PART 1: FORMULATION AND EVALUATION OF AN ARTIFICIAL LIPOPROTEIN GENE DELIVERY SYSTEM FOR TARGETED GENE DELIVERY TO GLIOMA CELLS

PART 2: EFFECT OF DUAL SURFACTANT SYSTEMS ON PROPERTIES OF ETHYL CELLULOSE MICROSPHERES PREPARED BY NON-AQUEOUS EMULSION-SOLVENT EVAPORATION METHOD

by

RANAJOY SARKAR

(Under the Direction of JAMES C. PRICE)

ABSTRACT

In the first part of the dissertation, the formulation and development of an artificial lipoprotein gene delivery system is discussed. Cancer cells have a higher requirement for cholesterol than normal cells since they are growing at a faster rate and have over-expressed LDL receptors. This difference can be used to selectively target cancer cells. Apolipoprotein B-100 is the ligand recognized by the LDL receptors and an artificial lipoprotein, composed of a lipid nanoemulsion having Apolipoprotein B-100 on its surface, can be used for targeted gene delivery to cancer cells. Apolipoprotein B-100 was conjugated to the lipid nanoemulsion and the gene delivery complexes were assembled by incubating the artificial lipoprotein, modified poly-L-lysine and reporter gene at various ratios. The transfection efficiency of the complexes were determined in human glioma cell line SF-767. It was observed that the artificial lipoprotein with Apolipoprotein B-100 had better transfection efficiency than nanoemulsion (without

Apolipoprotein B-100) and commercial reagent Lipofectamine^{TM} because of the presence of Apolipoprotein B-100.

In the second part of the dissertation, the effect of dual surfactant systems on the physical and drug release properties of ethylcellulose microspheres prepared by non-aqueous emulsion-solvent evaporation method was studied. Specifically, the effect of RHLB and surfactant type was investigated. Low and high HLB surfactants can be combined in different ratios to obtain intermediate HLBs (called RHLB). Different batches of microspheres were made at different RHLBs by combining two low HLB surfactants (Span 80, Arlacel 83 and Span 85) and two high HLB surfactants (Tween 61 and Brij 30) in different ratios. The geometric mean diameter of the batches decreased with an increase in RHLB. The dissolution rate and dose dumping in the microsphere batches increased with an increase in RHLB. . The dissolution rate and initial drug release is less in batches made with Span 80 than Span 85 and Arlacel 83 indicating that the number of chains in the surfactant structure is important in determining the drug release characteristics. The type of linkage in Brij 30 and Tween 61 also seems to influence the release characteristics.

INDEX WORDS: Gene delivery, glioma, artificial lipoprotein, transfection, plasmid DNA, LDL receptors, microspheres, surfactants, dissolution, non-aqueous emulsion-solvent evaporation.

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DEDICATION

To my Family……..thank you for everything

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Chapter 1

Introduction and Literature Review

A. Gene therapy

Human gene therapy refers to the administration of genetic material into human patients with the intention of correcting a specific genetic defect. The main reason for the growing interest in gene therapy is because of the large number and severity of known genetic diseases. There are over 2000 known genetic diseases and, for many of these, the involved gene has been identified (McKusick, 1983). Recombinant DNA technology can be used to make copies of the therapeutic gene, which can then be administered to the patients using modern gene delivery approaches.

Gene therapy is thus a paradigm which will enable physicians to treat the cause of a disease rather than just the symptoms. Human genetic diseases are caused due to mutation or removal of genes that lead to impaired metabolic pathways, impaired cell cycle regulation, or structurally and functionally defective proteins. Gene therapy was originally developed for treating metabolic disorders. Intermediates in various metabolic pathways are synthesized through the action of specific proteins (enzymes). A large number of diseases are caused by the accumulation or loss of intermediates or products from various metabolic pathways. The fields of genetics and molecular biology have demonstrated that many heritable diseases are caused by genetic mutations. These mutations lead to defects in those proteins involved in metabolic pathways. The defective enzymes in metabolic pathways result in toxic intermediates or absent

products which cause the resultant disease states. For many such diseases, existing treatments are unsatisfactory and it was proposed that these diseases should be treated at the genetic level rather than at the protein level to replace the defective or missing enzyme.

Diseases that can be treated by gene therapy can be divided into two categories: (a) *genetic diseases* and (b) *acquired diseases*. Genetic diseases are caused by the deletion or mutation of a single gene. Examples include severe combined immunodeficiency, hemophilia, familial hypercholesterolemia, cystic fibrosis and muscular dystrophy. Acquired diseases may be caused by defects in one or more genes Examples include cancer, neurological disorders like Parkinson's and Alzheimer's diseases, some cardiovascular conditions like defective blood vessels and infectious diseases like HIV.

Gene therapy can take several forms:

- *Gene insertion*: A new version of the gene is introduced into the cell.
- *Gene modification*: A gene already in place is altered.
- *Gene surgery*: A specific defective gene is either cut or replaced by its normal form.

The first step in a general approach to treat a disease by gene therapy is by identifying the abnormal gene involved in the disease. This can be done by analyzing the patterns of inheritance of the disease, studying the metabolism of the patients who have the disease or analyzing the genes of these patients using recombinant DNA technology. After the disease causing gene has been identified, the next step involves the identification and isolation of the corresponding normal gene. Although the abnormal gene and its normal counterpart are functionally different, they seldom differ greatly in overall sequence.

The next step comprises of making numerous copies of the identified and isolated normal gene. The process of making multiple copies of a single gene is called *cloning*. In this process,

the gene of interest is combined with DNA sequences that enable it to be copied in organisms such as bacteria or yeast. This modified DNA, containing the gene of interest is introduced into bacteria or yeast cells. These cells are allowed to grow in culture medium and as the cells grow, the DNA is copied. The copied DNA is purified to remove traces of other cellular components and the gene of interest can be excised from the unwanted DNA sequences. This gene is now combined with DNA that is compatible for introduction into human cells. The DNA, containing the gene of interest, can be administered to human cells using gene delivery systems. These include viruses, cationic lipids and cationic polymers as discussed in the following section.

History of gene therapy

One of the earliest attempts of gene therapy was recorded between 1970 and 1973 when Dr. Stanfield Rogers infected three women with Shope papilloma virus. The women suffered from a genetic disease arginemia and it was believed that the virus had activity similar to a key enzyme missing in these women. The attempt proved unsuccessful.

In 1980, Martin Kline, at U.C.L.A. attempted gene therapy using recombinant DNA for the first time. This was done to treat two patients suffering from thalassemia. Bone marrow samples were withdrawn from the patients and treated with DNA containing a normal hemoglobin protein gene. The treated bone marrow cells were then returned to the patients. The ethics behind these experiments were widely questioned and Dr. Kline was roundly criticized for having conducted these experiments without prior approval.

As it can be seen, the early attempts at human gene therapy were unsuccessful and the subject as whole faced a plethora of technological as well as ethical challenges.

B. Factors affecting gene therapy

Since gene therapy involves the introduction of foreign genes into the cells, the cells resist this gene transfer. For successful transgene expression, the gene delivery vector has to enter the blood circulation and reach the target cells followed by entry of the transgene into the cell cytoplasm. From the cytoplasm, the transgene must enter the nucleus and express the desired protein or enzyme (Kaneda, 2001). At each of these steps, the transgene and the delivery vector must face and overcome a number of biological barriers that protects the body (and the cells) against the invasion and expression of foreign genes. The physicochemical properties of the gene delivery system also play a major role in the success of gene therapy. Properties like size, electric charge, hydrophile/lipophile balance and presence of targeting moiety are important in this regard (Opanasopit et al., 2002). A brief discussion of these factors follows.

1. Physicochemical properties of the gene delivery system

i. Size

The particle size of the gene delivery vector largely determines its biodistribution and blood circulation times (Opanasopit et al., 2002). Vectors with particle size of 7 μm or above, such as microsperes and multi lamellar liposomes, get trapped in the capillaries of the lungs (Fidler et al., 1980). A particle size of less than 5 μm will ensure its passage through the blood capillaries while a size of 30-500 nm allows its passage through gaps between discontinuous endothelial cells. A diameter of 70-300 nm allows the vector to circulate in the plasma for long periods. It has been noted that liposomes of size less than 70 nm are accumulated in the liver as such small particles are easily able to pass through the fenestrae of the endothelial lining of the

liver (Litzinger et al., 1994). Liposomes with diameters above 300 nm are removed by mechanical filtration by the spleen followed by phagocytosis (Klibanov et al., 1991).

ii. Surface charge

The surface charge of the vector influences its biodistribution. The presence of large amounts of glycoproteins and glycolipids makes the surface of the cell membrane negatively charged and hence it can interact with positively charged compounds, such as cationic lipids and polymers, by electrostatic interactions. Therefore positively charged compounds have increased cellular uptake compared to neutral or anionic compounds (Miller et al., 1998).

iii. Surface properties

The surface properties of particulate gene carriers, especially liposomes and emulsions, greatly influence their biodistribution. In this regard, the lipid composition of the carrier, such as presence of a saturated phospholipid like sphingomyelin in the liposome, is important as it reduces the membrane fluidity and increases the blood circulation time (Opanasopit et al., 2002).

iv. Targeting moiety

The presence of a targeting ligand on the surface of the gene carrier can greatly facilitate its recognition and interaction with the corresponding receptor. This will be discussed in greater detail in a following section.

2. Biological factors

i. Blood flow rate to the tissue

Once the gene delivery vector reaches the systemic circulation, it is distributed to the tissues. The delivery rate of the vector to the target tissue is determined by the rate of blood flow to the tissue since, in humans, the blood flow rate to different tissue is different. In general, the

blood flow rate is higher in highly perfused organs like kidney, liver and brain compared to skin, muscle and fat.

ii. Capillary structure

Another important factor that governs the delivery of the vector to the tissue is the structure of the blood capillary wall, which varies greatly in different organs and tissues. The capillary endothelium is of 3 types depending on the continuity of the endothelial layer and basement membrane.

Continuous endothelium occurs in skeletal, cardiac, smooth muscles, lung, skin and subcutaneous tissue. Tight junctions and a continuous basement membrane characterize this type of endothelium. Molecules of size 6 nm or less can extravasate.

Fenestrated endothelium occurs in intestinal mucosa, endocrine and exocrine glands and glomerulus. These allow molecules of size 40-80 nm to extravasate.

Discontinuous endothelium occurs in liver, spleen and bone marrow. As the name suggests, the intercellular junctions in these capillaries have a diameter between 30-500 nm and the basement membrane is either absent or discontinuous. Therefore these tissues are relatively easier to be accessed by gene delivery systems.

iii. Interaction with blood components

Blood acts as an important barrier for gene delivery as it provides a very harsh environment. Plasmid DNA, if injected directly into the bloodstream, is subject to attack by nucleases that can rapidly degrade it (Dash et al., 1999). Positively charged cationic lipids and polymers are used to condense the DNA to prevent attack by nucleases. But the net positive charge of the gene-polymer/lipid complex in the blood stream makes the gene delivery system susceptible to opsonization. Opsonization is a process by which certain plasma proteins like

albumin, immunoglobulins, proteins of the coagulation cascade bind to the vector to tag it for clearance by macrophages of the mononuclear phagocytic system. Opsonization is a major barrier, especially for lipid vectors as 80-90% of these are opsonized and removed within minutes of intravenous administration (Pouton, 1999).

iv. Cellular internalization

Once the gene delivery vector reaches the target tissue, it must cross the cell membrane. Uptake of most macromolecules into cells occurs by adsorptive endocytosis, receptor-mediated endocytosis, pinocytosis and phagocytosis (Pouton, 1999). DNA itself can be internalized by cultured cells through pinocytosis but the transfection efficiency is very low. This implies that either the DNA is degraded prior to uptake or by lysosomal degradation following passive uptake. If the net charge of the DNA delivery system is positive, it may bind with the negatively charged cell membrane by electrostatic interactions. It has also been suggested that if a ligand can be used that is known to be internalized by receptor-mediated mechanism, as in the example of LDL internalization, then one can achieve a rapid rate of uptake (Pouton et al., 1999, Goldstein et al., 1985).

v. Lysosomal degradative pathway

Once the delivery complex is internalized, fusion with lysosomes occurs and this may result in degradation of the DNA by lysosomal enzymes. For effective transfection, the DNA must escape without endosomal degradation into the cytoplasm. Chloroquine is an endosomolytic agent that is widely used to disrupt endosomal function. It is a weak base and diffuses easily into endosome. Since endosome has low pH, Chloroquine becomes protonated there. This results in neutralization of the endosomal proton pump and disrupts endosome

function. Polyethyleneimine and dendrimers are some other agents used with the similar mechanism of action (Pouton, 1999).

vi. Cytoplasmic transport

Once the DNA escapes from the endosomes to the cytoplasm, it must then approach the nuclear envelope to enter the nucleus. For this to happen the DNA must transport across the cytoplasm and it is an extremely slow process since cytoplasm is a very viscous medium. Therefore it may become necessary for the DNA to use an active cytoplasmic transport system, such as the dynein system, which directs vesicles from the endosomes towards the perinuclear region.

vii. Nuclear transport

Plasmid DNA has poor access to the nucleus. This presents a major challenge to non-viral gene delivery and constitutes a rate limiting step in the transfection process. The nuclear pore, having a pore size of around 55Å, allows free diffusion of molecules of size 40kD or less. Larger molecules enter through the nuclear pore complex via a facilitated process.

C. Cancer

According to recently released figures by the American Cancer Society, cancer accounts for one-quarter of the deaths in the United States. In 2001 alone, the mortality rate was more than half a million and it is estimated that 1.37 million new cases will be diagnosed in the year 2004. The lung, prostate, breast and colon are the leading cancer sites that result in the deaths.

Conventional therapy of cancer involves surgical removal of the tumor combined with radiation therapy or chemotherapy. Radiation therapy preferentially affects rapidly dividing cells in malignant tumors but it is nonspecific in its action and kills both normal as well as cancer cells.

Some tissues like the eyes and the central nervous system may be especially susceptible to radiation and tumors in these areas cannot be treated by radiation therapy. Radiation therapy is also associated with several long-term side effects like edema, arthritis, skin changes and fibrosis Larson, 1983).

Chemotherapy refers to the concurrent administration of two or more cytotoxic drugs to the cancer patient. It has been demonstrated that cancer cells are more susceptible to a combination of drugs having different mechanisms of action. Although significant improvements have taken place in surgical procedures, chemotherapy and radiotherapy over the last few decades, the overall survival rate of cancer patients is still poor, with a high percent of tumor recurrence and distant metastases being reported. The main reason for this is that these conventional methods of cancer treatment suffer from the disadvantages of lacking specificity for the cancer cells and thus lead to nonspecific side effects, nonresponsiveness of certain tumors and tumor recurrence.

Fundamental insights into the biology of cancer have led to the development of specific, potent anticancer therapies including immune-based strategies and other new strategies. These include anticancer vaccines, gene-based agents, anti-angiogenesis agents and photodynamic agents. Cancer gene therapy is also emerging as a possibly viable alternative as it is expected to overcome the limitations associated conventional therapy by being more effective and more specific and as well as lacking side effects.

D. Cancer gene therapy approaches

Genetic modifications in previously normal cells lead to cancer. These genetic modifications include mutations in proto-oncogenes (which causes over-expression of proteins

that stimulate cell proliferation), down-regulation of tumor suppressor genes (which causes proliferation of mutant cell population) and abnormal expression of growth factors (which can lead to increased cell mass and angiogenesis). As cancer cells grow, they undergo genetic modifications such that they become heterogenous from each other. This diversification imparts resistance to chemotherapy and radiotherapy. Human gene therapy started off on the principle of replacing a single defective gene involved in the disease state. But in the case of cancer, where multiple abnormal genes are involved, such an approach would be too simplistic (O'Malley et al., 1999). As a result, several new strategies have been identified for cancer gene therapy including inactivation of oncogenes, replacement of tumor suppressor genes, inhibition of angiogenesis, immunocancer gene therapy, suicide gene therapy and drug resistant gene transfer to normal cells (Hughes, 2004).

1. *Inactivation of oncogenes*: Proto-oncogenes are expressed during the fetal development stage for generating enough cells in the body for organ formation and then they are silenced. In certain cases, cancer cells grow by activating proto-oncogenes and one can stop cancer cell growth by targeting gene therapy to disrupt tumor oncogenes using the following strategies: (a) preventing the transcription of oncogene into mRNA (b) reducing the translation of the mRNA into protein and (c) interfering with the transport and function of oncoprotein.

2. *Replacement of tumor suppressor genes*: If the DNA in a normal dividing cell gets damaged, the cell cycle is arrested and mechanisms for DNA repair are initiated. In case the DNA damage cannot be corrected, cell death or apoptosis occurs. Tumor suppressor genes are present in normal cells which encode for proteins that are involved in cell cycle regulation and DNA repair pathways. Cancer cells with DNA mutations, inhibit the expression of these tumor suppressor genes, avoid apoptosis and continue to grow uninterrupted. Thus, another strategy of

cancer gene therapy is to transfect the cancer cells with tumor suppressor genes in order to stop their growth and induce apoptosis. Examples of therapeutic tumor suppressor genes for gene therapy include p53 (helps in regulation of cell cycle and apoptosis), retinoblastoma gene Rb (helps in cell cycle regulation and differentiation) and PTEN (regulates cell survival) (Fueyo et al., 1998, Minaguchi et al., 1999, Boulay et al., 2000).

3. *Inhibition of angiogenesis*: Cancer cells require a continuous supply of blood and oxygen for their growth and therefore, the cancer needs an adequate blood supply to meet these demands. Another strategy for cancer gene therapy is to interfere with angiogenesis, i.e. the formation of blood vessels supplying the tumors. Integrins, which are cell adhesion receptors, bind matrix proteins to suppress the activation of protein kinase A which is an inhibitor of angiogenesis. Proteins like angiostatin and endostatin bind with integrins and thus protein kinase A activation is not suppressed. The process leads to an inhibition of endothelial cell migration, metastasis formation and angiogenesis (Sun et al., 2001, Shi et al., 2002).

4. *Immunocancer gene therapy*: This strategy involves enhancing the body's immune system to kill cancerous cells. This can take 2 forms: passive and active. The former involves improving the effectiveness of the body's natural immune response while the latter involves the activation of immune response against a previously unrecognized tumor. Passive immunotherapy can be achieved by transfecting the immune cells of the body's defense mechanism (like cytotoxic T cells, NK cells, macrophages and dendritic cells) with genes that encode for cytokines and growth factors. The transfected cells are allowed to grow in population and returned to the patient (Paul et al., 2002). Active immunotherapy can be achieved by genetically modifying the tumor cells to express a variety of tumor antigens. These tumor cells are then

irradiated to reduce their malignancy and to improve immunogenicity and injected into the patient (Simons et. al, 1998).

5. *Suicide gene therapy*: In this kind of gene therapy, the cancer cells are transfected with those genes that encode for enzymes that catalyze the in situ conversion of non-toxic pro-drugs to active cytotoxic forms. Since the pro-drug is non-toxic, it can be given to the patient in high amounts and these are then converted to the cytotoxic drug in the transfected cancer cells (Yazawa et al., 2002). As the concentration of the cytotoxic drug in the transfected cancer cells increase, the drug diffuses into the adjacent non-transfected cells till it has entered into most of the adjoining cancer cell mass (Freeman et al., 1993). Once the concentration of the cytotoxic drug reaches a critical toxic level, it kills not just the transfected cell but also the adjacent cancer cells as well. One advantage of this therapy is that only a small fraction of the cancer cells need to be transfected with the suicide genes in order to achieve tumor regression.

6. *Drug resistant gene therapy*: A lot of cancer cells become resistant to anti-cancer drugs due to the overexpression of a multiple drug resistant (MDR) gene (Chaudhary et al., 1991, Gottesman et al., 1993). Chemosensitization has been achieved in such cases through the disruption of the activity of the MDR gene by directing antisense, ribozyme or intracellular antibody against it (Piche et al., 1999). Alternatively, another strategy using drug-resistance genes and genes that encode for drug metabolizing enzymes is being studied for conferring protection to normal cells (Lui et al., 2003). As a result, a higher dose of the cytotoxic drug can be administered to the patient for better anti-tumor activity without endangering the normal cells.

E. Overview of gene delivery systems in cancer gene therapy

Gene therapy involves the successful in vivo transfer of the desired genetic material to the targeted tissue. Preclinical and clinical studies have suggested that the success of cancer gene therapy is largely governed by the gene delivery system.

Vectors being used currently for gene delivery can be categorized as viral and non-viral vectors. 70% of the clinical trials for cancer gene therapy use viral vectors like retrovirus, adenovirus, pox virus, adeno-associated virus, herpes simplex virus and vaccinia virus. The greater use of viral vectors in clinical trials is because it offers advantages like a high level of gene expression that can be sustained for long periods. But the focus of delivery systems is shifting towards non-viral vectors since the death of a patient in a clinical trial involving adenoviral vector (Marshell, 1999). This raised a question about the safety of viral vectors and several toxicities were found to be associated with these vectors. These included the ability of viral vectors to integrate with the host genome as well as causing inflammatory and immunogenic reactions. Moreover, preparation and large scale production of two of the most widely used viral vectors, retrovirus and adenovirus, are difficult.

Non-viral vectors, on the other hand, are easier to handle, have low immunogenicity and are easier to produce on a large scale. Therefore, non-viral vectors are emerging as a comparable, if not better, alternative to viral vectors and their use in clinical trials is gradually increasing.

F. Non-viral vectors for cancer gene delivery

1. Cationic lipids

Certain lipids, above a specific critical concentration, form self-assembling spherical structures called liposomes and micelles. These structures have been successfully demonstrated

to be highly effective as drug carriers. Positively charged cationic lipids can form these self-assembling structures and bind negatively charged DNA by charge interactions. Cationic lipids have a cationic headgroup (responsible for binding the DNA) and a lipid tail (responsible for the hydrophobic collapse of the lipid-DNA complex (Ogris et al., 2002). These DNA-lipid complexes, called lipoplexes, have been widely used for DNA delivery. Such complexes can protect the DNA from nuclease attack from the time it is administered till the time it reaches the target cell nucleus. One major problem associated with the use of cationic lipids is that the size of both liposomes and micelles are usually quite large and hence can be recognized and cleared by the reticuloendothelial system of the body. Some examples of cationic lipids include DOTAP {N-[-1-(2,3 dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate}, DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide) and DOPE (1,2-dioleoyl phosphatidylethanolamine).

Transfection of the lipid-DNA complex occurs in the following steps:

- *Lipoplex entry into the cell*: Lipoplexes with a net positive charge can bind to negatively charged cell membrane by charge interactions and are then internalized by endocytosis. Cell membranes are negatively charged due to the presence of proteoglycans and it has been reported that cell lines that have a higher negative charge density on their surface show better in vitro transfection efficiency (Mounkes et al., 1998, Mislick et al., 1996). The net charge of lipoplexes can be varied by varying the ratio of positively charged cationic lipid to negatively charged DNA. Although the net charge of the lipoplex plays an important role in transfection in vitro, serum components can also influence the in vivo transfection by changing the size or stability of the complex (Li et al., 1998). In such cases, the lipid composition of the liposome is very

important and addition of a helper lipid like cholesterol to the cationic lipid can greatly improve the stability of the lipoplex in serum and thus improve its in vivo transfection.

- *Lipoplex entry into cytoplasm*: After endocytosis, the lipoplex enters the endosomal compartment and from here either the DNA or the lipoplex must quickly pass on to the cytoplasm in order to enter the nucleus. Lysosomal nucleases in the endosomes will otherwise degrade the DNA. DNA release from the cationic lipid/DNA complex can be explained by a model proposed by Szoka et al. The cytoplasmic monolayers of the cell membranes have anionic lipids associated with them and when the positively charged lipoplex is endocytosed, it destabilizes the endosomal membrane. As a result of this destabilization, anionic lipids diffuse into the complex and displace the DNA and itself associates with the cationic lipid to form a charge neutral ion pair. The displaced DNA is then released in the cytoplasm for entry into the nucleus.

2. Cationic polymers

Polycationic polymers interact with DNA by electrostatic interactions that finally lead to hydrophobic collapse to form polyplexes in the nanometer range (Ogris et al., 2002). These polymers can condense the DNA into very small particles of size 100 nm or less. The polyplexes also have a high positive charge density thus facilitating the cellular uptake of DNA. The polyplexes can also protect the DNA from attack by nucleases. Because of these advantages, cationic polymers assist systemic DNA delivery and extravasation to the target tissue.

Examples of cationic polymers used for DNA delivery include poly-L-lysine (PLL), protamine, polyethylenimine (PEI), dendrimer and poly-L-lysine graft hyaluronic acid copolymer (Kouraklis, 1999, Asayama et al., 1998, Li et al., 2000, Kichler et al., 1999). Certain controlled

release polymers like poly(D,L-lactide-co-glycolide) (PLGA), gelatin and chitosan have been recently introduced for DNA delivery (Luo et. al, 2000, Leong, 1999).

G. Shortcomings of existing non-viral vectors for cancer gene delivery

- Poor transfection efficiency
- Lack of specific targeting
- Lack of prolonged gene expression
- Toxicity
- Immunogenicity
- Instability

H. Properties of an ideal gene delivery system for cancer cells

Some of the essential properties of an ideal gene delivery system for cancer cells are:

- It should be specific for the tumor cells.
- There should be efficient expression of the therapeutic gene.
- It should not be recognized and cleared by the body's immune systems.
- It should be stable in the body.
- It should not be toxic in nature.
- It should be easy to prepare and scale-up.

I. Strategies for targeting tumors

Targeted delivery of the therapeutic gene to tumors can be achieved in two major ways: physical targeting, which includes physical methods of targeting the gene to the tumor cells, and

biological targeting, which utilizes the characteristic biological and pharmacological properties of the tumors (Wagner et al., 2004). A brief discussion of these methods follows.

1. Physical targeting methods

This strategy for tumor targeting involves the use of physical forces such as an electric or a magnetic field, light, hydrodynamic pressure and mechanical forces. The physical force is localized in the tumor region resulting in the targeting. The following methods are commonly used.

a. Naked DNA

Naked DNA or free DNA can be administered through various routes and high level of gene expression can be achieved in highly perfused organs like liver, lung, heart, kidney and spleen. The mechanism by which naked DNA is transfected has not yet been elucidatd and several mechanisms have been suggested including receptor-mediated endocytosis, large membrane disruption and small membrane pore formation (Budker et al., 2000). The intramuscular route is most attractive, especially for naked DNA vaccines, as this results in high level and prolonged gene expression and this can confer humoral and cellular immunity for more than a year (Rhodes, 1999). Other routes that have been reported for administering naked DNA include intradermal, intra-arterial, intravenous and intratumoral (Raz et al., 1994, Liu et al., 1999, Zhang et al., 1997, Coll et al., 1998). Electroporation, which involves the introduction of exogenous substances into cells by high voltage electrical pulses, has also been used for transfection of cultured cells. A significant reduction in tumor size was observed when intratumor injection of naked DNA encoding toxic genes was followed by electroporation in animals with gliomas and colon cancer (Goto et al., 2000). Despite its successes, naked DNA is

highly prone to tissue clearance and is an inefficient way to deliver genes (Kawabata et al., 1995).

b. Gene gun

The gene gun is a physical method of gene delivery that involves coating of the DNA on very fine gold or tungsten particles. Helium gas of variable pressure is used to apply a compressed shock wave for accelerating the particles for penetration into the target tissue. The gene gun was primarily designed for gene delivery to plant cells but now its application has been extended for in vivo gene delivery to tissues like skin, muscle, liver and spleen (Yang et al., 1999, Yang et. al, 1990). Some of the advantages offered by using the gene gun include the small amounts of DNA needed to achieve high transfection efficiency, no limits to plasmid size and only minimal degradation of DNA since the DNA is delivered directly across the cell membrane thus bypassing the endosomes, lysosomes and the nuclear barrier. One disadvantage associated with this method is the resultant tissue damage due to its bombardment with metal particles. The gene gun has been used for tumor vaccination study and this method has shown significant shrinkage of liver and lung metastasis (Chen et al., 1999). Besides vaccination, the gene gun has also been used for the delivery of therapeutic genes to tumors with a successful reduction in primary and metastatic tumors in mice (Rakhmilevich et al., 1996, Wang et. al, 1999).

c. Hydrodynamic gene delivery

It has been observed that rapid injection of a large volume of DNA solution lead to high level of gene expression in the hepatocytes of mice and this led to the use of this technique in cancer (Wagner et al., 2004).

d. Electroporation

In this technique, electric pulses are applied for short durations which cause the destabilization of the cell membrane. This enables the DNA to pass directly into the cytoplasm bypassing the endocytotic pathway. The transgene expression is increased many-fold and this increases the therapeutic effect (Lucas et al., 2002).

e. Ultrasound

Ultrasound facilitated gene delivery to tumor cells has been successful in vivo. Ultrasonic waves cause cavitation which increases the permeability of the cell to DNA (Anwer et al., 2000, Huber et al., 2000). An extension of this technique involves the encapsulation of DNA as well as microbubbles in microspheres (Seemann et al., 2002). Presence of microbubbles facilitates the entry of the DNA carrier into the target cells when ultrasonic waves are applied and when the vehicle enters the cells, the microbubbles inside the carrier cavitate causing the release of the DNA.

f. Jet injection

This technique utilizes has been used to deliver a solution of DNA into deep seated tissues by forcing the liquid through a small orifice within a fraction of a second. This method has been used to introduce plasmids into lung carcinoma tumors and a penetration depth of 5-10 mm was achieved, which is greater than that for gene gun (Walther et al., 2001).

g. Magnetofection

This technique involves the reversible coupling of DNA with magnetic nanoparticles and then using high energy magnetic fields to direct them to desired tissue (Scherer et al., 2002).

h. Photochemical internalization

This method of targeted gene transfer involves the simultaneous internalization of the DNA and a photosensitizer into the endocytotic compartment. The photosensitive agent is then light activated which leads to the collapse of the endosome structure and release of the DNA into the cytosol (Hogset et al., 2003).

2. Biological methods for targeting tumors.

Biological targeting methods utilize the biological differences in tumors from normal tissues to target the genes (Wagner et al., 2004). This can be accomplished in several ways.

a. Transductional targeting

For a therapeutic gene to express its effect, it has to be transported into the tumor cell nucleus. This strategy can be exploited for targeting cancer cells. It has been reported that efficiency of gene transfer increased rapidly by cells that were in the G2 phase of their cell cycle (Bruner et al., 2000). Since cancer cells are continuously dividing, it is possible to target cancer cells while they are in the G2 phase.

b. Transcriptional targeting

The gene expression cassette contains certain promoters/enhancers that help in efficient expression of the gene. In the case of tumor targeting tumor specific promoters can be included in the gene expression cassette that will be active only in tumor cells. For example, a synthetic promoter was designed that had high activity only in metastatic colon cancer (Lipinski et al., 2001).

c. Receptor mediated targeting

The presence of a homing device on the surface of a delivery system can help in targeting the genes to specific cells or organs (Opanasopit et al., 2002). Active targeting of the delivery

system can be done using a ligand recognized by a specific receptor which then takes up the system by receptor-mediated endocytosis. Some of the ligands commonly used in this strategy for targeting tumors and the receptors they target are Apolipoprotein B-100 (LDL receptors), epidermal growth factor (EGF receptor), somatostatin peptide analog (somatostatin receptor), transferring (transferrin receptors) and folate (folate receptors).

The following chapters describe the use of receptor-mediated targeting strategy based on the Apolipoprotein B-100-LDL receptor system for the development of an artificial lipoprotein gene delivery system for targeting the LDL receptor on cancer cells. The objectives of my study were as follows:

1. Review LDL receptor mediated drug targeting to malignant tumors.
2. Formulate an artificial lipoprotein composed of Apolipoprotein B-100 conjugated to a lipid nanoemulsion to mimic natural lipoproteins.
3. Characterize the artificial lipoprotein for size, surface charge and staining properties.
4. Amplify and purify plasmid DNA by molecular cloning techniques.
5. Test the artificial lipoprotein for DNA carrying capacity and surface charge.
6. Test the targeting ability and transfection efficiency of the artificial lipoprotein in glioma cell line SF-767.

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Chapter 2

LDL-Receptor mediated drug targeting to malignant tumors

R. Sarkar, D. S. Halpern, S. K. Jacobs and D. R. Lu published in "Biomedical Aspects of Drug Targeting" by V.R. Muzykantov and V.P. Torchilin (Eds.), Kluwer Academic Publishers, 2002, pg 327-345. With kind permission of Spinger Press and Academic Media

INTRODUCTION

Malignant tumors are composed of abnormal cells which usually grow very aggressively. It is extremely difficult to treat many of these malignant tumors with conventional methods, including surgical resection, radiation therapy, chemotherapy and combinations of these modalities. To enhance the efficacy of tumor treatment with chemotherapy, targeted drug delivery resulting in high concentrations of therapeutic compounds in tumor cells and relatively low concentrations in neighboring normal cells has been attractive and many approaches for drug targeting have been extensively evaluated.

Rapidly dividing cells, such as those found within malignant tumors, have a high cholesterol requirement because cholesterol is utilized to construct the cell membranes. Cells can obtain cholesterol either by taking up plasma LDL (low-density lipoprotein) via receptor-mediated endocytosis or by *de novo* synthesis. The majority of cholesterol, however, is obtained from the receptor-mediated route. It is known that, for many malignant tumor cells, the expression of the LDL-receptor is upregulated on cell surfaces in order to acquire more cholesterol carried by LDL in blood circulation. The elevated LDL-receptor expression on tumor cells provides a rationale for targeted drug delivery to malignant tumors, using drug-loaded LDL in blood circulation containing either cholesterol-based antitumor compounds or non-cholesterol-based antitumor compounds.

LIPOPROTEINS AND CHOLESTERYL ESTERS

Lipids, including cholesteryl esters, are carried from one part of the body to another by various lipoproteins as the primary transport form. Most of these lipids function as structural components of membranes (such as phospholipids and cholesterol) or as storage units of chemical energy (primarily triglycerides). These lipids are in general not soluble in aqueous

solution and therefore need to be transported in blood through a suitable vehicle, i.e. lipoproteins. Lipoproteins also transport minor but important lipids such as steroid hormones, carotenoids, and tocopherols.

Structures and Functions of Lipoproteins

Lipoproteins are macromolecular complexes and generally have a spherical shape. Their structures consist of a hydrophobic core and a polar shell. Water-insoluble lipids are stored within the core and the polar shell allows the lipoprotein particles to float in blood circulation. Figure 2.1 shows the simplified structure of LDL. The lipid core is made up mostly of triglycerides and cholesteryl esters in varying proportions, depending on the type of lipoprotein. A group of polar molecules forms the outer polar shell which mainly contains phospholipids, such as phosphatidylcholine and sphingomyelin, and specific proteins, referred to as apolipoproteins. Unesterified cholesterol molecules can also be present in the polar shell. Apolipoproteins are partially exposed at the surface. There are many types of apolipoproteins which recognize and bind specifically to enzymes or receptor proteins on cell membranes and thus are responsible for directing the lipoproteins to their sites of function and metabolism.

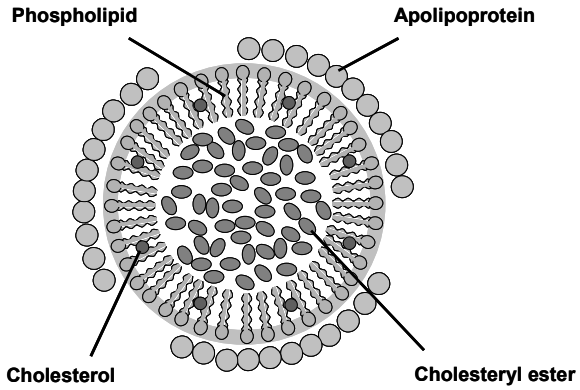


Figure 2.1: Structure of Low-Density Lipoprotein (LDL)

Table 2.1 lists those apolipoproteins which have been identified so far. The functions of the apolipoproteins are diversified. Apo A-I, apo A-IV, apo C-I, apo C-II, and apo C-III function as activation or inhibition modulators for extracellular enzymatic reactions involved in lipid homeostasis. Apo B, apo E, apo J and apo A-I, on the other hand, recognize the cell surface receptors that mediate lipid uptake and work as receptor-specific ligands.[1] The specific functions of many other apolipoproteins including apo C-IV, apo D, apo F, apo G and apo H remain unclear.

Table 2.1: Types of Apolipoproteins and their classifications and functions

Apolipoprotein	**Lipoprotein**	**Function**
A-I	LDL, HDL	LCAT activation
A-II	LDL, HDL	LCAT inhibition, hepatic lipase activation
B-100	VLDL, LDL	Cholesterol clearance
C-I	VLDL, HDL	Possibly LCAT activation
C-II	VLDL, HDL	LPL activation
C-III	VLDL, HDL	LPL inhibition, possibly LCAT activation
D	HDL	Unknown
E	HDL	Cholesterol clearance

LDL = Low Density Lipoprotein; HDL = High Density Lipoprotein; VLDL= Very Low Density; LCAT = Lecithin:cholesterol acyltransferase; LPL = Lipoprotein lipase

Various lipoproteins can be isolated according to their densities through ultracentrifugation with a salt gradient. Based on the separation, the plasma lipoproteins are divided into five major classes. Since they continuously undergo a metabolic course, lipoprotein particles have variable properties in chemical composition, apolipoprotein percentage, hydrated density and other physicochemical characteristics.

The five major classes of lipoproteins have different physicochemical properties and functions (Table 2.2). Chylomicrons act as carriers to bring exogenous (dietary) triacylglycerols and cholesterol from the absorption site (intestines) to the tissues and liver. Very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) are a group of related lipid carriers responsible for transporting triacylglycerols and cholesterol from the liver to various tissues. LDL is the most important lipid carrier among this group and transports more than 60% of the plasma cholesterol (primarily as cholesteryl esters) in humans. High density lipoprotein (HDL) carries endogenous cholesterol from the tissues back to the liver.

Structure and Functions of Cholesteryl Esters

As shown in Figure 1.1, the majority of the molecules in the core of lipoproteins are the esterified form of cholesterol. Cholesterol is one of the major structural components of cell membranes and sub-cellular organelle membranes. Cholesterol molecules in plasma and various tissues exist either in the unesterified form in which the polar hydroxyl group is exposed or in the esterified form in which the hydroxyl group is esterified with long chain fatty acids. About 90% of the total cholesterol in animal tissue is present as unesterified cholesterol located within cell membranes, myelin and the polar shell of plasma lipoprotein particles. However, in plasma, about two-thirds of the cholesterol molecules are esters located in the lipid core of lipoproteins.

Table 2.2: Physicochemical properties of lipoproteins

Features	**HDL**	**LDL**	**IDL**	**VLDL**	**Chylomicrons**
Molecular weight(x 10^5)	1.9-3.9	20-25	35-45	50-100	10000-100000
Mass (kD)	175-360	2300	5-10000	10-80000	400000
Diameter (Å)	50-120	180-250	250-350	300-800	750-12000
Density (g/cm^3)	1.063-1.21	1.019-1.063	1.006-1.019	0.95-1.006	<0.95
Protein	40-55%	20-25%	15-20%	5-10%	1.5-2.5%
Total Lipids	45-60%	75-80%	80-85%	90-95%	97-99%
Cholesterol	3-4%	7-10%	8%	5-10%	1-3%
Cholesteryl esters	12%	35-40%	30%	10-15%	3-5%
Phospholipids	20-35%	15-20%	22%	15-20%	7-9%
Triglycerides	3-5%	7-10%	22%	50-65%	84-89%

The high proportion of cholesteryl esters, as opposed to unesterified cholesterol, in plasma results in high transport efficiency by lipoproteins. The cholesteryl esters are also in a chemical form that does not interact unnecessarily with plasma membranes. In addition, cholesteryl esters in the adrenal cortex and gonad cells provide the lipid storage as a reservoir of cholesterol in a physiologically inactive form. The general chemical composition of cholesteryl esters can be seen in Figure 2.2. The structure can be divided into two major parts: cholesterol and fatty chains both of which are linked by an ester bond. This simple but versatile composition presents us with an opportunity to develop the mimics of native cholesteryl esters linked with drug molecules, which can be carried by lipoproteins, especially LDL, for targeted drug delivery. The details of such a targeting strategy are described in the following sections.

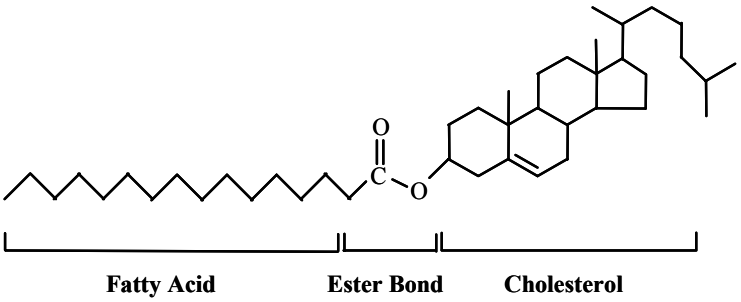


Figure 2.2: The general chemical composition of cholesteryl esters

All animal cells require cholesterol for cell growth and maintenance. The primary function of cholesterol is to stabilize the constituents of the cell bilaminar membrane. As such, it is well known that the requirement for cholesterol is much greater in cells that are rapidly dividing or growing than in those cells in a resting state. Consequently, the cholesterol requirement of tumor cells is greater since they divide rapidly. This presents a therapeutic rationale for cholesterol-based drug targeting. It should be emphasized that although there is a

large amount of cholesterol molecules in the body, only a small portion of these molecules is present in blood and they are primarily in the form of cholesteryl esters. The mimics of cholesteryl esters conjugated with antitumor compounds can be administered systemically to compete with the native cholesteryl esters for targeted drug delivery to tumor cells.

TRANSPORT OF CHOLESTERYL ESTERS BY LIPOPROTEINS

Cholesteryl esters are synthesized with enzymatic assistance from Acyl CoA:cholesterol acyl transferase. The absorbed dietary cholesterol is thus esterified with fatty acids within intestinal and other cells. Chylomicrons are formed by intestinal mucosa and secreted from the intestinal cells into lymph and subsequently into the blood. The function of chylomicrons is to deliver dietary cholesterol to the liver as well as dietary triacylglycerols to muscle and adipose tissue. During the passage through the capillaries of adipose and other tissues, the apo C-II protein on the chylomicron surface activates lipoprotein lipase (LPL) and the component triacylglycerols of the chylomicron are progressively hydrolyzed. Consequently, the size of chylomicrons is reduced and they become cholesterol-enriched chylomicron-remnants containing a relatively high proportion of cholesteryl esters. The apo E protein on the surface enables them to bind to specific receptors on liver cells. During the interaction, these remnants are absorbed by liver cells through receptor-mediated endocytosis and are degraded within the cells.

VLDL is assembled in liver cells. During the process, microsomal triglyceride transfer protein in the lumen of endoplasmic reticulum within the liver facilitates the transfer of lipids to apolipoprotein B-100 by complexing with protein disulfide isomerase. This complexation step is essential for the assembly of triglyceride–rich lipoproteins. There are several apolipoproteins associated with VLDL including apo B-100, apo C-I, apo C-II, and apo C-III. VLDL is further metabolized and the triglyceride content is hydrolyzed. The VLDL becomes smaller with higher

density and a higher proportion of cholesterol esters. As a result, VLDL is gradually transformed into IDL.

IDL contains a larger portion of apo E protein which enables it to bind to the LDL receptors on liver cells for internalization and degradation. However, only half of the IDL is taken up by liver cells. The other half experiences further metabolic processing and loses more triglycerides to eventually become LDL.

The lipid core of the LDL particle is mainly composed of cholesteryl esters. There are about 1500 cholesteryl ester molecules per LDL particle, surrounded by 500 cholesterol molecules and 800 phospholipid molecules as the polar shell. With the capacity of carrying large amounts of cholesteryl esters, LDL is the major vehicle to transport cholesteryl esters to peripheral cells. LDL contains one major apolipoprotein, apo B-100, which is associated with the surface monolayer of LDL. Apo B-100 allows LDL to bind the LDL receptors on the peripheral cell surfaces and to be internalized by these cells through a receptor mediated endocytosis.

In contrast to LDL, the function of HDL is to deliver excess cholesterol from various tissue cells back to the liver or to cells in demand of cholesterol. HDL is a smaller particle but contains a higher proportion of proteins. It is secreted by the liver and intestines as nascent HDL. Apo A-I protein on the nascent HDL activates the enzyme, lecithin:cholesterol acyl transferase (LCAT), to catalyze the esterification of cholesterol by transfer of an acyl group from lecithin to cholesterol. HDL receptors, named scavenger B1, exist on the surface of many different cells, including liver cells and the cells in demand of cholesterol. Through the recognition and binding of Apo A-I protein to the HDL receptors, cholesteryl esters in the HDL core are transferred to these cells without the internalization of HDL itself. After delivering its

cholesterol content HDL again returns to the circulation to scavenge more cholesterol. The mediation process for the cholesteryl ester transfer from HDL to VLDL and LDL is provided by cholesteryl ester transfer protein (CETP).

LDL RECEPTORS AND TUMOR TARGETING

LDL receptors are transmembrane glycoproteins present on cell surfaces which recognize and internalize LDL to obtain cholesterol from blood.[2] This receptor family includes LDL-receptor (LDL-R), VLDL-receptor (VLDL-R), apolipoprotein E receptor 2, LDL-R Related Protein (LRP) and megalin. These receptors share several common structural and functional features. All members of this family show cell surface expression. They all have an extracellular binding domain that helps in recognition and binding of apo E-containing lipoproteins. LRP and gp330 also bind several other extracellular ligands. While most signaling receptors have a single large intracellular domain, the members of the LDL-R family are characterized by large extracellular and comparatively shorter intracellular domains. For LDL-R, the N-terminal domain is on the exterior side of the membrane and interacts with apo B-100 protein or other apolipoproteins on LDL. The C-terminal domain is on the cytosolic side of the membrane to interact with adapter proteins mediating the formation of the clathrin coat.

The LDL-R Family Members and Subtypes

LDL-R regulates the plasma cholesterol by mediating uptake and catabolism of plasma LDL. In normal tissues, the majority of the LDL-R is expressed on hepatic tissue and adrenal cortex. Several regions of the CNS including the blood-brain barrier (BBB) also express the LDL-R. Many types of tumor cells were found to have elevated LDL-R expression.

The presence of certain structural features like a particular complement- type and EGF precursor homology repeats of the LDL-R play a major role in the binding of apo E and apo B. It was found that complement-type repeats 3-7 and EGF precursor repeat A were both essential for the optimal binding of apo B while only complement-type repeat 5 was needed to bind apo E. The main function of the LDL-R is to bind and internalize those lipoproteins containing apo B-100 and apo E from the plasma.

In terms of structure the very low density lipoprotein receptor differs from LDL-R only in the presence of an extra complement-type repeat in the ligand-binding domain present at the N-terminal. The VLDL-R has broad ligand binding ability. The majority of the VLDL-R is expressed in extrahepatic tissues such as the heart, muscle and adipose tissue. The VLDL-R may function in the uptake of triglyceride-rich, apo E containing lipoproteins in tissues where fatty acid metabolism occurs.

Cellular Uptake of LDL through LDL Receptor

Rapidly growing cells need a large amount of cholesterol. 90% of the cholesterol required by cells is acquired from receptor-mediated endocytosis while the remaining 10% is obtained by de novo synthesis. The endocytotic process begins by the formation of coated pits initiated by the binding of dephosphorylated adaptor protein to the plasma membrane. The coated pits are named because they are covered by the protein clathrin. Receptors from other regions of the plasma membrane move to the newly formed coated regions for internalization. Ligands containing apo B-100 and apo E are recognized and bound by the LDL-R to form a complex which is internalized into the coated pits.

After internalization of the LDL, the coated pits are pinched off and within a very short time, they shed their clathrin coating. The released clathrin can participate in the formation of

new coated pits. The LDL particle which has been internalized is then transferred to endocytotic vesicles or endosomes. Due to the acidic pH within the endosomes, the LDL dissociates from its receptor. This is followed by the fusion of the endosomes with lysosomes which contain hydrolases. The protein component of the LDL, apo B-100, is broken down to free amino acids while the cholesteryl ester component is cleaved by lysosomal lipase. The free cholesterol is released and incorporated into the cell membrane. Excess cholesterol is re-esterified by the action of enzyme ACAT and the cholesteryl ester formed is stored for later use.

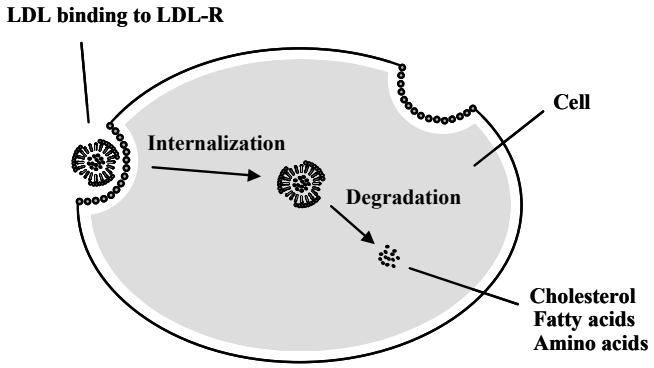


Figure 2.3. LDL receptor-mediated endocytosis of LDL particles

Elevated LDL Receptor Expression on Tumor Cells

Evidence has demonstrated an elevated LDL receptor activity on brain tumors. The human malignant glioma cell line (U-251 MG) was found to internalize and degrade LDL. The study by Murakami et al. indicated the presence of LDL receptors on the membrane of U-251 MG, which were responsible for the transport of cholesterol into the cell by receptor mediated endocytosis.[3] A recent study was conducted by Maletinska et al. on seven human glioma cell lines to determine the levels of LDL receptors on the cell surface.[4] It was found that all the cell

lines had elevated LDL receptor expression. For example, the SF-767 glioma cells had 300,000 LDL receptors per cell with a very high binding affinity. Other glioma cells also showed high LDL receptor levels (128,000-950,000 LDL receptors per cell) with variable binding affinity.

Gueddari et al. studied the A-549 human lung adenocarcinoma cell line and found an over-expression of LDL receptors as compared to normal human fibroblasts.[5] Yen et al. studied the Daudi Burkitt's Lymphoma cells and determined that the level of LDL receptors in these cells were much higher than normal peripheral blood lymphocytes and a majority of these receptors were not subject to downregulation.[6] Chen et al. concluded from a study on human prostate cancer cells that over-expression of LDL receptors by cancer cells was important for obtaining essential fatty acids via the LDL receptor pathway.[7] This led to an increased production of prostaglandins which in turn stimulated cell growth. Seven murine tumors were studied in vivo by determining the uptake of radio-labeled LDL.[8] The high relative uptake of the radio-labeled LDL by the murine tumor cells in vivo corresponded to an elevated LDL receptor activity.

Vitols et al. studied 59 patients with acute leukemia and suggested a correlation between hypocholesterolemia in such patients and elevated LDL receptor activity in malignant cells.[9] They also proposed utilization of this pathway for targeted delivery of LDL-associated anti-cancer drugs to malignant cells. In another study, a patient diagnosed with adrenal tumor was found to suffer from severe hypocholesterolemia. To investigate whether there was any relation between a low cholesterol level and the tumor, Nakagawa et al. established a cell culture line of the adrenal tumor and found that these cells had twice the LDL receptor activity in these cells when compared to Hep G2 cells.[10] This elevated LDL receptor activity resulted in low

cholesterol levels in the patient. Furthermore, it was found that these receptors were not susceptible to downregulation.

LDL Transport Across Blood Brain Barrier

Cholesterol in the brain can be derived either from the de novo synthesis or from the plasma by transport across the BBB. The presence of LDL receptors on the brain capillaries has been demonstrated. These LDL receptors are responsible for the transcytosis of LDL from the plasma across the BBB by a receptor-mediated mechanism.

Meresse et al. demonstrated the presence of LDL receptors on endothelial cells of brain capillaries. When radiolabeled LDL postmortem was injected into bovine brain circulation, it was found to bind to a specific LDL receptor.[11] This LDL receptor was similar in characteristic to the LDL receptor on human fibroblasts. Lucarelli et al. suggested that LDL receptors on brain microvessels may be responsible for transport of lipids across the BBB.[12] Dehouck et al. further demonstrated that the LDL receptors on the BBB capillary endothelial cells were responsible for the delivery of essential lipids to brain cells.[13] LDL particles were specifically transcytosed across the BBB and this transcytotic process ceased when the receptor was blocked using a monoclonal antibody.

CHOLESTEROL-BASED DRUG TARGETING THROUGH LDL RECEPTORS

As indicated above, cholesterol is the essential component of cell membranes and is in high demand by rapidly dividing malignant tumor cells. Many types of tumor cells thus have elevated LDL receptor expression in order to acquire more cholesterol compared to corresponding normal cells. This phenomenon forms the basis of a cholesterol-based drug

targeting approach through the synthesis of antitumor compounds which mimic native cholesteryl esters.[14] As these compounds share similar chemical and physical characteristics with native cholesteryl esters, they can interact well with LDL. They may transfer effectively into LDL in the physiological environment and, thus, utilize the elevated LDL receptor expression on tumor cells for targeted drug delivery. The cholesterol-based drug targeting to tumor cells can be schematically illustrated in Figure 4.

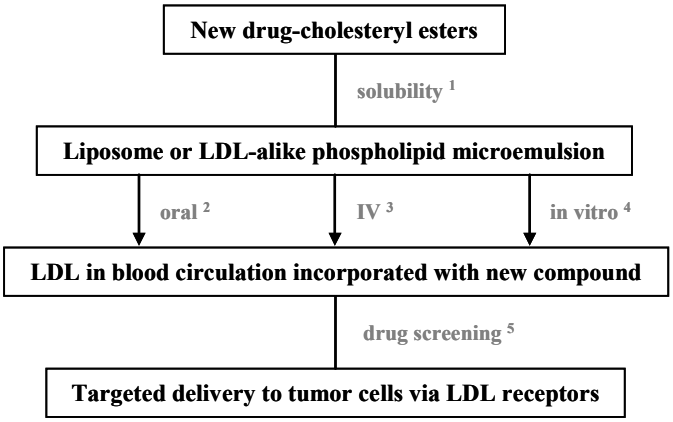
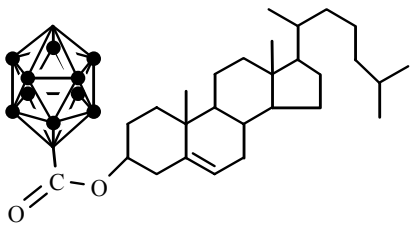


Figure 2.4. Schematic description of the cholesterol-based drug targeting approach. [1]These drug-cholesteryl esters are in general water-insoluble and thus require pharmaceutical formulations. [2-4]The formulations containing drug-cholesteryl esters are administered by the oral or IV route or incubated in vitro with freshly-isolated LDL for drug loading before IV administration. [5]A series of such drug-cholesteryl esters are screened, in vitro or in vivo, to maximize the targeting capability.

To observe the therapeutic potential of the cholesterol-based drug targeting approach and to examine the interactions of such compounds with LDL and malignant tumor cells, our laboratory recently synthesized a series of compounds mimicking native cholesteryl esters for targeted drug delivery to tumor cells. One of these compounds, cholesteryl 1,12-dicarba-closo-dodecaboranel-carboxylate (BCH, see Figure 2.5), is a carborane ester of cholesterol designed for use in boron neutron capture therapy (BNCT).

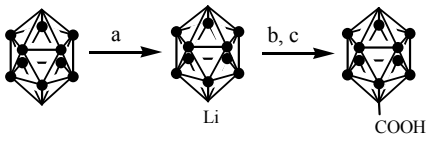


- Boron

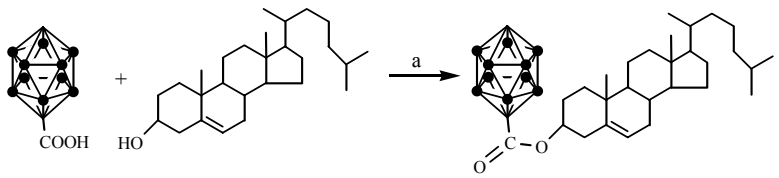
Figure 2.5. The chemical structure of a carborane ester of cholesterol (BCH). The second carbon atom on the carborane allows the further addition of functional groups to generate a large number of similar compounds for drug screening.

The chemical reactions for making such a compound involve several steps as briefly described in Scheme 1, 2 and 3.[15] The resulting compound contains 10 boron atoms to maximize the amount of boron per molecule. The compound also possesses two carbon atoms on the boron cage allowing the formation of a cholesteryl ester bond on one carbon atom and further chemical modifications on the second carbon atom to generate a series of cholesteryl esters of carborane. Feakes et al. also reported the synthesis of boron-containing cholesterol derivatives for incorporation in liposomes.[16]

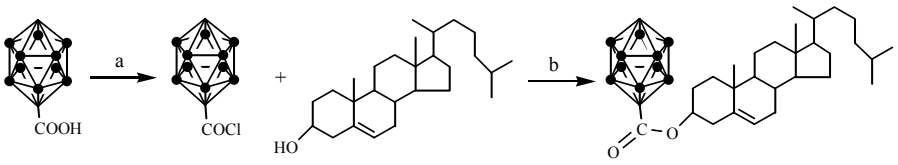
BNCT is an experimental therapy that has been used to treat glioma, melanoma and other malignant tumors. The basis for BNCT is a nuclear reaction which occurs when a stable isotope of boron, ^{10}B, is irradiated by a beam of low energy neutrons to yield high energy and short-range tumor-destroying α particles and ^{7}Li nuclei. For BNCT to be successful, the boron must preferentially localize in the tumor cells, compared to surrounding normal cells.[17] Therefore, utilizing the cholesterol-based drug targeting approach by linking cholesterol to boron compounds to make the mimics of cholesteryl esters may result in a higher concentration of boron in tumor cells and thus enhance the efficacy for BNCT treatment. BCH is extremely hydrophobic and thus we have formulated the compound in liposomes and VLDL-resembling phospholipid-submicron emulsions for cell culture studies.[18] In addition to solubilizing BCH, these formulations may also serve as suitable carriers to interact with LDL in the physiological environment. The formulations can be administered as indicated in Figure 2.4. Experiments involving tumor-bearing animal model may also supply direct information for the in vivo tumor-uptake of boron compounds formulated in these formulations.[19]



Scheme 1. (a) *n*-BuLi in ether at room temperature (RT); (b) Dry ice at –78 °C; (c) HCl.



Scheme 2. (a) DCC and DMPA.



Scheme 3. (a) Reflux in $SOCl_2$; (b) DMAP in CH_2Cl_2 at RT.

Experiments were carried out in our laboratory to examine the cellular uptake of BCH from both liposomal formulation and VLDL-resembling phospholipid-submicron emulsion. Based on the studies using 9L rat glioma cell lines, sufficient levels of boron in the cells (about 50 μg boron/g of cells) were achieved with these BCH formulations. Maletinska et al. showed that seven human glioblastoma multiforme cell lines, including SF-767 and SF-763, had very high numbers of LDL receptors per cell.[4] With the available information regarding the elevated LDL receptor expression on human tumor cells, further experiments were carried out in our laboratory using two human glioma cell lines (SF-767 and SF-763). The results indicated that extensive BCH uptake occurred in these human glioma cells. Although the concentration of BCH in the cell culture medium was low due to the limit of BCH formulation, the boron uptake reached 264 μg boron/g cells for the SF-767 cells, about 10 times higher than the required boron level (≥ 20-25 μg boron per g cells) for successful BNCT. For SF-763 cells, the boron uptake reached 283.3 μg boron/g cells, about 11 times higher than the required boron level.

In addition to the requirement for the cells to obtain an adequate amount of boron, it is also essential for boron to remain in the cells for a sufficient time period so that the neutron radiation may be effectively applied. In vitro cell incubation experiments were carried out on SF-767 and SF-763 human glioma cells. The results showed that a majority of the BCH taken up

in the human glioma cells was retained in the cells after the subsequent 24-hour incubation without the presence of BCH.

With the understanding of how LDL and LDL receptors work, better cholesterol-based compounds will be generated which can be used for the targeted delivery of anti-tumor agents. Clarifying the functions of LDL receptors within the blood-brain barrier may also aid in the development of other cholesterol-based therapeutical compounds for effective drug delivery across the blood-brain barrier.

NON-CHOLESTEROL-BASED DRUG TARGETING THROUGH LDL RECEPTORS

Non-cholesterol-based pharmaceutical compounds may also be incorporated into LDL for targeted delivery. The necessary requirement is that these compounds be hydrophobic in order to facilitate the loading. The incorporation is usually carried out in vitro. In general, human LDL is isolated from fresh serum by differential density ultracentrifugation. The incorporation of LDL with hydrophobic compounds is performed according to well-investigated protocols. The success of the incorporation requires the effective loading and a process to avoid the denaturation of apolipoproteins. The latter is a critical step because even a minor change in structure or conformation of apo B protein results in the rapid clearance of the modified LDL by the reticuloendothelial system. Studies have been conducted on drug-loading LDL in cell culture, as well as within preclinical and clinical experiments.

Lundberg successfully incorporated a steroid mustard carbamate, which is a lipophilic anti-cancer drug, into the core of reconstituted LDL and then evaluated its biological activity.[20] The incorporation was first carried out using detergents but a newer method using enzymatic hydrolysis provided a milder process. The structure as well as the cellular uptake were found to

be similar to those of native LDL. The cytotoxic activity of these compounds were tested using cultured human fibroblasts or neuroblastoma cells and it was found that the drug delivered to the cells via the LDL pathway was able to kill 100% of the cells. It was also found that inhibitors of LDL uptake blocked the cytotoxic activity of drug-lipoprotein complex indicating that the drug-LDL complex followed the same pathway as native LDL.

Lestavel-Delattre proposed that drugs from the 2-(aminomethyl) acrylophenones (AMA) class could be specifically targeted to cancer cells using LDL as the drug carrier.[21] This class of drugs shows in vitro anti-leukemic activity but is ineffective in vivo since they are actively bound by blood proteins. When an AMA compound was loaded into LDL the loading was about 100-300 drug molecules per LDL particle. The drug-LDL complex formed was highly electronegative. The drug-LDL complex was bound, internalized and degraded through the LDL receptor of neoplastic A-549 cells but to a slightly smaller extent compared to native LDL. The drug-receptor interaction was demonstrated to induce in vitro cytotoxicity as evidenced by growth inhibition of the A-549 cells.

In vitro studies were conducted by Kerr et al. on human squamous lung tumor cells to assess the effectiveness of daunomycin-LDL receptor complex.[22] The efficiency of the complex was compared with daunomycin itself and it was found that both were equally cytotoxic in vitro. Samadi-Baboli et al. loaded eliptinium oleate (OL-NME) into LDL, about 400 molecules per LDL particle.[23] Their results indicated that the complex enhanced the anti-tumor activity against B 16 melanoma model.

The success of anti-tumor treatment using photodynamic agents is dependent on the localization of the agent within tumor cells. The agents are required to be specifically delivered to tumor tissue followed by their activation after exposure to light. This approach is similar in

certain extent to BNCT as described above. The selectivity of photodynamic agents for the tumors can be enhanced when LDL is used as the carrier. It was found that Haematoporphyrin (Hp), a photodynamic agent, was non-covalently bound with LDL.[24]When administered, this Hp-LDL complex showed killing potential on tumor cells upon being internalized by the cells. Pharmacokinetic studies of a photodynamic agent, Zinc-phthalocyanate (Zn-Pc) were conducted by Reddi et al. using LDL as drug delivery system.[25] These studies were carried out in mice bearing a transplanted MS-2 fibrosarcoma. It was found that the LDL approach resulted in a higher Zn-Pc uptake by the tumor as well as improved selectivity.

The potential of boron neutron capture therapy for the treatment of brain tumors has been extensively investigated. The treatment is based on the principle of interaction of boron atoms localized in brain tumors with thermal neutrons generated from an external source. The efficiency of this method can be further enhanced when the selectivity of the boron compound is increased so that it is preferentially taken up by the brain tumor cells. Laster et al., at University of California, San Fransisco, prepared a boronated analogue of LDL containing almost 12,000 boron atoms per LDL.[26] In vitro experiments were carried out using cell culture lines to investigate the biological efficacy when carborane carboxylic acid esters of fatty alcohols were used to boronate LDL. The boronated-LDL was incubated with hamster V-79 and CHO cells. On being irradiated with thermal neutron beams from the Brookhaven Medical Research Reactor, it was found that the boron was distributed intracellularly through a receptor-mediated process. Boron concentration achieved inside the cells was found to reach 10 times the concentration required for BNCT.

De Smidt incorporated the oleoyl derivatives of two anti-cancer drugs, methotrexate and floxuridine, into LDL particles.[27] Three incorporation methods were used, namely the dry film

method, the transfer protein method and the delipidation-reconstitution method. It was found that the drug loading was the highest with the delipidation-reconstitution method, resulting in about 50-70 dioleoyl-FdUrd molecules per LDL particle. In vitro studies were carried out using hepatocellular carcinoma cell line Hep G2 and it was found that the drug-LDL complex competed effectively with native LDL for binding to LDL receptors. In vivo studies in rats showed that the half-life of the drug-LDL complex was prolonged when compared to the free drug. Vitols et al. incorporated a water insoluble mitoclomine derivative (WB 4291) into LDL.[28] The drug loading was about 1500 drug molecules per LDL particle. The drug-LDL complex was tested in vivo in Balb-C mice with experimental leukemia. After intraperitoneal administration the median survival time was prolonged 2-5 fold. Versluis et al. prepared a liposomal formulation containing apo E and found that it behaved similar to native LDL in vivo.[29] When cultured with B 16 melanoma cell lines the apo E containing liposome was bound 15 times more by the LDL receptor than native LDL. A lipophilic derivative of Daunorubicin was incorporated into this liposomal formulation and when tested in B 16 tumor-bearing mice it was found that the tissue distribution of the complex was comparable to that of native LDL.

A clinical study was conducted at Karolinska Hospital in Sweden.[30] Eleven adult patients diagnosed with acute myelogenous leukemia were administered drug-LDL formulation containing ^{14}C-sucrose labeled LDL. From the results of this study it was concluded that LDL could potentially be used as drug carrier for targeting lipophilic anti-cancer drugs to leukemia cells.

FUTURE DEVELOPMENT

As indicated in the literature, it is feasible to deliver therapeutic compounds specifically to malignant tumors through the LDL-receptor mediated route. Significant progress has been

made in many laboratories in both fundamental research and practical applications. To enhance the efficacy of LDL-receptor mediated drug targeting, new compounds mimicking the physical and chemical properties of native cholesteryl esters, either cholesterol-based or non-cholesterol-based compounds, need to be further developed. Suitable pharmaceutical formulations for these compounds are essential for effective interactions with lipoproteins and subsequent drug loading into lipoproteins in either in vivo or in vitro environment. Based on the experimental results at the molecular level and the cellular level, preclinical animal studies as well as clinical studies need to be carefully arranged to evaluate the efficacy of the novel therapeutic compounds carried by various pharmaceutical formulations for LDL-receptor mediated drug targeting to malignant tumors.

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Chapter 3

Formulation and evaluation of an artificial lipoprotein containing Apolipoprotein B-100 for targeted gene delivery to glioma cells[1]

[1]R. Sarkar, J. C. Price and D. R. Lu. To be submitted to *Pharmaceutical Research*

ABSTRACT

Purpose: In the present study, an artificial lipoprotein was formulated, consisting of Apolipoprotein B-100 (Apo B-100) conjugated to a nanoemulsion, for targeted gene delivery to glioma cells. **Methods:** A nanoemulsion (NE) consisting of lipids found in naturally occurring lipoproteins was formulated to resemble the lipid core of the lipoprotein. Surfactant-solubilized Apo B-100 was slowly added to the nanoemulsion to conjugate it to the nanoemulsion to form the artificial lipoprotein (AL). Extensive dialysis was carried out to ensure complete detergent removal. The AL was characterized by measuring particle size, lipid-protein staining after agarose gel electrophoresis and zeta potential measurement. Positively charged poly-L-Lysine, which served to hold the negatively charged DNA, was chemically modified by attaching palmitoyl chains to form palmitoyl poly-L-lysine (PPLL). Increasing ratios of PPLL were incubated with a fixed quantity of AL and 5μg DNA to form the PPLL/AL/DNA complex. PPLL is positively charged and serves to condense the negatively charged DNA while the palmitoyl chains on PPLL help in anchoring the poly-L-lysine to the lipid nanoemulsion by hydrophobic interactions. pSV β-Galactosidase, which contains a reporter gene for β-galactosidase, is used as a model plasmid DNA. PPLL/AL/DNA complex was characterized through measuring the charge of the complex by zeta potential and determining the DNA holding capacity by agarose gel electrophoresis. Transfection efficiency of the complexes was determined in SF-767 Glioma Cell lines using nanoemulsion (without Apo B-100) and Lipofectamine® as control. **Results:** Artificial lipoprotein was successfully formulated and characterized. Staining by lipid and protein stains confirmed the conjugation of the protein (Apo B-100) to the lipid nanoemulsion. Agarose gel electrophoresis showed that PPLL/AL at the ratios of 0.125, 0.25, 0.5 and 1 are capable of holding 5μg of DNA. When transfected in glioma cell line SF-767, the complexes at

0.125, 0.25 and 0.5 ratio of PPLL/AL showed successful transfection, with the complex at 0.25 ratio showing the highest efficiency. When the transfection of this complex was compared to transfection of PPLL/NE (without the targeting ligand Apo B-100) at 0.25 ratio and to commercial reagent Lipofectamine®, the transfection efficiency of the PPLL/AL/DNA complex was highest. This confirmed the targeting ability of the AL due to the presence of targeting ligand Apo B-100. **Conclusions:** An artificial lipoprotein containing Apo B-100 was successfully formulated and evaluated for *in vitro* transfection efficiency in glioma cells.

Keywords: Gene delivery, artificial lipoprotein, nanoemulsion, apolipoprotein B-100, LDL receptors, glioma cells, gene transfection.

Abbreviation: AL: Artificial Lipoprotein, PLL: Poly-L-Lysine, PPLL: Palmitoyl poly-L-Lysine, Apo B-100: Apolipoprotein B-100, ONPG: o-nitrophenyl- β-D-galactopyranoside, LDL: Low Density Lipoprotein

Introduction

The introduction of exogenous genetic material into specific cells is termed as gene delivery. The subsequent expression of functional genetic material can be utilized for manipulating the genetic profile of the target cell as in cases of metabolic disorders, AIDS and cancer. Although the field of oncology has seen many scientific advances and innovations, more than 20% of all deaths are still caused by cancer (Ogris et al., 2002). In most cases, surgery has to be resorted to as the first course of action. In advanced stages of tumors, surgery has to be frequently combined with radiation therapy and chemotherapy. But these forms of treatment

suffer from a number of drawbacks, being non-selective for tumor cells and development of resistance by the tumors. Gene therapy of malignant tumors, an emerging area wherein research and development, is focused on the search for an ideal delivery system and specific therapeutic gene. Intensive research is ongoing to identify and select specific genes involved in anti-tumor effect but the development of suitable vehicles and vectors for targeting these genes to their specific sites is equally important. Gene delivery systems can be broadly classified as viral and non-viral. Viral vectors have been used for the purpose of gene delivery owing to their high transfection efficiencies and their ability to integrate the exogenous DNA into the host genome. These advantages are negated by the fact that viral vectors have poor safety profiles, are immunogenic, are difficult to handle and produce on a large scale basis and are limited in their capacity to carry large DNA. They have also been reported to cause long term effects like tumorigenic mutations and can regenerate active viral particles through recombination with endogenous viruses. As a result, the focus for gene delivery has now shifted to non-viral systems.

Cationic lipids and liposomes, that can electrostatically interact and condense negatively charged DNA, have been the primary focus of synthetic gene delivery systems owing to their relative safety, stability, lack of immunogenicity and ease of handling and preparation. But these are effective only if they are injected locally. Other disadvantages include cytotoxicity at higher concentrations, limited delivery and gene expression efficiency and interactions with negatively charged plasma components which limits the ability of cationic complexes to reach the target tissues .

Many laboratories have reported on the use of cationic polymers as vectors for carrying plasmid DNA with encouraging *in vivo* results. These polymers include chitosan, hydroxylated nylons, polyethyleneamine, dendrimers, protective interactive non-condensing polymers, gelatin

nanospheres and biodegradable microspheres. But one major disadvantage of these systems is their low transfection efficiencies. Polyamines like poly-l-lysine have been used as DNA complexing and condensing agents, either alone or conjugated to ligands, to achieve receptor mediated gene delivery. But gene delivery by poly-l-lysine is complicated by the heterogeneity of the complexes, thus making them difficult to characterize. The size of the DNA-poly-L-lysine complex particles were found to be >1500nm which could be explained by the fact that the complexes are unstable in aqueous solution and the aggregates precipitate out due to charge neutralization. Poly-L-lysine is toxic to cells and, without a targeting ligand, they have poor transfecting ability. The concept of targeting cancer cell-surface receptors seems an attractive proposition to achieve specific binding and internalization of the therapeutic gene. This can be achieved by incorporation of a cell-binding ligand to a gene carrier system. The overexpression of Low Density Lipoprotein receptors on cancer cells provides a unique opportunity to target cancer cells.

Malignant tumors are composed of rapidly dividing cells which have high cholesterol requirement for cell membrane synthesis. The major part of the cholesterol obtained by the cells is via taking up plasma LDL through LDL-receptor mediated endocytosis. Overexpression of LDL receptors in case of brain tumors, human lung adenocarcinoma cells, Daudi Burkitt's lymphoma cells and human prostate cancer cells has been reported by several groups. Thus, the overexpression of LDL receptors on tumor cells provides a rationale for selective targeting. This pathway has been used extensively for the targeted delivery of cytotoxic drugs to tumor cells.

From the aforementioned, we can conclude that an ideal gene delivery system should be able to carry sufficient amount of DNA into the target cells selectively. They should be biocompatible, safe, small in size so as to escape clearance by reticuloendothelial system. They

should also be non-toxic, non-immunogenic and have prolonged plasma circulation. Lipoproteins are natural macromolecular complexes found in the blood that meet all these criteria as ideal gene carriers. Hara et al. prepared hydrophobic complexes of DNA and incorporated them into the core of an emulsion resembling chylomicron remnants to target hepatocytes. Kim et al. directly used a terplex complex comprising of natural Low Density Lipoprotein, hydrophobised PLL and plasmid DNA to achieve transfection in murine smooth muscle cell line and bovine artery wall cells.

Recently, our lab developed an artificial lipoprotein gene delivery system in the form of a nanoemulsion that mimics the lipid core of natural lipoprotein and has palmitoyl poly-L-lysine attached to the surface, which serves to condense the DNA molecules. When tested in human glioma cell line SF-767, this system showed similar transfection efficiency but much lower cytotoxicity compared to commercially available Lipofectamine system.

Based on this encouraging result, we attempt, in this paper, to formulate an artificial lipoprotein that will specifically target the LDL receptors on cancer cells. Several studies have reported the overexpression of LDL receptors in malignancies and the LDLR pathway has been exploited for cytotoxic drug targeting purposes.

The artificial lipoprotein will consist of a lipid nanoemulsion associated with Apolipoprotein B-100, the ligand that is recognized and bound by the LDL receptor (Scott, 1989, Brown et al., 1986). Apo B-100 is one of the largest known polypeptides and it is the surface protein of Low Density Lipoproteins (LDL). The rationale is to combine the targeting properties of Apo B-100 with the reduced cytotoxicity of the nanoemulsion previously formulated to develop an effective and improved gene delivery system to cancer cells.

MATERIALS AND METHODS

Materials

Triolein (99%), egg yolk phosphatidylcholine (99%), cholesterol (99%), cholesterol oleate (98%), L-α-lysophosphatidylcholine (98%), poly-L-lysine hydrobromide (M.W. 48100 based on viscosity), palmitoyl chloride were purchased from Sigma (St. Louis, MO, USA). Electrophoresis grade agarose was purchased from Bio-Rad (Hercules, CA, USA). Lipofectamine^{TM} 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Apolipoprotein B-100 was purchased from Athens Research Center (Athens, GA, USA). All other chemicals were of analytical grade obtained from Sigma or J.T. Baker (Phillipsburg, NJ, USA).

Preparation of artificial lipoprotein

Artificial lipoprotein, like natural lipoproteins, was composed of two parts: a lipid part, consisting of a phospholipids nanoemulsion, and a protein part, composed of apolipoprotein B-100. The nanoemulsion was prepared as described earlier (Pan et al., 2003, Alanazi et al., 2004). Egg yolk phosphatidylcholine (22.7%), lysophosphatidylcholine (2.3%), cholesterol (2%), triolein (70%) and cholesteryl oleate (3%) were dissolved in chloroform separately, mixed, and the solvent was evaporated under nitrogen. Sodium chloride (2.4M) solution was added to the dried lipids (to give a concentration of 10mg lipids per ml sodium chloride solution) in a jacketed vessel which was maintained at temperature of 55°C. The nanoemulsion was formed by sonication of this mixture in a closed nitrogen atmosphere for a period of 45 minutes and the temperature of 55°C was maintained throughout this period. Sonication was carried out using a Model 450 Sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA) with a duty cycle setting of 90% at output of 40 watts. After sonication, the particle size of the emulsion was further reduced and made uniform by immediately passing 10 times through an Emulsiflex B3

device (Avestin, Ontario, Canada) at a pressure of 70 psi. The nanoemulsion (NE) was dialyzed to pH of 7.4 by dialyzing against Phosphate Buffer Saline (pH 7.4) using Spectra/Por® 2 molecular porous membrane tubing with a molecular weight cut-off of 6000-8000 Daltons (Spectrum Medical Industries Inc., Houston, TX, USA).

Artificial lipoprotein (AL) was formulated by the association of Apolipoprotein B-100 (Apo B-100) to the nanoemulsion using the method described by Ginsburg (Ginsburg et al., 1984). The NE was dialyzed to a pH 10 by dialyzing against 0.05M sodium chloride, 0.05M sodium carbonate buffer. Sodium deoxycholate (NaDC)- solubilized Apo B-100 was introduced into the NE (2mg protein per 8mg phospholipid) at a very slow rate (0.5ml/hours) using a peristaltic pump which leads to the binding of Apo B-100 to NE to form the artificial lipoprotein (AL). Extensive dialysis was done at 4°C to ensure complete removal of NaDC. Control experiments were carried under identical conditions. The conjugation of the protein with the lipid nanoemulsion to form AL was characterized by gel electrophoresis followed by lipid and protein staining (Lundberg et al., 1984). Electrophoresis was carried out in a 1% agarose gel for 1 hour at 90V, 20mA. The fluorescent dye, Nile Red, was used as the lipid stain while Simply Blue™ Safe Stain (Invitrogen, Carlsbad, CA, USA) was used as the protein staining dye. The AL was further characterized by measuring its particle size by Submicron Particle Sizer Model 370 (Nicomp Particle Sizing System, Santa Barbara, CA, USA) and zeta potential by Submicron Particle Size Analyzer 90Plus (Brookhaven Instruments Corporation, Holtsville, NY, USA). For conducting the following experiments, the AL at pH 10 was dialyzed against PBS to bring pH to 7.4. Electrophoresis was conducted to confirm that there was no change in the AL due to this change in pH.

Lipidization of poly-L-lysine

Poly-L-lysine (PLL) was modified by attaching lipid chains to it as described previously (Kim et al., 1997, Alanazi et al., 2004). Thirty five mg of palmitoyl chloride was dissolved in 4ml of dioxane. In a separate flask, 100mg of PLL was dissolved in 4ml of dimethyl sulfoxide (DMSO) and 400µl of 1N sodium hydroxide. The former solution was added to the latter and the mixture was allowed to react for 24 hours at room temperature with constant stirring. At the end of this period, the reaction was stopped and the reaction mixture was filtered. The filtrate was added to an excess of diethyl ether which caused the precipitation of the palmitoyl poly-L-lysine (PPLL). The precipitate was redissolved in DMSO and reprecipitated in diethyl ether. PPLL was purified by dialyzing in distilled water overnight at 4°C, lyophilized and stored at -20°C till further use. The lipid modification of the PLL to yield PPLL was characterized by H^1-NMR using d-DMSO. The PPLL was also characterized qualitatively by agarose gel electrophoresis as follows. PPLL solution of concentration 1mg/ml was prepared from the lyophilized product. A similar solution was prepared of unmodified PLL at a concentration of 1mg/ml. Ten µl of fluorescent dye Nile Red solution in acetone (100µg/ml) were added to two test tubes and acetone was allowed to evaporate off to leave the dried dye behind. Samples were prepared by adding 20µl of the PPLL and PLL solutions to the dried dye and incubated for 30 minutes at room temperature. Six µl of glycerin was added to each sample to increase their density so that that they remain in the wells and do not float into the running buffer during electrophoresis. A 0.4% agarose gel was prepared in TAE buffer (40mM Tris-Acetic Acid, 1mM EDTA, pH 8) and then 20µl of each sample was loaded in the sample wells. Electrophoresis was conducted in a Horizontal Mini-Gel system (CBS Scientific Company Inc, CA, USA) for 1 hour at room temperature at 90V, 20mA. The binding of the fluorescent lipid dye, Nile Red, by the samples

was visualized by ChemiImager™ system (Alpha Innotech Corporation, San Leandro, CA, USA).

Amplification and purification of plasmid DNA

To amplify the plasmid DNA, pSV-β-Galactosidase Control Vector (Promega, Madison, WI, USA) was introduced into XL-1 Blue MRF' Supercompetent cells (Strategene, CA, USA) using standard transformation protocol. The transformed cells were grown in Luria-Bertani (LB) medium containing 50μg/ml ampicillin by incubating at 37°C overnight in a shaking incubator. The cultured cells were harvested the next day by centrifugation and the plasmid DNA was extracted and purified using Wizard®Plus SV Minipreps DNA purification system (Promega, Madison, WI, USA). The purity of the DNA was determined by taking the ratio of the absorbance of the DNA solution at 260nm and 280nm. The DNA is deemed to be pure if this ratio is higher than 1.8. The identity and purity of the amplified DNA was further confirmed by restriction digestion with Hind III and Bam H1 restriction enzyme followed by agarose gel electrophoresis on a 1% gel. The DNA was stained with ethidium bromide in the gel (0.25μg/ml) and DNA bands were visualized using ChemiImager™ system (Alpha Innotech Corporation, San Leandro, CA, USA).

Assembly of the PPLL/AL/DNA complex

Artificial lipoprotein (50μl) was diluted with phosphate buffer saline (PBS) and incubated with increasing amount of PPLL for 1 hour at 37°C. The weight ratios of PPLL, calculated with respect to triolein (the major component of AL) were: 1:32, 1:16, 1:8, 1:4, 1:2 and 1:1. After 1 hour, 5μg DNA (pSV-β-Galactosidase Control Vector) was added to the mixture and incubated

at room temperature for 15 minutes. To determine the DNA holding capacity of the complexes, electrophoresis was carried out on a 0.4% agarose gel as described above (under lipidization of poly-L-lysine). Zeta potential and mobility of the PPLL/AL/DNA complexes, suitably diluted with HPLC water, were measured by Submicron Particle Size Analyzer 90Plus (Brookhaven Instruments Corporation, Holtsville, NY, USA) as described earlier (Alanazi et al., 2004).

Transfection efficiency of the complexes

The transfection efficiency of the various PPLL/AL/DNA complexes were determined in human glioma cell line SF-767, obtained from the tissue bank of Brain Tumor Research Center (University of California-San Fransisco, San Fransisco, CA, USA). This cell line was used because of its reported over-expression of LDL receptors and aggressive growth characteristics. Plastic cell culture flasks (75 cm^2 , from A Cammbrex Company, Walkersville, MD, USA) were used to grow the cells. Eagle's Minimum Essential Medium (EMEM), supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin and 100µg/ml streptomycin was used as the medium and the cells were grown at 37°C and 5% CO_2. The cells were passaged every two days for 10 days in order to maintain an exponential rate of growth. Six-well culture plates (35mm diameter) were used to perform the transfection studies. Each well was seeded with $3x10^5$ cells and the cells were allowed to grow in EMEM (with serum and antibiotics) at 37°C and 5% CO_2 till the cells reach 95% confluency. After this period, the cells in each well were thoroughly washed with PBS to remove serum containing medium and 1ml of EMEM (without any antibiotics or serum) was added to each well. Transfection was carried out by preparing PPLL/AL/DNA complexes as described earlier and incubating with the cells for 12 hours at 37°C and 5% CO_2 after which EMEM, (containing 20% FBS but no antibiotics), was

supplemented and the cells were incubated for additional 24 hours. Additional transfection experiments were performed under identical conditions with the DNA, PPLL/AL and PPLL/nanoemulsion (without apo B-100) as negative controls and Lipofectamine^{TM} (Invitrogen, Carlsbad, CA, USA) as positive control.

Enzymatic assay for the detection of β-Galactosidase

The transfection efficiency of the PPLL/AL/DNA was determined by measuring the β-Galactosidase activity in the cells using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA). The growth medium was completely removed and the cells in each well of the 6-well plate were washed with PBS. Reporter Lysis Buffer (1X, 300µl) was added to each well and the plates were rocked to ensure complete coverage of the cells. The cells were incubated at 37°C for 30 minutes. Pipet tip or cell scraper was used to scrape off the wells and the cell lysates were transferred to microfuge tube and centrifuged for 10 minutes in order to obtain the clear cell lysate. The cell lysate (100 µl) was mixed with 100 µl of Assay 2X Buffer supplied with the assay kit and incubated for 6 hours at 37°C. Then reaction was stopped by adding 500 µl of 1M sodium carbonate and absorbance was taken at 420 nm, diluting if necessary. The protein concentration of the cell lysate was also determined using the Bradford Assay and β-Galactosidase enzymatic activity was defined as described in Pan et al. (Pan et al., 2003).

Results

Preparation of artificial lipoprotein

The nanoemulsion was prepared as described by Alanazi et al., 2004 and Pan et al., 2005 with a slight modification. The dried lipids were sonicated at 55°C, which was the phase

transition temperature of the highest melting lipid used and the sonication was done for 45 minutes instead of the previously reported 30 minutes, followed by passing through the Emulsiflex B3 device. The mean particle size (number weighted) of the nanoemulsion was found out to be 30.5±13.4 nm diameter.

The pH at which the NaDC-solubilized Apo B-100 interacts with the NE is highly critical (Ginsburg et al., 1984). This is because Apo B-100, under neutral conditions and in the presence of surfactant like NaDC, undergoes a pH-dependent aggregation which can be prevented by raising the pH. For this reason, the NE was dialyzed against sodium carbonate, sodium chloride buffer to a pH of 10 before the addition of NaDC-solubilized Apo B-100. The NaDC-solubilized Apo B-100 was added to the NE (at pH 10) at a very slow rate of 0.5ml/hour. This causes the concentration of the NaDC to fall below its CMC (critical micelle concentration) when it enters the NE, allowing the Apo B-100 to bind to the lipid NE. The association of the Apo B-100 with the NE was not expected to cause much structural and compositional change in the NE and a similar result has been previously reported (Hirata et al., 1999).

Figure 3.2 shows the electrophoretic mobility of the NE, AL and the various controls used in the experiments and stained by fluorescent dye Nile Red. Lane 1 shows the mobility of the control NE where pH 10 buffer was dripped into the NE (also at pH 10) while lane 2 shows the mobility of NE (at pH 10). This control was run to see if the experimental conditions had any effect on the NE. As can be clearly seen, both the control and NE have similar electrophoretic mobility confirming that the experimental conditions had no effect on the NE. Lane 3 shows the electrophoretic mobility of the AL. Two bands can be seen, one inside the well and one farther up as compared to the single band of NE.

The association of the Apo B-100 to the NE to form the AL was confirmed by agarose gel electrophoresis. Staining of the gel with both lipid and protein stains shows that the bands coincide (Lane 3, Figs 3.2 and 3.3). LDL was used as a positive control and it showed a similar staining characteristic for lipid and protein stain (Lane 7). The AL was considerably heterogeneous with a particle size of 37.1±25.5nm compared to 30.5±13.4nm for the NE and 20nm as reported for the LDL. The zeta potential of the AL was -34.06mV compared to -40.48 mV for NE and 13.3mV for the LDL. The reason that most of the AL band remains inside the wells of the gel compared to NE is that AL had larger size and less negative charge on its surface and therefore is attracted less towards the anode during electrophoresis. Lane 4 shows the band for the negative control. NaDC was dripped into NE under same conditions as for AL except that there was no Apo B-100 solubilized in the NaDC. The band is similar to the band for NE (Lane 2) clearly showing that the surfactant NaDC does not interact in any way with the NE and doesnot interfere with the binding of Apo B-100 to NE. Lane 5 had NaDC as a sample to check for its staining property. As it can be seen, NaDC does not stain for either protein or lipid stain and should not give false positive results when we stained AL with lipid and protein stains. Lane 6 is Apo B-100 and, as expected, it stains for the protein stain (Fig 3.3) but not lipid stain (Fig 3.2). Apo B-100 also does not show as a single narrow band but as a very broad band implying heterogeneity.

Lipidization of poly-L-lysine

The zeta potential of the AL was -34.06mV making it unsuitable for condensing DNA, which is also negatively charged, because of electrostatic repulsion. Positively charged poly-L-lysine has been widely used for condensing DNA. Previous studies have shown that incubation

of unmodified PLL with NE results in precipitation because of charge neutralization (Pan et al., 2003). Therefore, PLL was lipidized by attaching palmitoyl chains through N-alkylation of the free ε-amino groups to form palmitoyl poly-L-lysine (PPLL). It has been proposed that PPLL interacts with AL predominantly by hydrophobic interactions and does not result in precipitation Pan et al., 2003). PPLL can be differentiated from PLL by the presence of fatty (palmitoyl) chains. This difference was used to qualitatively characterize PPLL and PLL. Both PPLL and PLL were incubated with fluorescent dye Nile Red (which binds to lipids) and agarose gel electrophoresis was performed. When visualized under UV light, PPLL band emits fluorescence, while PLL does not (Fig 3.4). This is because the fatty chains on PPLL bind Nile Red while PLL does not.

Amplification and purification of plasmid DNA

Plasmid DNA was amplified and purified by standard molecular cloning techniques. Purity of the DNA was determined by taking the ratio of absorbances at 260nm and 280nm and this ratio should be above 1.8 for pure DNA. The A260/A280 was found to be 2.04. Fig 3.5 shows the agarose gel electrophoretic mobility of amplified DNA. Lanes 1 and 8 contains 1 kb DNA ladder (250-10,000 base pairs) while lanes 2-7 consist of different batches of amplified DNA. Three bands are seen in each of the samples. Plasmid DNA can exist in 3 forms: supercoiled, open circular and closed circular form. These 3 forms have different electrophoretic mobility and the 3 bands seen after electrophoresis of the samples could depict these 3 forms. But it may be possible that the DNA can be contaminated. A260/A280 gives the purity of the DNA but cannot tell us if we have amplified and purified the right DNA, in this case pSV-β-Galactosidase control vector.

In order to confirm that we have amplified and purified the correct DNA, we digested the DNA samples with restriction endonuleases followed by agarose gel electrophoresis (Fig. 3.6). The DNA was treated with the restriction enzymes Bam H1 and Hind III which cleave the pSV-β-Galactosidase control vector at location 4151 and 414 respectively to generate linear DNA. Although after amplification, DNA can exist in 3 forms (supercoiled, closed circular and open circular), all 3 forms can be cleaved by restriction enzymes to generate linear DNA. But these enzymes can only cleave pSV-β-Galactosidase control vector into linear form while any contaminating DNA will not be cleaved and this is an ideal tool to confirm the purity of the amplified DNA. Lanes 1 and 10 shows the DNA ladder while lanes 5 and 9 shows the amplified DNA (3 bands). Restriction digestion with Bam H1 yields a single band of 6.8kb (lanes 2 and 6) while digestion with Hind III also yields a single band of 6.8kb (lanes3 and 7) which corresponds to the reported size of pSV-β-Galactosidase control vector. Double digestion of the DNA with both Bam H1 and Hind III yields 2 fragments of sizes 3.7kb (4151-414) and 3.1kb (6.8kb-3.8kb) thus confirming the purity of the amplified pSV-β-Galactosidase control vector (Lanes 4 and 8).

Assembly of the PPLL/AL/DNA complex

Since AL is negatively charged it will repel negatively charged DNA. Hence, in order for the AL to carry the DNA, increasing ratios of PPLL were incubated with AL. PPLL interacts with AL through hydrophobic interactions while increasing the surface charge of the AL, making it more positive. Different ratios of PPLL to AL were prepared and their ability to condense 5μg DNA was determined by electrophoresis on a 0.4% agarose gel and staining by ethidium bromide to observe the DNA(Fig 3.7). Lane 1 and 9 are for plasmid DNA. Lane 2 consists of DNA and AL. As expected, the negatively charged AL is unable to condense the negatively

charged DNA and we can see the DNA bands. Lanes 3-8 consist of PPLL/AL complexes with 5μg DNA. These complexes had increasing amounts of PPLL to AL. The ratios tested were 1:32, 1:16, 1:8, 1:4, 1:2 and 1:1. Lanes 3 and 4, having complxes at ratios of 1:32 and 1:16 were not able to condense all of the 5μg DNA and DNA bands can be seen in these lanes. On the other hand, lanes 5, 6, 7 and 8 of ratios 1:8, 1:4, 1:2 and 1:1 respectively were able to condense all of the DNA as no DNA bands can be seen escaping from these complexes.

It has been reported that the charge of the transfection complexes play an important role in transfection. Therefore the zeta potential and mobilities of the complexes were determined as shown in Figs 3.8 and 3.9. It can be clearly seen that, with a fixed quantity of 5μg DNA, an increase in the ratio of positively charged PPLL to AL led to an increase in the zeta potential of the complexes.

Transfection efficiency of the complexes

The four complexes at different ratios of PPLL:AL (1:8, 1:4, 1:2 and 1:1) that were able to condense 5μg of DNA were tested for transfection efficiency in the human glioma cell line SF-767 along with appropriate controls. The transfection efficiencies are shown in Figure 3.10. As it can be seen, naked DNA, in the absence of a carrier, cannot transfect. Of the 4 complexes tested, three of the complexes were able to transfect with the complex at ratio 1:4 having the highest transfection efficiency. This complex was then used to determine the role of Apo B-100 on transfection into the glioma cells.

To determine if the Apo B-100 in the AL was helping in improving the transfection, PPLL was complexed with NE (without any Apo B-100) and tested for transfection. It was found that the PPLL/NE complex (at 1:4 ratio) expressed 370 milliUnits β-galactosidase per mg protein

which was comparable to that expressed by commercial reagent Lipofectamine (420 milliUnits/mg protein) and this was similar to the results reported previously (Pan et al., 2003). But the PPLL/AL (with Apo B-100) complex at 1:4 ratio expressed 950 milliunits of β-galactosidase per mg protein, clearly indicating its improved transfecting ability due to the presence of Apo B-100.

Discussion

Lipoproteins play a very important role in lipid transport in the body and 90% of the cholesterol required by the cells is obtained via receptor mediated endocytosis of LDL through the LDL receptors on cell surface. Rapidly growing cells like cancer cells require a large amount of cholesterol and hence they have elevated LDL receptors compared to normal cells (Sarkar et al., 2002). In a study by Maletinska et al., 2000, seven human glioma cell lines were found to have an over-expression of LDL receptors e.g. the SF-767 glioma cells had 300, 000 LDL receptor per cell (Maletinska et al., 2000) indicating that the LDLR pathway can be used to selectively target cancer cells. Therefore, the development of gene carriers that can mimic LDL and utilize the LDLR pathway to deliver therapeutic genes to cancer cells need to be developed. Kim et al., 1997 have developed and tested a terplex DNA carrier system comprising of natural LDL, DNA and hydrophobized poly-L-lysine. This complex was successful in condensing DNA and transfecting (Kim et al., 1997, 1998, Yu et al., 2001). This approach would involve withdrawing blood from the patient, isolating LDL by differential density ultracentrifugation, preparation of the terplex complex followed by injection back to the patient. This strategy suffers from a number of drawbacks including being tedious and time consuming (fractionation of lipoproteins to isolate LDL) besides causing discomfort for the patient (withdrawing blood and

reinjecting). These drawbacks can be overcome if, instead of withdrawing and isolating LDL from the patient, we can prepare artificial lipoproteins and that is the primary motive behind this study.

Initially, the lipid structure of the LDL was artificially mimicked by preparing protein free models which were basically microemulsions (Ginsburg et al., 1982, Maranhao et al., 1993, Via et al., 1982, Reisinger et al., 1990). This was followed by complexes of phospholipids, cholesterol ester and Apo B-100 which were used as models resembling human LDL for studying the molecular structure, interactions, functions and metabolism of LDL (Ginsburg et. al, 1984, Walsh et al., 1986, Lundberg et al., 1984, Chun et al., 1986).

Recently, our lab developed a protein free phospholipid nanoemulsion which was tested for in vitro gene transfection in glioma cell line SF-767 (Pan et al., 2003). This formulation was found to have similar transfection compared to commercially available Lipofectamine but was less cytotoxic. In this paper, we have attempted to improve the transfection efficiency of this phospholipids nanoemulsion by conjugating it with Apo B-100 to form an artificial lipoprotein. Apo B-100 is the ligand recognized by the LDL receptors and the presence of Apo B-100 will enable us to target the LDL receptors on cancer cells. Such a formulation will combine better transfection efficiency and targeting ability to cancer cells with the reduced toxicity of the previous phospholipid formulation. Another potential advantage is that this is an artificial lipoprotein formulated from commercially available lipids and protein, so there will be no need to isolate LDL from the patient by withdrawing blood. Since the AL mimics natural LDL, it would be stable in biological fluids, remain longer in circulation and not be cleared by the RES.

The artificial lipoprotein (AL), like native lipoproteins, consists of a lipid part and a protein part. The lipid part of the AL consists of a nanoemulsion made up of commercially

available lipids. Since our goal is to target the AL to the LDL receptors (LDLR) on cancer cells, the protein part of the AL consists of Apo B-100, which is the apolipoprotein found on LDL and, as mentioned above, it is the ligand that helps in binding LDL to the LDLR.

The nanoemulsion was made by sonicating the lipids for 45 minutes. Egg yolk phosphatidylcholine and lysophosphatidylcholine form the outer polar shell of the nanoemulsion and cholesterol helps to stabilize this layer. At the core of the nanoemulsion are triolein and cholesteryl oleate. The nanoemulsion was found to have a particle size of 30.5±13.4 nm diameter compared to 48-54nm as reported previously (Pan et al., 2003, Alanazi et al., 2004). This is likely due to the increased sonication time which resulted in smaller particle size for the nanoemulsion. It has been previously reported that LDL has a size of 23-24nm for maximum LDLR affinity and this is related to the conformation of the Apo B-100 (Rensen et al., 2001) and we wanted to make the particle size of the nanoemulsion as small as possible without affecting stability.

Apo B-100 has both polar and non-polar domains which allow it to associate with lipids in LDL as well as interact with the LDL receptor (Law et al., 1990, Mahley et al., 1983). Apo B-100 is supplied in frozen dried form together with a mild surfactant, sodium deoxycholate (NaDC). This is because Apo B-100 is obtained through delipidating LDL by substituting the NaDC for the naturally occurring amphiphilic ligands and lipids like phospholipids, cholesterol, cholesteryl ester and triglycerides (Watt et al., 1981). If NaDC were not present, upon solubilization in an aqueous medium the Apo B-100 would aggregate irreversibly. But in the presence of a bound amphiphile like NaDC, Apo B-100 is solubilized in NaDC micelles and also maintains its native dimeric state (Watt et al., 1981). The Apo B-100 was conjugated to the NE by the method employed by Hirata as well as Ginsburg for their studies (Hirata et al., 1999,

Ginsburg et al., 1984). The NaDC solubilized Apo B-100 was dripped at a very slow rate into the NE (at a pH of 10) (1mg Apo B-100 to 4mg phospholipids), which is kept in a dialysis tube while dialyzing against buffer of pH 10. Under such conditions, diffusion of NaDC occurs across the membrane resulting in the lowering of the NaDC concentration below its critical micelle concentration. This allows the Apo B-100 to bind with the NE to form Artificial Lipoprotein (AL). After extensive dialysis had been done to remove the NaDC, the AL was dialyzed back to pH 7.4 which was not expected to cause any major change in its integrity (Ginsburg et al., 1984). The pH of the NE had to be raised from 7.4 to 10 because it has been reported that at pH of 7.4 Apo B-100-amphiphile complexes had a tendency to aggregate (Watt et al., 1981, Dhawan et al., 1983). This aggregation was the result of interactions between the water soluble regions of the Apo B-100. Raising the pH to 10 helps to maintain a significant charge on the protein and this prevents aggregation.

Conjugation of Apo B-100 to the NE was confirmed by agarose gel electrophoresis with lipid and protein staining which showed that lipid band of AL and protein band of AL coincide (Lundberg et al., 1984). This staining property compares well with the positive control, LDL, which shows similar staining. The electrophoretic mobility of the newly formed AL is reduced because of the large size of the Apo B-100 on it and also because of its overall large size (37nm) compared to LDL (20-25nm). The zeta potential of the AL was negative (-34.06 mV) making it unsuitable for condensing negatively charged DNA (Fig 3.7, Lane 2).

Studies on the location of Apo B-100 in native and reconstituted LDL led to the conclusion that a portion of the protein is associated with the spherical surface while the remainder of the Apo B-100 is intercalated into the hydrophobic core (Dhawan et al., 1983). It was also found that the proportion of the Apo B-100 molecule associated with the surface and

the proportion that penetrates towards the core varies in LDL and also the different reconstituted LDLs. in each case (Ginsburg et. al, 1984, Walsh et al., 1986, Lundberg et al., 1984, Chun et al., 1986). In vitro experiments have demonstrated the ability of the Apo B-emulsion conjugates to bind to the LDLR with the same affinity as native LDL (Hirata et al., 1999).

This led to the conclusion that Apo B-100 is capable of conformational adaptability (Reynolds, 1976). It therefore appears that Apo B-100 has the ability to adopt grossly different morphological structures depending on the amphiphiles associated with it (Dhawan et al., 1983). It has also been demonstrated that the secondary structure of Apo B-100 does not change much by the type of ligand bound to it as long as an appropriate hydrophobic environment is maintained (Watt et al., 1981). All this leads us to conclude that the Apo B-100 will bind to the NE to form AL with no significant change in its secondary structure and thus its conformation and this binding will not affect its receptor binding properties.

Since the negatively charged AL was unable to condense negatively charged DNA (Fig 3.7, Lane 2), modified poly-L-lysine (PPLL) was interacted with it. Modified PLL has palmitoyl chains attached to some of the lysine residues and these palmitoyl chains interact with phospholipids of the NE through hydrophobic interactions (Pan et al., 2003). Therefore, when increasing amounts of PPLL were incubated with the AL followed by the incubation with DNA, the surface charge of the PPLL/AL/DNA complex became increasingly positive as shown by the increase in zeta potential and mobility (Figure 3.8 and 3.9). Figure 3.7 also shows that PPLL/AL/DNA complexes at ratios 1:32 and 1:16 (Lanes 3 and 4) are unable to condense 5µg DNA while the complexes at ratios 1:8, 1:4, 1:2, an 1:1 can (Lanes 5, 6, 7, 8).

When transfection of these complexes was carried out in SF-767 glioma cell lines, the complexes at 1:8, 1:4 and 1:2 were able to transfect with the ratio at 1:4 being the highest. Naked

DNA was unable to transfect while the positive control, Lipofectamine showed transfection. To see if the Apo B-100 was making a difference in transfection, nanoemulsion, with no Apo B-100, was incubated with PPLL at ratio of 1:4 (PPLL:NE) followed by incubation with 5μg DNA. Transfection of this complex in SF-767 was compared with PPLL/AL/DNA (at 1:4) and Lipofectamine and it was found that PPLL/AL/DNA (1:4, with Apo B-100) had 2.5 times the transfection efficiency of PPLL/NE/DNA (1:4, no Apo B-100) and more than twice that of the commercial reagent Lipofectamine. This clearly demonstrated the role of Apo B-100 in improving the uptake of DNA into glioma cells.

Conclusions

An artificial lipoprotein containing Apo B-100 for targeting the LDL receptors on cancer cells was successfully formulated and characterized. When tested for transfection efficiency in human glioma cell line SF-767, the artificial lipoprotein was found to be twice as successful as the commercially available reagent Lipofectamine.

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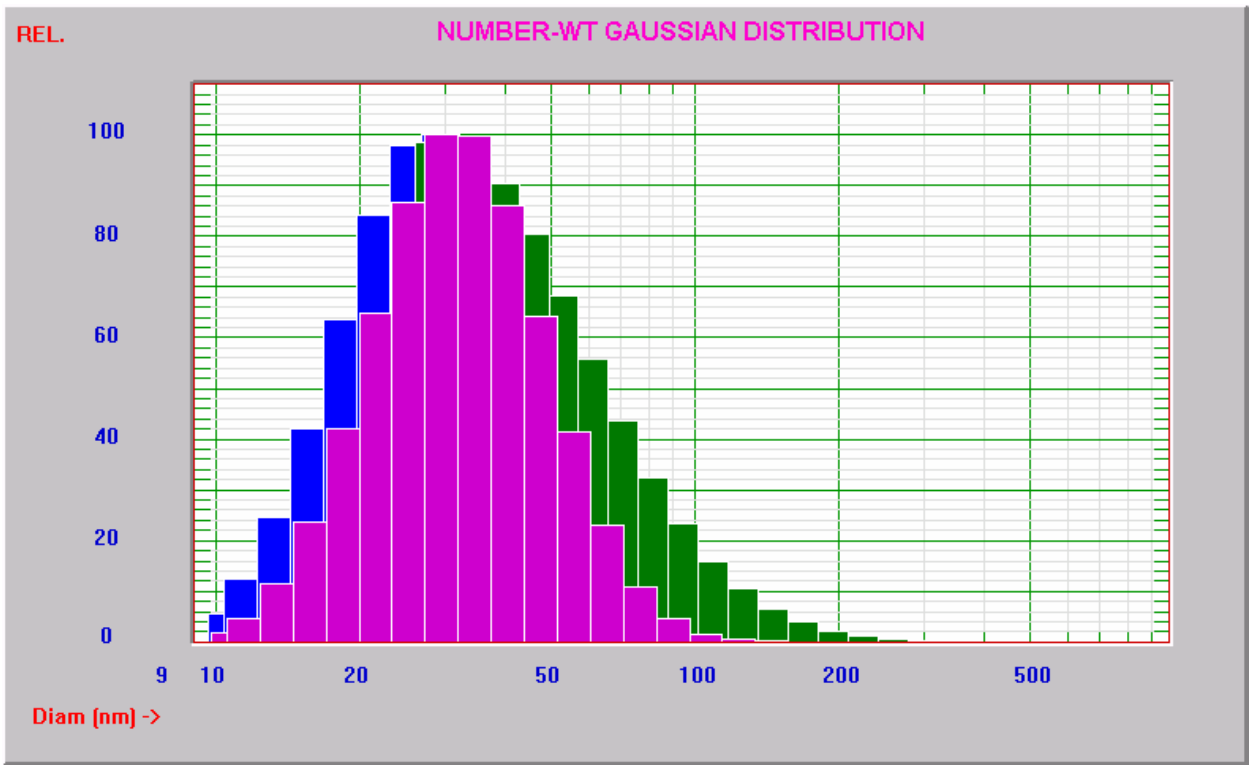

Figure 3.1: Particle size distribution of nanoemulsion (Blue), AL (Green) and Control (Purple)

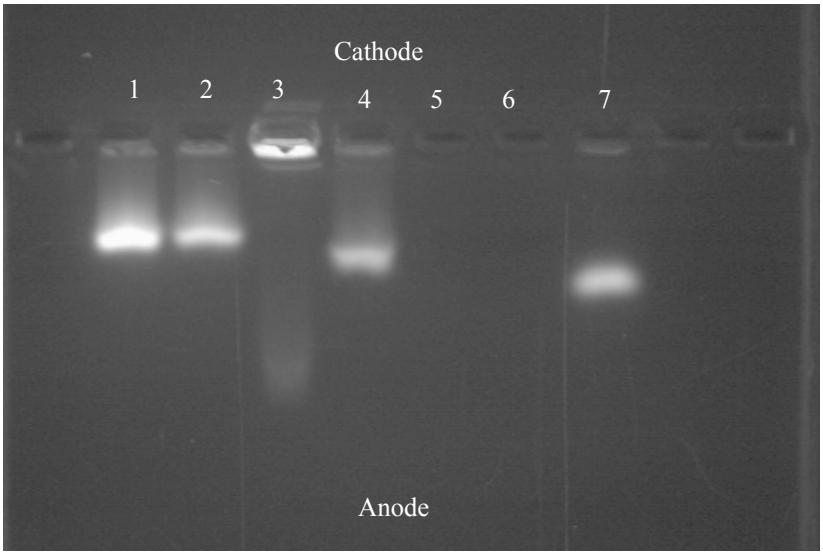

Figure 3.2: Agarose Gel Electrophoresis of artificial lipoprotein with experimental controls (Stained with Nile Red lipid stain) (Lane 1: Control 1, Lane 2: nanoemulsion, Lane 3: Artificial Lipoprotein, Lane 4: Control 2, Lane 5: sodium deoxycholate, Lane 6: Apo B-100, Lane 7: LDL)

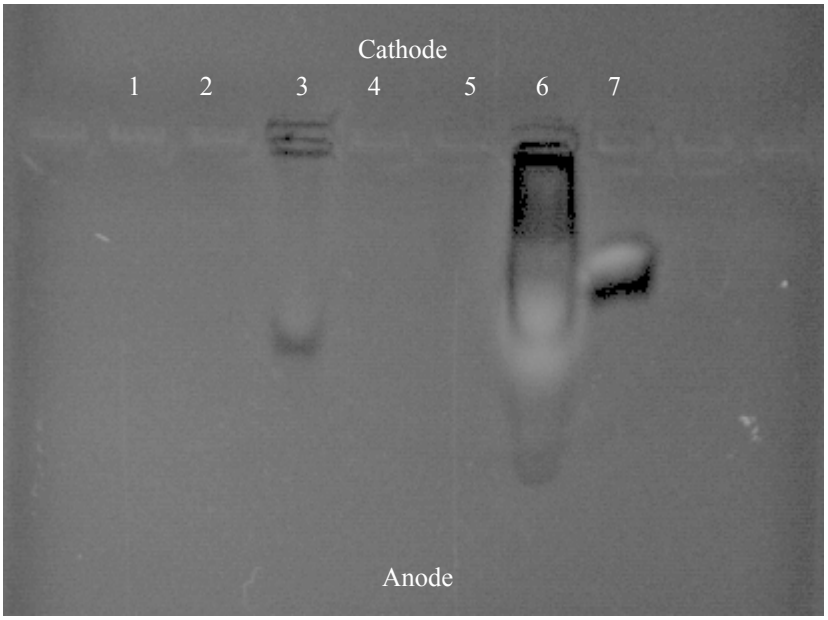

Figure 3.3: Agarose Gel Electrophoresis of artificial lipoprotein with experimental controls (Stained with protein stain) (Lane 1: Control 1, Lane 2: nanoemulsion, Lane 3: Artificial Lipoprotein, Lane 4: Control 2, Lane 5: sodium deoxycholate, Lane 6: Apo B-100, Lane 7: LDL)

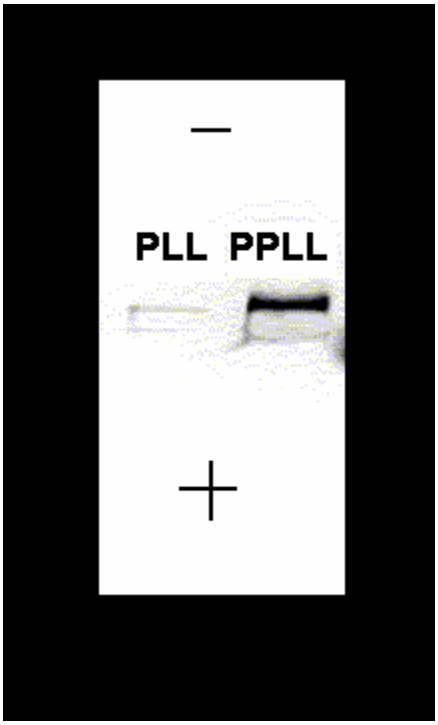

Figure 3.4: Agarose Gel Electrophoresis of PPLL and PLL with staining by Nile Red Lipid dye.

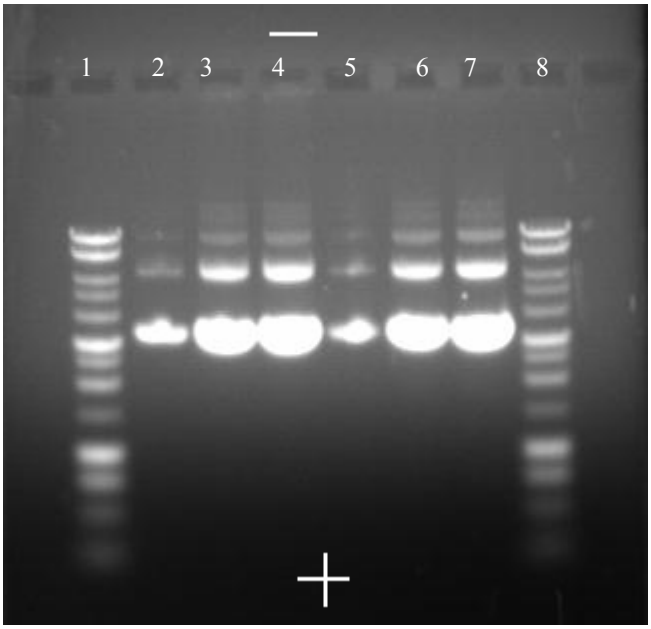

Figure 3.5: Agarose Gel Electrophoresis of amplified DNA with ethidium bromide staining

(Lanes 1 & 8: DNA ladder, Lanes 2-7: Different batches of amplified DNA).

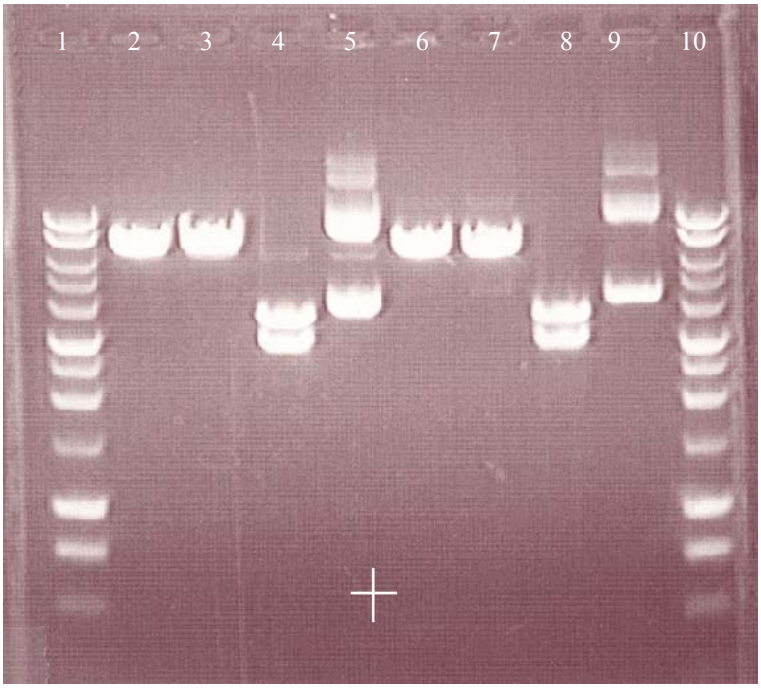

Figure 3.6: Restriction digestion of amplified DNA with ethidium bromide staining (Lanes 1 & 10: DNA ladder, Lanes 2 & 6: Bam HI digested DNA, Lanes 3 & 7: Hind III digested DNA, Lanes 4 & 8: Double digested DNA, Lanes 5 & 9: Amplified DNA)

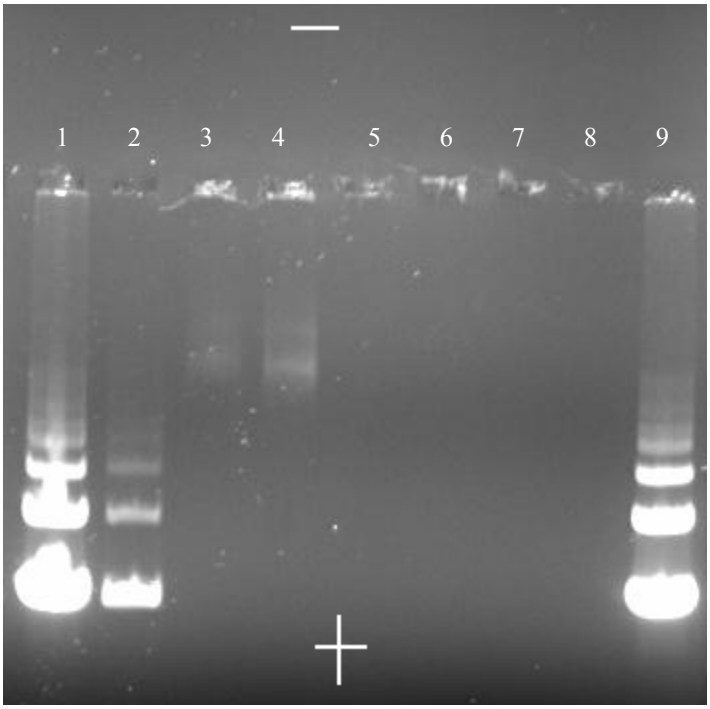

Figure 3.7: Agarose Gel Electrophoresis of PPLL/AL/DNA complexes stained with ethidium bromide (Lanes 1 & 9: DNA, Lane 2: AL & DNA, Lanes 3-8: PPLL/AL/DNA at ratios 1:32, 1:16, 1:8, 1:4, 1:2, 1:1)

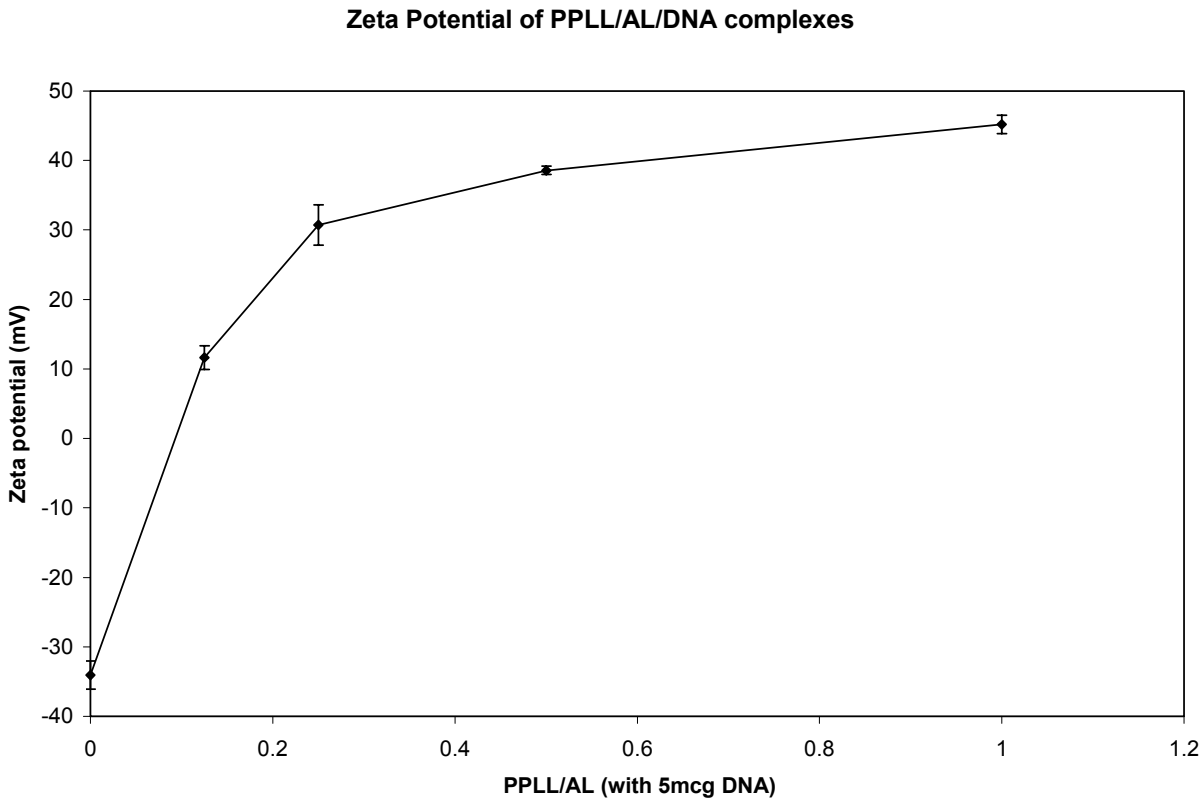

Figure 3.8: Zeta potential of the PPLL/AL/DNA complexes

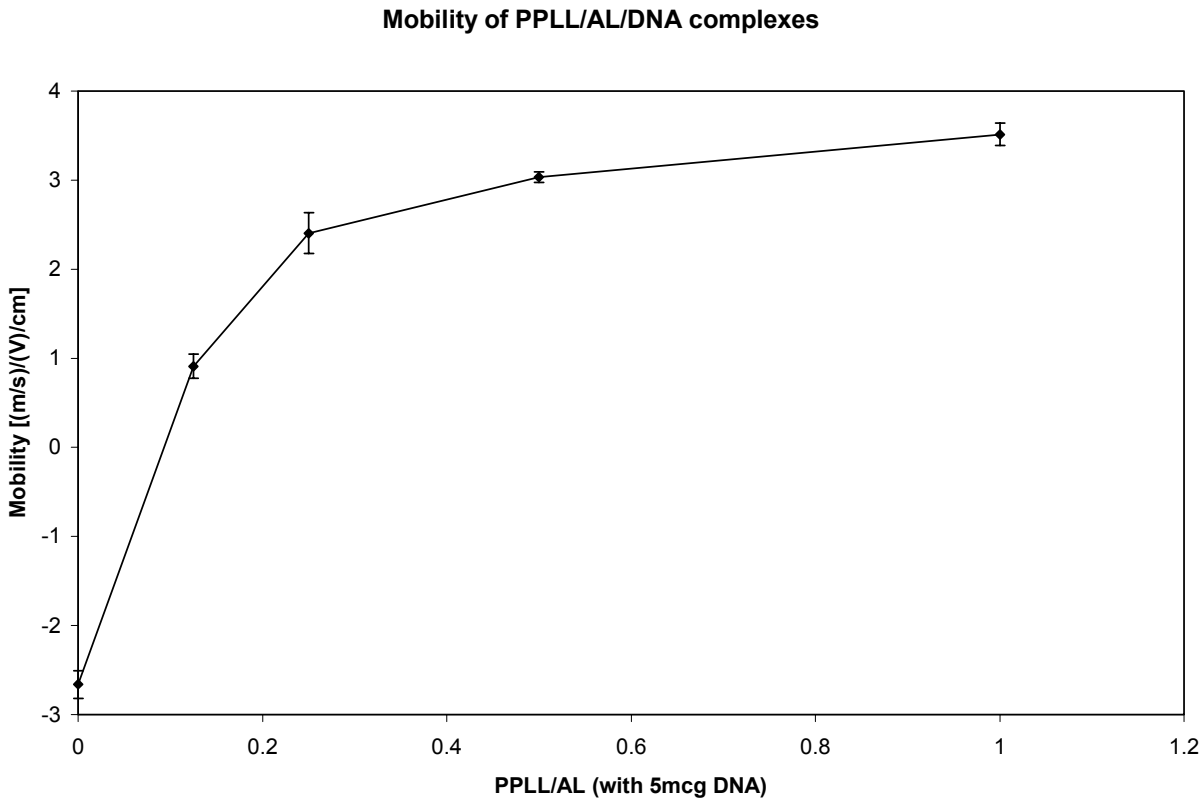

Figure 3.9: Mobility of the PPLL/AL/DNA complexes

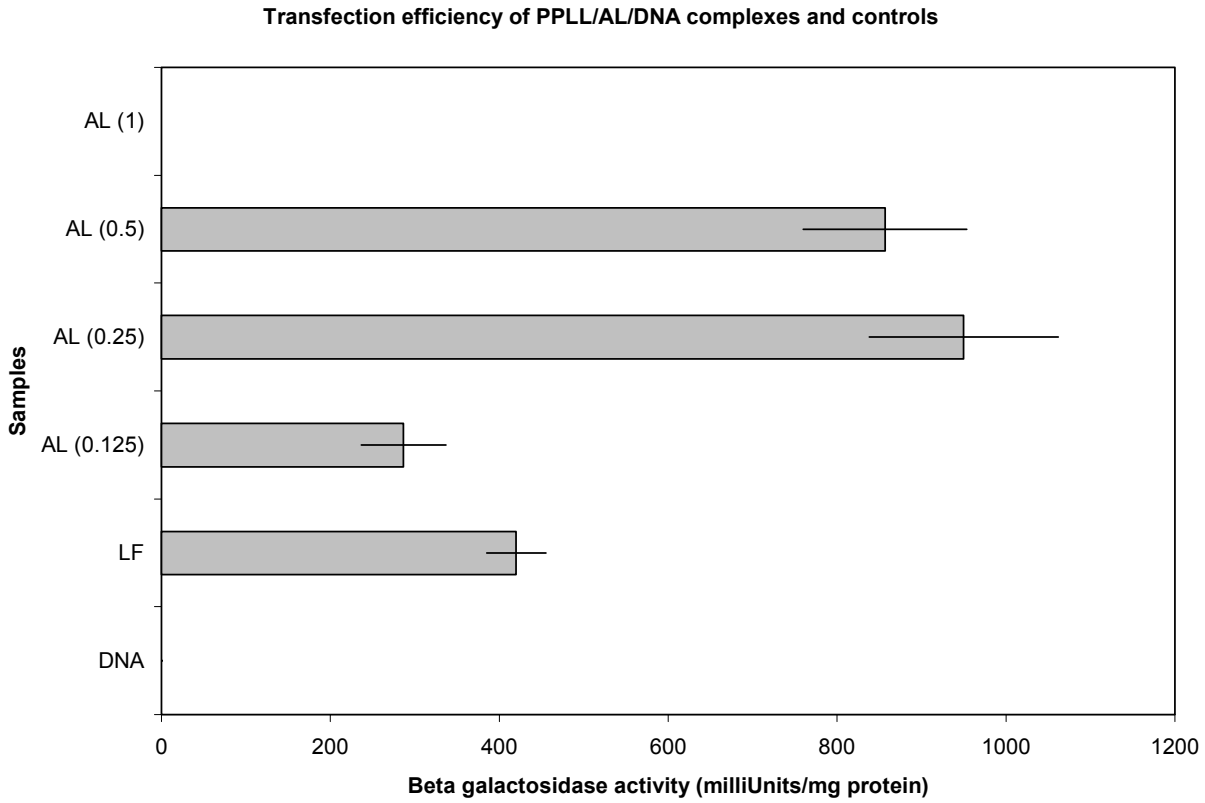

Figure 3.10: Transfection efficiency of the complexes as measured by amount of β-Galactosidase expressed (LF: Lipofectamine, AL: Artificial Lipoprotein, DNA: naked DNA)

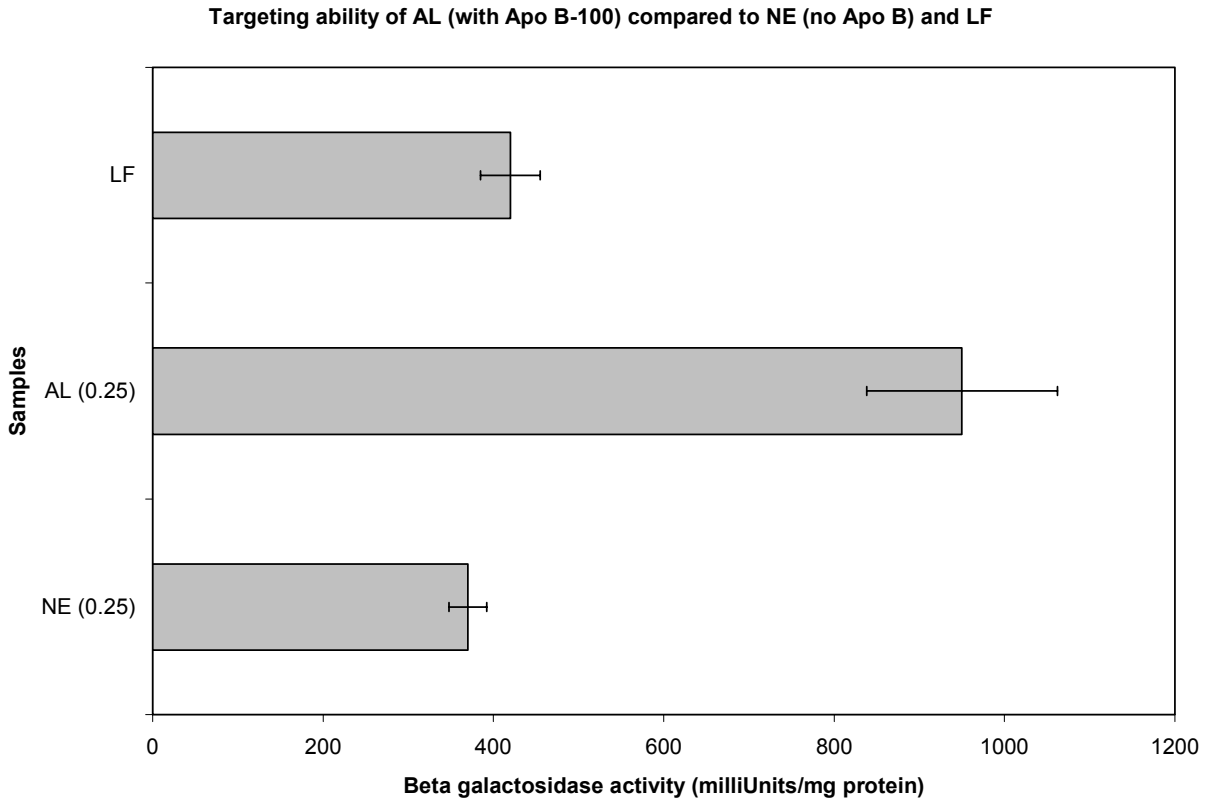

Figure 3.11: Effect of Apo B-100 on transfection efficiency (NE: Nanoemulsion, AL: Artificial Lipoprotein, LF: Lipofectamine)

Table 3.1: Properties of artificial lipoprotein and controls

	Lipid stain	Protein stain	Particle size (nm)	Zeta Potential (mV)	Electrophoretic mobility
NE	+	-	30.5±13.4	-40.48	+
AL	+	+	37.1±25.5	-34.06	-
Control 1 (NaDC)	+	-	35.1 ± 14.6	-45.03	+
Control 2 (conditions)	+	-	31.2 ± 11.7	-40.96	+
NaDC	-	-	N/A	N/A	N/A
Apo B-100	-	+	N/A	N/A	-
LDL	+	+	20-25*		+

* reported

Part 2

Effect of using dual surfactant systems on properties of ethyl cellulose microspheres prepared by non-aqueous emulsion-solvent evaporation method

Chapter 4

Introduction and Literature Review of Controlled release drug delivery systems

It is well known that the conventional dosage forms are unable to control the rate or the site of action of the drug release and often result in poor therapeutic results with the drug. This can be overcome by the use of controlled release dosage forms that help in achieving precise temporal and spatial placement of the drug within the body and thereby achieving the two main objectives (Robinson et al., 1987):

1. Improved therapeutic efficacy and safety of drugs.
2. Improved patient compliance.

Advantages and disadvantages of controlled release drug delivery systems

(Welling et al., 1987, Robinson et al., 1978, Ranade et al., 1996)

1. Decreased fluctuations of plasma concentration of drug leading to improved efficacy of the drug.
2. Improved bioavailability of some drugs.
3. Minimize local or systemic side effects by employing less total drug.
4. Reduced toxicity due to minimal drug accumulation in case of chronic therapy.
5. Reduction in dose size and number helps in decreased cost to the patient over long dosing periods.

Potential disadvantages of controlled release systems (Welling et al., 1987, Robinson et al., 1978, Ranade et al., 1996)

1. Poor and unpredictable in vitro-in vivo correlation.
2. Longer time period required to achieve effective therapeutic concentrations.
3. Chances for increased variation in bioavailability after oral administration.
4. Increased potential for first-pass effect.
5. Dose dumping (a large quantity of medication released rapidly) leading to toxicity.
6. Reduced potential for dosage adjustment.
7. Effective drug release period is influenced and limited by GI residence time.

Despite the above shortcomings, improved patient compliance and increased safety and efficacy justify the need for controlled release drug delivery systems.

Types of oral controlled release drug delivery systems

Among all the routes, oral routes have been most popular and successful because of patient acceptance of oral dosage forms and their inherent convenience. On the other hand, oral route is constrained by short and variable GI transit time, first pass metabolism, limited absorption in the lower part of the GI tract, and the size of dosage form.

Oral controlled delivery systems (Wise et al, 2000, Robinson et al., 1987) can be broadly divided into following categories, based on their mechanism of drug release:

1. Polymer Dissolution-controlled release

- Encapsulation dissolution control
- Polymer Matrix dissolution control

2. Diffusion controlled release

- Reservoir devices
- Matrix devices

3. Diffusion and Dissolution controlled systems
4. Ion exchange resins
5. pH-independent and dependent formulations
6. Osmotically controlled release
7. Altered density formulations/Gastroretentive systems

Polymer Dissolution-controlled release

Dissolution controlled release can be obtained by slowing the dissolution rate of a drug in the GI medium and this can be achieved by either incorporating the drug in a polymer matrix or by coating the drug particles or granules with polymeric materials of varying thickness. One method for drug delivery using rate of dissolution of a protective polymer as a controlled release mechanism is by encapsulation (encapsulation-dissolution control) of a drug- excipient matrix with a slowly dissolving polymeric membrane. The coated beads are either compressed into tablets (as in Spacetabs) or put in capsules (as in Spansule products). Since the time required for the membrane coat to dissolve is a function of membrane thickness, granules with varying thickness can be employed to achieve sustained release of the drug. Encapsulation can be achieved by several methods such as coacervation/phase separation, interfacial polymerization, electrostatic coating, precipitation, hot melt, salting out and solvent evaporation methods.

Another method, called matrix dissolution control, involves incorporation of the drug in a hydrophobic matrix such as waxes and compressing into tablets. The rate of drug availability is controlled by the rate of penetration of dissolution fluid into the matrix. This, in turn, can be

controlled by the porosity of compressed structure, presence of hydrophilic additives, and the wettability of the structure and particle surfaces.

Diffusion controlled release

Diffusion of drug molecules through a polymeric membrane forms the basis of these controlled drug delivery systems. Similar to the dissolution controlled systems, the diffusion-controlled devices are manufactured by encapsulating the drug particle in a polymeric membrane (reservoir devices) or by dispersing the drug in a polymeric matrix (matrix devices). Unlike the polymer-dissolution controlled systems, the drug is made available as a result of diffusion through pores in the membrane or partitioning through the polymer. In reservoir devices, a water-insoluble polymeric material encapsulates a core of drug. When placed in dissolution fluid, drug either partitions into and diffuses through the polymer to exchange with the fluid surrounding the device or dissolves in fluid that has diffused through pores in the membrane and then the drug molecules diffuse out through pores. Press coating and air suspension coating and coacervation are methods that can be used to prepare these devices. The drug release from such devices depends on polymer ratio in the coating, film thickness and porosity of the microcapsule. In matrix diffusion controlled devices, a solid drug is dispersed in an insoluble polymer matrix and the rate of drug release is dependent on drug diffusion from the device. Matrix diffusion control can be achieved through the use of plastic, fatty or hydrophilic matrices.

Diffusion and Dissolution controlled system

In this system, the drug core is enclosed with a partially soluble membrane. As the membrane partially dissolves, diffusion of the contained drug through the pores in the polymer

coat occurs. The major factor controlling drug release is the fraction of soluble polymer in the coat. One such system provides zero order release of KCl from a tablet and thereby reduces the gastrointestinal irritating effects of this compound (Robinson et al., 1987).

Ion-exchange resins

Ion-exchange drug delivery is based on the principle that when high concentration of an appropriately charged ion is in contact with the drug carrying ion-exchange resin, the drug molecule is exchanged and diffuses out of the resin to the bulk solution. The resins consist of water-insoluble materials containing anionic or cationic groups in repeating position on their chain. The resin is loaded with the drug by mixing it with drug solution either in a column or by keeping the resin in contact with the drug solution for extended period of time. The resin particles are then washed to remove contaminant ions and the resins are dried to form particles or beads. The Pennkinetic system is an improved ion-exchange drug delivery system in which the drug containing resin granules are treated with an impregnating polymer such as PEG 4000 to retard the rate of swelling in water followed by coating with a water permeable polymer such as ethyl cellulose, which acts as a rate limiting barrier to control drug release.

pH-independent and dependent formulations

pH independent formulations are designed for the oral controlled release of basic or acidic drugs at a rate independent of the pH in the GI tract. Such formulations are prepared by mixing the drug with an appropriate buffering agent, granulating with excipients and finally coating with a permeable film former. The buffering agents adjust the fluid entering the

formulation to a suitable constant pH, thereby allowing a constant rate of drug release which is independent of the pH of the fluid entering.

pH dependent formulations are intended for those drugs which are acid labile, drugs which cause gastric irritation or drugs which are intended to be delivered for the colon. This is usually achieved by coating the dosage form (tablet or pellets) with an enteric polymer such as shellac, cellulose acetate phthalate, methacrylates\ and hydroxypropyl methylcellulose phthalate. These polymers have the common characteristic of being acid esters and are insoluble in gastric fluids upto a pH of 4. But these polymeric films lose their integrity in intestinal fluid, which has higher pH, and allow the release of the drug in the intestine or colon.

Osmotically Controlled Release

These systems use osmotic pressure as the driving force and are fabricated by applying a semipermeable membrane around a core of an osmotically active drug or a core of an osmotically inactive drug in combination with an osmotically active salt. The system possesses an optimized delivery orifice drilled by laser or by high speed mechanical drill. When exposed to water or any body fluid, an osmotic pressure develops across the coating membrane leading to the water flow into the core. Initially, the drug is pumped out of the device at a zero order release rate until the concentration of the osmotically active salt is below saturation solubility, after which a non-zero order release pattern takes place. But one major disadvantage of this system is the blockage of the delivery orifice which can lead to the failure of the device.

Several modifications of osmotic controlled release have been developed including an osmotic bursting device with no delivery orifice and the osmotic pressure controlled release with bioerodible polymer on the external surface of semipermeable membrane.

Altered-Density formulations/Gastroretentive system

This system is based on the principle of achieving long term oral controlled release by prolonging the residence time of drug delivery system in the GI tract. Several approaches are used to meet the above objective. The bioadhesion approach is based on the adherence of bioadhesive polymers to the mucin/epithelial surface of GI tract. Another approach is to modify the formulation's density by using high or low density pellets. High density pellets should have a density at least 1.4 times greater than that of normal stomach contents in order to prolong the residence time of delivery system in GI tract. This can be achieved by coating of the drug on a heavy core or mixed with heavy inert materials e.g. barium sulphate or titanium oxide. Low density formulations consist of an empty globular shell of density that is lower than gastric fluid. The final product floats on gastric fluid for a prolonged period, while slowly releasing drug. The application of buoyancy principle for formulating buoyant tablets or capsules by incorporating gas-filled flotation chambers into a microporous compartment that houses a drug reservoir.

Microspheres

Microparticulate drug delivery systems have been extensively studied for the past several decades owing to numerous advantages as compared to other delivery systems (Rathbone et al., 2003):

- The physicochemical characteristics of microparticulate systems can remain unaltered for long periods because of good physical stability. They can also protect the drug from enzymatic or pH –dependent degradation.
- Microparticulate drug delivery systems can be administered by several routes (oral, intramuscular, subcutaneous, etc) depending on their composition.

- Microparticulate drug delivery systems are industrially scalable.
- Microsphere-based formulations can be formulated to provide a constant drug concentration in the blood or to target drugs to specific cells or organs.

Microparticulate drug delivery system includes microparticles that are polymeric entities falling in the range of 1-1000 μm covering two types of forms (Benita et al., 1996):

Microcapsules (reservoir systems)

Microspheres (matrix systems)

In the present study, we would confine our discussion to matrix type of microparticles called microspheres. Microspheres are solid, spherical particles containing dispersed drug molecules either in solution or crystalline form and represents multi unit dosage forms. Microspheres are multi-particulate controlled drug delivery system having many advantages over single unit drug delivery system:

- Microspheres spread over large areas in the GI tract.
- Release rates of microspheres are less variable
- Microspheres are less dependent on their GI transit time.
- Microspheres cause less GI irritation
- Failure of one or a few microspheres has little effect on overall release properties (catastrophic failure less likely than with single unit doses).

Methods of preparation

Several microencapsulation methods have been developed for the preparation of microspheres. Such methods include (Deasy et al., 1984, Benita et al., 1996):

1. Solvent evaporation process (Emulsification-Evaporation)

2. Coacervation-phase separation method using aqueous or non-aqueous vehicles
3. Interfacial polymerization
4. Solvent Extraction method (Emulsification-Extraction)
5. Electrostatic method/ Spray drying methods (Nebulization)
6. Precipitation
7. Hot melt
8. Salting out

1. Emulsion-Solvent Evaporation process

The emulsion-solvent evaporation technique is based on the evaporation of the internal phase of an emulsion by movement of the globules to the surface by agitation and also by diffusion of solvent molecules into the external phase followed by evaporation of the solvent from the external phase at the surface. At the beginning, the polymer is dissolved in a volatile organic solvent. The active moiety to be encapsulated is then dispersed or dissolved in the organic solution to form a suspension, emulsion or a solution. Then the organic phase is emulsified by agitation in a dispersing phase consisting of a nonsolvent of the polymer that is immiscible with the organic solvent and contains an appropriate tensioactive additive. Once the emulsion is stabilized, agitation is maintained and the solvent evaporates after diffusing through the continuous phase, resulting in the formation of microspheres. Microspheres held in the continuous phase are recovered by filtration or centrifugation and washed and dried (Watts et al., 1990). Several systems have been investigated based on the nature of the external phase (aqueous or nonaqueous), the incorporation mode of the active principle in the organic solution

of the polymer (dissolved, dispersed or emulsified) and the elimination procedure of the organic solvent (evaporation or extraction). These include (Aftabrouchad et al., 1992):

Oil-in-Water emulsion: In this system, the polymer is dissolved in an organic solvent such as methylene chloride or chloroform and the drug is dissolved or dispersed in the same medium, and then the entire mixture is emulsified in an aqueous solution containing an appropriate surfactant. This method is advantageous in being economical and negates the recycling of external phase. However, the aqueous emulsion evaporation method leads to low microencapsulation of hydrophilic drugs due to partitioning of the drug in the external phase (Beck et al., 1979).

Multiple emulsions (Water-in-Oil-in-Water): In the multiple emulsion technique, drusg to be encapsulated are incorporated in an aqueous solution, which is poured into an organic solution of the polymer to form a water-in-oil (W/O) emulsion. The primary emulsion is itself emulsified in an external aqueous phase leading to a multiple emulsion of the type water-in-oil-in water (W/O/W). The organic phase acts as a barrier between the two aqueous compartments preventing the diffusion of the drug towards external aqueous phase (Bodmeier et al., 1991). This method yields high encapsulation efficiency of water soluble active moieties.

Nonaqueous emulsion solvent evaporation method (Jalil et al., 1990): The technique involves replacement of the aqueous continuous phase by an oily phase and is sometimes called an oil-in-oil emulsion. In this method the organic solution of the polymer with the active principle is prepared which is then dispersed into the external phase containing surfactant (mineral or vegetable oil or other liquid that is not a solvent for the polymer phase). Continuous agitation is used to form an emulsion and to promote evaporation of the organic solvent and the formation of solid microspheres. This system shows not only elevated microencapsulated yields for water

soluble components, but also prevents hydrolysis of the medicine or polymer. A number of disadvantages are related to the use of nonaqueous solvents in that they are expensive and need to be recycled. Moreover, trace residues are often difficult to eliminate, which pose technological problems.

2. Coacervation/Phase separation

Coacervation involves the formation of a polymer-rich separate phase that is called a coacervate. This coacervate phase can form a film around insoluble liquid or solid entities that can be congealed to form a capsule. When the coacervate is gelatin-based, the system can be cooled to cause the coacervate coating to gel. The gelled coacervate can then be crosslinked to form the capsule wall. Water insoluble drugs can be encapsulated with gelatin by this method. Gelatin coacervates can be induced by adding salts such as sodium sulfate to gelatin solutions or by adding an oppositely charged substance like acacia and adjusting the pH of the solution for optimum coacervate formation. Coacervates of some drug water-insoluble polymers can be induced by solvent change or by temperature change of a polymer solution. If an insoluble separate particulate phase is present, the coacervate can form a film around the particles that can be gelled or solidified to form microcapsules.

3. Interfacial polymerization

The interfacial polymerization method involves dispersing the organic phase containing drug into the aqueous phase containing monomers that react at liquid/liquid interface to form a capsule wall (Kondo et al., 1978). A crosslinking agent is incorporated to the continuous phase to cause interfacial polymerization.

4. Solvent extraction method (Emulsification-Extraction method)

In solvent extraction method, continuous phase is chosen in such a way that it immediately extracts the solvent(s) of the dispersed phase making the evaporation stage unnecessary. In this method large volumes of dispersing medium is used with respect to the dispersed phase or a dispersed phase consisting of cosolvents is chosen, of which at least one has great affinity for dispersion medium. One may formulate a dispersing phase with two solvents in which one acts as a solvent extractor of the dispersed phase (Singh et al., 1991).

5. Electrostatic method

The electrostatic method (Langer et al., 1964) is suitable in cases where the coating material and the drug to be encapsulated are both aerosols and oppositely charged. The drug and coating material are atomized resulting in the formation of microspheres. These are cooled and collected by a suitable aerosol collection system.

6. Precipitation process

The precipitation process involves precipitation or congealing of a preformed polymer around the drug being encapsulated and involves numerous techniques, for instance precipitation of ethylcellulose from cyclohexane by cooling, gelation of sodium alginate with aqueous calcium salt solutions and desolvation of water soluble polymers with water miscible solvents.

7. Hot melt method

Hot melt coatings are composed of relatively low molecular weight lipids that involves mechanical drop formation at an elevated temperature followed by cooling step. The hot melt

coatings have low melt viscosities at reasonable operating temperatures and can be readily sprayed. This method is usually used in cases where thermally stable drugs are to be encapsulated.

8. Salting out method

In salting out method salt is added to an aqueous polymer solution thereby separating the polymer from the solution. The drawback of this method is that there is possibility of incorporating a relatively high concentration of salt into the capsule wall.

Reported factors affecting properties of microspheres made by emulsion-solvent evaporation

The emulsion-solvent evaporation method has been extensively used for the formulation of microspheres. Several polymers have been used for this purpose including cellulose acetate butyrate, polylactide, polylactide-co-glycolide and ethyl cellulose. The properties of these microspheres have been reported to be greatly affected by formulation and process variables such as stirring speed, drug solubility, drug-polymer ratio, solvent type, temperature, polymer molecular weight, polymer viscosity, polymer concentration and emulsifier concentration (Hincal et al., 2000)

Factors not studied/reported

During microsphere formation by the non-aqueous emulsion solvent evaporation process, the polymer is dissolved in a suitable organic solvent and the drug is either dissolved or dispersed in this solution. This constitutes the internal phase and is emulsified in a non-aqueous

external phase, usually mineral oil, under vigorous stirring. A low HLB surfactant is often added to the external phase to stabilize the emulsion.

When the internal phase (polymer solution in organic solvent with dissolved or dispersed drug) is added to the external phase, due to the stirring the internal phase is sheared into small globules by the stirring to form an emulsion. As the organic solvent gradually partitions into the mineral oil and evaporates off, the polymer solution viscosity increases and eventually gels and hardens. This process continues till all the solvent has evaporated leaving behind the rigid polymer microspheres. Formation of a stable emulsion is a critical step in the formation of microspheres by this method. This is because it is important for the polymer solution globules to remain stable and not coalesce while the solvent is evaporating in order to get discrete, individual microspheres.

Several papers have reported the use of a single low HLB surfactant during this process (Roy et al.,1989, Wu et al., 2003). Although the use of two surfactants has been reported for making buoyany theophyline microspheres, the authors could not find any reports on the use of surfactant combinations at different RHLBs for stabilizing the emulsion during microsphere formation by the non-aqueous emulsion solvent evaporation process (Stithit et al., 1998). Dual surfactant systems, obtained by combing a high HLB and a low HLB surfactant, are commonly used for stabilizing pharmaceutical emulsions containing an aqueous phase, as explained in the following sections. Hence it would be interesting to investigate the effect of using such combinations on physical properties and release characteristics of microspheres prepared by the non-aqueous emulsion solvent evaporation process.

Role of surfactants in emulsification

Surface-active agents or surfactants are molecules that are adsorbed at interfaces and are regarded as amphiphile as they show affinity for both polar and non- polar solvents (Martin et al., 1995). Depending on the number and nature of the polar and non-polar groups present, the surfactants may be hydrophilic or lipophilic or reasonably balanced between the two extremes. Surfactant molecules consists of hydrophilic heads and lipophilic tails that cause them to get adsorbed and oriented at the interface in such a way that the hydrophilic heads are located in the more polar phase and the lipophilic tails in the less polar phase (oil, non-polar solvents or air) making them excellent emulsion stabilizers. Surface active agents form a monolayer of adsorbed molecules at the oil/water interface. The presence of these interfacial molecules reduces the interfacial energy resulting in a more stable emulsion. Moreover, the emulsion stability is further enhanced as the droplets are surrounded now by coherent monolayer that reduces coalescence between approaching droplets (Lachman et al., 1991).

Type of surfactant

Surfactant molecules may or may not possess a charge. Depending on the charge present on the surfactants, they are classified as (Nielloud et al., 2000):

1. Anionic surfactants
2. Cationic surfactants
3. Non-ionic surfactants

Anionic surfactants

In this group, the surfactants bear a negative charge on the hydrophilic part. Anion surfactants may be with carboxylate ions, or with sulfate ions or with sulfonate group (Remington et al., 1995). The most frequently allied cations are sodium, calcium, magnesium

and zinc, with multivalent ions often creating marked water insolubility. The straight chain is a saturated or unsaturated C12-C18 aliphatic group. Anionic surfactants may be irritating depending on the chemical class and concentration. Anionic surfactants are used in pharmaceutical preparations as solubilizers, wetting agents, emulsifiers, suspending agents or as inert tablet lubricants. Examples of anionic surfactants are sodium, potassium, ammonium, aluminum, calcium or zinc salts of stearates and oleates, sodium lauryl sulfate and sodium docusate (dioctyl sodium sulfosuccinate). The monovalent salts of stearates and oleates have a high affinity for water, wheras the di- and trivalent salts are practically insoluble in water and have more affinity for non-polar phases.

Cationic surfactants

The cationic surfactants bear a positive charge and are absorbed strongly by negatively charged products such as hair, skin and microorganisms. This type of surfactant is important pharmaceutically because of their bacteriocidal properties but may be irritating to the eyes or skin. Some examples of cationic surfactants are trimethyltetradecylammonium bromide (Cetrimide), Cetrimonium bromide, Benzalkonium chloride, etc. Owing to the presence of quaternary ammonium groups, cation surfactants show incompatibility with soaps, many anionic compounds and certain inert polymers such as polyacrylate and carboxymethyl cellulose.

Nonionic surfactants

Nonionic surfactants do not bear any charge on the molecule. They have the advantages of being less irritating than anionic or cationic surfactants; show compatibility with other types of surfactants; have excellent ability to solubilize poorly soluble drugs and have lower toxicity than other classes of surfactants. But they diminish the antimicrobial activity of some preservatives (Reynolds et al., 1996). The properties of nonionic surfactants depend on the

proportions of hydrophilic or hydrophobic groups in the molecule. The hydrophilic portion typically consists of the polyoxyethylene, polyoxypropylene, or polyol derivatives and hydroxyl groups. The hydrophobic part usually consists of saturated or unsaturated fatty acids or fatty alcohols. A wide range of hydrophilic-lipophilic balance (HLB) values can be obtained by varying the number of hydrophilic groups or the length of lipophilic chain. Griffin invented a scale in order to classify nonionic surfactants and to select mixtures for emulsification of particular oils (Attwood et al., 1983). According to this classification, lipophilic surfactants with HLB values greater than zero and less than ten (i.e. $0<HLB<10$) can be used as antifoaming agents, water-in-oil emulsifiers or wetting agents. On the other hand, hydrophilic surfactants with HLB values lying between ten and twenty (i.e. $10<HLB<20$) are generally oil-in-water emulsifiers or soulubilizing agents. Due to their conditions of their fabrication, the nonionic surfactants are usually mixtures of associated substances, so there are variations in properties between different manufacturers.

The principal groups used in this class are polyol derivatives, polyoxyethylene esters and ethers and polaxamers. Amongst the different classes of nonionic surfactants, polyol derivatives are most frequently used and include the groups: glyceryl esters and sorbitan esters. Glyceryl esters, owing to their good stabilizing properties, are used as stabilizers both in water-in-oil and oil-in-water emulsions. Sorbitan esters are esters of cyclic anhydrides of sorbitol with fatty acid (C12-C18). They are lipophilic surfactants used as water-in-oil emulsifiers. Examples of sorbitan fatty esters are sorbitan monolaurate (Span 20), sorbitan mono palmitate (Span 40), sorbitan monostearate (Span 60), sorbitan monooleate (Span 80) and sesquioleate (Span 65), trioleate (Span 85). When sorbitan esters are polyoxyethylened they are called polysorbates or Tweens. A large range of properties may be obtained by varying the umber of oxyethylene groups in the

molecule and are have mainly oil-in-water emulsifying and solubilizing properties. Examples of polysorbates are polysorbate 20, 60 and 80.

Hydrophilic Lipophilic Balance (HLB) system

Griffin (Martin, 1995) devised an arbitary scale of values to serve as measure of the hydrophilic-lipophilic balance (HLB) of surface-active agents. HLB scale is numerical scale extending from 1 to approximately 50. The more hydrophilic surfactants have high HLB numbers (in excess of 10), while surfactants with HLB numbers from 1 to 10 are considered to be lipophilic. Surfactants with a proper balance in their hydrophilic and lipophilic affinities are effective emulsifying agents since they concentrate at the oil/water interface. The relationship between HLB values range and surfactant application is given below:

HLB range	**Use**
0-3	Antifoaming agent
4-6	W/O emulsifying agent
7-9	Wetting agent
8-18	O/W emulsifying agent
13-15	Detergents
10-18	Solubilizing agents

Griffin defined the HLB of a nonionic surfactant, whose only portion is polyoxyethylene, is calculated using the formula:

HLB = E/5 where E is the percent by weight of ethylene oxide.

A number of polyhydric alcohol fatty acid esters such as glyceryl monostearate, may be estimated by using the formula:

HLB = 20(1-S/A) in which S is the saponification number of the ester and A is the acid number of the fatty acid.

Griffin evolved a series of "required HLB" (RHLB) values; i.e. the HLB value required by particular material if it is to be effectively emulsified; that is to effectively disperse and stabilize in the external phase. The required HLB value differs depending on whether the final emulsion is O/W or W/O. The HLB values are algebraically additive and by using a low HLB surfactant with one having a high HLB it is possible to prepare blends having HLB values intermediate between those of the individual emulsifiers.

Theories of emulsification

When two immiscible substances are mixed together, they show a tendency to separate (i.e. emulsified droplets tend to coalesce) on standing to produce minimum possible surface area of contact between the phases. The cohesive forces between the molecules of each separate liquid are greater than the adhesive force between the two liquids. The cohesive force of the individual phases is manifested as an interfacial energy or tension at the boundary between the liquids. When a liquid is broken into small particles, the interfacial area of the globules constitutes a surface that is enormous compared with the surface area of the original liquid. The increase in surface free energy is sufficient to make the system thermodynamically unstable, hence the droplets have the tendency to coalesce.

The process of coalescence can be reduced to insignificant levels by the addition of the third component-the emulsifying agent or emulsifier. The emulsifying agent increases the physical stability of emulsion by showing the following properties:

Reduction in interfacial tension: The emulsifying agent is surface active in nature and reduces the interfacial tension between the two immiscible liquids leading to the corresponding decrease in surface free energy which helps to maintain the surface area generated during the dispersion process and therefore makes the emulsion system thermodynamically stable.

Film formation: The potential characteristic of an emulsifier is that it is adsorbed quickly around the dispersed phase droplets to form a film that prevents coalescence. The main purpose of the film –which may be monolayer, a multilayer or a collection of small particles adsorbed at the interface- is to form a barrier that prevents the coalescence of droplets that come into contact with one another. For the film to be an efficient barrier, it should have some degree of elasticity and should not be thinned out or rupture when sandwiched between the two droplets. If broken, the film should have the capacity to reform rapidly. Some of the theories related to stability of emulsion are:

1. Monomolecular adsorption-Surfactants are adsorbed at oil-water interfaces and form monomolecular films that reduce interfacial tension resulting in a stable emulsion. Besides reducing interfacial tension, surfactants increase emulsion stability by surrounding the droplets by coherent monolayer that prevents coalescence between approaching droplets. Also, a surface charge on surfactants increases system stability. If the surfactant forming the monolayer is ionized, the presence of mutually charged and repelling droplets further increase the emulsion stability. Even with the nonionic surfactants, particles still carry charge from adsorption of specific ion or ions from the solution. The type of emulsion that is

produced i.e. oil-in-water or water-in-oil depends on the hydrophilic-lipophilic balance or polar-nonpolar nature of emulsifier. According to rule of Bancroft (Martin, 1995), the type of emulsion is a function of the relative solubility of the surfactant, the phase in which it is more soluble being the continuous phase. Therefore, an emulsifier with high HLB is preferentially soluble in water and forms oil-in-water emulsion and likewise emulsifier with low HLB forms water-in-oil emulsion. Boyd (Boyd et. al., 1972) has reported that blends of high and low HLB emulsifiers further improve emulsion stability by interacting at the interface to form more stable films than single HLB emulsifiers.

2. Multimolecular adsorption- Hydrated lipophilic colloids form multimolecular films around droplets of dispersed phase. Though these agents are adsorbed at an interface, the amount of lowering of surface tension is not enough by itself to stabilize the emulsion. However, they form strong coherent multimolecular films in contrast to the monomolecular films formed by small molecule surfactants. The strong multimolecular films act as a coating around the droplets and render them highly resistant to coalescence even in absence of high surface potential. Hydrocolloids not adsorbed at the interface enhance the emulsion stability by increasing the viscosity of the continuous aqueous phase. Since the emulsifying agents that form multilayer films around low polarity droplets in aqueous emulsions are hydrophilic, they promote the formation of oil-in-water emulsions.
3. Solid Particle adsorption- Finely divided solid particles that are wetted to some degree by both oil and water act as emulsifiers by being concentrated at the interface and produce a particulate film around the dispersed droplets therefore preventing coalescence. Powders that are preferentially wetted by water form oil-in-water emulsions, whereas those more easily wetted by oil form water-in-oil emulsion.

Electric potential: Some emulsifying agents promote emulsion stability by imparting an electric potential to droplets of the two immiscible liquids in the emulsion to generate repulsion between the approaching drops.

Viscosity: Emulsifiers that add sufficient viscosity to the continuous phase of the emulsion, help preventing creaming and coalescence and thus enhance emulsion stability.

The objectives of the present study were:

1. To prepare microspheres using a dual surfactant system and compare their properties with microspheres prepared with a single surfactant.
2. To investigate the role of RHLB on physical properties and drug release properties of ethyl cellulose microspheres.
3. To investigate the role of surfactant molecular type on the physical properties and drug release properties of ethyl cellulose microspheres.

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Chapter 5

Effect of dual surfactant systems on physical and drug release properties of ethyl cellulose microspheres prepared by a non-aqueous emulsion solvent evaporation method[1]

[1] R. Sarkar and J. C. Price. To be submitted to Journal of Microencapsulation

Abstract: Non-ionic surfactants are assigned an HLB value depending on the balance between the hydrophilic and lipophilic parts of its molecule. The objective of the present study was to investigate the influence of using different combinations of low HLB and high HLB surfactants (to obtain intermediate HLB numbers, or RHLB numbers) on the physical properties as well as the drug release of ethyl cellulose microspheres. Previous studies with the ethyl cellulose-acetone-mineral oil system have reported the use of single low HLB surfactants for microsphere formation by the non-aqueous emulsion solvent evaporation method. The authors did not find any reports that studied surfactant combinations as formulation factors for microspheres prepared by this technique. The influence of RHLB on microsphere properties like yield, particle size distribution, geometric mean diameters, initial drug release, drug release characteristics and drug release mechanism was studied. Different batches of microspheres were made at different RHLBs by combining two low HLB surfactants (Span 80 and Span 85) and two high HLB surfactants (Tween 61 and Brij 30) in different ratios. The microspheres were then evaluated for particle size distribution and geometric mean diameter, drug loading, drug dissolution characteristics, initial drug release and drug release mechanism. The geometric mean diameter of the batches decreased with an increase in RHLB. The dissolution rate and initial drug release in the microsphere batches increased with an increase in RHLB.

Introduction

Drugs such as theophylline, caffeine and salicylic acid are loaded into microspheres by the non-aqueous emulsion solvent evaporation technique because they cannot be efficiently loaded by the o/w emulsion method due to some amount of the drug being lost to the external phase. The resultant matrix microspheres that are formed are rugged in nature, making them suitable for tableting without damaging their controlled release properties (Sayed and Price, 1986). Production of microspheres that meet a specific therapeutic objective requires the optimization of formulation and processing factors. In order to do so, it is necessary to identify those variables that critically affect microsphere production and properties.

Several factors have been identified that affect the formulation and properties of matrix microspheres. The critical formulation factors include the type and molecular weight of the polymer, core drug particle size, drug:polymer ratio and solubility of the drug in the polymer while the critical processing factors for matrix microspheres prepared by emulsion solvent evaporation method include the phase ratios, the mixing intensity, the temperature during processing and the polymer phase viscosity (Suzuki and Price, 1985, Sprockel and Price, 1990, Hariharan and Price, 2002, Shukla and Price, 1989 and Obeidat and Price, 2003).

Griffin proposed the HLB scale as a measure of the hydrophile-lipophile balance (HLB) of a surfactant (Martin, 1993). The higher the HLB value of a surfactant, the more hydrophilic it is. A low HLB and a high HLB surfactant can be blended together in order to stabilize an emulsion at a particular HLB (called the RHLB). Previous studies dealing with the production of ethyl cellulose microspheres by non-aqueous emulsion solvent evaporation method have reported the use of a single, hydrophobic, low HLB surfactant like Span 80, Span 85, aluminum stearate, magnesium stearate or Synperonic L-61 for stabilizing the emulsion (Roy et al., 1989, Lin and

Wu, 1999, Zinutti et al., 1994, Wu et al., 2003, Palomo et al., 1996). In this paper, we investigate the effect of using combinations of low and high HLB surfactants and the role of RHLB on the physical and drug release properties of ethyl cellulose microspheres prepared by this method.

Experimental

Materials

Ethyl cellulose (100cps, 49% ethoxyl content, Scientific Polymer Products, New York), micronized theophylline (Knoll AG), light mineral oil, Span 85, Tween 61, Span 80, Brij 30 and Tween 80 (Ruger Chemical Company Inc., Irvington, NJ), methylene chloride (Fisher Scientific, NJ), acetone, monobasic potassium phosphate and sodium hydroxide (J.T. Baker, Phillipsburg, NJ).

Instruments

Stirrer (Lab. Stirrer LR 400D, Yamato Scientific Company Ltd., Tokyo, Japan), USP Dissolution Apparatus II (Dissolution Test system 5100, Distek Inc., North Brunswick, NJ), Aquamate (UV Spectrophotometer, Thermo Electron Corporation, Mercer's Row, Cambridge, UK), Accumet 5 pH meter (Fisher Scientific, NJ and, standard sieve series.

Preparation of microspheres

Different batches of ethyl cellulose microspheres containing anhydrous micronized theophylline were prepared by the non-aqueous emulsion-solvent evaporation method under identical conditions. Preliminary experiments were done to optimize the polymer concentration, drug concentration, stirring speed, ratio of internal to external phase and total surfactant

concentration; these were kept constant for all the batches. The only variable was the ratio of the low HLB surfactant and the high HLB surfactant used for each batch (Table 5.1). Microsphere batches were prepared using the single low HLB surfactants alone (Batch 31-Span 85 and Batch 59-Span 80). Batches were also prepared at different RHLBs from 4.5 to 7.5 by combining Span 85 and Tween 61 (Batches 34 to 37) and Span 80 and Brij 30 (Batches 60 to 63). Microsphere batches for both combinations were prepared over same RHLB range (4.5 to 7.5) in order to compare results.

The polar internal phase consisted of a 5% solution of ethyl cellulose in acetone also containing the higher HLB surfactant. Micronized theophylline was then dispersed in this solution to give a theoretical drug loading of 33%. This slurry was added to light mineral oil containing the low HLB surfactant at room temperature under constant stirring at 2000 r.p.m. The stirring was continued for 16 hours to enable complete evaporation of the acetone and formation of microspheres. The microspheres were separated from the oil phase by decantation, washed extensively with mineral spirits to ensure complete removal of oil on the surface of the microspheres and dried at 50°C overnight.

Particle size distribution

The particle size distribution of the microsphere batches were studied by sieve analysis using a set of standard sieves of range 90-710μm. The samples were placed on the topmost sieve and tapped by hand till no change in weight was observed in the sieves. The weight of microspheres retained on each sieve was recorded. The geometric mean diameter (d'g) and the geometric standard deviation were determined for each batch. The 355μm fraction of each batch

was deemed to be representative and the following tests were carried out on this fraction of each batch.

Drug loading

The actual drug loading of the 355µm fraction of each batch of microspheres was determined by taking accurately weighed samples (in triplicate) in 10ml volumetric flasks and dissolving in methylene chloride. Drug concentration was then determined by taking absorbance at 276.5nm. Ethyl cellulose did not cause any spectrophotometric interference at this wavelength.

In vitro dissolution

The release of theophylline from the 355µm fraction of each batch of microspheres was evaluated using the USP Dissolution Apparatus II. Microsphere samples (triplicate for each batch) were suspended in 900 ml of dissolution medium (Simulated Intestinal Fluid with 0.2% Tween, no enzymes) and dissolution carried out at 37±0.5°C at 100 r.p.m for 12-24 hours. Three ml of sample was withdrawn at specific time intervals and replaced with fresh medium. The drug released was determined spectrophotometrically at 272 nm. The dissolution data was evaluated for initial release (dose dumping), dissolution rate and the drug release mechanism.

Results and discussion

Effect of surfactant combinations and RHLB on particle size distribution and geometric mean diameter

Figure 5.2 shows the effect of using a combination of Span 85 and Tween 61 at different RHLBs on microsphere size distribution compared to a single surfactant. The batch prepared with Span 85 alone at HLB of 1.8 (Batch 31) showed a bimodal distribution with a high percentage of the 600µm (43.6%) and 355µm (46.4%) fractions. When viewed under a light

microscope, the 600μm fraction from the batch made with Span 85 appeared to be clumps of microspheres (not shown). The microspheres were observed to be fused together and were hard to break even when considerable pressure was applied with a spatula. The 355μm fraction appeared to be predominantly individual, discrete microspheres with few fused ones.

In the non-aqueous emulsion solvent evaporation method for making microspheres, the polar organic phase is emulsified in the non-polar mineral oil phase. The internal phase is a solution of the polymer in a volatile organic solvent and as the solvent evaporates, the polymer solidifies to form the microspheres. The presence of fused microspheres could be because of the fact that the single surfactant, Span 85 (HLB=1.8) does not form a sufficiently strong interfacial film during the emulsification of the polar internal phase in the non-polar mineral oil phase. As a result of this, the droplets of the internal phase (ethyl cellulose solution in acetone with dispersed theophylline) coalesced while acetone diffused into the mineral oil prior to evaporation, resulting in fused microspheres.

When Span 85 was combined with Tween 61 (HLB = 9.6) to give RHLBs from 4.5-7.5, the percentage of 600μm fraction decreased and the percentage of the 355, 250 and 150μm fractions increased. This effect is more pronounced as the RHLB increases from 4.5 to 7.5. The two surfactants in combination appeared to stabilize the emulsion by forming a stronger interfacial film and this effect becomes more pronounced as RHLB increases. This results in a retardation of coalescence of the internal phase and therefore we get microspheres with smaller geometric mean diameters with increasing RHLB (Figure 5.1).

A similar trend is shown when Span 80 is combined with Brij 30 (HLB = 9.7) (Figures 5.1 and 5.3). Span 80 alone (HLB=4.3) shows a bimodal distribution with a high percentage of the 600μm (32.6%) and 355μm (48.7%) fractions. When Brij 30 is combined with Span 80 at

RHLB of 4.5, there doesn't appear to be much change in the percent of the 600μm fractions geometric mean diameter of this batch (540μm) compared to the batch with Span 80 alone (520μm). This is because the HLB of Span 80 is 4.3, which is quite close to the first combination of Span 80 and Brij 30 at RHLB of 4.5. But with a further increase in RHLB, the percentage of the 600 μm fraction decreases and 355μm fraction increases indicating that with an increase in RHLB, stabilization of the emulsion occurs and coalescence is retarded, leading to smaller microsphere particles. The retardation of coalescence by appropriate combinations of low and high HLB surfactants is described and explained by Boyd (Boyd et. al., 1972) as due to the interaction of two different surfactants to form a more stable film around the globules.

Effect of surfactant combinations and RHLB on drug loading

Table 5.2 shows the drug content in the 355 μm fraction of the microsphere batches with a theoretical loading of 33.3% (1 part theophylline to 2 parts ethyl cellulose). The drug content of batches prepared with surfactant combinations showed no particular relation with the RHLB. It was however noticed that the drug content of batches 59, 60 and 63 were higher than the theoretical loading. This could be due to the fact that some microspheres encapsulated air instead of the drug and floated to the top and were lost during decantation (polymer loss). The loss of these microspheres increased the drug to polymer ratio resulting in higher than theoretical drug loading.

Effect of surfactant combinations and RHLB on dissolution characteristics

Figure 5.4 describes the dissolution characteristics of the microsphere batches prepared with Span 85 and Tween 61. Figure 5.6 describes the change in the dissolution rate with a change in RHLB. Dissolution rate was characterized by determining the $t_{50\%}$, which was defined as the time taken for 50% of the drug to be released from the microspheres. As can be seen, the

dissolution rate of the batch prepared with Span 85 alone is slowest and increases with an increase in RHLB from 4.5 to 7.5.

Figure 5.5 describes the dissolution of microsphere batches prepared with Span 80 and Brij 30 while the change in $t_{50\%}$,with increase in RHLB is described in Figure 5.6. As was observed previously, the batch made with the single hydrophobic surfactant, Span 80, had the slowest release and when combined with the hydrophilic surfactant Brij 30, the dissolution rate increased.

When the low HLB surfactant is combined with a high HLB, hydrophilic surfactant like Tween 61 or Brij 30, some of the hydrophilic surfactant may dissolve in the polar polymer solution phase and become trapped in the microspheres. Therefore the hydrophilic surfactant will dissolve in the dissolution medium and facilitate medium entry into the system. With an increase in RHLB, the amount of hydrophilic surfactant in the microsphere matrix also increases and therefore the dissolution rate increases with an increase in RHLB. Another factor which may contribute to the faster drug dissolution is that the surfactant may act as a solubilizer for the drug.

A possible reason for the slower dissolution rate of the microspheres made with the single hydrophobic surfactant, that is, with Span 85 alone or Span 80 alone, could be because some of this surfactant collects at the interface of the polar phase droplets and the external mineral oil phase. Even though the microspheres were thoroughly washed with mineral spirits to remove surface mineral oil, some of the hydrophobic surfactant may still remain in the microsphere and during dissolution testing and may impede the entry of the dissolution medium into the microspheres resulting in slow dissolution from these microspheres. It was also observed that the batches prepared with Span 80 and Brij 30 had release profile more suited for sustained drug delivery compared to batches prepared with Span 85 and Tween 61. The $t_{50\%}$ of the former

batches ranged from 2-6.7 hours while for the latter batches, it ranged from 0.2-1.4 hours making them unsuitable for practical applications. Tween 61 and Brij 30 have similar HLBs (9.6 and 9.7 respectively) while Span 80 and Span 85 have different HLBs (4.3 and 1.8 respectively). This seems to indicate that the difference in the drug release from microspheres prepared by the 2 different combinations could be because of the difference between Span 80 and Span 85 rather that Tween 61 and Brij 30.

Span 80, with a HLB of 4.3 is better able to partition at the interface of acetone/mineral oil compared to Span 85. Span 85 is extremely is hydrophobic with an HLB of 1.8 and may not be able to partition and collect at the interface as well as Span 80. Therefore, in batches made with Span 80, the interfacial film is stronger and the acetone is able to diffuse into the mineral oil and evaporate in a more controlled manner compared to batches with Span 85. This allows the drug to be located more towards the core of the microsphere than towards the surface resulting in prolonged dissolution and longer $t_{50\%.}$ for the batches made with Span 80.

Effect of surfactant combinations and RHLB on dose dumping

The dose dumping of the 355μm fraction of each batch of microspheres was evaluated by determining the percentage of drug released in the first 30 minutes of dissolution and the data is summarized in Table 5.3. For reasons explained above, batches made with Span 80 have more of the drug concentrated towards the core and less on the surface resulting in less dose dumping compared to batches made with Span 85.

Also, the batches made with a single surfactant show lower dose dumping possibly because of the hydrophobic surfactant trapped in the microsphere matrix which may impede the dissolution of the drug. With an increase in RHLB, the hydrophilic surfactant proportion

increases and this does not hinder the surface drug dissolution and in fact may assist drug dissolution by solubilizing effect.

Effect of surfactant combinations and RHLB on drug release mechanism

The dissolution data for all batches were fitted to the Higuchi equation for spherical matrices (Figures 5.7 and 5.8) and the regressed values are shown in Table 5.4**.** The data indicates that the drug release from the microspheres was primarily by diffusion.

Conclusions

From this study, it can be concluded that use of a combination of high and low HLB surfactant influences the physical properties as well as the drug release properties of microspheres prepared by non-aqueous emulsion solvent evaporation method. With an increase in RHLB the geometric mean diameter of the microsphere batches decrease. Since the proportion of the high HLB surfactant increases as the RHLB is increased, some of this surfactant may get trapped in the microsphere matrix which results in a high initial drug release and faster drug dissolution from these batches.

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Table 5.1: Batches of microspheres prepared using 2 different surfactant combinations

Surfactants used	Batch #	RHLB	Ratio of surfactants
Span 85 (HLB=1.8) + Tween 61 (HLB=9.6)	31	1.8	100
	32	2.5	91:9
	33	3.5	78:22
	34	4.5	65:35
	35	5.5	53:47
	36	6.5	40:60
	37	7.5	27:73
Span 80 (HLB=4.3) + Brij 30 (HLB=9.7)	59	4.3	100
	60	4.5	96:4
	61	5.5	78:22
	62	6.5	59:41
	63	7.5	40:60

Table 5.2: Actual Drug loading (in percent) of 355μm fraction microspheres made from two different combinations of surfactants (Theoretical loading is 33.3%)

RHLB	Span 85 + Tween 61	Span 80 + Brij 30
4.5	29.2 ± 0.3 (#34)	33.7±1.7 (#60)
5.5	32.1 ± 1.7 (#35)	28.9±1.0 (#61)
6.5	28.5 ± 1.1 (#36)	30.1±1.3 (#62)
7.5	27.5 ± 1.6(#37)	33.3±1.7 (#63)
1.8 (Span 85)	33.1 ± 1.5 (#31)	
4.3 (Span 80)	37.2 ± 1.7 (#59)	

Table 5.3: Initial drug release (defined as % drug released in the first 30 minutes of dissolution studies) **by the 355μm fraction of the microspheres prepared by the two different surfactant combinations**

RHLB	(Span 85 + Tween 61)	Span 80 + Brij 30
4.5	34.2	18.2
5.5	35.2	16.7
6.5	49.1	21.9
7.5	37.2	31.7
1.8 (Span 85)	31.6	
4.3 (Span 80)	17.0	

Table 5.4: Regression analysis of the dissolution data of the 355µm batches of microspheres by fitting to the Higuchi equation for spherical matrices

RHLB	R^2 (Span 85 + Tween 61)	R^2 (Span 80 + Brij 30)
4.5	0.9958	0.9997
5.5	0.9307	0.9977
6.5	0.9626	0.9972
7.5	0.8971	0.9801
1.8 (Span 85)	0.9700	
4.3 (Span 80)	0.9990	

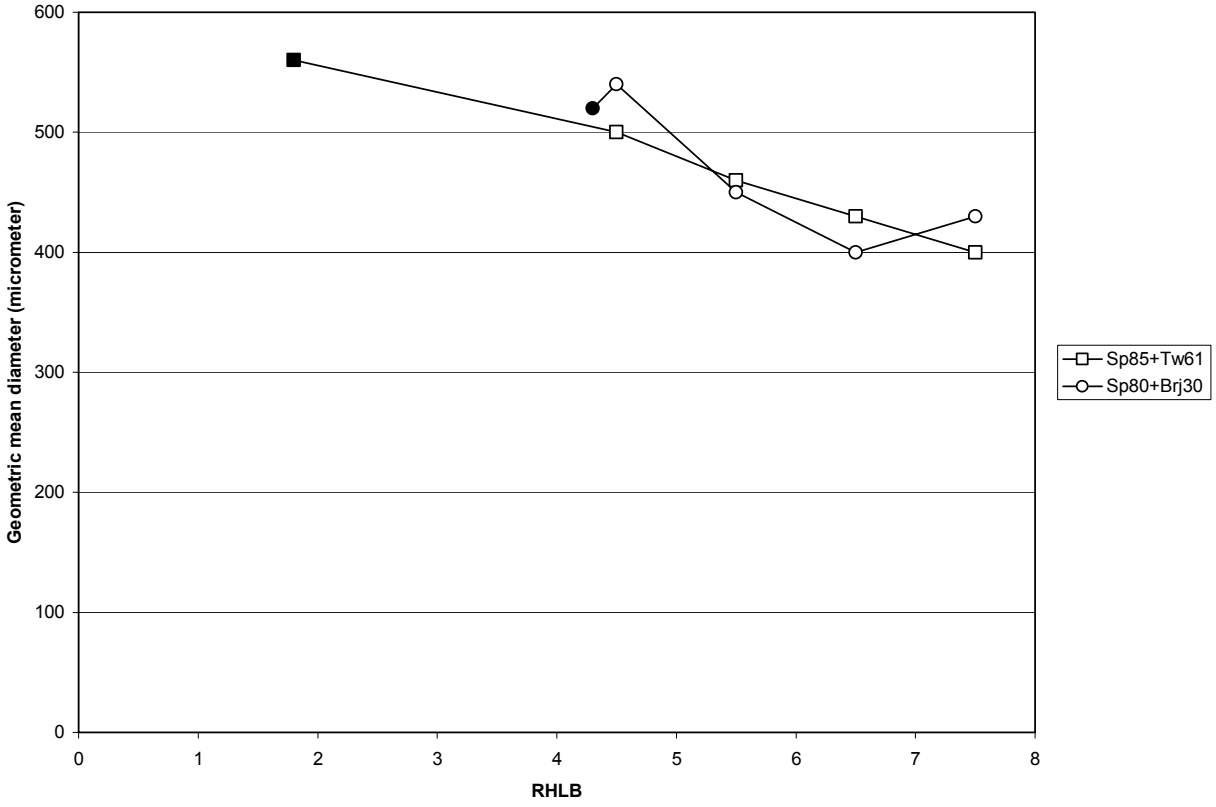

Figure 5.1: Change in geometric mean diameter (d'g) of microsphere batches with an increase in RHLB (The dark data points indicate the batches made with single low HLB surfactant while the light data points indicate batches made with surfactant combinations).

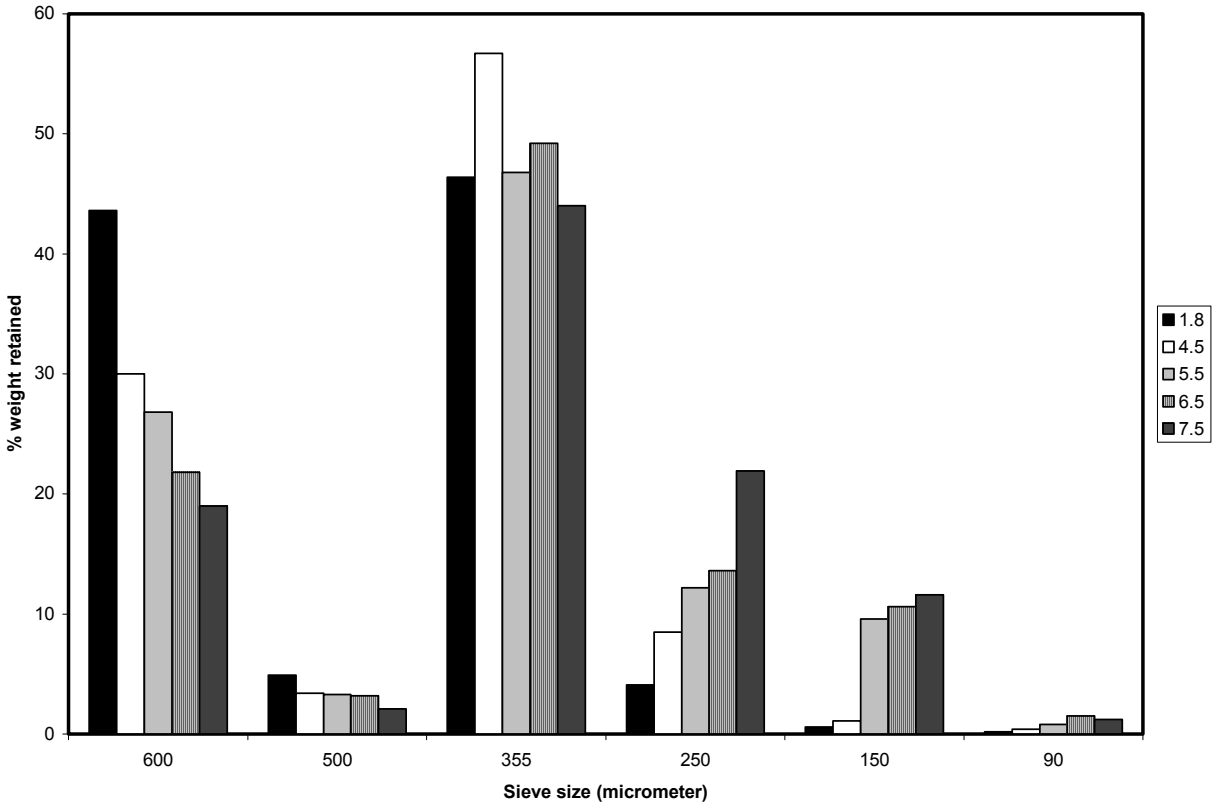

Figure 5.2: Particle size distribution of microsphere batches prepared with Span 85 + Tween 61 at five different RHLB values

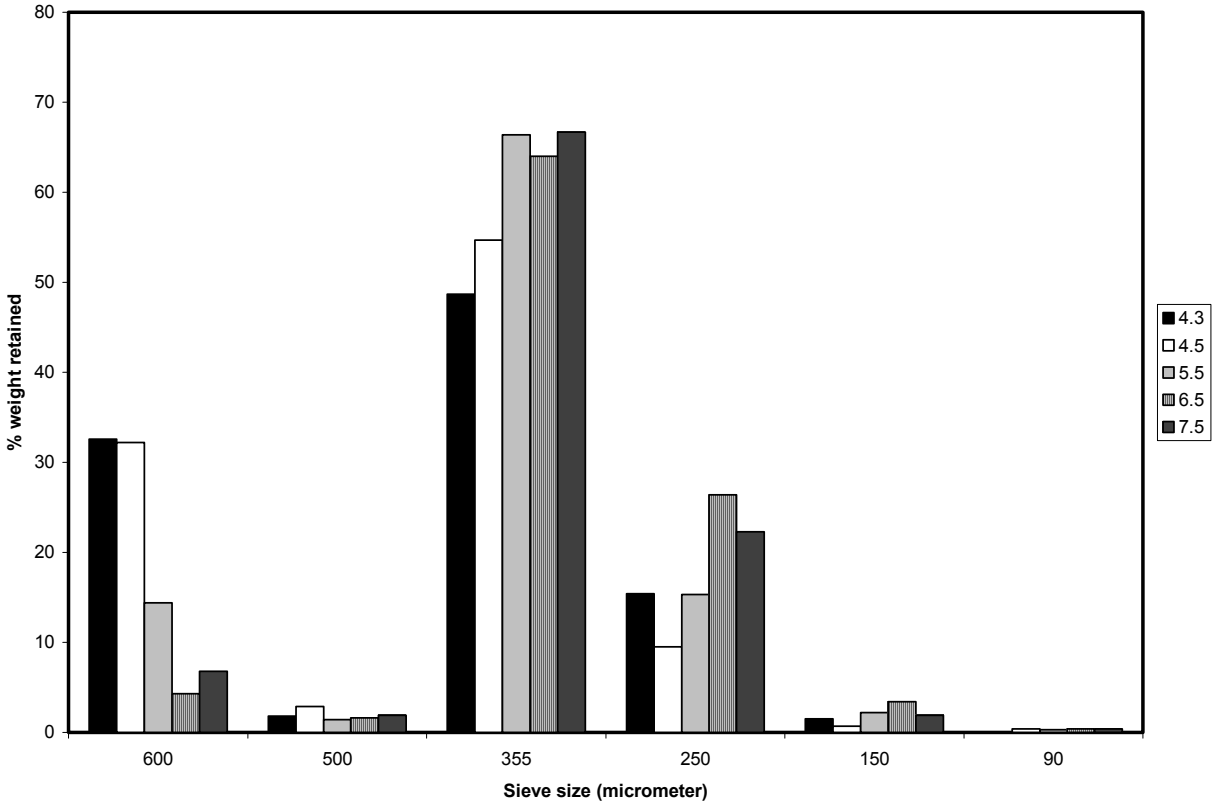

Figure 5.3: Particle size distribution of microsphere batches prepared with Span 80 + Brij 30 at five different RHLB values

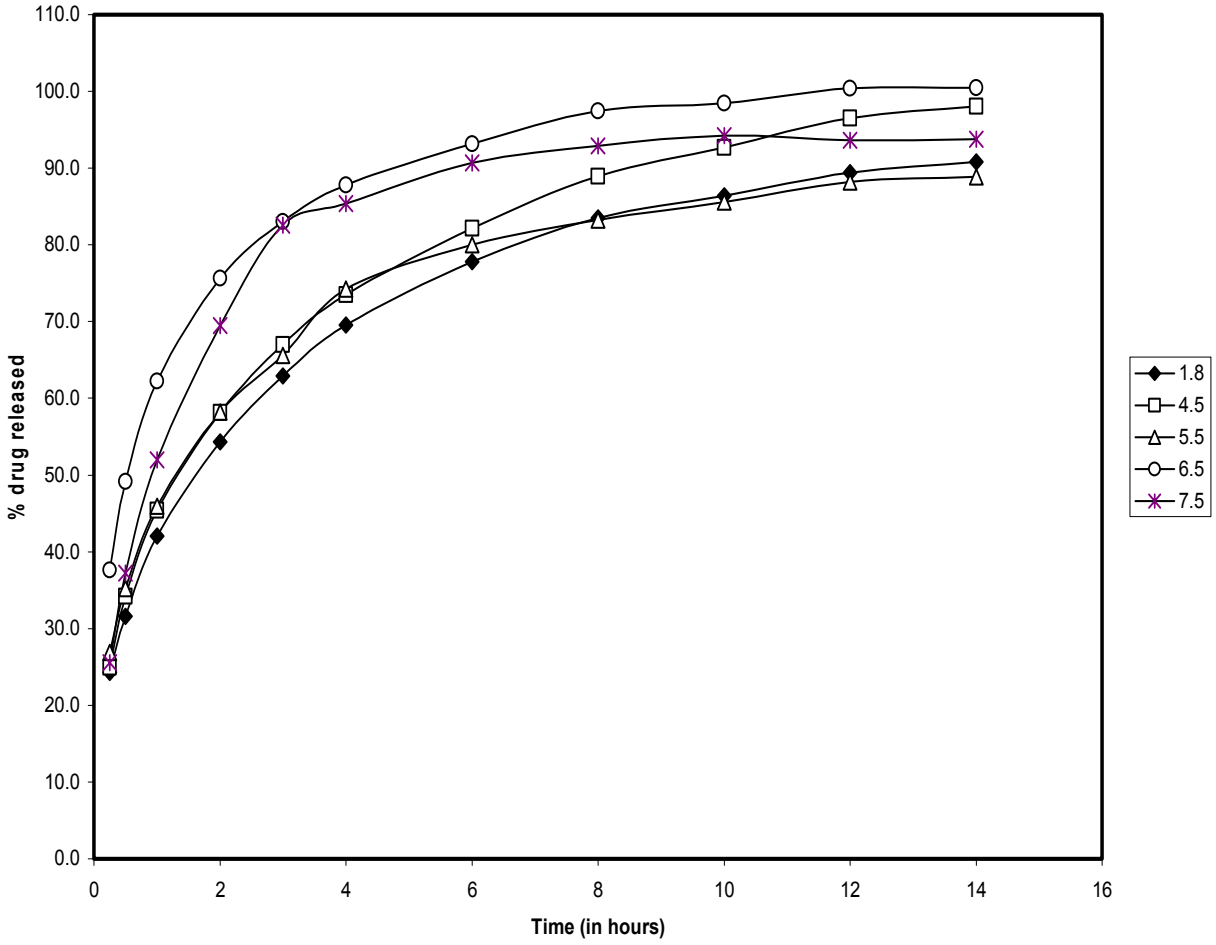

Figure 5.4: Dissolution profiles of microsphere batches prepared with Span 85 + Tween 61 at different RHLB

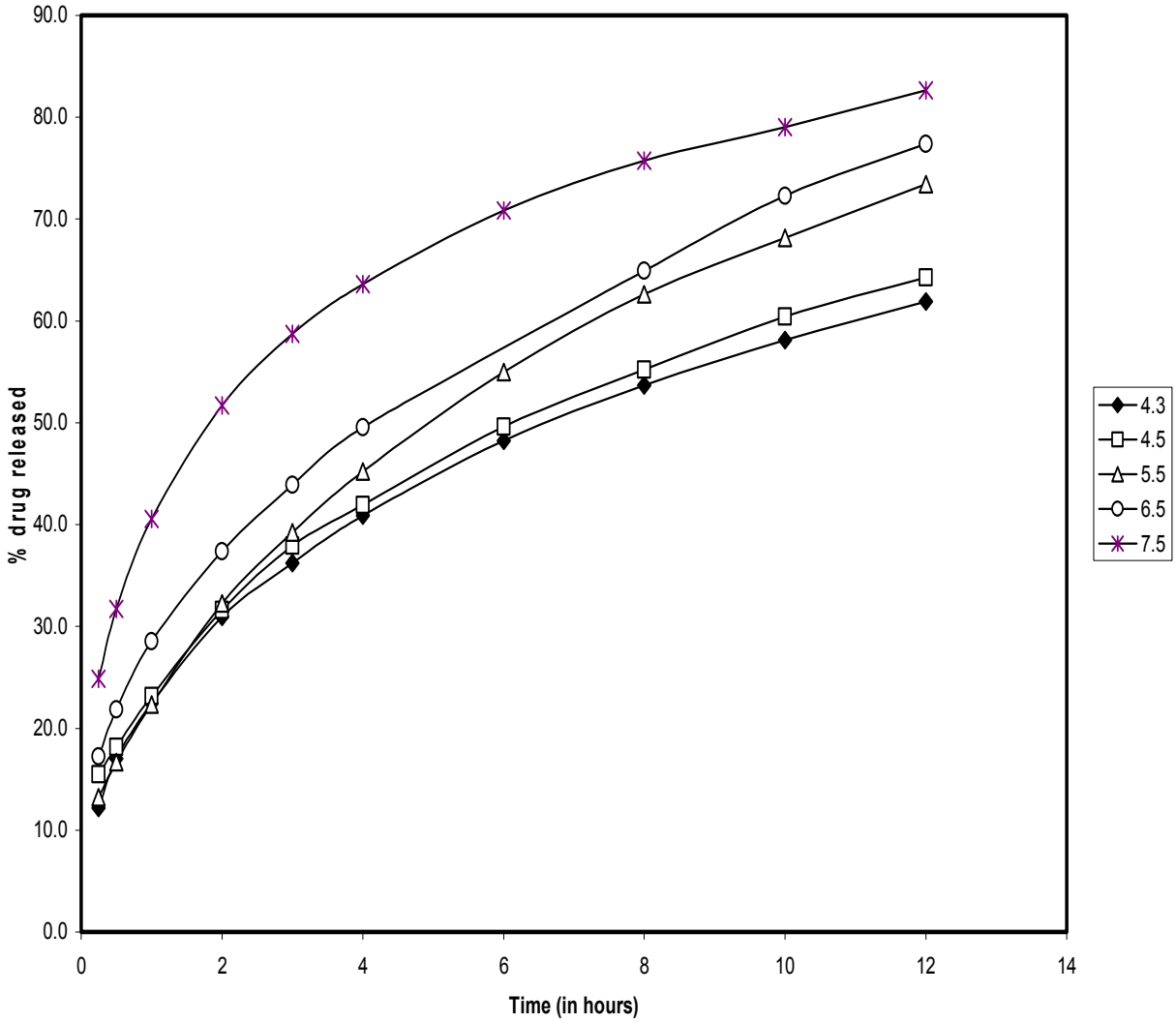

Figure 5.5: Dissolution profiles of microsphere batches prepared with Span 80 + Brij 30

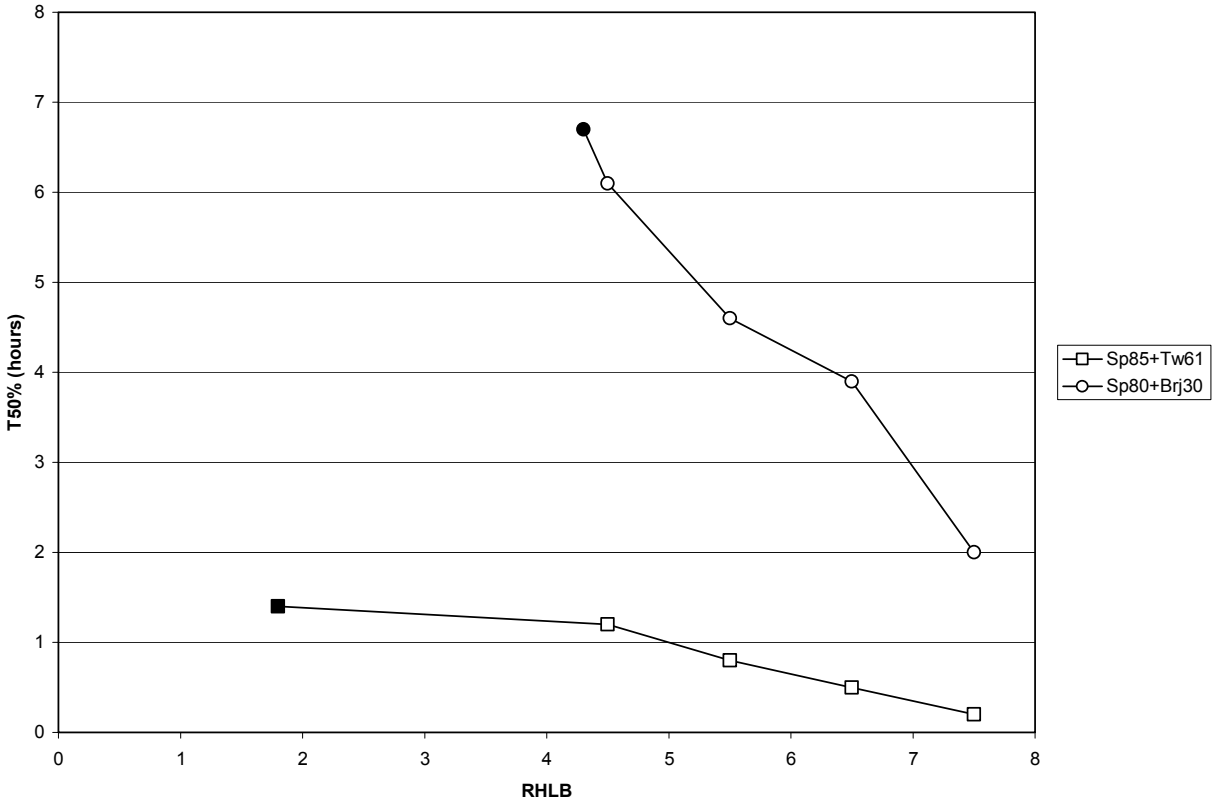

Figure 5.6: Change in dissolution rate (characterized by T50%) with a change in RHLB (The dark data points indicate the batches made with single low HLB surfactant while the light data points indicate batches made with surfactant combinations).

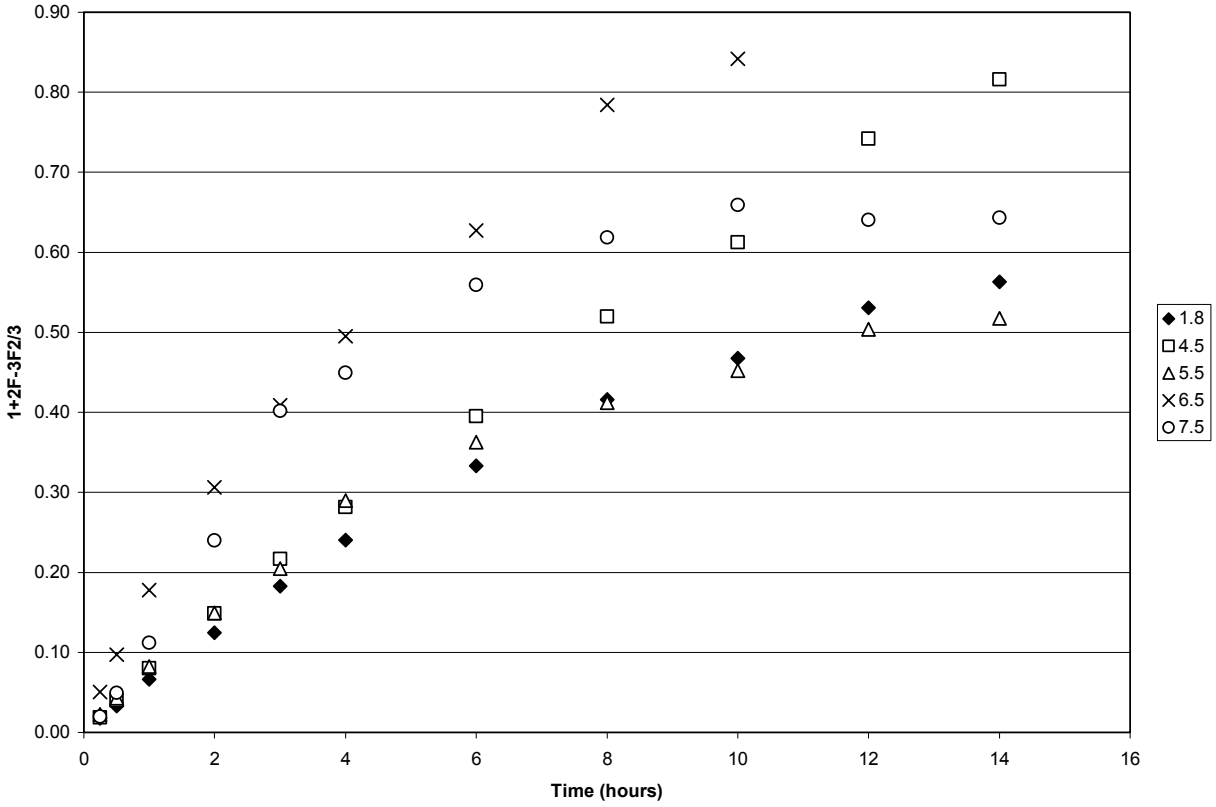

Figure 5.7: Higuchi plots for dissolution of 355 micron fraction of microsphere batches prepared with Span 85 & Tween 61

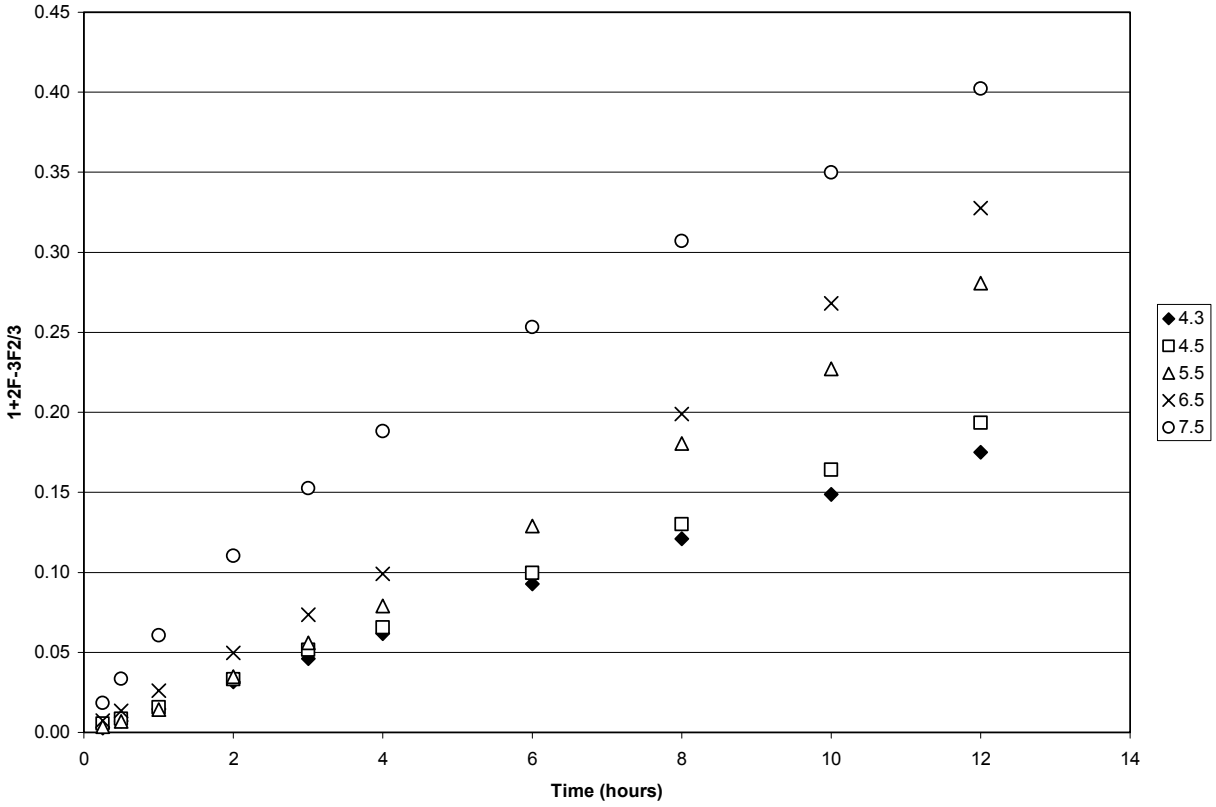

Figure 5.8: Higuchi plots for dissolution of 355 micron fraction of microsphere batches prepared with Span 80 & Brij 30

Chapter 6

Effect of low and high HLB surfactant type on the properties of microspheres prepared by the non-aqueous emulsion solvent evaporation method using dual surfactant systems

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Abstract: The objective of the present study was to investigate the influence of the structure of the low HLB and high HLB surfactants on the physical properties as well as the drug release of ethyl cellulose microspheres. Previous studies in our lab with the ethyl cellulose-acetone-mineral oil system have shown the effects of using combinations of low HLB and high HLB surfactants on microsphere formation by the non-aqueous emulsion solvent evaporation method. In this study we investigated the effect of surfactant structure on microsphere properties like yield, particle size distribution, geometric mean diameters, initial drug release and drug release mechanism. Different batches of microspheres were made at different RHLBs by combining low HLB surfactants (Span 80, Arlacel 83 and Span 85) and two high HLB surfactants (Tween 61 and Brij 30) in different ratios. The microspheres were then evaluated for the various parameters. The geometric mean diameter of the batches made with Span 80 were less than the batches made with Arlacel 83 and Span 85. The drug loading was minimally affected by the surfactant structure. The dissolution rate and initial drug release is less in batches made with Span 80 than Span 85 and Arlacel 83 indicating that the number of chains in the surfactant structure is important in determining the drug release characteristics. The type of linkage in Brij 30 and Tween 61 also seems to influence the release characteristics.

Introduction

Matrix microspheres are rugged in nature, making them suitable for tableting without compromising their drug release characteristics (Sayed and Price, 1986). Production of microspheres that meet a specific therapeutic objective requires the optimization of formulation and processing factors. In order to do so, it is necessary to identify those variables that critically affect microsphere production and properties.

Several factors have been identified that affect the formulation and properties of matrix micropsheres. Critical formulation factors include the type and molecular weight of the polymer, core drug particle size, drug:polymer ratio and solubility of the drug in the polymer. Critical processing factors for matrix microspheres prepared by emulsion solvent evaporation method include the phase ratios, the mixing intensity, the temperature during processing and the polymer phase viscosity (Suzuki and Price, 1985, Sprockel and Price, 1990, Hariharan and Price, 2002, Shukla and Price, 1989 and Obeidat and Price, 2003).

Previous studies dealing with the production of ethyl cellulose microspheres by non-aqueous emulsion-solvent evaporation have reported the use of a single, hydrophobic, low HLB surfactants like Span 80, Span 85, magnesium stearate, aluminum stearate and Synperonic L-61 for stabilizing the emulsion (Roy et al., 1989, Lin and Wu, 1999, Zinutti et al., 1994, Wu et al., 2003, Palomo et al., 1996). In a previous study, we reported the effect of using a combination of a low and high HLB surfactant and the role of RHLB on the physical and drug release properties of ethyl cellulose microspheres prepared by this method (Sarkar and Price, 2005).

In this paper, we investigated the influence of low HLB and high HLB surfactant type on the physical and drug release properties of ethyl cellulose microspheres prepared by non-aqueous emulsion solvent evaporation method.

Experimental

Materials

Ethyl cellulose (100cps, 49% Ethoxyl content, Scientific Polymer Products, New York), micronized theophylline (Knoll AG), light mineral oil, Span 85, Tween 61, Span 80, Brij 30 and Tween 80 (Ruger Chemical Company Inc., Irvington, NJ), methylene chloride (Fisher Scientific, NJ), acetone, monobasic potassium phosphate and sodium hydroxide (J.T. Baker, Phillipsburg, NJ).

Instruments

Stirrer (Lab. Stirrer LR 4000, Yamato Scientific Company Ltd., Tokyo, Japan), USP Dissolution Apparatus II (Dissolution Test system 5100, Distek Inc., North Brunswick, NJ), Aquamate (UV Spectrophotometer, Thermo Electron Corporation, Mercer's Row, Cambridge, UK), Accumet pH meter 5 (Fisher Scientific, NJ) and, standard sieve series.

Preparation of microspheres

Different batches of ethyl cellulose microspheres containing anhydrous micronized theophylline were prepared by the non-aqueous emulsion solvent evaporation method. The microspheres were prepared under identical conditions keeping the polymer concentration, drug concentration, stirring speed, ratio of internal to external phase and total surfactant concentration constant. Three low HLB surfactants were individually combined with different high HLB surfactants as shown in Table 1. Microsphere batches for all combinations were prepared over the same RHLB range (4.5-7.5) in order to compare results.

The polar internal phase consisted of a 5% solution of ethyl cellulose in acetone also containing the higher HLB surfactant. Micronized theophylline was then dispersed in this solution to give a theoretical drug loading of 33%. This slurry was added to the light mineral oil external phase containing the low HLB surfactant at room temperature under constant stirring at 2000 r.p.m. The stirring was continued for 16 hours to enable complete evaporation of the acetone and formation of microspheres. The microspheres were separated from the oil phase by decantation, washed extensively with mineral spirits to ensure complete removal of oil on the surface of microspheres and dried at 50°C overnight.

Particle size distribution

The particle size distribution of each microsphere batch was determined by sieve analysis using a set of standard sieves of range 90-710µ. The samples were placed on the topmost sieve and tapped by hand till no change in weight was observed in the sieves. The weight of microspheres retained on each sieve was recorded. The geometric mean diameter (d'g) was determined for each batch. The 355µ fraction of each batch was deemed to be representative and the following tests were carried out on this fraction of each batch.

Drug loading

The actual drug loading of the 355µ fraction of each batch of microspheres was determined by placing accurately weighed samples (in triplicate) in 10ml volumetric flasks and dissolving in methylene chloride. Drug concentration was then determined by taking absorbance at 276.5nm. Ethyl cellulose did not cause any spectrophotometric interference at this wavelength.

In vitro dissolution

The release of theophylline from the 355µ fraction of each batch of microspheres was evaluated using the USP Dissolution Apparatus II. Microsphere samples (triplicate for each batch) were suspended in 900 ml of dissolution medium (Simulated Intestinal Fluid with 0.2% Tween, no enzymes) and dissolution carried out at 37±0.5°C at 100 r.p.m for 12-24 hours. 3ml of sample was withdrawn at specific time intervals and replaced with fresh medium. The drug released was determined spectrophotometrically at 272 nm. The dissolution data was evaluated for initial drug release, dissolution rate and the drug release mechanism.

Results and discussion

Effect of low HLB surfactant on particle size (d'g)

Figure 6.1 shows the effect of the low HLB surfactant type on the geometric mean diameter of microspheres. In general, at a given RHLB, microspheres prepared using Span 80 have a smaller d'g compared to those prepared with Span 85.

Effect of high HLB surfactant on particle size (d'g)

The effect of high HLB surfactant structure on microsphere particle size is shown in Figure 6.2. No well defined effect could be observed. Microsphere batches made with Brij 30 and Span 80 decrease in size as RHLB increases from 4.5 to 6.5. The d'g then suddenly increases with increase of RHLB from 6.5 to 7.5. On the other hand, d'g of batches made with Tween 61 and Span 80 decreases with an increase in RHLB from 4.5 to 7.5.

The d'g of microsphere batches made with Brij 30 and Arlacel 83 show no clear trend, decreasing from 4.5 to 5.5, remaining constant from 5.5 to 6.5 and increasing from 6.5 to 7.5.

Tween 61 with Arlacel 83 shows the surprising trend of increasing d'g with an increase in RHLB from 4.5 to 6.5 and then decreasing from 6.5 to 7.5. This ambiguity of results could be because of the composition of Arlacel 83, which is an equimolar mixture of sorbitan mono-oleate and di-oleate and hinders the identification of a clearly defined trend.

Effect of surfactant structure on drug loading

Table 6.2 shows the drug content of 355µ fraction of the microsphere batches. No clear effect of the surfactant type on drug loading can be seen. In some of the cases, the drug loading was observed to be more than the theoretical drug loading of 33.3%. This was possibly due to the loss of micropheres that entrapped air and were lost during removal of the oil. This increased the drug to polymer ratio resulting in an analyzed drug content that is more than the theoretical drug content.

Effect of low HLB surfactant structure on dissolution

Figures 6.3a, 6.3b and 6.3c show the effect of low HLB surfactant structure on drug release from the 355µ fraction of microspheres prepared using these surfactants. It can be observed, that at all RHLBs (3a-Low HLB surfactants used alone, 3b-surfactants combined with Tween 61 to give RHLB of 4.5, 3c-surfactants combined with Tween 61 to give RHLB of 5.5), microspheres prepared with Span 80 have a slower release profile compared to those prepared with Arlacel 83. Also, microspheres prepared with Arlacel 83 have slower release than microspheres prepared with Span 85. Another way to visualize this effect is by comparing the T50% (time required for 50% of the drug to be released from microspheres) of these dissolution curves. Figure 6.5a shows how the T50% is greatest for the Span 80 batches followed by Arlacel

83 and then Span 85 formulation having the lowest T50% (fastest dissolution). This clearly indicates that low HLB surfactant structure affects drug release.

Effect of high HLB surfactant on dissolution

Microspheres were prepared by using Tween 61 combined with Span 80 and Arlacel 83 to give RHLBs of 4.5 and 5.5. Brij 30 was similarly combined with Span 80 and Arlacel 83 to give RHLBs of 4.5 and 5.5. Figure 6.4a and 6.4b shows the effect of high HLB surfactant structure on drug release from the 355µ fraction of microspheres. Batches prepared with Brij 30 had slower release profiles compared to batches prepared with Tween 61 at RHLBs of 4.5 and 5.5. In terms of T50%, Figure 6.5b depicts how the T50% values are higher for the microspheres prepared with Brij 30. Both Tween 61 and Brj 30 have very similar RHLB values (9.6 for Tween 61 and 9.7 for Brij 30) which indicates that it is the difference in their structure which may be responsible for the difference in the release characteristics of microspheres prepared using these surfactants.

The ether linkage in Brij 30 [Polyoxyethylene (4) lauryl ether] is less polar than the ester linkage in Tween 61 [Polyoxyethylene (4) sorbitan monostearate]. This may enable Brij 30 to partition better at the interface than Tween 61, which in turn could result in less permeable encapsulation of the drug. This could be the reason why drug release is slower from batches made with Brij 30.

Effect of low HLB surfactant on initial release

Table 6.3a shows the effect of low HLB surfactant structure on initial release from the microspheres. As was explained earlier, Span 80 forms a tighter interfacial film that results in

better internalization of the drug compared to Arlacel 83 and Span 85. Therefore, microsphere batches prepared with Span 80 have less drug on their surface and consequently show lower initial release.

Effect of high HLB surfactant on initial release

Table 6.3b shows the effect of high HLB surfactant structure on dose dumping from microspheres. Microspheres prepared using Brij 30 as the high HLB surfactant show lower initial release compared to batches prepared with Tween 61. This could be because of the ability of Brij 30 to partition at the interface and internalize the drug better than Tween 61. This results in less drug on the surface of such microspheres and therefore less initial drug release.

Discussion

When ethyl cellulose microspheres are prepared by the emulsion-solvent evaporation process, the formation of a stable emulsion of the polymer solution in the mineral oil phase is a critical step. Surfactants play an important role in maintaining the dispersed polymer solution as stable droplets in the mineral oil by forming an interfacial film around the droplets. It is of utmost importance to prevent coalescence of the polymer solution while solvent diffusion and evaporation occurs in order to get discrete microspheres. The quality of interfacial film not only prevents coalescence during microsphere formation, it also affects the physical and drug release properties of the microspheres.

It has been reported that at the interface, the hydrocarbon portions of the two surfactants lie in the non-polar oil phase while the more polar parts of the surfactants lie in the polar phase (Martin, 1995). It has been suggested that the hydrocarbon chains of the Span and Tween orient

themselves alternately and such an orientation results in effective van der Waals attraction between the hydrophobic parts of the surfactants. This leads to a stronger interfacial film that stabilizes the emulsion during microsphere formation and retards coalescence.

Span 80 has a single oleate chain and it is able to orient itself at the interface in such a way that it is able to bond effectively with hydrophobic part (stearate chain) of Tween 61 through van der Waals interactions. This results in a tighter and more compact interfacial film which retards coalescence more effectively than Span 85, which has three oleate chains and may not be able to associate effectively with the hydrocarbon tail of Tween 61 due to steric hindrance (Figure 6.6). As a result, the interfacial film formed by Span 85 and Tween 61 may not be as strong in retarding coalescence as the interfacial film formed by Span 80 and Tween 61. Therefore we get microspheres with a larger d'g with the former combination. Also, as noted in our previous study, the d'g decreases with an increase in RHLB.

Arlacel 83 shows an arbitrary effect on d'g with an increase in RHLB. This ambiguity could be because it is an equimolar mixture of sorbitan mono-oleate and di-oleate, and the effect of interfacial film on d'g is not as well defined as for Span 80 or Span 85.

Span 85 (sorbitan tri-oleate) has 3 hydrocarbon chains (Figure 7) compared to Span 80, which has one hydrocarbon chain. Because of this structural difference, Span 85 is more hydrophobic (HLB of 1.8) than Span 80 (HLB of 4.3) and it is not able to partition at the interface as well as Span 80. Therefore, Span 80 is better able to maintain intact globule than Span 85 and this results in a slower release profile (Figure 3a) and larger T50% (Figure 5a). Arlacel 83 is an equimolar mixture of sorbitan mono-oleate and di-oleate, has a HLB value between Span 80 and Span 85 (HLB of 3.7) and shows an intermediate dissolution rate.

When these low HLB surfactants are combined with a high HLB surfactant, Tween 61 (HLB of 9.6), to give RHLBs of 4.5 and 5.5, the dissolution rate shows that same trend as when these surfactants are used alone (Figures 6.3b, 6.3c and 6.5a). Batches prepared with Span 80 release the drug more slowly than Arlacel 83 which, in turn, is slower than Span 85. This can also be explained by the difference in the type of interfacial film these surfactants form based on their differing abilities to partition at the interface, as explained above. Another factor that comes into play is the bonding between the hydrophobic parts of the low and high HLB surfactants. As was explained earlier, the Spans orient at the interface such that their hydrophobic part lies in the mineral oil and its sorbitan part lies in polar phase. Tween 61 is also expected to orient itself such that its hydrophobic part is in the oil phase and hydrophilic part is in the polar phase. The two surfactants arrange themselves such that the Span and the Tween lie alternately and this allows their hydrocarbon parts to interact effectively through van der Waals attraction. Span 80, with a single oleate chain, is able to form a tighter and more compact interfacial film with Tween 61, partly because of its ability to partition better at interface and partly because of having a single oleate chain. Span 85, with three oleate chains, is not able to partition as well at the interface. Also, the three oleate chains may hinder effective van der Waals interactions with the Tween 61, possibly because of steric hindrance which may hamper the approach of Tween 61 close enough to associate effectively. The same is the case when Arlacel 83 is combined with Tween 61. As a result, the interfacial film with these combinations is not as effective as with Span 80 and Tween 61. Thus microspheres formed with Span 80 and Tween 61 are able to protect the internal phase globules resulting in less permeable microspheres with slower release profiles.

Conclusions

It can be concluded from this study that the type of low HLB and high HLB surfactant used for the production of microspheres by the non-aqueous emulsion solvent evaporation method has an impact on the properties of such microspheres. In case of the low HLB surfactants, Span 80, with a single oleate chain, form a stronger and more compact interfacial film with Tween 61 compared to Arlacel 83 and Span 85. In case of the high HLB surfactants, Brij 30 appears to form a better interfacial film at the interface than Tween 61. As a result of the changes in interfacial films, the microsphere properties are affected in the following manner. Batches made with Span 80 and Tween 61 show slower dissolution and least initial drug release sice they are better capable of protecting the internal phase globules during microsphere formation which results in less permeable microspheres.

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Table 6.1: Batches of microspheres prepared using different combination of low and high HLB surfactants

	Batches	RHLB	Ratios
Span 85 (1.8) + Tween 61 (9.6)	31	1.8	100
	34	4.5	65:35
	35	5.5	53:47
	36	6.5	40:60
	37	7.5	27:73
Arlacel 83 (3.7) + Tween 61 (9.6)	39	3.7	100
	40	4.5	86:14
	41	5.5	70:30
	42	6.5	53:47
	43	7.5	36:64
Span 80 (4.3) + Brij 30 (9.7)	59	4.3	100
	60	4.5	96:4
	61	5.5	78:22
	62	6.5	59:41
	63	7.5	40:60
Arlacel 83 (3.7) + Brij 30 (9.7)	64	4.5	87:13
	65	5.5	70:30
	66	6.5	53:47
	67	7.5	37:63
Span 80 (4.3) + Tween 61 (9.6)	68	4.5	96:4
	69	5.5	77:23
	70	6.5	58:42
	71	7.5	40:60

Table 6.2: Drug content (in percent) of 355μ fraction microspheres made from five different combinations of surfactants

RHLB	(Span 85 + Tween 61)	Span 80 + Brij 30	Span 80 + Tween 61	Arlacel 83 + Brij 30	(Arlacel 83 + Tween 61)
4.5	29.2 ± 0.3	33.7±1.7	30.1±1.3	26.2±0.8	27.7 ± 1.4
5.5	32.1 ± 1.7	28.9±1.0	26.4±0.6	32.0±1.1	31.5 ± 0.4
6.5	28.5 ± 1.1	30.1±1.3	31.7±2.1	33.2±1.3	27.9 ± 1.5
7.5	27.5 ± 1.6	33.3±1.7	29.8±0.7	32.5±2.4	27.5 ± 1.3
1.8 (Span 85)	33.1 ± 1.5				
3.7(Arl 83)	31.7 ± 1.6				
4.3 (Span 80)	37.2 ± 1.7				

Table 6.3a: Initial drug release (% of drug released in 30 minutes) in microspheres prepared with three different Low HLB surfactants

RHLB	Span 80 + Tween 61	Arlacel 83 + Tween 61	Span 85 + Tween 61
4.5	20.5	32.2	34.2
5.5	24.5	38.1	35.2
	4.3 (Span 80)	**3.7(Arl 83)**	**1.8 (Span 85)**
	17.0	31.3	31.6

Table 6.3b: Initial drug release (% of drug released in 30 minutes) in microspheres prepared with two different high HLB surfactants

RHLB	Span 80 + Brij 30	Span 80 + Tween 61		Arlacel 83 + Brij 30	Arlacel 83 + Tween 61
4.5	18.2	20.5		21.2	32.2
5.5	16.7	24.5		25.8	38.1

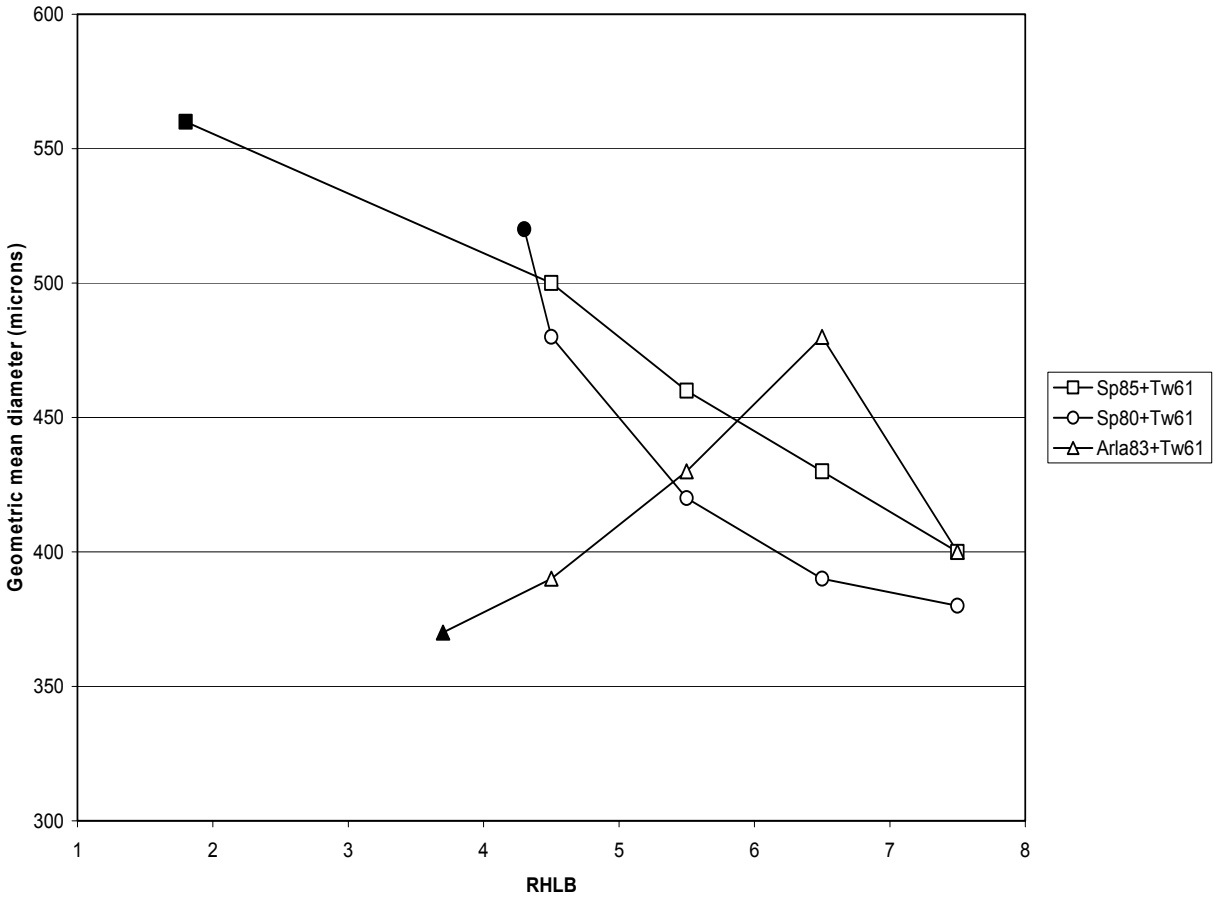

Figure 6.1: Effect of low HLB surfactant type on geometric mean diameter (The dark data points indicate the batches made with single low HLB surfactant while the light data points indicate batches made with surfactant combinations).

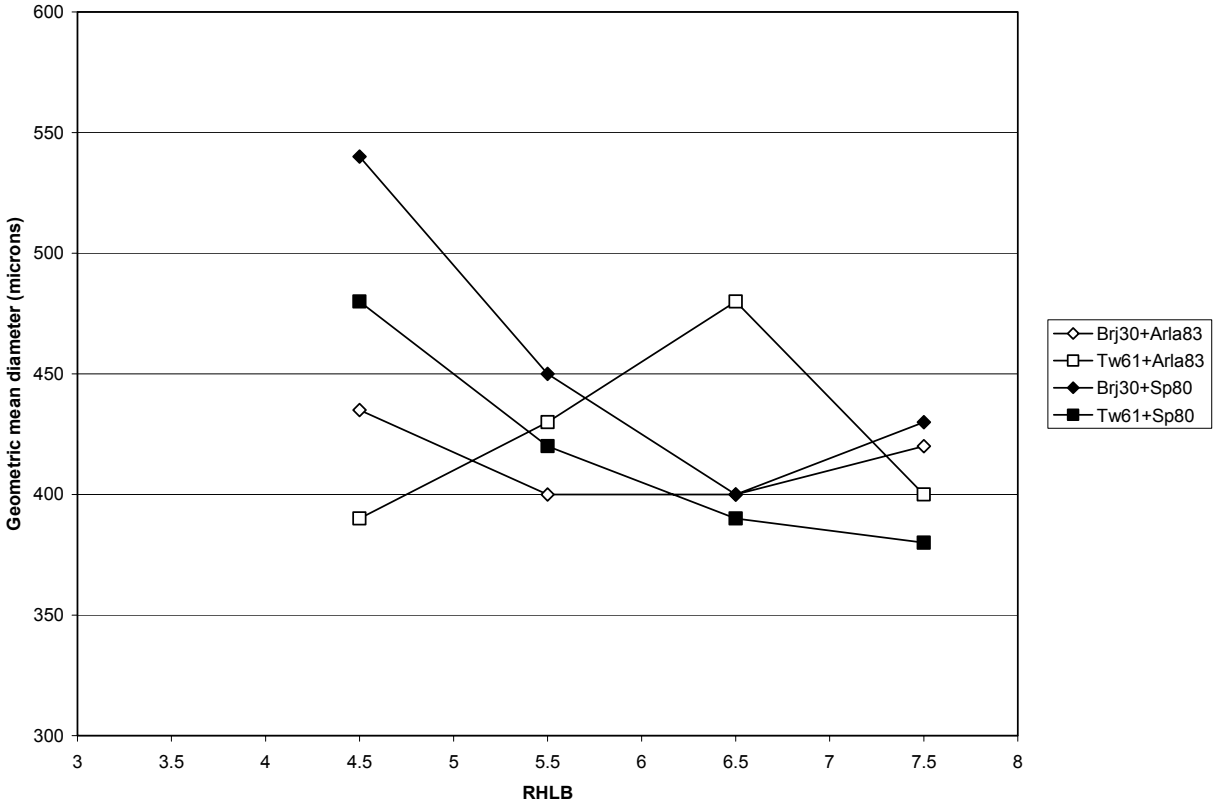

Figure 6.2: Effect of high HLB surfactant type on the geometric mean diameter of the microspheres.

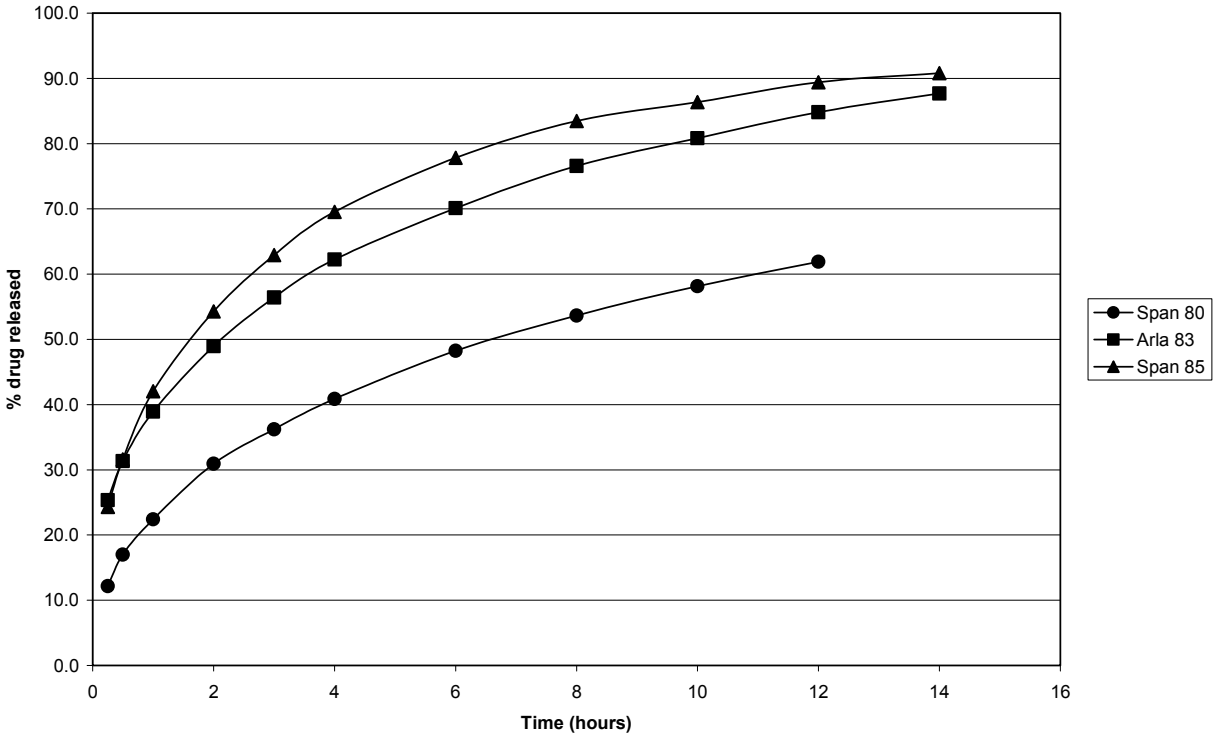

Figure 6.3a: Effect of low HLB surfactant type on dissolution profiles of microspheres (single surfactants used).

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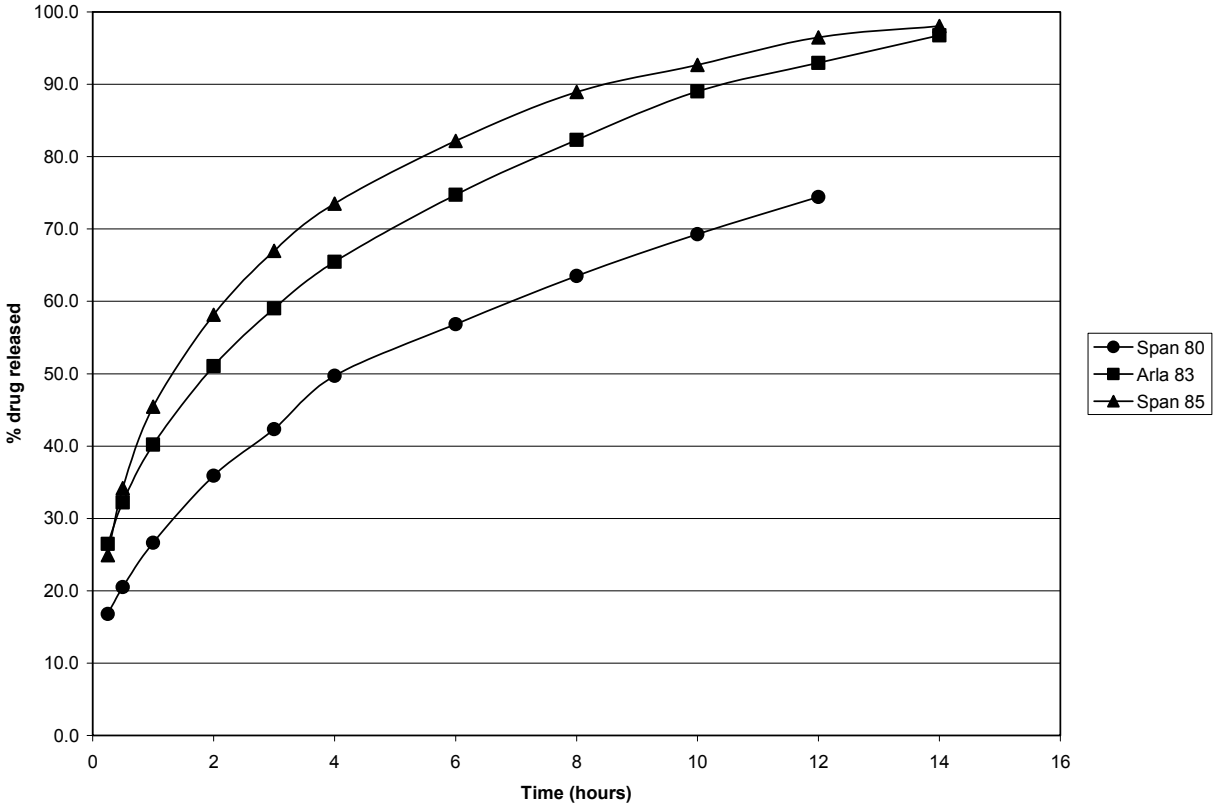

Figure 6.3b: Effect of low HLB surfactants on dissolution profiles when combined with Tween 61™ for an RHLB of 4.5.

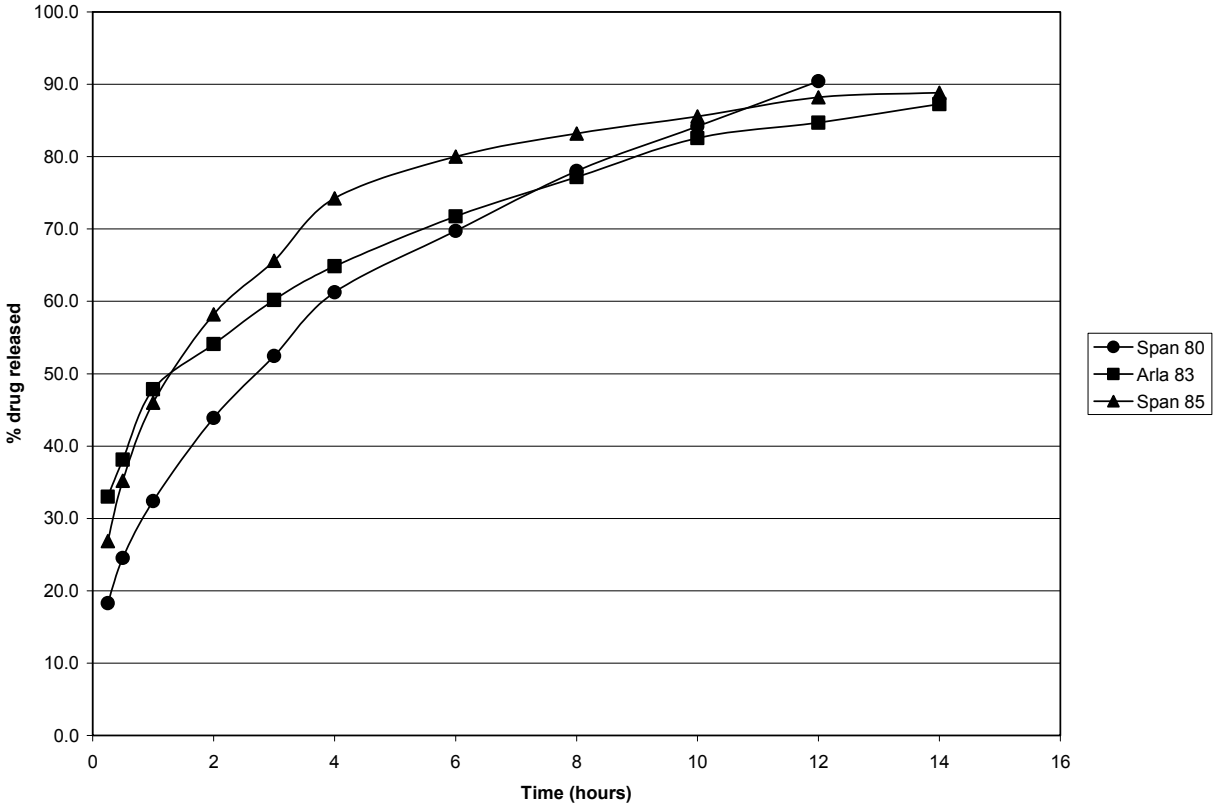

Figure 6.3c: Effect of low HLB surfactants on dissolution profiles when combined with Tween 61™ for an RHLB of 5.5.

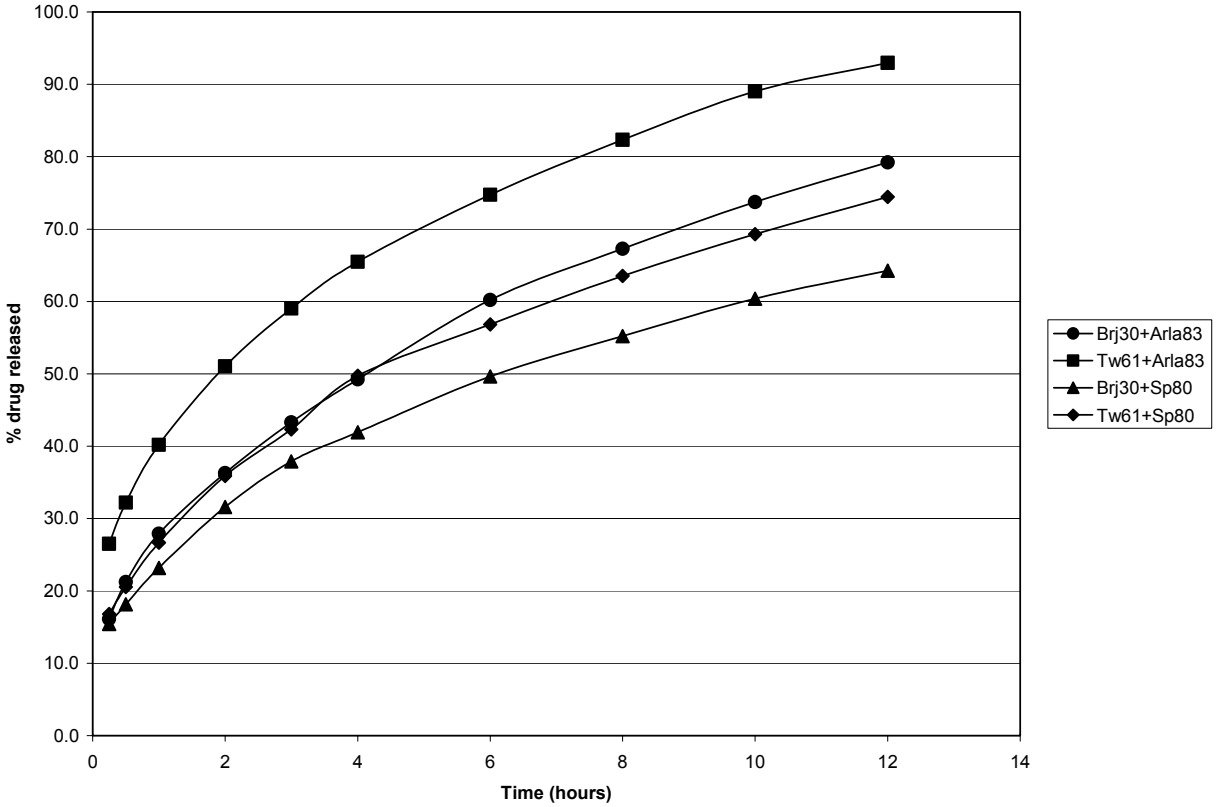

Figure 6.4a: Effect of high HLB surfactant type on dissolution profiles at RHLB = 4.5.

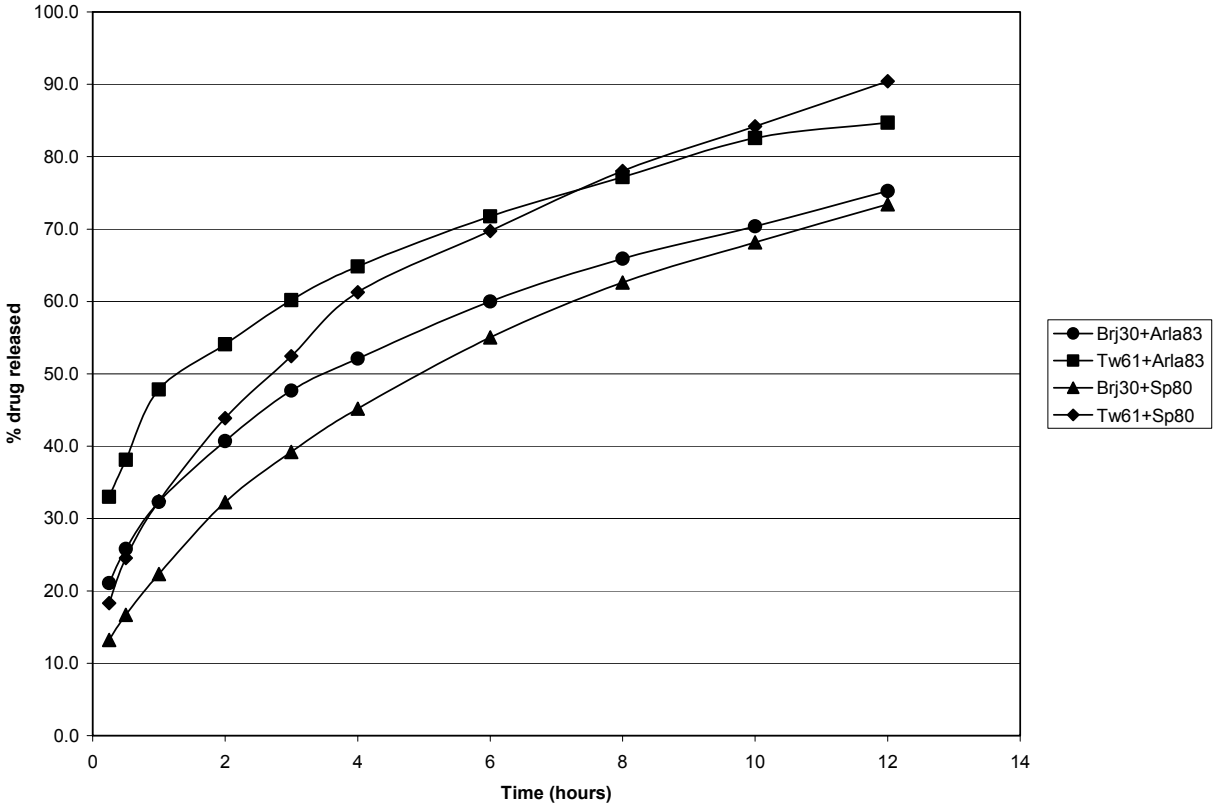

Figure 6.4b: Effect of high HLB surfactant types on dissolution profiles at RHLB = 5.5.

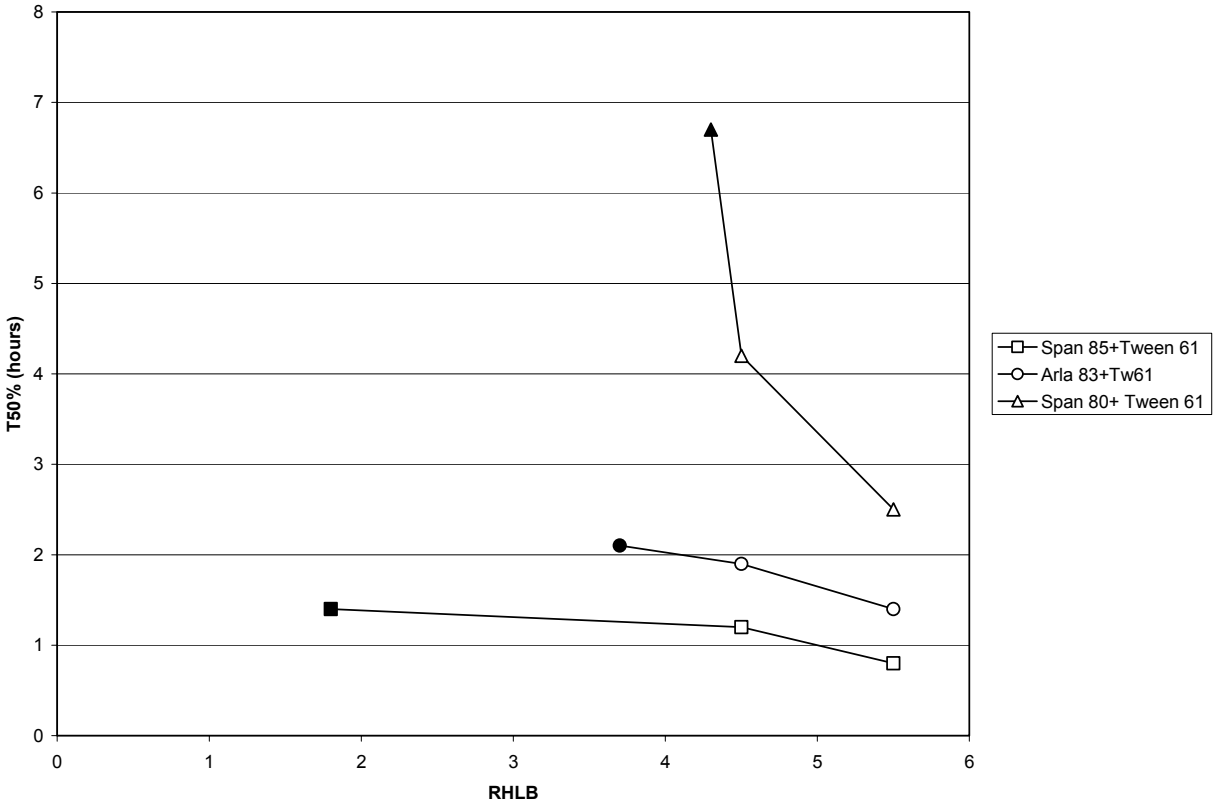

Figure 6.5a: Effect of low HLB surfactant structure on dissolution T50% (The dark data points indicate the batches made with single low HLB surfactant while the light data points indicate batches made with surfactant combinations).

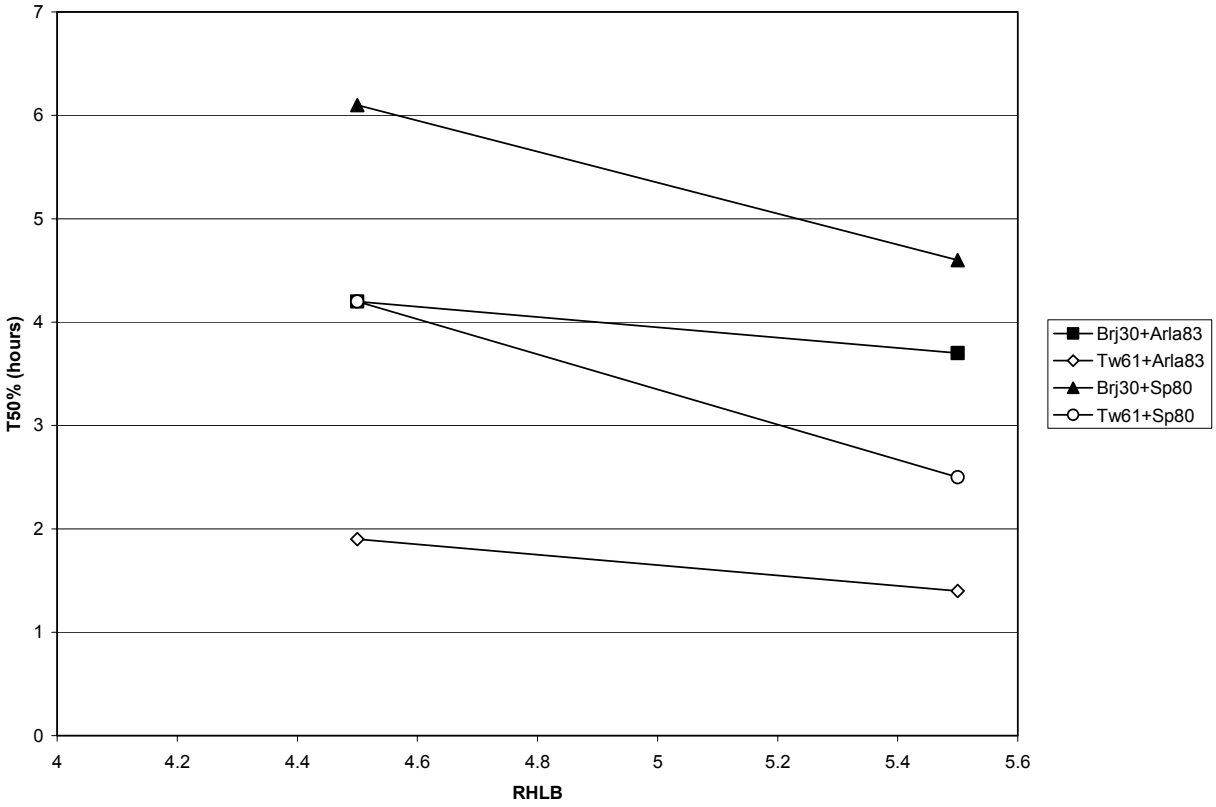

Figure 6.5b: Effect of high HLB surfactant type on dissolution T50%.

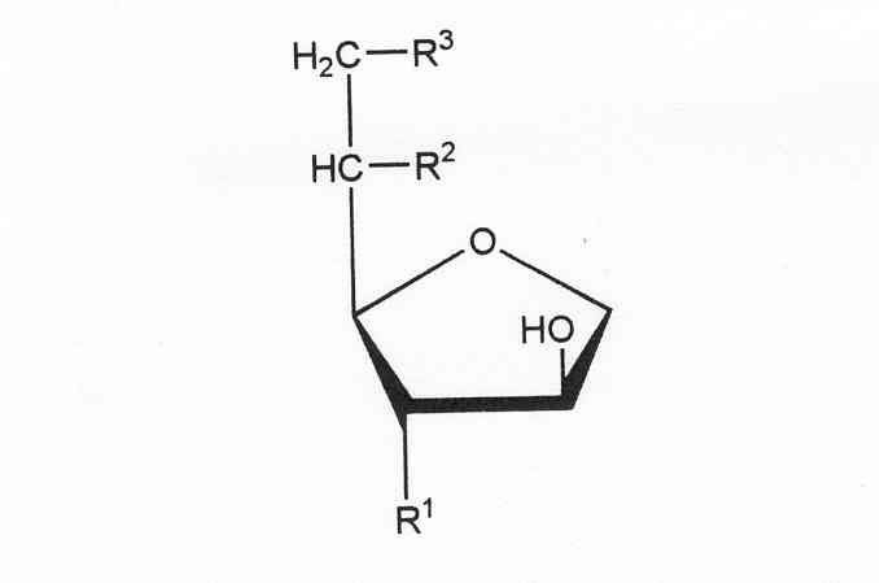

Figure 6.6: Structure of Spans (Span 80: $R^1 = R^2 = OH$, $R^3 = C_{17}H_{33}$, Span 85: $R^1 = R^2 = R^3 = C_{17}H_{33}$)

Chapter 7

Significance of formulation factors on the release of a weakly acidic and a weakly basic drug from HPMC matrix tablets[1]

[1]R. Sarkar, J. C. Price, D. R. Lu, D. Kaushik and S.C. Mehta. To be submitted to International Journal of Pharmaceutics

Introduction

One of the essential components of the drug development process is the delivery system for new as well as existing drugs. The design of such systems depends on a number of intercalated elements on which more information is required. An in-depth understanding of such systems will enable us evolve drug delivery to such a stage that we can formulate drug delivery systems which conform to individual patient requirements. In this regard, administration of controlled release dosage forms through the oral route has received most widespread attention as it is more amenable to design manipulation. To achieve sustained release of a drug in the gastrointestional tract, most oral controlled release systems use the mechanisms of dissolution, diffusion or a combination of both (Robinson & Lee, 1987).

Monolithic systems, consisting of a swellable matrix-forming material compressed with the drug using established tableting technology, are the most common oral drug delivery systems (Colombo et al., 2000). When a water swellable polymer is used to form the matrix tablet, on placing it in dissolution medium, a gelatinous layer is formed at the tablet surface across which the drug diffuses out (Velasco et al., 1999). The polymer particles on the tablet surface, which get hydrated first and form the gel layer, gradually dissolve or erode. As a result, for hydrophilic matrix tablets, 2 types of mechanisms control drug release (Velasco et al., 1999).

1. The gelatinous polymer layer controls drug release by retarding the diffusion of the drug across it. This mechanism is predominant for water-soluble drugs (diffusion controlled).
2. As the gel layer gradually dissolves and erodes, drug particles are released in the surrounding media. Most water insoluble drugs are released exclusively by this mechanism

(erosion controlled). This mechanism may contribute a minor part in the release of water soluble drugs also (Ford et al., 1987).

Drug release from a hydrophilic matrix tablet is a complex phenomena affected by the properties of the polymer forming the matrix, properties of the drug, properties of the excipients present in the formulation and the dimensions of the tablet. To achieve sustained release of a drug, the rate of formation of the gel layer is critical. To prevent instantaneous dissolution of the drug, the particles of the selected polymer must form the gel layer rapidly. The various polymer related factors which affect drug release from hydrophilic matrix tablets include polymer type, polymer concentration, polymer viscosity and particle size (Velasco et al., 1999).

Although other polymers have shown comparable performance [poly(ethylene oxide), Carbopol, xanthan and guar gums], HPMC is still one of the most widely used polymers for sustained release formulations (Colombo et al., 2000). Its properties have been extensively studied and documented (Ford et al., 1985a, Ford et al., 1985b).

The properties of the drug being formulated into SR matrix tablets also affect its release. Water solubility of the drug plays a major role in determining which release mechanism will predominate. As mentioned above, water-soluble drugs are released by diffusion across the gel layer. For water insoluble drugs, erosion of the gel layer controls drug release.

But in many cases, drugs are weak acids or bases (or their salts) and such compounds demonstrate a pH-dependent solubility in the gastrointestinal tract (GIT) (Streudel et al., 2000). Since controlled release dosage forms of these drugs release the drug over several hours, they are expected to encounter a wide range of pH in the GIT that could result in bioavailability problems. But to the best of the authors' knowledge, most studies demonstrate the effect of formulation factors on drug release from HPMC matrix tablets in a single dissolution medium

while neglecting how the effect of these factors might change from one medium to another (Ford et al., 1985 a,b, Ford et al., 1987, Velasco et al., 1999, Huang et al., 2003, Sung et al., 1996, Rekhi et al., 1999). The purpose of the present study was to investigate how the various formulation factors affect drug release of weakly acidic and basic drugs from matrix tablets in different dissolution medium using a statistical design of experiments.

Verapamil hydrochloride was chosen as the model weakly basic drug as it has high solubility in acidic pH and solubility decreases as pH increases (Streubel et al., 2000). Ketoprofen was chosen as the weakly acidic drug that has a high solubility in intestinal pH but low solubility in acidic medium (Solinis et al., 2002). Factorial design of experiments has been widely used in tablet formulations for screening purposes or to identify different formulation factors in complex systems (Khanvilkar et al., 2002, Vatsaraj et al., 2002, Bolton, 1990, Huang et al., 2003). A five-factor, half-factorial design of experiment was used in the present study. The five factors chosen were polymer content, drug content, type of drug, type of filler and surface area to volume ratio.

Materials and Methods

Design of Experiement

A five-factor, half-factorial design of experiment was chosen to study the effects of HPMC content, drug content, drug type, filler type and surface area to volume ratio on drug release from HPMC matrix tablets. These five formulation factors were the independent variables and were studied at two different levels, +1 and -1 (Table 1). Sixteen experiments were performed according to the design generated by DesignExpert6® (Table 2). M_{3hours} and $M_{12hours}$, the percentage of drug released in three hours and 12 hours respectively, were chosen as the dependent variables.

Manufacture of tablets

Sixteen tablet formulations were prepared according to Table 2. All the ingredients except magnesium stearate were passed through a 600 micrometer sieve. Drug, HPMC and filler were weighed out and transferred to a plastic tube and mixed thoroughly for fifteen minutes by inverting the tube. Magnesium stearate was then added to this mixture and mixed for an additional thirty seconds. An appropriate amount of the blend was weighed and tablets were prepared by direct compression on a single-punch Carver press at a constant compression pressure of 2500 pounds. Nine tablets were prepared per formulation. Deep cup concave tooling of two different dimensions (3/8 inch and ½ inch) were used to vary the surface area to volume ratio of the tablet

In vitro *dissolution studies*

Tablet dissolution was carried out at 37°C and 100 rpm in the USP dissolution apparatus II (Paddle Method) (Distek Inc.) in 1000 ml of dissolution medium. The dissolution was carried out in Simulated Gastric Fluid (SGF, without enzymes) for the first three hours. At the end of this period, the tablets were transferred to a second vessel containing 1000 ml of Simulated Intestinal Fluid (SIF, without enzymes) at 37°C and dissolution carried out for nine more hours. Three tablets were tested per formulation. Stainless steel sinkers were used to prevent the tablets from sticking to the bottom of the vessels but allowing the tablets to swell freely.

Three ml samples were withdrawn at specific time intervals and analyzed spectrophotometrically (Aquamate, Thermo Electron Corporation). Verapamil HCl was analyzed at 278.5nm (both SGF and SIF) while ketoprofen was analyzed at 259.5nm (SGF and 261.5nm

(SIF). Three ml of fresh dissolution medium was added to each vessel in order to keep the volume constant.

Mechanism of drug release

Several mathematical models can be used to describe the kinetics of a drug released from matrix tablets (Siepmann and Peppas, 2001). To determine which model is best suited the dissolution data was fitted to the Higuchi equation (1) and the Ritger-Peppas equation (2) and the best fit was determined (Higuchi, 1962, 1963, Ritger and Peppas, 1987).

$$M_t / M_\infty = Kt^{0.5} \quad (1)$$

$$M_t / M_\infty = kt^n \quad (2)$$

where M_t / M_∞ is the fraction of drug released at time t, is the K is the Higuchi rate constant, k is a constant that considers the structure and geometry of the tablet and n is the drug release exponent which is indicative of the drug release mechanism.

Results and Discussion

Effect of HPMC level

The polymer level had a significant effect on the release of both ketoprofen and verapamil hydrochloride in SGF as well as in SIF (p-value of 0.0001 and 0.0008 respectively). This can be attributed to the fact that the release of a soluble drug from a hydrophilic matrix tablet is a dynamic process that occurs in 4 sequential steps: polymer wetting followed by hydration and swelling of the matrix, drug dissolution within the matrix and finally diffusion of the dissolved drug across the hydrated (gel-state) polymer layer. To elaborate, placing the matrix tablet in dissolution medium causes surface wetting. This wetting process progresses by the entry

of water molecules into the microscopic pore spaces inside the matrix. HPMC particles, on wetting, absorb water, swell, block the existing pores and form a gel like structure. During this process, a penetration front and a dissolution front are formed within the system (Sung et al., 1996). The dissolution front is formed at the interface of the dissolution medium and the gel layer while the penetration front is formed at the interface of the gel layer and unwetted polymer. The distance between the 2 fronts determines the diffusional path length and controlling its thickness determines the rate of drug diffusion and drug release. An increase in the polymer level from 20% to 40% leads to the formation of a stronger gelatinous layer which results in an increase in the diffusional path length and thus retards drug release. In case of poorly soluble drugs, which are released by polymer erosion, increasing the polymer level would slow down the polymer erosion process and retard the drug release.

Effect of Drug level

The effect of varying the amount of drug loaded into matrix tablets on drug release warrants close study. This information becomes very crucial especially when a drug needs to be formulated in different strengths. Several studies have stressed upon the importance of the HPMC to drug ratio (Ford et al., 1985 a, b, Velasco et al., 1999). In these studies the HPMC to drug ratio is varied by changing the HPMC content all the while keeping the drug content constant. Such studies neglected the effect of varying the drug content (while keeping polymer level constant) on drug release as well as the effect the drug may have on the distribution of water in the polymer matrix.

The drug loading in the matrix tablets appears to have a significant effect on the drug release in both SGF and SIF (p value of 0.0019 and 0.0017 respectively) (Tables 7.4 and 7.5). It

was observed that as the drug loading is increased from 8% to 40% for ketoprofen, the drug release rate decreases in both SGF and SIF. This is also true in the case of verapamil hydrochloride where an increase in drug loading lead to a decrease in release rate. This could possibly be explained by the fact that the presence of the drug helps to maintain the gel structure of the HPMC (Mitchell et al., 1993). This improves the integrity of the matrix and results in slower drug release from the tablet. As the drug level is increased, this effect becomes more pronounced and the drug release becomes even slower.

Effect of drug type

It has been suggested that water soluble drugs are released from hydrophilic matrix tablets primarily by diffusion while water insoluble drugs are released primarily by erosion of the polymer matrix. But for acidic and basic drugs, the solubility varies according to the pH of the dissolution media. Therefore, for HPMC matrix tablets formulations containing these drugs, the release mechanism will vary depending on which part of the gastrointestinal tract the tablet is in.

In this study, the dissolution was carried out for 3 hours in SGF followed by 9 hours in SIF. In SGF, the type of drug (acidic or basic) in the matrix tablet has a significant effect on the drug release (p value of 0.0001). Ketoprofen, which is a weakly acidic drug, has low solubility in SGF. When we determine the release mechanism of ketoprofen from matrix tablets in SGF (Table 7.6), the dissolution data fits best to the Ritger and Peppas equation than to the Higuchi equation. In most cases, the release exponent, n, is close to or above 0.89. For cylindrical devices, such as a tablet, this is clearly indicative of Case II drug release mechanism which is characterized by linear drug release kinetics (Mitchell et al., 1993). Case II transport or zero order release occurs when the rate controlling step of drug release is the relaxation of the HPMC polymer chains upon fluid uptake (Siepmann et al., 2001. Such a situation occurs when the drug

has poor solubility in the penetrating fluid, making drug diffusion redundant. The penetrating fluid decreases the glass transition temperature of the polymer till the polymer chains have undergone a transition from glassy to rubbery state (Siepmann et al., 2001). As the polymer chains change to the rubbery state, they become more mobile and this allows the drug to be released into the dissolution medium.

The drug release data of verapamil hydrochloride from HPMC matrix tablets in SGF, on the other hand, fits best to the Higuchi equation (Table 7.7). Verapamil hydrochloride is a weakly basic drug and has high solubility in SGF (Streubel et al., 2000). It is a well known fact that the main mechanism for the release of soluble drugs is by diffusion and this mechanism follows the Higuchi equation (Mitchell et al., 1993). The dissolution fluid enters the HPMC tablet, dissolves the drug and the dissolved drug diffuses across the gel layer into the dissolution medium.

When the tablet dissolution is carried out for 9 hours in SIF after 3 hours in SGF, an interesting phenomenon is observed. In SIF, the type of drug in the matrix does not have a statistically significant effect on drug release. This implies that the solubility of the drug is not the rate limiting step in drug release as it was in SGF and indicates a diffusion controlled mechanism of release for both ketoprofen and verapamil hydrochloride.

Examination of the dissolution data reveals that ketoprofen release in SIF fits best to the Higuchi equation than the Ritger and Peppas equation (Table 6.6). This is because the solubility of ketoprofen increases in SIF compared to SGF. As a result, as the SIF enters the tablet, ketoprofen dissolves in it and then diffuses out across the gel layer. This probably explains a better fit of the release data to the Higuchi equation as ketoprofen release in SIF is a diffusion controlled process.

The dissolution data for verapamil hydrochloride shows the best fit to the Higuchi equation indicating that diffusion across the gel layer is the rate controlling step for drug release. This is contrary to reports where it has been suggested that for a weakly basic drug, as the pH value of dissolution medium exceeds its pKa, precipitation of the free base occurs (Streubel et al., 2000) an the precipitated drug is unable to diffuse out of the matrix and is not released. One reason why diffusion is occurring in verapamil hydrochloride formulations even in SIF (where it is expected to precipitate as free base) can be the presence of SGF in the matrix. Since the tablet had spent 3 hours in SGF, some of the SGF may still remain at the core of the matrix and maintain the pH value of the core low. At a low core pH, verapamil hydrochloride would exist in ionized form which would be soluble and hence its release is diffusion controlled. This explanation agrees well with reports that mention the use of organic acids such as citric or adipic acid in formulations of weakly basic drugs. These organic acids help to in maintain a low pH in the matrix core and constant drug release is achieved over wide pH range depending on type and amount of organic acid (Streudel et al., 2000).

But the dissolution data for verpamil hydrochloride in SIF has to be treated with caution because in most cases, the amont of drug released drops drastically and only about 20% of the drug remaining in the matrix is released in SIF. Thus it would be difficult to predict whether the diffusion controlled drug release would hold over the rest of the release.

Effect of filler type

Statistical analysis of the data showed that changing the filler type from lactose to avicel had a significant effect on the release of both the drugs in SGF as well as SIF (p-value of 0.0379 and 0.0376 respectively). Formulations containing avicel give slower drug release compared to

formulations containing same proportion of lactose. This can be attributed to the fact that a soluble filler like lactose will wet, dissolve and diffuse out while an insoluble filler will be held in place until the polymer dissolves or erodes (Rekhi et al., 1999). This leads to an increase in the tortuosity of the matrix thus increasing the diffusional path length for the water-soluble drug to diffuse out. On the other hand, a soluble filler like lactose decreases the tortuosity allowing for faster diffusion of the drug Williams et al., 2002).

Effect of surface area to volume ratio

The importance of surface area to volume ratio is realized when the need arises to increase the dose of a drug in a controlled release formulation while maintaining a similar release profile (Reynolds, 2002). In this study the SA/V ratio was found to have a statistically significant effect on the release of both the type of drugs in SGF and SIF (p-values of 0.0337 and 0.0095). 300mg tablets have a greater SA/V ratio compared to 600mg tablets and hence release the drug faster. The release of a soluble drug from a HPMC matrix tablet can be quantitated by the equation given by Higuchi (Ford et al., 1985b):

$W_t/W_0 = 2(S/V) (D'/\pi)^{0.5} t^{0.5}$

Where W_t = drug released in time t, W_0 = initial drug loaded into the tablet, (S/V) = surface area to volume ratio for the tablet and D' = the apparent diffusion coefficient of the drug in the hydrated matrix. .

Conclusions

The polymer level, drug level, filler type and SA/V ratio plays a statistically significant effect on the release of a weakly acidic and a weakly basic drug in both SGF and SIF. The type of drug (acidic or basic) is a significant factor on drug release in SGF but does not seem to be as important for drug release in SIF. This could be because of the solubility characteristics of the two drugs. In SGF, verapamil is freely soluble while ketoprofen is not and hence the type of drug is significant. But when the tablets are transferred to SIF, ketoprofen solubility increases. Verapamil has limited solubility in SIF but some of the SGF may still remain in the hydrated matrix and could possibly be serving as a buffering agent to decrease the microenvironmental pH and maintain adequate solubility of verapamil. Therefore, in SIF, the type of drug may not be statistically significant. As the polymer level increases, the drug release slows down considerably in both SGF and SIF since the increased polymer can maintain gel structure for longer time and therefore can retard drug release for longer time. An increase in the drug level in the formulation also slows down the drug release as the drug may help in maintaining the integrity of the gel. In case of filler, a soluble filler like lactose dissolves in the penetrating fluid creating channels in the tablet matrix which increases the drug release compared to an insoluble filler like lactose. Increased surface area to volume ratio, as in the case of a smaller tablet, increases the drug release compared to a larger tablet which has a smaller ratio.

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Table 7.1: Factors to be studied and their respective levels

Factors	Level 1	Level 2
Polymer level	-1 (Low) (20%)	+1 (High) (40%)
Drug level	-1 (Low) (8%)	+1 (High) (40%)
Drug solubility	-1 (Acidic)	+1 (Basic)
Filler type	-1 (Insoluble filler) (Avicel)	+1 (Soluble Filler) (Lactose)
SA/Vol	-1 (600mg tablet))	+1 (300mg)

Table 7.2: Five-factor, half-factorial design of experiments generated by DesignExpert6®

Run	Factor A Polymer level (%)	Factor B Drug Level (%)	Factor C Drug type	Factor D Filler Type	Factor E SA/Vol
1	20	40	Acidic	Avicel	-1
2	40	8	Basic	Avicel	+1
3	20	8	Basic	Avicel	-1
4	40	8	Acidic	Lactose	+1
5	40	40	Acidic	Lactose	-1
6	20	40	Basic	Lactose	-1
7	20	8	Basic	Lactose	+1
8	20	8	Acidic	Lactose	-1
9	40	8	Basic	Lactose	-1
10	40	8	Acidic	Avicel	-1
11	40	40	Acidic	Avicel	+1
12	20	40	Basic	Avicel	+1
13	20	40	Acidic	Lactose	+1
14	40	40	Basic	Lactose	+1
15	20	8	Acidic	Avicel	+1
16	40	40	Basic	Avicel	-1

Table 7.3: Response parameters for the sixteen formulations

Run	**M_{3hrs}** (% released in SGF)	**M_{12hrs}** (% released in SIF)
1	10.4	39.0
2	35.2	52.4
3	49.2	68.3
4	11.8	83.9
5	4.3	32.8
6	45.1	71.5
7	68.2	98.5
8	23.8	94.8
9	36.5	53.1
10	8.1	57.4
11	6.8	59.1
12	41.5	68.2
13	19.3	76.7
14	35.2	50.4
15	26.8	87.7
16	25.3	36.4

Table 7.4: Analysis of variance table (partial sum of squares) for the selected factorial model for the response M_{3hrs}

Source	SS	DF	MS	F	P
HPMC level	916.58	1	916.58	50.09	0.0001
Drug level	321.31	1	321.31	17.56	0.0019
Drug type	3161.25	1	3161.25	172.75	0.0001
Filler type	104.55	1	104.55	5.71	0.0379
SA/Vol	110.78	1	110.78	6.05	0.0337
Residual	183.00	10	18.3		
Total	4797.45	15			

Table 7.5: Analysis of variance table (partial sum of squares) for the selected factorial model for the response M_{12hrs}

Source	SS	DF	MS	F	P
HPMC level	2007.04	1	2007.04	20.65	0.0008
Drug level	1640.25	1	1640.25	16.88	0.0017
Filler type	542.89	1	542.89	5.59	0.0376
SA/Vol	954.81	1	954.81	9.83	0.0095
Residual	1068.97	11	97.18		
Total	6213.96	15			

Table 7.6: Result of fitting the dissolution data of ketoprofen formulations (in SGF and SIF) to the Higuchi equation and Ritger-Peppas equation (Figures in bold indicate the the model that best fits the data).

	Ketoprofen release in SGF			**Ketoprofen release in SIF**		
	Higuchi	**Ritger and Peppas**		**Higuchi**	**Ritger and Peppas**	
	R^2	R^2	Slope (n)	R^2	R^2	Slope (n)
1	0.9806	**0.9978**	0.7853	**0.9986**	0.9961	0.7294
4	0.9532	**0.9999**	1.0938	**0.9933**	0.9704	1.0003
5	0.949	**0.9993**	1.1622	**0.9979**	0.9838	1.0619
8	0.9742	**0.9980**	0.7721	**0.9891**	0.9795	0.7354
10	0.9535	**0.9990**	0.9852	**0.9992**	0.9879	0.9917
11	0.9419	**0.9981**	1.0579	**0.9987**	0.9858	1.1056
13	0.9769	**0.9963**	0.8444	**0.9981**	0.9944	0.6964
15	0.9745	0.9795	0.4836	**0.9962**	0.9947	0.5962

Table 7.7: Result of fitting the dissolution data of Verapamil HCl formulations (in SGF and SIF) to the Higuchi equation and Ritger-Peppas equation (Figures in bold indicate the the model that best fits the data).

	Verapamil HCl release in SGF			**Verapamil HCl release in SIF**		
	Higuchi	**Ritger and Peppas**		**Higuchi**	**Ritger and Peppas**	
	R^2	R^2	Slope (n)	R^2	R^2	Slope (n)
2	**0.9988**	0.9953	0.6512	**0.9782**	0.9610	0.1961
3	**0.9964**	0.9838	0.7458	**0.9733**	0.9591	0.1886
6	**0.9999**	**0.9999**	0.6655	**0.9922**	0.9874	0.3623
7	**0.9978**	0.9931	0.6193	**0.9964**	0.9926	0.2383
9	**1.000**	0.9970	0.7397	**0.9993**	0.9983	0.2211
12	**0.9998**	0.9989	0.6920	**0.9573**	0.9559	0.3861
14	0.9978	**0.9998**	0.7342	**0.9961**	0.9922	0.2473
16	**0.9990**	0.9982	0.7732	**0.9923**	0.9859	0.2588

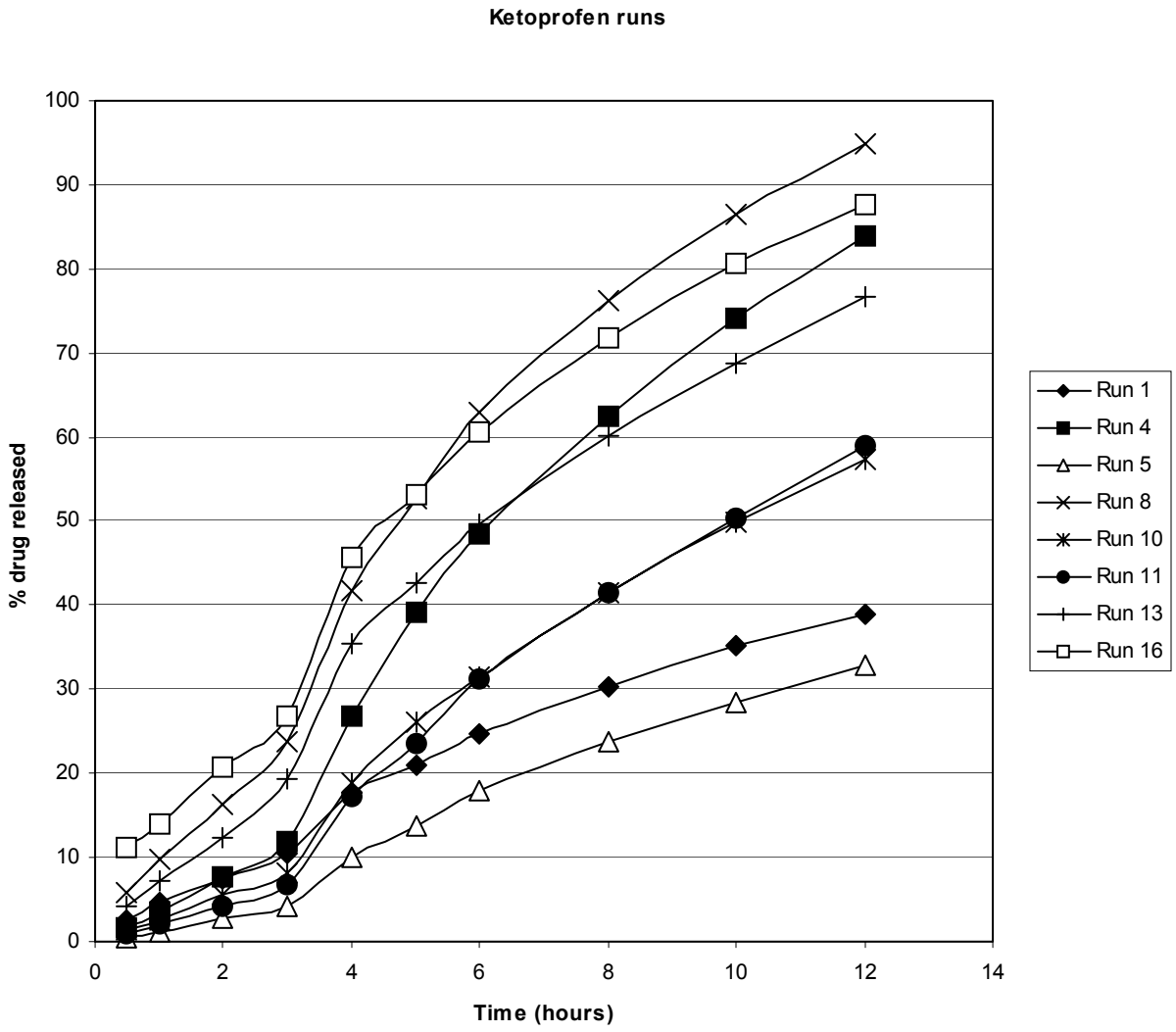

Figure 7.1: Dissolution curves for the ketoprofen runs (0-3 hours SGF, 3-9 hours SIF)

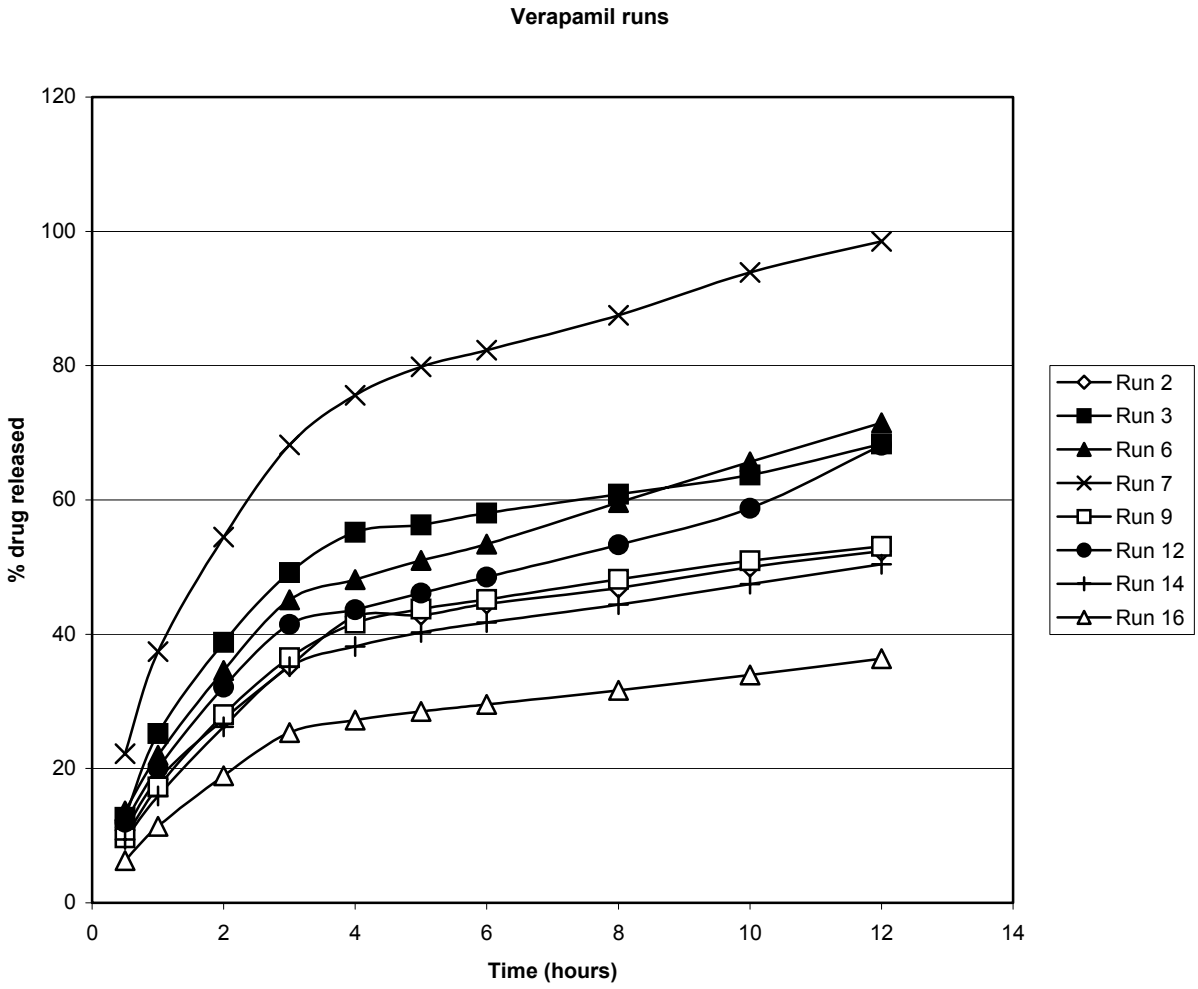

Figure 7.2: Dissolution curves for the verapamil hydrochloride runs (0-3 hours SGF, 3-9 hours SIF)