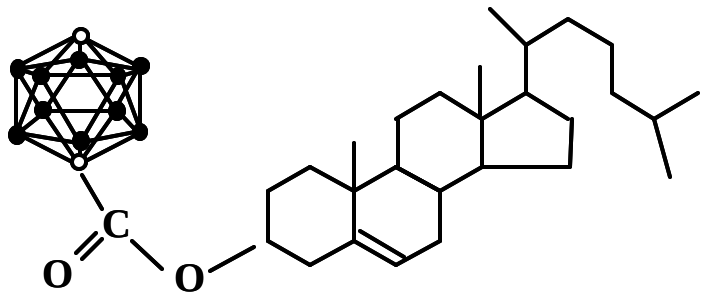

Figure 2.1: Cholesteryl –Carborane (BCH), cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate for BNCT

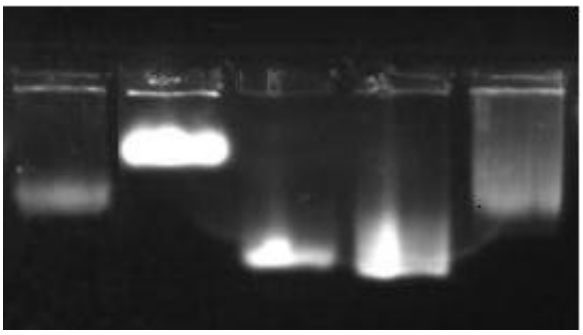

Figure 2.2: Agarose electrophoresis (the anode is at the bottom of the gel), from left to right: PSME (3rd fraction)+ LDL, LDL alone, PSME (3rd fraction) alone, PSME (2nd fraction) alone, and PSME (1st fraction) alone.

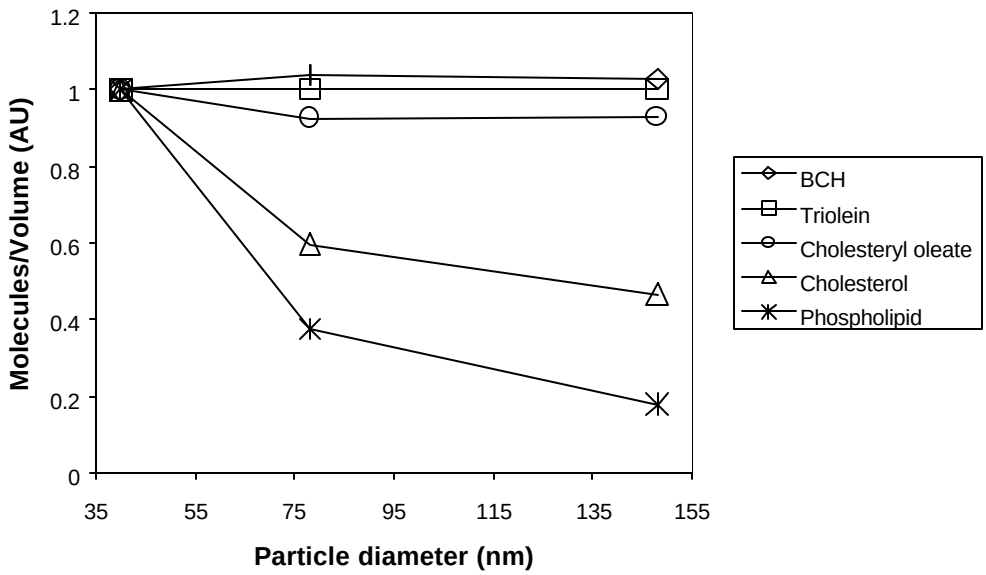

Figure 2.3: The location of BCH in different size PSME as a core component. The y-axis represents an arbitrary unit (AU) in which the molecules to volume ratio of the third fraction (smallest PSME) is set at 1.0.

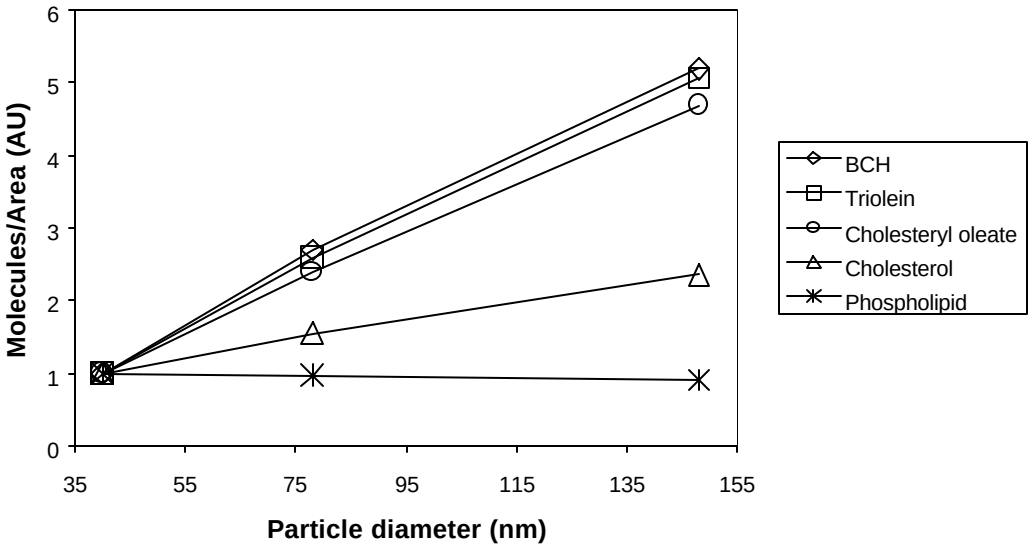

Figure 2.4: The location of BCH in different size PSME as a core component. The y-axis represents an arbitrary unit (AU) in which the molecules to surface area ratio of the third fraction (smallest PSME) is set at 1.0.

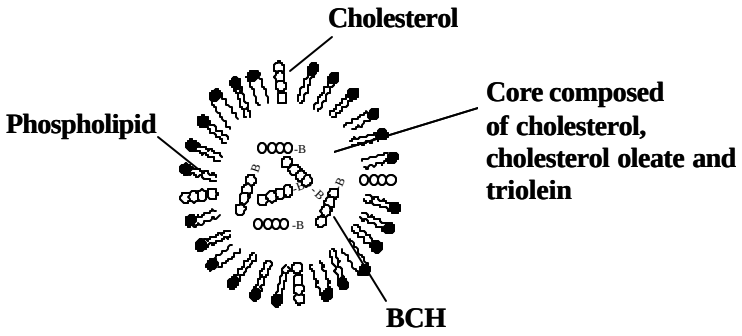

Figure 2.5: Schematic representation of VLDL-resembling phospholipid submicron emulsion containing cholesterol-based drug, BCH.

Abbreviations

LDL, low-density lipoprotein; VLDL, very low-density lipoprotein, BCH, cholesteryl 1,12-dicarba-closo-dodecaborane1-carboxylate; BNCT, boron neutron capture therapy; PSME, phospholipid submicron emulsion; PL, phospholipids; Ch, cholesterol; CO, cholesterol oleate; T, triolein.

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CHAPTER 3

IN VITRO DISTRIBUTION AND CELLULAR UPTAKE OF BORONATED CHOLESTEROL FORMULATED IN PHOSPHOLIPID SUBMICRON EMULSION[1]

[1] Shawer, M., P. Greenspan, R. Sidwell, R. Lu. To be submitted to *Journal of Pharmaceutical Sciences*.

Abstract

The purpose of this paper is to study the efficiency of delivering newly synthesized boronated cholesterol, BCH, for boron neutron capture therapy to human glioma cells SF-767. It's known that certain types of cancer cells have increased LDL receptor activity due to the increased demand of cholesterol for synthesizing new cell membranes. Accordingly, the drug (BCH) was formulated in a lipoprotein-resembling submicron emulsion. The similarity in structure between these BCH-containing submicron emulsion particles and native lipoproteins may contribute to the transfer of drug between different lipoproteins in the body and ultimately to the transfer of the boronated cholesterol to the cancer cells via the LDL pathway. BCH-containing submicron emulsion was prepared by sonication at 55°C of various lipids, corresponding to the components of native lipoproteins, along with the boronated cholesterol (BCH). *In vitro* transfer of BCH to LDL was evaluated in LPDS and PBS. *In vitro* distribution of BCH in human plasma to IDL/LDL, and HDL was also examined. BCH uptake by the human glioblastoma multiforme cell line SF-767 was compared when FBS or LPDS were used in the culture media. BCH was successfully incorporated in lipoprotein-resembling submicron emulsion. BCH appears to transfer from the submicron emulsion to the LDL only in the presence of serum, LPDS, but not in PBS. Similar findings were observed with human plasma, as the drug distributed to both IDL/LDL, and HDL fractions. Cell uptake data showed significant difference in BCH uptake when incubated in the presence of lipoproteins. Significantly higher uptake was observed when the media contained FBS. This suggests that lipoproteins participate in the cellular uptake of the drug.

KEY WORDS: Drug targeting, low-density lipoprotein, submicron emulsion, cholesteryl ester transfer protein, cholesteryl derivatives, BNCT.

Introduction

Low-density lipoprotein is the main plasma component for cholesterol transport in the body. It is composed of submicron emulsion component and a protein, apolipoprotein B100. The submicron emulsion is composed of monolayer of phospholipid and cholesterol containing a core of cholesteryl esters and triglycerides. Apolipoprotein B100 is believed to interact with LDL-receptors present at the surface of the cells and initiate receptor-mediated endocytosis of the whole particle.[1] Cells process LDL particles in the lysosomal compartment where cholesteryl esters are hydrolyzed to cholesterol and fatty acids for use in the synthesis of new cell membranes. Cells have the ability to generate their own cholesterol via *de novo* synthesis. The latter constitute only 10% of the cellular needs of cholesterol.[2] Due to the increased need of cholesterol in rapidly dividing cancer cells, LDL receptor activity has been reported to increase in certain types of cancer. Goldstein *et al.* reported that leukemic cells isolated from patients with acute myeloid leukemia had 3-100 times higher LDL receptor activity than normal white blood cells.[3] Elevated LDL receptors have been also reported in colon, kidney, and liver tumors.[4] Increased cholesterol consumption in certain intracranial tumors was also reported by Collins *et al*.[5] In a recent study, Forte *et al.* measured a relatively high number of LDL receptors in seven human glioma cell lines, suggesting the potential of using the LDL pathway for drug targeting.[6]

Utilizing human lipoproteins, especially LDL, as a drug carrier has several advantages: 1) biocompatibility, 2) the oil core can be a suitable media to solubilize hydrophobic compounds, 3) anticancer compounds can be targeted via the increased LDL receptor in some cancer cells, 4) ability to modify the pharmacokinetic profile of incorporated compounds in these particles.

Previous methods for loading LDL with drugs in vitro were unsuccessful possibly because apolipoprotein B-100 became denatured and inactivated by the process.[7]

Another limitation of using the *in vitro* LDL loading approach is that sufficient incorporation of drug may not be achieved.

Boron neutron capture therapy (BNCT) is a binary cancer therapy that is composed of boron, ^{10}B, containing compound and a beam of low energy neutrons. For successful BNCT, boron must first localize preferentially in tumor cells, and then irradiated by a beam of low energy neutrons to yield high energy and short-range α particles and ^{7}Li nuclei.[8] Kahl and Callaway were the first to exploit the LDL pathway for targeted delivery intended for BNCT. They reconstituted human LDL with long-chain unsaturated fatty alcohol esters of carborane carboxylic acid.[9] Their findings indicated superior boron uptake, retention, and efficacy in comparison with other boron compounds previously studied in vitro.[10]

Due to the limitations and practical problems of using human LDL for drug delivery we suggested a surrogate system with the ability to interact with human lipoproteins for targeted delivery of newly synthesized boronated cholesterol, BCH (cholesteryl 1,12- dicarba-closo-dodecaborane-1-carboxylate) for BNCT, Figure 3.1.[11] Our strategy is to incorporate cholesteryl ester of carborane into phospholipid submicron emulsion resembling, in lipid composition and structure, the human very low-density lipoprotein (VLDL). The VLDL core is mainly composed of triglycerides, which can be a good solvent for many hydrophobic drugs to achieve sufficient incorporation. It is known that in the biological system, lipoprotein lipase interacts with VLDL and this results in the liberation of fatty acids from the triglyceride core. Consequently the VLDL remnant particle is either taken up by the LDL receptor in the liver or mostly converted to LDL in the vascular space via intermediate density lipoprotein (IDL), as the case in humans.[12-14] We have prepared the VLDL-resembling phospholipid submicron emulsion (PSME) to function as a carrier solubilizing the hydrophobic BCH compound and to facilitate the interactions with LDL leading to the drug transfer to LDL. Earlier uptake experiments carried with 9L rat glioma cells, showed sufficient uptake of the compound

needed for successful BNCT (>20μgB^{10}/g cells). In this paper, we investigate the transfer of BCH into human LDL in vitro and its distribution in human plasma lipoproteins. We have also conducted cellular uptake experiments in the presence and absence of lipoproteins in the media to further study the possible mechanism of uptake of BCH incorporated in VLDL-resembling PSME into human glioblastoma multiforme cells SF-767.

Materials and Methods

Materials

Triolein (99%), egg yolk Phosphatidylcholine (99%), cholesterol (99%), and human low-density lipoprotein (LDL, 5.2 mg protein/ml) were purchased from Sigma (St. Louis, MO). L-α-Lysophosphatidylcholine (99%) was from Avanti (Alabaster, AL). Cholesterol oleate (99%) was obtained from Acros (NJ, USA). Human glioblastoma multiforme cell line SF-767 was obtained from the tissue bank of the Brain Tumor Research Center (University of California-San Francisco, San Francisco, CA). Dulbecco's Modified Eagle's Media (DMEM), Dulbecco's phosphate-buffered saline (PBS) and trypsin-EDTA were obtained from VWR Scientific Products, Inc. (Suwanee, GA). Fetal bovine serum (FBS), and bovine and human lipoprotein deficient serum (LPDS) were obtained from BioCell Laboratories, Inc. (Rancho Dominguez, CA). Human Analytical grade chloroform, methanol and other chemicals were obtained from J.T. Baker (Phillipsburg, NJ). BCH (cholesteryl 1,12-dicarba-closo-dodecaborane1-carboxylate) was prepared in our laboratory according to the method described by Ji and Lu.[15]

Preparation of VLDL-resembling phospholipid submicron emulsion containing BCH

Stock solutions of individual lipid and BCH were prepared in chloroform. Various lipids and BCH were mixed and were composed of the following ratio (w/w): Triolein: egg Phosphatidylcholine: Lysophosphatidylcholine: cholesterol oleate: cholesterol: BCH, 70: 22.7: 2.3: 3.0: 2.0: 2.0, respectively. [11] All components were

combined and chloroform was evaporated under a stream of nitrogen. The preparation was then desiccated overnight at 4°C to remove residual solvent. Following addition of 10 ml of 2.4 M NaCl to 102 mg of lipid and BCH mixture, the preparation was sonicated under nitrogen for 30 min using a probe sonicator (Branson Sonifier 450) at output 5, while the temperature was maintained at 55°C.

Particle size and chemical composition

Different particle-size populations of BCH-containing PSME were separated by density gradient ultracentrifugation as described by Marranhao *et al*.[16] The fractions of different sizes of the PSME were dialyzed (Spectra/Por® membrane; MWCO 12-14 kDa) against PBS and particle size of each fraction was measured by photon correlation spectroscopy (PCS, NICOMP particle sizing systems, Santa Barbara, CA). Particle size was also calculated according to the chemical composition as described earlier.[11] Non-aqueous reverse phase HPLC chromatography was used to determine the chemical composition (BCH, cholesterol, cholesterol oleate and triolein) of each fraction with different particle sizes. Separation of different chemical components was achieved by C-18 HPLC column using isocratic elution with 50:50 (v:v) methanol:2-propanol, and was detected by UV absorption at 202 nm.[17] The phospholipid content in each fraction was measured as phosphorous concentration by inductively coupled plasma (ICP).

Stability of the BCH-containing PSME formulation

Dialyzed BCH-containing PSME fractions were incubated at 4°C to study possible hydrolysis of the drug (BCH) and the stability of these different particles. Samples were collected on day 1, 3, 10, and 38 for each fraction and analyzed for BCH by HPLC. Particle size of each sample was also measured by photon correlation spectroscopy.

Transfer of BCH from PSME to LDL in PBS and LPDS

LDL (100 µl) was incubated with 200 µl of the first or second fraction of BCH-containing PSME in 2 ml of either phosphate buffer saline (PBS) or lipoprotein deficient

serum (LPDS) for 1 hr at 37°C. After the incubation, the density of each tube was adjusted to 1.08 g/ml with solid KBr. All samples were placed into centrifuge tubes and the remainder of the tube was filled with KBr solution of 1.006g/ml density. Samples where subjected to 285,000 g for 1 hr at 11°C and centrifugation was allowed to stop without use of brake. Additional samples were added to tubes containing dried Nile Red before centrifugation and subsequently detected under UV lamp to identify the volume fractions separated for PSME and LDL. The top 1.5 ml and the bottom 2 ml, where PSME and LDL were present, respectively, based on the Nile Red method, were collected from each tube. Quantitative analysis of BCH was conducted by HPLC as described before. Control samples were also analyzed, which had the same procedure described above except no LDL was involved.

In vitro distribution of BCH in serum lipoproteins

250µl of the second fraction of the emulsion was incubated with 1.25ml of fresh human plasma at 37°C for 5, 30, and 120 min. Incubation was stopped by the addition of 2.5ml of ice cold PBS. The density was adjusted with solid KBr to 1.21gm/ml. Density gradient ultracentrifugation was performed as described by Redgrave, 1975.[18] In brief, the plasma-emulsion mixture was placed at the bottom of nitrocellulose centrifuge tube and layered with 3ml of KBr solution of 1.063g/ml density, followed by another 3 ml of KBr solution of 1.019g/ml and finally the tubes were filled with 1.006g/ml solution. Centrifugation was carried at 285,000 g for 18hrs at 4°C. Emulsion fraction was collected from the top of the tubes. Fractions of 0.6ml where collected from the top of the tube. IDL and LDL were recovered in the fractions corresponding to volume containing between 1.006 and 1.063 g/ml density. HDL (light yellow band, at the interface of 1.063 and 1.21g/ml densities) was recovered at fractions 5-10 respectively as described by Bijsterbosch, 1998.[19] Lipoprotein fractions where analyzed by HPLC as described before.

Cellular uptake of BCH formulated in phospholipid submicron emulsion

Human SF-767 glioma cells were grown in 150 cm^2 plastic cell culture flasks containing 26 ml of DMEM with 10% FBS and 0.1ml gentamicin (50 mg/ml) (referred to as the growth medium). In subsequent experiments, flasks containing 26 ml of growth medium were seeded with approximately 1-2 x 10^6 cells and placed in a humidified, 5% CO_2 –95% air incubator at 37°C. After 24 hours, when cell culture flasks were about 60% confluent, growth medium was removed and replaced with 24 ml of pre-warmed DMEM medium containing 10% of either FBS or LPDS and 2ml of the second fraction of the prepared BCH-containing phospholipid submicron emulsion.

After 18 hours of incubation at 37°C, the media were removed; the cells were rinsed 3 times with PBS, then harvested with trypsin-EDTA and counted under microscope with hemocytometer. The cells were then pelleted by centrifugation at 3500 rpm for 10 minutes and stored at 4°C until analyzed by HPLC for BCH. Student's t-test was used to compare the uptake of BCH in media containing either FBS or LPDS.

Results

Preparation of BCH-containing phospholipid submicron emulsion

BCH was incorporated in sufficient quantities into phospholipid submicron emulsion resembling VLDL in lipid composition. The mean particle size of the formulation before fractionation was 155 ± 5 nm as measured by PCS. Density gradient ultracentrifugation was used to fractionate the emulsion into three particle size populations of the same density, and the mean particle sizes were 161 ± 2, 76 ± 1 and 40 ± 3 nm, respectively. A fourth fraction was also detected that didn't move to the top even after 5 hrs of centrifugation at 285,000g and was recovered at the interface formed between the lower two density layers (1.065 and 1.02 g/ml). The particle size of the fourth fraction, however, was not detected by photon correlation spectroscopy indicating that the size was very small. The chemical composition of each fraction with different

particle size, separated by density gradient ultracentrifugation, was examined by HPLC and is shown in Table 3.1.

Stability of BCH-containing submicron emulsion.

After 38 days, incubation at 4°C, the formulation appeared to be unchanged. The particle size remained without any significant change during this period (data not shown). HPLC analysis of the BCH showed that after 38 days at 4°C about 85±4% (n = 3) of the initial BCH was recovered from the formulation. However, for this study, all preparations were used within five days of preparation.

BCH Transfer to LDL in PBS and LPDS.

After separating the submicron emulsion particles from the LDL, analysis of the LDL particles revealed that a significant amount of BCH originally incorporated in the first and second fractions of the submicron emulsion was transferred to the human LDL after 1hr incubation at 37°C in LPDS media, Table 3.2. This transfer was not observed when the incubation was carried in PBS (no BCH was detected at the LDL fraction after incubation).

In vitro distribution of BCH in serum lipoproteins

BCH appeared to distribute selectively into IDL/LDL fraction and HDL after incubating BCH-containing PSME with fresh human plasma, Figure 3.2. The amount of BCH transfer to IDL/LDL and HDL appeared to increase with increasing the incubation time. A total of 19% of BCH has transferred after 2 hrs into human lipoproteins (IDL, LDL, and HDL). IDL and LDL fractions were considered together in this study to prevent the possibility of contamination of these closely related particles with each other.

Cellular uptake of BCH formulated in phospholipid submicron emulsion

To further investigate the use of this formulation in vitro we studied the cellular uptake of BCH in the presence and absence of lipoproteins. Results showed that uptake in FBS (lipoproteins are present) were more than triple the amount when the media

contained no lipoproteins (LPDS) as shown in Figure 3.3. The difference in BCH uptake in the FBS and LDPD was significant ($P<0.05$).

Discussion

Lipoproteins and corresponding submicron emulsion systems resembling natural lipoproteins have several advantages over liposomes as drug delivery systems. The ability to utilize the natural pathway of lipoprotein uptake in the body comprises a unique targeting tool to deliver different compounds in the body. This pathway is of special importance as several reports have shown a significant increase in LDL receptor activity to meet the elevated cholesterol demand by some types of cancer cells. Another advantage relies on the physical nature of these lipoproteins or PSME particles, in which they have a core of oil that serves as a good solvent for hydrophobic compounds that suffer from low water solubility. In this paper we investigated this pathway for selective delivery of newly synthesized, and extremely water insoluble boron-containing compound, BCH, for boron neutron capture therapy.

We described in an earlier paper the preparation of these lipoprotein-resembling submicron emulsion particle and the possibility of association when incubated with LDL, as evident in agarose electrophoresis. In this paper we demonstrate that the incorporated drug, BCH, was transferred to LDL after incubation. The results also indicate that the association that was detected under agarose electrophoresis is of a physical nature since the PSME and LDL particles re-separate under ultracentrifugation. Lipid-exchange between different classes of lipoproteins has been established by others to be dependent on the presence of certain exchange proteins like cholesteryl ester transfer protein (CETP) and phospholipid transfer protein.[20] BCH was transferred from the submicron emulsion particles to human LDL only in the presence of serum proteins. No transfer was detected while in PBS, Table 3.2. This indicates that the transfer is an active process and not merely due to physical contact between these particles. It also indicates that certain molecule(s) in the plasma, probably cholesteryl ester transfer protein CETP, was

responsible for the interaction and transfer of BCH to the human LDL. CETP was described as less selective to its substrate as it transfers not only cholesteryl esters (CE) but also triglycerides.[21] Morton *et al*. reported the activity of CETP to be dependent on the transition temperature of the lipoprotein core.[22] The activity of CETP increases when the temperature is above the transition temperature of the core. In the described VLDL carrier system, the high amount of triolein decreases the phase transition of the core, a criteria that make these particles susceptible for CETP. This can be advantageous since drug molecules of similar structure of CE may also be a substrate for this enzyme and be transferred to body's lipoproteins when incorporated in this submicron emulsion due to the unique resemblance between both particles. Drug transfer to LDL can be another attribute of this formulation for selective delivery via the LDL pathway.

BCH distribution in human plasma is another indication of such transfer process to human lipoproteins. The transfer was specifically carried into HDL and IDL/LDL. A total of 19% of BCH has transferred after 2 hrs into human lipoproteins (IDL, LDL, and HDL). HDL is known to be the major substrate for cholesteryl ester transfer protein, in which the net transfer of CE occurs from HDL to VLDL and IDL. Yet the transfer process is not unidirectional, and can occur from other lipoproteins to HDL as reported by others.[20] We have to emphasize that the exact mechanism of BCH transfer may require further investigation

Cellular uptake results supported the ability of this system to deliver BCH to human glioma cells when lipoproteins were present. BCH uptake was lower when the media didn't contain lipoproteins, in LPDS. These results, in FBS, also meet the requirement for successful boron neutron therapy (>20μg B/gm cells).[7, 8] The number of cells per flask was not affected in the presence or absence of lipoproteins. These results support the role of lipoprotein in the drug transfer and eventual uptake into the cells.

Conclusion

The unique resemblance between these submicron emulsion particles and serum lipoproteins may facilitate drug/lipids exchange with natural lipoproteins in the presence of serum proteins. The incorporation of cholesteryl ester mimicking drugs in this submicron emulsion system can be advantageous for selective delivery of these compounds via the lipoproteins pathway.

Table 3.1: Composition and particle size of different fractions of BCH-containing PSME

PSME Fraction	First	Second	Third	Fourth
Particle Size (nm, Measured)	161±2	76±1	40±3	NA
Particle Size (nm, Calculated)	158	81	31	NA
BCH (%w/w)	2.4±0.1	2.2±0.1	1.8±0.1	0.5±0.1
Cholesterol (%w/w)	1.9±0.1	2.3±0.1	3.1±0.2	3.7±0.2
Cholesteryl oleate (%w/w)	3.0±0.2	2.8±0.1	2.5±0.1	0.3±0.1
Phospholipids (%w/w)	6.5±0.6	12.6±0.4	27.4±0.4	89.3±0.8
Triolein (%w/w)	86.3±0.5	80.1±0.2	65.3±0.9	6.1±0.1

[a] All value are means ± SEM of three experiments.

Table 3.2: *In vitro* transfer of BCH into human LDL in PBS and LPDS

Sample	Percent of BCH transfer into LDL after 1hr incubation at 37^{o}C
F1 + LDL in PBS	0%
F1 + LDL in LPDS	36±5%
F2 + LDL in PBS	0%
F2 + LDL in LPDS	20±4%

All value are means ± SEM of three experiments.

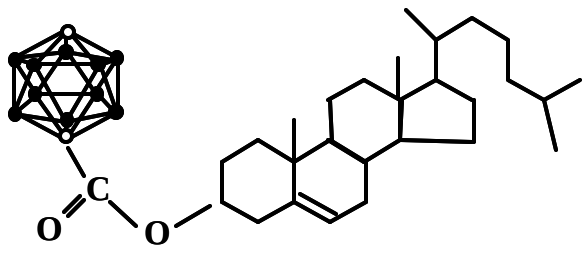

Figure 3.1: Cholesteryl –Carborane (BCH), cholesteryl 1,12-dicarba-closo-dodecaborane-1-carboxylate for BNCT

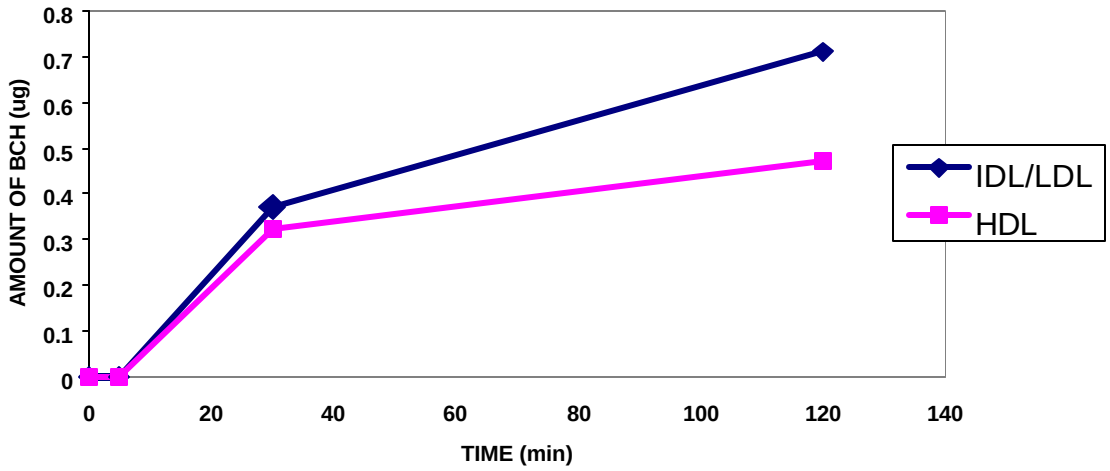

Figure 3.2: *In vitro* distribution of BCH into IDL/LDL, and HDL as a function of time. Incubation with human plasma was conducted at 37°C.

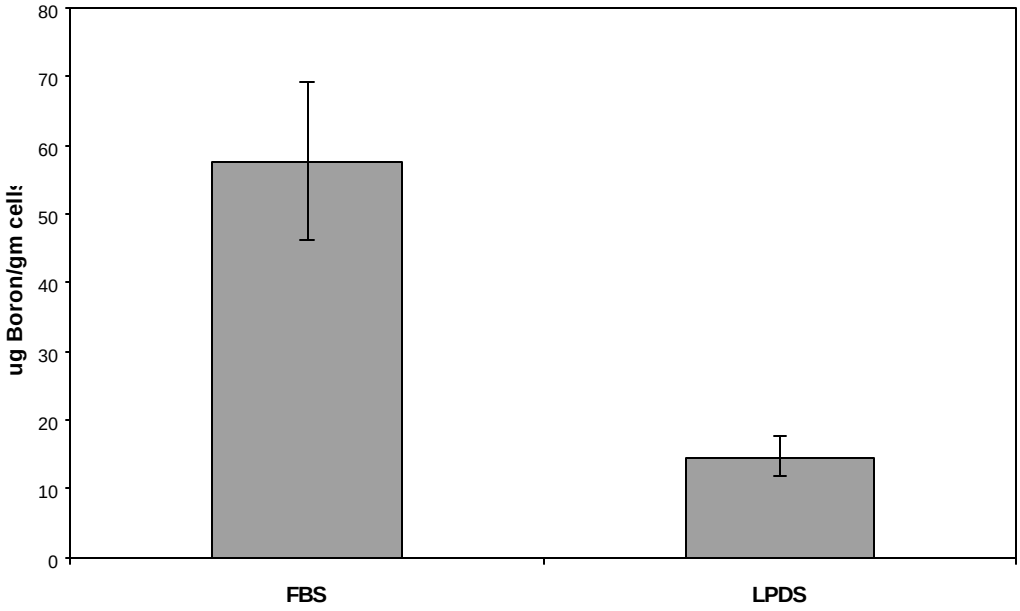

Figure 3.3: Cell uptake of BCH by 767-SF human glioma in presence and absence of lipoproteins in the incubation media.

Abbreviations

LDL, low-density lipoprotein; VLDL, very low-density lipoprotein, BCH, cholesteryl 1,12-dicarba-closo-dodecaborane1-carboxylate; BNCT, boron neutron capture therapy; PSME, phospholipid submicron emulsion; CETP, cholesteryl ester transfer protein; LPDS, lipoprotein deficient serum; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; CE, cholesteryl esters.

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CHAPTER 4

POLYMER ASSOCIATED SUBMICRON EMULSION FOR GENE DELIVERY[1]

[1] Shawer, M., G. Pan, R. Lu. To be submitted to *Journal of Applied Science and Research*

Abstract

The objective of the current study was to develop and evaluate a novel polymer associated submicron emulsion for gene delivery. Cationic liposomes and cationic polymers have been the center of attention for nonviral gene delivery system. Kim *et al.* was among the first to attempt combining the advantages of cationic polymers with low-density lipoprotein creating a targeted gene delivery system, the terplex system.[1] In this paper we studied the possibilities of developing palmitoyl polylysine associated with lipoprotein resembling submicron emulsion. One advantage over the terplex system is that the lipid particles can be easily formulated from similar lipids present in natural lipoproteins. The ability of incorporating different compounds inside these emulsion particles is another advantage for combining gene and drug delivery in the same system. Polylysine was chemically hydrophobized with palmitoyl chloride. Lipoprotein-resembling submicron emulsion (SME) was formulated by sonication. Agarose gel electrophoresis showed the interaction between palmitoyl polylysine (p-PLL) and the emulsion particles, as evident by change of particle charge. Density gradient ultracentrifugation was used to evaluate the amount of p-PLL associated with emulsion particles. When DNA plasmid was added to p-PLL/SME complex, efficient association with the polymer/lipid particles was observed and quantified. In conclusion, this p-PLL/SME system has the capability to carry DNA in vitro, and may assist the gene delivery into cells and tissues.

KEY WORDS: Gene delivery, polylysine, submicron emulsion, lipoprotein.

Introduction

Gene therapy has a great potential for treating different types of diseases. With the completion of the human genome project, we understand more about the functions of these genes and thus significantly increase the possibility for curing a wide range of diseases. One of the main obstacles for gene therapy, however, is to effectively deliver these genes into target cells and tissues. Many research groups have investigated different techniques for delivering genetic material into cells. Two main strategies have been adopted for gene delivery system involving either viral vectors or nonviral carrier systems. Viral vectors are very efficient, but their use is narrowed by the potential side effects of the virus itself, such as over-expression, immunogenicity and activation of oncogenes in the human body [2]. On the other hand, the nonviral systems in general are less efficient but have a better safety profile, if properly formulated, than the virus-based systems. Nonviral carrier systems, contrary to viral vectors, are not limited by size and amount of DNA material that can be incorporated.

The main approaches for nonviral gene delivery involve cationic liposomes and cationic polymers. Both systems depend on the ionic interaction between the negatively charged DNA and the positively charge lipid or polymer. Such interaction was shown to have a condensing effect on the DNA molecules. Accordingly several novel cationic lipid and polymer molecules have been synthesized in search for the efficient carriers to deliver the genetic material. Some of the cationic lipids used in preparing cationic lipid systems include: Lipofectin™ (1:1 ratio of dioleoylphosphatidyleehtanoleamine (DOPE) and 2,3-dioleyloxypropyl-1-trimethylammonium bromide (DOTMA)) [3-5], Lipofectamine™ (3:1 ratio of 2,3-dileyoxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminuim trifluoroacetate (DOSPA) and DOPE) [6,7], and Transfectam™ (diocta-decylamidoglycylspermine (DOGS)) [8]. Most of these lipid carrier systems are liposomes and prepared from these cationic molecules in addition to neutral lipids, such as cholesterol and DOPE. Cationic liposomes complexed with DNA, called lipoplexes,

have been used in clinical trials for treatment of cancer [9,10]. Studies showed that these lipid/DNA complexes have a good safety profile, yet they suffer from low transfection efficiency in vivo, lack of target specificity, and short duration of expression.

Recently, cationic polymer-based gene delivery systems have drawn significant attention. Cationic polymers have been reported with better capability to condense DNA than cationic lipid [11]. Some of the polymers, mainly polyamines, used for gene transfection include poly-L-lysine (PLL), polyethenimine (PEI), and polyamidoemine (PAMAM). Many of these polymer carrier systems have been conjugated with different targeting moiety (ligand) for site-specific gene delivery. The ligand may interact with a cell receptor and initiate a receptor-mediated endocytosis of the polymer/DNA complex (polyplexes). Examples of these targeted systems include transferin-PLL [12], folate-PLL [13], and asialoglycoprotein-PLL [14]. Other class of polymers include cationic block copolymer, which have the ability to self-assemble into micelle-like aggregates in aqueous solutions, similar to amphiphilic block copolymers. They are pH and salt sensitive with the ability to form a stable complex with DNA [15, 16]. Polyethylene glycol-polylysine (PEG-PLL) is an example of these polymers that showed superior protection of DNA from nucleases compared to PLL when complexed with DNA [17].

Combination of cationic lipid and polymer has been studied in an attempt to increase the efficiency of these nonviral systems. Huang et al, (1996) reported potentiation of these lipoplexes (liposome/DNA complex) transfection efficiency when they were combined with polycationic polymers to produce virus-like particles [18]. Kim et al, was the first group to combine low-density lipoprotein (LDL) with chemically hydrophobized polylysine to examine the ability of natural lipoprotein, LDL, to deliver genetic material via the LDL pathway. [1,19, 20] This novel system, called terplex system, is based on a balanced hydrophobicity and net surface charge among LDL, hydrophobized polylysine and DNA. Delivery via receptor mediated endocytosis, biocompatibility, and effectiveness are among the advantages the terplex system has proved.

In this paper we prepared a nonviral gene carrier system composed of palmitoyl polylysine associated with lipoprotein-resembling submicron emulsion prepared from commercially available lipids. It is an artificial lipoprotein system containing chemically modified polylysine, instead of apoE or apoB-100 proteins; to interact with DNA materials.[21] The interaction between the chemically modified polymer and the submicron emulsion is through hydrophobic interactions, and between the polymer and DNA through charge interaction. This artificial lipoprotein system allows us to control the particle size or charge, and to solubilize different compounds in the core of the submicron emulsion (hydrophobic drugs or transfection enhancers). Other advantages include: 1) the capability of using this artificial lipoprotein for *in vitro* or *in vivo* transfection, 2) easiness of preparation of this system, and 3) the consistency in the production of our surrogate system are attributes that may enhance the efficacy of this gene delivery system.

Materials and Methods

Materials

Triolein (99%), egg yolk phosphatidylecholine (99%), cholesterol (99%), human low-density lipoprotein (LDL, 5.2 mg protein/ml), and Poly-$_L$-lysine hydrobromide (av. MWt 50,000 Da) were purchased from Sigma (St. Louis, MO). L-α-Lysophosphatidylcholine (99%) was from Avanti (Alabaster, AL). Cholesterol oleate (99%) was obtained from Acros (NJ, USA). Analytical grade chloroform, methanol and other chemicals were obtained from J.T. Baker (Phillipsburg, NJ). DNA plasmid, (pIRES-hrGFP-1a, 5.0 kb) was obtained from Stratagene (La Jolla, Ca).

Preparation of lipoprotein-resembling submicron emulsion SME

Stock solutions of individual lipids and BCH were prepared in chloroform. Various lipids were mixed. The lipid mixture was composed of the following ratio (w/w): Triolein: egg phosphatidylecholine: lysophosphatidylcholine: cholesterol oleate: cholesterol, 70: 22.7: 2.3: 3.0: 2.0, respectively. [22] All components were combined and

chloroform was evaporated under a stream of nitrogen. The preparation was then desiccated overnight at 4°C to remove residual solvent. Following addition of 10 ml of 2.4 M NaCl to 100 mg of lipid mixture, the preparation was sonicated under nitrogen for 30 min using a probe sonicator (Branson Sonifier 450) at output 5, while the temperature was maintained at 55°C. Different particle-size populations of SME were separated by density gradient ultracentrifugation as described by Marranhao *et al*.[23]. The fractions of different sizes of the submicron emulsion were dialyzed (Spectra/Por® membrane; MWCO 12-14 kDa) against PBS and particle size of each fraction was measured by photon correlation spectroscopy (PCS, NICOMP particle sizing systems, Santa Barbara, CA). Non-aqueous reverse phase HPLC chromatography was used to determine the chemical composition (cholesterol, cholesterol oleate and triolein) of each fraction with different particle sizes. Separation of different chemical components was achieved by C-18 HPLC column using isocratic elution with 50:50 (v:v) methanol:2-propanol, and was detected by UV absorption at 202 nm [24]. The phospholipid content in each fraction was measured as phosphorous concentration by inductively coupled plasma (ICP) spectroscopy.

Lipidization of poly-$_L$-lysine

Lipidization of polylysine (PLL) was achieved as described by Kim et. al (1997) with modification.[1] In brief, 30 mg PLL was dissolved in 2 ml DMSO. Triethylamine (10µl) was added to the mixture. Palmitoyl chloride (20 mg) was used to react with the ε-amino group of the lysine in the PLL polymer. The mixture was allowed to react at room temperature for 2 hrs. The mixture was then filtered and acetone was added to the filtrate to precipitate the lipidized polymer, palmitoyl polylysine (p-PLL). The product was dissolved in methanol, re-precipitated by acetone, and dried under vacuum overnight. The modified polymer was characterized by proton NMR.

Interaction of palmitoyl polylysine with lipoprotein-resembling submicron emulsion

The different size fractions of the submicron emulsion (50 µl of each fraction: F1, F2 and F3) were incubated individually with either 50 µg or 100 µg of polylysine (PLL) or palmitoyl polylysine (p-PLL) for 1 hr in 2 ml PBS at 37°C with gentle shaking. As a comparison, human LDL (20 µl LDL+30 µl PBS) was also incubated with either 50 µg or 100 µg of p-PLL in the same experiment condition. Agarose gel electrophoresis was performed according to the method described by Greenspan *et al.* [25] using Nile Red as the fluorescent dye to determine the electrophoretic mobility of the lipoprotein-resembling submicron emulsion or human LDL and to examine their interactions with p-PLL. In brief, 0.6% agarose gel was prepared in 50 mM barbital buffer, pH 8.6. Five ìl of Nile Red in acetone (100 ìg/ml) was dried out in test tube. Samples were then added to the tube individually (50 ìl sample to each tube) and mixed until Nile Red was in solution. Five ìl of sucrose solution (30%, w/v) was added. Each electrophoretic well was loaded with 11 ìl of sample preparation. Electrophoresis was conducted for 1 hr at 56 V at room temperature. Different electrophoretic bands on the gel were visualized under UV lamp.

Degree of palmitoyl poly lysine association with SME

In order to measure the amount of p-PLL associated with the submicron emulsion particles (SME), 250 µl of second fraction of the emulsion (containing 2.39 mg triolen/ml, triolein was used to reflect the amount of emulsion used) were incubated with 2 mg of palmitoyl polylysine (p-PLL) in 2 ml PBS for 1 hr at 37°C with gentle shaking. The density of the mixture was then adjusted to 1.08 g/ml with solid KBr and placed in the bottom of 13.5 ml centrifuge tubes. The remaining of the tube was filled with KBr solution of 1.063 g/ml. The mixture was subjected to density gradient ultracentrifugation at 285,000 g for 2 hrs, and centrifugation was allowed to stop without use of break. The top 4 ml, where the emulsion particles are recovered, and the bottom 5ml of the tubes were collected and assayed for content of lipidized polylysine using the modified Lowry method [26]. Tubes containing p-PLL only or SME only were centrifuged along with the mixture samples and used as controls.

Interaction of p-PLL associated submicron emulsion with DNA.

Varying amounts of p-PLL associated emulsion (second fraction), of fixed weight ratio (triolein: p-PLL, 1: 0.5) were incubated with 200 ng of DNA plasmid. Incubation was carried at room temperature for 15 minutes. Samples were then loaded into 0.6% agarose gel, containing 0.5 μg/ml ethidium bromide, as described above. DNA bands were visualized on the gel under UV lamp according to standard method [27]. Control groups containing DNA by itself, DNA incubated with p-PLL, and DNA incubated with SME were treated similarly and carried on the same gel.

Results

Particle size and chemical composition

Density gradient ultracentrifugation was used to fractionate the emulsion into three particle size populations of the similar density (<1.006 g/ml). These three fractions (as F1, F2 and F3) were used individually to examine their ability to interact with the modified polymer, p-PLL. The mean particle sizes for F1, F2, and F3 were 155 ± 5, 76 ± 3 and 44 ± 4 nm, respectively. The chemical composition of each fraction with different particle size was examined by HPLC and is shown in Table 4.1. The majority of the lipid was recovered in the second fraction (37%). This fraction had an average particle size of 76nm with lipid composition as follows: 2.1% cholesterol, 6.2% cholesteryl esters, 12.6 % phospholipid, and 79.1% triolein. This resembles human VLDL, as it's size ranges from 35-80nm, which has lipid composition 5% cholesterol, 12% cholesteryl esters, 18% phospholipid, and 65% triglycerides.

Interaction of lipidized polylysine with lipoprotein-resembling submicron emulsion

Due to the association of p-PLL with submicron emulsion particles the surface charge of the particles was completely reversed. Before addition of p-PLL the emulsion particles had slight negative charge as the case with human LDL too. After the addition of 50 μg p-PLL that has a positive charge, owing to the free ε-amino groups of lysine, the particles showed small movement toward the negative electrode suggesting the charge of

the p-PLL associated submicron emulsion is positive (see Figure 4.1). Similar results were obtained when the incubation was carried with 100 µg p-PLL. Human LDL, which has negative charge, showed similar results when incubated with p-PLL and its charge has changed into positive charge as shown in the Figure 4.1. When the SME was incubated with polylysine (unlipidized), precipitation of the emulsion particles was observed immediately, contrary to results with p-PLL.

Degree of palmitoyl polylysine association with SME

The amount of 0.55±0.5mg of p-PLL per 1mg of triolein associated with the second SME fraction. This amount was calculated after subtracting the amount of p-PLL from control tube of m-PLL alone that floats to the top and the interference of emulsion turbidity on the analysis.

Interaction of p-PLL associated submicron emulsion with DNA.

Figure 4.2 showed that DNA plasmid migrated towards the positive anode. When DNA plasmid was incubated with p-PLL and p-PLL associated submicron emulsion, complete reversal of DNA migration was observed indicating the ability of p-PLL/SME system to carry DNA plasmid. As a control, DNA was incubated with SME only, and SME by itself showed no effect on the electrophoretic mobility of DNA, Figure 4.2.

By varying the amount of p-PLL associated SME to DNA, we were able to determine the system capacity to carry DNA. At 1µg triolein of the polymer /lipid complex incubated with 200ng DNA, some of the DNA started to escape from the system as the DNA showed electrophoretic mobility toward the positive electrode, similar to naked DNA. Such behavior was not detected at 5µg triolein of the polymer/lipid complex.

Discussion

Efficient gene therapy and transfection is dependent on the ability of the carrier system to overcome numerous obstacles in vivo. This include; 1) transport in blood circulation from the site of injection to the targeted tissue, 2) uptake by cells, 3) release

from endosomal compartment, 4) dissociation of DNA from carrier system, 5) transit from cytoplasm to nucleus, 6) and gene transcription. [28] Different synthetic carrier systems have been investigated, mainly cationic liposomes. Unfortunately, DNA/liposome complex is inactive or less effective in the presence of serum due to dissociation of DNA from the complex or formation of large aggregates that are taken up by the reticuloendothelial system rapidly. Kim et al described a novel terplex system based on the natural lipid carrier, LDL, associated with hydrophobized polylysine capable of condensing DNA and subsequently transfecting cells in vitro and in vivo. [1,19,20] Huang et al, 1997, described the use of reconstituted chylomicrons remnants (RCR) as nonviral vector. [29] DNA was complexed with cationic lipid and solubilized in the core of these RCR particles. Both of these lipoprotein-based systems appeared to offer resistance against serum effect, which is critical for in vivo systemic application. In an earlier paper we investigated a lipoprotein-resembling submicron emulsion for targeted delivery of boronated cholesterol .[230] The advantage of these lipoprotein-resembling particles is their ability to interact with the LDL receptor and taken up via LDL receptor pathway in vitro and in vivo.[22] In this paper we prepared the lipoprotein-resembling particles from commercially available lipids, and attempted to combine the ability of polyamine polymer to condense DNA with these highly characterized lipid particles for gene delivery. As shown in Table 4.1, three fractions of the submicron emulsion has been isolated and characterized. All of these SME fractions had a density of less than 1.006g/ml, similar to chylomicrons and VLDL with regard to lipid composition and particle size. Hydrophobization of polylysine was achieved through N-alkylation of the free ε-amine groups with palmitoyl chloride and confirmed via proton NMR (data not shown). The ratio of palmitoyl chloride: lysine monomer combined was 1: 4. This ratio assures that not all of the free ε-amine groups are conjugated, to maintain the ability of polylysine to condense DNA. [1]

All of the SME fraction and LDL had negative surface charge as shown in agarose gel, Figure 4.1. When SME was combined with polylysine (unmodified) immediate precipitation was observed, indicating the formation of large aggregates. When p-PLL was incubated with SME particles, no precipitation or change in the turbidity was observed. These results indicate that with hydrophobized polylysine the interaction is not merely through charge, but also through hydrophobic interaction between the added palmitoyl chains to PLL and the surface of SME. The charge of the SME particles and LDL was reversed when p-PLL was added, Figure 4.1. The p-PLL associated SME or LDL barely interred the gel.

To confirm and quantify the amount of p-PLL associated with SME (2^{nd} fraction) excess amount of p-PLL was incubated with SME particles and subjected to density ultracentrifugation. P-PLL associated SME was recovered at the top, where the unassociated portion of the polymer was detected in the bottom of the tube. A control of SME alone was used to avoid the effect of SME turbidity on the spectrophotometric detection of p-PLL. The amount of p-PLL associated averaged 0.55 mg /mg of triolein. Such kind of interaction is believed to occur at the surface of the SME with the hydrophobic chain (palmitoyl groups) added to polylysine. When DNA was added to pre-incubated p-PLL/SME complex, the electrophoretic mobility of DNA was toward the cathode end of the gel, Figure 4.2. These results indicate that the DNA was complexed with p-PLL/SME particles and traveled according to the net charge of the DNA/p-PLL/SME complex, Figure 4.3. Naked DNA showed electrophoretic mobility toward the anode end of the gel. SME alone had no effect on the mobility of the DNA as shown in Figure 4.2. Different amounts of p-PLL/SME complex were added to the same amount of DNA in order to determine the maximum amount of DNA that can be carried with the p-PLL/SME system. As presented in Figure 4.2, with decreasing the amount of p-PLL/SME added, the movement of DNA, visualized with ethidium bromide, was decreased toward the cathode, until it reached a point where some of the DNA appeared

traveling toward the anode. The DNA bands become significantly dimmer when complexed with p-PLL/SME due to the loss of ethidium bromide fluorescence on complete complexation of DNA with polymer. A maximum amount of 200ng of DNA can be incorporated per at least 5μ of triolein. The ability of DNA to move into the gel after complexation with the p-PLL/SME system implies the small size of the complex, which is an attribute that may facilitate gene delivery. Changing the ratio of DNA to p-PLL/SME can control the net charge of the final complex. Transfection abilities of lipoplexes were shown to be related to the charge carried by these complexes. It is necessary to have positively charged complexes to interact with the negatively charged cell surface.[31] Other cationic polymer can be similarly hydrophobized and used to condense DNA on the surface of these SME particles. For example, polyethyleneimine (PEI) has an intrinsic endosome-buffering property that can protect DNA from degradation via endosomal enzymes once it enters the cells.

Conclusions

In this paper, we described the formulation and characterization of a lipoprotein–based system for gene delivery. The main advantage of this system is that it is easily formulated from commercially available lipids, with the ability to control the size and charge of the complex generated for gene delivery. The hydrophobic core of the SME provides a good solubilizing media for hydrophobic drugs or transfection enhancers for a combined drug and gene therapy. All of these parameters are of importance for optimization of transfection process in vitro and in vivo.

Table 4.1: Composition and particle size of different fractions lipoprotein resembling submicron emulsion (SME)

Fraction	Particle size (nm)	Cholesterol (w/w%)	Cholesteryl oleate (w/w%)	Triolein (w/w%)	Phospholipid (w/w%)
F1	155±5	1.9±0.1	6.4±0.2	84.4±0.5	7.3±0.6
F2	76±3	2.1±0.1	6.2±0.1	79.1±0.4	12.6±0.4
F3	44±4	2.7±0.2	5.1±0.2	64.8±0.9	27.4±0.5

All values are means ± SEM of three experiments.

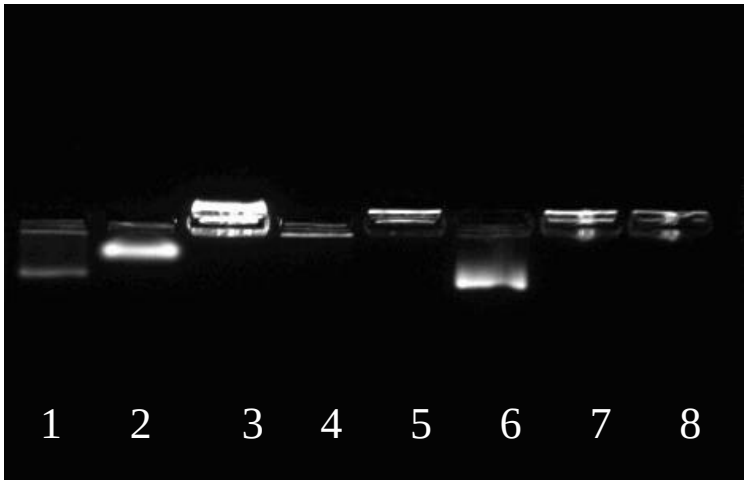

Figure 1: Agarose electrophoresis (the anode is at the bottom of the gel),

1: Fraction 3, 2: LDL, 3: LDL/p-PLL, 4: F1, 5: F1/p-PLL, 6:F2, 7: F2/p-PLL,

8: F3/p-PLL, (F represents the fraction of SME used).

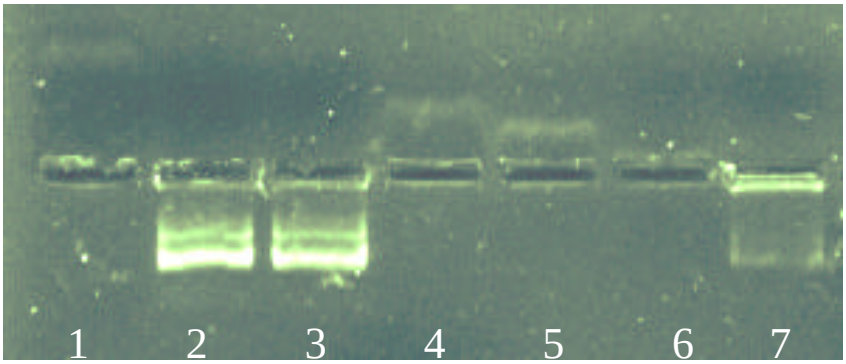

Figure 2: Agarose electrophoresis (the anode is at the bottom of the gel), from left to right: 1:DNA + p-PLL, 2:DNA alone, 3:DNA + SME F2 (25µg triolein), 4:DNA + p-PLL-SME F2 (25µg triolein) , 5: DNA + p-PLL-SME F2 (12.5µg triolein) 6:DNA + p-PLL-SME F2 (5µg triolein) , 7:DNA + p-PLL-SME F2 (1µg triolein).

The final weight ratio of p-PLL to triolein =0.5

200ng of DNA was used in each lane

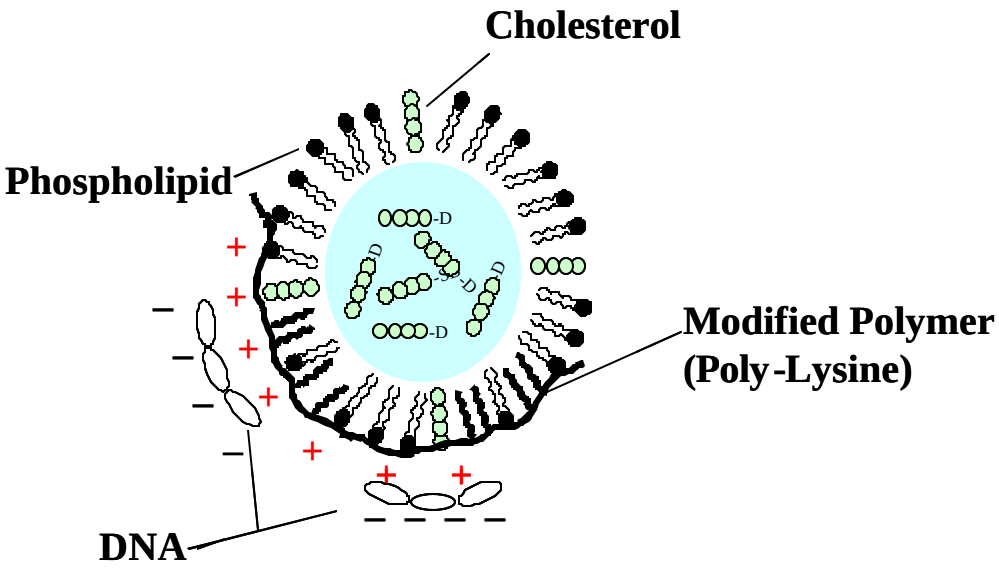

Figure 3: Schematic diagram of DNA/p-PLL/SME system.

Abbreviations

LDL, low-density lipoprotein; PLL, polylysine; p-PLL, palmitoyl polylysine; SME, submicron emulsion; VLDL, very low-density lipoprotein; T, triolein.

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CHAPTER 5

LYSOZYME LIPIDIZATION AND DETECTION BY MALDI-TOF MASS SPECTROMETRY[1]

[1] Shawer, M., R. Lu. To be submitted to *Journal of Colloid and Interface Sciences*

Abstract

Purpose. To develop an efficient method for chemical lipidization of proteins and its detection by MALDI-TOF mass spectrometry, using lysozyme as the model protein. Lipidized proteins are intended for association at the surface of submicron emulsion, which can target the whole particle to certain tissues.

Methods. Lysozyme, used here as a model protein, was chemically hydrophobized by N-hydroxysuccinimide active ester of stearic acid. Molecular weight analysis was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Lysozyme lytic activity was measured against *M. lysodeikticus.* The ring method was used to measure surface tension.

Results. The mass spectrometry results showed that the molecular weight of lysozyme (14,300 Da) has shifted to higher values (15,200 Da), indicating attachment of 3 fatty acid molecules. Modified lysozyme maintained 75% of its lytic activity. The surface tension of modified lysozyme solution was lower than that of native lysozyme.

Conclusion. The method for lipidizing lysozyme succeeded in producing derivatized, relatively undenatured lysozyme with approximately three molecules of bound stearate.

Introduction

Several attempts have been made to modify proteins with lipids. The difficulties of lipidization arise from protein's limited solubility and risk of denaturation in organic solvents and from lipid's limited solubility in the aqueous phase.

Using AOT (dioctyl sulfosuccinate) as a surfactant and acyl chlorides as the lipidizing agent, chemotrypsin and other proteins were lipidized in reverse-phase micelles formed in octane.[1] Others performed the reaction in an aqueous micellular dispersion using a reactive fatty acid derivative.[2,3] Hashimoto *et al* (1989) has also attempted such conjugation to insulin in organic solvent.[4] Each of these methods has its advantages and disadvantages for the conjugation reaction and preserving the protein from denaturation.

It has been reported that lipidized proteins have higher ability to translocate through the cell membrane, and to be taken up by cells both *in vitro* and *in vivo.*[5,6] Torchilin *et al*, (1986), Niedermann *et al.*, (1991), and Baszkin *et al*, (1999), have studied the effect of protein lipidization to enhance the association with liposomal structure.[7-9] Others carried the conjugation reaction between proteins and liposomes using reactive phospholipids.[10, 11]

In this paper lysozyme is our model protein, which we lipidize with stearic acid. The purpose of lipidization is to enhance the protein's association with drug-containing colloidal carriers so that these may facilitate tissue targeting for different compounds. This system is based on the natural carrier of cholesterol in the body, lipoproteins, especially low-density lipoprotein (LDL). This lipoprotein is composed of submicron emulsion droplet that is surrounded by a protein component, apolipoprotein B100, which is responsible for targeting the particle to the LDL receptor. Similarly, we created a submicron emulsion resembling the lipid part of these natural lipoproteins, and we are attempting to generate different surface ligands on these particles to target different receptors in the body.[12]

The concept may be illustrated by lipidized avidin, which on the surface of submicron emulsion serves as a universal docking station for biotinylated antibody, drug, nucleic acid or polymer (Figure 5.1). The targeting ability of the complex (formed by interaction of emulsion, lipidized avidin and one of many biotinylated molecules) then depends on the specificity of the attached biotinylated molecules.

This paper presents a method for lipidization of proteins that achieves derivatization of stearates to free amino groups and minimizes protein denaturation.

Materials and Methods

Hen's egg lysozyme was purchased from Sigma (St. Louis, MO). Stearic acid (99%), *N*-hydroxysuccinimide (98+%) and dicyclohexylcarbodiimide (99%) were purchased from Lancaster (Pelham, NH). Ethyl acetate and *N*, *N*-dimethylformamide were purchased from J.T Baker (Phillipsburg, NJ).

Preparation of active ester

The preparation of stearic acid active ester was performed as described by Lipodot *et al*. (1967).[13] *N*-hydroxysuccinimide (3.45 g) was dissolved in dry ethyl acetate (150 ml). Then 8.53 g of stearic acid was added to that solution. A solution of dicyclohexylcarbodiimide (6.18 g) in dry ethyl acetate (10 ml) was added to the above reaction mixture and left overnight at room temperature.

Dicyclohexylurea was removed by filtration and the filtrate yielded white crystals of the stearic acid active ester under rotavapor. The crystals were then recrystallized in ethanol. Differential scanning calorimetry (Perkin Elmer DSC7, Norwalk CT) was used to determine the purity of the active ester and its melting point. The active ester of stearic acid was prepared in relatively pure form as evidenced by a melting point of 94^{o}C compared to the melting point of stearic acid 69^{o}C (Figure 5.2).

Lipidization of Lysozyme

Stearic acid active ester (10 mg) was dissolved in 1.5 ml of dimethylformamide (DMF) and then added drop wise while stirring to a solution of 10 mg of lysozyme dissolved in 2.5 ml of distilled water. The mixture was left overnight at 37^{o}C. The mixture was then dried under reduced pressure, re-dissolved in distilled water and passed through a 0.45 µ syringe filter.

A control reaction was also conducted under the same conditions but without the addition of stearic acid ester to rule out any effect of the reaction conditions on the molecular weight of lysozyme. In addition, the same reaction (with and without stearic acid ester) was carried out in DMF only.

MALDI-TOF analysis

The modified lysozyme sample was analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry using a Bruker Reflex time-of-flight mass spectrometer (Billerica, MA) retrofitted with delayed extraction. The matrix was a saturated solution of 3,5 dimethoxy-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) in a 50:50 mixture of water: acetonitrile with 0.1% triflouroacetic acid (TFA). The MALDI target was first spotted with nitrocellulose and allowed to dry. A 2-µL sample was applied next and dried. The sample was washed with a cold water solution of 0.1% TFA. After washing two times, 0.5 µL of solvent was added to each sample. The spectrum was acquired in linear mode by averaging 26 laser-shots and was externally calibrated using lysozyme MH^{+} and MH_2^{2+}(MH^{+} and MH_2^{2+} represent one molecule of lysozyme with one or two protons, respectively).

Lysozyme lytic activity

Lysozyme activity was measured from the rate of lysis of *Micrococcus lysodeikticus* in suspension at 570 nm as described by Shugar *et al* with modification.[14] In brief, 2 ml of 9 mg/25 ml solution of *M. lysodeikticus* in PBS was incubated at 25^{o}C. Lysozyme (60 µl of 140 µg/ml) was added to the cell suspension and the absorbance at

570nm was noted at different time points. This assay was carried out for both modified and native lysozyme and for lysozyme (control) exposed to similar reaction conditions but without the addition of the active ester.

Measurement of surface tension

A Fisher Surface Tensiomat, model 21, was used to determine the apparent surface tension. This model employs the ring method of measuring the surface tension. Temperature was controlled in all the experiments to be 22–23^{o}C. The platinum-iridium ring was rinsed in benzene between each measurement to remove hydrocarbons, then in acetone (to remove the benzene) followed by flaming until the ring was red-hot. Samples were dissolved in 0.01 M sodium phosphate buffer to form 0.3 mg/ml solutions, pH 7.0. The ring was lowered (6 mm) into the sample solution, and care was taken to ensure consistency in pulling the ring from the solution for each measurement. Measurements represent the average of 3 experiments taken at varying times over a 90-minute period.

Results and Discussion

Our method was to prepare an active ester of stearic acid (the fatty acid reagent) and to conduct its reaction with lysozyme in a mixture of aqueous and organic solvent, forming one phase to solubilize both the protein and the fatty acid reagent. The N-hydroxysuccinimide stearic ester has better reactivity and water solubility than stearic acid.[13] The chemical reaction for the preparation of the active ester of stearic acid and subsequent reaction with lysozyme is presented in Figure 5.3.

The mass spectrum of the modified lysozyme showed an obvious increase in the molecular weight of lysozyme (Figure 5.4). The shift of around 900 Da represents the attachment of three stearic acid chains. There was no shift in the spectrum of the control lysozyme, which was exposed to the reaction conditions without active ester (not shown). Lysozyme has 7 amino groups that are available for modification, one α-amino group of N-terminal lysine and 6 ε-amino groups of lysine residues.[15]

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was utilized to detect the chemical modification of lysozyme. Contrary to the spectrophotometric titration methods used to detect protein modification, the MALDI-TOF mass spectrometry depends solely on the molecular weight changes that occur after the chemical modification. And at the same time, MALDI-TOF mass spectrometry offers a good estimate of the degree of the modification (i.e., the number of fatty chains introduced into the protein). These estimates were made by dividing the increase in molecular weight of the protein by the molecular weight of the fatty group introduced by the reaction.

The yield of the modified lysozyme produced in water/DMF was 45%. This preparation was water soluble, whereas the lysozyme modified in medium containing DMF only had low water solubility. The MALDI-TOF spectrum of this weakly soluble preparation has a shoulder, indicating incomplete reaction (Figure 5.5).

Lysozyme lytic activity against *Micrococcus lysodiekticus* was measured after the reaction in water/DMF to be 75% of the native lysozyme (Figure 5.6). The reaction condition even without modification appeared to slightly affect the lytic activity of lysozyme (81% of native lysozyme). Native lysozyme activity was significantly different from modified lysozyme using t-test at $\alpha=0.05$ ($P=0.006$). Modified lysozyme activity was also significantly different at $\alpha=0.05$ from non-modified lysozyme that undergone the same reaction conditions without adding the active ester of stearic acid ($P=0.028$). This indicates that modification of lysozyme is occurring at a position that has slight effect on the lytic activity (6%), and that the reaction conditions contribute more to loss of this activity (19%).

Surface tension measurement showed a greater decrease in the air-water surface tension of a solution containing modified lysozyme than one containing unmodified lysozyme at a similar concentration (Figure 5.7). The explanation as described Wei *et al* (1990) is that the surface tension kinetic behavior at high protein concentration is a

reflection of the protein's surface hydrophobicity and its chain length.[16] Magdassi *et al*. (1997) showed that lipidizing glucose oxidase with 5 palmitate molecules enhanced the surface activity at solution/air interface compared to native enzyme.[9] Similar justification can be given here that modification of lysozyme with 3 stearate molecules causes distortion of the water structure and increases total free energy of the system to a higher extend than does the native lysozyme. Therefore, the transport of modified lysozyme to the water/air interface will benefit more from entropy gain than native lysozyme.

The surface tensions of both solutions of modified and native lysozyme appeared to decrease over time, similar to findings by Yamashita *et al*., (1968), and Wang *et al* (1997) until equilibrium was reached.[17, 18] Yamashita explained this behavior of native lysozyme solution by the time needed for lysozyme molecules to diffuse to the surface, become adsorbed and unfold.

Conclusions

Chemical lipidization of lysozyme was achieved. The presence of an aqueous phase during the reaction was important to get a complete reaction and to minimize protein denaturation. The degree of protein denaturation appeared to be minimal for lysozyme, since the enzyme retained about 75% of its lytic activity. Modified lysozyme was shown more capable than native lysozyme of reducing surface tension. This property will be utilized to enhance the association between macromolecules and colloidal carrier systems.

This method might be used to modify other proteins in a similar way. MALDI-TOF mass spectrometry is a very good tool to evaluate the extent of reaction. Different macromolecules with a targeting ability can be associated with the surface of drug carrier systems such as submicron emulsions. Lowering the surface tension at the interface of the submicron emulsion system is of significant importance for stabilizing the system and lowering the interfacial tension at the interface.

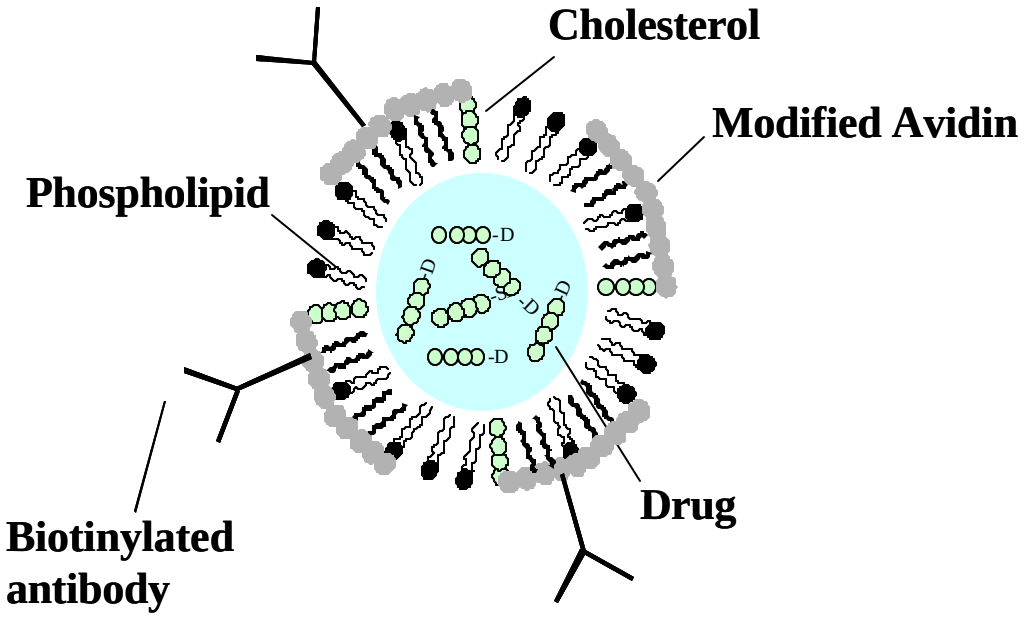

Figure 5.1: Schematic diagram of artificial lipoprotein for drug targeting.

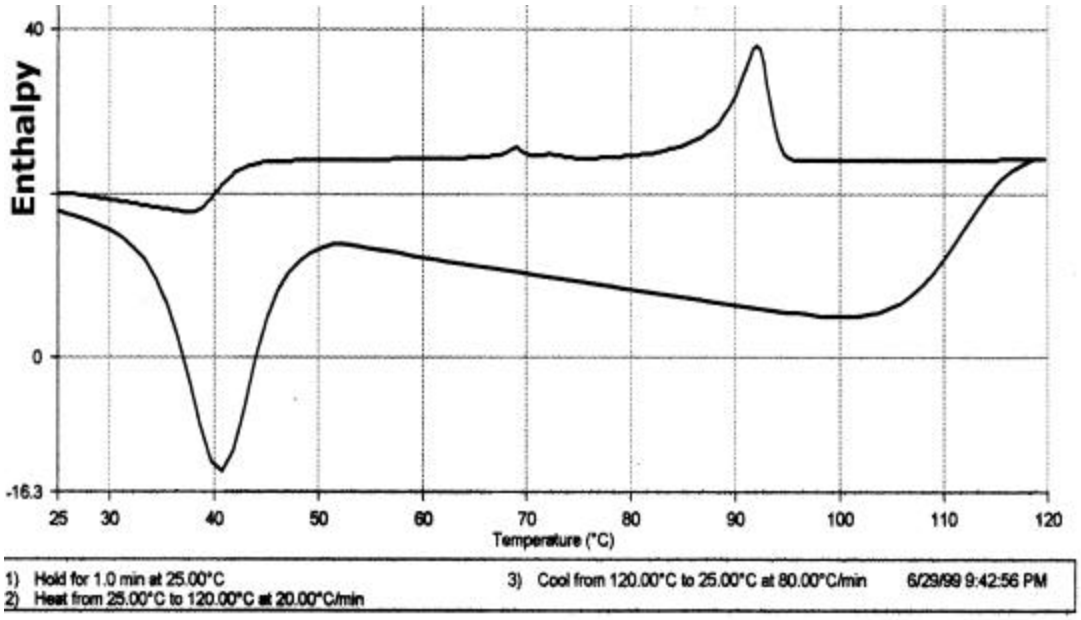

Figure 5.2: DSC spectrum of N-hydroxysuccinamide derivative of stearic acid with melting point 94^{o}C. A small peak of unreacted stearic acid m.p 69^{o}C was noticed.

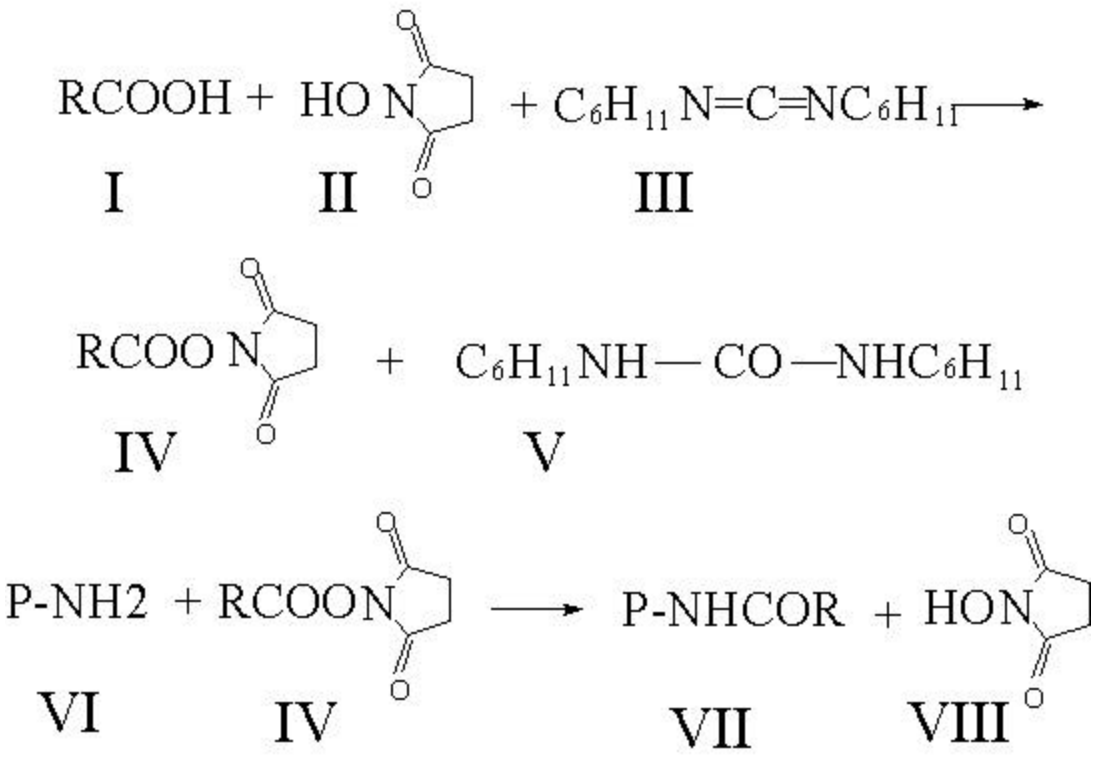

Figure 5.3: Chemical reaction for the formation of active ester of stearic acid and the reaction with lysozyme. I: stearic acid, II: N-hydroxysuccinimide, III: dicyclohexylcarbodiimide, IV: N-hydroxysuccinimide of stearic acid, V: dicyclohexylurea, VI: protein (lysozyme) containing primary amine group, VII: N-stearylamino derivative of protein, VIII: N-hydroxysuccinimide

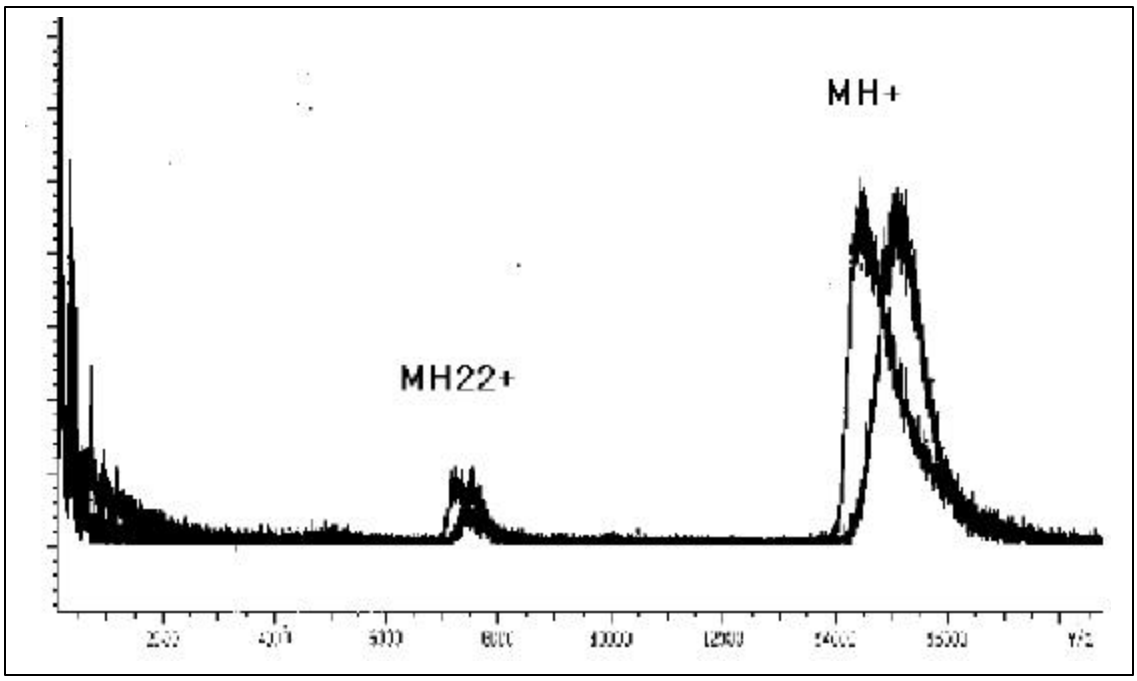

Figure 5.4: MALDI-TOF spectrum showing the shift in molecular weight following the lipidization reaction with N-hydroxysuccinimide active ester of stearate (right) compared to a control reaction (left). MH^+ and MH_2^{2+} represent one molecule of lysozyme with one or two protons, respectively. The x-axis represents molecular weight (da), and the y-axis represents an arbitrary intensity unit.

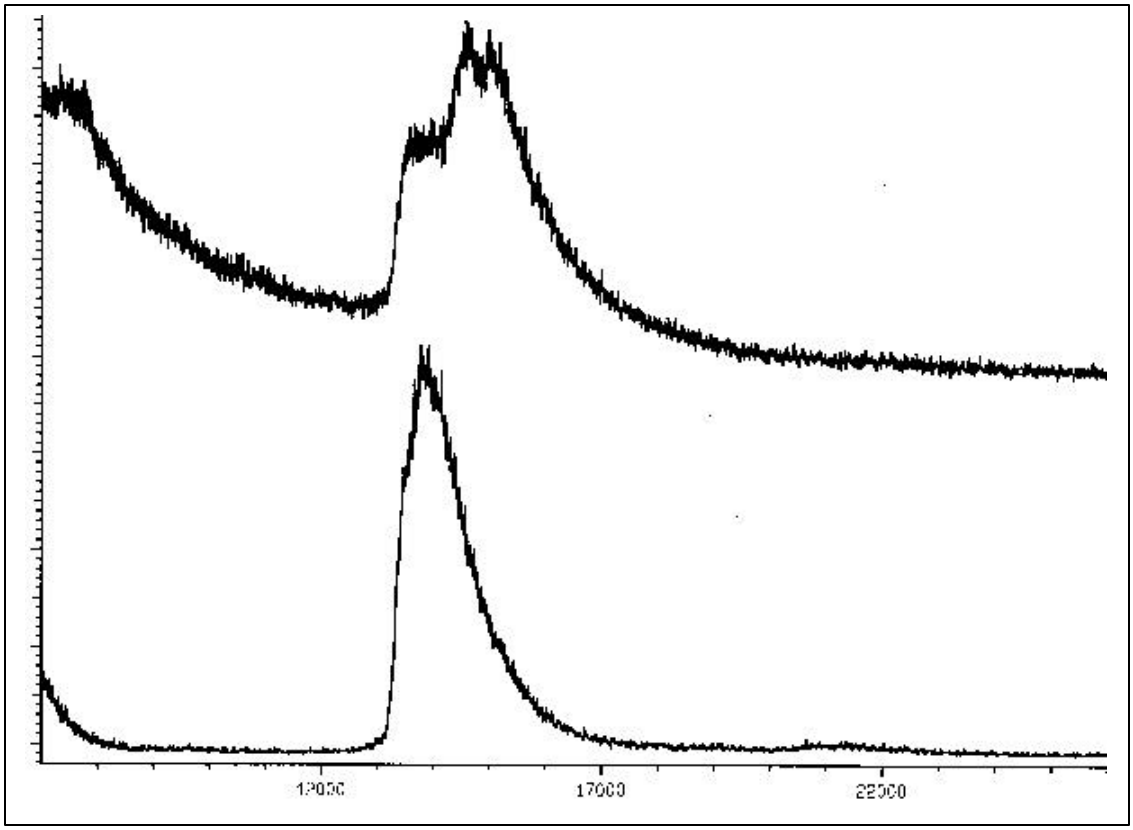

Figure 5.5: MALDI-TOF spectrum showing the incomplete reaction when carried in DMF only media (top), control (bottom). The x-axis represents molecular weight (da), and the y-axis represents an arbitrary intensity unit.

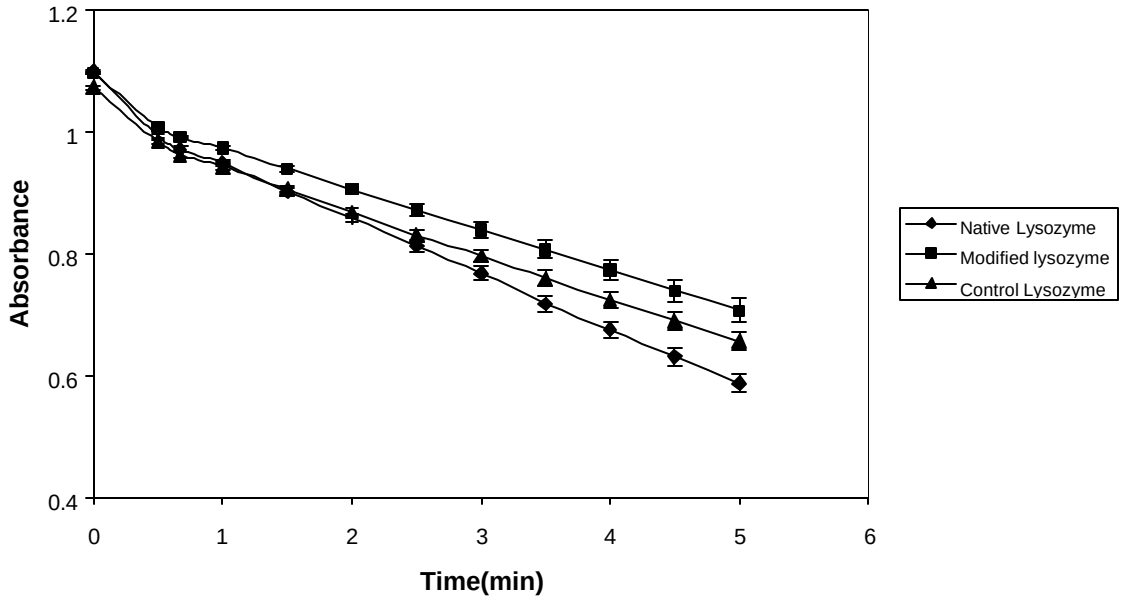

Figure 5.6: Lysozyme lytic activity measured from the rate of lysis of *Micrococcus lysodeikticus* in suspension at 570 nm in samples containing either native lysozyme, modified lysozyme, or control lysozyme which undergone same reaction conditions without the addition of active ester.

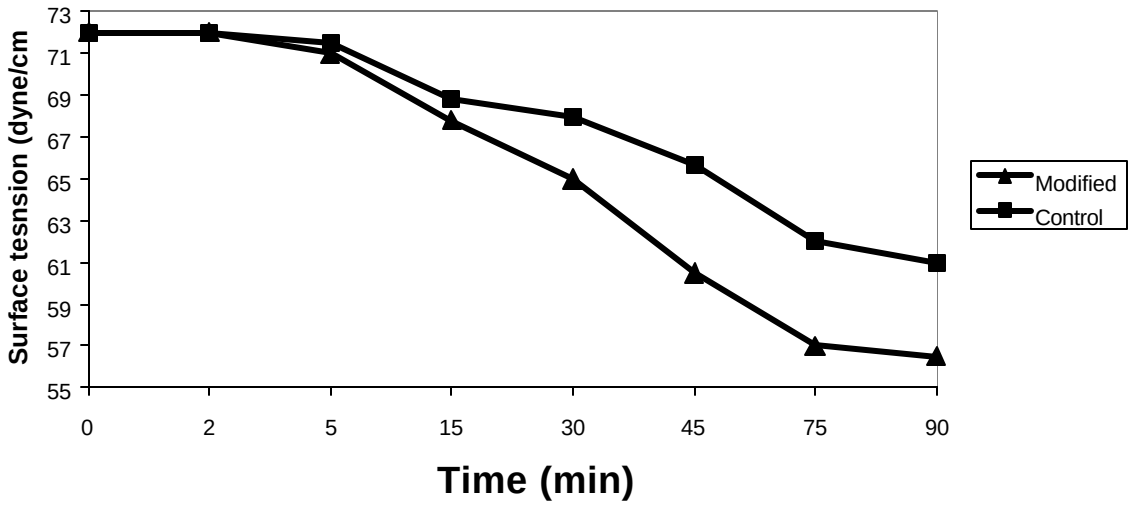

Figure 5.7: Represents the average surface tension measured (n=3) over 90-minute period of an aqueous solution containing 0.3mg/ml of native lysozyme of modified lysozyme.

Abbreviations

LDL, low-density lipoprotein; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; DMF, dimethylformamide; DSC, differential scanning calorimeter

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CHAPTER 6

CONCLUSIONS

In this research we have developed a new drug/gene delivery system. Our strategy was based on the natural cholesterol carrier in the human body, LDL. To mimic the structure of lipoproteins, we prepared submicron emulsion of similar chemical and physical properties of the lipid composition of lipoproteins. Having an oil core in these submicron emulsions was proven to solubilize sufficient quantities of water insoluble compound, BCH. We have demonstrated the ability of this system to deliver this compound into cancer cells in vitro. Drug was also transferred to human lipoproteins when these emulsion are incubated in vitro or in vivo with human lipoproteins. Such attribute may also assist in delivering the compound selectively to cancer cells via the LDL pathway.

We have also the ability of this system to carry DNA. By adding positively charged palmitoyl polylysine, the polymer appeared to associate with the submicron emulsion particles through hydrophobic interactions. This association created positively charged particles capable of carrying DNA. We quantified the amount of genetic material carried with these polymer/lipid particles by agarose gel electrophoresis.

We also described a detailed method to carry protein hydrophobization with minimal denaturation of the protein. The chemical conjugation was confirmed using MALDI-TOF mass spectrometry. Different targeting molecules can be associated with these emulsion particles to target different tissues depending on the hydrophobized protein/polymer used.