“DEVELOPMENT AND EVALUATION OF LIPOSOMES FORPOSTERIOR OPHTHALMIC DRUG DELIVERY”

A **PROJECT** SUBMITTED TO

NIRMA UNIVERSITY

In the partial fulfilment of the requirements for the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL TECHNOLOGY
BY

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UNDER THE GUIDANCE OF

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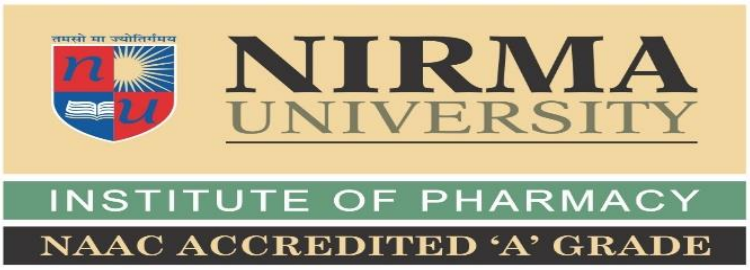

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May 2019

CERTIFICATE

This is to certify that the dissertation work entitled "Development and evaluation of liposomes for posterior ophthalmic drug delivery" submitted by Mr. Vatsal shah with Regn. No. (17MPH112) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutics" is a bonafide research work carried out by the candidate at the Department of Pharmaceutics, Institute of Pharmacy, Nirma University under my/our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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To whomsoever it may concern

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His project has been found satisfactory by the guide.

We wish him all the best in future endeavors.

Sincerely
For **Sun Pharma Advanced Research Company Ltd.**

Hardik Vaidya
Sr. Manager – Human Resources.

Date : 16-Apr-2019
Place : Baroda

CERTIFICATE OF ORIGINALITY OF WORK

*This is to undertake that the dissertation work entitled "**Development and evaluation of liposomes for posterior ophthalmic drug delivery**" Submitted by Vatsal shah (17MPH112) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutics" is a bonafide research work carried out by me at the "Pharmaceutics", Institute of Pharmacy, Nirma University under the guidance of "Dr. Shital butani". I am aware about the rules and regulations of Plagiarism policy of Nirma University, Ahmedabad. According to that, this work is original and not reported anywhere as per best of my Knowledge.*

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DECLARATION

I hereby declare that the dissertation entitled ***"DEVELOPMENT AND EVALUATION OF LIPOSOMES FOR POSTERIOR OPHTHALMIC DRUG DELIVERY"****, is based on the original work carried out by me under the guidance of Dr. Ajay Khopade, vice-president of FDD non-orals Sun Pharma Advanced Research Company. Dr. Shital Butani, Associate Professor, Department of pharmaceutics, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.*

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ABSTRACT

Diabetic retinopathy and Age related macular degeneration are major causes of blindness nowadays. The standard therapy for these diseases is the monthly or twice in a month intravitreal injection of anti-VEGF agents. But sight threatening complications are observed because of these therapies. Topical eye drops in the form of liposomal dispersion is non-invasive mode of treatment. Liposomes act as a non-toxic and biocompatible vesicular nanocarriers which can be used to encapsulate active pharmaceutical ingredients to deliver sustained and targeted delivery. It is reported that in the comparison of cornea, conjunctiva and sclera has many folds higher paracellular space and provide facility to permeate water soluble molecules. It is hypothesized that anionic lipid will provide more diffusion due to negatively charge of sclera and cationic lipid will retain on the epi-scleral region because of electrostatic interactions. First pre-formulation study done for the gradient identification of salt and based upon that ammonium sulphate salt identified as a gradient for active loading. After that particle size-based study carried out and against hypothesis, particle size achieved from 55 to 120 nm. Based upon that anionic, cationic and neutral charged liposomes were prepared with 0.5,1,1.5 mg/ml loading capacity and characterized for particle size, zeta potential, entrapment efficiency and in-vitro release. Entrapment efficiency of drug in to liposomes varied from 80 to 100%. It was found that as particle size of liposomes decreased, slow drug release was observed in in-vitro study.

Chapter 1. Aim of presentation investigation

Eye is one of the precious organs of body, when it gets diseased, must be cured as early and as effectively as possible. For that the use of many drugs and drug delivery systems are needed. Basically, the structure of eye divided into anterior and posterior segment.(Mitra A K, 2003). Eye is affected by various vision diseases. Diseases affecting anterior segment include, allergic conjunctivitis, anterior uveitis, cataract and glucoma etc. whereas, age-related macular degeneration (AMD) and diabetic retinopathy are themajor diseases affecting posterior section of the eye. (del Amo et al., 2017)

Especially For the treatment of anterior disease, topical eye drop is most suitableand patient compliant route of drug delivery. Distribution of medications to the targeted ocular tissues is constrained by various anatomical ocular barriers. Also, to maintain therapeutic drug levels are challenging task for the formulation scientist, so with the help of topical deliveries, it is difficult to treat posterior segment.(Mitra A K, 2003)

Because of an increasing need for treatment of posterior eye diseases, development of newerandmoresuitable systematic techniques are required. Currently, for the treatment of retina, intravitreal injection is most widely used(Mitra A K, 2003). But regular administration of drugs with the help of this route can cause detachment of retina, inflammation and increased intraocular pressure, and it is highly patient inconvenient. Although implants are available but surgical methods are required for that so if topical delivery available for posterior segments it will be highly beneficial for the patients.(Moisseiev & Loewenstein, 2017)

For formulation entry in to posterior ocular tissues, corneal and conjunctival routes are the two local pathways. Corneal pathway involves entryinto the cornea followed by interior tissues including iris, aqueous humour, lens, and irisciliary body. Conjunctival pathway involves drug penetrationacrossconjunctiva followed by entry into the sclera, choroidandretina. [27].The use of topical eye drops for the posterior section of eye is beneficial because it is non-invasive, self-administrative and highly patient compliance. But it is also very challenging task because of various static, dynamic and enzymatic barriers of eye.So goal of study is to develop ocular eye drops which can travel through peri-ocular route and give targeted delivery to the posterior part.

Chapter 2. Introduction

2.1 Anatomy of eye:

The anterior portion of eye subdivided in to Tissues such as cornea, conjunctiva, aqueous humour,lens, iris, ciliary body and. Posterior segment includes sclera, choroid, retinal pigment epithelium, neural retina, optic nerve and vitreous humour.(Mitra A K, 2003)

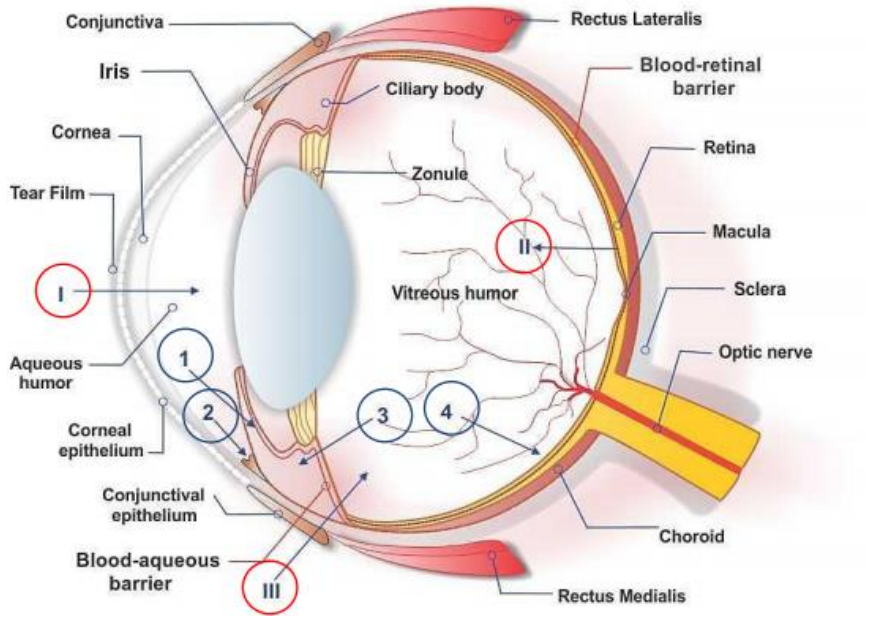


Figure 2.1 Schematic diagramshows main parts of the anterior and posterior sections and barriers to ophthalmic drug delivery. The position of ophthalmic barriers shows in red circlewhich includes I) Tear filmandthe cornea; II) bloodretinalbarriers(BRB); III) blood-aqueous barriers. Routes of elimination of drug from the vitreous shows in blue circle are; 1) venous blood flow after diffusing across the iris surface; 2)Out flow of aqueous humour; 3) diffusion into the anterior chamber (1, 2 and 3 are referred to diffusion through the blood-aqueous barriers); 4) diffusion through the BRB.

2.1.1 Composition of eye:

Normal composition of eye is comprised of following components.

- Water –97-98%,
- Solid -1.8-2%,
- Organic elements
- Protein - 0.6-0.7%,
- sugar - 0.6%,

- NaCl - 0.66%
- Other mineral element sodium, potassium and ammonia - 0.80%.

Structure of eye composed of series of barriers which are reason for sub therapeutic drug level at the targeted site. Actually, it is challenging task to determine how to surpass those barriers with the effective way with patient compliance.(Mitra A K, 2003)

2.1.2 Various constraints to the ocular\ drug delivery:

To prevent from various foreign toxic particles,ocular tissues are organised with various protective mechanisms. (Mitra A K, 2003)
Theseinclude,

- Surface of the eye continuously flushed because of tearsecretion.
- Surface epithelium which is impermeable.
- Retina actively cleared by various transport mechanisms.

But, these defensive mechanisms areone of the reason for the sub therapeutic levels of drug at targeted site. So major aim of ocular therapeutics is to overcome the protective mechanisms for achieve desired therapeutic effect. (Mitra A K, 2003) In the precorneal and corneal spaces,Physiological barriers to the diffusion and productive absorption of topically applied ophthalmic drugs exist.

Those Precorneal constraints include

- Drainage of solution,
- Lacrimation and tear dilution,
- Tear turnover rate,
- Conjunctival absorption

Drugs must cross the blood-ocular barrier in significant amounts to demonstrate therapeutic effect, for the effective treatment of diseases.(Mitra A K, 2003)

2.1.3 Transport barriers in eye:

In addition to precorneal barriers, eye has several other physiological barriers also which we will discuss below. Because of that doctors have to compulsory medicateregular high doses of drugs to achieve desired pharmacological effects. This pulsatile treatmentare major reason for extreme fluctuations and side effects. The cornea works as a mechanical and

chemical barrier to the intraocular tissues. The mammalian cornea, mainly consists of more than five separate layers, with a thickness of more than 300–500, (Mitra A K, 2003)
Through the limbus region, cornea is connected to sclera. The sclerahard in nature and mainly included collagen fibres. It maintains shape of eye.The conjunctiva is a thin, transparent mucous membrane lining the inside of the eyelids and is continuous with cornea.(S. H. Kim, Lutz, Wang, & Robinson, 2007)
The major difference between cornea and conjunctiva is that theconjunctiva has rich vasculature, the presence of many goblet cells, and its ability to Transdiscrimination. (Mitra A K, 2003)

The vascular uveal coat of the eye is made up of the iris, ciliary body, and choroid. The anterior part of iris is embedded in the aqueous humour and The ciliary body secretes aqueous humour into the posterior part, and then it flows via pupil into the anterior part.(Mitra A K, 2003)
The aqueous humour is a transparent, aqueousso1ution, The aqueous humour production and theintraocular pressure are maintained by membrane transport processes. (Mitra A K, 2003)

Iris endothelium and blood vessels of ciliary body and ciliary epithelium are important parts of blood aqueous barrier.(Mitra A K, 2003)

The lens is a transparent tissue, with major part consisting of waterand the remains are proteins. Anteriorly, the lens is in contactwith the pupillary portion of the iris, and posteriorly it fits into a hollowdepression of the anterior vitreous surface.(Mitra A K, 2003)
Capsule, epithelium, and lens fibric cells are the chief portions of the lens.(S. H. Kim et al., 2007)

The BRB (blood retinal barrier) is located at two levels: the outer BRB, consisting of theretinal pigment epithelium (RPE), and the inner BRBconsists of retinal capillaries. BRB plays acrucialfunction in the homeostasis of the neural retina by restrict the entry ofxenobiotic into the extravascular spaces of the retina and by preventtheloss of essential molecules.(Mitra A K, 2003)
RPE is a single layer of hexagonal cells, which seperates the outer surface of theneural retina from the chorio-capillaries. The role of RPE in regulating the microenvironment

adjacent the photoreceptors in the distal retina, where the photo transduction takes place. (Mitra A K, 2003)

Because of presence of tight junctions, RPE is extremely restrictive for Para cellulartransport of solutes. However, it is capable of a many of specializedtransport processes.

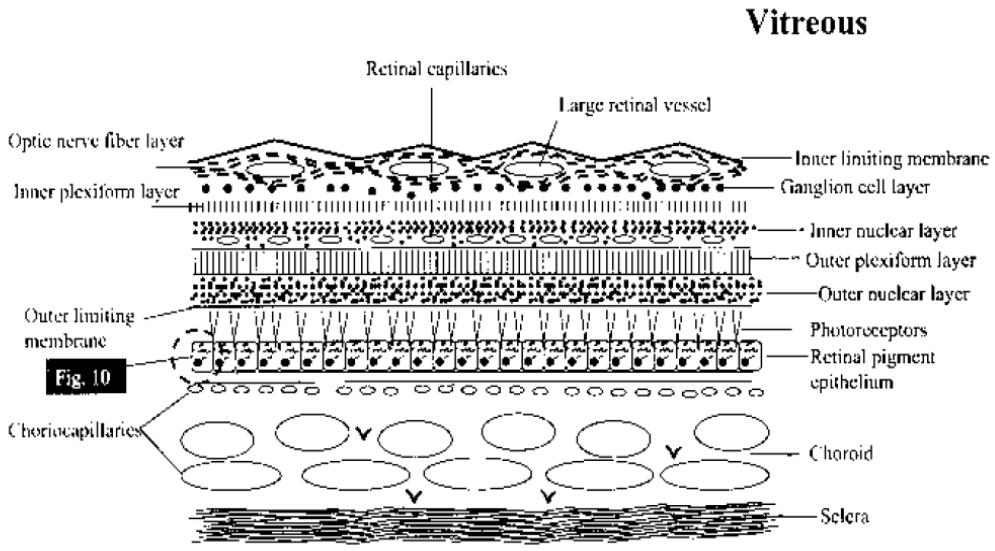


Figure 2.2 Different part of posterior portion

The sclera is covered by the conjunctiva which is clear mucous membraneandworkas lubricant to the eye. [3]Epi-sclera is loose connective tissue which covers both the sclera and the conjunctivaandorganised with rich blood supply.[5]

Choroid is a large, vascular and pigmented tissue, provide oxygen to the outer layers of the retina. [5]Retina is a large layer of nervous tissue that covers the inside of the back more than half of the eye, in which because of stimulation by light, initiation of the sensation of vision occurred. (Mitra A K, 2003)

The vitreous makes up the major volume of the eye, and is a clear, jelly-like substance. Anteriorly, the vitreous face sits behind the lens capsule, and is restricted by the retina posteriorly. The vitreous consists of is a framework of collagen, mucopolysaccharide, and hyaluronic acid. [5]

2.2 Posterior segment related diseases:

2.2.1 Age-related macular degeneration (AMD)

It is a major reason for irreversible loss of central vision in agedpeople. Gross vision loss observed because of choroidal neovascularization or RPE detachment.(S Natarajan, 2009)**At early stage:**Drusen and/or hypo and/or hyper pigmented macular injuries are considered.(S Natarajan, 2009)**AtLater stage:** Exudative deterioration of the macula isseen.But may not have the same degree of degenerative variations in both eyes.Through AMD, patient could lose central vision and cannot see fine details.(S Natarajan, 2009)

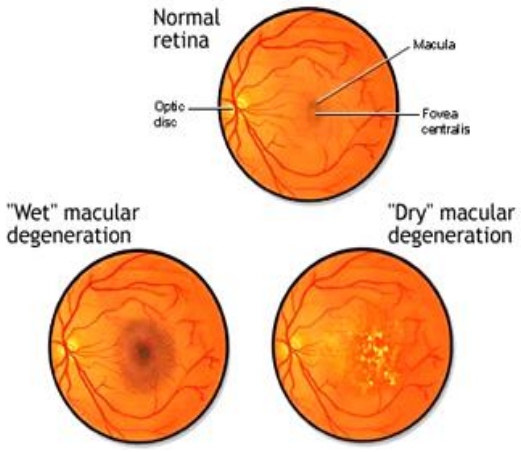


Figure 2.3 Difference between normal eye and diseased eye.

There are Two types of AMD observed:

A. **Non-exudative AMD**

It is revealed based on histopathological studies of choroid that during AMD a relative reduction in vessel density and a contraction of vessel lumens observed.(Porte, 2012)
Many people who have AMD have the dry form. In dry AMD, thinner macula and minute clumps of protein observe and Patient slowly loses central vision. (S Natarajan, 2009)

B. **Exudative AMD**

This form is less recurrent, but much more serious. In that production of new, irregular blood vessels observed. Macula scaring can be observed due toLeakageof blood or other fluids. (S Natarajan, 2009)The development of choroid neo vascularization (CNV) is the symbol of exudative AMD. CNV grows as a capillary-like assembly, usually with multiple

points of source, and further progresses into arterioles and venules. CNV can cause detachment of the RPE or retina, breaking of RPE, and lipid exudation.(Porte, 2012)

2.2.2 Diabetic retinopathy:

Diabetes is one of the major causes of blindness in many countries. Diabetic retinopathy observes when high blood sugar levels cause damage to blood vessels in the retina. These vessels can swell and leak or they can block blood from passing through. Sometimes abnormal new blood vessels growth observes on the retina. All of these changes can leads toloose patient's vision.(S Natarajan, 2009)

Pathogenesis of diabetic retinopathy:

Based upon severity and clinical appearance,it can be divided in to two stages.(Tarr et al., 2013)

A. NPDR (non-proliferative diabetic retinopathy)

Vascular changes are observed in initial stages in the form of dilation of veins and area of capillary non-perfusion. So that ischemia occurred. It stimulates the formation of micro aneurysms which is called red dots.(S Natarajan, 2009) At later stage, they mature and may become yellowish in colour. After that intra-retinal haemorrhages occurred because of bleeding from ruptured capillaries. The gathering of lipidsalso seen at the posterior end with leaking capillaries which is called hard exudates. These are the biomarkers of hyperlipidaemia and can result in visual loss. (S Natarajan, 2009)(Tarr et al., 2013)

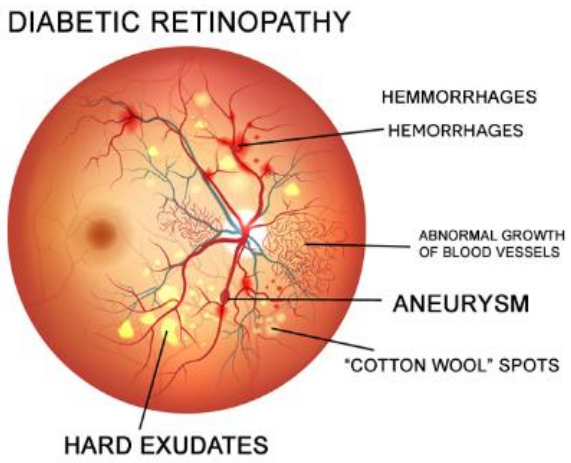


Figure 2.4 Diabetic retinopathy in retina.

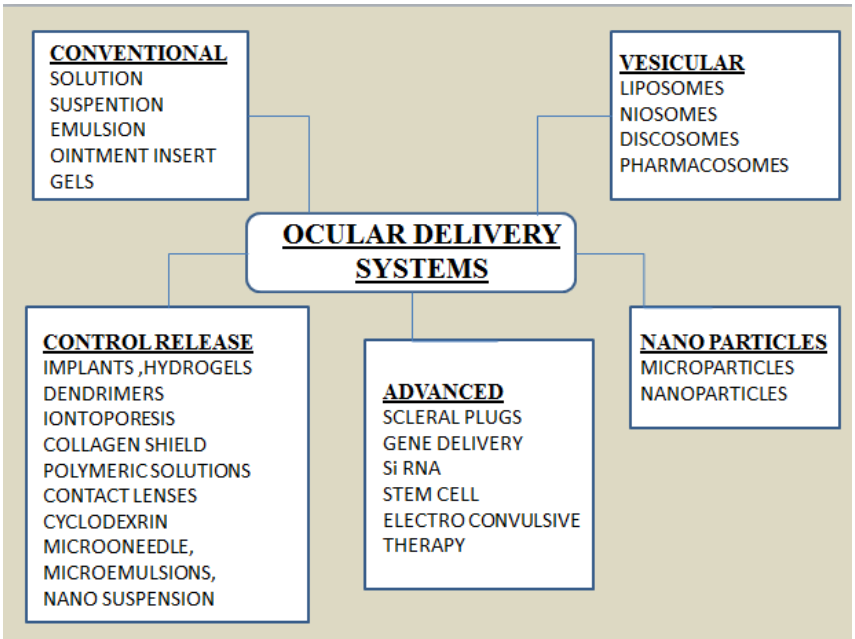
B. PDR (proliferative diabetic retinopathy)

It is the advancedphase of diabetic eye disease defined as the presence of newly formed blood vessels arising from the retina or optic disk called as neovascularization. These breakable new vessels often bleed into the vitreous. Because of these new blood vessels,scar tissues can form.Thesecan cause difficulties with the macula or lead to a ‘’Detached retina’’.(Tarr et al., 2013)

2.3 Ocular dosage forms:

Ophthalmic preparations are defined as sterile products, free from microbes, which are properly compounded and wrapped for instillation in to the ocular tissues. The main reason of developing strong attention of scientists in these dosage forms is the problem of a low bioavailability of medicinal ingredients after the application to the eyeball. The purpose for the advancement of ophthalmic drug forms is to achieve therapeutic index with in the place of absorption and sustaining it for suitably longer period of time, which in turn contributes to smaller application frequency. (Baranowski, Karolewicz, Gajda, & Pluta, 2014)

Table no. 1 Different ocular dosage forms



2.3.2 Topical eye drops for the posterior segment of the eye.

A topical drug delivery signifies the least invasive technique for targeting diseases like posterior segment macular degeneration (ARMD), Diabetic retinopathy, choroidal melanoma, retinitis pigmentosa of the eye. Because of high patient compliance, Dosage forms majorly delivered by eye drops.Although, there are various other techniques also available for the drug distribution to the posterior part of eye, like intravitreal, periocular injections,implantation of devices are effective but invasive, and governs by high risk of retinal detachment, inflammation of the tissue, haemorrhage and cataract.(Tahara, Karasawa, Onodera, & Takeuchi, 2017)These advancement developed because of limitations for eye drops to the drug distribution across the surface of the ocular in tear and periocular fluids and other pre-constraints like reflex blinking, naso-lachrymal drainage, Efflux of drug by the corneal and conjunctival epithelia, metabolism in ocular tissues and rapid clearance from ocular compartments.(Baranowski et al., 2014)

2.3.3 Eye drops

Although severalalternatedosage forms tried, theeyedropsremain the major technique of administration for the topical ocular site. These are byfar the most common dosage forms for delivering drugs to the eye and are one of the fewdosage forms not administered by

exact volume or weight dosage, yet this apparently imprecise method of dosing is quite well-established and accepted by the ophthalmologist.(Nanjwade, Life, & Pvt, 2016)
The area of topical ophthalmic preparations majorly covered by aqueous solutions. The ingredients must be completely soluble in solvent so that dose uniformity isn't issue.Majorly all ophthalmic therapeutic ingredients are water soluble or solubility can be increase by formulatedas water soluble salts. A homogeneous solution dosage form offers many advantages including the simplicity of large scale manufacture.(Nanjwade et al., 2016)While formulating aqueous solutions, the one has to consider some parameters like Ph, tonicity, viscosity, ionization constants, ocular comfort, choice of preservativeetc.But the disadvantage istheir relatively brief contact time between the API and the absorbing tissue of the external eye. Because of initial high drug quantity in tears, followed by a rapid decrease in quantity, poses a potential risk of toxicity.(Tadros, 2018)

"Suspensions are dispersions of finely divided, relatively insoluble drugsubstances in an aqueous vehicle containing suitable suspending and dispersing agents". Suspensions are used for those drugs which are proven to significant therapeutic activity but cannot use in ocular delivery due to low solubility. (Tadros, 2018)But due to insoluble substance, ocular irritation must not be observed. An ophthalmic suspension consists of many inactive ingredients such as dispersing and wetting agents, suspending agents, buffers and preservatives.(Tadros, 2018)

For formulating an ideal suspension, understanding of the interfacial properties, wetting properties, particle interaction, zeta potential, aggregation and sedimentation properties and rheological concepts is required for formulating an effective and elegant suspension. Non-homogeneity of dosage form,cake formation, settling, aggregation of particles are the major problems that formulator mustsolve during development phase.(Tadros, 2018)

Aqueousemulsionshavingabenefit of the capability to carry a poorly water-soluble drug in a solubilized form as an eyedrop. Here The drug is dissolved in a non-aqueous vehicle, and emulsified with aqueous phase, using a non-ionic surfactant and, if needed, an emulsion stabilizer. The one can resolve the problem of irritation by using water as the external phase than use of a purely nonaqueous media. (Barkat Ali Khan, 2012)

2.4 Topical routes for the posterior segment of the eye:

There are three majorpathsidentifiedfordiffusion of topically applied ophthalmic drugs to the posterior section.(Kompella, Bandi, & Ayalasomayajula, 2003)

1. In the trans-vitreous path,aftertrans-corneal diffusion entry into vitreous and then distribution to ocular tissues observed. (as blue arrow).
2. In periocular route,after diffusion around sclera trans scleral absorption observed. (as red arrow)
3. In uvea- scleral route,aftertrans corneal diffusion progression observed through the uvea-sclera (as green arrow).

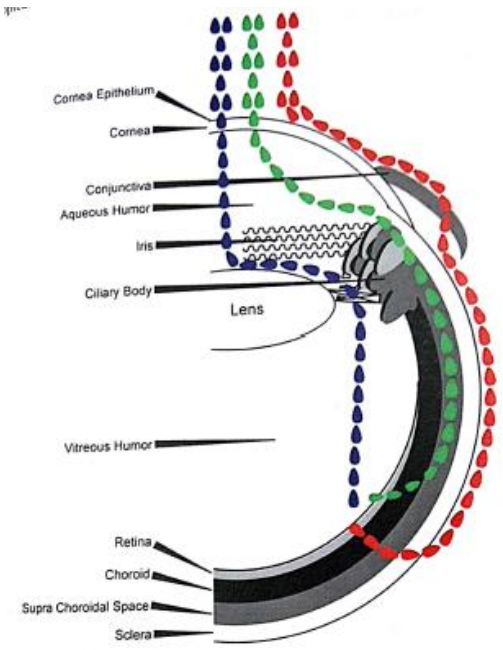


Figure 2.5 Topical routes for the posterior segment of eye

2.4.1 The Trans vitreous route:

According to Pharmacokinetic studies,firstdrug reaches its maximum concentration in the surface of ocularand aqueous humour, and after that the vitreous followed byretina/choroid.(Joseph et al., 2017)

2.4.2 The Uvea- scleral route:

Topically applied drug firstpenetrated the aqueous humour and thentransfer to the posterior segment via the uvea-scleral route andthenpossibly access choroid and retina.

Particlesprogressing along the uvea- scleral route are subject to vascular absorption and clearance in to the systemic circulation. This clearance route will most likely affect small molecules more than proteins.(Joseph et al., 2017)

2.4.3 The periocular route:

Several studies on beta-adrenergic receptor blockers (e.g., propranolol, atenolol, timolol, and nepradilol) provide insight on the transit of drug from ophthalmic drops to the retina. When timolol applied to the conjunctiva was shown to rapidly diffuse across the conjunctiva to gain access toperiocular fluid and the posterior sclera. Timolol levels in the cornea and aqueous humour were significantly less when the drug solution was denied contact with the cornea surface. This results support the periocular trans scleral route as the predominant route for timolol transit to the back of the eye, and that drug distribution via this path can be rapid.(Joseph & Venkatraman, 2017)

2.5 Nano carrier drug delivery system:

The major aim for any drug delivery system is to achieve therapeutic index at targeted sight and to control the drug delivery systems. Targeting will ensure high efficiency of the drug and reduce the side effects. The reduction of side effects can also be achieved by controlled release. Nanocarriers for drug delivery may be defined as sub-micron colloidal particles (10-1000nm) that contain a therapeutic agent either dispersed in a polymer carrier matrix, encapsulated within polymer shell, adsorbed to the particle surface, or encapsulated within a structure such as liposome. (Joseph et al., 2017)

Nanocarriers offer many advantages over free drugs.

1) Protect the drug from early degradation.
2) Prevent drugs from early interacting with the biological environment.
3) Enhance absorption of the drugs into a selected tissue. (ex. Solid tumors)
4) Control the p'kinetic and drug tissue distribution profile.(Joseph et al., 2017)

For cancer therapy, nanocarriers offer certain advantages like lower toxicity because of entrapment of the cytotoxic drug and also improved BA of the anti-cancer drug.

2.6 Liposomes as drug delivery system

Liposomes are phospholipid made, biodegradable, nontoxic vesicles of spherical shape. They are having self-centric property due to their hydrophilic head and hydrophobic tail.Due to their size and hydrophobic and hydrophilic characteristics and biocompatibility, liposomes are promising systems for drug delivery.Phospholipids having anamphiphilic structure in that hydrophilic head will attract towards aqueous phase and hydrophobic tail portion made up with acyl chain associate together to form a membrane of liposomes. Liposomes were discovered about 60 years ago by A.D Bangham.(Meisner & Mezei, 1995)

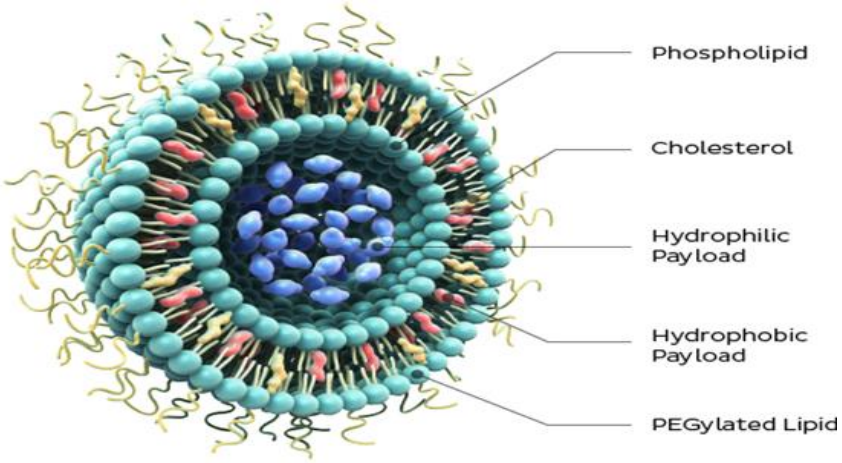


Figure 2.6 Structure of liposomes

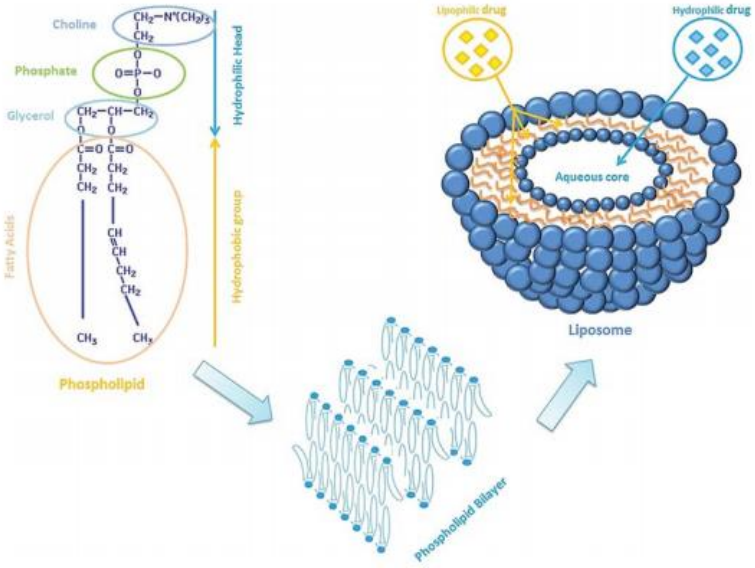


Figure :2.7 Schematic drawing of liposomes structure and lipophilic or hydrophilic drug entrapment models.

Vesicle Type	Abbreviation	Diameter Size	No of Lipid Bilayer
Unilamellar vesicle	UV	All size range	One
Small Unilamellar vesicle	SUV	20-100 nm	One
Medium Unilamellar vesicle	MUV	More than 100nm	One
Large Unilamellar vesicle	LUV	More than 100nm	One
Giant Unilamellar vesicle	GUV	More than 1 micro meter	One
Oligolamellar vesicle	OLV	0.1-1 micro meter	Approx. 5
Multilamellar vesicle	MLV	More than 0.5	5-25
Multi vesicular vesicle	MV	More than 1 micro meter	Multi com-partmental structure

Table no. 2 classification of liposomes

Structurally classification of Liposomes based on lipid bilayers

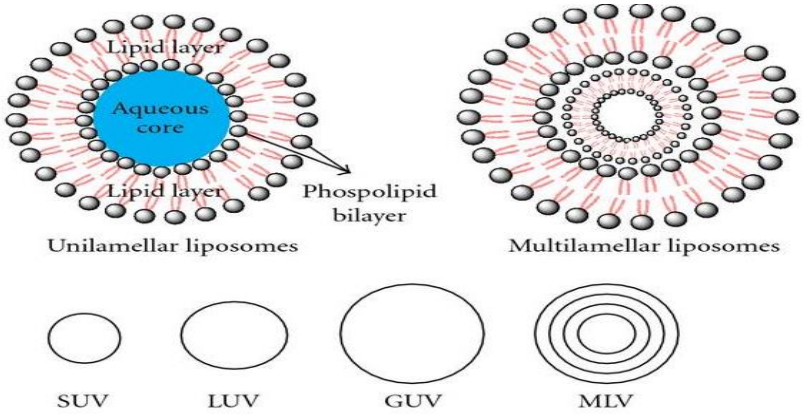


Figure 2.8 Classifications of liposomes

As Liposomes are having ability to bind both aqueous and lipid moiety in the form of polar head and non-polar tail. The polar end mainly composed of phosphoric acid and non polar end mainly composed of acyl chain. Liposomes are formed when the thin films are hydrated

and stacks of liquid crystalline players become fluid and swells. Once these vesicles get formed, a change in vesicle shape and morphology required energy input in the form of sonic energy to get SUVs and mechanical energy to get LUVs.(Meisner & Mezei, 1995)

2.6.1 Methods of preparation

The choice of liposome preparation method depends upon these parameters,(Samad, Sultana, & Aqil, 2007)

1) The physicochemical properties of liposomal ingredients and the material to be entrapped.
2) Nature of media in which the lipid vesicles are dispersed.
3) Entrapped substance's effective concentration and it's potential toxicity.
4) During application or delivery of the vesicles, any processes involved or not.
5) Polydispersity, optimal size and shelf life of the vesicles
6) Possibility of scale up production.

In general preparation methods, first step is solubilization of lipophilic materials in organic solvents of the constitutive lipid and then lipid hydration in compatible buffers at above gel liquid crystal transition temperature (Tm) and MLVs form instantly. Here solubilization required to assure homogeneity of lipid mixtures. There are various methods for the preparation of liposomes. Which is followed by mechanical dispersion methods and solvent dispersion methods.(Samad et al., 2007)

2.6.1.1 Mechanical dispersion methods (Akbarzadeh, Rezaei-sadabady, Davaran, Joo, & Zarghami, 2013)

First dissolve the lipids in to organic solvent to assure a homogeneous mixture of lipids. Generally as an organic solvent, chloroform or chloroform:methanol mixtures are used.

Then next step is Hydration of the dry lipid film/cake, it is carried out by adding an aqueous medium to the container of dry lipid with agitating. The temperature of the hydrating medium should be maintained throughout above the gel liquid crystal transition temperature (Tc or Tm) of the lipid during the hydration period. In case of high Tc lipids, first transfer the lipid suspension to a round bottom flask and then place the flask on a rotary evaporation system without a vacuum and Spin the flask in the warm water bath maintained at a temperature above the Tc.The hydration medium is generally determined by the application of the lipid vesicles. Suitable hydration media include distilled water, buffer solutions,

saline, and non-electrolytes such as sugar solutions.Theproduct of hydration is a large, multi-lamellar vesicle (LMV) analogous with high poly-dispersity index. Then reduction of particle size can be achieved with the various techniques including sonication and extrusion methods.

2.6.1.2 Sonication method (Akbarzadeh et al., 2013)

In this method, small uni-lamellar vesicles (SUV) obtained with the Disruption of LMV suspensions using sonic energy (sonication). For that bath and probe sonicator are use generally. Probe tip sonicatordeliver high energy input to the lipid suspension but there are some disadvantages like suffer from overheating of the lipid suspension can cause degradation andChances of contamination because of Sonication tips. For these reasons, bath sonicators are the widely used.Sonication of an LMV dispersion is accomplished byplacing a test tube containing the suspension in abathsonicator (or placing the tip of the sonicatorinthe test tube) and sonicating for 5-10 minutes above the Tc of the lipid. There are various parameters influenceMean size and distribution like composition and concentration of lipid dispersion, temperature of assembly, sonication time and Power and volume.

2.6.1.3 Extrusion method (Akbarzadeh et al., 2013)

Liposomes of defined size and homogeneity can be prepared by sequential extrusion of the usual multi-lamellar vesicles through polycarbonate membranes. The phospholipids must be handled at a temperature above their transition temperature (Tc) from gel to liquid crystallinephase The principle in extrusion technique is based on employment of moderate pressure to force MLVs through polycarbonate filters with defined pore size. At applied pressures (100-150 psi), MLVs display a reduced-size while maintaining their multi-lamillarity with homogeneity , but here important thing is to maintain transition temperature throughout the process because at above transition temperature, liposomes are more flexible and their outer lamellar vesicles can break easily and can pass through polycarbonate membranes at reduced size. Different size of Polycarbonate membranes are available like 0.2µm, 0.08µm and 0.05µm pore size. Here more than one passes are required for the Unprocessed MLVs have limited uses in in vivo studies because of their large diameter and heterogeneity of size (Gregoriadis, 2007). However, the techniques used to change these parameters may influence physical properties of liposomes. The conversion to SUVs from MLVs may result in vesicles with very low trapped volumes. Furthermore, SUVs can be

unstable and prone to fusion process due to the high curvature of the lipid bilayer (New, 1990). Extrusion technique used to produce LUVs may result in rupturing and resealing which leads further to leakage of the entrapped drug and the final vesicles may have lower amount of entrapped material, depending on the lipophilicity of the drug (Gregoriadis, 2007).

2.6.1.3 Solvent dispersion methods(Akbarzadeh et al., 2013)

In this method, a suspension of lipids dissolved in diethyl ether or ethanol is slowly injected to an aqueous solution of the material to be encapsulated at 55-65°C or under reduced pressure. The subsequent removal of organic solvent under vacuum leads to the formation of liposomes. The main disadvantages of the method are particles are having high polydispersity index (70-190 nm) and the exposure of compounds to be encapsulated to organic solvents or high temperature.

Chapter 3. Reported evidences and hypothesis

3.1 Reported evidences

1) Samolinetal.(1981) investigated the role of liposomes in ophthalmic drug delivery.
2) Schaeffer and Krohn explored the role of vesicle type on transcornealpermeationacrosstheexcisedrabbitcornea.
3) Zhang et al. utilized cytochrome-C (Cyt-C) loaded cationic liposomes for the treatment of selenite-induced cataract in rats.
4) Shen and Tu reported the application of liposomes for the delivery of ganciclovir (GCV) to the vitreous humor via topical administration in the rabbits.
5) Chetoni et al. reported acyclovir (ACV) containing positively charged unilamellar liposomes (LIPO-ACV), administered topically into rabbit eyes.
6) A.K mitra et.al. Investigated the effect of composition on the encapsulation efficiency. This study reported improvement in the entrapment efficiency (EE) with increasing phosphatidylcholine component.
7) Mark r. Prausnitzet.al.(1998) identified from the rabbit tissue and other sources that the sclera is much more permeable than the cornea and this may serve as a potential pathway to deliver a drug to the back of the eye.
8) Urttietal.(1990) found that when eye drops in a volume more than 25 µl comes in a contact with surface of cornea, drug loss observed due to overspill. Before drug comes in direct contact with surface of ocular, it is mixes with the precorneal film. It has been observed that even if the drug loss due to drainage is compensated by sustained drug delivery through a solid delivery system, because of surface of ocular barriers, the bioavailability reaches only up to 10%.
9) Based upon studies,Ha¨ma¨la¨inen et. Al. found thatcompared to cornea,The conjunctiva has more than 200 times greater paracellular spaces and 2 times larger pores and 16 times higher pore density, Hence due to greater surface area and leakier epithelium compared to cornea are to most important parameters for the absorption for large and hydrophilic molecules.
10) Lajunen et al. reported that conjunctival capillaries are fenestrated and that there are few barriers preventing the transfer of macromolecules or NPs from the epithelium to the blood vessels [3]. Below the retinal pigment epithelium, the capillaries are densely

fenestrated with pores with diameters of 75–85 nm, which may limit the size of the particles that can pass through.

11) Ahmed et al., 1987 reported that in the comparison of corneal permeability, conjunctival permeability to hydrophilic drugs is higher. For some sucrose,b-blockers, and inulin,scleral permeability was found to be higher than the corneal permeability.
12) According to Allen et al., 1981, for intra cellular drug delivery,Adsorption of liposomes to cell membrane is one of the important mechanisms. In the presence of cell surface proteins,the adsorbed liposomes, becomeleakier and release their contents in cell membrane. Because of that,higher concentration of drugobtainsadjacent to cell membrane and simplifies cellular uptake of drug by passive transport.
13) Lipowsky, 1995 reported that after theadsorption of liposomes on the surface of cell membrane, engulfment and internalization in to endosome proceeds. Endosomes carry liposomes to lysosomes. Later, because of lysosomal enzymes, degradation of the lipids observed and then release the entrapped drug into the cytoplasm.
14) Amrite*etal*.showed that the 20 nm particles were cleared fast from the episcleral surface, and were transported across the sclera to some extent.
15) Takechi-Haraya*et al*. found cholesterol to be detrimental to saturated lipids, causing an increase in the permeability coefficient.
16) Kohei Tahara et al reported that the scleral pore is hydrophilic and the diameter varies between 20 and 80 nm.
17) Amrite*etal*.showed that the 20 nm particles were cleared fast from the episcleral surface, and were transported across the sclera to some extent. In comparison, 200 nm particles were found to be retained at the site of action for more than a month post injection (due to slower clearance of the particles compared to the smaller 20 nm particles). [36]

3.2 Hypothesis:

The cornea has relatively poor permeability characteristics to both water soluble and sparingly soluble molecules. Conjunctiva and sclera is relatively permeable for the water soluble molecules. The conjunctiva has more than 200 times greater paracellular spaces in the comparison to corneaand 2 times larger pore size and pore density is 16 times higher. So because oflargesurface area and holier epithelium compared to cornea, conjunctiva appears to be more favoured as the route of absorption forlarge and hydrophilic particles.(Joseph et al., 2017)

The diameter of scleral pores varies between 20 to 80 nanometres. It is reported that 20 nm particles were cleared fast from the episcleral surface so that liposomal size should between 20 to 80nanometres.The outer surface of liposomes is hydrophilic which provides feasibility to penetrate sclera. The liposomes are able to show some diffusion into the scleral tissue based on the malleability and fluidity compared to the rigid polycarbonate nanoparticles. The distribution of the liposomes may be different among the different types of liposomes, based on their phospholipid type, presence of cholesterol (bilayer rigidity) and charge. Scleral surface is negatively charged so the positively charged liposomes, remained at the episcleral region because of electrostatic interations, so it may not provide good permeability.The negatively charged liposomes and neutral liposomes were able to diffuse to some extent intrasclerally. So the lipids which are using in liposomal preparation must be anionic. (Agarwal et al., 2016)

There are four expected mechanisms for intracellular drug delivery to the liposomes.(Agarwal et al., 2016)

1. **Adsorption:**for intra cellular drug delivery, cell membrane Adsorption of liposomes is important mechanism of intracellular drug delivery. In the presence of cell surface proteins, liposomes become leaky and release contents in cell membrane. Because of that, higher conc. of drug observed close to cell membraneand alsoenables cellular uptake of API by passive transport.

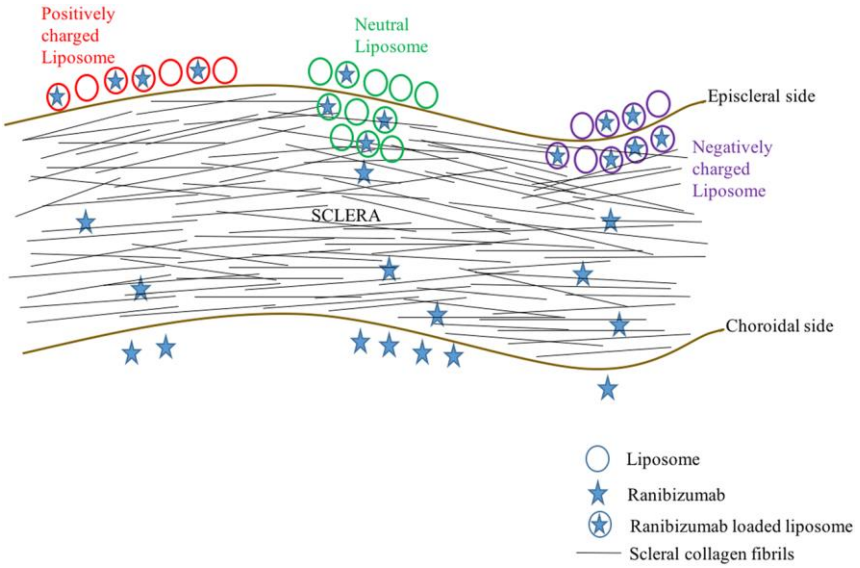


Figure 3.1 Diagram shows charge-based diffusion and drug releasein liposomes in the sclera.

2. **Endocytosis:**After the Adsorption of liposomes on the surface of cell membrane, further process observed is their engulfment and internalization into endosomes. Endosomes carry liposomes to lysosomes. After that, because of lysosomal enzymes degradationof lipids has observedand finally release the entrapped drug into the cytoplasm. (Lipowsky, 1995).

3. **Fusion:**In the process of fusion,intermixing of lipid bilayer of liposomes with lipoidal cell membrane observed that further results in adjacent diffusion of lipids and then direct delivery of liposomal contents into the cytoplasm (Knoll et al., 1988).

4. **Lipid exchange**: One of the important advantage of liposomes is that membrane of liposomes resembles cell membrane phospholipids, so that lipid transfer proteins in the cell membrane identify liposomes and subsequently cause lipid exchange. And this results inweakening of liposomal bilayer membranes and intracellular release of drug particles.

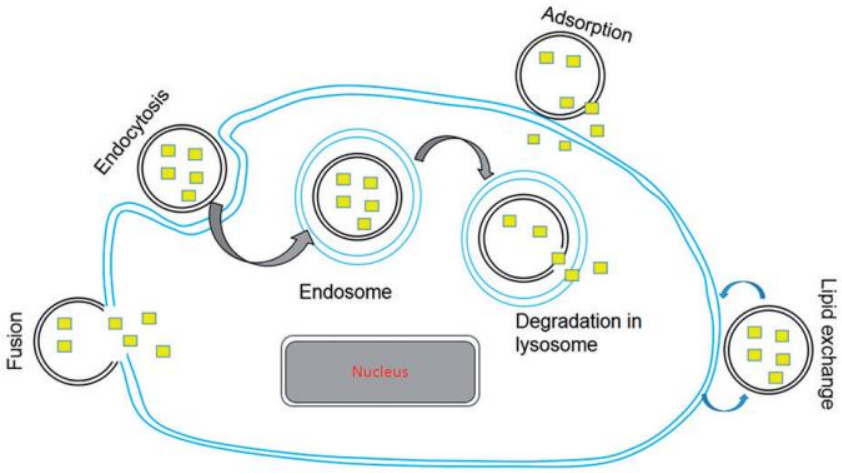


Figure 3.2 Schematicinformation of various mechanism of intracellular drug delivery by liposomes.Here Small squares indicate entrapped API in liposome.

Chapter 4. Envision Research plan

4.1 Physicochemical properties of SDO1:

4.1.1 Introduction

SDO1 is an multitargeted tyrosine kinase inhibitor with antiangiogenic and antitumor activities based on the inhibition of several related tyrosine kinase receptors like vascular endothelial growth factor receptors, platelet derived growth factor receptors which are implicated in tumor proliferation, angiogenesis, and metastasis.(Kavitha, Saidevaraj, & Lakshmi, 2016),(Alshetaili et al., 2018),(J. H. Kim et al., 2015)

4.1.2 Drug substance physicochemical properties

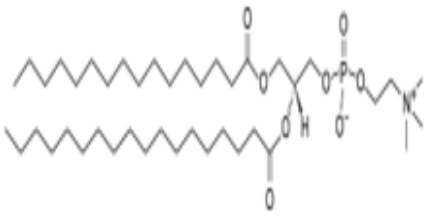
SDO1 is orange coloured substance with salt, which has poor solubility in ethanol and water. It is photosensitive substance. Dissociation constant of drug is 9.3 and partition coefficient of drug is 2.5.(Knickelbein et al., 2016),(Detry et al., 2013),(Hu et al., 2016),(Padervand, Ghaffari, Attar, & Nejad, 2017)

4.2 Excipient profile

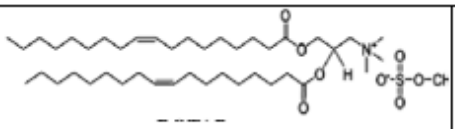
Table no. 3 Properties of lipids

4.2.2 Bilayer forming lipids

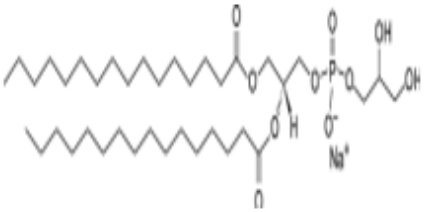
4.2.2.1 Neutral lipid

Sr. no	Lipids	Structure	Molecular weight	Application
1.	HSPC	 Empirical formula: $C_{44}H_{88}NO_8P$ IUPAC: 1,2-Distearoyl-rac-glycero-3-phosphocholine	790.161	-Brucine-loaded liposomes composed of HSPC and DPPC at different ratios: in vitro and in vivo evaluation. -Effect of oxymatrine HSPC liposomes on improving bioavailability, liver target distribution and hepatoprotective activity of oxymatrine.

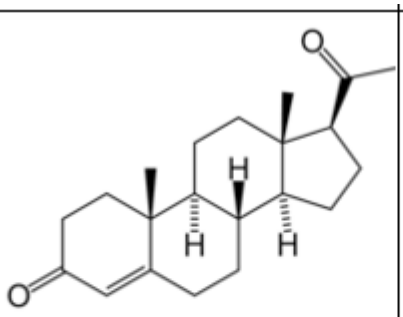
4.2.2.2 Cationic lipid

2.	DOTAP chloride	 Empirical formula: $C_{42}H_{80}ClNO_4$ IUPAC: 1,2-Dioleoyl-3-trimethylammonium-propane	698.555	-Cationic Liposomes Carrying siRNA: Impact of Lipid Composition on Physicochemical Properties, Cytotoxicity and Endosomal Escape. -Intranasal Immunization with DOTAP Cationic Liposomes Combined with DC-Cholesterol Induces Potent Antigen-Specific Mucosal Immune Responses in Mice.

4.2.2.3 Anionic lipid

3.	DPPG. Na	 Empirical formula: $C_{38}H_{74}NaO_{10}P$ IUPAC: 1,2-Dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol), sodium salt	744.964	-dppg liposomes as preferential vehicles for "human-identical" ceramides. -Effect of Lipid Composition on Activity of Liposome-Entrapped Ampicillin against Intracellular Listeria monocytogenes Tm: 41˚C

4.2.3 Fluidity buffer

4.	Cholesterol	 Empirical formula: $C_{27}H_{46}O$ IUPAC: Cholest-5-en-3beta-ol	386.664	Cholesterol is considered to be practically insoluble (in water) and basic. Moderately soluble in hot alcohol; oils, fats.

4.3 Research plan against hypothesis:

As discussed, particle size and charge of lipid are important parameter for diffusion of liposomes in choroid and retina. So major focus is to develop liposomes with particle size of less than 80nm and comparison of lipid formulations based on their charge with different entrapment efficiency. For the reduction of particle size, extrusion machine will be use.(Joseph et al., 2017)

Batches	Lipids for liposomes	Charge
1(A,B,C)	HSPC- Chol	Neutral
2 (A,B,C)	HSPC- DPPG- Chol	Anionic
3 (A,B,C)	HSPC- DPTAP- Chol	Cationic

Table no. 4 Charge of SDO1 loaded liposomes. (whereA,B,C indicates 0.5,1,1.5 mg/ml loading respectively).

Research plan

Steps:

1. Drug substance characterization with UV spectroscopy.
2. Pre-formulation study of liposomes with gradient salt identification.
3. Formulation of liposomes with different drug loading capacity

4. Characterization and analysis of formulation.
5. In-vitro release of formulation.

Chapter 5: Experimental work

5.1 list of materials and equipment

5.1.1 Chemicals and materials:

Table no. 5 List of Chemicals

Sr. no.	**Name of Chemical**	**Manufacturer**
1.	Ammonium sulfate	Merck ,Germany
2.	Di sodium EDTA	Merck ,Germany
3.	Ammonium hydrogen phosphate	Merck ,Germany
4.	Tri ammonium citrate	Merck ,Germany
5.	Copper gluconate	Merck ,Germany
6.	hydrogenated soybean phosphatidylcholine	Corden, India
7.	Cholesterol	Dishman ,Netherlands
8.	DPPG	Corden, India
9.	Sucrose	Merck ,Germany
10.	L-Histidine	Merck ,Germany
11.	Ethanol	Greenfield, Canada
	Material	**Manufacturer**
1.	Drain disc	GE healthcare, United Kingdom
2.	Poly-carbonate membrane filters	Merck ,Germany
3.	Centrifugal filters	Merck ,Germany
4.	Dialysis membrane	Sigma Aldrich, India
5.	Clips	Sigma Aldrich, India

5.1.2 Solutions:

The following solutions are given in examples of 1L volume:

PBS solution pH 7.4

1. Distilled water 800 ml.
2. Sodium chloride 8 g
3. Disodium hydrogen phosphate 1.44 g
4. Potassium chloride 0.2 g
5. Potassium dihydrogen phosphate 0.24 g
6. Adjust to pH 7.4 by adding HCL
7. Distilled water q.s 1000 ml.

5.2 Methods

5.2.1Drug substance characterization.

5.2.1.1 UV-Visible Spectrophotometric analysis:

1. Determination of absorption maxima and linearity of SDO1 in water: ACN system (5:5).

Preparation of stock solution:

10 mg of SDO1 was accurately weighed and transferred in 100 ml volumetric flask and diluted up to 100ml mixture containing 5 part of water and 5 part of ACN to get a concentration of 100µg/ml.From this, 5ml solution taken in 20 and 25 ml volumetric flask and diluted up to 20ml and 25ml with water:ACN system to get concentrations of 25 and 20 µg/ml respectively. Further sample solutions were prepared in between the range of0.1µg/ml to 15 µg/ml by suitably diluting stock solutions.Absorbance maxima of dilutions was noted on UV visible spectrophotometer at 430 nm. The UV scans ranges were taken between the wavelengths 200-800 nm. The graph of absorbance versus concentration of drug was plotted and analysed for regression coefficient. (Meisner & Mezei, 1995)

2. Determination of absorption maxima and linearity of SDO1 in PBS7.4 .

Preparation of stock solution:

The procedure for the preparation of stock solution were same as SDO1 in water:ACN. Except here as a dilution media, PBS buffer were used. Absorbance maxima of dilutions was noted on UV visible spectrophotometer at 430 nm. The UV scans ranges were taken between the wavelengths 200-800 nm. The graph of absorbance versus concentration of drug was plotted and analysed for regression coefficient. (Ramazani et al., 2015)

5.2.2 Pre-formulation study for liposomal formulation.

The aim behind Pre-formulation study was Gradientidentification of SDO1. For that precipitation trial of drug was performed in different salts solution. From that we can understand the physicochemical properties of the formed precipitates.

5.2.2.2 Preparation of stock solution

1. Preparation of 1.5 mg/ml of ammonium sulfate

1. 75 mg of ammonium sulfate was taken and transferred in 50 ml glass vial.
2. Ammonium sulfate completely dissolved in 25 ml of WFI with the help of sonicator.

2. Preparation of 3.8 mg/ml of disodium EDTA

1. 190 mg of disodium EDTA was taken and transferred in 50 ml glass vial.
2. Disodium EDTA completely dissolved in 25 ml of WFI with the help of sonicator.

3. Preparation of 1.3 mg/ml of ammonium dihydrogen phosphate

1. 65 mg of dihydrogen phosphate was taken and transferred in 50 ml glass vial.
2. Ammonium dihydrogen phosphate completely dissolved in 25 ml of WFI with the help of sonicator.

4. Preparation of 2.8 mg/ml of Tri ammonium Citrate

1. 140 mg of Tri ammonium Citrate was taken and transferred in 50 ml glass vial.
2. Tri ammonium Citrate completely dissolved in 25 ml of WFI with the help of sonicator.

5. Preparation of 5.2 mg/ml of copper gluconate

1. 260 mg of copper gluconate was taken and transferred in 50 ml glass vial.
2. copper gluconate completely dissolved in 25 ml of WFI with the help of sonicator.

6. Preparation of SDO1 stock solution

1. 75 mg of drug accurately weighed and transferred in 25 ml of WFI.
2. SDO1 completely dissolved in 25 ml of WFI with the help of sonicator.

Table no. 6 Composition of drug salt solution

1A SDO1ammoniumsulfate solution

Batch no.	Compound	M.wt (g/mole)	final mg/ml	mM	Molar ratio	Amount of solution taken(ml)	Concentration (mg)	observation
1A	SDO1	398	1.5	3.76	1.000	25	75	Precipitates observed.

1B SDO1 di sodium EDTA solution

batch	compound	M.wt (g/mole)	final mg/ml	mM	Molar ratio	Amount of solution taken (ml)	Concentration (mg/ml)	observation
1B	SDO1	398	1.5	3.76	1.000	25	75	No precipitates.
	Di sodium EDTA	336	3.8	11.31	3.004	25	190	

1C SDO1Ammonium dihydrogen Phosphate solution

Batch	compound	M.wt (g/mole)	final mg/ml	mM	Molar ratio	Amount of solution taken(ml)	Concentration (mg/ml)	observation
1C	SDO1	398	1.5	3.77	1.000	25	75	No precipitates
	Ammonium dihydrogen Phosphate	115	1.3	11.30	2.999	25	65	

1D SDO1Tri ammonium Citrate solution

Batch	Compound	M.wt (g/mole)	final mg/ml	mM	Molar ratio	Amount of solution taken(ml)	Concentration (mg/ml)	observation
1D	SDO1	398	1.5	3.77	1.000	25	75	Precipitation observed.
	Tri ammonium Citrate	243	2.8	11.52	3.057	25	140	

1E SDO1 copper gluconate solution

Batch no.	compound	M.wt (g/mole)	final mg/ml	Mm	Molar ratio	Amount of solution taken (ml)	Concentration (mg)	observation
1E	SDO1	398	1.5	3.77	1.000	25	75	No precipitates.
	copper gluconate	454	5.2	11.46	3.040	25	260	

5.2.3 Preparation of liposomes:

5.2.3.1 Preparation of lipid solutions

Accurate amount of lipidswere weighed and transferred in the 50 ml glass bottle, 10% of ethanol was added in the bottle. Lipid mixture was heated at 65°C (above transition temperature) using hot water bath with the help of stirring at 250 rpm.(Akbarzadeh et al., 2013)(Laouini et al., 2012)(Traïkia, Warschawski, Recouvreur, Cartaud, & Devaux, 2000)(Traïkia et al., 2000)

Table no. 7 Composition of lipid solutions

Lipid composition					
Batch no.	HSPC (mg/ml)	DPPG.Na (mg/ml)	DOTAP.Cl (mg/ml)	Chol. (mg/ml)	Ethanol (%)
2	12.5	-	-	4.16	10
3	15	1.5		3.5	10
4	15	-	1.5	5	10
5	15	1.5	-	5	10
6	15	-	-	5	10

5.2.3.2 Preparation of aqueous phase

WFI (200 ml) 80% of batch size was taken in 500 ml glass bottle Weighed ammonium sulphate was dissolved completely in approx. 80% of batch size(300 ml) of WFI. Final volume was adjusted to 100 ml with WFI to produce 250 mM ammonium sulphate solution. (Traïkia et al., 2000)

* same procedure was followed in every batch

Table no. 8 Composition of aqueous phase in every batch:

Batch no.	Ammonium sulphate (mM)	Quantity/ml (mg/ml) in water
2	250	33.03
3	300	39.64
4	300	39.64
5	300	39.64
6	300	39.64

5.2.3.3Lipid hydration

Once both phase i.e lipid phase and buffer phase attained 65˚C, the prepared lipid solution was added into ammonium sulphate solution under stirring (350 RPM) at 65˚C. Then lipid was rinsed with ethanol and injected in lipid dispersion kept in stirring by injection. Final volume = 330 ml.(Samad et al., 2007)

Process of stirring: total stirring time: 30 mins

* Same procedure was followed in every batches.

5.2.4 Size reduction of liposomes

5.2.3.4Extrusion

The hydrated lipid suspension was extruded 2 times at 65˚C, 200 psi nitrogen pressure through 0.2 µm polycarbonate membrane(90 mm diameter, whatman) and optimized times through 0.08 and 0.05µm poly carbonate membrane (90 mm diameter, whatman)Then dispersion was cooled to room temperature. lipid loss observed approx. 10 ml.The resultant

products were stored in the fridge at 4°C over night prior to Dia-filtration.(Samad et al., 2007)(Ong, Chitneni, Lee, Ming, & Yuen, 2016)

Table no. 9 Parameters followed during extrusion of batches.

Batch no.	Nitrogen pressurein PSI	No. of Extrusion passes (polycarbonate membeane)				Temperature (°C)	pH	Conductivity (ms/cm)
		0.2 µm	0.08 µm	0.08 and 0.05 µm				
2	200	2	12	-	-	65	4.87	29
3	200	2	12	-	10	65	5.12	31.87
4	200	2	8	5	3	65	4.67	30
5	200	2	8	5	3	65	4.85	34
6	200	2	8	5	3	65	5.01	32

5.2.5 Ultra-filtration:

5.2.5.1 Procedure for 10% sucrose solution:

1. S.S vessel (capacity:10 L) was kept on weighing balance and weight was tare.
2. 600 g sucrose weighed and transferred in S.S vessel.
3. WFI was added to adjust final weight 6 L to produce 10% sucrose solution.
4. Sucrose was completely dissolved using stirrer.

5. pH : 8 to 9
6. conductivity:7 to 11 µs/cm

5.2.5.2 Procedure for Ultrafiltration:

1. lipid suspension preparedwas passed through hollow fibre cartridge750 KDa and was concentrated to 60%. further the process was continued by maintaining the volume of liposome dispersion to certain volume against 10% sucrose solution till the conductivity of permeate matched to the conductivity of the 10% sucrose solution.

   After achieving the desired conductivity, the addition of 10% sucrose solution was stopped Further recovery cycle (washing of cartridge) was performed by using10% sucrose solution and the final volume of the liposomal dispersion was made up to 60% to batch size.

5.2.6 Drug loading

For loading process, eachbatches divided in to A,B,C sub part with the loading capacity of 0.5 ,1 ,1.5 mg/ml respectively. (Gubernator, 2011)(de la Fuente et al., 2010)(Wehbe et al., 2017)

5.2.6.1 Preparation of sucrose histidine buffer:

1. 75 Mg of L-histidine was taken and transferred in 25 ml clear glass vial.
2. 17 Ml of 10% sucrose solution was prepared and transferred in vial containing L-histidine.
3. L-histidine was completely dissolved in 10% Sucrose solution by sonication.(Cullis, Mayer, Bally, Madden, & Hope, 1989)(Gubernator, 2011)

Table no. 10. Composition of sucrose histidine buffer

Sr. no.	Ingredients	Qty/ml (mg/ml)	Qty / 50 ml (mg/ml)	Qty in ml
1.	Histidine	1.5	75	-
	Sucrose	-	-	17 ml

5.2.6.2 Procedure for drug solution in sucrose histidine buffer:

1. SDO1 accurately weighed and transferred in sucrose histidine buffer solution.
2. SDO1 was completely dissolved in buffer by stirring at 300 rpm. Time required 30 min.

5.2.6.3 Process for drug loading:

1. 33 Ml of 60 % concentrated placebo liposome was taken in 50 ml glass bottle and kept on stirring at 350rpm on hot water bath at 55˚C. (Water bath set temperature: 58 ˚C)
2. Prepared SDO1 solution was added in liposome kept in stirring. After stirring for 60 min, sample was cooled to 2˚-8˚C in refrigerator.

Table no. 11 Drug loading capacity of different batches of SDO1

Batch no.	Drug loading capacity (mg/ml)	SDO1 (mg/50 ml)
2A	0.5	33.55
2B	1	67.1
2C	1.5	100.65
3A	0.5	33.55
3B	1	67.1
3C	1.5	100.65
4A	0.5	33.55
4B	1	67.1
4C	1.5	100.65
5A	0.5	33.5
5B	1	67.1
5C	1.5	100.65
6A	0.5	33.5
6B	1	67.1
6C	1.5	100.65

5.2.7Characterization of liposomes.

5.2.7.1 Particle size of liposomes.

Particle size of dispersion was observed in Malvern zeta sizer ver. 7.11.Which is based on the principle of Dynamic light scattering and Brownian motion of particles in medium. When particles diffuse in the fluid, the collisions with the medium molecules causes a random movement of the particles. For the measurement of diffusion speed, the speckle pattern produced by illuminating the particles with a laser is observed. The scattering intensity at a specific angle will vary with time, and this is detected using an avalanche photodiode detector (APD). A digital autocorrelator generates a correlation function, and changes in intensity are analysed with it. (Laouini et al., 2012)(Danaei et al., 2018)(Johnston, Edwards, Karlsson, & Cullis, 2008)(Joseph et al., 2017)

5.2.7.2 Zeta potential of liposomes

Zeta potential is the charge acquired by a particle or molecule in a given medium and it depends upon the surface charge,concentration and types of ions in the solution. It is determined by measuring molecules velocity while they are moving due to electrophoresis.If a field is applied, Particles and molecules which are having zeta potential will migrate towards an electrode. And then it is analysed through laser doppler electrophoresis.As known that Particles of similar charge will repel each other, those with high charge will resist flocculation and aggregation.Suchsampleswill more stable for longer periods. (Alshetaili et al., 2018)(Joseph et al., 2017)

5.2.8 Separation of Unentrapped active ingredients.

5.2.8.1 Dialysis method.

Dialysis is based on diffusion during which the mobility of solute particles between two liquid space is restricted, mostly according to their size or molecular weight. (restriction of diffusion via charge or polarity is also possible).Amount of free drug from liposomal

dispersions was studied using a dialysis method. Dialysis bags (MWCO 12000-14000, sigma) were soaked before use in distilled water at room temperature for 12 hours to remove the preservative, followed by rinsing thoroughly in distilled water. Liposomal dispersion was placed in a dialysis bag of 9 cm initial length and 6.3 cm diameter. The bag was closed at both ends with cotton thread and tested for leakage. Final length of the bag after tying was 7±0.2 cm. Put the dialysis bag in to 100 ml milli Q water containing beaker. Put the magnetic stirrer in to beaker and 100 rpm provided. 5 ml Aliquots of the release medium were withdrawn for analysis at different time intervals and replaced with 5 ml fresh medium and absorbance checked.The reproducibility and efficacy of the release study were ensured through a control sample containing only the drug in the free form and drug salt complex. This could ensure that the dialysis membrane was not a barrier throughout the release study. The control samples in the case of SDO1 was released in about two hours. (Ramazani et al., 2015)(Kavitha et al., 2016)(City, 2007)

5.2.8.2 Ultra-centrifugal method

A centrifuge is a device for separating particles from a solution based upon to their size, shape, viscosity, density of the mediumand rotor speed. The particles whose density is higher than that of the solvent sediment and particles that are lighter than it float to the top. The ultra-centrifuge is a centrifuge optimized for spinning a rotor at very high speeds also capable of generating acceleration as high as 1000000 g (9800 km/s^2).

The sample is centrifuged in Beckman coulter optima XPN -100 Ultra-centrifuge for separation of free drug from liposomal dispersion.(Milan & Peal, 2013)(Pollard, Fellouah, & Data, 2014)(Ong et al., 2016)

Table no. 12 Ultra-centrifuge trials

For BATCH 2

Batch (A,B,C)	**Rpm**	**Time**	**Ml of sample**	**Drug concentration(µg/ml)**	**Results**

		(hrs)		A	B	C	
1.	60000	3	6.5	500	1000	1500	clear separation was not observed
2.	70000	3	6.5	500	1000	1500	clear separation was not observed
4.	80000	4	6.5	500	1000	1500	clear separation was not observed

For BATCH 3,4,5,6

Sample (A,B,C AND PLACEBO)	Rpm	Time (hrs)	Ml of sample	Drug concentration(µg/ml)			Results
				A	B	C	
1.	70000	3	6.5	500	1000	1500	clear separation observed

5.2.8.3 Centrifugal method.

Principle for the centrifugal method is same as the principle of ultracentrifugation accept relative centrifugal force (RCF). In centrifugal method, RCF is much lower than ultracentrifugation. Centrifugal filters (MWCO 10 Kd) were used to separate free SDO1 from Liposomal dispersion. 1.5 ml of SDO1 –liposomal dispersion was placed in the filter and the centrifugation was done at a speed of 12000 rpm for 15 mins for 3 cycles at 4°C using a centrifuge manufactured by Beckman Coulter (Allegra 64R). The solution at the bottom of the filter was carefully removed from the filter.

5.2.8.4 Determination of entrapment efficiency

the entrapment efficiency measurements were performed on UV spectrophotometer 1700 series. To quantify the content of SDO1 in supernatant, series of standard solutions were prepared. And analysis of free drug measured from supernatant with respect to these standard solutions. The absorbance was measured at 430 nm based on the spectral analysis.

5.2.9 In vitro release study

Dialysis bags were soaked before use in distilled water at room temperature for 12hours to remove the preservative, followed by rinsing thoroughly in distilled water. Liposomal concentrate (0.5,1,1.5 mg/ml) dispersed in one mL of histidine sucrose buffer (pH 6.66) was placed in a dialysis bag of 8 cm initial length and 5.4 mm diameter. The bag was closed at both ends with clips and tested for leakage. The final length of the bag after tying was 6 ± 0.2cm. placed dialysis bag into 100ml PBS buffer in glass bottles and placed these bottles into shaking water bath at 37ºC at 50 rpm. 3 ml Aliquots of the release medium were withdrawn for analysis at different time intervals and replaced with fresh 3ml medium. Sink condition was properly maintained with the help of 100 times external media. The reproducibility and efficacy of the release study were ensured through a control sample containing only the drug in the free form and the drug in the complex form with the ammonium sulfate salts.(Baranowski et al., 2014)(Joseph & Venkatraman, 2017)

Chapter 6: Results and discussion

6.1 UV-Visible Spectrophotometric analysis:

6.1.1 Determination of λ max of SDO1:

6.1.1.1 UV absorption maxima of SDO1 in water:ACN(5:5) system

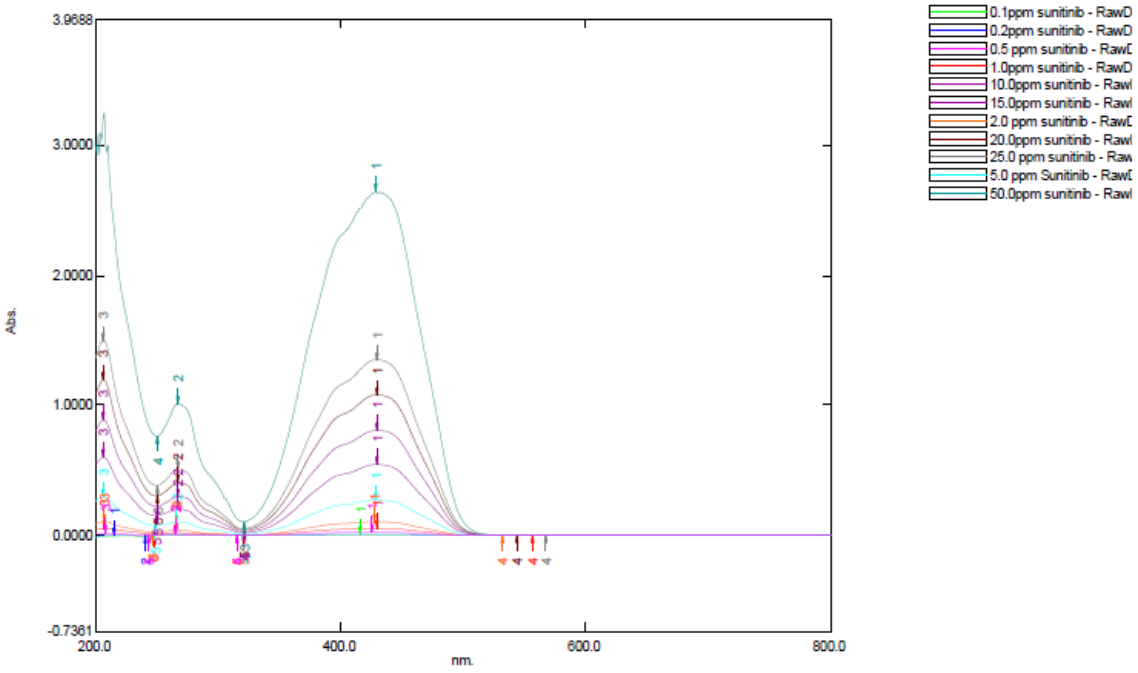


Figure no. 6.1 UV absorption spectra of SDO1 (absorption v/s wavelength)

6.1.1.2 Calibration curve of SDO1.

Preparation of stock solution.

The dilutions which are used for the preparation of linearity curve, same were used for the preparation of the calibration curve.

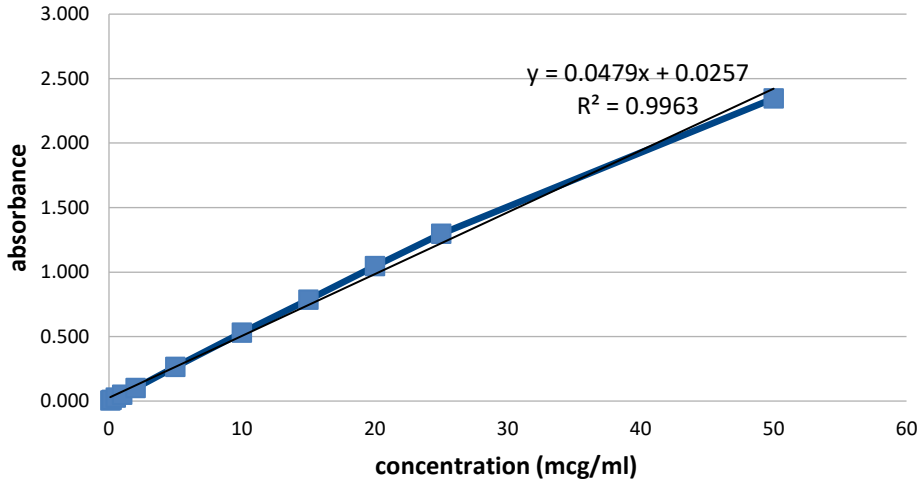


Figure no. 6.2 Standard calibration curve of drug SDO1 in water: ACN system(5:5).

Table no. 13 Absorbance at different concentrationin water: ACN system(5:5).

Sr. no.	Concentrations (µg/ml)	Absorbance			
		1 2	3	Average	
1	0	0	0	0 0	
2	0.1	0.004	0.004	0.003	0.004
3	0.2	0.009	0.008	0.009	0.009
4	0.5	0.026	0.026	0.027	0.026
5	1	0.049	0.050	0.048	0.049
6	2	0.101	0.100	0.102	0.102
7	5	0.264	0. 265	0.263	0.264
8	10	0.530	0.530	0.532	0.530
9	15	0.785	0.787	0.785	0.785
10	20	1.046	1.048	1.045	1.048
11	25	1.296	1.300	1.295	1. 296
12	50	2.346	2.348	2.346	2.346

6.1.1.3 Result:

The wavelength of maximum absorbance (λmax) was found to be 430 nm. Lower Limit of Quantification observed is 0.1 µg/ml.

6.1.1.4 Discussion:

The different concentrations of SDO1 was scanned in UV spectrophotometer UV-1700 series and was found absorbance maxima at 430 nm. Based on that linearity curve obtained, which shows linearity from 0.1 µg/ml to 25 µg/ml and after that from 50 µg/ml it is deviated which we can see in the standard calibration curve of SDO1.

6.1.1.5 UV absorption maxima of SDO1 in PBS 7.4

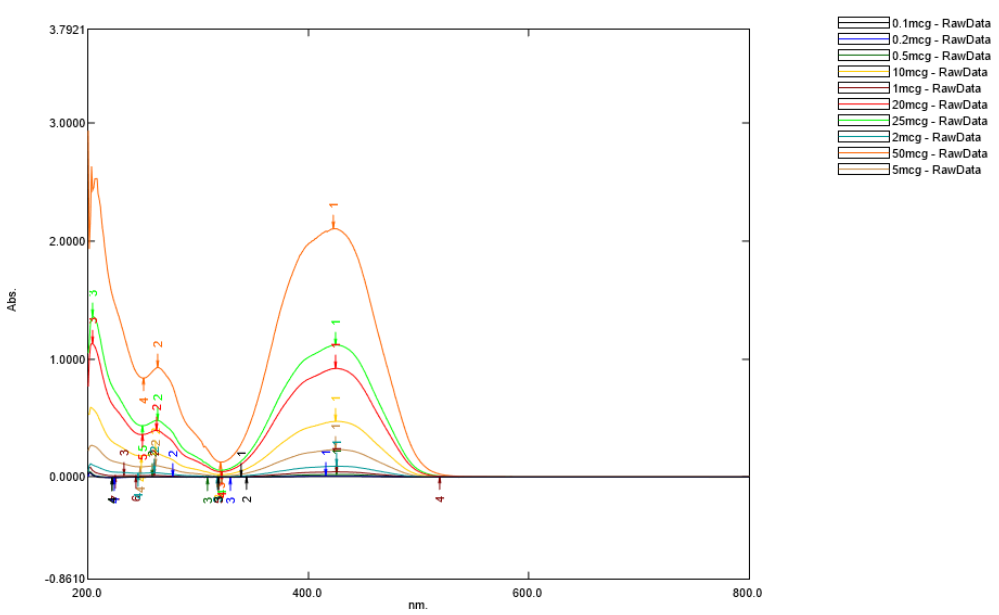


Figure no. 6.3 UV absorption spectra of SDO1 (absorption v/s wavelength) in PBS 7.4

6.1.1.6 Calibration curve of SDO1.

Preparation of stock solution.

The dilutions which are used for the preparation of linearity curve, same were used for the preparation of the calibration curve.

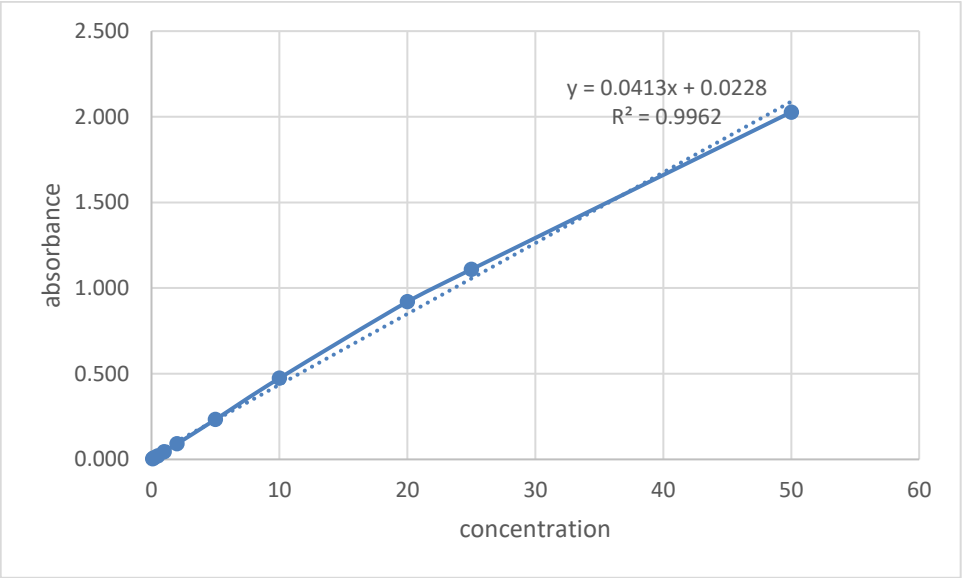


Figure no. 6.4 standard calibration curve of drug SDO1 in PBS buffer (pH 7.4)

Table no. 14 Absorbance at different concentration in PBS buffer (pH 7.4)

Sr. no.	Concentrations (µg/ml)	Absorbance			
		1 2	3	Average	
1	0	0	0	0	0
2	0.1	0.004	0.004	0.003	0.004
3	0.2	0.008	0.008	0.007	0.008
4	0.5	0.020	0.021	0.020	0.020
5	1	0.044	0.043	0.045	0.044
6	2	0.091	0.090	0.092	0.091
7	5	0.233	0. 235	0.233	0.233
8	10	0.474	0.475	0.474	0.474
10	20	0.9200.923	0.9250.920		
11	25	1.109	1.1111.109	1. 109	
12	50	2.027	2.028	2.027	2.027

6.2 Pre-formulation of liposomes

6.2.1 Calculation:

$$\text{mMole} = \frac{\text{mg/ml}}{\text{Molecular weight (mg/mM)}} * 1000$$

SDO1: $$\text{mMole} = \frac{1.5}{398} * 1000$$

$$= 3.76 \text{ mMole}$$

Ammonium sulphate $$11.36 = \frac{\text{mg/ml}}{132} * 1000$$

$$\text{mg/ml} = \frac{11.36*132}{1000}$$

$$= 1.5 \text{ mg/ml}$$

6.2.2 Preparation of precipitates:

To perform trials above salts solution are used with the drug stock solution as shown in the table.

6.2.3 The general procedure for performing the trial

In clean 50 ml glass vial add 25ml of salt solution, to this add 25 ml of drug salt solution with the help of syringe.

6.2.4 Analytical methods:

6.2.4.1 Microscopy:

Observe the precipitates with the help of microscope.

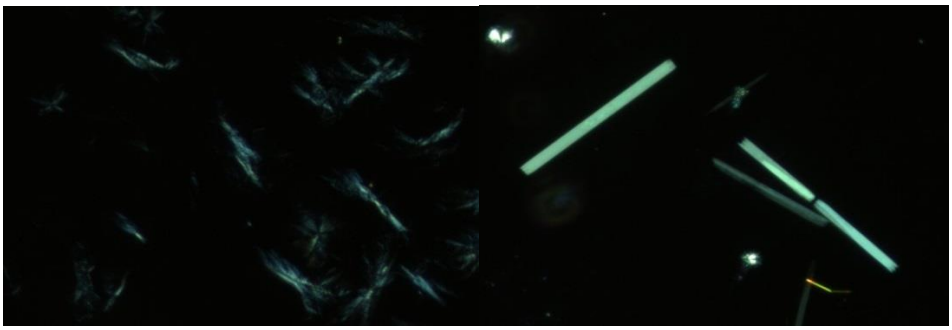
6.2.5 Results:

Table no.15 Observation of precipitates at different time points

Samples	Precipitates observed						
	0 hrs.	**1 hrs.**	**2 hrs.**	**3 hrs.**	**4 hrs.**	**5 hrs.**	**Shape**
1A	Yes	Yes	Yes	Yes	Yes	Yes	**Sun flower shape crystals**
1B	No	No	No	No	No	No	-
1C	No	No	No	No	No	No	-
1D	No	No	No	No	Yes	Yes	**Rod shape crystals**
1E	No	No	No	No	No	No	-

6.2.6 Discussion:

From 5 samples, in sample 1A (SDO1-ammonium sulphate solution) Precipitates shown immediately. And in sample 1D (SDO1- tri ammonium citrate solution) precipitates shown after 4 hours. Precipitation is essential in slow release of drug. In that We can apply these phenomena to the liposomal delivery in that drug salt solution which is present in central compartment of liposome can provide slow release.



(a) (b)

Figure 5.4 Phase contrast microscope (a) Sun flower shaped crystals of SDO1 ammonium sulphate precipitates.(b) Rod shaped crystals of SDO1 tri ammonium citrate precipitates.

In case of SDO1 tri ammonium citrate solution first we have to identify that what parameters triggers the formation of precipitates after 4 hours. It may be because of difference in pH or difference in temperature.

Table no. 16 Effect of pH on SDO1- tri ammonium citrate precipitates

ph	**Observation**
6.39	Precipitation observed
4.96	Precipitates disappears

Buffer used: 10% citric acid anhydrous.

Volume: 0.6 ml

But in case of low pH, precipitates disappear so it gives negative results.

Table no. 17 Effect of temperature on SDO1- tri ammonium citrate precipitates

Temperature (˚c)	**sunitinib- tri ammonium citrate precipitates**	
	Low Ph (4.96)	High pH (6.39)
25	Clear solution	Ppts observed
40	Clear solution	Clear solution

Precipitates get dissolved with the increase in temperature. So based upon above results, SDO1 ammonium sulfate precipitation is more promising so we will apply ammonium sulfate salt in our future studies.

6.3 Optimization of liposomal preparation method.

6.3.1 Extrusion

The extrusion process applied to reduce the particle size in more controllable manner.

6.3.1.1 Discussion:

Here for the BATCH 1 (sample A,B,C) the lipid suspension was extruded 2 times through 0.2 µm polycarbonate membrane and 12 times through 0.08 µm polycarbonate membrane for the optimization of size reduction. For BATCH 2 (sample A,B,C) same polycarbonate membranes were used but in further addition, lipid suspension was extruded 3 times further more with 0.05 µm polycarbonate membrane for the further reduction of particle size and was shown significant difference between particle size of BATCH 1 and 2.(discussed in characterization of liposomes.)

After the process of extrusion, the concentration of ammonium sulphate remained same at outer and inner environment of liposomal compartment. So the system remained thermodynamically stable (at lowest energy level).

6.3.2 Ultra-filtration

This technique is use for the removal salt from the outer compartment with the exchange of buffer in liposomal dispersion. For the drug to penetrate the layers of liposomes and to enter in the central liposomal compartmentsome kind of gradient is required, this technique is also called desalting.

6.3.2.1 Mechanism of ultra- filtration

An ultrafiltration membrane column retains molecules that are larger than the pores of the membrane while smaller molecules such as salts, solvents and water, which are 100% permeable, freely pass through the membrane. The process selectively utilizes permeable (porous) membrane filters to separate the components of solutions and suspensions based on their molecular size. The solution retained by the membrane is known as the concentrate or

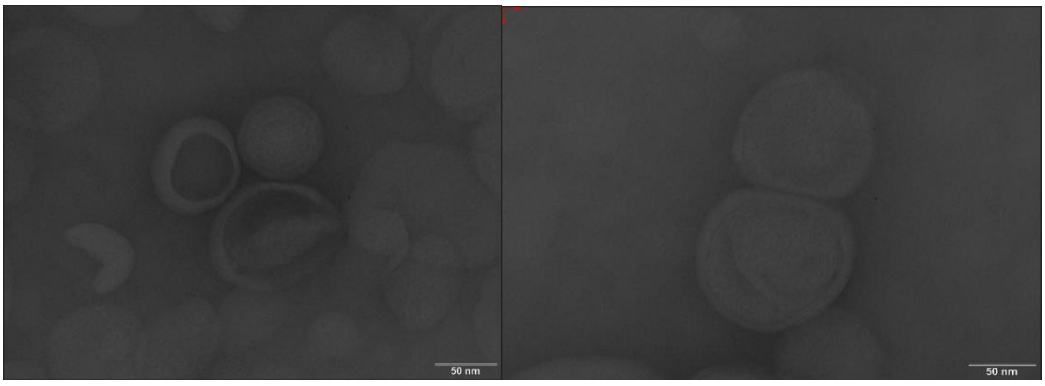
retentate. The solution that passes through the membrane is known as the filtrate or permeates.

Table no. 18 Parameters observed during ultra-filtration.

Conductivity of Sucrose solution	7 to 11 µs/cm
pH of sucrose solution	8 to 10
Inlet pressure	5 psi
Pump RPM	250
UF Cartridge surface area and serial number	420 cm^2, BATCH NO: 9698910
pH of Lipid suspension after UF	4-5
Total time for ultrafiltration	6 -8 hrs
Conductivity of lipid suspension after UF	9 to 15 µs/cm
Product temp. during UF.	8-15˚C

6.4 Morphology of liposomes(Demetzos, 2008)(Li et al., 1998)

Figures are TEM images for the 1 mg/ml SDO1 concentrations for HSPC liposomes. The TEM images were taken after extrusion diafiltration of liposomes. The size of SDO1 liposomes were approx. 80 nm and these values correlate with the size analysis study. Thus extrusion method applied in this study was successful at converting the MLVs into liposomes in the nanometre size range.



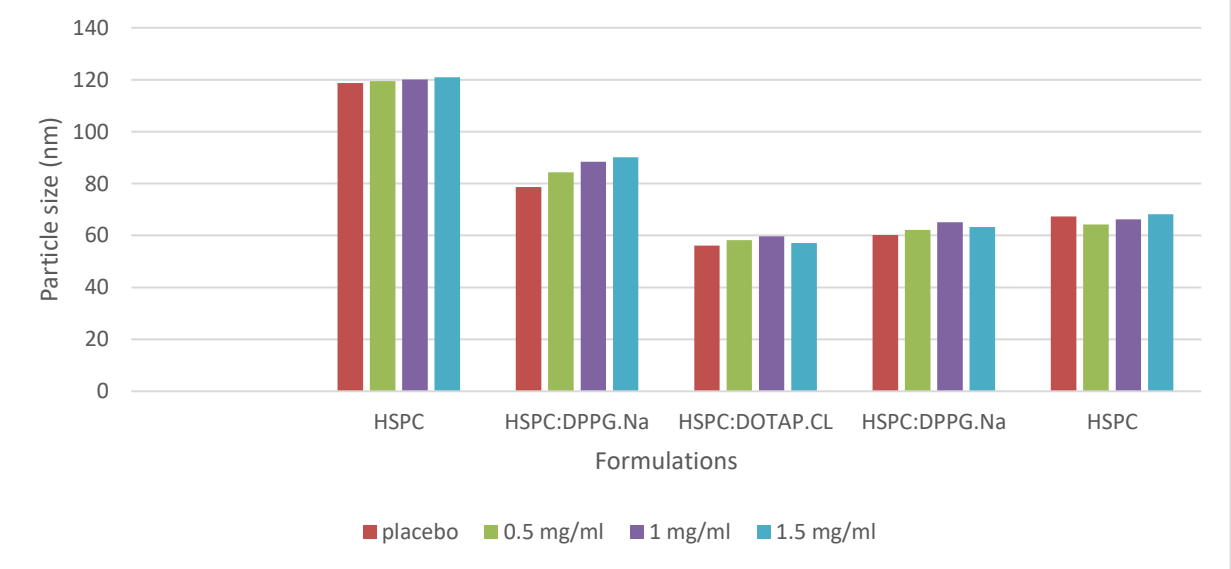
6.5 Physicochemical characterization of liposome formulations

6.5.1 Particle size and zeta potential:

The particle size and zeta potential of placebo liposomes and drug loaded liposomes was measured by Malvern Zetasizer ZS™ (Malvern Instruments, UK). 5 times diluted samples were used. And following results were obtained.

6.5.1.1 Particle size

Results:



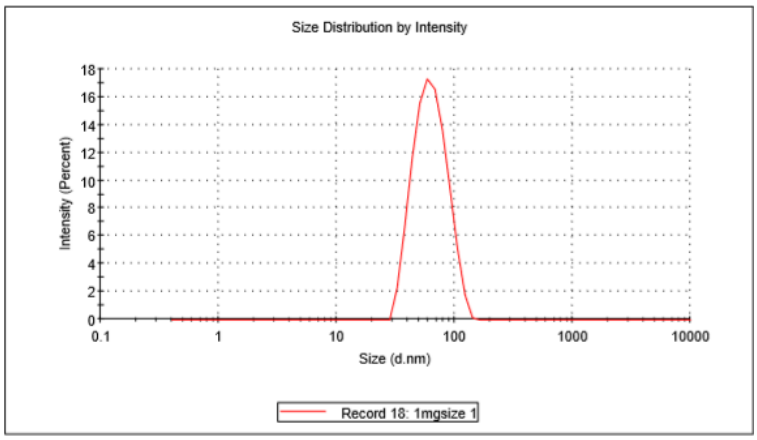


Figure: size distribution by intensity

Table no. 21 particle size parameters of liposomes

Discussion

The particle size of drug loaded liposomes as determined by Malvern was found to be 50-120 nm by extrusion diafiltration method. As shown in table. There are various factors responsible for the size reduction of liposomes but here I determined size reduction with the help of mechanical pressure. Here it is obvious that in the process of extrusion, particle size reduction depends upon transition temperature of lipids, number of passes and pore size of polycarbonate membrane. In first batch 0.2, 0.08 µm polycarbonate membrane used, and size observed around 120nm and PDI is optimum. In second batch because of addition of 0.05 µm and increase in number of passes, reduction of particle size observed but with increase in PDI so that optimization was required. So in third batch, with the optimization of passes and properuse of polycarbonate membranes reduction of particle size observed and PDI also decreased.

Zeta potential

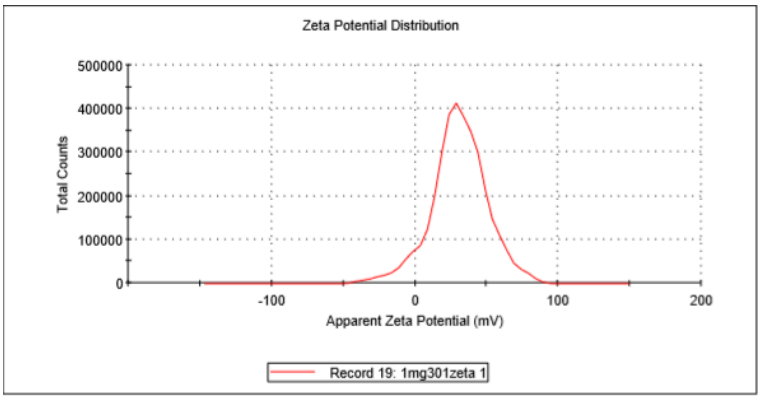
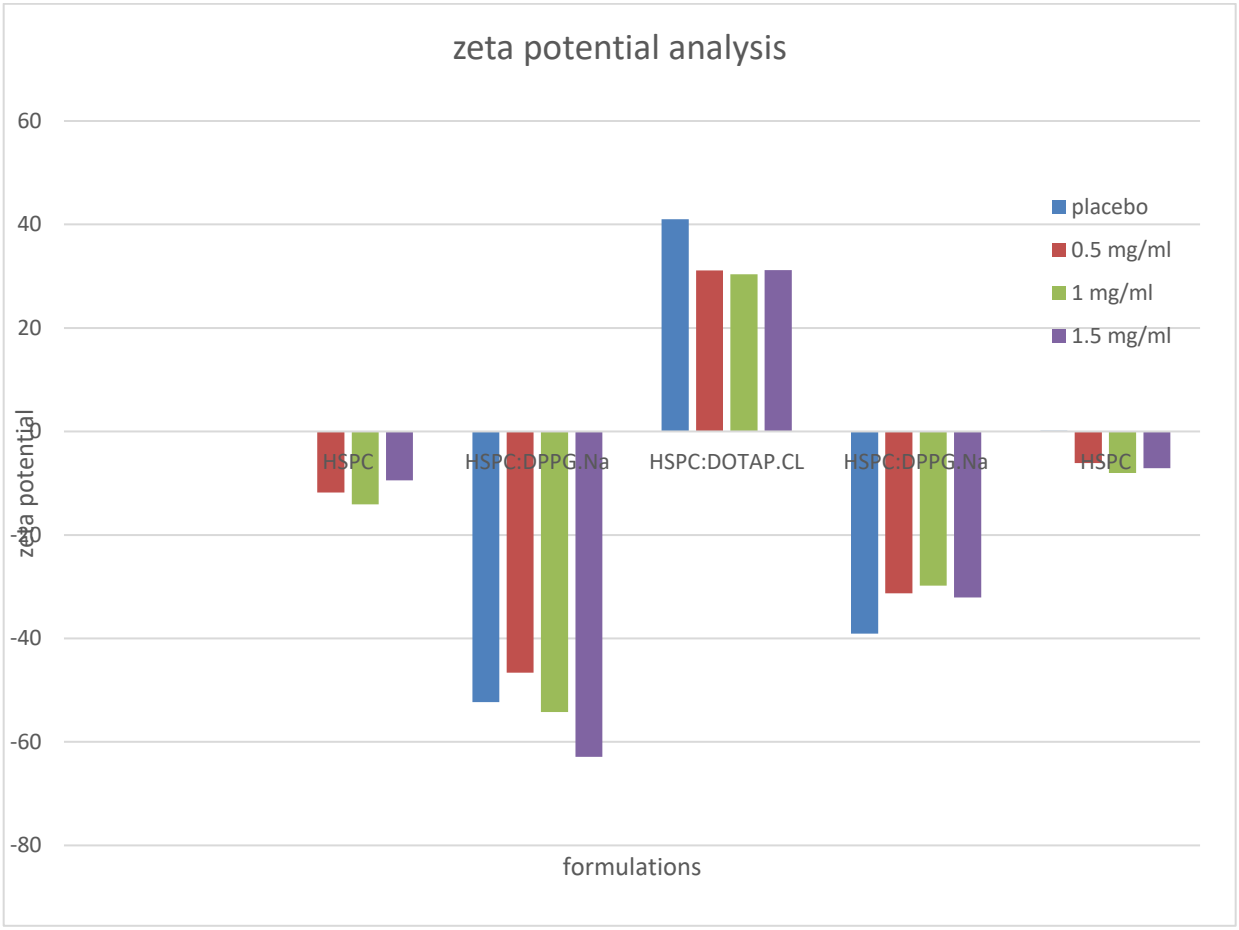


Figure: zeta potential distribution of cationic lipid.

Results:

Table no. 22 Zeta potential of different batches.



Discussion:

It is hypothesized that the cationic liposomes basically localised on the episcleral region due to electrostatic attraction with the negatively charged sclera. And negatively charged liposomes are able to demonstrate some digree of scleral diffusion but less than zwitterionic liposomes, because of repulsion with the negatively charged sclera. So based upon that batch 1 were made with neutral lipids and batch 2 were made with anionic lipids. And zeta potential were as expected.

6.6 Identification of % Assay.

Identification of % Assay is very important step before analysis of liposomes. It indicates actual amount of drug present in the liposomal dispersion. And helps to determine stability

study. All samples are diluted 100 times before interpretation. (Kompella et al., 2003)(Valsecchi et al., 2018)(Valsecchi et al., 2018)

Table no. 19 % Assay identification

For BATCH 2

Sr. no.	Absorbance	Ideal amount of drug present in sample. (Mcg/ml)	Absorbance	Actual amount of drug present in sample. (mcg/ml)	% ASSAY
Sample1(A)	0.264	5	0.181	4.31	68.56
Sample1(B)	0.530	10	0.401	8.49	75.66
Sample1(C)	0.785	15	0.643	13.24	81.91

BATCH 3

Sr. no.	Absorbance	Ideal amount of drug present in sample. (Mcg/ml)	Absorbance	Actual amount of drug present in sample. (mcg/ml)	% ASSAY
Sample1(A)	0.264	5	0.261	4.94	98.86
Sample1(B)	0.530	10	0.538	10.15	101.50
Sample1(C)	0.785	15	0.790	15.09	100.6

BATCH 4

Sr. no.	Absorbance	Ideal amount of drug present in sample. (Mcg/ml)	Absorbance	Actual amount of drug present in sample. (mcg/ml)	% ASSAY
Sample1(A)	0.264	5	0.258	4.88	97.6
Sample1(B)	0.530	10	0.513	9.67	96.79
Sample1(C)	0.785	15	0.805	15.38	102.54

BATCH 5

Sr. no.	Absorbance	Ideal amount of drug present in sample. (Mcg/ml)	Absorbance	Actual amount of drug present in sample. (mcg/ml)	% ASSAY
Sample1(A)	0.264	5	0.241	4.56	91.28
Sample1(B)	0.530	10	0.523	9.86	98.67
Sample1(C)	0.785	15	0.801	15.3	102.03

BATCH 6

Sr. no.	Absorbance	Ideal amount of drug present in sample. (Mcg/ml)	Absorbance	Actual amount of drug present in sample. (mcg/ml)	% ASSAY
Sample1(A)	0.264	5	0.258	4.88	97.6
Sample1(B)	0.530	10	0.513	9.67	96.79
Sample1(C)	0.785	15	0.805	15.38	102.54

6.7 Evaluation of separation method:

6.7.1 Optimization of Ultracentrifugation method.

6.7.1.1 Results

Table no. 20. Ultracentrifugation parameters.

BATCH 2

Results were not obtained because of uncleared separation.

BATCH 3,4,5,6

Sample	Total conc. of drug in dispersion(µg/ml)	Absorbance of supernatant	Free drug obtained in supernatant(µg/ml)	% EE
A	491	0.074	1.40	95 to 99
B	1015	0.130	2.46	93 to 99
C	1509	1.04	20	95 to 99

6.7.1.2 Discussion:

The ultracentrifuge technique is very fast and accurate technique for the determination of the free drug from the liposomal dispersion.Becauseofhigh relative centrifugal force high density liposomes will sediment and water soluble drug will remain as a supernatant. for batch 1 as such samples(A,B,C) were taken and centrifuged for 60000 rpm for 3 hrs. but clear separationwasn't observed. So in next trial five times diluted samples were used at 70000 rpm and for the same hours.but same results were observed. Soin next trial time were increased for the same rpm and for ten times diluted samples. But still clear separation was not observed. (samples were diluted with histidine sucrose buffer.)

Ultracentrifugation was performed for the 3 times with the different time and different RPM. Clear separation not observed.One of the reasons for that because of low conc. of liposomes resulted in lower density of liposomes against histidine sucrose buffer.So for the batch 2, The amount of lipid taken per ml were high so that because of high lipid density, sedimentation of liposomes were observed.And samples were interpreted with the help of UV series 1700;

6.7.1.3 Calculations:

$$EE\% = \frac{C(total)-C(free)}{C(total)} * 100$$

Where C (Total) is the total drug concentration before filtration containing both trapped and free SDO1, C (free) is the drug concentration in the filtered solution.

6.7.2 Centrifugal-Filtration method

Table no. 21 Centrifugal Filtration parameters

6.7.2.1 Results:

For BATCH 2,3,4,5,6

Sample	Total conc. of drug in dispersion(µg/ml)	Absorbance of filtrate	Free drug obtained in filtrate (µg/ml)	% EE
A	431	0.08	1.63	74-99
B	849	0.146	2.97	80-97
C	1324	0.201	4.10	85-98

6.7.2.2.Discussion:

The average molecular weight of liposomes is around 1000kds and molecular weight of drug SDO1 is in the range of 400-600 Daltons so10kd centrifugal filters were used and then samples centrifuged at 15000 rpm for 15 mins for 3 times in the THERMOSPECIFIC

HERAEUS CENTRIFUGE And then absorbance was measured respectively for sample A,B,C mg/ml samples in the comparison of standard drug solutions.

$$EE\% = \frac{C(total)-C(free)}{C(total)} * 100$$

Where C (Total) is the total drug concentration before filtration containing both trapped and free SDO1, C (free) is the drug concentration in the filtered solution

6.7.3 Dialysis membrane method

Table no. 22 Free drug analysis through dialysis membrane

6.3.3.1 Results:

BATCH 2

Sample	Total conc. of drug in dispersion(µg/ml)	**% ASSAY**	1hr	2hr	4hr	6hr
A	431	68.56	0.022	0.033	0.048	0.041
B	849	75.66	0.053	0.122	0.116	0.109
C	1324	81.91	0.037	0.056	0.076	0.105

Sample	**%Entrapment efficiency (+_5%)**
A	76.79
B	74.10
C	89.12

BATCH 3,4,5,6

RESULTS:

Sample	**Total conc. of drug in dispersion(µg/ml)**	**% ASSAY**	**1hr**	**2hr**	**4hr**	**6hr**
A	4.94	98.86	0.004	0.012	0.015	0.016
B	10.15	101.50	0.000	0.003	0.004	0.004
C	15.09	100.6	0.022	0.054	0.062	0.058

Sample	%Entrapment efficiency (+_5%)
A	94.23
B	99.26
C	92.21

Discussion:

The average molecular weight of liposomes is around 1000kds and molecular weight of drug SDO1 is in the range of 400-600 Daltons so here dialysis membrane used were 12,000 to 14000 MWCO. And drug has solubility 5mg/ml so free drug passed through membrane and results were interpreted with the help of UV 1800 series.

$$EE\% = \frac{C(total)-C(free)}{C(total)} * 100$$

Where C (Total) is the total drug concentration before filtration containing both trapped and free SDO1, C (free) is the drug concentration in the filtered solution.

6.8 In vitro release study

6.8.1 Discussion

Table no. 24 In vitro parameters

BATCH 2

Time	Absorbance		
Hrs.	Sample A	Sample B	Sample C
1	0	0.015	0.002
4	0.003	0.017	0.000
24	0.056	0.035	0.003
48	0.151	0.172	0.138
72	0.157	0.265	0.245
96	0.159	0.291	0.427
120	0.145	0.288	0.425

Time	% Cumulative release		
Hrs.	0.5mg	1mg	1.5mg
1	0	3.74	0.3
4	1.6	4.2	0
24	30.93	8.7	0.46
48	83.42	42.89	21.46
72	86.74	66.08	38.10
96	87.84	72.56	66.40
120	80.11	71.82	66.09

BATCH 3

Time	Absorbance		
Hrs	0.5mg	1mg	1.5mg
1	0.006	0.039	0.203
4	0.005	0.167	0.267
24	0.01	0.165	0.277
48	0.143	0.311	0.73
72	0.246	0.53	0.724
96	0.235	0.52	0.71

	% cumulative release		
Hrs	0.5 mg	1mg	1.5mg
1	2.29	7.24	25.69
4	1.91	31.04	33.79
24	3.83	30.66	35.06
48	54.7	57.80	92.40
72	94.25	98.51	91.64
96	90.03	96.65	89.87

BATCH 3

BATCH 1

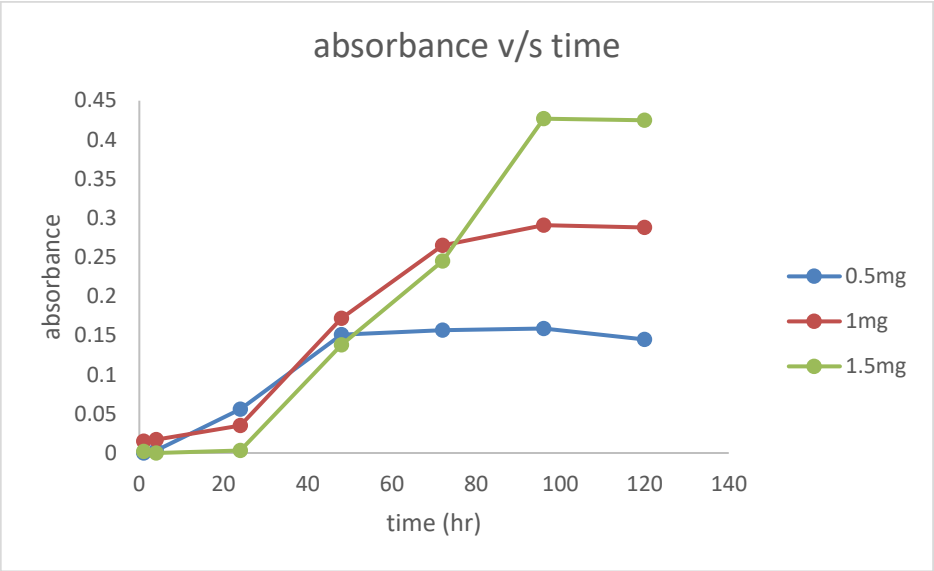


Figure The in vitro release profile for SDO1 from HSPC/cholesterol liposomes dialyzed under sink conditions(100- fold volume excess) Against PBS buffer at 37°C.

BATCH 2

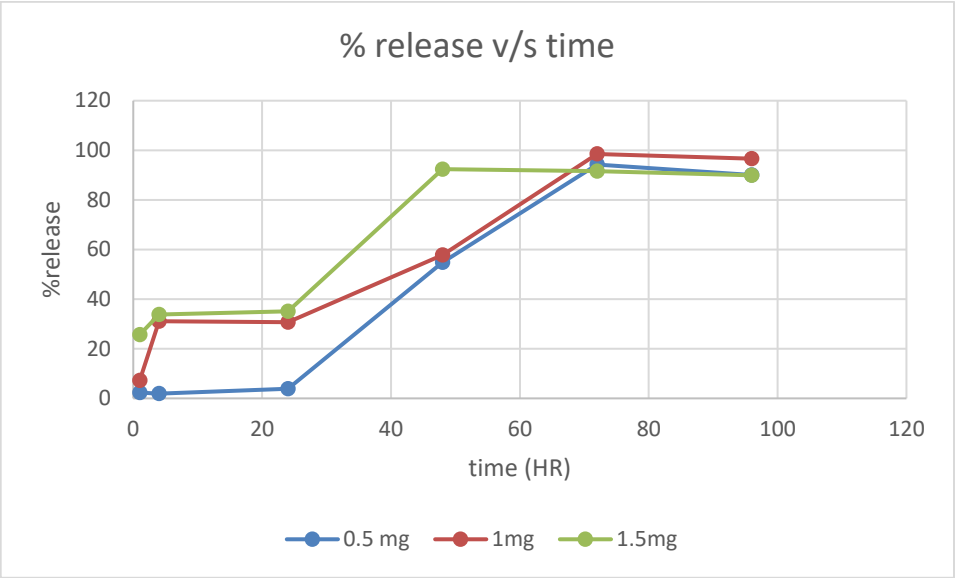


Figure6.3 The in vitro release profile for SDO1 from HSPC/DPPG/cholesterol liposomes dialyzed under sink conditions (100- fold volume excess) Against PBS buffer at 37°C.

An in vitro study was conducted, whereby release profile of five different formulations (1mg/ml solution from every batch) was compared with the standard SDO1 solution, SDO1 sulphate complex as a control. Cholesterol were mutual as a fluidity buffer in every batch, although concentration were different. Release of HSPC (BATCH 2) with particle size around 120were doneultil96 hrs, release were constant after study, release of HSPC/DPPG (BATCH 3) with particle size around 89were done until 24 hrs. Release of HSPC/DOTAP were done (BATCH 4,5,6) with particle size around 56 surprisingly until 8 days.

6.8.2 Results

6.8.3 Conclusion

Conclusion:

Here, from the results it can be identified that, drug release from the dialysis membrane depends upon parameters like particle size, concentration of cholesterol, drug to lipid ratio.In

the comparison of other batches, From the batches 2, drug release was observed very early within 1 day, because concentration of cholesterol waslesser than optimum concentration, so from the optimization, it was identified that one third part of cholesterol is required to provide membrane rigidity to drug delivery.

Chapter 7 Conclusion

In this study, SDO1 encapsulated liposomes were formulated and optimized with the extrusion and ultra-filtration mechanism. Characterization of SDO1 liposomes done with particle size and zeta potential analysis. Analysis of liposomes done with three methods, Ultracentrifugation, centrifugation and dialysis membrane method. And after that analysed with UV spectra.

In vitro study carried out and it was shown that the inclusion of cholesterol in liposomal membrane affected membrane rigidity as well as stability of vesicles. Particle size of liposomes is one of the important parameters which affected IVR. With reduction of particle size, more slower sustained release observed with the duration of 7 days in vitro compared to the other formulations.

Chapter 8 Future perspective

To optimize the stability of formulations is one of the important works need to be done in future. Although good results were identified in in vitro release, but promising release profile results of ex-vivo and in vivo is required. In future, analysis of formulation needs to be done with more accurate methods like HPLC. Toxicological study of Nano-formulation is required. This technology has the potential to replace the current invasive intravitreal injections.

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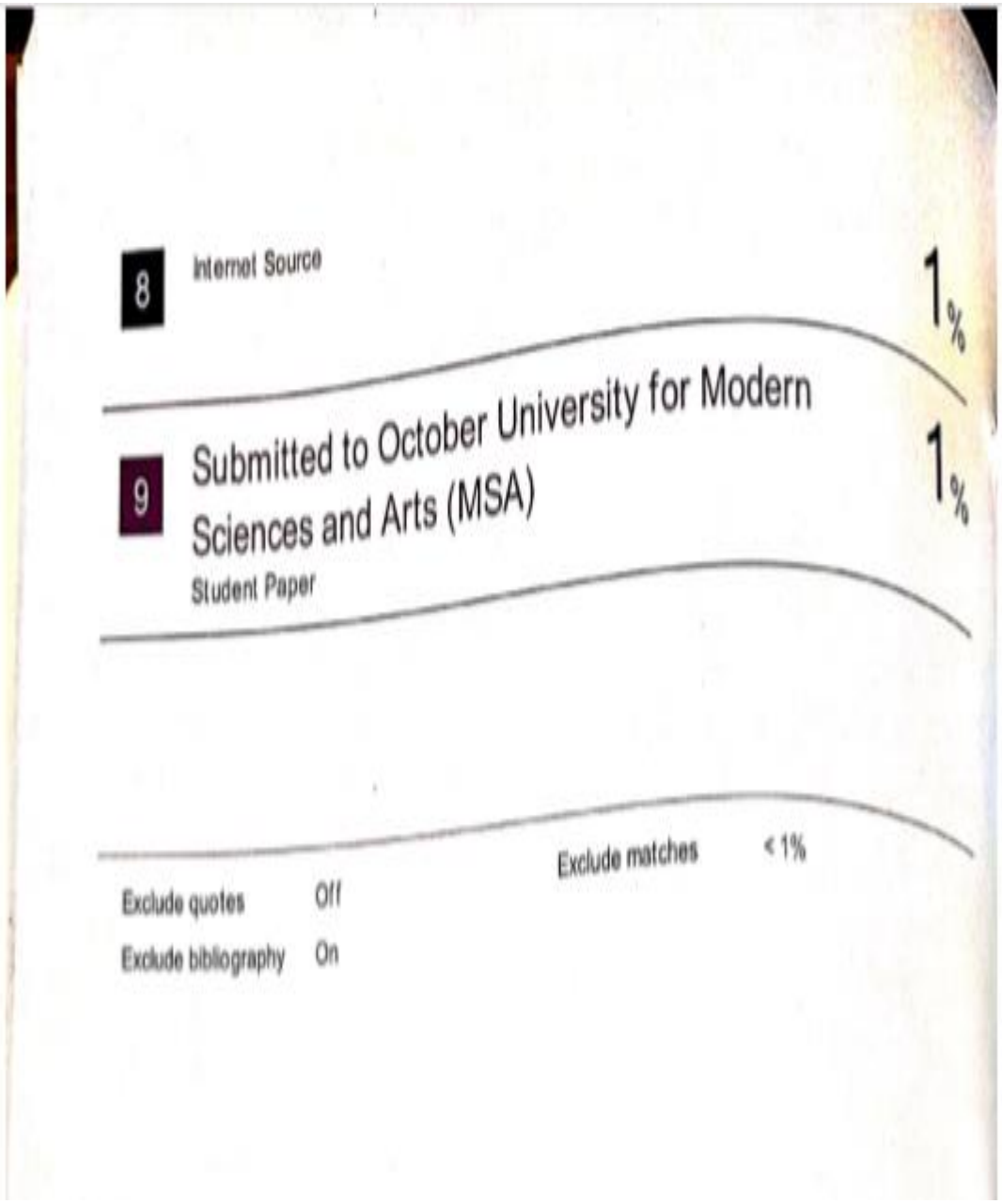
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