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CHANGING THE LIPOSOMAL SURFACE PROPERTIES BY MEANS OF LOW MOLECULAR WEIGHT HEPARIN

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ABSTRACT

CHANGING THE LIPOSOMAL SURFACE PROPERTIES BY MEANS OF LOW MOLECULAR WEIGHT HEPARIN

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This study was done to solve the problems of liposomes such as rapid uptake by phagocytic system and short half life in clinical practice and to be able to use them in the creation of biocompatible surfaces. In order to realize these stability of liposomes in blood should be increased and the interaction between the liposome and blood forming elements should be decreased.

In this study, MLVs containing phosphatidyl choline (PC) : cholestrol (CHOL): stearylamine (SA) in the molar ratio of 7 : 2 : 0.2 were prepared by thin film hydration method. Low molecular weight heparin (LMWH, MW : 3000) was conjugated with these MLVs by using carbodiimide (EDC). To determine the amount of heparin bound, toluidine blue (TB) method was used following column

chromotography. Moreover, infrared (IR), raman and nuclear magnetic resonance (NMR) spectra of each sample (MLV, LMWH and MLV-LMWH) were obtained. Thus it was possible to investigate the chemical changes occurred in the structure at the end of conjugation step.

Fluorescence microscopy and light scattering were used to detect the difference between the sizes of MLV and MLV-LMWH. In addition, the changes in the chemical structures of the conjugated samples were revealed by the use of elemental analysis method.

In order to study the effect of LMWH and the behavior of these three samples (MLV, LMWH and MLV-LMWH) in blood, osmotic fragility (in saline and plasma), hemolytic activity and plasma recalcification time (PRT) tests were applied and it was observed that osmotic stability and PRT's of MLV conjugated with LMWH were increased.

In the *in vivo* experiment, ^{14}C-arginine, ^{14}C-arginine containing MLV and MLV-LMWH samples were applied i.v. to three different rabbit group and it was concluded that conjugation of MLV with LMWH increases the stability and the half life of liposomes in blood.

Thus it was possible to construct liposomes that can avoid the reticuloendothelial system, carry drugs for long durations and form biocompatible surfaces.

Keywords : Liposome, MLV, conjugation, biocompatibility, biological half life

ÖZ

LİPOZOM YÜZEY ÖZELLİKLERİNİN DÜŞÜK MOLEKÜL AĞIRLIKLI HEPARİN ARACILIĞIYLA DEĞİŞTİRİLMESİ

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Bu çalışma, lipozomların klinik uygulamalarda yarı ömür kısalığı, fagosite olma gibi bazı problemlerini çözülebilmesi ve biyouyumlu (biocompatible) yüzeyler oluşturulmasında kullanılabilmesi için yapılmıştır. Bunları gerçekleştirebilmek için ilk olarak lipozomun kan içindeki dayanıklılığını arttırmak ve kanı oluşturan elementler ile etkileşimini azaltmak gerekmektedir.

Bu çalışma için 7 : 2 : 0.2 molar oranında fosfatidilkolin (PC): kolesterol (CHOL): stearilamin (SA) içeren MLV'ler ince lipid film hidrasyonu yöntemiyle hazırlandı. Bu MLV'lere karbodiimid (EDC) yardımı ile düşük molekül ağırlıklı heparin (LMWH, MW: 3000) bağlandı. Bağlanan heparin miktarının tayini için

kolon kromotografisinden sonra toluidin mavisi (TB) metodu uygulandı. Ayrıca, LMWH, MLV-LMWH ve MLV'lerin infrared (IR), raman ve NMR spektrası alındı. Böylece, konjugasyon nedeni ile kimyasal yapıda meydana gelen değişikliklerin incelenmesi mümkün oldu.

Floresan mikroskopisi ve ışık saçılma spektroskopisi (light scattering) kullanarak MLV ile MLV-LMWH büyüklüklerinin farkı gözlendi. Ayrıca elementel analiz metodu uygulanarak konjuge edilmiş MLV'deki kimyasal yapı farklılığı ortaya çıkarıldı.

LMWH'nin etkisi ve üç örneğin (MLV, LMWH ve MLV-LMWH) kandaki davranışlarını incelemek üzere ozmotik dayanıklılık (salin ve plazmada), hemolitik aktivite ve plazma rekalsifikasyon zamanı (PRT) testleri uygulandı ve bu testler sonucunda LMWH ile konjuge edilen MLV'lerin osmotik dayanıklılıklarının ve PRT'lerinin arttığı görüldü.

Hayvan deneylerinde üç tavşan grubuna ^{14}C-arjinin ile ^{14}C-arjinin içeren MLV ve MLV-LMWH i.v. olarak verildi. Sonuçta, LMWH ile konjuge edilmiş MLV'nin kan içerisindeki dayanıklılığının ve yarılanma süresinin arttığı anlaşıldı.

Böylelikle kanda retiküloendotelyal sistemden korunmayı başarabilen, uzun süreli ilaç taşıyabilecek ve biyo uyumlu yüzey oluşturabilecek lipozomların yapımı sağlanmış oldu.

Anahtar kelimeler : Lipozom, MLV, konjugasyon, biyouyumluluk, biyolojik yarı ömür.

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LIST OF ABBREVIATIONS

Chol	:	Cholesterol
EDC	:	Carbodiimide
HDL	:	High density lipoproteins.
IgA	:	Immunoglobulin A
IR	:	Infrared spectroscopy
LMWH	:	Low molecular weight heparin
MLV	:	Multilamellar vesicle
MPS	:	Mononuclear phagocytic system
MLV-LMWH	:	Multilamellar vesicles conjugated with low molecular weight heparin
NMR	:	Nuclear magnetic resonance spectroscopy
PC	:	Phosphatidylcholine
PRT	:	Plasma recalcification time test
PEG	:	Polyethylene glycol
RES	:	Reticuloendothelial system
SA	:	Stearylamine
SEM	:	Scanning electron microscopy
SH	:	Standard heparin
SUV	:	Small unilamellar vesicle
TB	:	Toluidine blue method

CHAPTER 1

INTRODUCTION

1.1. Introduction to Liposomes

Liposomes are artificially prepared spheres, consisting of concentric phospholipid bilayers separated by aqueous compartments. They are particularly attractive candidates as universal carriers for fat and water soluble species such as drugs, enzymes, immunomodulators and genetic material due to their suitable characteristics such as biodegradability, minimal toxicity, non-immunogenicity, and simplicity of use.

They were first proposed and tested twenty five years ago by A.D. Bangham, and his colleagues. They published a paper in 1965 in which they demonstrated for the first time that "the diffusion of univalent cations and anions out of spontaneously formed liquid crystals of lecithin is remarkably similar to the diffusion of such ions across biological membranes" (Bangham et al., 1965). The ability of liposomes to entrap solutes to which they were selectively permeable made the system a good model for cell membranes, and heralded a proliferation of studies on cell membrane biophysics, structure and function. This sequestration of solutes also formed the basis of the liposome drug-carrier concept that was proposed and demonstrated in the early 1970s by G. Gregoriadis and B.Ryman

(Gregoriadis, 1976). Subsequent work by Gregoriadis and colleagues established the feasibility of using the system with enzymes, anticancer and antimicrobial drugs, and also its ability to potentate immune responses to entrapped antigens (Allison and Gregoriadis, 1974). In the ensuing years, liposomes were adopted by numerous researchers as the vehicle of choice for drug and vaccine delivery and targeting. Extensive studies on the behavior of liposomes and its control within the biological environment have recently culminated in the development of several injectable liposome-based formulations that have been licenced for use in humans (Gregoriadis, 1993).

Liposomes are composed of one or more concentric lipid bilayers enclosing aqueous compartments ranging in size from 25 nm to several microns in diameter.

A broad range of amphiphilic lipids (usually phospholipids) can be used for the preparation of liposomes. Most commonly used phospholipids (Table 1.1) are positively charged long chain amines (e.g. stearylamine), long chain phosphates (e.g. dihexadecylphosphate) or negatively charged phosphoglycerides (e.g. phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine and phosphatidyl inositol). The properties of some phospholipids are represented in Figure 1.1.

When certain lipids like lecithins having suitably balanced sizes of their polar head-group and hydrophobic parts are dispersed in a water phase, they spontaneously form the liposome structure. These bilayers are permeable to molecules with sufficient hydrophobicity and to small non-electrolyte molecules with high polarity. Rate of diffusion across the membrane is dependent largely on the fluidity of the lipid bilayer. Decreasing the acyl chain length and increasing

Table 1.1. Most commonly used phospolipids in liposome preparation and their characteristics.

Lipids	Abbreviation	Charge	Tc
Egg phosphatidylcholine	PC	0	-15 to -7
Dilauryloyl phospohatidyl choline (C12:0)	DLPC	0	-1.8
Dimyristoyl phosphatidyl choline (C14:0)	DMPC	0	23
Dipalmitoyl phosphatidyl choline (C16:0)	DPPC	0	41
Distearoyl phosphophatidyl choline (C18:0)	DSPC	0	55
1-Myristoyl-2-palmitoyl phosphatidyl choline (C14:0, 16:0)	MPPC	0	27
1-Palmitoyl-2-myristoyl phosphatidyl choline (C16:0, 14:0)	PMPC	0	35
1-Palmitoyl-2-stearoyl phosphatidyl choline (C16:0, 18:0)	PSPC	0	44
1-Stearoyl-2-palmitoyl phosphatidyl choline (C18:0, 16:0)	SPPC	0	47
Dioleoyl phosphatidyl choline (C18:1)	DOPC	0	-22
Dilauryloyl phosphatidyl glycerol	DLPG	-1	4
Dimyristoyl phosphatidyl glycerol	DMPG	-1	23
Dipalmitoyl phosphatidyl glycerol	DPPG	-1	41
Distearoyl phosphatidyl glycerol	DSPG	-1	55
Dioleoyl phosphatidyl glycerol	DOPG	-1	-18
Dimyristoyl phosphatidyl ethanolamine	DMPE	-	50
Dipalmitoyl phosphatidyl ethanolamine	DPPE	-	60
Dimyristoyl phosphatidyl serine	DMPS	-	38
Dipalmitoyl phosphatidyl serine	DPPS	-	51
Brain phosphatidyl serine	PS	-	6-8
Brain sphingomyelin	BSP	0	32
Dipalmitoyl sphingomyelin	DPSP	0	41
Distearoyl sphingomyelin	DSSP	0	57

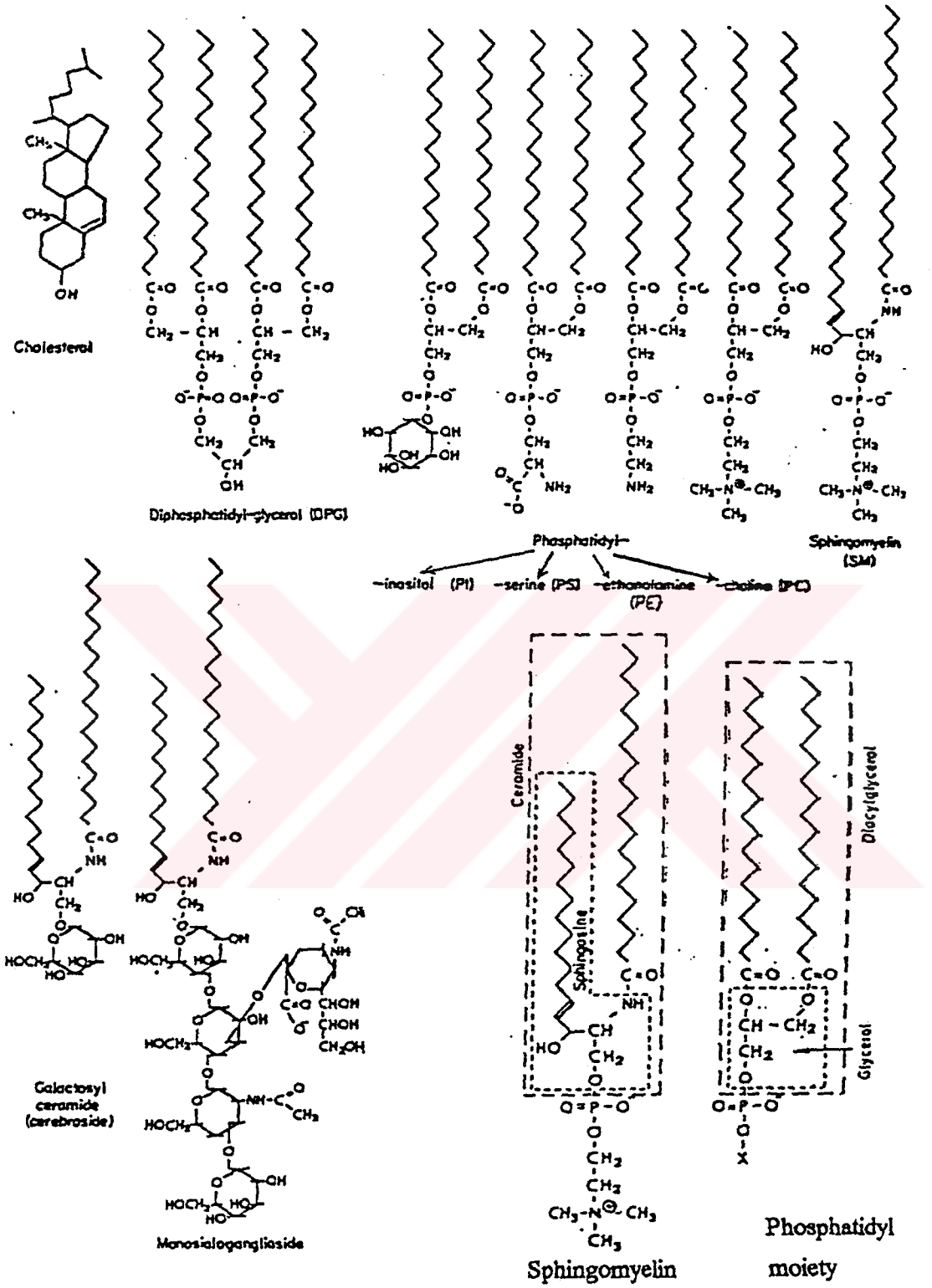


Figure 1.1. Chemical structure of some lipid molecules (from Finean et. al., 1974).

unsaturation, fluidity of the membrane can be enhanced. In contrast, cholesterol introduction reduces the fluidity and the permeation rate. Cholesterol, a sterol found in eukaryotic biological membranes, is one of the major modifiers of the phospholipid membrane structure. Gangliosides and lysolipids are examples of membrane lipids with a relatively large polar head group. When they are dispersed alone in water, micelle formation occurs instead of bilayer organization. However, the concentrations of these lipids in biological membranes are so low that the permeability characteristics is not altered drastically. In contrast, the concentrations of lipids with an opposite size balance, i.e. a small polar head group and an extended hydrophobic part are quite considerable in most membranes. Phosphatidylethanolamine belongs to this group of lipids. When dispersed in water, they adopt an inverted hexagonal phase. Incorporation of this type of lipid in the bilayer also affects the permeability of the barrier and results in increased ion permeability, trans-bilayer movement of the lipid molecules, fusion events and formation of non-bilayer domains like inverted-micelles or cubic and hexagonal phases.

At different temperatures, phospholipids can exist in different phases. The most consistently observed phase transition takes place at a particular temperature, Tc, called the "main transition temperature". This transition temperature is the minimum temperature required for the water to penetrate between the layers of the lipid molecules at which transition from the ordered "gel" state to the less ordered "liquid crystalline" state takes place. Phase behavior and fluidity of phospholipid membrane are important characteristics since they determine permeability, fusion, and aggregation, all of which can affect the stability and potential use of liposomes.

Transition temperature depends on the head group, hydrocarbon chain length, and degree and the type of unsaturation.

In general, lipids with:

i) more unsaturated chains have lower transition temperatures than more saturated ones,

ii) shorter chains have lower transition temperatures than longer ones,

iii) cis-unsaturated chains have lower transition temperatures than trans-unsaturated ones.

1.2. Application Areas of Liposomes

There are many proposed therapeutic applications that are based on successful results with experimental animal models of disease; these include: antimicrobial therapy of viral, bacterial and protozoan infections; gene and antisense therapy; enzyme-replacement therapy of inherited metabolic disorders, where substrates accumulate in tissues as a result of enzyme deficiencies; hormone replacement therapy; detoxification of tissues that contain intracellularly deposited metals; arthritis treatment; topical therapy, such as ophthalmic or skin treatment; the administration of haemoglobin-loaded liposomes as a blood surrogate (haemosomes) in emergencies where appropriate donor blood is not available; and for vaccinations (Gregoriadis, 1990).

In addition to these bioactive material release based applications, liposomes are put to use in basic research. Liposome-protein studies include proteins that are receptors, enzymes, antibodies, channels and encapsulated hemoglobin. Liposomes

are used as models of biological membranes in all of the above, and also in other biological-membrane studies, such as membrane transport processes with and without transport systems. They are also useful in investigation of physicochemical properties of lipid molecules and of lipids organized in a bilayer structure.

They are studied for several roles in immunology. Liposomes are candidates for gene transfection in two modes of interaction with the genetic material: encapsulation and complexation.

The most promising applications, however, rely either on the spontaneous uptake of liposomes by the RES (for example, in intracellular microbial infections and vaccination), or on the extended retention of small liposomes in the circulatory system for intravascular or extravascular access to cells, such as cancer or infected cells.

Table 1.2. shows the potential medical applications of liposomes with realistic expectations.

Table 1.2. Potential medical applications of liposomes with realistic expectations (Adapted from Gregoriadis, 1995)

Application	Liposome-associated drug	Route of administration	Desired key properties of liposomes	Research issues in vivo
Gene and antisense therapy	Plasmid DNA, antisense oligonucleotides	IV,IA, IM, IT, topical (oral?)	High-yield DNA incorporation; stability; controlled clearance rates; fusogenic (cationic, virosomes, pH-sensitive); targeted	Fate after administration (stability, clearance rates, cellular and subcellular distribution); control of duration of expression; mode of action; role of cationic charge; toxicity; immunogenicity of DNA and virosomes; comparison with viral vectors
Blood surrogate	Haemoglobin (Hb), synthetic oxygen transporters	IV	High yield Hb entrapment; good oxygen dissociation curve for Hb; acceptable viscosity; negative surface charge (?); stability, long-circulating	Stability; clearance rates; effect on blood (e.g. prothrombin time); heamodynamic blood gases and respiration data
Immunomodulation	Endogenous cytokines (e.g. IL, IFN, G-CSF, GM-CSF, TNF); exogenous products (e.g. LPS, lipid A trehalose diesters, MDPs)	IV, IM, SC, IT	High-yield incorporation; stability; controlled clearance rates; targeted	Targeting to immunocompetent cells (T and B lymphocytes, NK cells, PMN cells, mononuclear phagocytes); interaction with cell receptors and significance of entrapment versus vesicle surface coupling of cytokines

Table 1.2. (cont'd)

Desensitization	Allergens and allergen extracts	IM, SC, oral (?)	High yield allergen entrapment; proportional entrapment of all allergens in extracts; stability	Extent of IgE reduction and IgG increase; threshold dosage for immunological effect; safety of liposomal allergens (avoidance of anaphylactic shock)
Lung therapeutics (e.g. cancer, microbial infections asthma, cystic fibrosis)	Anticancer and antimicrobial drugs, anti-asthma agents, plasmid DNA	IT	Stable (in terms of drug retention) in aerosols; efficient vesicle formation with entrapped drug from fluorocarbon blends; efficient vesicle formation with entrapped drug from powders on rehydration in the respiratory tract	Levels of local drug concentration within the respiratory tract; effect of mucus layers and mucociliary clearance on vesicle penetration in the treatment of airways epithelium, vesicle protection of entrapped drugs from inhibitors in the bronchial mucus

Abbreviations: G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte/monocyte colony stimulating factor; IA, intraarterially; IFN, interferons; IgE, immunoglobulin E; IgG, immunoglobulin G; IL, interleukins; IM, intramuscular; IP, intraperitoneal; IT, intratracheal; LPS, lipopolysaccharide; MDPs, muramyl dipeptide and derivatives; SC, subcutaneous; TNF, tumour necrosis factor.

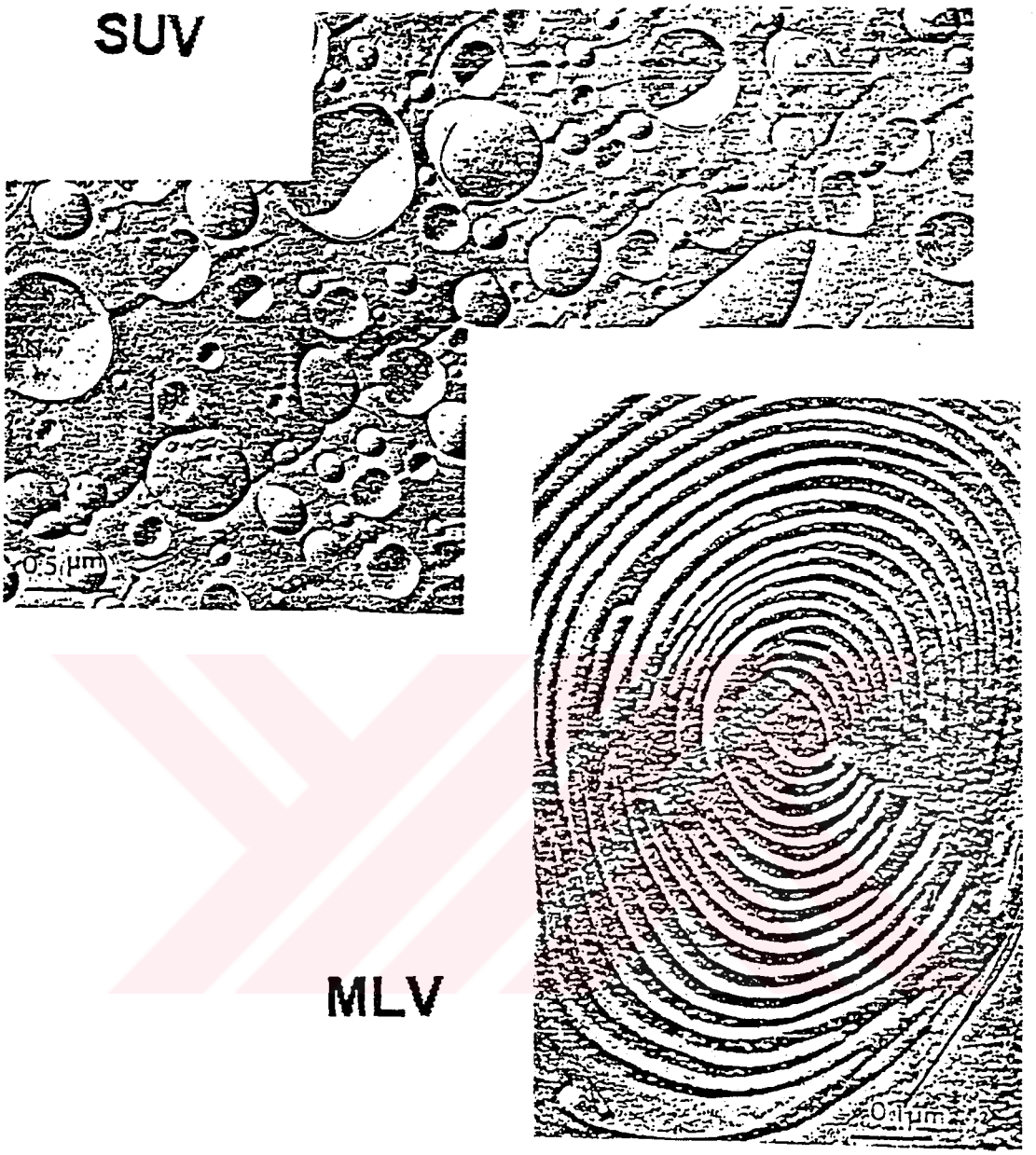
1.3. Classification of Liposomes

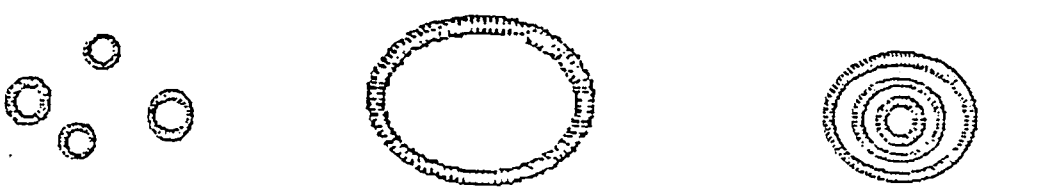
Liposomes can be classified according to their number of lamellae, size, and preparation method. The most common approaches to classification of liposomes in current use are based on the number of lamellae (Figure 1.2). The three major types of liposomes are: small unilamellar vesicles (SUV), multilamellar vesicles (MLV), and large unilamellar vesicles (LUV).

Small unilamellar vesicles (SUV) can vary in diameter from about 20 nm to 50 nm. They consist of a single lipid bilayer enclosing the aqueous phase. The small size, relatively narrow size distribution, and high surface to volume ratio of SUVs make them a good choice for some applications, especially if there is no need for efficient encapsulation of water soluble solutes. Since their circulation times in blood are longer compared to the other forms, they are considered to be a more suitable carrier for targeting in vivo.

Multilamellar vesicles (MLV) have a population of vesicles with a heterogeneous size distribution with diameters ranging from 170 to 5000 nm. They consist of alternating, concentric aqueous and lipid bilayer like an onion skin. Due to presence of several lipid bilayers, usage of MLVs is very advantageous when lipophilic materials are to be entrapped.

Large unilamellar vesicles (LUV) have a single lipid bilayer and diameters in the order of 1000 nm. They are particularly well suited for efficient encapsulation of water-soluble solutes because of their favorable aqueous volume to surface ratio.





SUV	LUV	MLV
20-50 nm	1000 nm	170-5000 nm

Figure 1.2. Types of liposomes

They are also appropriate for reconstitution and other studies of membrane permeability and fusion.

Liposomes can also be found in the following groups (Mayer et al., 1986; New, 1990): oligolamellar vesicles (OLV), medium-unilamellar vesicles (MUL) (>100 nm), giant unilamellar vesicles (GUV).

1.4. Preparation of Liposomes

Preparation methods of liposomes can be said to involve three or four basic stages: drying down of lipids from organic solvents, dispersion of lipids in aqueous media, purification of the resultant liposomes, and the analysis of the final product (Figure 1.3).

The different methods of preparation and characteristics of the resultant liposomes are shown in Table 1.3.

Hydration of dried phospholipids results in the spontaneous formation of multicompartment liposomes (MLVs). The method, developed by Bangham in 1965, involves the swelling of a thin lipid film. The film is deposited on the wall of a round bottom flask by rotary evaporating a chloroform stock solution of the phospholipids. Removal of the lipid film from the wall of the round bottom flask is accomplished by handshaking or by vortex mixing. Addition of glass beads facilitates this process.

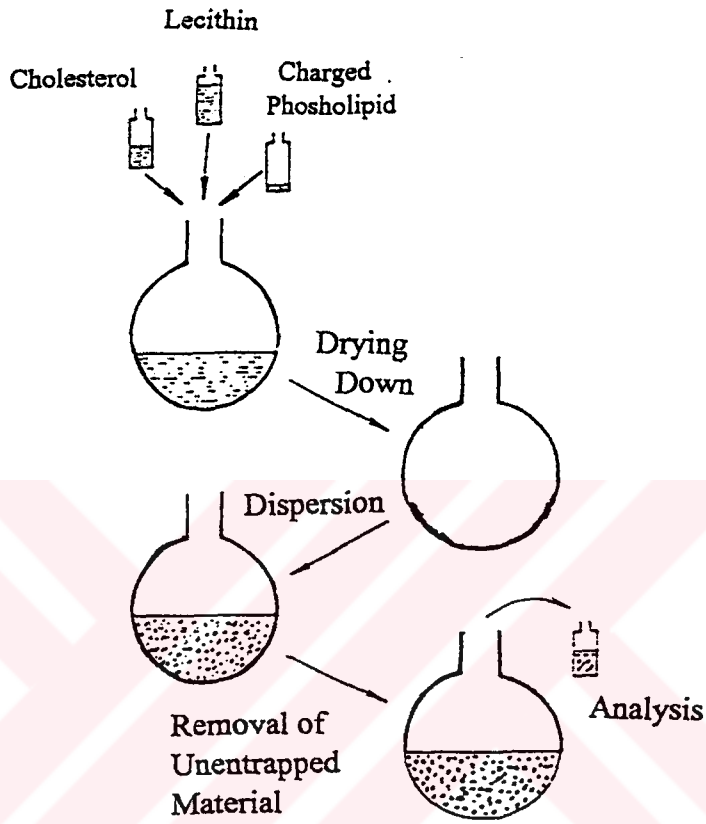


Figure 1.3. Stages common to all methods of preparation of liposomes (There are various intermediary steps depending on the specific method used)

Multicompartment liposomes (MLVs) of defined size and homogeneity have been prepared by extrusion through polycarbonate membranes (Olson et al., 1979). Liposome solutions, prepared in the usual way, were extruded through polycarbonate membranes with pore diameters of 1.0, 0.8, 0.6, 0.4, and 0.2 μm, under pressure. This process has to be carried out, however, below the phase transition temperature since above the phase transition temperature liposomes are not filterable (Brendzel and Miller, 1980).

MLVs have also been prepared by the introduction of an aqueous buffer into an ether solution of the phospholipid, followed by the removal of the organic solvent (Szoka and Papahadjopoulos, 1978). The proposed mechanism involves the collapse of reversed micelles into a gel-like state on the removal of the organic solvent, and this results in the assembly of bilayers.

Single-compartment liposomes (SUVs) can be formed by sonication (Huang, 1969). Either probe or a bath type sonicator can be used. The temperature in both types of sonication is maintained slightly above the phase transition temperatures of the lipid. Degradation of lipid through oxidation is prevented by carrying out the process in a nitrogen atmosphere. SUVs have also been prepared by ethanol injection (Batzri and Korn, 1973), French press extrusion (Barenholz et al., 1977), and detergent dialysis (Razin, 1972).

Slow swelling of a lipid film method can be used for the formation of large single-compartment bilayer liposomes (LUVs) (Reeves and Dowben, 1970). Another method is the controlled injection of an ether solution of phospholipids into warm aqueous solutions (Deamer and Bangham, 1976). Uniformity is achieved by membrane and gel filtration.

LUVs can also be formed by detergent removal technique (Ostro, 1983). The procedure involves formation of mixed micelles by dispersing the phospholipid and surfactant in an aqueous solution. Then, the mixed micelles are diluted to below critical micelle concentration (CMC) for the particular detergent by fast controlled dialysis, centrifugation or filtration.

Calcium induced fusion is another technique for preparation of LUVs (Papahadjopoulos et al., 1975). The procedure is initiated with the addition of calcium to the preformed SUVs causing fusion of SUVs into the cochleate structures. These structures are then transformed into LUV with the addition of chelating agent EDTA.

The technique known as reverse phase evaporation was developed by Szoka and Papahadjopoulos in 1978. In this method, the lipid in a mixed aqueous-nonpolar solution forms inverted micelles and then the nonpolar solvent is evaporated under vacuum. As a result, LUVs are formed.

Table 1.3. Preparation of Liposomes (adapted from Fendler, 1982)

Method of Preparation	Liposomes Formed	Advantages of Method	Disadvantages of Method	Key Reference
Handshaking or vortexing thin lipid film with aqueous solution	Multicompartment heterodisperse, diameter= 1800-8000 Å	Ease of preparation, large entrapped volumes	Nonuniformity precludes basic studies	Bangham et al., 1965; Kinsky, 1974
Extrusion of liposomes through polycarbonate membranes	Multicompartment	Defined size and homogenity, large entrapped volumes	Need of special equipment	Olson et al., 1979; Szoka et al., 1980
Introduction of an aqueous buffer into an ether solution of the phospholipid	Multicompartment	Entrapped volumes are larger than vortex liposomes	Difficult to control injection rate	Szoka and Paphadjopoulos, 1978; Schieren et al., 1978
Ultrasonic dispersal of lipid	Mostly single compartment, diameter=ca.250Å	Relative ease of preparation	Possible degradation, small entrapped volumes, need to remove titanium fragment, inhomogenity	Huang, 1969
Injection of an alcoholic phospholipid solution through a small bore syringe into thermostated water	Single compartment, diameters range between 150 and 500Å, depending on lipid concentration in alcohol	No degradation, fair degree of homogenity	Difficult to control injection rate, ethanol needs to be removed	Batzri and Korn, 1973; Kremer et al., 1977
Dilution of phospholipid detergent micelles by gel filtration or dialysis	Single-compartment, diameter = 150-250Å	No degradation, homogeneous liposomes, commercial equipment available	Detergents and rate of dialysis need to be controlled	Razin, 1972; Brunnert et al., 1976; Korenbrot, 1977; Milsmann et al., 1978; Rhoden and Goldin, 1979; Zumbuehl and Weder, 1981.

Table 1.3. (cont'd)

Method of Preparation	Liposomes Formed	Advantages of Method	Disadvantages of Method	Key Reference
Injection of lipid dispersion through the small orifice of a Power laboratory (French) press	Single compartment, diameter = 315-525Å	No degradation, high degree of reproducibility, large entrapped volumes	Special equipment needed	Barenholz et al., 1977, 1979; Hamilton et al., 1980
Slow swelling of a lipid film	Large, predominantly single-compartment, diameter = 0.5 - 1.0 μm	Possible to entrap large molecules	Difficult to control hydration rate	Reeves and Dowben, 1969, 1970
Slow swelling of protein containing lipid film	Large, predominantly single-compartment, diameter = 0.5 - 1.0 μm	Suitable model for reconstituted membranes	Inhomogenity, difficult to control rate of hydration	Darszon et al., 1980
Controlled injection of an ether solution into a warm aqueous solution	Large, predominatly single-compartment, diameter = 0.1-1.0 μm	Possible to entrap large molecules	Difficult to control injection rate	Deamer and Sangham, 1976; Deamer, 1978; Schieren et al., 1978
Removal of organic phase under reduced pressure from water-in-oil (w/o) microemulsions of lipids and buffer (REV)	Large, predominantly single-compartment, diameter = 0.2 - 09 μm	Possible to entrap large macromolecules	Exposure of material to be encapsulated to organic solvents	Szoka and Papahadjopoulos, 1978
Addition of Ca^{2+}, EDTA to single-compartment liposomes	Large spiral cylinders, diameter= 2000-10000Å	Possible to entrap large molecules	Needs to be characterized	Papahadjopoulos et al., 1975.

1.5. *In Situ*, *In Vitro* and *In Vivo* Stability of Liposomes

Liposomes have gained acceptance as potential drug carriers since they have many advantages, especially for the transport of water-soluble drugs, over other drug delivery systems. Several basic problems, however, must be overcome before a liposomal drug carrier can be employed in clinical practice. Greater stability must be attained both to achieve longer storage periods and longer half life in the circulation after in vivo administration.

Many recent reports have demonstrated that rapid uptake of liposomes in vivo by cells of the mononuclear phagocytic system (MPS) has restricted their therapeutic utility (Gregoriadis and Ryman, 1972; Beaumier and Hwang, 1983). The uptake is accomplished largely through a scavenger function of cells of the mononuclear phagocytic system (MPS) located in several tissues, originally known as the reticuloendothelial system (RES) which functions as an important host defense mechanism. Uptake by these cells usually results in sequestering the drug into the MPS, advantageous for diseases of these tissues or agents which are slowly released back into the blood. However, it mostly leads to irreversible accumulation and degradation. In addition, there is toxicity of these cells and/or acute impairment of their functions.

These events caused considerable efforts to be made to identify, critically address and evaluate the major limitations of liposomes for pharmaceutical applications. These limitations include rapid clearance from blood, inability to extravasate from the blood, restricted control of encapsulated molecule release, low or nonreproducing drug loading and physical and chemical instability.

Plasma, which normally accounts for 55 % of the blood volume is an aqueous solution containing several hundred different ions, molecules and macromolecules. Several studies have demonstrated that liposome stability in plasma depends on the relative concentrations, liposome size and lamellarity, lipid composition, and the incubation temperature (Jones and Nicholas, 1991; Scherphof and Wilschut, 1981; Scherphof and Morselt, 1984).

The interaction of liposomes with various proteins may involve simple ionic and/or hydrophobic interactions which lead to protein adsorption onto the liposome surface or its penetration into the bilayer. In the absence of MPS cells, these two types of interactions normally do not have much influence on liposome integrity. The latter one, however, and lipid exchange between liposomes and other particles, especially high density lipoproteins (HDL), can result in the loss of encapsulated or bound molecules, liposome disintegration or both. Although most lipoproteins may be involved in interactions with liposomes, the major interaction occurs with HDL, which is probably the most important for liposome disintegration *in vitro*. The net transfer of phospholipid molecules to HDL is greatly enhanced by a lack of cholesterol in the liposomes and by plasma phospholipid transfer proteins. After incubation with liposomes, liposomal lipid is found associated with the HDL.

Opsonins play an important role in enhancing the uptake of those particles through binding on the surface of foreign particles and bound opsonins are recognized specifically by the receptors on the surface of phagocytes. Principal opsonins and dysopsonins (which inhibit the uptake) for liposomes were reviewed intensively by Patel such as IgG, complements, fibronectin and IgA (Patel, 1992).

When target cells are in the RES (for example, in the case of a number of intracelluar microbial and storage diseases), the only criterion for successful drug delivery is that liposomes quantitatively retain their load while they are circulating. The problem of HDL-induced destabilization of bilayers has been resolved by manipulating their composition, for instance, by the addition of excess cholesterol and/or by using phospholipids with a high gel-liquid-crystalline transition temperature (Tc) (Gregoriadis, 1995; Gregoriadis and Davis, 1979; Allen, 1981). Both approaches reduce HDL action considerably, and circulating vesicles are, therefore, able to retain entrapped solutes quantitatively. However, as the RES (particularly the liver and spleen) takes up most of the injected liposomes, targeting to alternative cells requires strategies for delaying the involvement of the RES.

The stability of liposomes with a diameter ≤ 100 nm in blood at 37 °C appears to be directly related to their retention in the circulatory system, i.e. the more stable the liposomes (in terms of solute retention), the greater their half life. This can be explained by assuming that with decreasing HDL-promoted destabilization of the vesicle surface, there is a corresponding reduction in opsonin adsorption; this, in turn, dictates the rate of vesicle recognition and uptake by the RES (Gregoriadis, 1995).

Also the other factors, such as phospholipid composition, surface charge, and liposome size play an important role for liposomal stability in biological fluid.

A number of liposome physical properties have been found to influence their clearance. One of the most important is lipid composition. In the case of lipid composition, phase transition temperature of the phospholipid component and cholesterol content have the most profound influence. Highly saturated phospolipids

(with a high phase transition temperature) in combination with cholesterol, give rise to rigid bilayers. They exhibit slower clearance from blood when compared with unsaturated acyl forms of the same lipid (with a low phase transition temperature).

Particle size is also an important parameter influencing blood clearance. Generally, increasing particle size increases in vivo uptake (Woodle, 1988).

Finally, surface charge plays a role in blood clearance. The results indicate a general increase in clearance of the negatively charged liposomes. This negative charge has been attained by incorporation of a variety of lipids: PA, PS, PG, gangliosides, DCP and cardiolipin (Senior, 1987). However, positively charged phospholipids such as stearylamine (SA), DOTMA and BisHOP may decrease the blood clearance (Tan and Gregoriadis, 1989).

1.7. Modifications

Biocompatible surfaces, i.e., protein nonadsorptive and cell nonadhesive surfaces, are gaining increased attention recently. A common feature of all these materials is the need to minimize protein and/or cell adsorption.

It is well known that liposomes are rapidly cleared from circulation mainly due to two processes:

i) Lipid exchange with high-density lipoproteins (HDL), leading to liposome disintegration,

ii) Opsonization followed by macrophage uptake.

A hypothesis on the molecular mechanism of long-circulating liposomes put forth that stabilization results from local surface concentration of highly hydrated

groups that sterically inhibit both electrostatic and hydrophobic interactions of a variety of blood components at the liposome surface (Lasic and Papahadjopoulos, 1991).

Many approaches based on surface modification have been considered to decrease interaction with biological components and thereby increase the blood half life in the circulation. Some of the earliest attempts explored methods of coating liposomes with natural products: proteins, polysaccharides, and glycoproteins (Torchillin, 1980; Saito, 1988).

Recently, methods described for coating latex based colloidal particles with amphipathic polymers have been applied to lipid stabilized emulsions and liposomes (Senior, 1987; Woodle, 1992). The results indicated that these polymers do absorb and in some cases give rise to coatings similar to those obtained with latex. However, decreased retention of aqueous entrapped components was observed limiting this approach for drug delivery.

Moreover, many other approaches have been tried in order to minimize the adsorption. For example, surfaces were coated with various polymers by covalent grafting, block copolymerization with the base copolymer, direct adsorption or interpenetration (Sunamoto, 1986; Illium, 1987; Troster, 1990). The most widely studied polymers used in were polyvinylpyrrolidone, polyethyloxazoline, polyethylene glycols, block copolymers such as poloxamine or polymethylmethacrylate (PMMA) (Desai, 1991; Prime, 1991). Their efficiency was measured by their ability to minimize interactions with biological materials such as adhesion of fibroblasts, whole blood perfusion, and thrombus formation.

New formulation of liposomes has been developed by introducing ganglioside G_{M1} (Allen et al., 1987) and hydrogenated phosphotidyl inositol (Gabizon and Papahadjopoulos, 1988) to prolong the half-life up to 12 h after intravenous administration.

In 1990, a major advance was achieved when it was realized that inclusion of certain lipids in the bilayer can effectively prolong blood circulation times of liposomes. The first approaches were based on mimicking the outer composition of red blood cells. The next step was achieved by inclusion of phospholipids with a synthetic hydrophilic polymer head group. This work was done with proteins, on polyoxyethylene (or polyethylene glycol, PEG) chains with molecular weights from 120 to 5000 derivatized to PE (PEG-PE). They provide a hydrophilic shelter around the external layer of liposomes hindering the attachment of plasma proteins to its surface. Because of their reduced recognition and capture by cells of the MPS, these liposomes have been referred to as "Stealth Liposomes". PEG liposomes prolonged the circulation half-lives regardless of membrane fluidity or surface charge.

Reduced MPS uptake restores much of the original promise of liposomes and opens up entirely new applications: from drug delivery systems with dramatically changed pharmacokinetics and biodistribution to more realistic models of biological membrane surfaces (Ringsdorf, 1988) and red blood cells, cell to cell, protein to cell, and receptor interactions. In parallel with rapid development for pharmacological applications such as liposomes containing bioactive peptides (Mayhew, 1992) and anticancer drugs (Papahadjopoulos, 1991) show encouraging preclinical results.

1.7. Heparin: Properties and Uses

Heparin is a naturally occurring polysaccharide present in tissues of such diverse species as mammals and molluscs. It has a molecular weight ranging from 4000 and 30000 daltons. It is composed of repeating units of uronic acid, containing an 0-sulfate group at the C-2 position, and D-glucosamine, usually N-sulfated with an additional 0-sulfate group at C-6 (Figure 1.4).

The property of heparin which has attracted most attention and resulted in its widespread clinical use is its anticoagulant activity. Due to its unique structure and surface charge distribution, heparin is able to interact strongly with several different proteins in a specific manner. The significance of heparin's biological activities, especially its interaction with blood-clotting factors and human serum lipoproteins, has made this glycosaminoglycan the focus of many investigation (Chung, 1976).

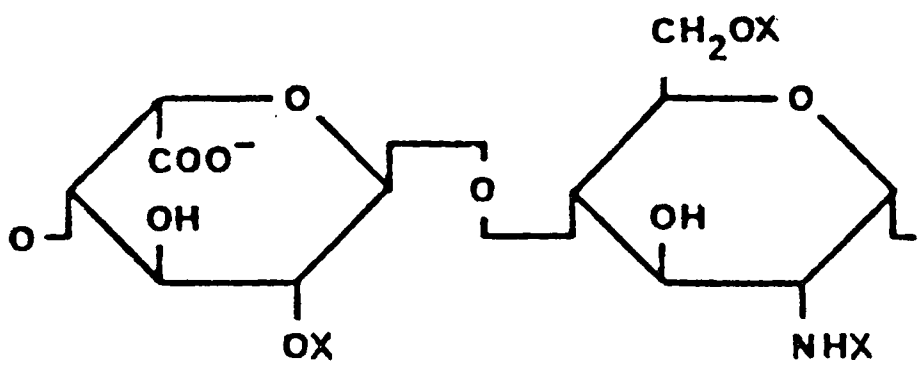


Figure 1.4. Structure of heparin (X= H, SO_3^-)

Heparin has a broad range of other biological activities, such as stimulation of angiogenesis, antibacterial and antiviral activity, and inhibition of tumor growth.

Low molecular weight heparins (LMWH) are fragments of commercial grade heparin produced by either chemical or enzymatic degradation. LMWH's developed commercially have mean molecular weights which vary from 3000 to 6500 daltons. Interest in LMWH's as potential antithrombotic agents came from the observations in the mid 1970s that LMWH fractions prepared from standard heparin (SH) progressively lose their ability to prolong the activated partial thromboplastin time but retain their ability to inhibit factor Xa (Johnson et al., 1976; Andersson et al., 1976). They are effective and safe antithrombotic agents and produce less bleeding than the standard heparin from which there were derived (Andriuoli, 1985). Normal plasma neutralizes the anti-factor Xa activity of standard heparin (SH) but not of smaller molecular weight fractions of heparin because SH binds to a number of plasma proteins. The binding of SH to these proteins contributes to its reduced plasma recovery (bioavailability) at low concentrations. LMWH's have much lower affinity for these proteins than SH. An observation could explain the superior bioavailability of LMWH's at low doses and the more predictable anticoagulant response of LMWH's. SH also binds to endothelial cells and macrophages, while LMWH's do not bind to endothelial cells in culture. This feature contributes to the longer plasma half life of LMWH's.

Table 1.4. Potential advantages of LMWH's over standard heparin (adapted from Green et al., 1994).

Potential advantage of LMWHs	**Proposed mechanisms for observed effect**
LMWHs have more stable dose response and, therefore, do not require laboratory monitoring.	LMWHs do not bind to heparin-binding proteins.
LMWHs have better bioavailability when injected subcutaneously in low doses.	LMWHs bind less to heparin-binding proteins.
LMWHs have longer plasma half-life	LMWHs bind less to endothelial cells and matrix proteins.
LMWHs associated with a lower incidence of heparin-induced thrombocytopenia.	LMWHs bind less to platelets.
LMWHs produce less bleeding for equivalent antithrombotic effect.	LMWHs bind less to platelets.

In short, LMWH's differ from SH in a number of important ways: they have different molecular weight distribution profiles, different rates of plasma clearance and different recommended dosage regimens. LMWH's also share properties which give them a number of advantages over SH (Table 1.4): they have longer plasma half life than SH and their anticoagulant response to weight-adjusted doses is less variable. Finally LMWH's produce less bleeding than SH.

1.8. The Approach in This Study

The presence of cholesterol, gangliosides and poly(ethylene glycol) has proved to reduced the rate of HDL and opsonin interactions, resulting in enhanced circulation times. It is well established that the beneficial effect of PEG or gangliosides on prolonging half-life is due to the fact that they provide a hydrophilic shelter around the external layer of liposomes hindering the attachment of plasma protein to its surface. Following this assumption, the possibility of using a polysaccharide was explored for this study to provide same effect. Coating the liposomal surface with a naturally occurring polysaccharide derivative such as low molecular weight heparin is another approach to decrease uptake of liposomes and increase their circulation time. This technique of polysaccharide-coating is more favorable for the larger sizes of liposomes which therefore more conveniently encapsulate a large amount of water soluble drugs in the interior water pool than do the smaller liposomes. Therefore, such polysaccharide-coated liposomes were considered to be very good candidate for use as a targetable cancer therapy. In this conjugation method, water soluble carbodiimide (1-ethyl-3[3-dimethylaminopropyl]-

carbodiimide) can be used to achieve conjugation of LMWH with liposome. Figure 1.5 shows the mechanism of one step carbodiimide activation of carboxyl group of heparin for binding to amino group (coming from stearylamine) carrying liposome.

Since it is known that liposome characteristics such as ingredients, size, charge and method of preparation also play an important role in the plasma clearance rate, the type and the ingredients of the liposomes were chosen according to these criteria. For example, the rate and extent of localization of liposomes in mononuclear cells of liver and spleen is dependent on liposome composition. Since the most important factor is the bilayer rigidity that renders liposomes more resistant to the destabilizing effects of serum components, in particular HDL, cholesterol was used. Also phosphotidyl choline (PC) was chosen as the main component of the liposome due to its neutral charge and chemical inertness. Finally, as a third component, positively charged stearylamine (SA) was used. Because of two reasons it was chosen as a final component of liposome:

i) Negatively charged liposomes are removed more rapidly from circulation than neutral or positively charged liposomes.

ii) According to the conjugation of LMWH with liposome procedure amino group carrying phospholipid was required.

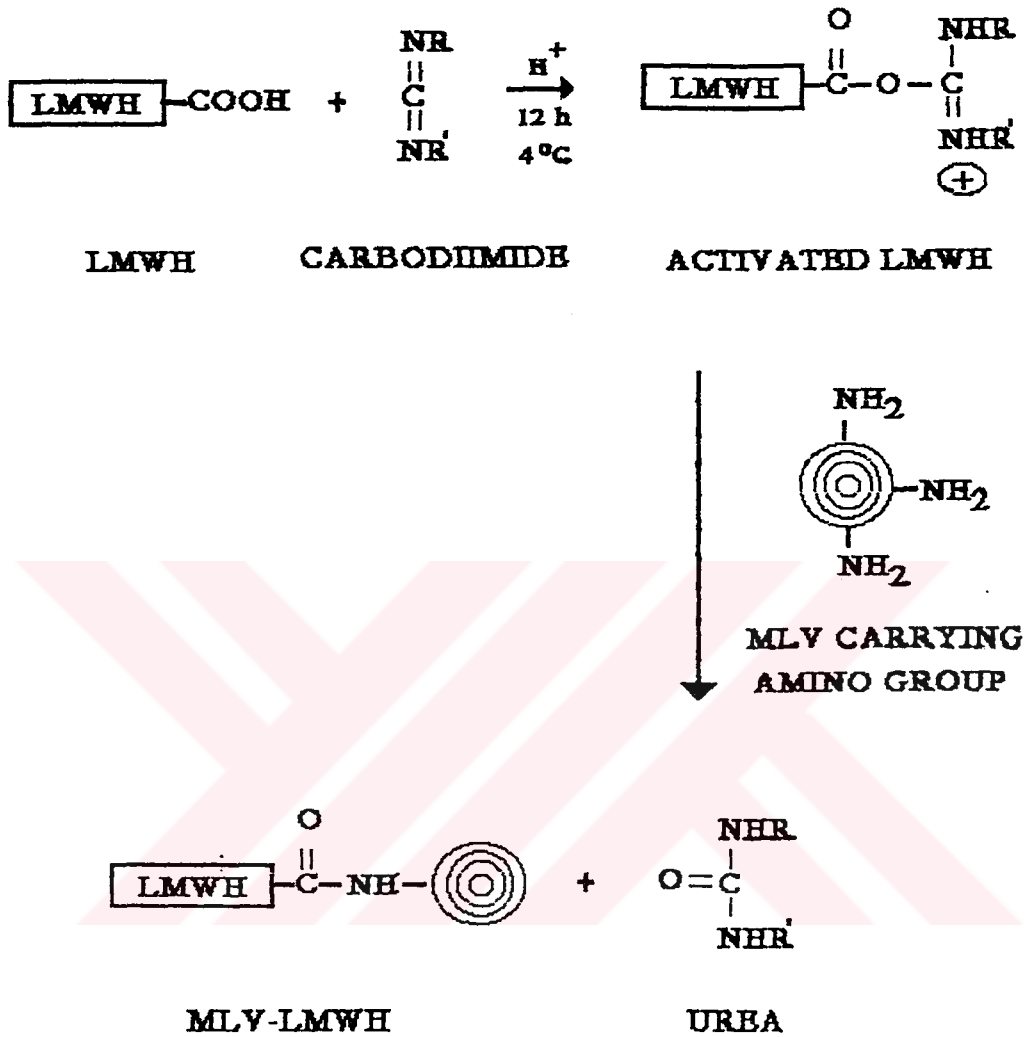


Figure 1.5. Carbodiimide conjugation method

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Cholesterol (5-cholesten-3β-ol) and positively charged stearylamine (octadecylamine) (SA) were purchased from Sigma Chemical Company (U.S.A) and used without further purification.

Lecithin (1,2-diacyl-sn-glycero-3-phosphocholine) (from egg yolk) was obtained from E. Merck (Germany) and used without further purification.

Sephadex G-25, Sephadex G-50, and dialysis sacks (cellulose tubing 25 cm long x 10 mm inflated diameter) were obtained from Sigma Chemical Company (U.S.A) and swollen in excess phosphate buffer (100 mM, pH: 7.4) before use.

Sodium chloride (NaCl), potassium hydroxide (KOH), hydrochloric acid (HCl), potassium dihydrogenphosphate (KH_2PO_4), chloroform and methanol were products of E.Merck (Germany) and used without further purification.

POPOP (1,4-bis [2-(5-phenyloxazolyl)] benzene) and PPO (2,5-diphenyl-oxazole) were obtained from Sigma Chemical Company (U.S.A).

^{14}C-L-Arginine HCl (0.8 mL 50μ Ci, 324 mCi/mmole) was purchased from Amersham (Japan).

Low molecular weight heparin: Sodium salt (from bovine intestinal mucosa,

molecular weight: 3000), carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (EDC), potassium bromide, glutaraldehyde (25%), FITC-dextran (MW: 4400), oil red O and potassium ferricyanate were products of Sigma Chemical Company (U.S.A).

Calcium chloride, toluidine blue, diammonium oxalate monohydrate, dipotassium oxalate monohydrate, n-decane and toluene were obtained from E. Merck (Germany).

2.2. Methods

2.2.1. Preparation of Liposomes

2.2.1.1. Preparation of MLVs

Multilamellar vesicles (MLVs) were prepared by employing the thin film hydration method. In this method phosphotidylcholine (PC) (600 μL, 116.2 mM in chloroform), cholesterol (CHOL) (600 μL, 33.2 mM in chloroform), and stearylamine (SA) (600 μL, 3.32 mM in chloroform and methanol 1 : 1) were mixed in a 50 mL round bottom flask in a molar ratio of 7:2:0.2. After flushing with nitrogen gas, the organic solvents were evaporated by using rotary vacuum evaporator (Bibby Sterilin, (U.K), Model RE-100). Any residual organic solvent was removed by flushing with nitrogen gas. After formation of a thin lipid film, phosphate buffer (3.5 mL, 100 mM, pH 7.4) was added into the flask and MLV formation was achieved by vortex mixing. The MLV was then used immediately

in the further stages of the study.

2.2.1.2. Preparation of Oil Red O Loaded MLV

Oil red O (10 mM) in chloroform was prepared and 0.1 mL of this solution was placed into the round bottom flask with the other phospholipids before the evaporation step (see section 2.2.1.1). After formation of the thin lipid film, FITC-dextran (1 mL, 1 mM, MW: 4400, fluoresces with λ_{ex} = 492.8 nm and λ_{em} = 515.2 nm) and phosphate buffer (2.5 mL, 100 mM, pH 7.4) were added into the flask. MLVs were prepared by vortex mixing. The free oil red O and FITC-dextran molecules were removed by centrifugation at 15000 rpm for 30 min in a Sorvall, (USA), Model RC-5B refrigerated superspeed centrifuge. The MLV pellets were taken and resuspended in phosphate buffer (3 mL, 100 mM, pH 7.4) before use.

2.2.1.3. Preparation of Potassium Ferricyanate Loaded MLV

Potassium ferricyanate (3.5 mL, 100 mM in 0.85 % w/v saline solution) was added into the round bottom flask containing thin lipid film. After preparation of the liposome, the free potassium ferricyanate molecules were removed by dialysis in saline solution (20 mL, 0.85 % w/v) overnight.

2.2.1.4. Preparation of Radiolabeled MLV

After preparation of thin lipid film as in section 2.2.1.1, ^{14}C-L-arginine HCl

(0.2 mL, $2.7.10^{-4}$ mCi/mmole) and saline solution (2.8 mL, 0.85% w/v) were added into the flask and MLV formation was completed by vortex mixing. The free (unentrapped) L-arginine molecules were removed by dialysis for 1 h. in saline (0.85 % w/v) solution.

2.2.1.5. Preparation of SUVs

a) Via Probe Sonication

After obtaining the MLV suspension by the thin film hydration method as described in section 2.2.1.1, it was sonicated in ice under nitrogen atmosphere with an ultrasonic homogenizer (Cole Parmer Instrument Co., (USA), Model 4710) at 20 W for 15 min with 30 s intervals (Figure 2.1). Titanium particles released from the probe of the ultrasonicator were removed by centrifugation (IEC Clinical Centrifuge, USA) at 4000 rpm for 20 min.

B) Via Bath Sonication

MLV suspension was sonicated in a bath sonicator (Branson, (USA), Model 2200 E4) at a frequency of 47 kHz, at 15 °C, under atmospheric pressure, for 50 min. (Figure 2.1)

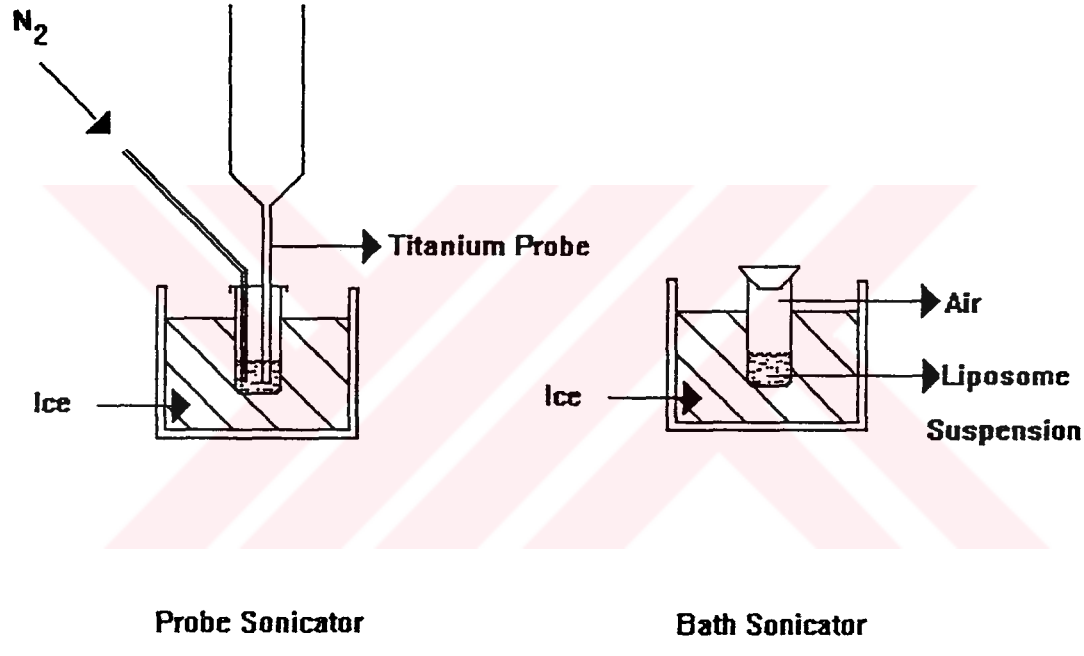


Figure 2.1. Preparation of liposomes by different methods of sonication.

2.2.1.6. Preparation of Mono-Size Liposomes

A) Column Chromatography

SUV suspension, prepared by either bath or proble sonication, was applied to a column (inner diameter 1.2 cm, 37 cm in length) to obtain an approximately equal size liposome population. Preswollen Sephadex G-50 was used as column packing material and equilibrated with (100 mM, pH 7.4) phosphate buffer. Elution rate was 1mL/min. Fraction collector (LKB HeliRac 2212) was used for collected eluents. Elution absorbances were determined by a UV-Visible spectrophotometer (Shimadzu, (Japan), Model 2100S) at 410 nm in order to determine the liposome presence.

B) Minicolumn Chromatography

A minicolumn (inner diameter: 2 cm and length : 10 cm) was packed with Sephadex G-50. Then, 1 mL of SUV-LMWH suspension was applied to it. Eluate (3.25 mL) was placed in to a dialysis sack and dialyzed in phosphate buffer (100 mM, pH 7.4) overnight. Content of the dialysis sack was taken and centrifuged in a centrifuge (IEC Clinical Centrifuge, USA) at 4000 rpm, for 5 min. Both pellet and supernatant were subjected to toluidine blue test.

2.2.2. Preparation of Conjugated Liposomes (MLV-LMWH or SUV-LMWH)

The pH of LMWH solution (1 mL, 0.33 mM) was adjusted to 4.75 with 1M HCl. Carbodiimide (EDC) (73 μL, 0.68 mM) was added to this solution and incubated for 6 h. at 4°C. MLV or SUV suspension (described in section 2.2.1.1 and section 2.2.1.5) was mixed with this solution, the container was stoppered and incubated overnight, at 4°C. For the elimination of free (unconjugated) LMWH molecules, dialysis against saline solution (0.85 % w/v) was carried out for 3 h.

2.2.3. Physicochemical Investigation of MLV and LMWH Conjugation

2.2.3.1. Toluidine Blue Colorimetric Method

A number of methods are known for the determination of the heparin in solution. Toluidine blue metachromatic shift assay is one of the most suitable methods for this purpose because other methods to directly determine heparin conjugated with liposome are limited or not applicable. Using this approach, the concentration of heparin in the reaction mixture prior to conjugation is compared to the free heparin amount left in the supernatant and wash solutions after conjugation. The difference is taken as the amount bound.

In this method, toluidine blue binds to the polysaccharide, makes a complex with it and undergoes a color change which is accompanied by a shift in the absorbance spectrum to lower wavelengths. The absorbance maximum of a dilute

toluidine blue solution in water occurrs at approximately 631 nm. (Smith, 1980).

Toluidine blue method was applied to two heparin containing samples.

i) Unreacted low molecular weight heparin (LMWH),

ii) Low molecular weight heparin conjugated with small unilamellar vesicles (SUV-LMWH)

For the determination of conjugated LMWH, SUV-LMWH (3 mL) was mixed with (2 mL, 0.001 M) toluidine blue. After 1 min, n-decane (2 mL) was added into this solution for the extraction of unreacted, excess toluidine blue and this was mixed with a vortex mixing for 2 min. After extraction, the absorbance in the visible range was determined at 631 nm with a UV-Visible spectrophotometer (Shimadzu, (Japan), Model 2100S) in order to determine the amount of conjugated LMWH.

The same procedure was applied to the aqueous phase of the centrifuged contents of the dialysis sack and to the dialysate prepared in secton 2.2.1.6/B.

A calibration curve was prepared in the 10 μg and 100 μg LMWH range and was used in converting the absorbances into concentrations and eventually to conjugated LMWH (Appendix A).

2.2.3.2. Infrared Spectroscopy

IR is the measurement of the amount of radiation absorbed by the molecules within the infrared region of the electromagnetic spectrum. The IR spectrum of a compound provides data on the amount of energy required to increase the vibrational energy levels. It is useful in the detection of bonds and therefore the

groups consitituting a molecule.

In this study, the following liposomal samples were prepared and studied with IR spectroscopy.

i) MLV (prepared as described in section 2.2.1.1),

ii) MLV-LMWH (prepared as described in section 2.2.2),

iii) LMWH (MW : 3000)

MLV and MLV-LMWH suspensions were centrifuged in a Sorval RC-5B refrigerated superspeed centrifuge for 30 min. at 15000 rpm. Pellets of MLV and MLV-LMWH were mixed with potassium bromide and after grinding them, KBr pellets were prepared. Finally, infrared spectra were obtained by using an infrared spectrophotometer (Philips, (UK), Model PU9700).

2.2.3.3. Raman Spectroscopy

In raman spectroscopy, pure rotational and vibrational-rotational frequencies of molecules can be studied. The reason for this is that a portion of the scattered light undergoes a wavelength change and this can be detected at angle perpendicular to that of incident light. Since vibrations and rotations which might not be active in the IR may be active in raman and therefore this method provides molecular data of a similar type, but largely complementary to that of IR.

Gases, liquids and solids can be studied by raman spectroscopy. Solids can be measured with a special holder which allows the solid samples to be suspended in the center of the laser beam.

This was applied to the samples given in section 2.2.3.2. The Raman was

obtained with a Nicolet (Model 910) which uses a Yag type Nd: YVO4 laser. The sampling geometry used was 180 degrees. Laser power was up to one watt.

2.2.3.4. Nuclear Magnetic Remonance Spectroscopy (^{1}H NMR)

A nucleus is composed of protons and neutrons, and both these nucleons have intrinsic angular momenta or spins, and therefore act as elementary magnets. In most nuclei these spins add to give a nonzero resultant nuclear spin. When a nucleus with spin is placed in a magnetic field, the magnetic moment precesses about the field direction and its component in the direction of the field is a measure of a definite energy. This consitutes the basis of NMR spectroscopy. It provides us characteristics of atoms and their arrangements in complex molecules. Interpretation of NMR spectra is usually feasible in greater detail than is the case for IR or ultraviolet spectra.

Same procedure (section 2.2.3.2) was applied to prepare the samples which were resuspended in deuterated water and NMR spectroscopy was carried out by using a Bruker Avance, Model DPX 400, NMR spectrometer.

2.2.3.5. Fluorescence Microscopy

MLV and MLV-LMWH loaded with oil red O were prepared as described in section 2.2.1.2.

A drop of each sample was placed on the microscope slide and covered with a coverslip where a drop of immersion oil was placed on for x 1000 magnification.

The samples were photographed by an Olympus, (Japan), Model BX50 with PM20 camera. Fujichrome 200 ASA film and B (Blue) filter mounted on an Olympus microscope were used.

2.2.3.6. Scanning Electron Microscopy (SEM)

MLVs and MLV-LMWHs were prepared as described in section 2.2.1.1 and in section 2.2.2, a drop of each was put on the coverslip and dried under vacuum. Then, one drop of oxalated blood was added onto each of these slides and also onto an clean slide to serve as positive control. After waiting for 10 min., the three samples (MLV with blood, MLV-LMWH with blood, only blood) were immersed into glutaraldehyde (8%) for fixation. After 5 min, they were removed and left to dry at room temperature. Finally, they were coated with gold and SEM was carried out in a Leitz (Germany), Model AMR 1000 SEM.

2.2.3.7. Elemental Analysis

MLV and MLV-LMWH were prepared according to sections 2.2.1.1 and 2.2.2 respectively, and centrifuged in a Sorvall, (USA), Model RC-5B centrifuge at 15000 rpm for 30 min. at 4°C. The C, H, N, O contents were determined with a Laco, Model CHNSO-932 Elemental Analyzer.

2.2.3.8. Light Scattering

Light scattering is used to determine the diameters of relatively homogenous populations with an error of up to ±20 %. The kind of errors inherent to this method make it more sensitive to changes in samples such as might occur upon surface adsorption or storage.

In this study, MLV and MLV-LMWH were prepared in phosphate buffer (100 mM, pH 7.4) filtered through cellulose nitrate membrane filters (pore size: 0.1 μm, diameter : 25 mm, MFS micro filtration systems, Japan). Then, the mean radius of both MLV and MLV-LMWH were determined by a Dawn B Laser Photometer (Wyatt Technology Corporation, USA). The configuration of specific calibration constant was 1.304 e^{-4} and the wavelength was 632.8 nm.

Five solutions having the following concentrations of the samples were used: 100, 200, 300 mg/L.

2.2.4. In Situ Liposome Stability Tests

2.2.4.1. Osmotic Fragility in Saline

After the formation of thin phospholipid film (see sections 2.2.1.3), potassium ferricyanate (3 mL, 100 mM) was put into a 50 mL flask and MLV was formed after vortex mixing. Potassium ferricyanate loaded MLV-LMWH were also prepared in this fashion followed by LMWH conjugation.

After the formation of potassium ferricyanate loaded MLV and MLV-

LMWH, samples were dialyzed against saline solution (0.85%) overnight to eliminate the unentrapped potassium ferricyanate. Different saline concentrations were prepared (Table 2.1) for both MLV and MLV-LMWH solutions.

A constant amount of liposome suspension (3 mL) was put into each tube and different amounts of saline solution (0.85 % w/v) and distilled water were added. Each test tube was incubated for 1 h. to produce the effect of various saline concentrations on the liposomes.

The samples were then centrifuged at 15000 rpm for 30 min. and the absorbances of the supernatant were determined by UV Visible spectrophotometer (Shimadzu (Japan), Model 2100S) at 420 nm. As a blank, 0.85% saline solution was used.

The amount of retained potassium ferricyanate was calculated from a calibration curve constructed at 420 nm. (Appendix B).

2.2.4.2. Osmotic Fragility in Plasma

Potassium ferricyanate loaded MLV and MLV-LMWH solutions were prepared as described in section 2.2.1.3

Fresh human blood was put into centrifuge tubes containing an anticoagulant solution prepared by dissolving diammonium oxalate monohydrate (0.15 g.) and dipotassium oxalate monohydrate (0.1 g.) in distilled water (5 mL). They were centrifuged in a clinical centrifuge (IEC, USA) at 4000 rpm, for 20 min. Supernatant (plasma) was taken and different concentrations were prepared using (100 fold diluted) plasma according to Table 2.1. Into each test tube, 0.3 mL of

liposome (7 tubes of MLV and 7 tubes of MLV-LMWH), and different amounts of plasma and distilled water were added and incubated for 1 h. All samples were centrifuged in a Sorvall RC-5B at 15000 rpm for 30 min. and absorbances were determined at 420 nm. The blank consisted of plasma and distilled water without liposome suspension.

Table 2.1. Composition of the samples used in the osmotic fragility test.

Salinity (%, w/v)	**Liposome (mL)**	**Saline (mL)**	**Distilled Water (mL)**
0.042	0.3	0.0	5.7
0.085	0.3	0.3	5.4
0.170	0.3	0.9	4.8
0.340	0.3	2.1	3.6
0.500	0.3	3.2	2.5
0.680	0.3	4.5	1.2
0.850	0.3	5.7	0.0

2.2.5. In Vitro Liposome Stability Tests

2.2.5.1. Hemolytic Activity Test

Oxalated blood, MLV and MLV-LMWH were prepared according to sections 2.2.4.2, 2.2.1.1 and 2.2.2; respectively. The only difference in the preparation of liposomes was the use of saline (0.85 % w/v) during the dialysis step instead of phosphate buffer (100 mM, pH 7.4)

As described in Figure 2.2, a constant amount of oxalated blood (0.2 mL) (prepared as described in section 2.2.4.2) was put into four test tubes. 3 mL of isotonic saline, distilled water, MLV, and MLV-LMWH were added into the tubes separately. All tubes were incubated at 37°C for 1h. and centrifuged at 3000 rpm for 20 min. to observe hemolytic activity of each solution.

Absorbances in the visible region were determined at 545 nm to detect the hemoglobin relased. Then percent hemolytic activity was calculated for each solution by subtracting the absorbance of the negative control and taking the absorbance of distilled water as 100 %.

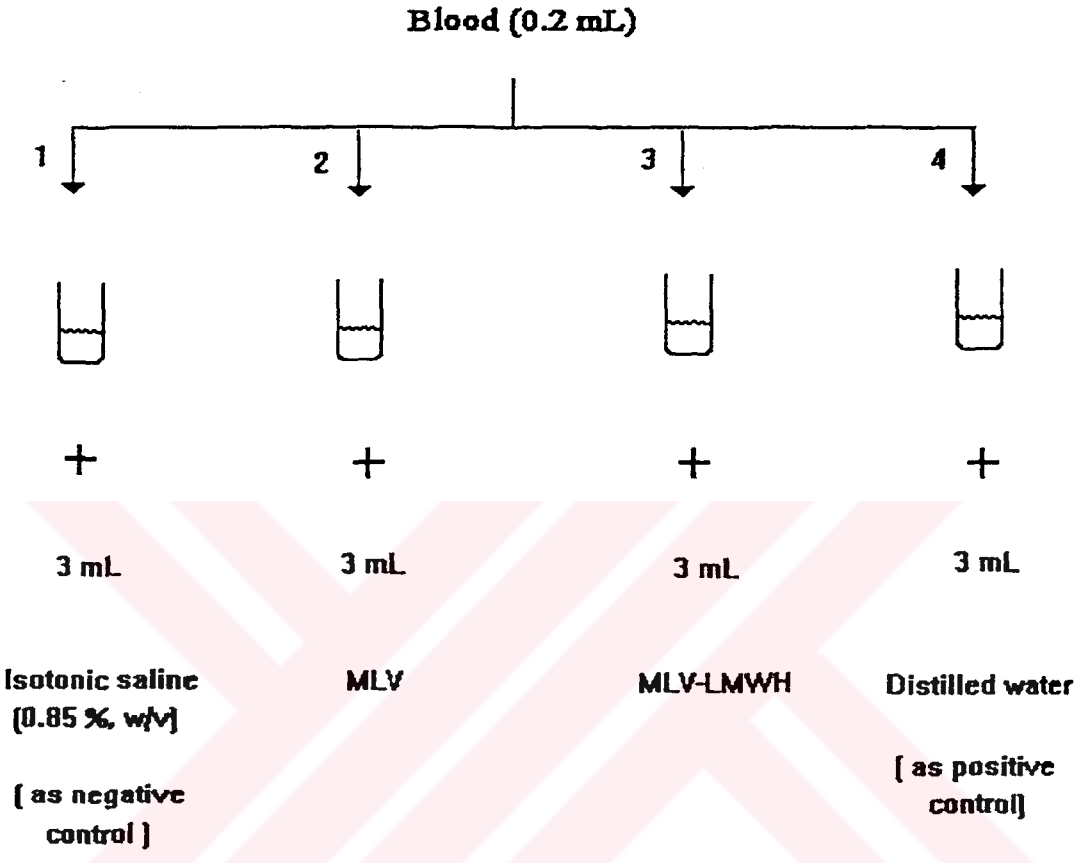


Figure 2.2. Scheme for the hemolytic activity test

2.2.5.2. Plasma Recalcification Time (PRT) Test

One drop of MLV and MLV-LMWH was put onto each coverslip, separately. Nothing was added onto the other coverslip (positive control). After drying, these three coverslips were then put into three different test tubes and 1 mL human plasma (prepared as described in section 2.2.4.2) was added onto each test tube (Figure 2.3). After waiting for 5 min, $CaCl_2$ (1 mL, 0.25 M) was added into each test tube. The test tubes were tilted gently each minute until formation of clumps was observed and time to clot was noted.

2.2.6. In Vivo Liposome Stability Test : Radiolabeled Amino Acid, ^{14}C-L-Arginine, in the Circulatory System of the Rabbit

For this test, three samples were prepared:

i) MLVs loaded with a radiolabeled compound, ^{14}C-L-arginine HCl (prepared as described in section 2.2.1.4),

ii) MLV-LMWHs loaded with ^{14}C-L-arginine HCl (as in section 2.2.1.4),

iii) ^{14}C-L-arginine HCl.

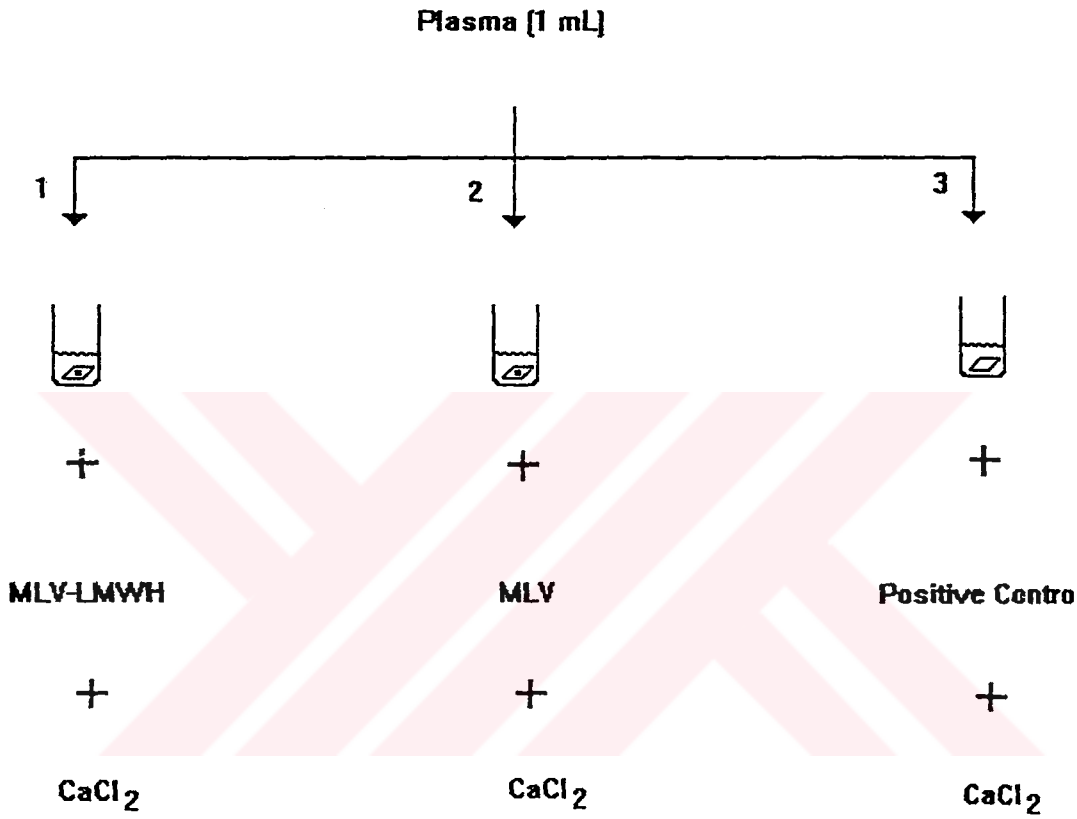


Figure 2.3. Preparation of plasma recalcification time test.

An aqueous scintillant, containing POPOP (1,4-bis[2-(5-phenyl oxazolyl benzene)] (0.48 g), PPO (2,5-diphenyl-oxazole) (0.002 g) in toluol (100 mL) was prepared for the counting of radioactivity emitted by the aminoacid.

In order to determine the radioactivity in the samples, 50 μL of MLV loaded with radioactive aminoacid, MLV-LMWH loaded with radioactive aminoacid and free radioactive aminoacid solutions were put into 20 mL vials and the volume was brought to 20 mL with the aqueous scintillant. The radioactivity was measured by a liquid scintillation counter (LKB, (UK), Model Wallac 1209 rackbeta).

1 mL of each radioactive sample was introduced intravenously to the ears of three different rabbits (New Zealand wild type, obtained from Tavuk Yetiştirme ve Geliştirme Çiftliği, Ankara). After about 30, 45, 60 and 90 min (Table 2.2), 1 mL blood was drawn from the ears of the rabbits, 100 μL of which was put into a vial, volume was brought to 20 mL with the aqueous scintillant and the radioactivity due to the β emitted was measured.

Table 2.2 Blood sample collection from rabbits

Sample collection time (min) after introduction of ^{14}C-L- Arginine into the rabbits		
MLV	MLV-LMWH	^{14}C-L-Arginine
15	25	28
50	63	48
80	85	75
113	115	105

CHAPTER 3

RESULTS AND DISCUSSION

Liposomes have gained a wide acceptance as potential drug carriers because they have many advantages over other drug delivery systems. However, several fundamental problems, such as the rapid uptake of liposomes by cells of the mononuclear phagocytic system (MPS), rapid clearance from the blood and their short half life in *in vivo* must be overcome before a liposomal drug carrier can be employed in clinical practice. Viable approach to overcome some of these problems may be to change the surface properties of the liposomes by coating the liposomal surface with molecules (e.g. PEG, low molecular weight heparin) that will enable them to avoid MPS.

In this study, liposomes were conjugated with LMWH in order to increase their biocompatibility and circulation time. The extent of conjugation was investigated by using methods like UV/Visible, IR, Raman and NMR spectroscopies as well as by microscopic methods like Fluorescence Microscopy and SEM. Moreover, Elemantal Analysis and Light Scattering methods were also used for this purpose. The behavior of the conjugated liposomes in the biological fluid was studied with *in situ* (Osmotic Fragility in saline and in plasma), *in vitro* (Hemolytic Activity and Plasma Recalcification Time), and *in vivo* tests (Clearance of a Radiolabeled amino acid from the circulatory system of rabbit).

3.1. The Extent of Conjugation of Liposomes with LMWH

The extent of conjugation was determined by using both spectroscopic and microscopic methods. Other physicochemical analysis methods like light scattering and elemental analysis were also applied.

3.1.1. Toluidine Blue Colorimetric Method

In this method, the amount of bound LMWH was compared with standard LMWH solutions to determine the concentration of LMWH in the solution.

Firstly, SUVs were prepared by either bath (at 47 kHz, 15°C, for 50 min., under atmospheric pressure) or probe (at 20 W, for 15 min. with 30 second intervals, at 4°C, under atmospheric pressure) sonication of MLVs. Bath sonication is more advantageous than probe sonication since in the former there is the risk of an interference of titanium particles relased from the probe.

In order to obtain a liposome population with a narrow size distribution, SUV solution was applied to column (diameter 1.2 cm, length 37 cm) packed with preswollen Sephadex G-50 (equilibrated with phosphate buffer pH 7.4, 100 mM). When SUVs were applied to the column, they eluted in the void as expected from their size. SUV-LMWH, however, was found to be minimal in void. It was found to have localized at the top 1 cm of the G-50 beads. Then, the packing material was changed to Sephadex G-25 beads and this time like the SUV, SUV-LMWH also came in void volume too.

Dialysis of pellet after centrifugation in the minicentrifugation method

(section 2.2.1.6/B) followed by toluidine blue test proved that the pellet definitely contains LMWH. The dialysate contained unbound LMWH (also shown by toluidine blue application). From this result, it was deduced that binding of LMWH to SUV was succesfully achieved.

The calibration curve prepared for LMWH was valid with unbound heparin, but a deviation was observed with the liposome samples possibly due to binding with the liposome (metachromic effect reported in the literature Smith et al., 1980).

Conjugation of LMWH with liposome prevented the agglomerate to go through Sephadex G-50 beads but it went through (in void) in G-25 (Figure 3.1). This could be a result of size increase but also of an interaction between the LMWH bound on the liposome and the column packing material.

Due to observation of aggregation of liposomes at the end of the conjugation step, separation with centrifugation was tried instead of chromotography. Due to extremely high g's required for precipitation of SUVs, it was decided to use MLVs instead.

It was decided that toluidine blue is good quantitative indicator of LMWH but it can not be used for quantitative measurements in the presence of liposomes.

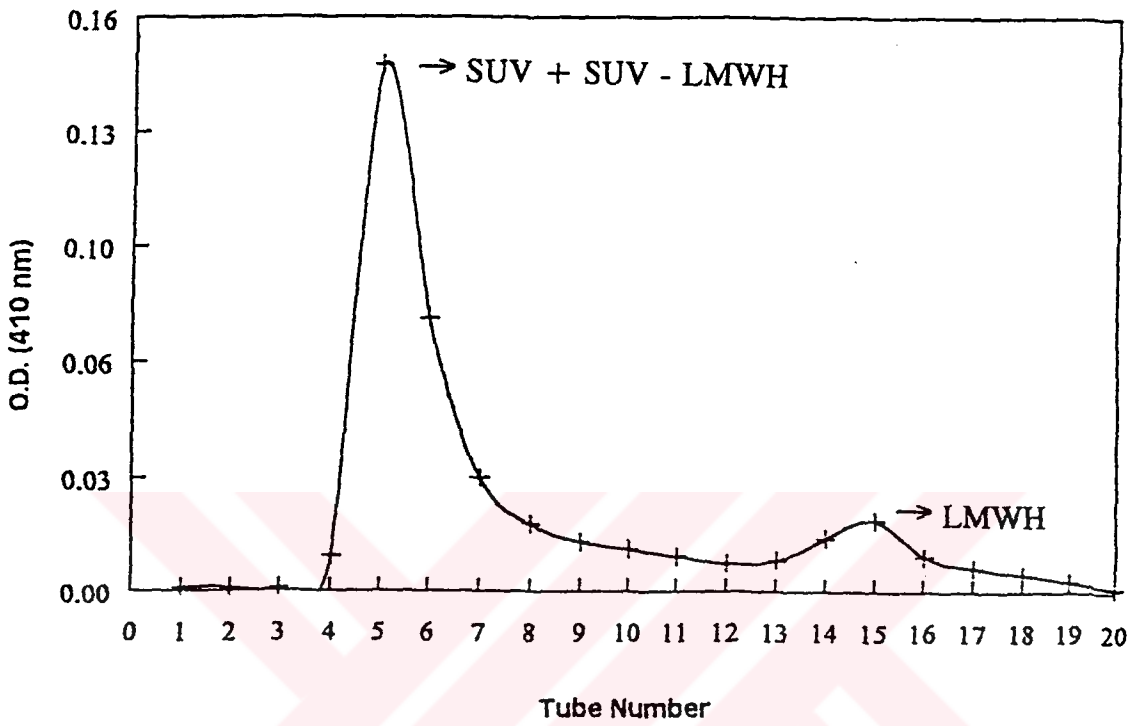


Figure 3.1. Elution profile of chromotographic separation products. Column length was 37 cm and diameter was 1.2 cm. It was packed with sephadex G-25 and elution rate was 1mL/min. Each tube contained 3 mL sample. Absorbances of each were determined at 410 nm.

3.1.2. Infrared Spectroscopy (IR)

The IR spectrum of a compound provides data on the presence or absence of certain structural features in the molecule. In this study, the structural differences of the conjugated liposome (MLV-LMWH) from the unconjugated one (only MLV) were determined. In order to achieve this, three samples (MLV, LMWH, MLV-LMWH) were analyzed.

In Figure 3.2 when the IR spectra of the three compounds were examined, it was observed that IR spectra of MLV-LMWH consisted mainly of liposome peaks. Very little effect of LMWH was seen. This presence was observed in the intervals 1100-1200 cm^{-1} and 1500-1600 cm^{-1}. Since the peaks between 1600-1550 cm^{-1} represent the presence of amide, from here it was deduced that the amino group of MLV was lost in MLV-LMWH and amide formation occurred because of the conjugation step (Figure 1.5). Also the disappearance of the peak in 1100 cm^{-1} represents the amide formation. These results give us a clue about the achievement of the conjugation of liposomes with LMWH.

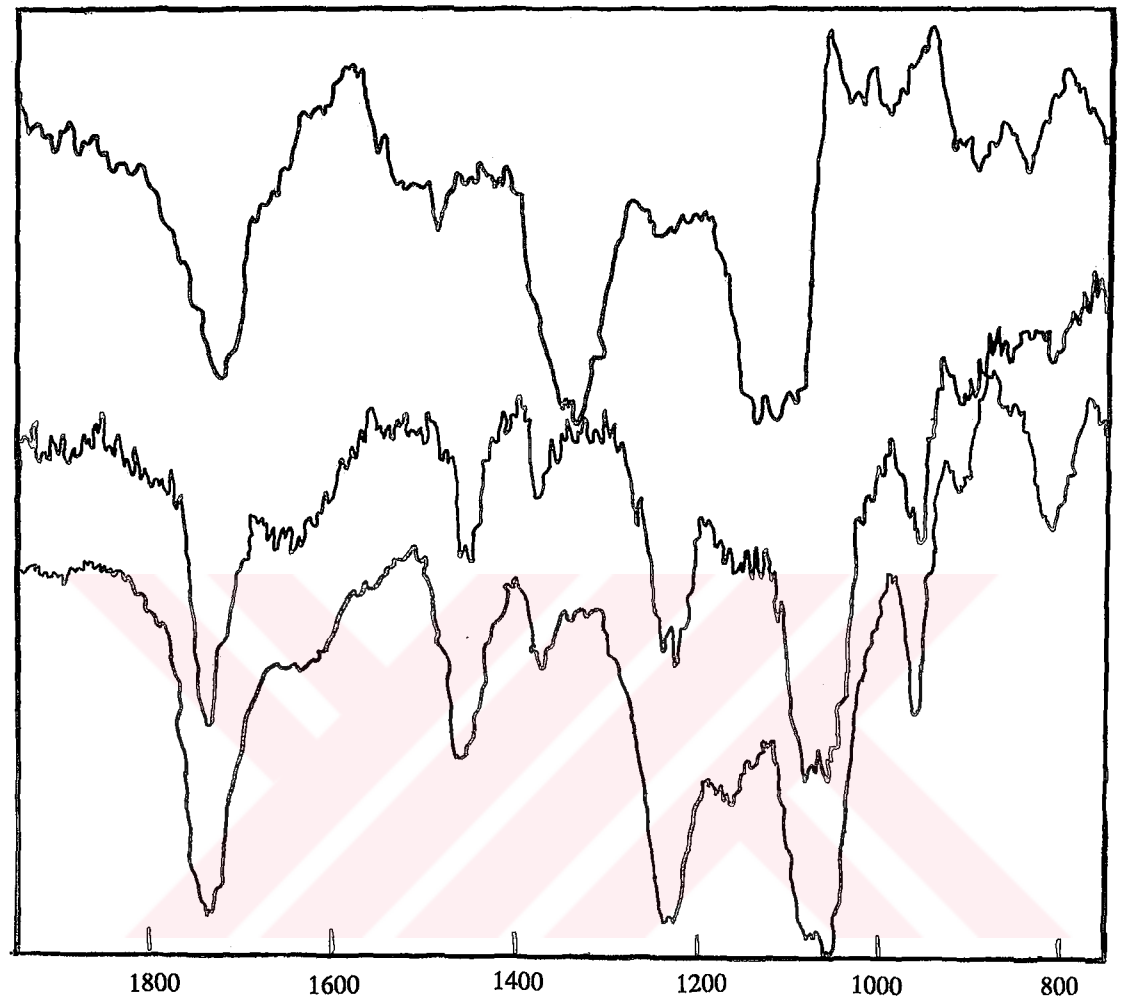


Figure 3.2. IR spectroscopy

3.1.3. Raman Spectroscopy

Raman spectroscopy provides molecular data of a similar type, but largely complementary to that of IR. In order to eliminate water interference faced with in IR spectroscopy, raman spectra of the MLV, LMWH and MLV-LMWH samples were taken and analyzed.

From Figure 3.3 and 3.4, the differences between MLV and MLV-LMWH can be seen from in the proportion of the peak sizes at 1295 cm^{-1}, 1440 cm^{-1} and 1656 cm^{-1}.

If the amide I (1668 cm^{-1}) is due to carbonyl group presence, in a MLV this could be due to the C=O in the phospholipids (fatty acid). The presence of the peaks in MLV and in MLV-LMWH at quite similar locations and the low degree of conjugation make explanation of the change in the peaks in raman spectra extremely difficult. For example, both have C=O, NH and C-N groups. The changes one has to look for are in amide formation and in introduction of sulfur. Sulfur peaks are at very low wave numbers so they are not available. The relative decrease in the peak at 1664 cm^{-1} with respect to the peaks at 1450 cm^{-1} and 1350 cm^{-1} could be due to creating more C-N linkages (due to the amide formation), while the amount of C=O and N-H is not significantly changed because they are present in almost equal quantities in both phosphatidyl choline as well as in LMWH.

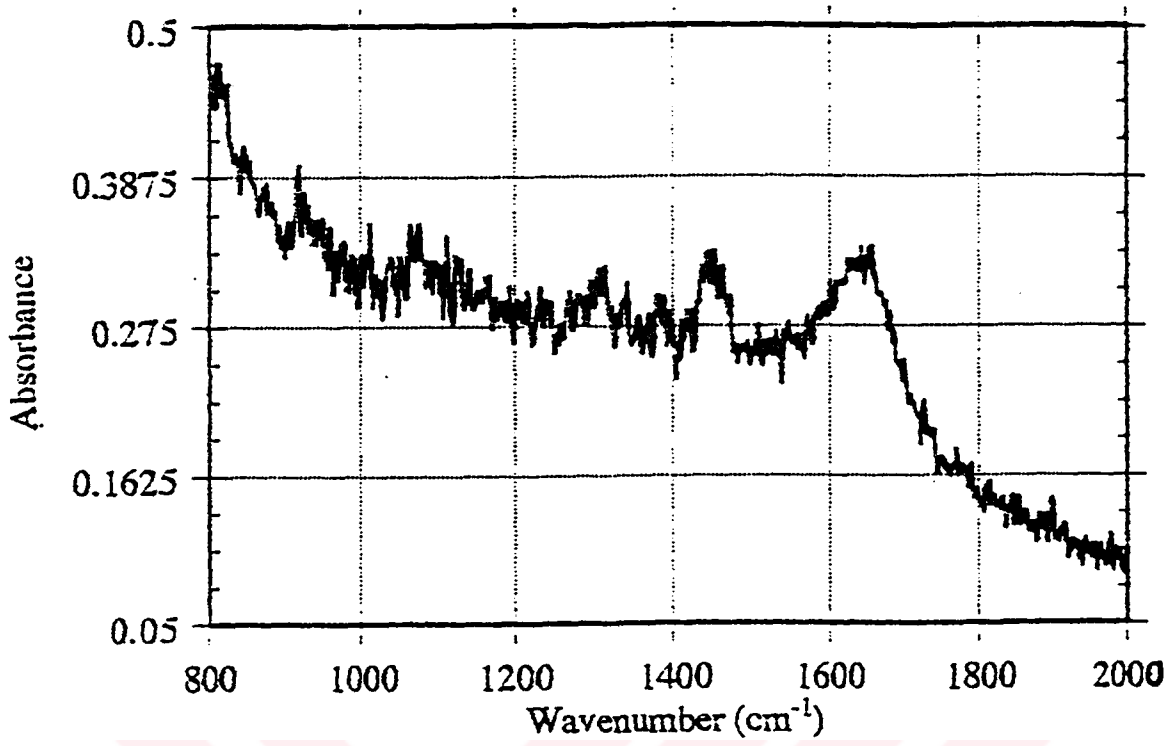


Figure 3.3. Raman spectroscopy of MLV

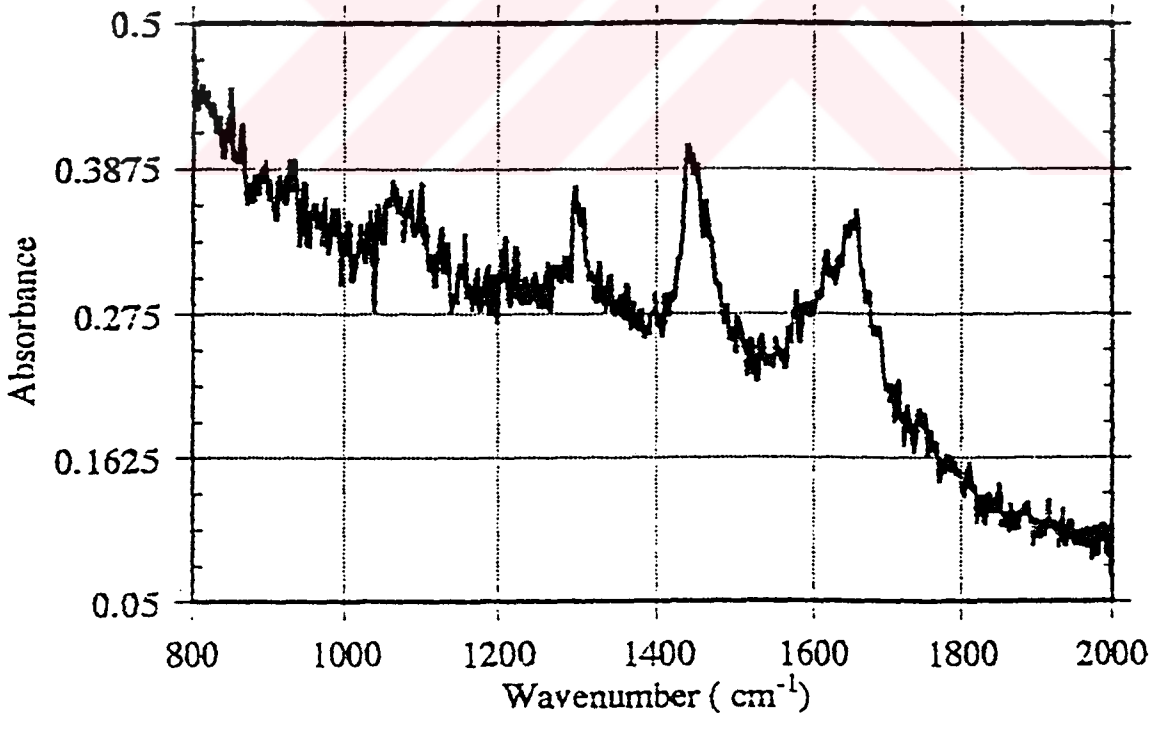


Figure 3.4. Raman spectroscopy of MLV-LMWH

3.1.4. Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

The interpretation of NMR spectra, merely by inspection, is usually feasible in greater detail than is the case for infrared or ultraviolet spectra. NMR spectra is especially a powerful tool for studying hydrogen bonding. In this study, it was used to observe chemical differences between the MLV and MLV-LMWH.

In the interpretation of the ^{1}H-NMR results of MLV (Figure 3.5) and MLV-LMWH (Figure 3.6) samples, the chemical structures of both the MLV and the LMWH have to be taken into account. In the MLV, the dominant component is phosphotidyl choline (PC), and there is also cholesterol and to a significantly smaller extent stearylamine (SA). Stearylamine provides an amine, PC contributes quaternary amine and phosphate, and cholesterol contributes hydroxyl group (OH), in addition to various CH_2 and CH_3 groups.

LMWH, on the other hand, contributes OH's, carboxylic acid and N-acetyl groups with sulphonation.

When the ^{1}H-NMR of the MLV and MLV-LMWH were compared, two regions in the MLV-LMWH NMR were significant. One is the appearance of a peak at 8.2 ppm which can be interpreted as the appearance of OH groups of carboxylic acids. This could very well be introduced by the many unreacted carboxyl groups (COOH) of the LMWH conjugated with the MLV.

The other is the change and intensification of the peak at 3.2 ppm which can be interpreted as alcoholic OH groups. They are available in cholesterol and also in the LMWH structure.

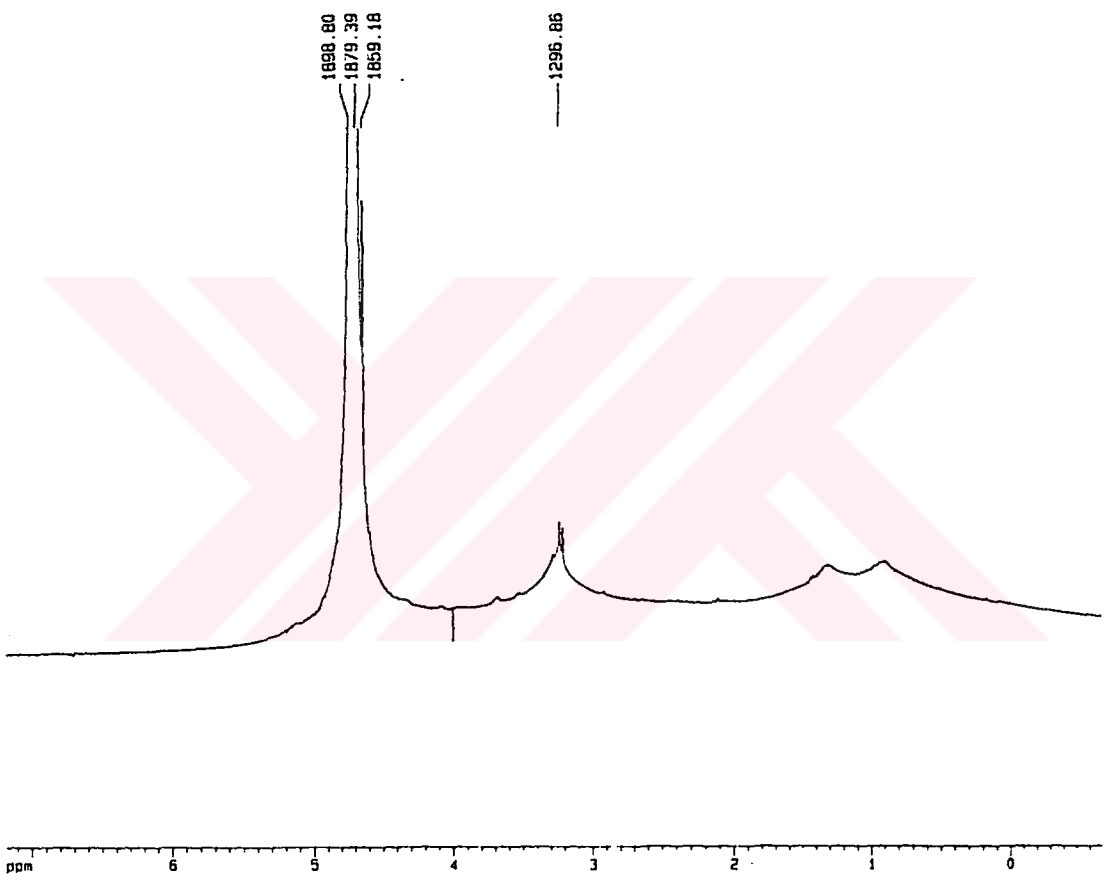


Figure 3.5. NMR spectroscopy of MLV

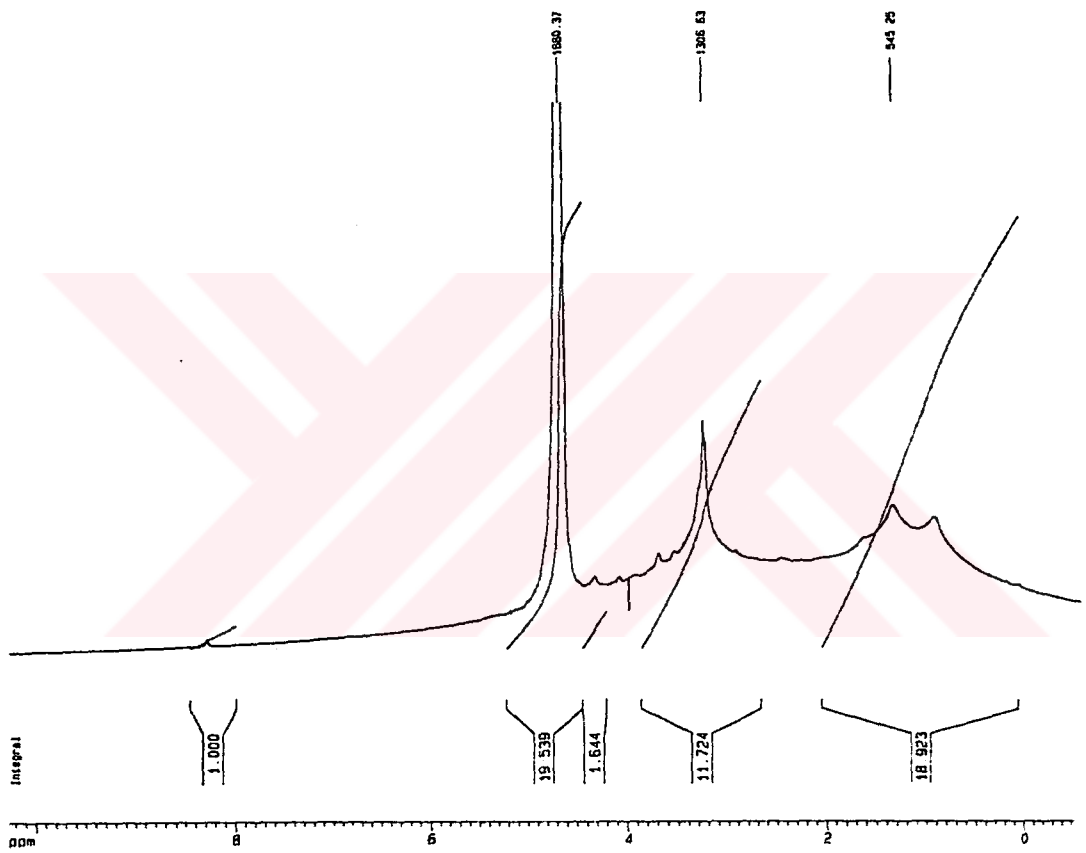


Figure 3.6. NMR spectroscopy of MLV-LMWH

This peak's presence in MLV was probably due to the cholesteric OH which was then intensified and modified by the contribution of the LMWH's OH groups.

Both these changes (at 3.2 and 8.2 ppm's) are good indicators of the success of the conjugation attempt. The conjugation site of stearylamine represents only 2.2 % of all the molecules in a MLV and only a minute fraction of these are on the outside layer of the MLV. Thus, the detection of such small scale changes are quite difficult but were nevertheless achieved in this work.

3.1.5. Fluorescence Microscopy

MLV (section 2.2.1.1) and MLV-LMWH (2.2.2) were loaded with FITC-dextran and oil red O and incorporated in phospolipid lamellae as described in section 2.2.1.2. They were viewed and photographed by PM20 camera with 1000x magnification (section 2.2.3.5). Thus both the MLV lamellae and the aqueous interior were expected to fluoresce.

Figure 3.7 presents fluorescent compound labelled MLV (PC: Chol: SA, 7: 2: 0.2) and Figure 3.8 shows fluorescent compound labelled MLV-LMWH.

Although a statistical study was not conducted, it was observed that the average diameter of MLV-LMWH sample was higher than that of MLV. However, due to its qualitative nature, this result was not sufficient to prove an increase in

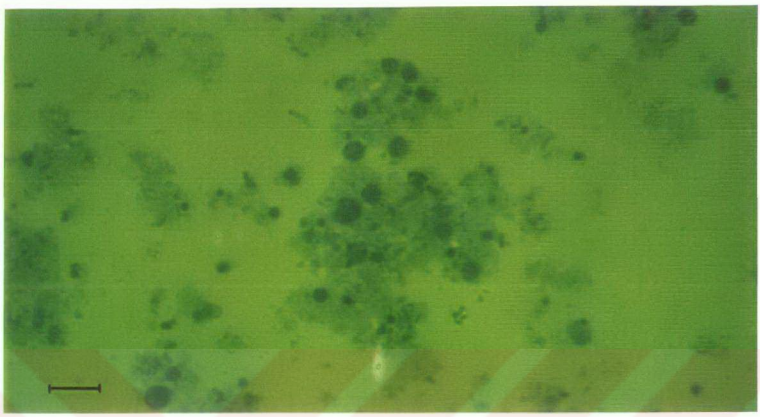


Figure 3.7. Fluorescence micrograph of multilamellar vesicles (MLV) (PC: Chol: SA, 7 : 2: 0.2 containing 10 mM oilred O and 1 mM FITC-dextran) Magnification x 1000. Bar = 2 μm

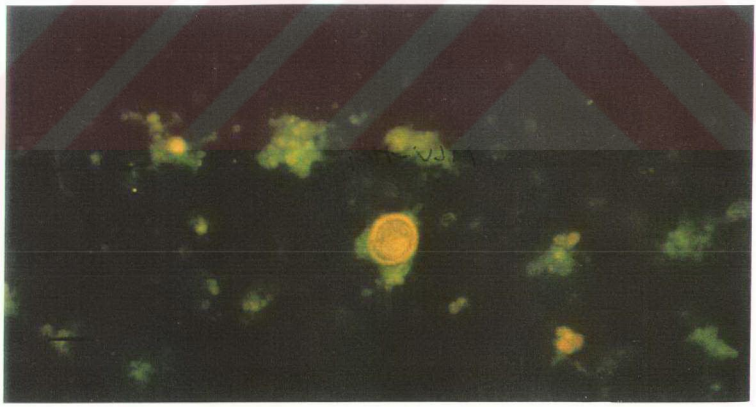


Figure 3.8. Fluorescence micrograph of conjugated multilamellar vesicles (MLV-LMWH) (containing 10 mM oil red O and 1 mM FITC - dextran) Magnification x 1000. Bar = 2 μm

diameter upon conjugation. Therefore light scattering measurement was employed as the method to quantify the change in the diameter.

3.1.6. Light Scattering

As indicated in section 2.2.3.8, light scattering was used to calculate the average diameters of the MLVs and the MLV-LMWH. Although this method has some drawbacks such as low resolution etc., it is possible to measure dimensions and thus to have an idea about the change in diameter upon conjugation.

In this study, both the solvents of the liposomal preparations and the LMWH solution were filtered through cellulose nitrate membranes (pore size: 0.1 μm, diameter: 25 mm, MFS microfiltration systems, Japan) (section 2.2.3.8) because the scattering of light by dust or other extraneous materials could have easily outweighed the scatter by the desired particles or colloids.

The average radius of MLV was determined to be 77.4 $\pm$ 18.3 nm. This radius is much smaller than typical MLV diameters which are in the range of 170-5000 nm.

Figure 3.9 represents the Zimm plot of conjugated MLV (MLV-LMWH). From this Zimm plot, the average radius was calculated to be 222.1 $\pm$ 89.4 nm. This indicates an almost three fold increase in size. This result was expected because the diameter of MLV should increase after conjugation of liposomes with LMWH. A layer of hydrophilic polysaccharide on the surface of a liposome should definitely lead to an increase in the light scattered. This result is also a confirmation

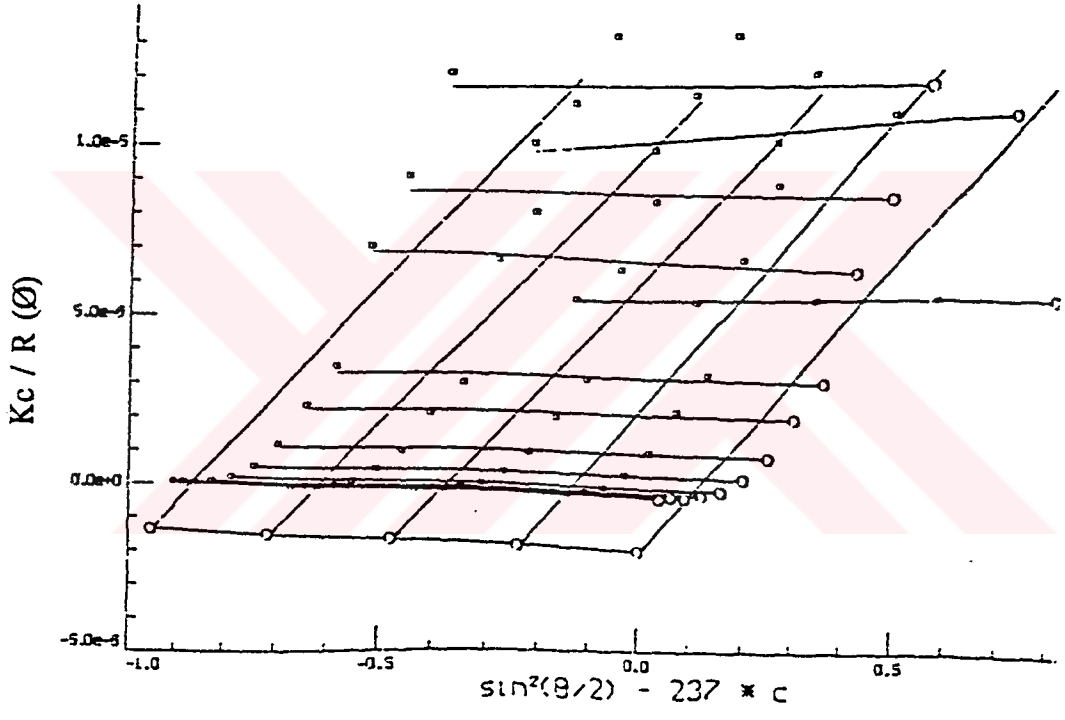


Figure 3.9. The Zimm plot of MLV-LMWH

and quantitation of the qualitative size increase indications of flourescence microscopy and column chromotography. It is also conformation of the successful conjugation which was shown to exist by infrared, raman and nuclear magnetic resonance spectroscopies.

3.1.7. Elemental Analysis

This analysis was used to determine the differences in the chemical composition (C,N,H,O and S contents) of the MLVs and MLV-LMWHs.

LMWH is a linear polysaccharide composed of repeating units of uronic acid, containing an O-sulfate group at the C-2 position, and D-glucoseamine, usually N-sulfated with an additional O-sulfate group at C-6 (Figure 1.4). MLV-LMWH complex therefore should carry sulfur (S) atoms. Since MLV does not have any S atoms, this constitutes a big difference when comparing the chemical composition of MLV and MLV-LMWH. Also because of conjugation procedure, a decrease in the amount of carbon (C) and hydrogen (H), an increase in the amount of oxygen (O) and very little change in the amount of nitrogen (N) were expected in the chemical structure of MLV-LMWH.

In Table 3.1 both the theoretical (% , mole) and experimental (%, mole) amounts of O,C,N,H and S atoms in MLV and MLV-LMWH are presented.

In the calculation of the theoretical values, both monomeric units were assumed to be fully sulphated.

It was observed that for the MLV, the experimental carbon content was about 6 % lower, hydrogen was 0.7 % higher, oxygen was 1 % lower than that of the theoretical and no sulfur was found. Thus, they are close enough to be accepted. Then, when the results of the conjugated samples were analyzed, quite a good match between the theoretical and experimental was found. This is remarkable because the MLV-LMWH calculations were based on an assumption.

The most important result in the experiment was the presence of sulfur (S) in MLV-LMWH sample. Although the amount was much lower than expected, it indicates the presence of conjugation.

Table 3.1. Elemental analysis of MLV and MLV-LMWH

THEORETICAL (%)

	O	C	N	H	S
MLV-LMWH	18.2	65.0	1.7	11.0	1.6
MLV	14.6	69.1	1.6	11.3	0

EXPERIMENTAL (%)

	O	C	N	H	S
MLV-LMWH	16.1±0.013	59.8±0.035	1.2±0.035	10.6±0.030	0.086±0.06
MLV	13.6±0.004	63.0±0.068	1.4±0.018	12.0±0.022	0±0.0

3.2. *In Situ* Liposome Stability Tests

The effect of LMWH on liposomal stability in both human plasma and serum were determined by UV spectrophotometry.

3.2.1. Osmotic Fragility in Saline and Plasma

MLV and MLV-LMWH were loaded with potassium ferricyanate (100 mM) to serve as a non-leaking solute as described in section 2.2.1.3. According to the Table 2.1, different saline and plasma concentrations were prepared and osmotic fragility of MLV and MLV-LMWH were tested and compared as described in section 2.2.4.1.

Figure 3.10 represents the osmotic fragility of MLV and MLV-LMWH in both saline and plasma. As can be seen in Figure 3.10, when the medium is isotonic there is no leakage of the potassium ferricyanate. Upon decrease of salinity, the liposomes start becoming leaky due to swelling of their interiors and instabilization of their membranes. The increase in instability is quite linear until about 0.1 % salinity beyond which stability loss is more rapid than before.

When the stabilities of MLV and MLV-LMWH are compared, it can be easily seen from the figure that MLV-LMWH is much more stable than MLV in both saline and plasma. It appears that conjugation introduced a certain stability into the structure of the conjugated MLVs. It was also concluded from this figure that the stability of liposome solutions were less in plasma than in saline, especially in

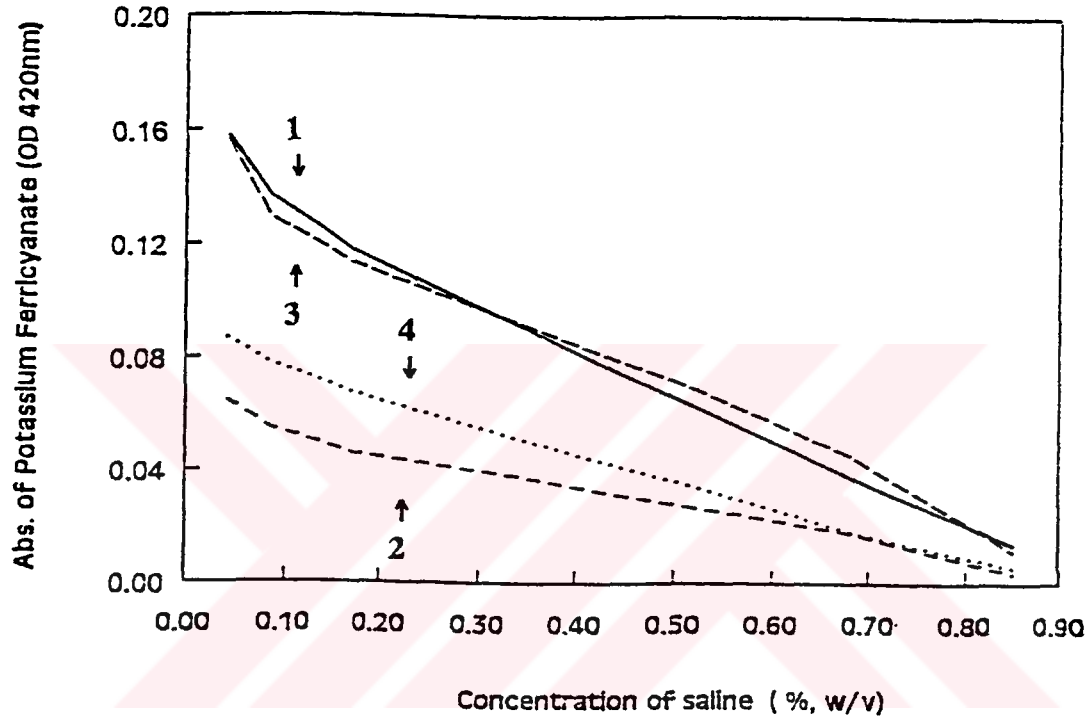


Figure 3.10. Osmotic fragility in saline and plasma —1— MLV in saline -2- MLV-LMWH in saline -3- MLV in plasma ····· 4 MLV-LMWH in plasma.

the case of conjugated samples. Even though the plasma used in this study was quite diluted with saline, still its influence was detectable. This result was expected because in plasma, there are opsonins and high density lipoproteins (HDL) which cause removal of liposomes from the circulation and also lead to decrease in the circulation time.

3.3. *In Vitro* Liposome Stability Tests

Tests were used to observe LMWH anticoagulant activity. For this purpose, hemolytic activity test, plasma recalcification time test and blood coagulation test by using scanning electron microscop (SEM) were carried out.

3.3.1. Hemolytic Activity Test

This test was done to observe the effect of surface modification on liposomes and to determine the possibility of using these liposomes in creating hemocompatible surfaces.

Hemolytic activities of distilled water, MLV and MLV-LMWH were measured and calculated as described in section 2.2.5.1. Isotonic saline solution was used as the blank.

In Table 3.2, it can be observed that complete hemolysis occurred in distilled water (positive control) and negligibly in saline (negative control).

When the hemolytic activities of MLV and MLV-LMWH are compared, it can be seen that hemolysis occurred less with MLV-LMWH than with MLV. When MLV was introduced to blood, 1.05 % hemolysis was observed. However, by using MLV-LMWH, even this low degree of hemolysis was further decreased to 0.7 %. Thus, conjugation proved to have a significant positive effect.

Table 3.2. Hemolytic activity test. (Data was normalized with distilled water after subtracting the absorbance due to 0.85 % saline)

Sample	**% Hemolysis (10 times diluted)**
Distilled Water	100±0.0
MLV	1.05±0.5
MLV-LMWH	0.7±0.5

3.3.2. Plasma Recalcification Time (PRT) Test

The anticoagulant activity of LMWH and the effect of surface modification were determined by using Plasma Recalcification Time test.

Three different coverslips containing MLV + plasma, MLV-LMWH + plasma and only plasma were prepared as described in section 2.2.5.2. $CaCl_2$ (0.25 M) was used to remove oxalate ions present in plasma. Afterwards, the time to clot was noted.

Table 3.3 shows the clotting time of each coverslip containing sample. From the results, it can be concluded that presence of conjugated liposomes increase the PRT. Moreover it can be observed from the figure that even the unconjugated MLV delay the coagulation time of the blood. It is possible that the positive charges (coming from stearylamine) present on the liposome may interact with the blood interfere with the blood coagulation cascade and can increase the coagulation time.

Table 3.3. Plasma recalcification time test for MLV (PC: Chol: SA, 7 : 2 : 0.2 with plasma), MLV-LMWH (with plasma) and only plasma (positive control)

Sample	Time to clot (min)
Plasma	8 ± 0.0
Plasma + MLV	9 ± 0.5
Plasma + MLV-LMWH	11 ± 0.5

3.3.3. Blood Coagulation Test by Using Scanning Electron Microscopy (SEM)

In contrast to optical microscopy, SEM combines very high resolution with great depth of field. With SEM it is possible to obtain topographic, chemical, crystallographic, electrical and magnetic information on a microscale with a resolution often approaching 10 nm.

In this test, the effect of surface modification and anticoagulant activity of LMWH were studied.

Three coverslips containing a) MLV + blood, b) MLV-LMWH + blood and c) only blood were prepared as described in section 2.2.3.6. After covering with gold, their microstructures were studied under the LEITZ-AMR 100 SEM and photographed.

Effects of MLV and MLV-LMWH on blood can be seen in Figure 3.11 and Figure 3.12 respectively. The positive control which contains only blood is shown in Figure 3.13.

In Figure 3.13, quite thick layers of RBC clusters were observed.

In Figure 3.11, however, an evenly distributed layer of RBC was observed on the coverslip. This may be due to the positive charge of the liposomes.

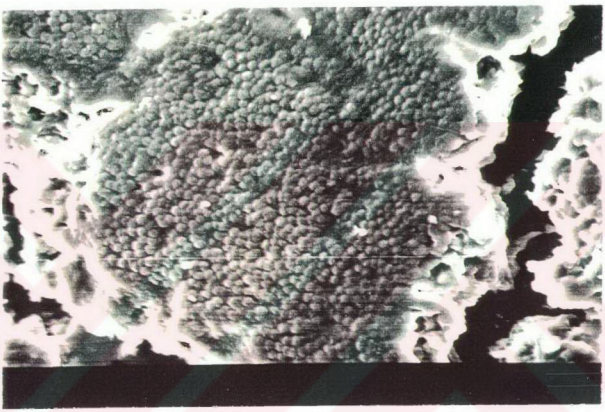


Figure 3.11. SEM of blood + MLV (PC: Chol: SA, 7 : 2 : 0.2) on glass surface (Magnification x 1000)

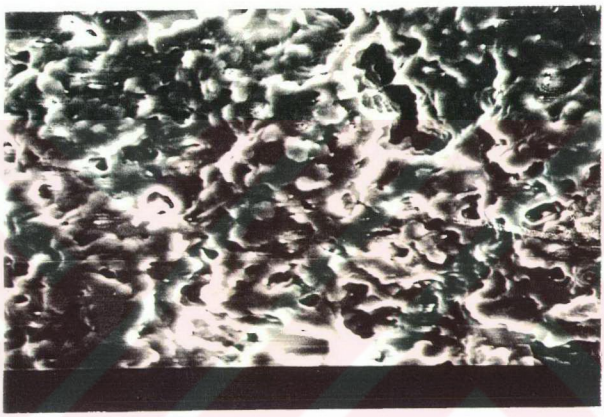


Figure 3.12. SEM of blood + MLV-LMWH on glass surface (Magnification x 1000)

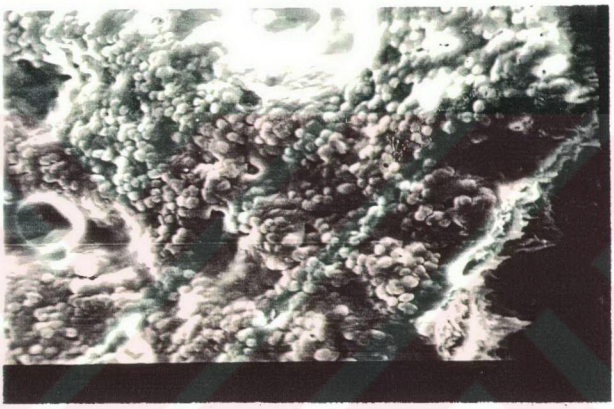


Figure 3.13. SEM of only blood (positive control) on glass surface (Magnification x 1000)

Relatively low concentration of RBC was found in Figure 3.12 possibly because LMWH has an anticoagulant effect and thus RBCs are not accumulated in a fibrin mesh along with platelets.

At the end of this study and the other two *in vitro* studies, it is quite possible to state that LMWH is a good anticoagulant and it carries this property to liposomes when it is conjugated with them.

3.4. *In Vivo* Test : Radiolabelled Amino Acid, ^{14}C-L-Arginine, in the Circulatory System of Rabbit

For this test, three female rabbits (New Zealand wild type) having quite similar ages and weights were used and the kinetics of disappearence of MLV and MLV-LMWH from the rabbit circulation were investigated.

MLV and MLV-LMWH loaded with radiolabelled amino acid (section 2.2.1.4), and the free ^{14}C-L-arginine were introduced intravenously through the ear vein of three different rabbits as previously described in section 2.2.6. Blood samples were taken at times shown in Table 2.2 and the radioactivity due to emitted β particles was measured in a liquid scintillation counter. Figure 3.14 represents the behavior of the radiolabelled MLV, MLV-LMWH and amino acid *in vivo*.

It can be observed from Figure 3.14 that when the free radiolabelled amino acid is introduced to the circulatory system, its level in the blood linearly decreases with time.

When radiolabelled MLV was introduced to blood, a very sharp decrease in radioactivity level was observed between 15-50 minutes. This rapid clearence

may be due to the elimination of large MLVs by the phagocytic system. The level of radioactivity stayed almost constant between 50-113 minutes. Since small particles or structures are not seeked by the phagocytic system, it appears that they stayed in the circulation after the elimination of the large MLV's.

When radiolabelled MLV-LMWH was introduced to blood, a steady level of radioactivity was observed from the beginning to the end for the same duration as the MLVs and amino acid.

These mean that conjugation of MLV with LMWH significantly increases liposomal half life possibly by preventing the rapid clearance of liposomes from the circulation.

In the literature there are reports about liposomes coated with O-palmitoyl pulluan, O-palmitoylamylopectin (Sunamoto, 1986) and collagen (Fonseca et.al., 1996). In these, it was observed that conjugation (or coating) increased the rate of removal of the liposomes or left it unchanged (Fonseca et. al, 1996). The increase was explained by removal of liposomes by macrophages. In the collagen case, insufficient coating or weak binding were reported as the cause for the observed behavior.

The MLV-LMWH prepared in this study are significantly better in their performance in *in vivo* and can be considered as "Stealth Liposomes".

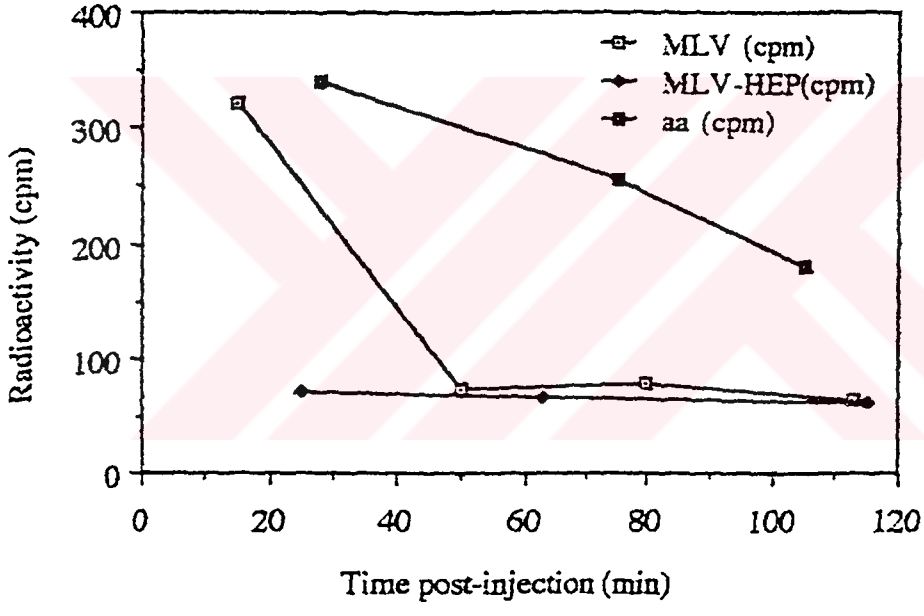


Figure 3.14. The behavior of radiolabeled aminoacids in the circulatory system of rabbit.

CHAPTER 4

CONCLUSIONS

The most important drawback of liposomes as potential drug carriers is their rapid clearance from the circulation partially due to uptake by the cells of reticuloendothelial system (RES) and their structural instability. Numerous attempts have been made to prolong liposome lifetime and biocompatibility in the circulation.

In this study liposomes were coated with heparin through a conjugation approach employed for the first time with heparin, which is naturally occurring polysaccharide present in tissues of diverse species as mammals and molluscs. It is an effective and safe antithrombotic agent.

The conjugation of liposomes with LMWH was determined by spectroscopic methods such as toluidine blue metachromic shift assay, infrared, raman and nuclear magnetic resonance spectroscopies.

Change in the liposomal size due to conjugation was shown by fluorescence microscopy. It was further proved and quantified by light scattering which indicated a nearly three fold increase in liposomal size due to conjugation with heparin.

Elemental analysis was used to determine and compare the chemical composition of MLV and MLV-LMWH. MLVs do not contain sulfur (S) in their structure and presence of conjugation was proven by observing this element in the

chemical structure of this complex. Also, changes in level of C, N, H, and O added further credibility to conjugation.

Osmotic fragility tests in saline and plasma showed that MLV-LMWH are more stable than MLV in both media.

Hemolytic activity test, plasma recalcification time test and blood coagulation test by using scanning electron microscope were used to observe anticoagulant activity of LMWH and also the effect of modification of surfaces by liposomes (MLVs). From this result, it was concluded that degree of hemolysis can be decreased and the coagulation time of blood increased by the use of MLV-LMWH complex. From the SEM photographs it was observed that MLV-LMWH complex causes less deposition of red blood cells possibly due to the good anticoagulant activity of LMWH.

An *in vivo* test was also carried out to see the behavior of MLV-LMWH complex in the circulation. This test showed us that circulation time and biocompatibility of liposomes in the blood can be increased significantly by conjugating them with LMWH.

It thus appears that conjugation of liposomes with LMWH will be very important for their use as a drug release system and in the creation of biomimetic surfaces.

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APPENDIX A

CALIBRATION CURVE OF LMWH

The calibration curve of LMWH was constructed at 631 nm by using toluidine blue method. It was used in the conversion of the absorbances into concentrations of free and conjugated LMWH.

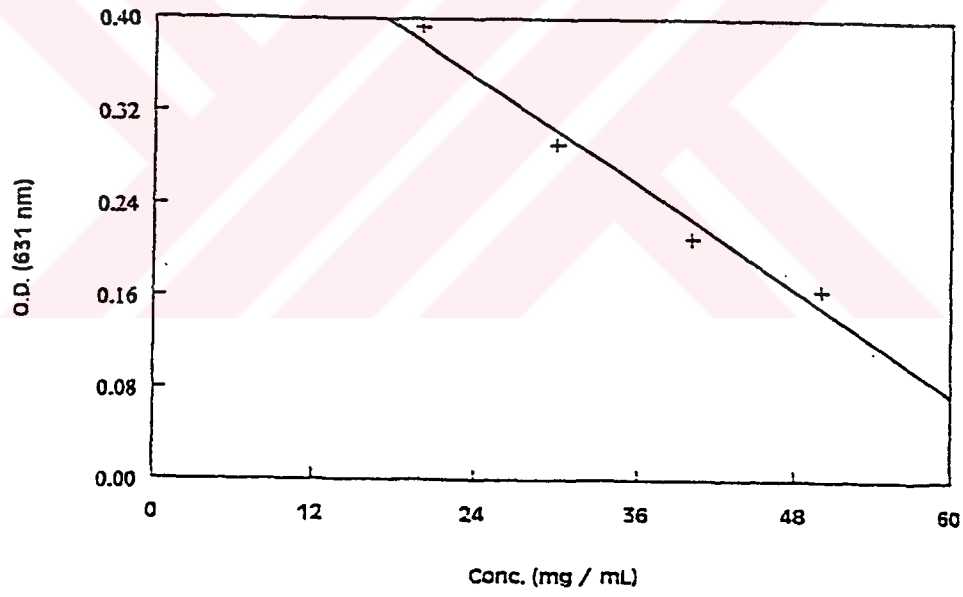

Figure A.1. Calibration curve of LMWH

APPENDIX B

CALIBRATION CURVE OF POTASSIUM FERRICYANATE

The amount of potassium ferricyanate retained in the liposomes in the osmotic fragility test was calculated from the calibration curve (below) which was constructed at 420 nm.

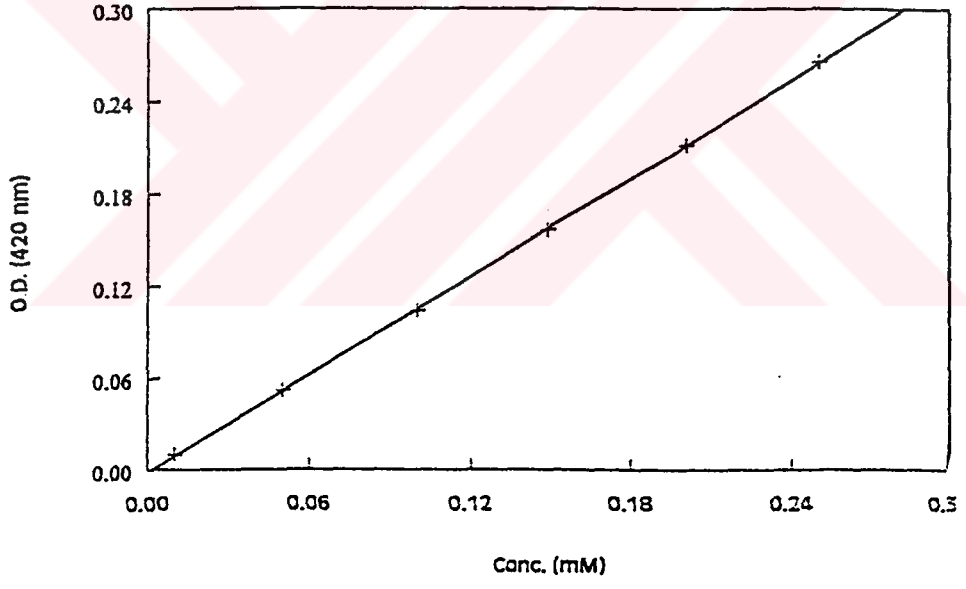

Figure A.2. Calibration curve of Potassium Ferricyanate