Dissertation

Development of liposomal drug delivery systems for inhalative application of iloprost

submitted by
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for the academic degree of

Doctor of Philosophy
(Ph.D.)

at the

Medical University of Graz

Ludwig Boltzmann Institute for Lung Vascular Research

under Supervision of

Prof.Dr. Andrea Olschewski

2014

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, [date & signature]

Acknowledgements

Firstly, I would like to thank my supervisor Prof. Dr. Andrea Olschewski for providing me a chance to work at the Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria. Further, I would like to sincerely thank my co-supervisors Prof. Dr. Ruth Prassl, Dr. Regina Leber and Dr. Leigh Marsh without their excellent scientific input and knowhow of the project it wouldn't have been possible. Hence, once again I would like to acknowledge them which made me think like a good researcher. I really learned a lot during these four years and it was a privilege to be a part of this highly skilled research personnel. Then, I would like to specially thank Daniela Frascione and Yasemin Manavbasi for showing me around Graz during my initial years. A very special thanks to Caroline Vonach, Gebhard Schratter and Thomas Fuchs for their excellent technical assistance without them this wouldn't have been possible at all. Next, I wish to thank Bernhard Lehofer for all his excellent scientific discussion during lab meetings. Further, a big 'Thank You' to Chandran Nagaraj whose constant motivation and critique made me learn THE Wire Myograph. Then, I would like to specially thank Valentina Biasin for all her scientific and non-scientific expertise throughout my PhD experience and YES of course not to forget Bence M. Nagy for his most encouraging behavior and continue to move on every day in life. Then, I would I like to thank my besties Manjula Vinod and Shailaja Rao for only listening to all the jibber jabber throughout and supporting me always whenever I needed them. Then, I would like to finally thank all the people at LBI or Institute of Biophysics who were directly or indirectly involved in getting me through this stage. Next, my exceptional thanks to my parents who gave me a chance to pursue higher education and explore myself. Without them, I wouldn't have attained this level where I currently stand. Therefore, a big Thank You to you. Lastly, I would like to thank my fiancé Swetha for her kind understanding and patiently believing in me during the last years of my PhD. And definitely Almighty God, Thank You for everything.

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Abstract in English

Prostacyclin analogues (prostanoids) are standard therapeutic options for vasoconstrictive diseases, including pulmonary hypertension and Raynaud's phenomenon. Although effective, the systemic application is expensive and has several side effects. For the therapy of pulmonary arterial hypertension, inhaled application of stable analogues of prostacyclin represents a well established alternative treatment leading to selective vasodilation of well-ventilated regions of the lung. However, the short half-life of the drugs reduces patient compliance. To improve drug efficiency we tested liposomal nanoparticles as carrier systems. In this study, we synthesized liposomal nanoparticles tailored for the prostacyclin analogue, iloprost, and evaluated their pharmacologic efficacy on mouse intra pulmonary arteries using wire myograph.

Six formulations for iloprost were optimised and termed as LI-1 to LI-6. The use of cationic lipids, stearylamine or 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP) in liposomes promoted iloprost encapsulation to at least 50%. Addition of cholesterol modestly reduced iloprost encapsulation. Liposomal nanoparticle formulations were characterized using biophysical techniques and tested for toxicity and pharmacologic efficacy *in vitro* and *ex vivo,* respectively. Liposomes did not affect the viability of human pulmonary artery smooth muscle cells. Compared to an equivalent concentration of free iloprost, four out of the six polymer-coated liposomal formulations exhibited significantly enhanced vasodilation on mouse pulmonary arteries. Encapsulated iloprost in PEGylated liposomes exhibited concentration dependent relaxation of arteries. Strikingly, half the concentration of iloprost in liposomes elicited similar pharmacologic efficacy as non-encapsulated iloprost. Nebulisation data revealed that the vibrating mesh nebuliser can efficiently nebulise cationic liposomes and had the minimum impact on the integrity of liposomal formulations. Cationic liposomes can encapsulate iloprost at high efficacies and can serve as potential iloprost carriers to improve its therapeutic efficacy.

Abstract in German (Zusammenfassung)

Prostazyklin-analoge Wirkstoffe werden als Standardtherapeutika zur Behandlung von gefäßverengenden Erkrankungen, wie der pulmonalen Hypertonie oder dem Raynaud-Syndrom, eingesetzt. Obwohl sie sehr gut wirken, ist ihre systemische therapeutische Anwendung sehr teuer und wird außerdem von vielen Nebenwirkungen begleitet. Um die Effizienz der Wirkstoffe zu erhöhen, wurden liposomale Nanopartikel als Trägersysteme für Prostazyklin-Analoga getestet. In dieser Studie wurden liposomale Trägerpartikel für die Anwendung des gefäßerweiternden Wirkstoffes Iloprost entwickelt und ihre pharmakologische Wirksamkeit mit Hilfe von intrapulmonalen Mausarterien am Myographen getestet.

Die Zusammensetzung der liposomalen Membran hatte einen großen Einfluss auf die Verkapselungsrate von Iloprost. Der Einbau von kationischen Lipiden, wie Stearylamin oder DOTAP, in die Liposomenmembran erhöhte den Einschluss von Iloprost auf mindestens 50%. Hingegen führte die Zugabe von Cholesterol zur Lipidschicht der Liposomen zu verringerten Verkapselungsraten von Iloprost. Die verschiedenen liposomalen Formulierungen wurden auch auf ihre Toxizität getestet. Die Lebensfähigkeit von pulmonal-arteriellen glatten Muskelzellen war in Gegenwart der Liposomen nicht vermindert. Verglichen mit einer äquivalenten Menge an freiem Iloprost, konnten vier der sechs neuen Iloprost-Formulierungen eine deutlich verbesserte Blutgefäßerweiterung an pulmonalen Mausarterien erreichen. Das in PEGylierten Liposomen eingeschlossene Iloprost wies außerdem eine konzentrationsabhängige Wirkung an den Arterien auf. Damit konnte gezeigt werden, dass zur Erreichung desselben pharmakologischen Effektes nur halb soviel vom liposomalen Iloprost benötigt wird wie vom Iloprost in wässriger Form. Vernebelungsversuche zeigten, dass der „vibrating mesh“- Inhalator Liposomen am effizientesten vernebeln kann und außerdem den geringsten Einfluss auf den Einschluss von Iloprost in die Liposomen hatte. Kationische Liposomen könnten somit effiziente Wirkstoffträgersysteme für Iloprost darstellen um dessen therapeutische Effizienz *in vivo* zu steigern.

Abbreviations

6MWD	6Minute Walking Distance
A549	Alveolar epithelial cell line
cAMP	cyclic adenosine mono phosphate
cGMP	cyclic guanosine mono phosphate
COX	Cyclooxygenase
CTEPH	chronic thrombo embolic pulmonary hypertension
C/M	Chloroform:Methanol
DOTAP	1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane
DMSO	Dimethylsulfoxide
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPE-PEG2000	[methoxy (polyethyleneglycol)-2000]-dipalmitoyl-phosphatidylethanolamine
DLS	Dynamic Light Scattering
ERA	Endothelin receptor antagonist
EE	Encapsulation Efficiency
GUV	Giantunilamellar vesicles
GPCR	Guanosine protein coupled receptor
HSA	Human Serum Albumin
hPASMC	human pulmonary artery smooth muscle cells
HPβCD	hydroxyl-propyl-β-cyclodextrin
IV	Intravenous
IPAH	Idiopathic Pulmonary Hypertension
LUV	Large unilamellar vesicles
MLV	Multi lamellar vesicles
MPS	Mononuclear phagocyte system
NO	Nitric Oxide
POPC	1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine
PEI	Polyethyleneimine
PPAR	Peroxisome proliferative activated receptor
PVP	Polyvinylpyrrolidone
PH	Pulmonary Hypertension

PAH	Pulmonary Arterial Hypertension
PDI	Polydispersityindex
PA	Pulmonary artery
PDE-5	Phosphodiesterase-5 inhibitors
PSS	Physiologic Salt Solution
SA	Stearylamine
SC	Subcutaneous
sGC	soluble guanyl cyclase
SUV	Small unilamellar vesicles
TLC	Thin Layer Chromatography
TEM	Transmission Electron Microsocpy
TTCW	Total Time to Clinical Worsening

1 Introduction

1.1 Introduction to Pulmonary Hypertension

Pulmonary Hypertension (PH) is a severe disease with poor prognosis. The diagnosis of PH is confirmed by right heart catheterization demonstrating mean pulmonary arterial pressure of 25mm Hg or higher. The characteristics of PH include remodeling of small pulmonary arteries, right ventricular hypertrophy and might lead to right ventricular failure and death (1). Progressive breathlessness represents PH (2). PH is classified in 5 groups. Group 1: Pulmonary arterial hypertension can be idiopathic, heritable, drug induced or related to other diseases like congenial heart disease, connective tissue disease, HIV infection or portal hypertension, schistosomiasis. Group 2: PH due to left heart disease. Group 3: PH due to lung disease and/or hypoxia. Group 4: Chronic thromboembolic pulmonary hypertension. Group 5: PH with unclear or multifactorial mechanisms (3). An updated classification of Pulmonary Hypertension is shown in **Table 1** (3).

Table 1: Updated Classification of Pulmonary Hypertension (3)*.

1	Pulmonary arterial hypertension 1.1 Idiopathic PAH 1.2 Heritable PAH 1.2.1 BMPR2 1.2.2 ALK-1, ENG, **SMAD9**, **CAV1, KCNK3** 1.2.3 Unknown 1.3 Drug and toxin induced 1.4 Associated with: 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Congenital heart diseases 1.4.5 Schistosomiasis 1’ Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis 1’’ **Persistent pulmonary hypertension of the newborn (PPHN)**
2	Pulmonary hypertension due to left heart disease 2.1 Left ventricular systolic dysfunction 2.2 Left ventricular diastolic dysfunction 2.3 Valvular disease 2.4 **Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies**
3	Pulmonary hypertension due to lung diseases and/or hypoxia

	3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4 Sleep-disordered breathing 3.5 Alveolar hypoventilation disorders 3.6 Chronic exposure to high altitude 3.7 Developmental lung diseases
4	Chronic thromboembolic pulmonary hypertension (CTEPH)
5	Pulmonary hypertension with unclear multifactorial mechanisms 5.1 Hematologic disorders: **chronic hemolytic anemia**, myeloproliferative disorders, splenectomy 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders 5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, **segmental PH**

*5th World Symposium Pulmonary Hypertension, Nice 2013. The DANA point classification was maintained with modifications marked in **bold** based on recently published data.
BMPR = bone morphogenic protein receptor type II; CAV1 = caveolin-1; ENG = endoglin; HIV = human immunodeficiency virus.

Continuous research in understanding the mechanisms behind the pathophysiology of the disease is ongoing and exploring novel therapeutic targets have improved disease prognosis. However, progression of the disease cannot be stopped eventually resulting in right heart failure and ultimately death (4). New medications have increased the survival of the patients to 83% (1 year) and 58% (3 years) (5)(6). The majority of research has been focused to pulmonary arterial hypertension (PAH) (7). An imbalance between vasodilatory (nitric oxide or prostacyclin) and vasoconstrictory (endothelin) elements has been observed in all types of PAH. The current pharmacotherapy aims to target this imbalance (8)(9)(10)(11)(12).

1.2 Pathophysiology of PAH

PAH in infants arise from inability to dilate the pulmonary vascular bed and pathophysiological alterations in blood vessels are apparent at beginning of life. Aberrant muscularization of distal pulmonary arteries (PA) within alveoli and marked reduction in number of PA are mostly evident (13) (14). In adults, intimal hyperplasia causes occlusive changes and plexiform lesions in pulmonary arteries. Several experimental studies have shown that changes in the endothelial cells of PA exceed the muscularization of distal PA

(15)(16). Endothelial cells from Idiopathic PAH (IPAH) patients demonstrated increased expression in Angiopoieten-1 (Tie2) receptor which promotes serotonin release causing smooth muscle proliferation (17). Abnormalities in the endothelial cells can either stimulate or inhibit the proliferation of smooth muscle cells either by FGF-2 release or diminished production of anti-suppressive agents (18,19).

The pathology of PAH involves an intricate relationship between vasoconstriction, hypertrophy and fibrosis (12,20-23). Pathologic reports have shown intimal hyperplasia, fibrosis, medial hypertrophy of the PA (23). To date, it was thought that imbalance between vasoconstriction and vasodilation mediators were the cause for the symptoms. However, studies have also demonstrated that irregular proliferation, infiltration and fibrosis of smooth muscle and endothelial cells, inflammatory cells and vascular elements are involved in the pathologic processes of PAH. These alterations are related to increased or decreased endothelins and nitric oxide or prostacyclins. In addition to affecting other processes these mediators cause constriction, dilation and abnormal proliferation (22). Experimental evidence has shown that the pulmonary system is affected by abnormal proliferation and apoptosis of vascular cells. Hyperproliferation of the endothelial cell is believed to a vital contributing factor towards vascular remodeling (22, 24) perturbing several cells like endothelial, smooth muscle and fibroblasts within the pulmonary arteries (22). An overexpression of the potent vasoconstrictor, endothelin-1 (25) which diminishes the production of nitric oxide and prostacyclins, further alter the vascular homeostasis (22, 26-28). Several reports suggest that over expression of endothelin-1 is presumed to augment inflammation (29) and fibrosis (30).

Studies have shown that genetic mutations might be involved in pathology of PAH of which bone morphogenetic protein receptor type II (BMP), serotonin transporter and activin-like kinase type I have been investigated as key players towards developing PAH. Approximately, 50% of familial and ≤25% of IPAH patients have been reported to have BMP genetic mutations (31, 32). The carriers of BMP mutations have 15-20% chance of developing PAH (33)(31). Since BMP is involved in the apoptotic process of several cells, which might be the reason for hyperproliferation of the endothelial cells in PAH (34). Activin-like kinase type 1 mutations have been found in PAH patients related to hereditary hemorrhagic telangiectasia mostly affecting the vasodilation of small PA (35). Studies demonstrated that higher

percentage of IPAH patients showed hypertrophy of pulmonary artery smooth muscle cells, which is associated with serotonin transporter gene than healthy ones (36).

Apart from genetic mutations, there are several risk factors which can be associated with PAH. Scleroderma patients have an incread risk for PAH. Patients suffering from congenital heart disease and systemic to pulmonary shunts can develop PAH if they are not treated (24). Finally, HIV patients have a higher risk towards PAH development than healthy people (24).

Several drugs have been developed for the pharmacotherapy of the disease; however none of them can completely cure the disease. The next section will deal with available therapies.

1.3 Approved Therapies for PAH

Currently, the US-FDA has approved nine therapeutics for the therapy of PAH. They are classified based on mechanism of action. Prostacyclin analogues induce the synthesis of prostacyclins; endothelin receptor antagonists prevent the binding of the vasoconstrictor endothelin; phosphodiesterase 5 inhibitors prevent the metabolism of cGMP. PAH therapeutics can be administered by oral, inhaled, subcutaneous and intravenous routes. The approved medications are listed in **Table 2.**

Table 2: US-FDA approved medications for PAH (37)

Generic Name	Brand Name	Route of Administration	Drug Class	Indication
Epoprostenol	Flolan	IV	Prostacyclin derivative	Treatment of PAH (WHO group 1) to improve exercise capacity
Epoprostenol	Veletri	IV	Prostacyclin derivative	Treatment of PAH (WHO group 1) to improve exercise capacity
Iloprost	Ventavis	Inhaled	Prostacyclin derivative	Treatment of PAH (WHO group 1) to improve composite end point of exercise tolerance, symptoms (NYHA class), and lack of deterioration
Treprostinil	Remodulin	IV or SC	Prostacyclin derivative	Treatment of PAH (WHO group 1) to diminish symptoms associated with exercise Patients requiring transition from Flolan to reduce rate of clinical deterioration
Treprostinil	Tyvaso	Inhaled	Prostacyclin derivative	Treatment of PAH (WHO group 1) to improve exercise ability
Bosentan	Tracleer	Oral	ERA	Treatment of PAH (WHO group 1) to improve exercise ability and decrease clinical worsening
Ambrisentan	Letairis	Oral	ERA	Treatment of PAH (WHO group 1) to improve exercise ability and delay clinical worsening
Sildenafil	Revatio	Oral	PDE-5 inhibitor	Treatment of PAH (WHO group I) to improve exercise ability and delay clinical worsening
Tadalafil	Adcirca	Oral	PDE-5 inhibitor	Treatment of PAH (WHO group 1) to improve exercise ability

1.4 Prostacyclin and Prostacyclin analogues

Prostacyclin, an endogenous prostanoid, is synthesized from arachidonic acid in a series of steps utilizing the prostacyclin synthase and cyclooxygenase (COX) enzymes (38,39). Endothelial cells in the pulmonary arteries release it. Physiologically, prostacyclins exert their effects via surface receptors (prostacyclin receptors) present on endothelial cells, smooth muscle cells and platelets. These IP receptors are G-protein coupled receptors (GPCR) (40). Prostacyclin binds to these receptors, activates G-proteins, and thereby increases the intracellular cAMP further activating the protein kinase A. In this way, prostacyclin can counter balance the endothelin mediated vasoconstriction, which is active in PAH patients, thereby serving as potent vasodilator of pulmonary circulation. In addition, prostacyclins act as anti-thrombotic, anti-proliferative and immunomodulators (41). As mentioned previously, a decreased synthesis of prostacyclin is a factor related to PAH development (42). Since, prostacyclin have short biological half-life usually in minutes, the use of prostacyclins is clinically challenging.

The first synthetic prostacyclin, epoprostenol, is approved for PAH. It is a strong vasodilator and has been reported to have anti-thrombotic and anti-proliferative effects (43). Experimental data suggested that continuous epoprostenol infusion in humans significantly improves exercise capacity, hemodynamic parameters and survival (44). Unfortunately, its short serum half-life of about 6 minutes prevents its clinical utility. Epoprostenol required continuous infusion by catheter which was inconvenient, with risk of infection, and also death if pump failed or the dose was interrupted (41). The most common tolerable adverse effects include flushing, nausea, vomit, jaw pain and diarrhea. To overcome the potential side effects and instability, a novel and more stable formulation of epoprostenol, Veletri (Actelion, Allschwil, Switzerland) was developed. Intravenously (IV) administered epoprostenol remains a backbone in pharmacotherapy of severe PAH patients. To improve the pharmacokinetics profile, several prostacyclin analogues have been developed, inhaled iloprost and treprostinil, subcutantious (SC) and IV treprostinil. The safety and efficacy of each medication must be taken into consideration before use.

1.4.1 Iloprost

Iloprost is a synthetic derivative of prostacyclin PGI_2 **(Figure 1)** with higher stability and high affinity towards the IP receptor. Structurally, Iloprost possesses a methyl group at C16, C18 and C19 positions that are sp-hybridized resulting in triple bond formation. When the enol oxygen in upper ring of PGI2 was substituted with methylene group, longer half-life was attained.

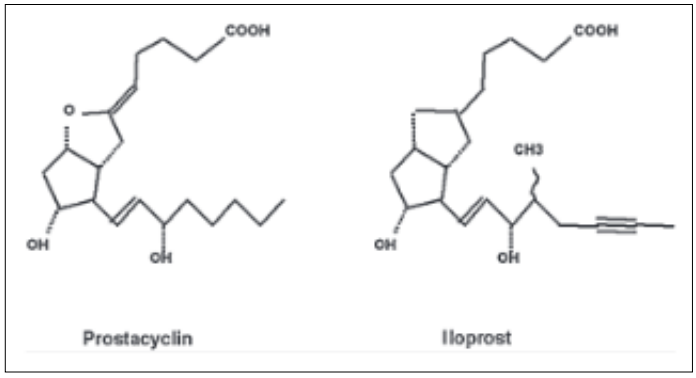


Figure 1: Chemical structures of endogenous prostacyclin and iloprost (45)

1.4.1.1 Prostacyclin receptors

Prostacyclin receptors are G-protein coupled receptors which bind to the G-proteins like the Gs, Gq and Gi to trigger the appropriate intracellular signaling pathways (46). Depending upon the type of G-protein, the prostacyclin receptors can act either as relaxants or constrictors. The relaxant receptors like the IP, DP, EP2 and EP4 couple to Gs protein to produce cAMP and thereby promoting smooth muscle vasodilation. The contractile receptors like EP1, FP and TP couple to Gq to mobilize calcium and thereby promoting smooth muscle vasoconstriction. The inhibitory receptor couple to Gi protein to reduce cAMP thereby reducing smooth muscle vasodilation (47,48).

Iloprost binds to membrane bound IP receptors or other receptors present on the cell surface or nucleus (peroxisome proliferator-activated receptor, PPAR) resulting in an increase of intracellular cyclic AMP (cAMP). This leads to potent inhibition of platelet aggregation and all associated reactions. With respect to vasodilation, increase in secondary messenger cAMP in smooth muscle cells not only activates calcium pumps causing removal of calcium from cytoplasm but also opens the potassium channel causing hyperpolarization. In addition to this, increased cAMP inhibits myosin kinases together causing vasodilation (49).

Iloprost not only exhibits high binding affinity towards the IP receptors but also to EP1 and EP3 receptors in both humans and murine (50)(51). From the radioligand studies of iloprost on the human IP prostanoid receptor, the binding affinities (Ki) of iloprost as reported by two research groups was found to be very similar Ki 4nM (52) and 11nM (53). A similar binding affinity Ki 11nM of iloprost was reported for murine IP receptor (51) indicating that iloprost is equipotent at both the murine and human IP receptor. Iloprost possesses lower binding affinities towards other prostanoids like FP, EP4 and very low binding affinities towards DP1, EP2 and TP receptors (52). A similar binding affinity of iloprost was reported on rat EP receptors (54).

The expression pattern of the different prostanoid receptors on isolated pulmonary arteries has not been clearly shown. In the current study, differential expression pattern of prostanoid receptors on murine pulmonary arteries has been determined.

1.4.1.2 Pharmacokinetics of Iloprost

When administered intravenously, concentration of iloprost in plasma drops biphasically, 2.8 ± 1.6min and 26 ± 7.2 mins (55). The second half-life demonstrates the metabolism of iloprost. β-oxidation is responsible for metabolism of iloprost. Majority of metabolic products of iloprost are removed via the kidneys (55) . The pharmacokinetics of iloprost has been investigated in isolated rabbit perfused lung model using two different doses, 75ng and 900ng (56). The bioavailability of inhaled iloprost (75ng deposited) in vascular compartment was determined to be 63% however only 14% for 900ng. The increased dose did not elevate the perfusate concentration. It was speculated that the decrease in availability of iloprost might be hindered by inhibition of perfusion or carrier mediated transport processes. This needs to be fully elucidated. In addition to this, it was reported that metabolism of inhaled iloprost is faster by β-oxidation when compared to infused iloprost (56).

1.4.1.3 Efficacy of inhaled iloprost

To reduce systemic vasodilation, the pulmonary route was chosen for organ specific action in pulmonary vessels. Clinically, it was observed that inhaled iloprost improved the hemodynamic parameters in PAH patients as measured by right heart catheterization. Furthermore, it was reported that daily inhalations of iloprost induced a sustained pulmonary response after 1 year with tolerable side effects without tachyphylaxis (57). Frequent iloprost inhalations together with overnight breaks might fluctuate the serum

concentrations of iloprost, probably allowing sufficient time for the regeneration of prostacyclin receptor which could be a possible reason for the fluctuating serum levels. *In vitro* studies have reported that IP receptor becomes desensitized within minutes when exposed to agonists by receptor phosphorylation which is predominantly facilitated by protein kinase C (58) following receptor sequestration and removal from cell surface (58,59).

The concept that inhaled iloprost is a selective pulmonary vasodilator was also validated in PH patients secondary to lung fibrosis (60). When compared to intravenous prostacyclin, inhaled iloprost produced prominent vasodilation maintaining gaseous exchange, arterial oxygen saturation and systemic arterial pressure in the fibrosis patients (60).

A pilot study comparing the pharmacokinetics of inhaled NO, inhaled and IV administered epoprostenol and inhaled iloprost in six PAH patients showed that inhaled iloprost and nitric oxide had comparable hemodynamic effects. It was reported that the effect of inhaled epoprostenol lasted only for 30 minutes; however, effects for inhaled iloprost lasted for 120 minutes (57). Iloprost was nebulized at a concentration of 10µg/mL using a jet nebuliser (IloNeb with Aerotrap reservoir) manufactured by Nebutec generating an aerosol particle size of about 2.9±3.1µM (57).

Another clinical study compared the hemodynamic effects of single inhalation of NO (40ppm) and iloprost (14-17µg) in 35 IPAH patients (61). Aerosolization was performed using the same jet nebuliser as mentioned above. The efficacy of inhaled iloprost was greater in reducing the pulmonary artery pressure, vascular resistance and significant increase in cardiac output when compared to inhaled NO. Thus, the potency of inhaled iloprost was greater than inhaled nitric oxide as vasodilator in PAH (61).

A pharmacokinetic study was performed using aerosolized iloprost, 5µg dose at the mouth piece using different jet nebulisers, Ilo-Neb/Aerotrap™,Nebutec, Germany; Ventstream™ and HaloLite™ Murrysville, PA. All the jet nebulisers displayed comparable pharmacokinetic and pharmacodynamic effects (62). It was observed that Iloprost rapidly entered the systemic circulation reaching peak levels when the inhalation was ceased. Pulmonary vasodilation was persistent even after iloprost was cleared from systemic circulation possessing serum half-lives of iloprost between 6-10 minutes supporting the hypothesis that locally deposited inhaled drug contributes to the preferential pulmonary vasodilation. The pharmacotherapy of inhaled iloprost for PH therapy necessitates between six-nine

inhalations daily because the effect of pulmonary vasodilation per inhalation levels off within 1 hour (62).

Another study compared the delivery of inhaled iloprost using jet (IloNeb™) and ultrasonic nebuliser (Multisonic Compact™, Germany) in 18 severe PH patients (63). The final inhalation dose was 2.8µg for both nebulisers; however, the time required for inhalation using the jet nebuliser was 12 minutes while for ultrasonic nebuliser it was 4 minutes. Inhaled iloprost induced preferential pulmonary vasodilation via both systems with minor systemic hypotension and few side effects in some patients. The study reported that ultrasonic nebulisers are more efficient with greater output rates, minimal drug wastage and shorter inhalation times, thus making them eligible for delivery of inhaled iloprost in PH patients.

Continuous development has been made to produce inhalation devices (64) and clinical studies have been performed to demonstrate the efficiency of such inhalation devices (63)(62). The traditional jet nebulisers have been replaced by modern ultrasonic devices that deliver constant doses of iloprost at mouth piece (2.5 or 5µg).

A couple of reviews summarize the data about the inhaled iloprost (64) (65) suggesting that iloprost exhibits an equivalent or even better effect on hemodynamic parameters in comparison to other vasodilatory therapeutics.

1.4.1.4 Safety and Regulatory affairs of inhaled iloprost

Based on current data reported, inhaled iloprost used singly or together with other PAH therapeutics, is safe to use with clinically acceptable side effects. Common side effects include coughing, headache, flushing and dry mouth (66) (67).

Based on fundamental clinical data for inhaled iloprost, European Medicines Agency (EMA) approved inhaled iloprost for the pharmacotherapy of IPAH functional class NYHA III patients in September 2003. Further, it was approved for PAH functional class NYHA III and IV by the US-FDA in 2004. Moreover, its use was also approved for severe IPAH, CTEPH by Australian Therapeutics Good Administration in 2004. Inhaled iloprost is only legally approved for selective patients with PH (Idiopathic or familial PAH) in Europe.

Apart from iloprost, other prostacyclins were also developed and will be briefly discussed.

1.4.2 Treprostinil

Another prostacyclin analogue, treprostinil, is more stable and with longer half-life (4 hours) than iloprost and epoprostenol. Treprostinil can be administered subcutaneously (SC), IV, orally or by inhalation. Remodulin® is a commercially available preparation of treprostinil from United Therapeutics (ResearchTriangle Park, NC, USA) for IV or SC route. Since continuous IV infusion shows risk of infection, continuous SC has been suggested if patients can tolerate SC route. SC treprostinil improved the pulmonary hemodynamics and exercise capacity in dose dependent fashion in severe PH patients (68). A majority of studies were performed using SC treprostinil. Several clinical data have reported that continuous SC infusion of treprostinil has therapeutic benefits. Diarrhoea, nausea and swelling of feet are common side effects of treprostinil (69). It was demonstrated that IV and inhaled treprostinil displayed slight improvements in hemodynamic effects having higher tolerability (70) (71) (72). A recent clinical trial using oral treprostinil therapy on PAH patients did not show any improvement in the 6-min walking distance (6MWD) which is the primary end point in FREEDOM-C trial; 22% patient withdrawal was also reported (73). Oral treprostinil was further investigated in another clinical trial, FREEDOM-M on PAH patients which reported a preliminary observation that 23metre improvement in 6MWD while secondary end points like total time to clinical worsening (TTCW) and NYHA FC remained similar in drug and placebo http://www.prnewswire.com/news-releases/freedom-m-trial-of-oral-treprostinil-in-pulmonary-arterial-hypertension-meets-primary-endpoint-123212773.html. Treprostinil can be used as first line medication to replace epoprostenol due to its longer half-life, SC route and stability at room temperature (74). Treprostinil cassettes are replaced every alternate day while epoprostenol cassettes are changed daily (74). As recommended by experts, optimum effective dose for treprostinil varies between 40-110ng/kg/min which is much higher than doses of epoprostenol which may be because of the difference between the formulations (74).

Rationale for inhaled prostacyclins

To achieve selective targeting, the inhaled prostacyclins, iloprost and treprostinil were developed. Since alveoli and resistant pulmonary arterioles are in close proximity to each other, the inhalation mode of delivery seems very logical from pathological point of view (75). Due to short half-life of iloprost, frequent inhalations of iloprost are needed. This

reduces the patient's compliance (74). Inhaled treprostinil was developed since it demonstrates relative selectivity towards pulmonary circulation in comparison to iloprost (75). It was observed that inhaled iloprost and treprostinil induce comparable decrease in pulmonary vascular resistance in a cross over trial. Although, differences in peak effects, treprostinil effect appears later than iloprost (18 vs 8 minutes) but a longer duration of action was observed (76). A clinical study investigated that a higher patient satisfaction and 66% reduction in administration time was observed when inhaled iloprost was transitioned to treprostinil (77). Inhaled medications appear to be efficacious in PAH patients. Infusion therapy can be replaced by inhalation therapy with careful decisions in selected patients however this has not been evaluated systematically (75).

Iloprost is a prototype of prostacyclin. It is not only approved for inhaled therapy of PAH, Europe but also for other vasoconstrictive diseases like Raynauds phenomenon (78). It is well known from the literature that iloprost is a potent vasodilator and causes preferential vasodilation of intra pulmonary arteries (79). Further, the inhaled doses of iloprost are much lower than the intravenous or subcutaneous doses (80). Despite several advantages, iloprost exhibits a short plasma half-life with limited sustainability of its hemodynamic effects (30 -90 minutes) requiring 6-9 inhalations per day. Thus, there is a need to improve the half-life of iloprost.

1.5 Endothelin and Endothelin antagonists

Endothelin-1 (ET) is a strong vasoconstrictor and causes proliferation of vascular smooth muscles. It binds to two types of endothelin receptors: ET_A present on pulmonary artery smooth muscle cells (PASMC) and ET_B present on endothelial and PASMC (81) (82).

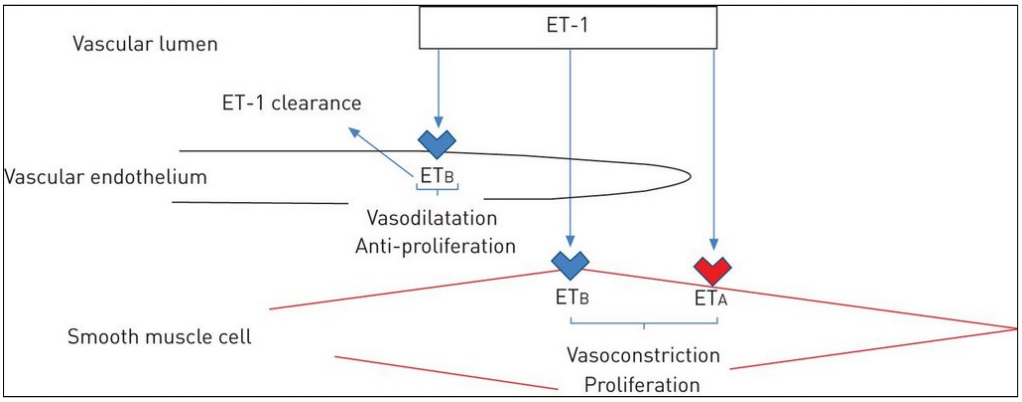


Figure 2: Schematic representation of endothelin pathway in pulmonary vasculature (83)

Binding of ET-1 to ET_A and ET_B causes contraction and proliferation of vascular smooth muscle cells (84). ET-1 is cleared via ET_B receptors in vasculature of lungs and kidney and might promote NO and prostacyclin mediated vasorelaxation from the endothelium (85)(86). It was observed that patients with IPAH and PAH with scleroderma and congenital cardiac shunt lesions have high concentrations of ET-1 in their lungs (87).

The therapy aims to antagonize the endothelin receptor which began in late 1990s for Group I PAH. Enothelin receptor antagonists (ERA) target both ET_A and ET_B receptors. Hence, both selective and non-selective ERA has been approved for therapy of PAH. Selective antagonists for ET_A include ambrisentan and sitaxsentan and non-selective dual acting receptor antagonists include bosentan and macitentan. Bosentan and macitentan (non-selective) and ambrisentan (selective) are the approved ones.

1.5.1 Bosentan (Tracleer; Actelion, Switzerland)

It is an oral non-selective ERA utilized for the pharmacotherapy of PAH. Oral daily doses of bosentan (125mg and 250mg) demonstrated improvement in 6MWD and NYHA FC in 213 PAH patients enrolled in BREATHE study (88). 13% of patients showed 3 times increase in liver transaminases. Hence, the therapy was started with lower doses (62.5mg, twice a day) which were later elevated (125mg twice a day) for 4 weeks with careful monitoring of liver transaminases. Improved pulmonary vascular resistance (PVR) and significantly delayed TTCW when compared to placebo was reported in EARLY clinical study who enrolled patients having mild symptoms of PAH (89). It was demonstrated that Bosentan improved the hemodynamics for Eisenmenger patients (90), HIV associated PAH (91), portal PH (92) and recently for NYHA FC II and III patients (93).

1.5.2 Ambrisentan (Volibris; GlaxoSmithKline, Brentford, UK)

It is an oral selective ET_A antagonist displaying showing minimal hepatic damage (94). Two large clinical trials for ambrisentan called ARIES-1 and ARIES-2 were performed. Both trials showed improved 6MWD. Improved NYHA FC and longer TTCW were reported for ARIES-1 and ARIES-2 respectively (95). The results of 6MWD were confirmed by further extension of the trial for 2 years which also demonstrated good tolerability (96). Ambrisentan in ARIES-3 trial showed successful results in PH patients with different aetiologies (97), which lead to its approval for NYHA FC II, III and IV patients in USA.

1.6 Nitric Oxide (NO) pathway

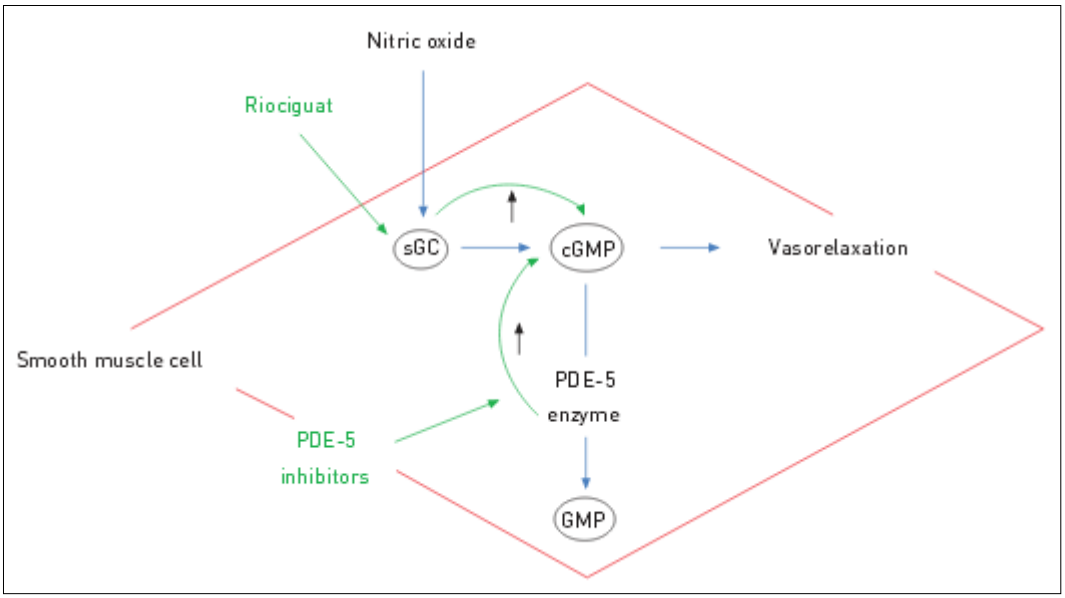


Figure 3: Schematic representation of nitric oxide mediated vasorelaxation (83)

Nitric oxide (NO) is synthesized in endothelial cells via NO synthase. Since NO exhibits neutral charge, it can easily diffuse through cell membranes (98)(99)(100). Thus, after being produced in endothelial cells in pulmonary circulation, NO diffuses into pulmonary smooth muscles. Here, it binds to soluble guanylate cyclase (sGC). Activation of sGC stimulates the production of intracellular secondary messenger, cyclic GMP (cGMP). The cGMP has several target proteins. In smooth muscles, it activates cGMP dependent protein kinase which in turn phosphorylates inositol 1,4,5-tri-phosphate which in turn reduces elevated intracellular calcium concentration promoting smooth muscle vasodilation (101) (102).

cGMP is rapidly metabolized by phosphodiesterase type 5 (PDE-5). By inhibiting the enzyme activity using sildenafil and tadalafil, acute and long term beneficial therapeutic effects have been demonstrated in PAH patients (103)(104). The pharmacotherapy of sildenafil and tadalafil is dependent on basal expression of NO which is normally reduced in PAH patients. Therefore, drugs that can directly stimulate sGC could have higher therapeutic efficacy then sildenafil and tadalafil (105). Riociguat, directly activates sGC is currently under trials for PAH and CTEPH patients (106).

1.6.1 Sildenafil (Revatio, USA)

Sildenafil demonstrated sustained progress in 6MWD, NYHA FC and hemodynamics for SUPER-1 trial (104) for 1 year independent of tested dose. Sildenafil group showed side effects like headache and flushing. Further, extension of trial called SUPER-2 study for 3 years showed similar progress thereby increasing the life expectancy up to 3 years (79%) (107). Sildenafil is currently approved for NYHA FC II, III and IV patients in USA and Canada at dose 20mg three times a day.

1.6.2 Tadalafil (Adcirca, USA)

Tadalafil demonstrated improvements at 16 weeks in 6MWD, hemodynamics and TTCW applying highest dose, 40 mg in naïve patients or patients on additional bosentan therapy (103). Nevertheless, less marked progress was observed in patients with additional bosentan therapy. Similar side effects were observed as that of sildenafil. Results from first trial, PHIRST-1 were maintained in second trial, PHIRST-2 with good improvements in 6MWD and tolerability.

1.6.3 Riociguat

Riociguat was tested on PAH and CTEPH patients in phase II clinical trials for 12 weeks. It improved the NYHA FC, 6MWD and hemodynamics. Further, the efficacy of riociguat was evaluated in 445 PAH patients in phase III PATENT-1 trial and it demonstrated significant improvements in primary and secondary end points (108). Results from the Phase III clinical trials were sustained for 12 weeks at 2.5mg max dose (109) suggesting that it exhibits a good safety profile with syncope as side effect.

1.7 Combination therapy

Table 3: List of few combined drugs used in the clinical trials modified from (110)

Drugs	Combination used	Functional Classification	Improvement in 6 minute walk distance	Change in hemodynamic parameters at highest tolerated dose
Bosentan	Sildenafil	II	11.2 metres @ 24 weeks (ns)	mPAP = - 2.7mmHg PVR = -141 dynes CI = 0.09 L/min/m^2
Sildenafil	Epoprostenol	I-IV (71% class II-III)	29.8 metres @ 16 weeks	mPAP = - 2.8mmHg PVR = -150.6 dynes CI = 0.9 L/min/m^2
Iloprost	Bosentan	II-IV (94% Class III)	30 metres @ 12 weeks	mPAP = - 6mmHg PVR = -164 dynes CI = 0.1 L/min/m^2 (ns)

CI: Cardiac Index, mPAP: mean pulmonary artery pressure, PVR: Pulmonary Vascular Resistance, ns: not significant

It has been anticipated that integrating therapeutic drugs with different modes of action may elicit an additive or similar effect at reduced doses of each drug. This combined strategy was adopted in clinical trials to evaluate the primary and secondary end points in PH treatment (110) and few examples are listed in **Table 3**.

1.8 Selection of therapeutic agent for therapy of PH

Continuous developments have been made towards the pharmacotherapy of PH. The decision to use an appropriate therapy becomes more complex. Selection can be based on an algorithm which was established at 4th World Symposium on PH (111). Nevertheless, based on clinical experience by clinicians, therapy may deviate from developed algorithm.

WHO functional Class with **Preferred therapeutics**
II
Ambrisentan, bosentan, macitentan, sildenafil, tadalafil or riociguat
III
Ambrisentan, bosentan, macitentan, IV epoprostenol, IV or SC treprostinil, inhaled iloprost, sildenafil, tadalafil or riociguat
IV
IV epoprostenol, IV treprostinil as alternative, inhaled iloprost if IV not tolerable

Combination therapy is applicable if monotherapy is not justified (93)(111). Basically, drugs with different modes of action should be combined which means drugs from any 2 of the subsequent classes, prostacyclins, ERA and PDE-5 inhibitors.

2 Liposomes as drug delivery systems

The main goal of any therapy is to use drugs with high therapeutic index and minimal side effects. The clinical application of most of the therapeutics is hindered by insufficient drug concentrations at target sites or toxic effect on normal tissues. Several approaches have been explored to bypass these difficulties e.g. by creating selective drug delivery to the target site. An ideal situation would be to specifically target the organ affected by the disease. Molecular conjugates and colloidal particles can be appropriate for the above mentioned problem. Incorporating drug moieties in colloidal systems like liposomes, niosomes, micelles and erythrocytes can generate colloidal particulates (112). Of all these carriers, liposomes have been explored extensively. The composition of liposome bilayer makes them biocompatible and biodegradable. Phospholipids when dispersed in aqueous medium self-assemble to form spherical vesicles of multiple bilayers made of natural or synthetic lipids with an aqueous core. Liposomes made of natural lipids are physiologically inert and inadequately immunogenic with minimal toxicity (112).

Bangham first reported liposomes in England in 1960 while investigating phospholipids and blood clotting (113). He observed that phospholipids when dispersed in aqueous medium spontaneously form spherical vesicles. This rearrangement of phospholipids is because of the hydrophobic effect which arranges the phospholipids in order to reduce unfavorable interactions between acyl chains and aqueous medium (114) (115). The hydrophobic effect is supported by electrostatic interactions, hydrogen bonds and Van der Waals forces (116). Liposomes are artificial vesicles composed of aqueous interior core surrounded by one or multiple lipid bilayers (lamellae).

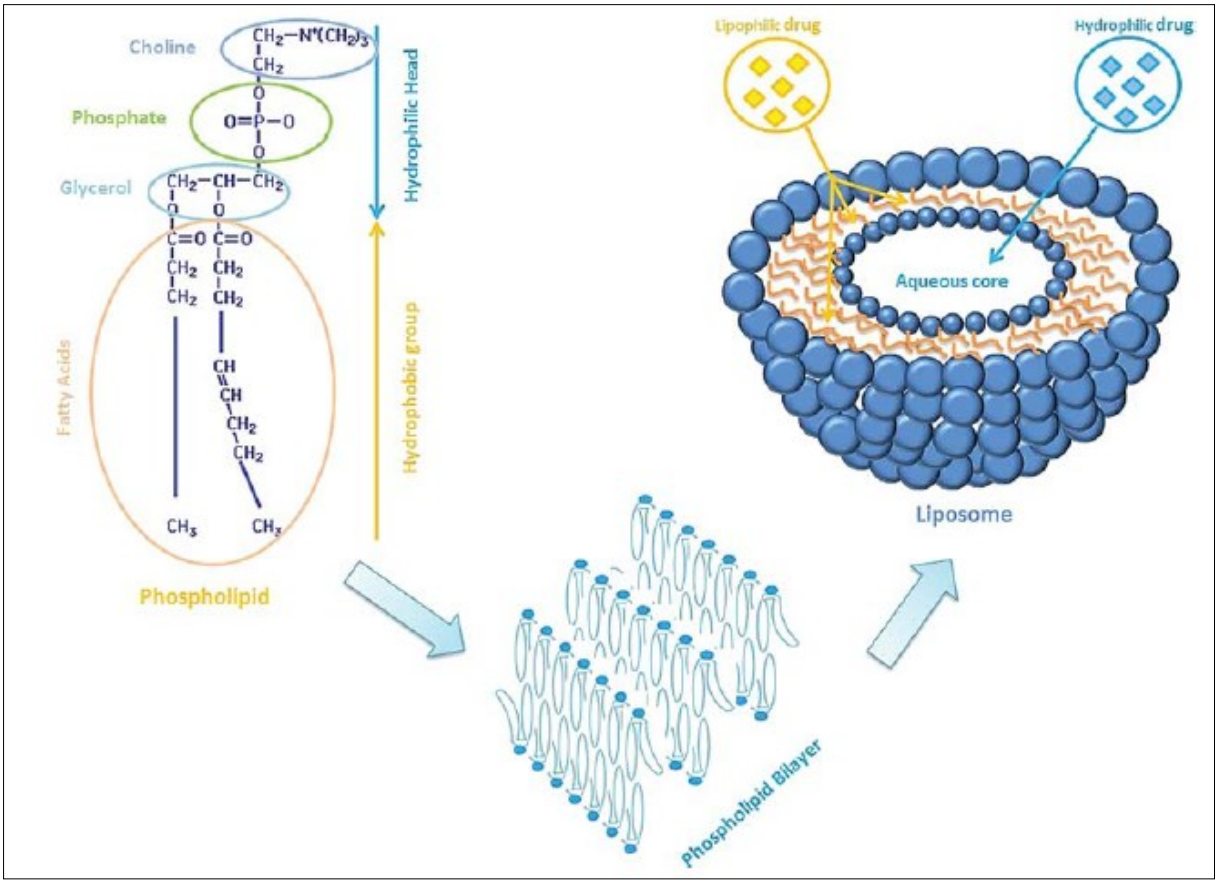


Figure 4: Liposome assembly (117)

Encapsulation of therapeutic agents within liposomes depends upon the lipophilicity of drug. Hydrophilic, lipophilic and amphiphilic drugs are entrapped in liposomal aqueous core, lipid bilayer and between aqueous and and lipid phase, respectively, making liposomes as the most promising carriers (118).

Based on composition and mechanism of delivery, liposomes are classified in five categories, conventional, pH -sensitive, cationic, immune and stealth (119). The liposome's size determines its fate and the liposome size and lamella affect the drug entrapment within liposomes. Therefore, liposomal vesicles are classified in terms of their size and lamella; Small unilamellar vesicles (SUV, 20-100nm), Large unilamellar vesicles (LUV, >100nm), Giant unilamellar vesicles (GUV, >1000nm), Oligolamellar vesicles (OLV, 100-500nm) and Multilamellar vesicles (MLV, >500nm)(120).

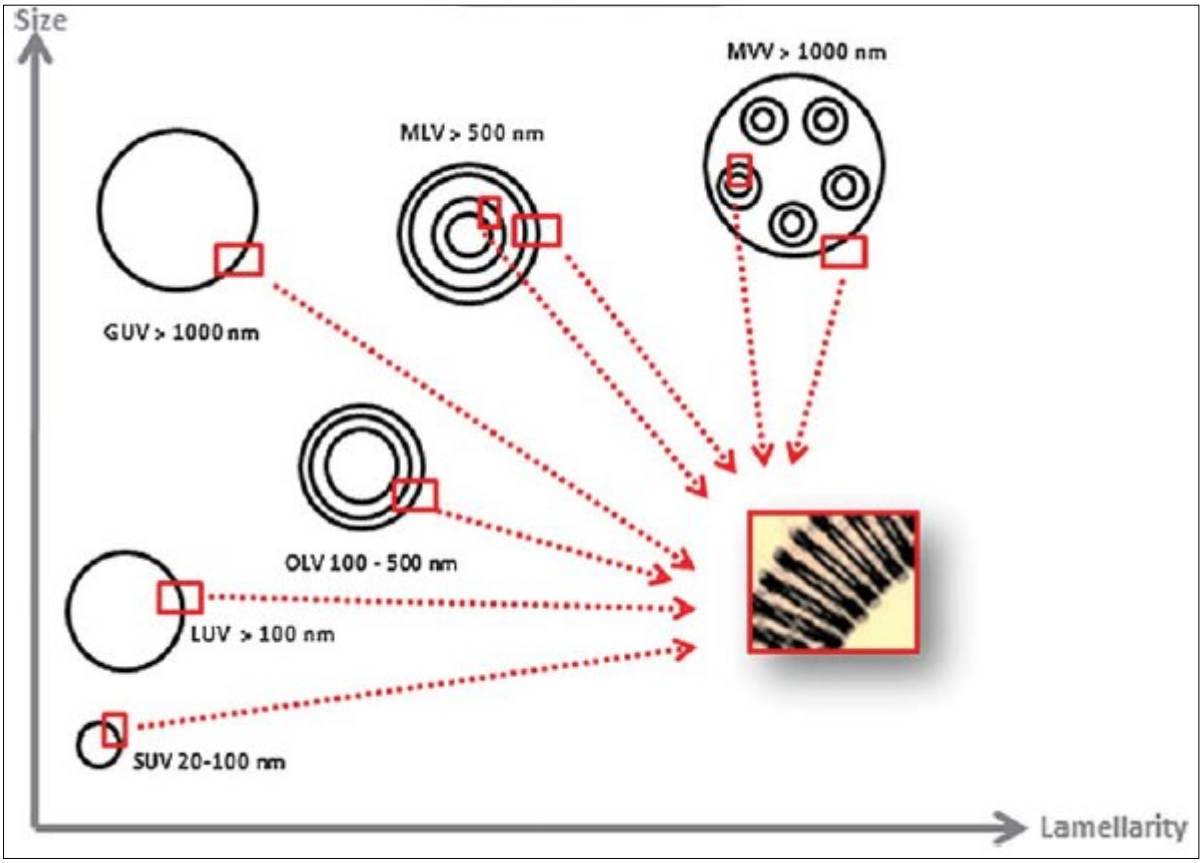


Figure 5: Classification of liposomes based on size and lamellarity (117)

Liposomes are prepared by four classical methods, hydration of lipid film (Bangham method), reverse phase evaporation (REV), ether or ethanol injection and detergent dialysis technique. The thin film hydration method will be described since this was used for the liposome preparations throughout the entire study.

Thin film hydration method (Bangham)

Liposomes were initially synthesized by this method (121). Phospholipids (natural or synthetic) with or without cholesterol are mixed in organic solvent. Further, the solvent is eliminated by evaporation using rotary evaporator or vacuum. Then, the dried thin lipid cake remaining on walls of flask was hydrated using aqueous buffers with continuous agitation above the phase transition temperature of phospholipid used. It is the most widely used method and very handy to operate. Liposomes thus formed after hydration are multilamellar liposomes (MLV) with heterogeneous size 1-5µm. Downsizing of liposomes is performed to produce SUV (sonication) or LUV (122) (123) (extrusion through polycarbonate filters) having homogenous size distribution **(Figure 6,7,8).**

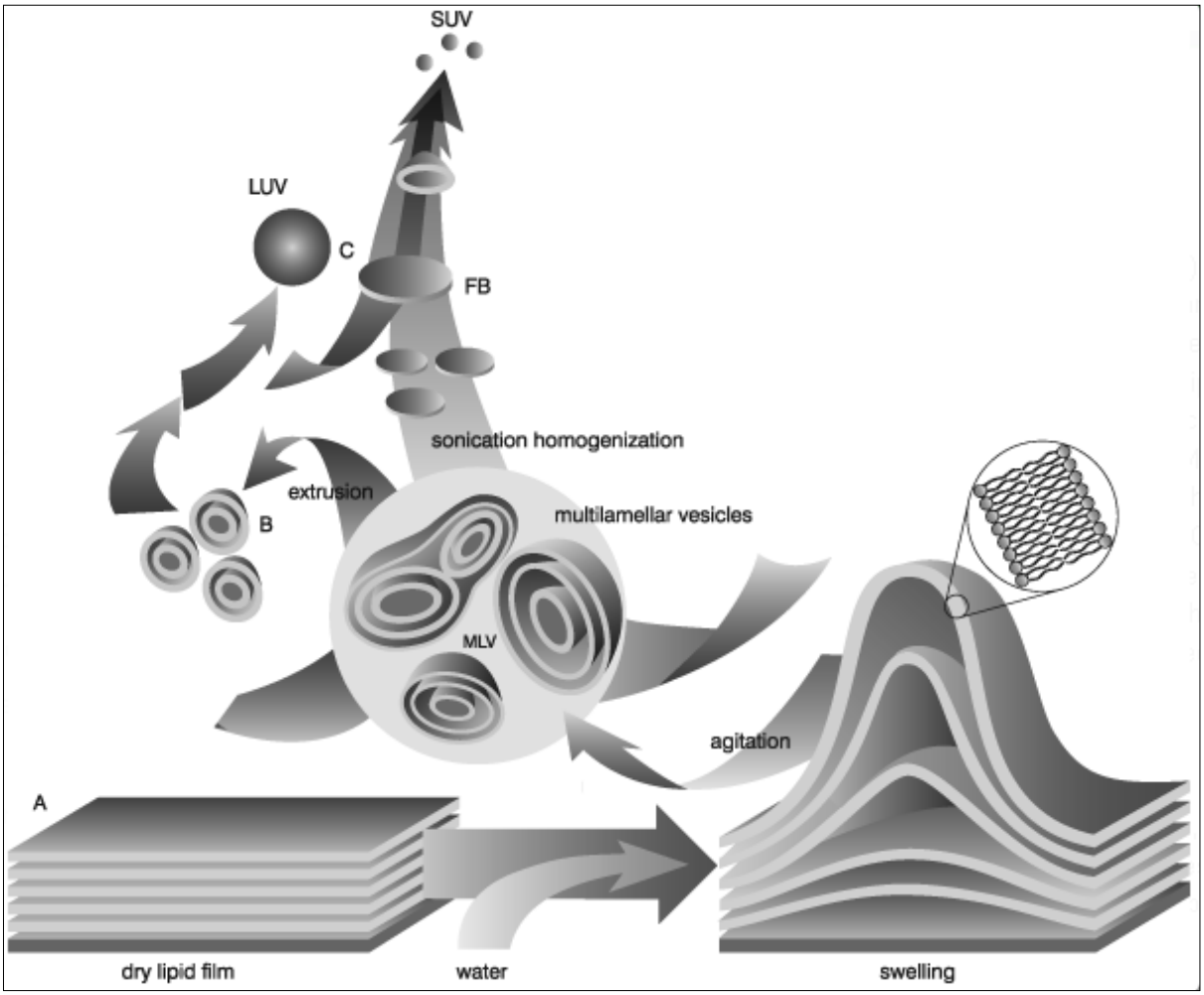


Figure 6: Thin film hydration method for the preparation of liposomes (www.avantipolarlipids.com)

Figure 7: Mini extruder with syringes containing liposomes (www.avantipolarlipids.com)

Figure 8: Extrusion of liposomes (www.avantipolarlipids.com)

2.1 Conventional liposomes

Several liposomal formulations with promising results have been developed trying to reach the clinical trial status. Liposomes help to protect the encapsulated drug from degradation and also aim to target specific tissues or organs. When liposomes are administered intravenously, they are approached by the mononuclear phagocyte system (MPS) and subsequently removed from the body (124)(124). This was used as a strategy for delivery of antiparasitic and antimicrobial therapeutics targeting the MPS (125) (126) (127) .

If the target site is other than the MPS, liposomes are efficiently engulfed by macrophages following rapid elimination of liposomes from circulation which is a drawback for potential applications of liposomes for drug delivery purposes. MPS directly cannot identify liposomes, in fact the serum proteins (opsonins) bind liposomes and these bound opsonins to liposomes are recognized by MPS. Few serum opsonizing proteins have been identified which influence the fate of liposomes. These include immunoglobulins (128), fibronectin (128) (129), β2-glycoprotein (130), C-reactive protein (CRP) (131)and β 2-macroglobulin (132).

Nevertheless, serum proteins that suppress phagocytosis of foreign particles called dysopsonins have been reported. Dysopsonins like HSA and IgA when present on particle surface have been shown to minimize identification and phagocytosis. The stability of

liposomes is influenced by charge, hydrophobicity, size, membrane fluidity, and bilayer packaging (133) (134). To prevent the rapid drug release from liposomes, the components of liposomal membrane can be manipulated to alter the fluidity of the membrane. It was reported that cholesterol in the liposomal membrane increased the bilayer packaging of phospholipids thereby reducing the transfer of lipids to HDL (135). It was shown that liposomes become unstable when they interact with HDL and LDL. Liposomes composed of saturated phospholipids exhibiting a higher phase transition temperature (Tm) are more stable than liposomes composed of unsaturated fatty acids (136). Thus, liposome bilayer rigidity and stability can be improved by incorporation of cholesterol and phospholipid with higher Tm. The molar ratio of cholesterol in liposomes is a critical factor for appropriate liposome morphology. The liposome morphology is changed when molar ratio of cholesterol exceeds 30 mol%. Cholesterol can help to capture encapsulated therapeutics within liposomes (137) (138). Further to reduce the MPS recognition and liposomal removal from the circulation, liposomal size and surface charge have also been altered. For instance, larger liposomes are cleared more rapidly than smaller liposomes (136). SUVs exhibit a longer half-life then MLVs suggesting that MPS can discriminate between sizes. Therefore, it was apparent that size alterations influence the binding of opsonins to liposomes. As a result, the fate of liposomes in liver is probably size dependent (139). Anionic liposomes exhibit shorter half-lives in the blood then zwitterionic liposomes, however conflicting data has been reported (140)(141). On the other hand, cationic liposomes may be toxic and can be rapidly eliminated from circulation (142).

2.2 Long circulating liposomes

Neutral liposomes cannot completely prevent the binding of opsonins and reduce recognition by MPS. To overcome this, the surface properties of liposomes have been modified using polymers or lipid conjugated polymers to create a steric barrier.

Polymers should possess basic characteristics like high hydrophilicity and flexibility in order to prove stealth properties. Examples are shown in **Table 4.** However briefly only poly(ethylene glycol) and PVP will be discussed below.

Table 4: List of natural and synthetic polymers for steric protection of liposomes

Natural polysaccharides	Synthetic polymers
Dextran, Polysialic acid, Hyaluronic acid, Chitosan, Heparin	Polyvinylpyrolidone, Polyvinyl alcohol, Polyacrylamide, Polyethylene glycol, Poloxamers, Poloxamines, Polysorbates

2.2.1 Polyethylene glycol (PEG) coated liposomes (Stealth liposomes)

It is the most widely used polymer for production of stealth liposomes. Because it is neutral, hydrophilic and flexible, it can create surface barriers that can prevent the binding of opsonins to liposomes making liposomes invisible to the MPS. This steric repulsion conferred by PEG was investigated by freeze –fracture TEM (143). Since desorption of PEG from liposomes can occur within bloodstream, PEG was covalently conjugated to phospholipids to retain its steric characteristics.

2.2.2 Polyvinylpyrrolidone (PVP)

PVP has a hydrophobic moiety and has been used to coat liposomes. It is the hydrophobic part of the polymer which acts as an anchor allowing polymer retention on particle surface. PVP liposomes have the potential to demonstrate steric characteristics *in vivo*. The extent of steric stabilization depends on hydrophobic chain length, molecular weight and density (144,145).

3 Pulmonary drug delivery

Pulmonary drug delivery has several advantages over oral and intravenous routes of administration. It prevents first-pass hepatic effects thereby reduces the therapeutic dose and adverse effects. Additionally, the large surface area (>100m^2), thin epithelium, and highly vascular properties allow rapid absorption and quick onset of action of a drug (146) thus making pulmonary drug delivery very attractive. Moreover, the minimal enzyme activity within lung increases the bioavailability of therapeutics (147). The patient compliance is good since the lung delivery is non-invasive (148) (149) (150).

Selective targeting of the drug within the lung is really challenging and it was found that aerosols exhibiting an aerodynamic diameter about 1-2µm are predominantly deposited in the alveoli region with high efficiency (151). Aerosols exhibiting a particle size greater than 5µm are deposited in airways or esophagus while smaller particles <1µm are exhaled immediately minimizing deep lung deposition (151).

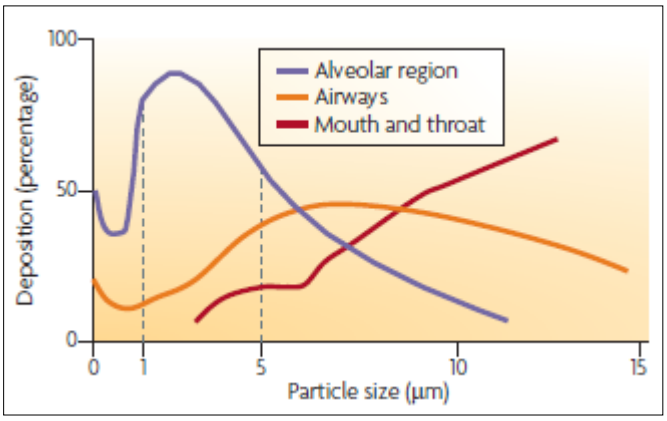


Figure 9: Deposition pattern of aerosols in human lungs (151).

3.1 Liposomes as suitable carriers for pulmonary delivery

Medications are inhaled directly for therapy of lung diseases like asthma and chronic obstructive pulmonary disease (COPD) to reach the target site at effective doses to avoid introducing large doses systemically attaining the same local concentration of therapeutic within lung. Since dosage can be potentially reduced by aerosol therapy, the systemic side effects or adverse effects can be consequently minimized and allow the development of efficacious therapies. There are several reasons to combine liposomes with inhalation: Liposomes can accommodate lipophilic drugs enabling inhalation without the need of

solubilizing excipients resulting in improved safety profile (152). The main objective is to alter the release kinetics of the therapeutic agent, extending its residence time within the lung thereby resulting in its prolonged duration of action. This claim is justified by the pharmacokinetic and pharmacodynamic data for aerosolized liposomal antibiotics. The controlled release of therapeutics further minimizes the systemic concentrations. Therapeutic agents that inherently have bad taste or induce cough are not generally tolerated, liposomes can develop a higher tolerability profile for these agents thus reducing the adverse effects. This claim was further justified by exposure data of inhaled amphotericin and antibiotics to humans (153).

Until now, for the inhalation use, neutral lipids like phosphatidylcholines and anionic lipids like phosphatidylglycerols were used for encapsulation of beclomethasone (154,155), fasudil (156) and VIP (157), respectively. A liposomal formulation composed of neutral and pegylated lipids have also been developed for pulmonary vasodilator iloprost which reported very low encapsulation efficiency (158). Nevertheless, none of the liposomes have been approved for inhalation route. On the other hand, ten liposome formulations have been acknowledged and approved by USA as an attractive therapeutic carrier for parenteral route of administration. The first approved parenteral liposomal product was amphotericin B (AmBisome®, USA, 1990) and the generic liposomal doxorubicin in 2013. Another recent liposomal vincristine formulation (Marqibo®, USA, 2012) was approved for the pharmacotherapy of leukemia. Such long history for liposomes encourages in continuously developing novel inhaled liposomal therapeutics with acceptable stability to sustain reasonable pharmaceutical industry (159). Several liposomal inhaled formulations have been developed, however only few have successfully reached later stages of clinical level, e.g. (ARIKACE®, USA) for cystic fibrosis and (Pulmaquin™, USA) for lung infections (159).

3.2 Fate of inhaled liposomes

Liposomes can be synthesized using biodegradable substances that are part of lung surfactant. This could potentially avoid toxicity issues which might occur if foreign components are used for liposomes. Cholesterol and phospholipids constitute major parts of the lung surfactant. Lung surfactant is composed of 90% of phospholipids of which >50% is saturated fatty acid, di-palmitoylphosphatidylcholine (DPPC), stearic acid 4-15% and monounsaturated oleic acid >10% and others with less percentage (160).

The fate of inhaled liposomes has been reviewed by Schreier's group (161,162). Liposomes when released their drug load, are cleared by alveolar macrophages when deposited within alveoli (161,162), when deposited in conducting airways they are cleared by mucociliary mechanisms (162). Studies showed that the aerosol droplet size determines the site of deposition, while the clearance rate was similar independent of the size of liposomes (163). Studies have suggested that there is no unnecessary accumulation of liposomes within the lung and that the phospholipids used for liposome synthesis are probably treated and recovered by alveolar type I and II cells analogous to endogenous phospholipids (161,162).

4 Inhalers

For a successful pulmonary delivery, liposomes should be effectively delivered to the airways. For this purpose, delivery technologies have been developed. Ideally, these technologies should generate aerosols within a narrow respirable size range such that maximum dose arrives at the targeted area, and minimum deposition in the oropharynx. Since no liposomes have been approved for inhalation therapy so far, extensive research has been performed to evaluate aerosols containing the delivery systems. Independent of delivery technology, the liposomes should sustain the aerosolisation process maintaining their vital properties like size, drug loading capacity and *in vitro* release kinetics to correlate the *in vivo* release. Dry powder inhalers, metered dose inhalers and nebulisers are the three important delivery technologies for aerosol generation of which only nebulisers will be introduced. If the nebulisation process alters the vital properties of liposomes, the *in vivo* profile of the therapeutic agent would be changed. Due to the differences in the breathing pattern of patients and incorrect use of nebulisers, a variable performance of inhaled drug may occur. To diminish this variability, robust liposomal formulations need to be developed.

Nebulisers in general convert liquid into droplets either by compressed air or piezoelectric vibrations. They are easily operable because the formulations are directly placed in the nebuliser and the aerosol is generated during normal respiration. The disadvantage of nebulisers is that they are not quite handy. They require longer treatment times and external adjustments like compressed air for jet nebulisers when compared to metered dose inhalers and dry powder inhalers. Three different types of nebulisers are available employing different working principles namely, air jet, ultrasonic and vibrating mesh nebulisers.

Previous work from our laboratory has demonstrated that the vibrating mesh nebuliser is the most efficient nebulisers to aerosolize liposomes (Manuscript in preparation). Therefore, we used the mesh nebuliser, m-Neb which was supplied by Nebu-TEC, Elsenfeld, Germany.

4.1 Vibrating mesh nebuliser

In the vibrating mesh nebuliser, the liquid is only passed once through the mesh of nebuliser to generate aerosol droplets (164). Continuous use of the mesh nebuliser may clog the mesh. Therefore, it is a good practice to clean and sanitize the mesh thoroughly to warrant a

robust performance (164). It has been shown that vibrating mesh nebulisers have a higher efficiency in generating aerosols than the jet nebulisers (165). They are superior than jet and ultrasonic nebulisers in terms of minimal potential to disrupt the liposomes because they avoid the re-exposure of liquid to aerosolisation process. Several comparison studies have shown that mesh nebuliser has minimum impact on liposome integrity and drug leakage than jet nebulisers. Nevertheless, the generated aerosol and liquid left in the reservoir which does not pass through the mesh still shows some loss of drug (158) (166,167). Thus, it means that even if the liposomes are not passing through the mesh, the nebulisation process may disrupt the residual liposomes remaining in the reservoir of the nebuliser which implies that the energetics of the mesh in the vibrating mesh nebuliser not only affects the upstream aerosol but also the liquid in the reservoir. The vibrating mesh nebuliser has been tested in clinical trials for delivery of liposomes containing cyclosporine A to 12 lung transplant patients which was well accepted (168). Another liposomal formulation of amikacin demonstrated excellent safety and tolerability profile when liposomes were delivered by mesh nebulisers to the cystic fibrosis patients (169).

5 Rationale for the hypothesis

Iloprost, prostacyclin analogue, is currently the only approved inhaled medication for pharmacotherapy of PH in Europe. Since iloprost exhibits short half-life, its pharmacologic efficacy is reduced. Hence, there is a need to improve its efficacy. The proof of principle that iloprost can be encapsulated within liposomal carriers was recently shown (158). Still, there is scope for further improvements in terms of drug loading capacity, stability and release kinetics.

Aim: To encapsulate iloprost in liposomes in order to improve its pharmacologic efficacy.

Objectives:

1) Establishment of a semi-quantitative method for the detection of iloprost.

2) Develop liposomes containing a high amount of iloprost.

3) Selection of lead formulations with a detailed biophysical characterisation.

4) Evaluate the viability of PASMC cells in presence of liposomes.

5) Test the pharmacologic efficacy of liposomes using a wire myograph *ex vivo.*

6) Nebulisation performance of liposomal formulations using the vibrating mesh nebuliser.

6 Materials and Methods

6.1 Materials

The lipids 1-palmitoyl-2oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (chol), [methoxy (polyethyleneglycol)-2000]-dipalmitoyl-phosphatidylethanolamine (DPPE-PEG 2000), 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP), 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-cholesterol) were purchased from Avanti Polar Lipids, USA. Human Serum Albumin (HSA), Polyethyleneimine (PEI), Hydroxypropyl-ß-cyclodextrin (HPßCD), Stearylamine (SA), sodium nitroprusside (SNP) was purchased from Sigma Aldrich, Vienna, Austria. U-46619 was purchased from Enzo® Life sciences (Lause, Switzerland) and dissolved in 99% dimesthyl sulfoxide (DMSO). Polyvinyl pyrrolidone (PVP) was a kind gift from BASF (Ludwigshafen, Germany). Dextran T-40 was purchased from Carl Roth, Austria. Pure Iloprost was a generous gift from Bayer Health Care, Germany). Iloprost was prepared as a stock solution of 500µg/mL in chloroform:methanol 2:1, v/v (C/M). Multi Wire Myograph System 620M was purchased from DMT, Denmark supplied with Lab Chart software (PowerLab, ADInstruments).

6.2 Liposomes

Liposomes were prepared by the thin film hydration method. The lipids were dissolved in C/M 2:1 in appropriate molar ratios. The organic solvent was then evaporated under inert nitrogen to obtain a thin dried lipid film which was further dried under overnight vacuum. Following this, the lipid film was rehydrated with rehydration medium. To begin with the development of liposomes for iloprost, initially aqueous iloprost (Ventavis®) 10µg/mL pH8.1 was used as rehydration medium. This rehydration step was performed for 2 hours to yield multilamellar liposomes (liposomes of various sizes having multiple bilayers). To have large unilamellar liposomes, the multilamellar liposomes were passed 21 times through a polycarbonate membrane (Whatman Inc., Clifton, NJ) having specific pore diameter using a mini extruder (Avanti Polar Lipids, Alabama, USA). These liposomes were then stored at 4°C for further analysis. Since the objective was to improve the encapsulation of iloprost, aqueous iloprost (Ventavis®) was pre-cincubated with complexing agents like HSA and cyclodextrins and then the encapsulation was evaluated. This strategy was adopted at the beginning to continuously improve the encapsulation of iloprost.

Later, instead of using aquoeus iloprost, we incorporated anhydrous iloprost 50µg/mL as part of the lipid film and continued to develop liposomes for iloprost. The protocol to prepare liposomes remained similar except the iloprost is now present in the lipid film and the rehydration medium used was 10mM Tris/HCl buffer, pH7.0. After extensive screening of different liposomes, we came up with optimized six different formulations for iloprost termed as LI-1 to LI-6 and the protocol to prepare them remained constant as shown in **Figure 10.**

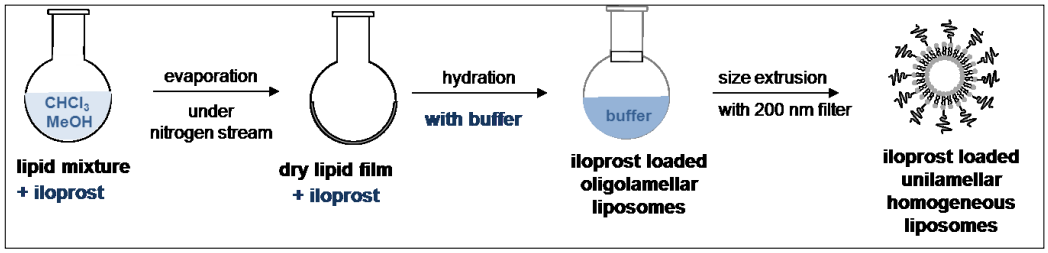


Figure 10: Thin film rehydration technique for preparation of liposomes (LI-1 to LI-6)

6.3 Separation of non-encapsulated iloprost from liposomes

Non-encapsulated iloprost was separated from liposomes by centrifugation for 10 minutes at 4000g using an Amicon Ultra filter device (cut off-100kDa) (Merck Millipore, Darmstadt Germany) **Figure 11**. An aliquot of freshly prepared liposomes was placed in amicon filter device and centrifuged. The filtrate 1 was collected and stored in glass pyrex tubes. The liposomes in supernatent were washed with appropriate buffer and re-centrifuged. The filtrate 2 and liposomes in supernatent were collected and stored in glass pyrex tubes. Finally, the amicon device was washed with 70% ethanol. To semi quantitatively estimate the amount of iloprost in liposomes; the liposomes, filtrates (1,2) and the ethanol wash were subjected for Folch extraction to determine the encapsulated and non-encapsulated iloprost as explained below.

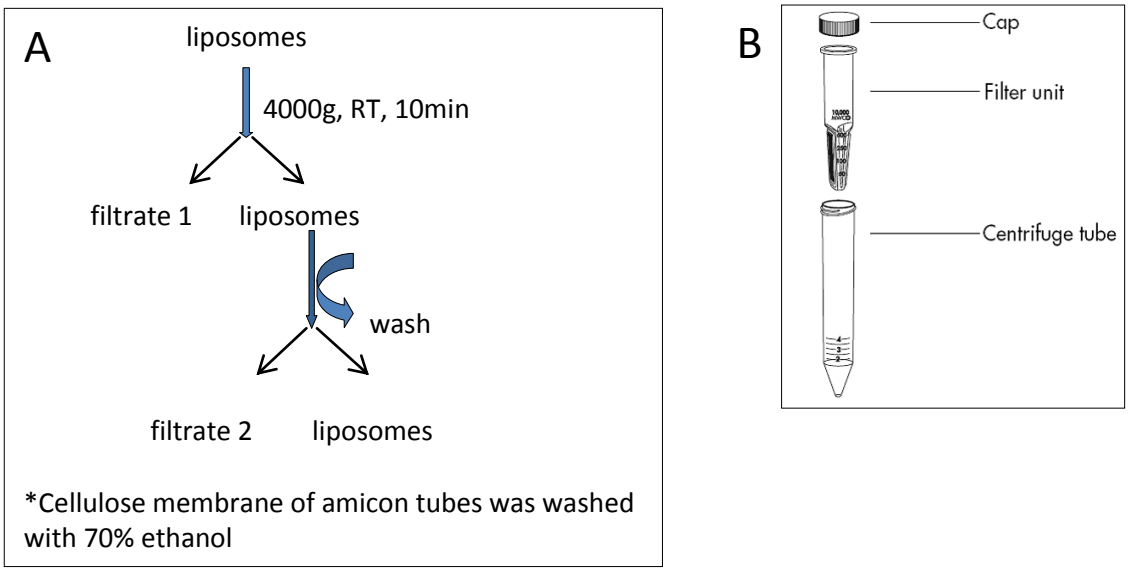


Figure 11: A) Scheme for separation of non-encapsulated iloprost from liposomes, B) Amicon ultra centrifugation device.

6.4 Determination of encapsulation efficiency of iloprost in liposomes

The amount of iloprost in liposomes was determined by semi quantitative Thin Layer Chromatography (TLC). Iloprost loaded liposomes, filtrates (1,2) and ethanol wash were first subjected to Folch extraction(Folch et al., 1957). Briefly, the lipids were extracted in C/M to final volume, 20 times the volume of liposomes added with intermittent vortexing. To this organic extract, 0.2 volumes of 0.034% Magnesium chloride were added. The mixture was centrifuged at 2500rpm, 5 mins, at RT to separate the two phases. Upper aqueous phase was removed by siphoning while lower organic phase containing lipids and iloprost was subjected to evaporation under inert nitrogen to obtain the dried lipid extracts. The lipids were resolved in 35µL C/M (2:1) and immediately applied on TLC plate in form of bands. The classical TLC developments were performed in glass chromatographic chambers at RT. The component separation was stopped when the solvent front was 1cm from the top edge of the plate. The distance between 2 bands was approximately 1cm. The solvent front was allowed to run for 6 cm from the point of application of sample. The lipids were then separated on a pre-coated silica TLC plate (TLC Silica Gel 60, Merck Millipore) using chloroform:methanol:ammonia (65:35:1.5, v/v/v) as the optimized mobile phase. The plate was then dried, dipped in 20% copper sulfate solution containing 9% phosphoric acid and heated at 190°C to visualize the bands. Semi quantitative estimation was determined by comparing the bands of reference iloprost and iloprost band associated with liposomes.

Liquid chromatography (LC-MS/MS, TSQ Quantum Ultra) mass spectroscopy was used for accurate quantifications of iloprost within liposomes (limit of detection 5ng-5µg).

6.5 Particle Size

Average particle size and polydispersity index (PDI) were measured by Photon Correlation Spectroscopy using Zetasizer 3000 HS (Malvern Instruments, Herrenberg, Germany). Empty and iloprost loaded liposomes were measured after dilution to a lipid concentration of 0.03mg/mL using ultra-pure water. Particle size was analysed by calculating the auto correlation function of the detected intensity. The PDI value of liposome preparations is obtained by the width of size distribution.

6.6 Transmission Electron Microscopy (TEM)

A volume of 4.5µL of pure (empty) and iloprost loaded liposomes (0.6mg/mL) was placed on carbon coated copper grids. After 1 minute incubation, the excess sample was blotted off and replaced by the staining reagent (1 % aqueous uranyl acetate). This treatment was repeated twice each with 30 seconds incubation time. Samples were visualized using a FEI TECNAI 20 Transmission Electron Microscope at 120kV as acceleration voltage with the help of a GATAN Ultrascan 1000CCD camera at 2k x 2k resolution. The study was performed in collaboration with Dr. Gerd Leitinger from the Medical University of Graz, Austria.

6.7 Freeze Fracture

Pure and iloprost loaded liposomes (6 mg/mL) were mixed with 30% glycerol and were frozen in liquid propane. Samples were fractured in a Balzers BAF400D freeze fracture apparatus (Balzers, Leichtenstein) at pressures around $1.3x10^{-5}$ Pa and replicas were made. The replicas were cleaned by introducing them into 12% sodium hypochlorite solution for approximately 3 hours with further overnight storage in 50% NaOH. Replicas were washed 3 times with double distilled water before loading them on uncoated copper grids. Samples were visualized as previously mentioned in section TEM. The study was performed in collaboration with Dr. Gerd Leitinger from the Medical University of Graz, Austria.

6.8 Zeta (ζ) potential

The ζ-potential was determined by laser Doppler electrophoresis using a Zetasizer NanoZS/ZEN3600 (Malvern Instruments, Germany). All measurements were performed at room temperature using samples diluted to a lipid concentration of 0.3mg/mL with Tris/HCl

buffer (10 mM Tris/Cl pH 7.0, 2 mM CsCl) and were carried out in triplicates with at least 10 runs.

6.9 Differential Scanning Calorimetry (DSC)

Phase transition studies were performed using a differential scanning calorimeter (MicroCal Inc Northampton, USA). Each scan was carried out with a scan rate of 30°C/h in the temperature range between 20°C and 50°C. The instrument was equilibrated overnight with Buffer A. Three heating and cooling cycles were executed. Before the DSC measurements, the total lipid concentration was adjusted to 1mg/mL using the Buffer A (reference).

Enthalpy (ΔH_{cal}) was calculated after baseline correction and normalized to the lipid concentration by integrating the peak areas using MicroCal LLC Origin software (OriginLab Corporation, Northampton, USA). The phase transition temperature was evaluated at the peak maximum in the heat functions and the half width of the transition peak (ΔT½) was determined which indicates the co-operativity of the transition. 1mL Buffer A (pH 8.1) contains 0.121mg Tris/HCl, 9mg sodium chloride, ethanol 99.9%, HCl and distilled water.

6.10 Cell viability

Human PASMCs were isolated from pulmonary arteries from non-transplanted donor lungs as previously described (170). A549 cells were purchased from Cell Line Service, Eppelheim, Germany. The effect of liposomes on cell viability was evaluated using an MTS assay according to the manufacturer's instructions (CellTiter96 Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany). A549 cells were cultured in DMEM/Hams-F12 medium (Gibco, Paisley, UK) containing glutamine and 10% FCS (A549) and hPASMC in VascuLife medium (Lifeline Cell Technology, Carlsbad, CA, USA) and seeded on 96-well plates at a density of 4x104 cells (hPASMC) and 2x104 cells (A549) per well. The cells were incubated with either 0.2µM iloprost (free or encapsulated) (8.5µg lipid/mL) or 1µM iloprost (free or encapsulated) (42.5µg lipid/mL) for 24 hours. Control liposomes (lipid concentration 6 mg/mL) were added at the same dilution factor as their respective liposomes. SDS, 0.1% and 0.5% w/v were used as positive controls for hPASMC and A549 cells, respectively. Post incubation, MTS/PMS was added with further incubation for 2 hours. Absorbance of the produced formazan was recorded at 490 nm using a 96-well plate reader (GloMax®-Multi Detection System – Promega, Madison, United States).

6.11 Isolation of tertiary pulmonary arteries

BALB/c mice (male or female) 20 to 25 weeks old were used in the study. Mice were pre anaesthetized with isoflurane and euthanized by an intraperitoneal injection of ketamine (500mg/kg) and xylazine (70mg/kg) according to the Austrian national guidelines. The heart and lungs were removed and the tertiary intra pulmonary arteries isolated with help of a stereo microscope (Olympus, Vienna, Austria). The arteries were cleaned of surrounding adipose and connective tissue and cut into segments 3-4mm in length for use in myograph studies. All tissues were preserved in the ice cold physiologic salt solution (PSS) 140.5mM NaCl, 5.5mM KCl, 1.5mM $CaCl_2$, 1mM $MgCl_2$, 10mM glucose, 0.5mM Na_2HPO_4, 0.5mM KH_2PO_4 and 10mM HEPES pH 7.4, solution throughout the dissection procedure.

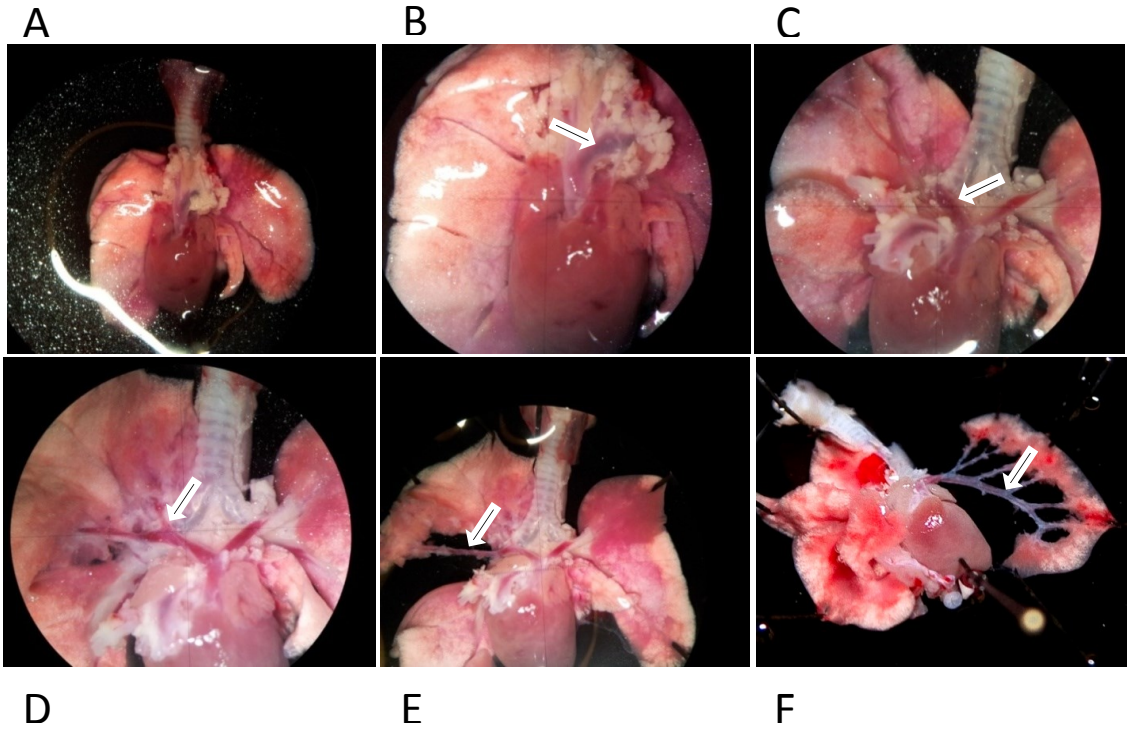


Figure 12: Steps (A-F) involved in the isolation of mouse tertiary pulmonary arteries; white arrow indicates pulmonary arteries.

6.12 Isometric tension measurements in pulmonary arteries using wire myograph

The pulmonary arteries were positioned between jaws with the help of tungsten wires in myograph chambers (Danish MyoTechnology, Arhus, Denmark) containing PSS and continuously aerated with 21% O_2, 5% CO_2 and balanced N_2 at 37°C. The myograph

chambers were connected to force transducers for isometric measurements (PowerLab, ADInstruments). The pulmonary arteries were incubated at 37°C and a basal tension of 2mN was applied. The arteries were allowed to stabilize for 45 minutes. PSS containing 120mM KCl was used to determine viability and adequate contractility of tissues. Arteries were stimulated three times using 120mM KCl to obtain reproducible contractions. Arteries which did not respond to these repeated stimuli were not included in the study.

6.13 Preparation of iloprost for wire myograph

Iloprost dissolved in C/M was dried by evaporation under inert nitrogen and dissolved in 96% ethanol and diluted 1:1000 with PSS. The final concentration of ethanol was 0.096 vol%.

6.14 Iloprost-induced relaxation of pre-constricted pulmonary arteries

Initially, concentration response-curves for mouse pulmonary arteries were established by adding cumulative doses of Phenylephrine (0.001-10µM), U-46619 (0.001-3 µM) and Serotonin (0.001-30µM). Initial experiments were performed by pre-contracting the pulmonary arteries using Phenyephrine followed by non-cumulative applications of free iloprost (1µM, 3µM, 10µM and 30µM). Further, only preliminary experiments were performed using serotonin as the pre-constrictor followed by application of 5µM free iloprost.

For subsequent experiments arteries were contracted with the half maximal effective concentration (EC50) 0.03 µM U-46619. Following stable contraction (~30 minutes post addition of U-46619) arteries were challenged with one concentration of free iloprost (1 µM, 3 µM, 10 µM and 30 µM), liposomal iloprost, or control (empty) liposomal formulations as indicated. Control liposomes were added to give the same final lipid concentration as their corresponding encapsulated formulation. In a sub-set of experiments sodium nitroprusside (10 µM) was added 60 minutes post iloprost addition to confirm vessel relaxation viability.

6.15 RNA isolation and real-time PCR measurements

Total RNA was isolated using the RNeasy Mini kit (Peqlab) from mouse pulmonary arterial sections. Total RNA was reverse transcribed using the iScript kit (BioRad, Hercules, CA, USA) according to manufacturer's instructions. Real-time PCR was performed using a LightCycler 480 (Roche, Wien, Austria). The PCR reactions were set up using a QuantiFast SYBR PCR kit (Qiagen). Cycling conditions were as follows: 5 minutes at 95°C, [5 seconds at 95°C, 5

seconds at 60°C, and 10 seconds at 72°C] x45. Due to the non-selective double strand DNA binding of the SYBR®Green I dye, melting curve analysis and gel electrophoresis were performed to confirm the specific amplification of the expected PCR product. PBGD and B2M were used as the reference genes. The ΔCt values for each target gene were calculated as ΔCt = Ct Reference gene - Ct target gene. Primer sequences were as follows (5'-3') EP1 GAA CCT CAG TCC TTG GCG GG, CAC CCC ATG CAT GTC TGC TC; EP2, ATA CTT AGG CCA CCG GTC CT, GGC ACT GGA CTG GGT AGA AC; EP3, ATT GCA GTT CGC CTG GCT TC, TTC AGG TTG TTC ATC ATC TGG C; EP4, CAC ACC GGG CTC CTA ACC C, GTT GAC TCC GGG GAT GGA CA; IP, CTA TCT TGG GGA ACT GCG CT, AAG CAT GCC TGT GAA GTC CT; TP, GAC TGC GAG GTG GAG ATG ATG, AGG TGG TGT CTG CAA CAA AGT; PBGD, GGT ACA AGG CTT TCA GCA TCG C; ATG TCC GGT AAC GGC GGC; B2M. CGG CCT GTA TGC TAT CCA GAA AAC C, TGT GAG GCG GGT GGA ACT GTG. The amplicons for all prostacyclin receptors were between 107-111bp.

6.16 Nebulisation of iloprost loaded liposomes

For the nebulisation experiments, three promising cationic liposomal formulations with high iloprost encapsulation efficiency were prepared and nebulised, LI-3 (containing stearylamine and cholesterol), LI-4 (containing DOTAP without cholesterol) and LI-36 **Table 5**. Basically, LI-36 was one of the newly developed liposomal formulations which is a modification of LI-6 having DC-cholesterol, a cationic cholesterol derivative and Dextran-T40 as sugar excipient as well as PVP as polymer component. Dextran was part of rehydration buffer. Liposomes were prepared in a similar way as mentioned in section 6.2. For the separation of non-encapsulated iloprost, liposomes were centrifuged just once at 4000g, 10 mins, 4°C and then the nebulisation was performed.

Table 5: Liposomes for nebulisation

Liposomes	Composition	Molar ratio	Rehydration buffer (per mL)
LI-3	POPC:SA:DPPE-PEG2000:cholesterol	75.6:8.7:2.5:13	10mM Tris/154 NaCl, pH7.4
LI-4	POPC:DOTAP:DPPE-PEG2000	87:10:3	10mM Tris/154 NaCl, pH7.4
LI-36	POPC:DC-cholesterol:PVP	77:20:3	10mg Dextran T-40 in 10mM Tris/154mM NaCl, pH 7.4

Protocol for nebulisation:

An aliquot of 600µL of pure liposomal dispersion 6mg/mL was placed in the vibrating mesh nebuliser. The entire volume was nebulised and the aerosol was collected. The total time for complete nebulisation was noted. The collected aerosol was analysed for size, PDI, phosphatidylcholine (PC) concentration and encapsulation efficiency of iloprost after nebulisation.

6.17 Data Analysis

The results are expressed as mean ± SEM, n indicates number of arteries. Concentration response curves for phenylephrine, U-46619, and serotonin are expressed as percentages of contraction relative to 120mM KCl. Relaxation values are expressed as the percentage change from Phenylephrine and U-46619 -induced contraction. To obtain relaxation values for encapsulated formulations, the relaxation values of empty liposomes were subtracted from the respective liposomal iloprost formulation. Multi-group comparisons were performed with a One-way ANOVA with either Tukey's or Dunnett's (for comparisons to a control group) post hoc test as appropriate. Comparison between two groups was performed with a Student's t-test. Graphpad Prism 5 (GraphPad Software, San Diego, CA) was used. Values of $p<0.05$ were considered statistically significant.

7 Results

7.1 Establishment of method of detection for iloprost

The primary objective was to establish a method for the detection and estimation of the iloprost concentration encapsulated in liposomes. For this purpose, we used a semi-quantitative method, Thin Layer Chromatography (TLC). For effective component separation, we optimized the mobile phase which is explained below.

7.1.1 Optimization of mobile phase for separation of iloprost from lipids

In order to separate iloprost from phospholipids on TLC plates, two different solvent systems were tested. The major components of liposomes (DPPC, POPC, cholesterol) and iloprost were applied in different amounts on TLC plate and allowed to develop.

a) Chloroform:methanol:ammonia (25%) (v:v:v): 65:35:1.5

It was observed that the retardation factor (Rf) was different for all the applied components. Iloprost was able to be well separated from lipids as seen in TLC **(Figure 13A)**.

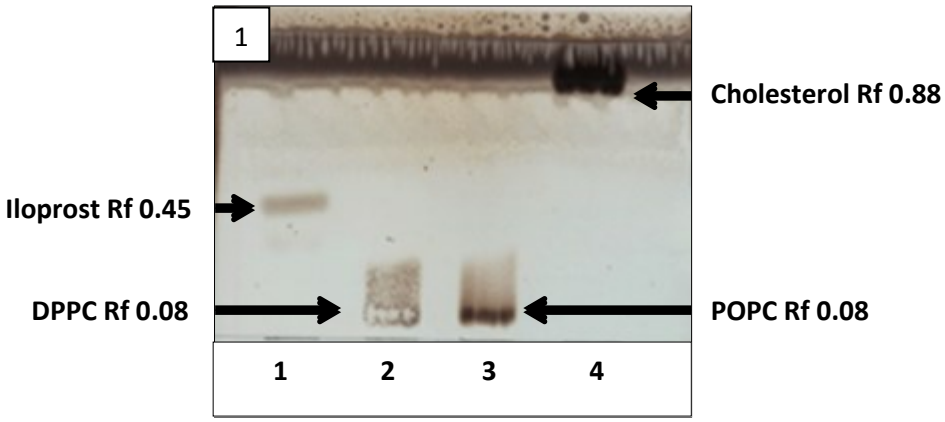


Figure 13 A: Thin Layer Chromatogram demonstrating the separation of iloprost (1µg) and phospholipids DPPC 50µg, POPC 23µg and cholesterol 100µg using the mobile phase, chloroform:methanol:ammonia (25%) (v:v:v): 65:35:1.5. The Rf value was calculated as distance travelled by the compound divided by distance travelled by mobile phase.

b) Chloroform:methanol:ammonia (25%) (v:v:v): 65:40:3

It was observed that the Rf was different for all applied components. Iloprost was able to be well separated from lipids. Nevertheless, Rf values of compounds were slightly different from the previous TLC **Figure 13A.**

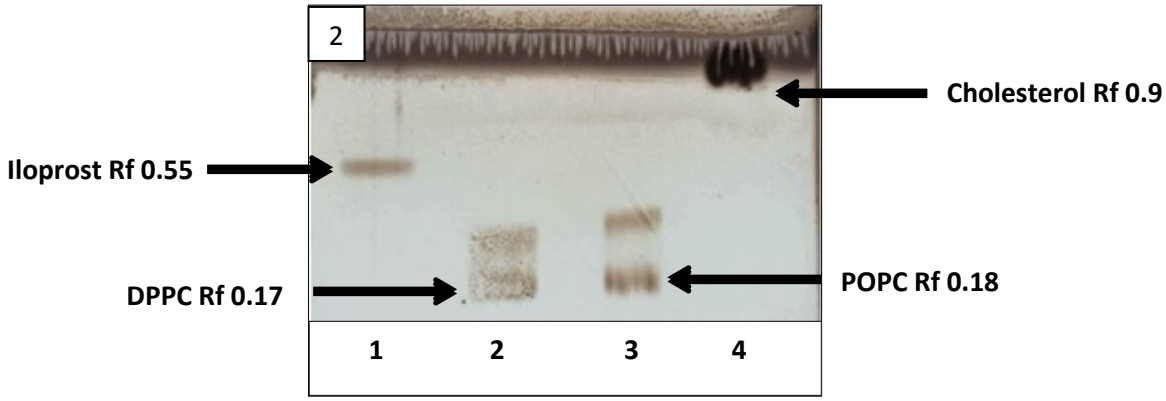


Figure 13 B: Thin Layer Chromatogram demonstrating the separation of iloprost (1.5µg) and phospholipids DPPC 3.3µg, POPC 1.53µg and cholesterol 6.6µg using the mobile phase, chloroform:methanol:ammonia (25%) (v:v:v): 65:40:3

By comparing the results of the two TLCs (**Figure 13 A,B**) we found that the phospholipid bands in **Figure 13B** were at slightly higher positions and may overlap with the iloprost band during the separation process and may interfere with quantification. Thus, we chose the mobile phase chloroform:methanol:ammonia25% (65:35:1.5) as the optimized mobile phase for the separation of lipids and iloprost for all the developmental experiments to enable iloprost quantification.

7.2 Estimation of iloprost in liposomes using aqueous iloprost Ventavis®

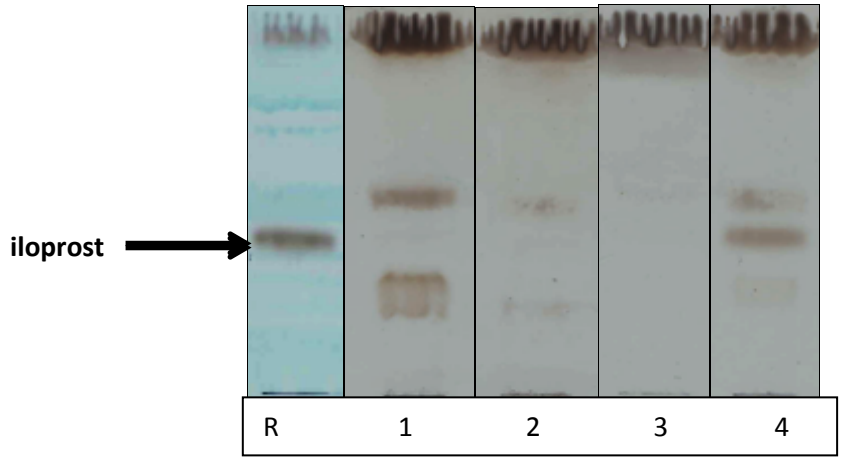
7.2.1 Conventional liposomes (DPPC and cholesterol)

Each film was rehydrated with 1mL Ventavis® pH 8.1 at 50°C for 2 hours followed by extrusion using a 200nm polycarbonate filter and subsequently the encapsulation of iloprost (Ventavis®) was determined as shown in **Table 6**.

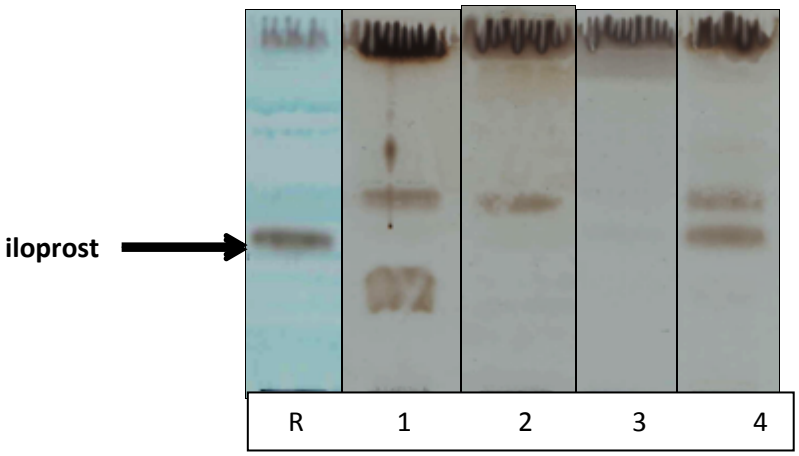
Table 6: Composition of conventional liposomes composed of DPPC with varying concentrations of cholesterol.

Number	Lipids (2mg/mL)	molar ratio	Rehydration medium	Size [nm]	PDI
1	DPPC	100	Ventavis®	193	0.203
2	DPPC/Cholesterol	80:20	Ventavis®	242	0.059
3	DPPC/Cholesterol	70:30	Ventavis®	237	0.055
4	DPPC/Cholesterol	60:40	Ventavis®	227	0.077

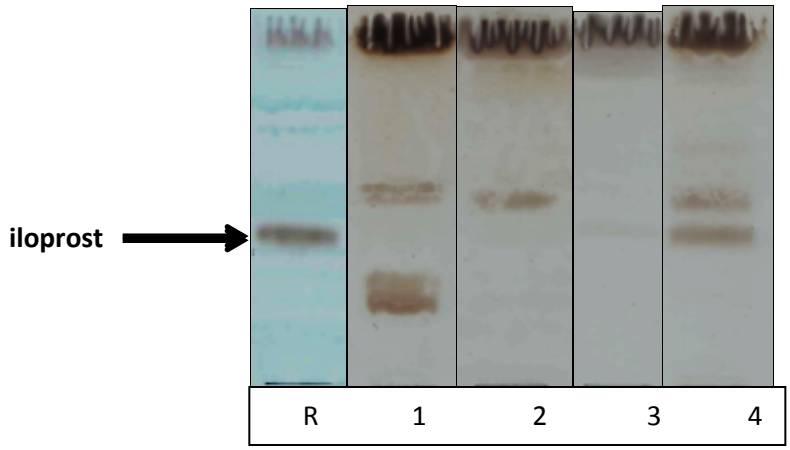
1) Liposome DPPC



2) Liposome DPPC/Cholesterol (80:20)



3) Liposome DPPC/Cholesterol (70:30)



4) Liposome DPPC/Cholesterol (60:40)

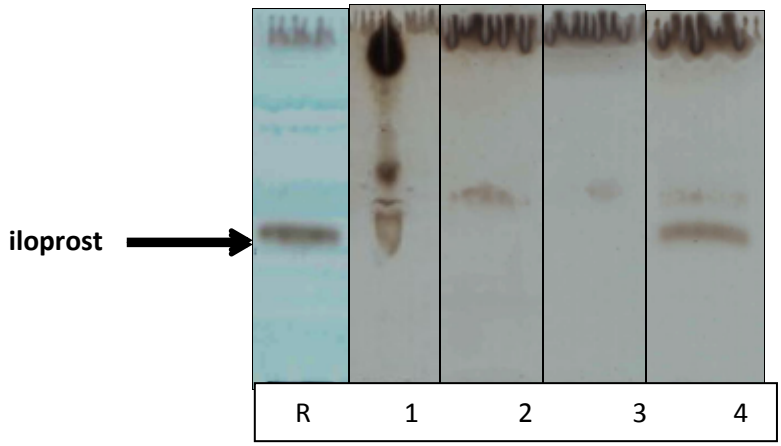


Figure 14: Thin Layer Chromatograms showing the encapsulation of iloprost (Ventavis®) in conventional DPPC liposomes with different concentrations of cholesterol where R is reference Iloprost (10µg) extracted from Ventavis®, 1 is liposomal iloprost, 2 and 3 are filtrates and 4 is the ethanol wash. Note that liposomal fractions of all the four liposomes in Table 4 were applied on the same TLC. Similarly, the filtrates 1, 2 and the ethanol washes of all the four liposomes were run on separate TLC. To enable comparison between liposomes and filtrates and ethanol wash, individual lanes were cut and placed. Iloprost was separately extracted from Ventavis® and placed to enable direct comparison.

From the above **Figure 14**, no iloprost band was observed in liposome lane (1) of all the four TLCs. Since iloprost was not encapsulated and was free, it might have adhered to the cellulose membrane of the amicon device due to its amphiphilic nature. Accordingly, , washing the cellulose membrane of the amicon device with ethanol, resulted in a complete

elution of iloprost as seen in lane 4 of all the TLCs which indicated that non-encapsulated iloprost sticks to the cellulose membrane. For direct comparison purpose, reference iloprost was separately extracted from Ventavis® and TLC was placed next to liposomal ilopost. Therefore, conventional DPPC liposomes cannot encapsulate iloprost.

7.2.2 Conventional liposomes with complexing agents

In the next step, we attempted to improve the encapsulation of iloprost in conventional liposomes using complexing agents like human serum albumin (HSA), cyclodextrin, polyethyleneimine (PEI) to complex with iloprost.

Tsai et al reported that HSA has the ability to stabilize iloprost (171). Therefore, we incubated HSA with iloprost and determined the encapsulationafter rehydration of the lipid film with the iloprost/HSA mixture. Cyclodextrins act as guest molecules for lipophilic moieties because of theinherent conical structure of the molecules (172). Again, we incubated cyclodextrins with iloprost and evaluated the encapsulation after rehydration of the lipid film with the iloprost/cyclodextrin mixture. Polyethyleneimine is a polycationic substance and since it bears a positive charge we took the advantage of electrostatic interactions between the negatively charged iloprost and the polycation PEI.

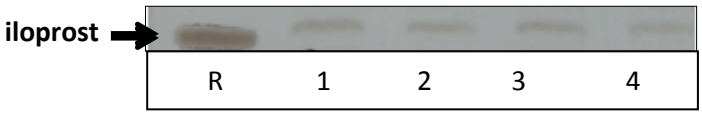
7.2.2.1 Complexation of Iloprost and Human Serum Albumin (HSA)

Here, in an attempt to improve the encapsulation, we first incubated 1mL Ventavis® with 10mg defatted HSA at RT for 15 minutes followed by rehydration, extrusion and subsequently determined the encapsulation as shown in **Table 7**.

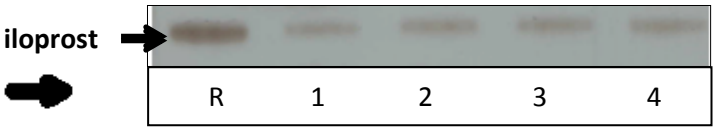
Table 7: Composition of conventional liposomes composed of POPC with varying concentrations of cholesterol.

Number	Lipids 2mg/mL	molar ratio	Rehydration medium	Size [nm]	PDI
1	POPC	100	Ventavis® + HSA	207	0.171
2	POPC/Cholesterol	80:20	Ventavis® + HSA	221	0.186
3	POPC/Cholesterol	70:30	Ventavis® + HSA	221	0.174
4	POPC/Cholesterol	60:40	Ventavis® + HSA	235	0.214

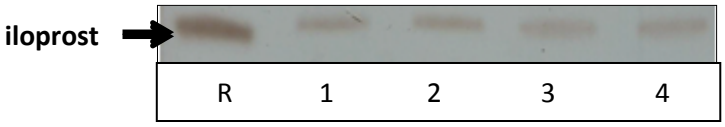
1) Liposomes of 1,2,3,4



2) Filtrate 1 of 1,2,3,4



3) Filtrate 2 of 1,2,3,4



4) Ethanol wash of 1,2,3,4

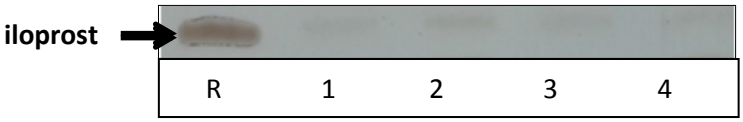


Figure 15: Thin Layer Chromatograms showing the encapsulation of iloprost (Ventavis®) in conventional POPC liposomes with different concentrations of cholesterol using HSA as complexing agent where R is reference iloprost 2µg (2µg is 100% encapsulation).

From **Figure 15** it was observed that only a very small quantity of iloprost was encapsulated as shown in TLC 1 (Liposomes 1,2,3,4). Altering the cholesterol concentration in liposomes did not influence the iloprost encapsulation efficiency as shown in TLC 1 (Liposomes 1,2,3,4). Both filtrates 1 and 2 of all liposomes contained significant amounts of iloprost complexed with HSA. Ethanol wash showed hardly any traces of iloprost which means that iloprost is complexed with HSA and does not adhere to the cellulose membrane of the Amicon device.

7.2.2.2 Complexation of iloprost with cyclodextrin

Further, we tried to complex iloprost using hydroxyl-propyl-β-cyclodextrin (HPβCD) in liposomes composed of POPC only. We first incubated 1mL Ventavis® with 10mg defatted HPßCD at RT for 15 minutes followed by rehydration, extrusion and subsequently determined the encapsulation efficiency.

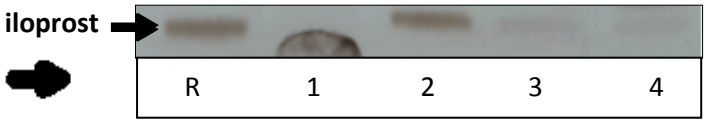


Figure 16: Thin Layer Chromatograms showing the encapsulation of iloprost (Ventavis®) in POPC liposomes using HPβCD as complexing agent, R is reference iloprost 1.5µg, 1 is liposomal iloprost, 2 and 3 are filtrates and 4 is ethanol wash in TLC lane.

From **Figure 16** it was observed that the entire iloprost was detected in filtrate 2 and traces of iloprost in filtrate 3 and ethanol wash and no iloprost was associated with liposomes in lane 1 (only part of lipid band seen). Therefore, cyclodextrin could not improve the encapsulation efficiency of iloprost in liposomes.

7.2.3 Pegylated liposomes with complexing agents

We next attempted to improve encapsulation of iloprost using pegylated lipids (phospholipid conjugated to polyethylene glycol, DPPE-PEG2000) in liposomes together with the complexing agent, Human Serum Albumin (HSA). Different amounts HSA were dissolved in 1mL Ventavis® and lipid films were hydrated at RT followed by extrusion and the iloprost encapsulation efficiency was determined as shown in **Table 8**.

Table 8: Composition of Pegylated liposomes with HSA as complexing agent

Number	Lipid (2mg/mL)	Molar ratio	Rehydration medium	Size (nm)	PDI
1	POPC:chol:DPPE-PEG2000	69:30:1	2mg HSA in Ventavis®	178	0.112
2	POPC:chol:DPPE-PEG2000	69:30:1	10mg HSA in Ventavis®	172.5	0.187

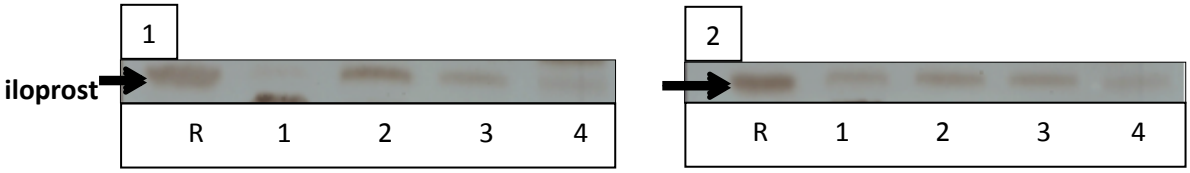


Figure 17: Thin Layer Chromatograms showing the encapsulation of iloprost (Ventavis®) in pegylated liposomes using HSA as complexing agent, R is reference iloprost 1.5µg, lanes 2 and 3 are filtrates and lane 4 is the ethanol wash.

From **Figure 17** it was observed that rehydration with 2mg HSA (1) did not encapsulate iloprost since most of the iloprost was observed in filtrates 2 and 3. However, an increase or the HSA concentration in the rehydration buffer slightly improved the encapsulation

efficiency to approximately 5-10% as evident in liposomal lane 1 of (2). Rest of iloprost was observed in filtrates 2 and 3, respectively.

We know that iloprost is negatively charged at physiologic pH because of its carboxylic acid group. Therefore, we thought of incorporating cationic lipids in liposomes which might improve the encapsulation due to electrostatic interactions between positive and negative charges.

7.2.4 Cationic liposomes

7.2.4.1 Cationic liposomes with complexing agents (HSA and cyclodextrins)

Therefore, in an attempt to improve the encapsulation, we used a positively charged component i.e, stearylamine (SA) together with complexing agents, HSA and cyclodextrins. Required amounts of HSA and cyclodextrin were dissolved in 10µg/mL Ventavis® and lipid films were rehydrated at RT without extrusion and subsequently we determined the encapsulation efficiency of iloprost (**Table 9**)

Table 9: Composition of cationic liposomes with complexing agents, HSA and cyclodextrins

Number	Lipid (2mg/mL)	Molar ratio	Rehydration medium
1	POPC:SA	90:10	10mg HSA in Ventavis®
2	POPC:SA	90:10	10mg cyclodextrins in Ventavis®

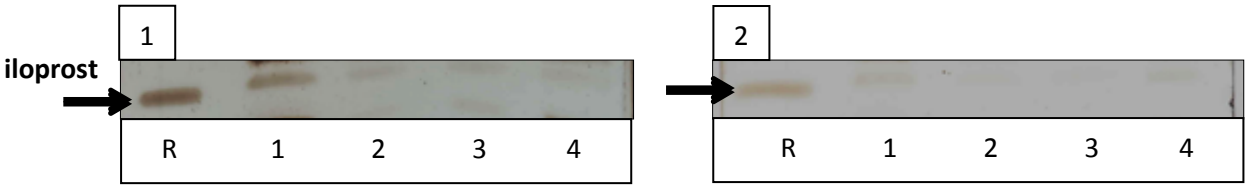


Figure 18: Thin Layer Chromatograms showing the encapsulation of iloprost (Ventavis®) in cationic liposomes composed of stearylamine in combination with complexing agents HSA (1) and cyclodextrin (2), R is reference iloprost 2µg, 2 and 3 are filtrates and 4 is ethanol wash in TLC lane (4µg iloprost is 100% encapsulation)

From **Figure 18** (1) an iloprost band was observed in liposomal lane 1 of (1) with encapsulation efficiency (~25%) when iloprost was complexed with HSA together with stearylamine (1). On the other hand, encapsulation of iloprost was ~10% (band is clearer to see in original TLC plate) lane 1 of (2) when it was complexed with cyclodextrin together with

SA. It was at this time, we realized that a positive charge in liposomes might promote iloprost encapsulation.

7.2.4.2 Liposomes with polyethylene imine (PEI)

Further, with an aim to improve encapsulation, we used the polycation polyethyleneimine (PEI) as component in liposomes. Iloprost (1mL Ventavis®) was incubated with either 100µg or 500µg PEI (hyper branced PEI 25kDa, hyPEI25K, Germany) at RT and the the lipid film was rehydrated, followed without extrusion and subsequently the iloprost encapsulation efficiency was determined (see **Table 10**).

Table 10: Composition of conventional liposomes with PEI as complexing agent

Number	Liposome	Molar ratio	Rehydration medium
1	POPC:chol	70	Ventavis®+100µgPEI
2	POPC:chol	30	Ventavis®+500µgPEI

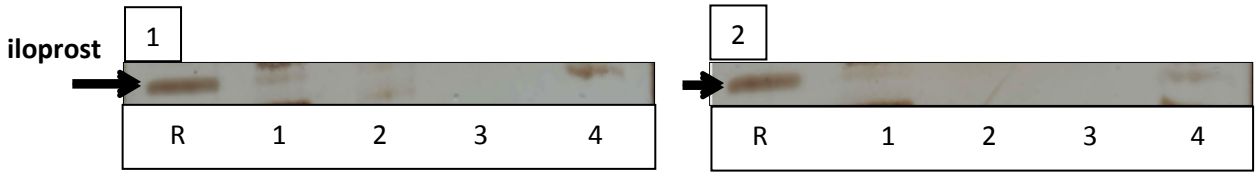


Figure 19: Thin Layer Chromatograms showing the encapsulation of iloprost (Ventavis®) in liposomes together with the polycation PEI (1) 100µg and (2) 500µg, R is reference iloprost 2µg, 2 and 3 are filtrates and 4 is ethanol wash in TLC lane (4µg iloprost is 100% encapsulation)

From **Figure 19** it was observed that addition of the polycation PEI in liposomes slightly (~5%) showed some encapsulation lane 1 of (1). Increasing the concentration of PEI resulted in no improve of iloprost encapsulation (see lane 1 of (2)). Most of iloprost was found to be fress, determined in the ethanol wash (see line 4).

7.3 Estimation of iloprost in liposomes using iloprost as part of lipid film

Due to unavailability of pure anhydrous iloprost, most of the initial experiments were done using the marketed preparation of iloprost, Ventavis®. Pure anhydrous iloprost was later received from Bayer and liposomes were tested for improved encapsulation of iloprost when iloprost was incorporated as part of the lipid film. From previous results, it was clear that a positive charge in the lipid film of liposomes might promote iloprost encapsulation.

Therefore, together with a positive charge we had the opportunity to increase the iloprost concentration in the lipid film and then to determine the encapsulation efficiency.

7.3.1 Cationic liposomes with iloprost in the lipid film

50µg/mL Iloprost was incorporated as part of the lipid film and rehydrated at RT using 10mM Tris/HCl pH 7 buffer followed without extrusion and then the encapsulation efficiency was determined (see **Table 11)**.

Table 11: Composition of liposomes using stearylamine as cationic component

Number	Lipids	Molar ratio	Rehydration medium
1	POPC:SA	90:10	10mM tris, pH7
2	POPC:SA	80:20	10mM tris, pH7

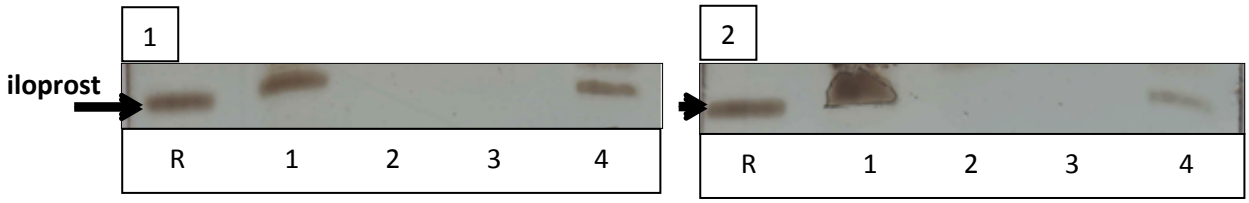


Figure 20: Thin Layer Chromatograms showing the encapsulation of anhydrous iloprost in cationic liposomes composed of different concentrations of stearylamine (SA), R is reference iloprost 2µg, 2 and 3 are filtrates and 4 is ethanol wash in TLC lane (5µg iloprost is 100% encapsulation)

From **Figure 20** it was observed that the presence of positively charged constituents like stearylamine in liposome enhanced the encapsulation efficiency of iloprost to ~40% lane 1 of (1) and ~ 80% lane 1 of (2), respectively. Later on we wer able to show that even Without the use of complexing agents, the positive charge in liposomes could retain iloprost.

7.3.2 Cationic liposomes with iloprost in lipid film + complexing agents

Until now, we observed that positive charges are essential for an efficient iloprost encapsulation. Further, with an aim to improve the stability, again complexing agents like HSA, cyclodextrin and PEG-1000 were used. The required amounts of the complexing agents were dissolved in the rehydration buffer and the lipid films were rehydrated at RT without extrusion followed by determination of encapsulation efficiencies (see **Table 12)**.

Table 12: Composition of liposomes using stearylamine as cationic component in combination with several complexing agents

Number	Liposome	Lipid concentration (mg/mL)	Molar ratio	Rehydration buffer
1	POPC:SA	2	90:10	10mg HSA in 10mM tris, pH7
2	POPC:SA	4	90:10	10mg cyclodextrin in 10mM tris, pH7
3	POPC:SA	4	90:10	10mg PEG-1000 in 10mM tris, pH7

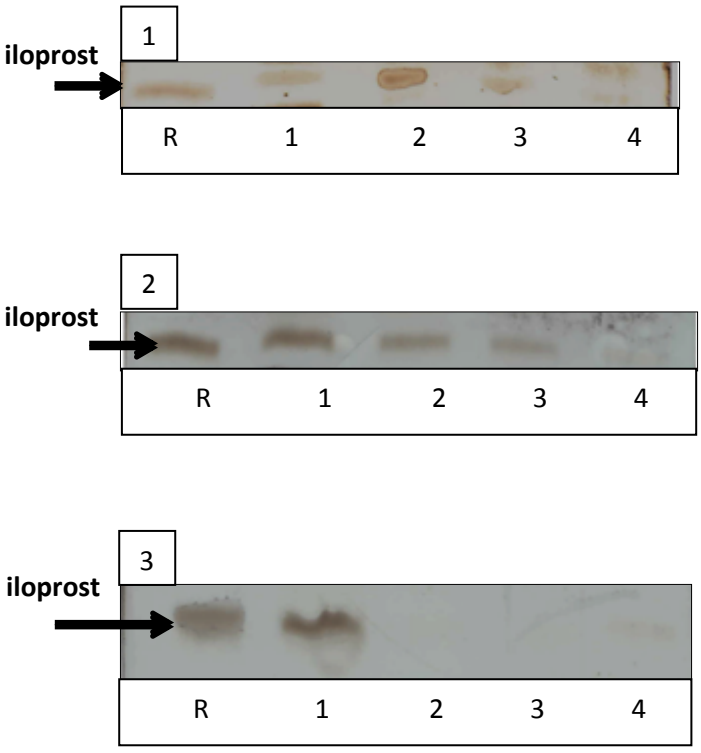


Figure 22: Thin Layer Chromatograms showing the encapsulation of anhydrous iloprost as part of lipid film of cationic liposomes in combination with complexing agents like (1) HSA, (2) cyclodextrins and (3) PEG-1000, R is reference iloprost 2µg, 2 and 3 are filtrates and 4 is ethanol wash in TLC lane (5µg is 100% encapsulation)

From **Figure 22**, an iloprost band was observed in lane 1 of all the three TLCs with varying encapsulation efficiencies varying from ~20%, 40% and 80% in lane 1 of (1), (2), and (3), respectively. A similar high encapsulation efficiency (~25%) was achieved when aqueous iloprost together with HSA was used in cationic liposomes (see **Figure 18)**. Comparing all the

three TLCs, the addition of the polymer PEG-1000 induced a drastic increase in encapsulation of iloprost providing the best results so far. Hence, iloprost as part of the lipid film together with a positively charged component is a pre-requisite for successful iloprost encapsulation. Keeping this in mind, we next proceeded with using PEG conjugated lipids in liposomes and evaluated the encapsulation of iloprost

7.3.3 Cationic liposomes with iloprost in lipid film + polymers PEG conjugated lipids/PVP

Since we know that the polymer PEG can retain iloprost within liposomes, we next attempted to use iloprost together with cationic lipids and PEG conjugated phospholipids or the polymer polyvinylpyrroldione (PVP) as part of the lipid film. Following simply rehydration with 10mM tris buffer, pH7 the encapsulation efficiency for iloprost was determined.

Table 13: Composition of cationic liposomes with polymers

Number	Liposome	Lipid concentration	Molar ratio	Rehydration medium	Size	PDI
1	POPC:SA:DPPE-PEG2000	6	87:10:3	10mM,tris, pH7	165	0.093
2	POPC:SA:PVP	6	87:10:3	10mM,tris, pH7	164.4	0.116

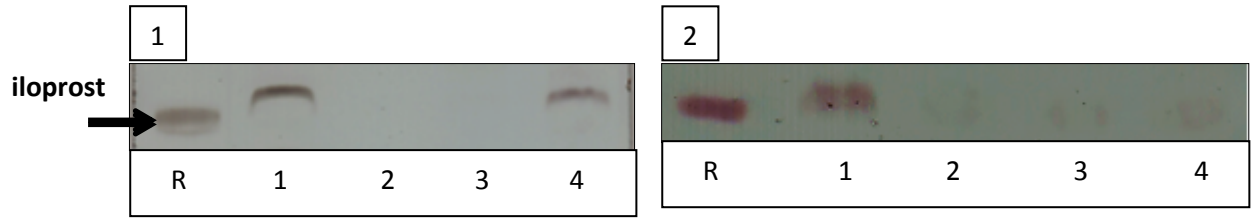


Figure 23: Thin Layer Chromatograms showing the encapsulation of anhydrous iloprost in cationic liposomes in combination with polymer conjugated phospholipid (DPPE-PEG2000) (1) and polymer Polyvinylpyrrolidone (PVP) (2) , R is reference iloprost 2µg, 2 and 3 are filtrates and 4 is ethanol wash in TLC lane (4µg iloprost is 100% encapsulation)

From **Figure 23** it was observed that the majority (~75%) of iloprost was associated with liposomes lane 1 of (1) and the rest of the non-encapsulated iloprost was observed in the ethanol wash lane 4 of (1) indicating that the combination of cationic and pegylated lipids in the lipid film could retain high amounts iloprost within liposomes. We confirmed these

results by repeating it several times. Hence, this was considered as our first formulation termed as LI-1 for iloprost with maximum iloprost loading capacity. Further, replacement of pegylated lipids in liposomes with PVP resulted in an iloprost encapsulation efficiency of about 70% lane 1 of (2). Rest of non-encapsulated iloprost may be present in filtrates and ethanol wash lane 4 of (2), but here the low concentration is difficult to observe in TLC. The results indicate that PVP can help to retain iloprost within liposomes. Hence, this formulation was then termed as LI-2 and was considered as our second formulation for iloprost with high drug loading efficiency. We then modified our two formulations (LI-1 and LI-2) by adding cholesterol (LI-3, LI-5), replacing SA with another cationic phospholipid DOTAP (LI-4), PVP plus DOTAP (LI-6) to evaluate any changes in iloprost loading capacity.

Table 14: Composition of cationic liposomes with polymers and cholesterol

Number	Liposome	Lipid concentration	Molar ratio	Rehydration buffer	Size	PDI
3	POPC:SA:DPPE-PEG2000 +cholesterol	6	87:10:3 + 13mol%	10mM,tris, pH7	185	0.06
4	POPC:DOTAP:DPPE-PEG2000	6	87:10:3	10mM,tris, pH7	171	0.136
5	POPC:SA:PVP +cholesterol	6	87:10:3 + 15mol%	10mM,tris, pH7	185	0.111
6	POPC:DOTAP:PVP	6	87:10:3	10mM,tris, pH7	169	0.07

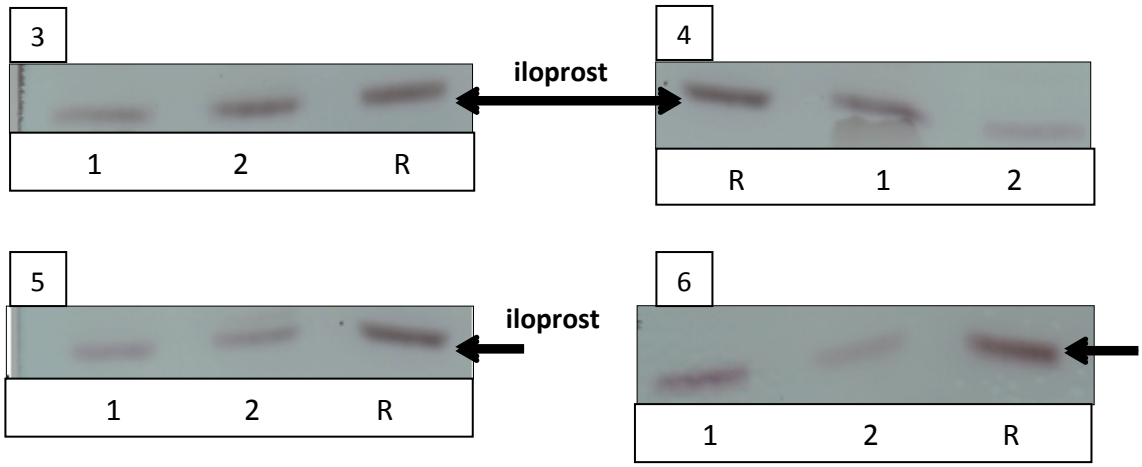


Figure 24: Thin Layer Chromatograms showing the effect of cholesterol (3,5) and the positive phospholipid DOTAP (4,6) on the encapsulation of iloprost, R is reference iloprost (2µg), 1 is liposomal lane and 2 is ethanol wash. Arrow indicates iloprost

From the **Figure 24** it was observed that integration of cholesterol in the formulations modestly reduced iloprost encapsulation (LI-3, LI-5) lane 1 of (3), (5), while the cationic phospholipid DOTAP and PVP helped to retain iloprost within liposomes (LI-4, LI-6) lane 1 of (4), (6).

Taken together, we have in all optimized six promising liposomes for iloprost (LI-1 to LI-6) with encapsulations ranging between 50-80% as estimated by TLC. These optimised liposomes were then further characterised in detail for their morphology, effect of cationic lipids on cell viability and pharmacologic efficacy.

Table 15: Characterization of pure (control) liposomal formulations (- iloprost) and iloprost loaded liposomes (+ iloprost). Data represent mean ± SEM, n=≥3.

Liposome formulation	Composition (Molar ratios)	Iloprost	Encapsulation efficiency (%)	Size (nm)	Polydispersity index	Zeta potential (mV)
LI-1	POPC:SA:DPPE-PEG2000 (87:10:3)	-	-	168.9±6.1	0.12±0.02	10.9±0.2
		+	64.7±4.4	168±1.6	0.1±0.0	12.8±0.2
LI-2	POPC:SA:PVP (87:10:3)	-	-	171.7±3.3	0.11±0.01	35.6±0.2
		+	59.6±3.3	169±3.6	0.1±0.0	36.2±0.4
LI-3	POPC:SA:DPPE-PEG2000:cholesterol (75.6:8.7:2.5:13)	-	-	174±6.9	0.07±0.0	11.4±0.3
		+	47.7±4.3	173.2±4.1	0.08±0.01	8.8±0.1
LI-4	POPC:DOTAP:DPPE-PEG2000 (87:10:3)	-	-	167.7±4	0.12±0.02	8.3±0.7
		+	66.2±6.4	167.8±2.7	0.1±0.01	9.6±0.0
LI-5	POPC:SA:PVP:cholesterol (73.2:8.5:2.5:15.6)	-	-	176.9±3.6	0.05±0.0	33.9±0.8
		+	53.2±7.7	178.3±4.4	0.09±0.01	38.6±0.2
LI-6	POPC:DOTAP:PVP (87:10:3)	-	-	173.8±8	0.05±0.01	37.1±1
		+	60.6±5.7	175±7.5	0.09±0.01	33.5±1.3

7.4 Liposome Morphology

The morphology of two of liposomes (LI-4, LI-6, +/- iloprost) was studied using electron microscopy applying two different techniques, negative staining and freeze fracture. The average liposome size was found to be between 100-200nm. Freeze fracture clearly depicted that liposomes are spherical in shape.

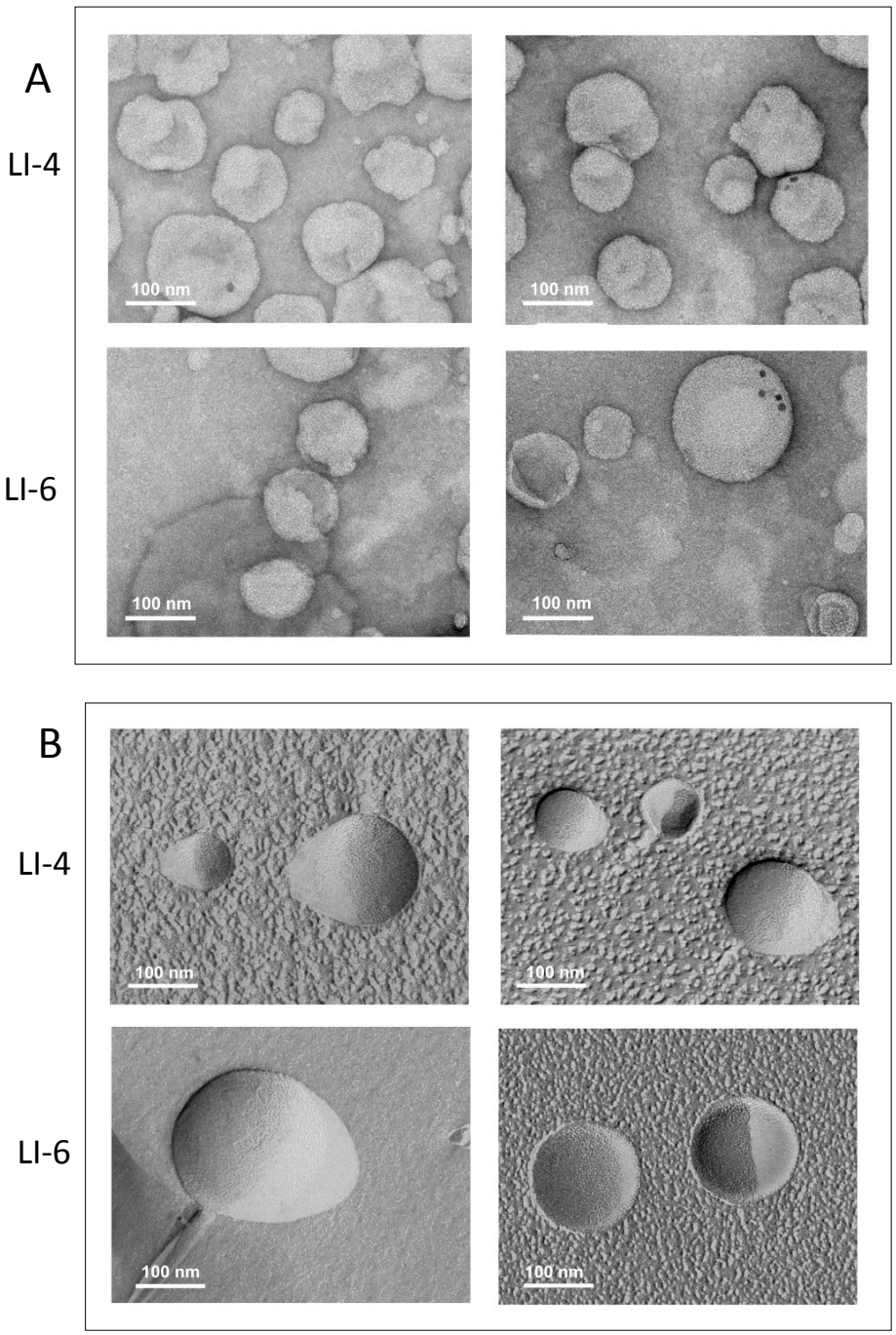


Figure 25: Transmission electron micrographs of two of liposomal formulations, LI-4 and LI-6 using negative staining (A) and freeze fracture (B) of iloprost loaded liposomes (left panels) and pure (empty) liposomes (right panels). Magnification is 19,000x. The data presented here was obtained in collaboration with Dr Gerd Leitinger, Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Austria.

7.5 Thermal behaviour of liposomes

To examine the influence of iloprost on the thermal phase behaviour of liposomes we have implied calorimetry. To evaluate the phase behaviour of liposomes in an easily manageable temperature range 20°C-50°C, we have replaced POPC for DPPC, as POPC has a phase transition trremperature below 0°C

The thermograms of multilamellar liposomes composed of DPPC and iloprost encapsulated in DPPC were recorded. The DSC data exhibited a sharp phase transition which is a characteristic to the main chain-melting transition from ripple ($P_{\beta'}$) phase to the fluid (L_{α}) phase at 41.6°C in accordance with published data (173).

The specific heat capacity curves of iloprost encapsulated in liposomes displayed similar features in comparison to control as shown in **Figure 26 and Table 16.**

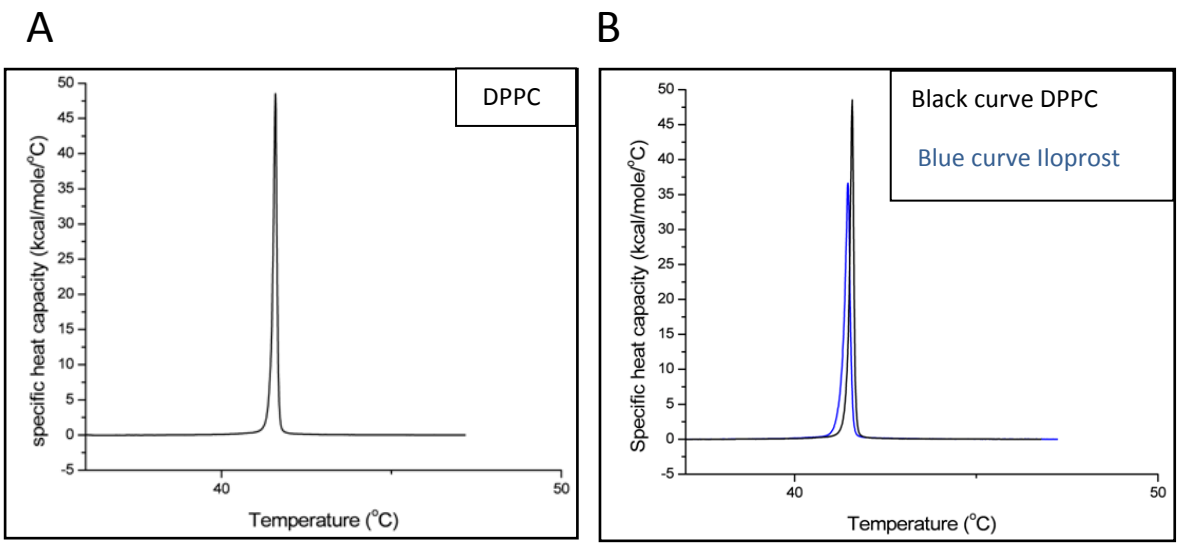


Figure 26: Specific heat capacity curves of multilamellar liposomes. A) Thermogram of liposomes composed of DPPC. B) An overlay of thermogram of DPPC liposomes and iloprost encapsulated in DPPC liposomes (5µg iloprost /mg DPPC).

Table 16: Thermodynamic data of phase transitions of liposomes composed of DPPC and iloprost encapsulated in DPPC.

Liposomes (± iloprost)	Tm (°C)	ΔH (kcal/mol)	ΔT½ (°C)
DPPC liposomes	41.6	8.3	0.1
Iloprost encapsulated in DPPC liposomes	41.5	8.5	0.1

Tm Phase transition temperature, ΔH Change in enthalpy, T Temperature

7.6 Cell Viability

To determine if cationic lipids used in liposomes had any detrimental effects on viability of hPASMC and A549 cells, the cell lines were treated with 2 different lipid concentrations, 8.57µg/mL (0.2µM iloprost) and 42.8µg/mL (1µM iloprost) for 24 hours. A similar result was observed when 42.8µg lipid/mL was used on A-549 cell line (not shown). Cationic lipids used in liposomes did not affect the viability of hPASMC **(Figure 27 A and B)** and A-549 **(Figure 27 C)** indicating that these applied lipid concentrations were found to be non-toxic to the cells.

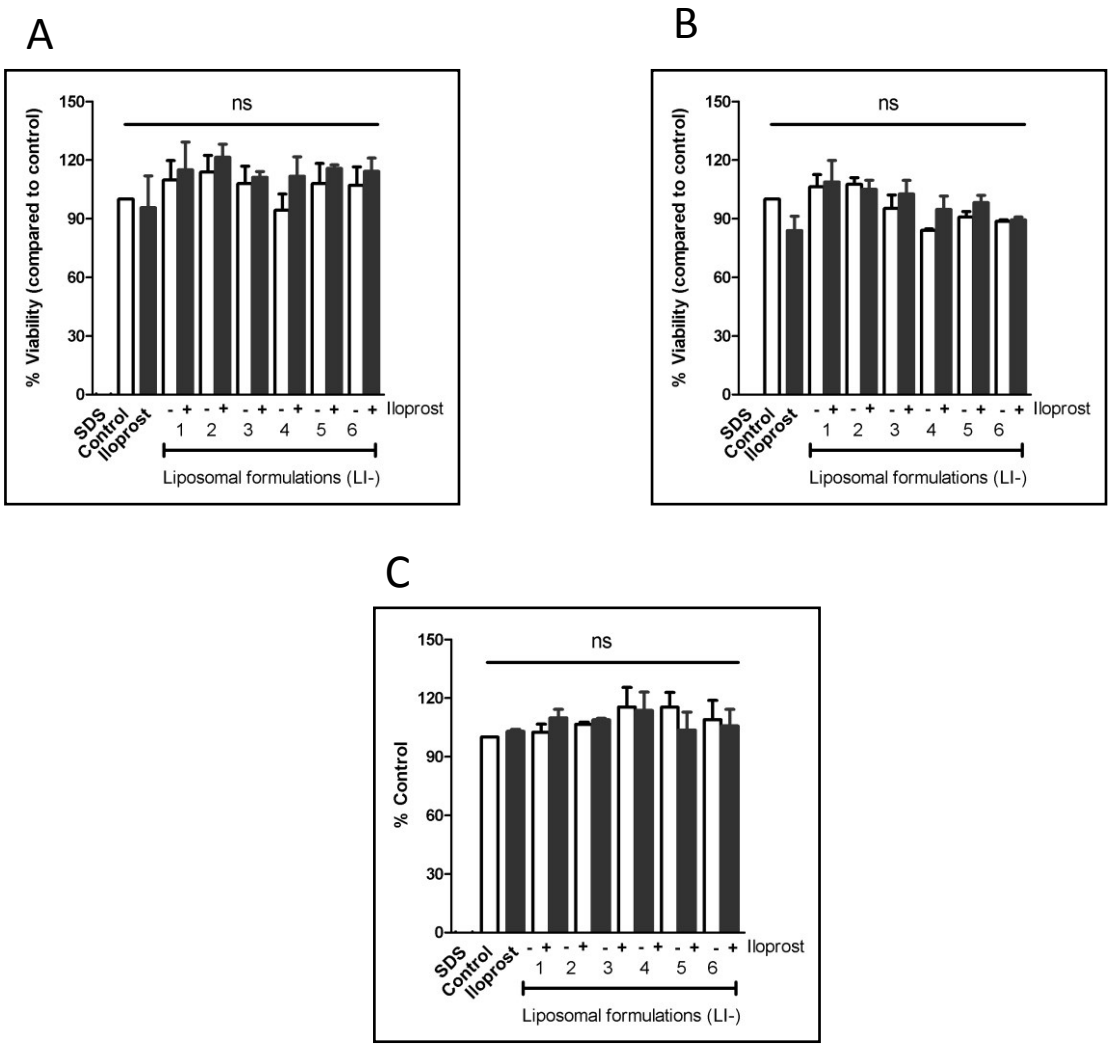


Figure 27: Viability studies of liposomes containing A) 8.57µg lipid /mL, B) 42.8µg lipid/mL, C) 8.57µg lipid/mL on the viability of (A,B) human pulmonary artery smooth muscle cells and C) A-549 cells for 24 hours, n=3. Data represent mean ± SEM. One-way ANOVA followed by Dunnett's Multiple Comparison Test was performed to calculate statistical significance, ns- not significant.

7.7 Pharmacologic studies of encapsulated iloprost using wire myograph

Pharmacological testing of encapsulated iloprost and free iloprost was determined by measuring changes in vascular tone of mouse intra-pulmonary arteries using a wire myograph as shown below schematically **(Figure 28)**.

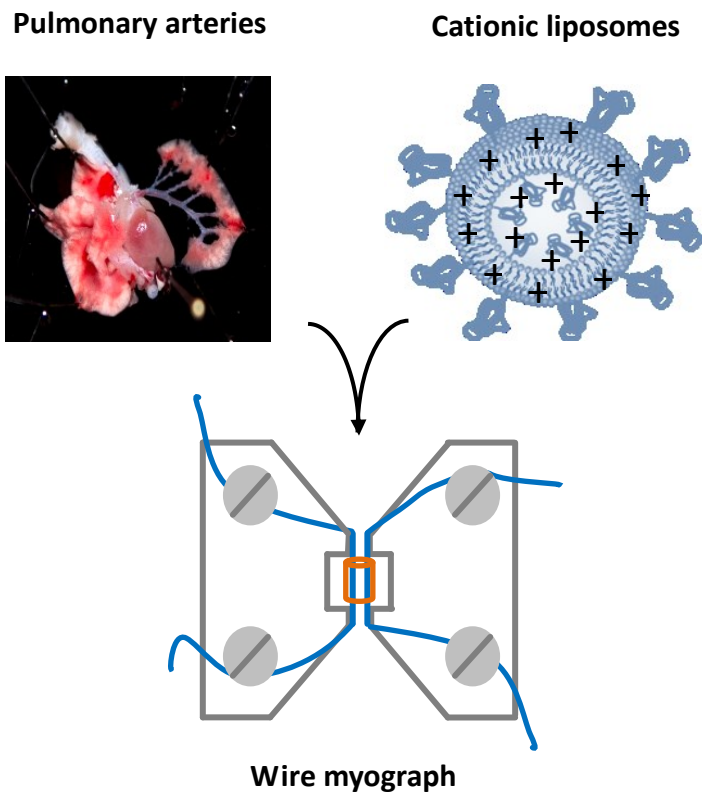


Figure 28: Graphical abstract demonstrating the methodology used to evaluate the pharmacalogic efficacy of encapsulated iloprost.

Cumulative concentrations of phenylephrine (0.001µM-10µM), U-46619 (0.01µM-3 µM) and serotonin (0.001µM-30µM) produced a concentration-dependent contraction **(Figure 29A, C and E).** The half maximal effective concentrations (EC_{50}) of phenylephrine, U-46619 and serotonin were determined to be 0.03 µM, 0.03µM and 0.07µM, respectively.

We observed that non-cumulative applications of free iloprost exhibited similar relaxation values independent of the concentration used upon phenylephrine pre-contsrictions **(Figure 29 B)**. Strikingly, application of 5µM free iloprost failed to produce any vasodilation upon Serotonin pre-constricted arteries **(Figure 29 F).** These are very interesting observations and can be further explored. Next, we proceeded with thromboxane analogue, U-46619.

We chose the thromboxane analogue U-46619 as the vasoconstrictor for our study because at this concentration (EC_{50}) a stable constriction was maintained for up to 2 hours **(Figure 30A).** For all subsequent experiments this EC_{50} dose of U-46619 was used to pre-contract the arteries. Following pre-contraction addition of iloprost produced a rapid relaxation and vasodilation was maintained **(Figure 29D).** The viability and smooth muscle-induced relaxation could be confirmed by the addition of the nitric oxide donor, sodium nitroprusside

(Figure 30B). Upon non-cumulative applications iloprost exhibited a non-linear concentration-dependent relaxation **(Figure 29D).** On the contrary, non-cumulative applications of iloprost elicited almost similar relaxations independent of the concentration used for phenylephrine pre-constricted arteries (Figure 29 B). For U-46619 pre-constrictions, maximal relaxation was observed with 10 µM of iloprost with no further increase at higher concentrations. Therefore, we chose half of this concentration (5 µM) for all further experiments. Together these results confirm the validity of the test system and that iloprost acts as a potent vasodilator on mouse pulmonary arteries.

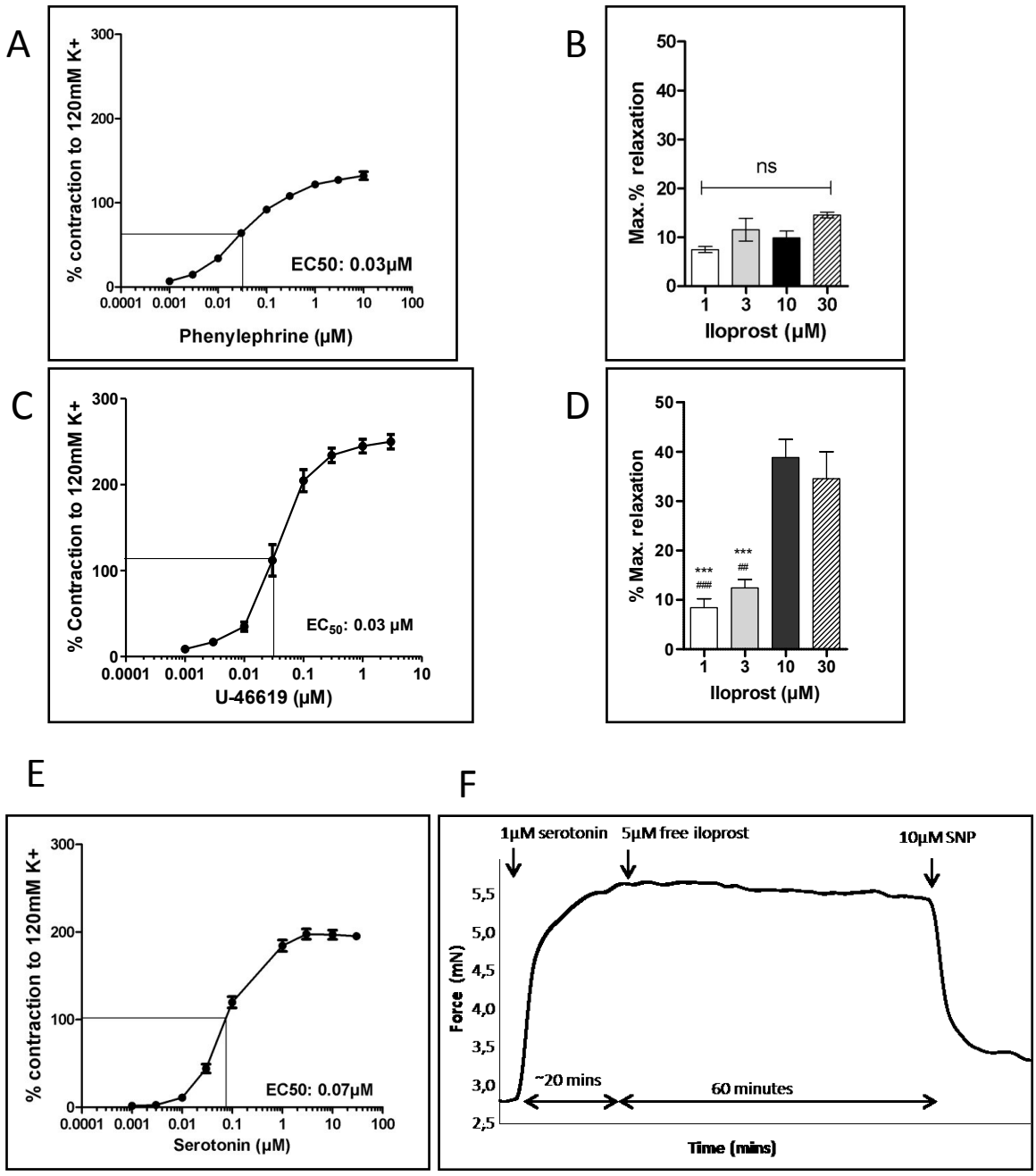


Figure 29: Concentration dependent contraction of mouse intra pulmonary arteries in response to A) phenylephrine (n=8), C) U-46619 (n=8) and E) serotonin (n=10) compared to 120mM potassium chloride. Percentage maximum iloprost relaxation on B) Phenylephrine (n=2-10) and D) U-46619 (n=5-7) pre-constricted mouse intra pulmonary arteries. F) Representative curve showing effect of iloprost on serotonin pre-constricted arteries followed by SNP as NO donor. Data represent mean ± SEM, ***p<0.001 compared to 10µM iloprost; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared to 30µM iloprost. One-way ANOVA followed by Tukey's Multiple Comparison Test was performed to calculate statistical significance.

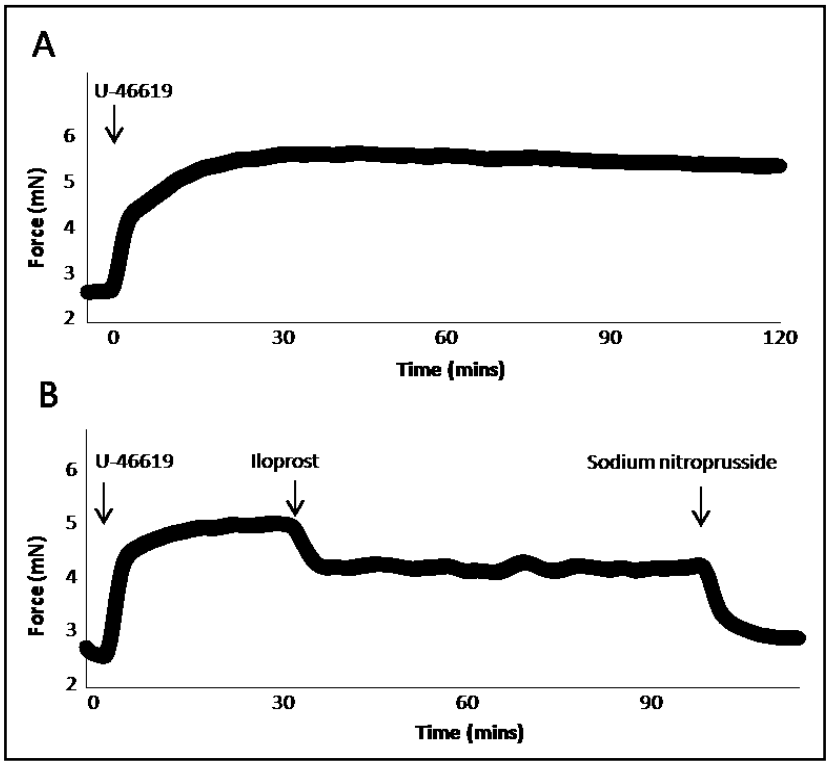


Figure 30: Representative real time curve from isometric tension measurements using a wire myograph. A) Duration of vasoconstriction following U-46619 (0.03µM) induced vasoconstriction. B) Vasorelaxation induced by addition of free iloprost (5 µM) and the nitric oxide donor sodium nitroprusside (10 µM) on U-46619 induced pre-constricted mouse pulmonary arteries.

As the binding of iloprost to different prostanoid receptors produces different vasoactive effects, we next investigated the expression levels of the prostanoid receptors in isolated mouse pulmonary arteries using real-time PCR. Highest expression was observed for EP1 together with TP, followed by the IP receptor. The complete expression levels were as follows EP1=TP>IP≥EP4=EP3>>EP2 **(Figure 31).**

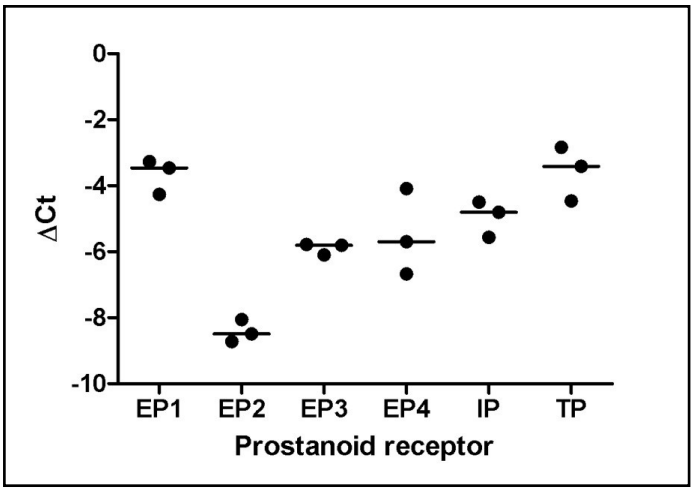


Figure 31: Real-time PCR analysis of prostanoid receptor expression in isolated mouse pulmonary arteries, lines indicate median, n=3

To compare the pharmacologic efficacy of free and encapsulated iloprost, mouse pulmonary arteries were initially pre-constricted with 0.03 µM U-46619 and treated with either free or encapsulated iloprost. Free iloprost exhibited 16% maximal relaxation within 18 minutes, which was maintained for 1 hour. Liposomes (without iloprost) produced a slight vaso-relaxation after 20 minutes **(Figure 32).** Liposomal iloprost formulations, LI-1 and LI-2 exhibited a non-significant increase in vasodilation (~25%) as compared to free iloprost **(Figure 33A,B and G).** Integration of cholesterol (LI-3, LI-5) or DOTAP (LI-4, LI-6) in the liposomal formulations significantly enhanced maximum vasodilation up to ~29% **(Figure 33C-G).** No significant differences in time to maximal relaxation were observed **(Figure 33H).** The same relaxation curve of free iloprost is depicted in all figures to enable direct comparison between free iloprost and encapsulated iloprost **(Figure 33A-F).**

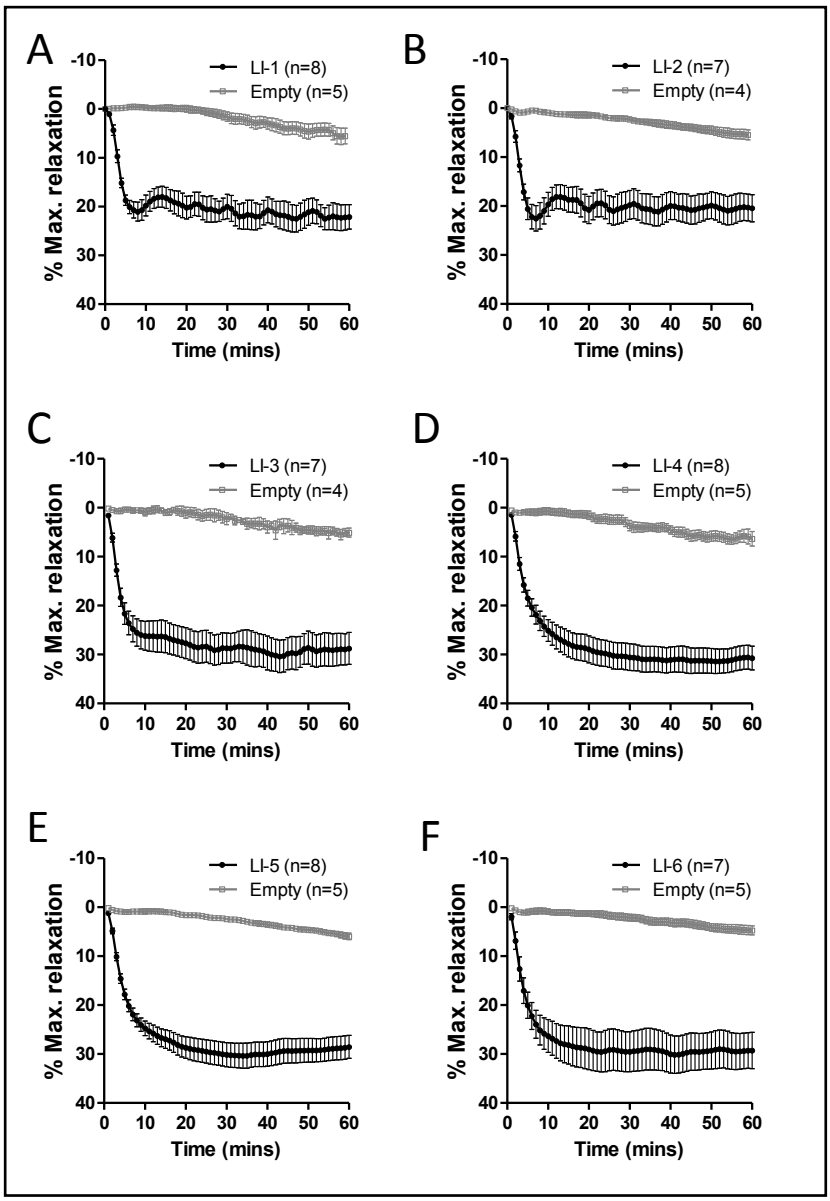


Figure 32: Relaxation kinetics of empty liposomes (empty) and encapsulated iloprost (LI-, 5µM iloprost) on isolated mouse pulmonary arteries following U-46619 pre-constriction A) LI-1, B) LI-2, C) LI-3, D) LI-4, E) LI-5, F) LI-6. Relaxation values obtained by empty liposomes were subtracted from the respective liposomal iloprost formulation to determine relaxation for each formulation as shown in Figure 33. Data represent mean ± SEM.

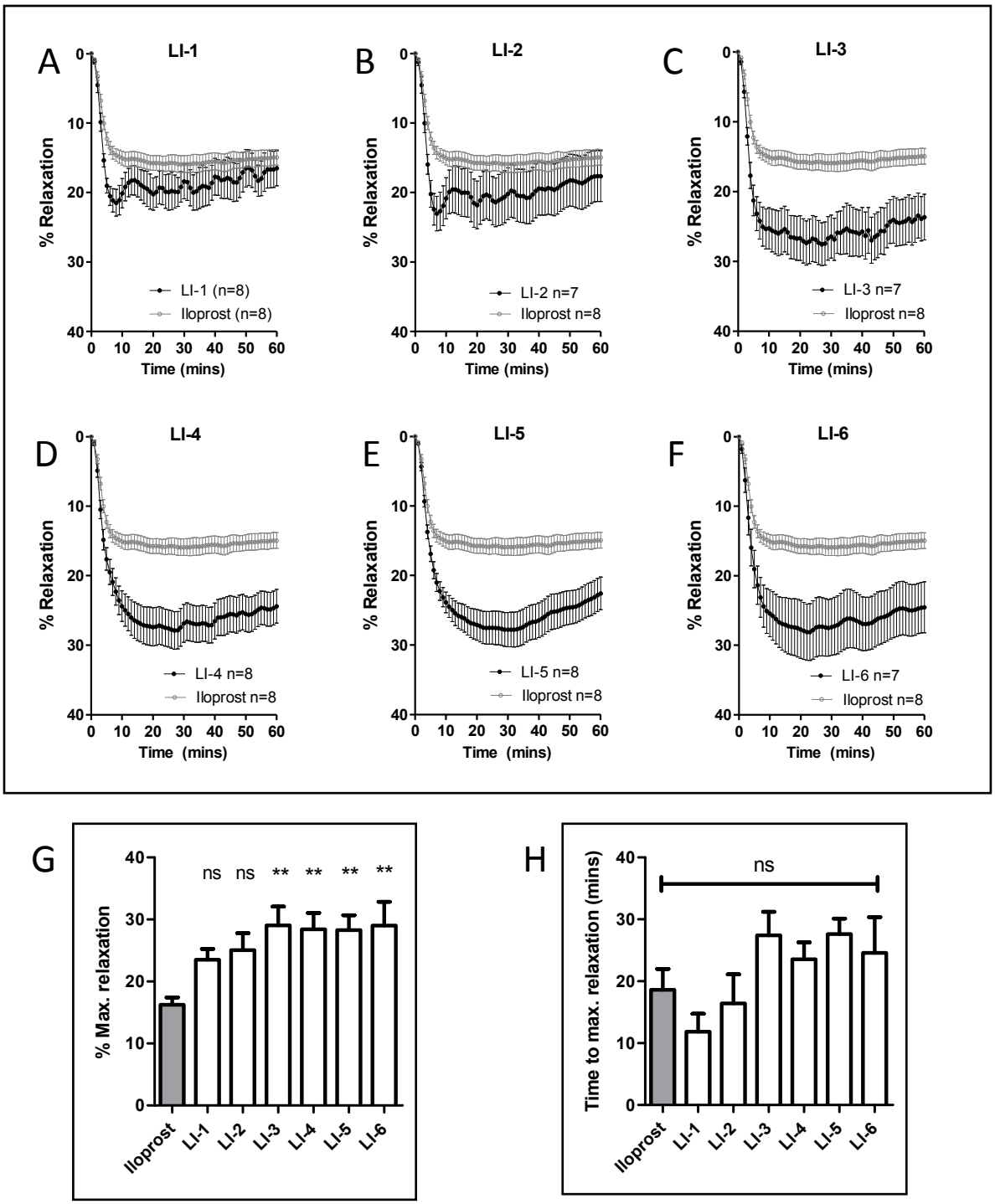


Figure 33: Relaxation kinetics of free and different encapsulated iloprost (5µM) formulations on isolated mouse pulmonary arteries following U-46619 pre-constriction A) LI-1, B) LI-2, C) LI-3, D) LI-4, E) LI-5, F) LI-6 (black symbols) compared to free iloprost (grey symbols). For easy comparison the same relaxation curve of free iloprost is depicted in all figures. G) Percentage change from U-46619 induced contraction. H) Time to maximum relaxation. Data represent mean ± SEM. **p<0.01. One-way ANOVA with Dunnett's multiple comparison tests was used to determine degree of significance between free and encapsulated iloprost.

Values presented in Figure 33G are obtained from corresponding relaxation and time required as indicated by line in Figure 33A.

We then selected one formulation (LI-4) that demonstrated an enhanced effect for further analysis. At 1µM both free and encapsulated iloprost (LI-4) produced similar levels of relaxation **(Figure 34A).** At 2.5µM and 5µM, encapsulated iloprost significantly increased the maximal relaxation when compared to free iloprost **(Figure 34B and C).** In order to provide direct comparison between the effects of different iloprost and LI-4 doses, the data from **Figure 33D** is presented again in **Figure 34C**. Strikingly, liposomal iloprost required half the concentration of iloprost to achieve similar levels of vasodilation as 5 µM free iloprost.

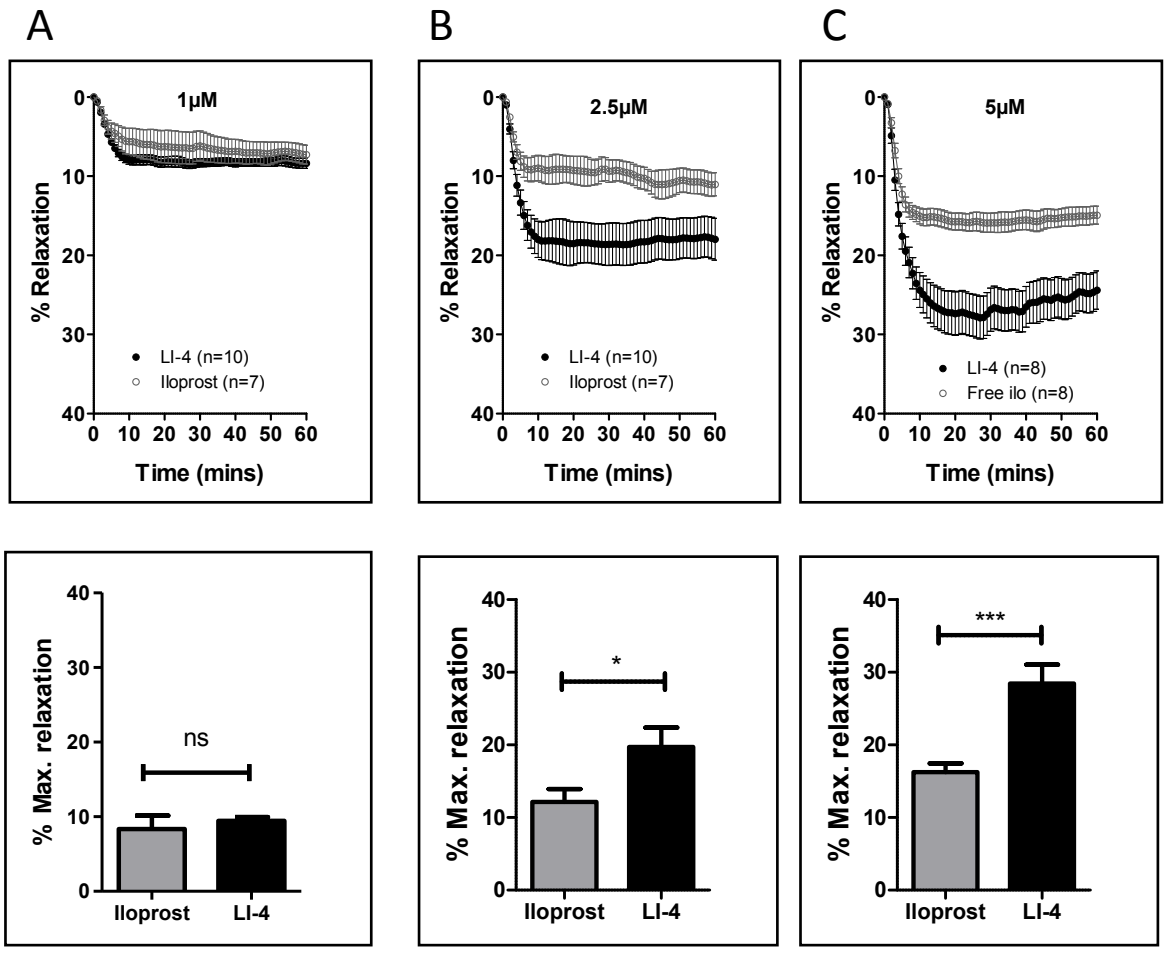


Figure 34: Relaxation kinetics of LI-4 compared to free iloprost on U-46619 pre-constricted mouse pulmonary arteries. A) 1µM iloprost, B) 2.5µM iloprost, C) 5µM iloprost. For direct comparison of the different iloprost concentrations, a modified version of Figure 32D is presented in panel C. Data represent mean ±SEM, ns not significant, *p<0.05, ***p<0.001. Unpaired t-test was used to calculate statistical significance.

7.8 Nebulisation of iloprost loaded liposomes

7.8.1 Characterisation of liposomes for nebulisation

The liposomes for nebulisation were prepared and characterised as shown in **Table 17**.

Table 17: Characterisation of liposomes, Data represent Mean±SEM, n=3

Liposome formulation	Composition (Molar ratios)	Iloprost	Encapsulation efficiency (%)	Size (nm)	Polydispersity index (PDI)
LI-3	(POPC:SA:DPPE-PEG2000) : cholesterol (75.6:8.7:2.5:13)	-	-	166.5 ± 1.56	0.07 ±0.01
		+	77±1.77	168.8 ± 0.85	0.08 ± 0.00
LI-4	POPC:DOTAP:DPPE-PEG2000 (87:10:3)		-	128.4± 1.44	0.21 ±0.00
		+	88.2±3.36	147.6 ± 6.08	0.13 ±0.01
LI-36	(POPC:Dc-Chol:PVP) +10 mg/ml Dextran 40 (77:20:3)	-	-	184.9 ± 4.48	0.09 ±0.00
		+	81.9±3.61	181.4 ± 2.62	0.11 ±0.00

7.8.2 Transport efficiency

To demonstrate the transport efficiency of cationic liposomes using the mesh nebuliser, M-neb, all the liposomes were subsequently nebulised. It was observed that phospholipid concentration (%PC) determined in the condensed aerosol was almost 100% for all the three liposomal formulations indicating that vibrating mesh nebuliser, M-neb can efficiently nebulise cationic liposomes **Figure 35.**

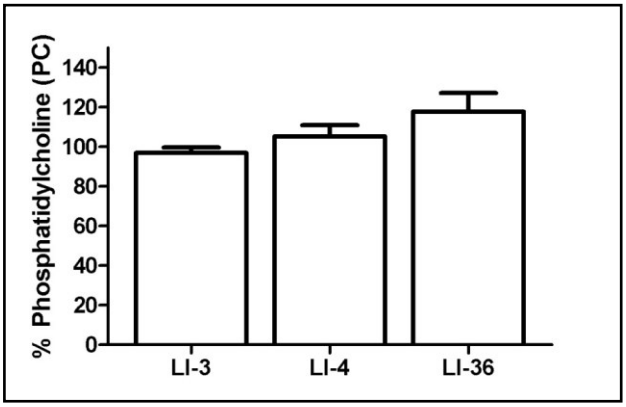


Figure 35: Transport efficiency of cationic liposomes, LI-3, LI-4 and LI-36. The phosphatidylcholine (PC) content was determined in the condensed aerosol. %PC was calculated by taking the PC content before nebulisation as 100%, Mean±SEM, n=3.

7.8.3 Stability of liposomes upon nebulisation

To demonstrate the impact of nebulisation on the stability of liposomes, liposomes were nebulised with the vibrating mesh nebuliser and size distribution curves of aerosol containing the liposomes were analysed. The mean particle size of the liposomes essentially remained the same before and after nebulisation. But, we observed some differences in mean PDI value for two of our formulations, LI-3 and LI-4 before (0.09, 0.15) and after nebulisation (0.47, 0.54). The mean PDI value for LI-36 remained almost the same before (0.08) and after nebulisation (0.13). A detailed analysis of the size distribution curves for the two formulations (LI-3, LI-4) showed that more than 90% and 60 % of the liposomes in LI-3 and LI-4, respectively, retain their original size post nebulisationt. The remaining fraction of liposomes aggregate to form large particles with diameters between 1-2µm. Therefore, to enable better understanding, independent curves are shown in **Figure 36**. This indicates that the mesh nebuliser does not affect the size distribution of liposomal formulation (LI-36) but causes some particle aggregation in case of LI-3 and LI-4.

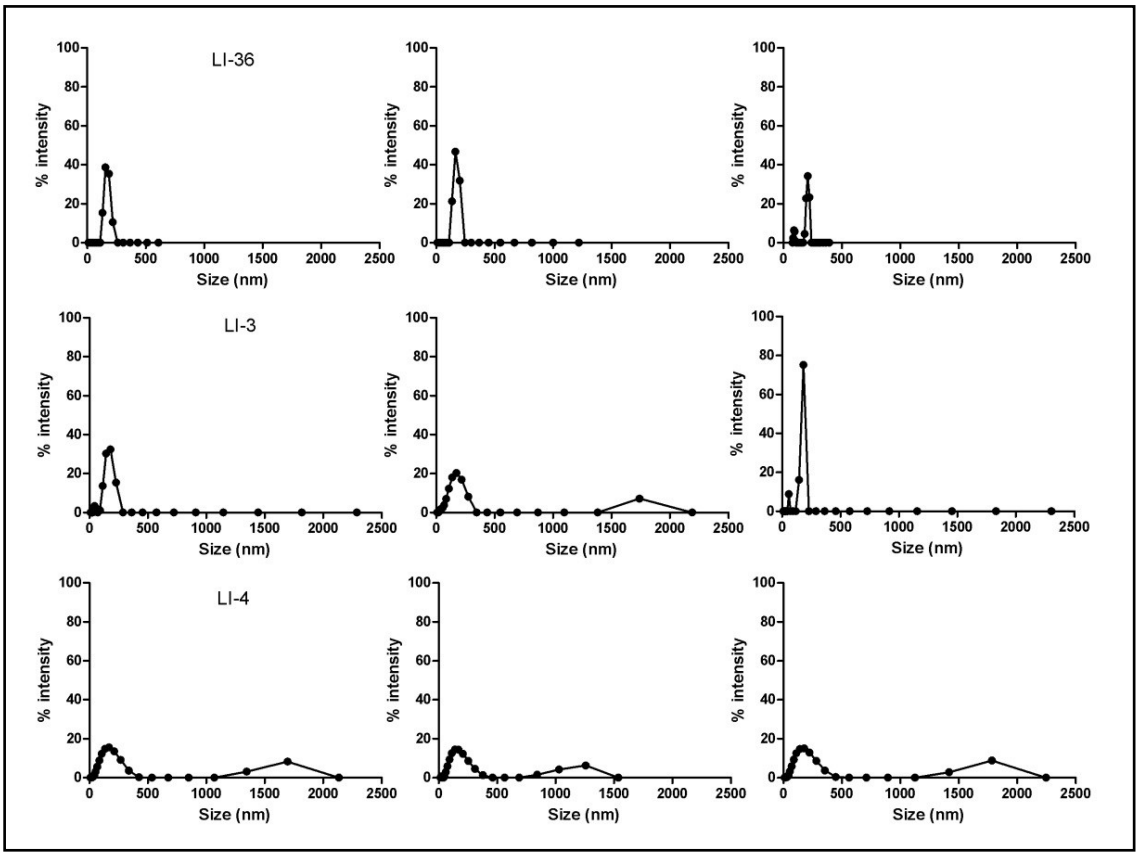


Figure 36: Size distribution curves of the aerosol containing liposomes LI-3, LI-4, and LI-36 (three independent experiments are shown).

7.8.4 Retention of iloprost post nebulisation

To determine the impact of mesh nebuliser on retention of iloprost post nebulisation, iloprost loaded liposomes were nebulised and iloprost content was determined. It was found that after nebulisation, the iloprost retention was ~95% in liposome formulation (LI-36) and ~80% in both liposome formulations (LI-3, LI-4) indicating that vibrating mesh nebuliser had least impact on (LI-36) and marginal impact on others (LI-3, LI-4). The data is shown in **Figure 37.**

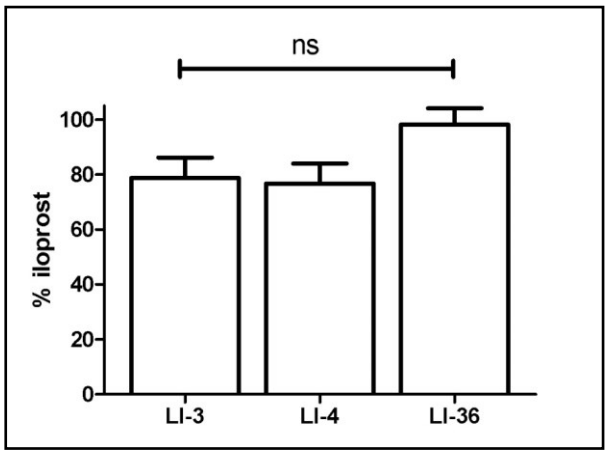


Figure 37: Retention of iloprost upon nebulisation, LI-3, LI-4, LI-36. % retention was calculated by taking iloprost content before nebulisation as 100%, Mean±SEM, n=2

8 Discussion

The rationale of this study was to create liposomal nanoparticles tailored for the transport of the prostacyclin analogue iloprost, and to determine the pharmacologic efficacy of iloprost loaded fromulations *ex vivo*. Previous reports have described very low (~4%) encapsulation efficiencies of iloprost into liposomes (158). This low encapsulation may be a limiting step for the creation of novel therapeutic strategies. Here we have developed several different liposomal formulations, which exhibit very high encapsulation efficiencies (50-65%, **Table 15**). Our data indicate that cationic excipients are pre-requisite for successful and efficient iloprost encapsulation. Four of six iloprost-liposomal formulations exhibited enhanced relaxation efficacies of pre-constricted mouse intra-pulmonary arteries.

To date the use of cationic liposomes has been mostly limited to the encapsulation of negatively charged nucleic acids or oligonucleotides for gene delivery. Recently, Bai et al used cationic liposomes to encapsulate low molecular weight heparin at higher efficiencies (174) than that reported for neutral liposomes (175). We here have used stearylamine and the cationic phospholipid DOTAP as the positively charged constituents in our formulations. Differences in the liposomes' surface charge (zeta potential) was dependent on the presence of PVP (LI-2, 5, 6) or PEG (LI-1, 3, 4), with PVP-liposomes possessing higher zeta potentials (**Table 15**). Liposomes containing stearylamine (SA) or DOTAP exhibited similar zeta potentials. The higher zeta potentials in PVP-liposomes, presumably originates from the nitrogen bearing pyrrolidone ring in PVP. The lower zeta potentials of PEG liposomes (LI-1, 3,4) may be due to charge neutralisation by ethoxylate chain present in DPPE-PEG_{2000}.

The presence of charged lipids presumably increases the electrostatic interaction between negatively charged iloprost and the liposome, thereby promoting itsencapsulation. Furthermore, the encapsulation efficiency for iloprost was greatly affected by the choice of phospholipid. Saturated phospholipids like DPPC form densely packed bilayer structures, which have very limited void volumes between the acyl chains. In contrast, unsaturated phospholipids such as POPC possess higher void volumes in the bilayer due to lower packing densities of the acyl chains. Liposomes composed of unsaturated phospholipids can therefore intercalate more amphiphilic drug molecules within the bilayer and are therefore beneficial for iloprost encapsulation. Cholesterol has been reported to induce a

conformational change in the lipid bilayer, imparting cohesive strength to the bilayer and reducing leakiness from liposomes (176). Furthermore, cholesterol can increase liposome membrane rigidity and may improve stability *in vitro* and *in vivo* (177). Incorporation of cholesterol (~15 mole %) in two of our stearylamine containing formulations resulted in reduced iloprost encapsulation. This result leads us to speculate that a part of iloprost is preferentially intercalated within the liposome bilayer and we propose that cholesterol in LI-3 and LI-5 may act as bilayer stiffener, which reduces iloprost capture.

To further promote liposome stability, we modified the surface of liposomes by integrating polymers (PVP) or polymer conjugated phospholipids (DPPE-PEG_{2000}). The presence of DPPE-PEG_{2000} has been described to provide an external shield thereby promoting steric stabilization (176). PVP has been used to coat liposomes and has been reported as an effective steric protector for liposomes *in vivo* (178-180). Incorporation of PVP within a DPPC lipid film promotes interaction with the DPPC acyl chains and consequently liposome incorporation (181). Therefore, we speculate that a similar interaction occurs with POPC integrating PVP within our liposomes. Thus, the chosen polymers should improve stability and help to maintain the structural conformation of liposomal nanoparticles.

At both lower and higher iloprost concentrations (0.2 µM and 1 µM iloprost) the liposomal formulations exhibited no adverse effects on cell viability (**Figure 27**). This is in line with other studies, which reported that instillation of cationic liposomes loaded with low molecular weight heparin did not significantly elevate lung injury markers (174). Due to the difficulty in obtaining human material, we have analysed the pharmacologic efficacy of liposomal formulations on mouse pulmonary arteries. The thromboxane analogue U-46619 was chosen as the pre-constrictor since elevated levels of thromboxane have been reported in PH patients (182). At 10 and 30 µM iloprost produced similar relaxation kinetics, which indicates ~10 µM as the maximal effective concentration; therefore, experiments directly comparing free and encapsulated iloprost were performed at 5 µM. The iloprost-induced relaxation of pre-constricted arteries (max. 40%) can be attributable to several factors: In mice and humans iloprost preferentially binds the IP receptor, thereby evoking vasodilation, but also possesses high affinity for the vasoconstrictive EP1 and EP3 receptors (50,51,183). In murine pulmonary arteries we observed high expression of the EP1 receptor followed by IP then EP3 (**Figure 31**). It is possible that human pulmonary arteries may have different

expression levels of these receptors, which may alter the response to iloprost. Our expression data together with the receptor binding affinity of iloprost indicates that the strong vasodilative response induced by IP is attenuated by activation of the EP1 and/or EP3 receptors.

One of our major findings was that the pharmacologic efficacy of 5 µM free iloprost was equipotent to half the concentration of liposomal encapsulated iloprost (2.5 µM) (**Figure 34**). Studies from Woo and colleagues demonstrated that liposomal encapsulation of deguelin enhances the anti-tumour efficacy by reducing the required dose from 4 mg/kg to 0.4 mg/kg and thus may prevent potential side effects (184). The exact mechanism why liposomal iloprost possesses higher activity is currently unclear; however, it may arise from a combination of factors like shielding the drug from degradation, docking of liposomes to the pulmonary artery or presentation of iloprost in an orientation that facilitates IP receptor binding. Based on our findings we propose that liposomal iloprost formulations can enhance the pharmacologic efficacy of iloprost and therefore may reduce the therapeutic dose.

9 Discussion (Nebulisation)

The objective was to determine the feasibility of iloprost loaded liposomes for aerosolisation. Iloprost loaded liposomes for the pharmacotherapy of PAH were reported by (158) and showed a very low encapsulation efficiency of ~4% which can be considered as a limiting step for a successful pulmonary delivery.

Our liposomal formulations for inhalation contained stearylamine, DOTAP and DC-cholesterol as the cationic excipients which resulted in higher encapsulation efficiencies for iloprost **Table 17**. We suppose that this positive charge is a pre-requisite for retaining iloprost within liposomes due to electrostatic interactions between positively charged components and iloprost being negatively charged at physiological pH.

One of our liposomal formulations contained cholesterol (LI-3). Integration of cholesterol in formulation had a slightly negative impact on the encapsulation efficiency of iloprost. Since cholesterol intercalates within the bilayer it replaced the iloprost and therefore we observed a reduction in %EE. A similar effect was reported by (185). They reported that incorporation of the amphiphilic drug rifampicin in liposomes composed of saturated lipids gave higher encapsulation efficiencies. However, addition of cholesterol reduced the encapsulation thereby replacing rifampicin from the liposomal membranes.

Further, our liposomes contained polymers like DPPE-PEG2000 or PVP. These polymers have been reported to act as steric barriers. Polymers are added to increase the circulation times of liposomes in body when administered intravenously. The *in vitro* stability of liposomes for pulmonary delivery was tested by (186). They showed that PEGylated liposomes upon nebulisation are more stable and can retain more than 80% of the drug in presence of lung surfactant when compared to non-PEGylated liposomes. Therefore, polymers seem to be beneficial for liposome retention within the lungs.

Inhaled iloprost remains the therapy for PH in Europe. To improve the pulmonary delivery, liposomal formulations were developed and tested for stability upon nebulisation. The transport efficiency of model liposome formulations using three different nebulisers namely, air-jet nebuliser (MicroDrop® Pro), ultrasonic nebuliser (Optineb®-ir) and the vibrating mesh nebuliser (eFlow® rapid) was tested in our group with mesh nebuliser being the most suitable nebuliser to completely transport the liposomes in aerosol demonstrating that

vibrating mesh nebuliser is most suitable for pulmonary delivery. Obviously, both the surface properties of liposomes and the physico-chemical properties of encapsulated drug should be taken into consideration for successful aerosol delivery. All liposomes for iloprost mentioned in **Table 4** were nebulised and the aerosol was analysed for size, stability and iloprost retention.

The size distribution curves of liposomal aerosols were analysed by DLS. This is an important aspect since large changes in the size or PDI would indicate liposomes that are not robust enough to survive the nebulisation process. From the size distribution curves, we found that the mean size of all liposomes remained the same before and after nebulisation. But, a fraction of larger particles was observed for the liposome formulations LI-3 and LI-4, which lead to an increase in the mean PDI value. As mentioned previously, PDI indicates the homogeneity of the particles in size. The higher PDI values thus give the impression that liposomal particles aggregate upon nebulisation. A detailed analysis of curves, however, showed that the majority of liposomes retain their original shape and that a small fraction of liposomes aggregate to form larger particles which might contribute to this high PDI value. This does not necessarily mean that the liposomal particles are unstable. Therefore, it is equally important to look at both the mean values and the size distribution curves.

Another important point to be considered is the encapsulation efficiency (EE) of iloprost after nebulisation. The retention of iloprost was greater in PVP/dextran liposomal formulation (LI-36) when compared to PEGylated liposomes (LI-3, LI-4) (**Figure 37)**. The membrane integrity of liposomes can be influenced by drug bilayer and lipid-lipid interactions. It was reported (158) that integration of DPPE-PEG in liposomes reduced the phase transition temperature of lipids which led to the speculation that DPPE-PEG might induce some membrane defects within liposome bilayer. This could be the reason why PEG liposomes in their study exhibited poor stability during nebulisation. In agreement with this study, we also observed that PEG liposomes (LI-3, LI-4) have less iloprost retention in comparison with dextran liposomes (LI-36), suggesting that dextran might improve the stability in terms of drug encapsulation.

10 Summary and Outlook

To summarize, addition of positively charged excipients significantly improved the iloprost loading capacity within liposomes. Cationic lipids at applied concentrations did not affect the viability of hPASMC and A-549 cells. Six formulations for iloprost were optimized and screened thoroughly and we found that four out of six liposomal formulations exhibited enhanced vasodilation (LI-3 to LI-6) when compared to free iloprost. The composition related differences arise due to integration of cholesterol (LI-3, LI-5) and cationic DOTAP (LI-4, LI-6). Nevertheless, the mechanism by which iloprost in liposomes demonstrates enhanced relaxation remains unclear.

Further, we found that the liposomal formulation containing a cationic cholesterol derivative in combination with the polysaccharide dextran as well as the polymer PVP was the most stable liposomal formulation which demonstrated excellent nebulisation performance using the vibrating mesh nebuliser.

The next steps will be to demonstrate the *in vivo* activity of such liposomes in a more physiologic model such as the isolated perfused lung model.

Publications

1) Pritesh P Jain, Regina Leber, Chandran Nagaraj, Gerd Leitinger, Bernhard Lehofer, Horst Olschewski, Andrea Olschewski, Ruth Prassl, Leigh M. Marsh

Liposomal nanoparticles encapsulating iloprost exhibit enhanced vasodilation in pulmonary arteries

(International Journal of Nanomedicine, 2014, Manuscript accepted and *In Pre-Press*, Impact Factor 3.46)

2) S Crnkovic, B Egemnazarov, P Jain, U Seay, N Gattinger, L M Marsh, Z Bálint, G Kovacs, B Ghanim, W Klepetko, R T Schermuly, N Weissmann, A Olschewski, G Kwapiszewska

NPY/NPY1R mediated vasoconstrictory and proliferative effects in pulmonary hypertension

(British Journal of Pharmacology, 2014, Impact Factor 5)

Awards

Best Poster Prize for poster session at PhD Doctoral Day 2013, Medical University of Graz, Austria

Abstracts (Poster Presentations)

International Liposome Society, London, 2013

Development of liposomal drug delivery system for inhalation application of iloprost.

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Pulmonary hypertension (PH) is a severe disease with poor prognosis. Current therapy includes the inhalable prostacyclin analogue, iloprost. Since, iloprost possesses a short plasma half-life, patients need frequent inhalations. Literature suggests that liposomes have strong potential to prolong the pharmacologic effects of anti-asthma drugs such as, salbutamol. We therefore investigated whether liposomes could serve as a depot and prolong the pharmacological half-life of iloprost. Furthermore, we analyzed the influence of liposome composition and nebulization on performance of liposomes.

Iloprost was encapsulated in liposomes with an efficiency of 45 to 65%. Cationic lipids used for liposome preparation promoted iloprost encapsulation. Liposomes exhibited no effects on cell viability at the applied concentrations 8.57µg lipid/mL. Iloprost formulations were tested for their pharmacologic efficacy using a wire myograph. Four out of six liposomal formulations exhibited significantly enhanced vasodilation of mouse pulmonary arteries compared to free iloprost.

The effect of nebulization on liposome integrity was then determined using three different nebulizer technologies (air-jet, vibrating mesh and ultrasonic). The mesh nebulizer demonstrated highest nebulization efficiency. Liposomes containing the polymer polyvinylpyrrolidone were most morphologically stable, while liposomes containing the polymer polyethylene glycol retained more iloprost. Conversely, nebulization of model liposomes containing the hydrophilic fluorophore-quencher system, ANTS/DPX, revealed a

positive correlation between structural changes and drug release. Aerosol particle size could be influenced by salt concentration but not by liposome formulation.

Here we showed that iloprost can be encapsulated at high efficiencies and nebulized without loss of activity. Further studies are required to demonstrate efficacy in vivo.

Ludwig Boltzmann Society, Vienna, 2013

Title: Development of liposomal iloprost formulations for inhalative application of Pulmonary Hypertension

Pritesh P. Jain, Regina Leber, Chandran Nagaraj, Bernhard Lehofer, Bakytbek Egemnazarov, Horst Olschewski, Andrea Olschewski, Ruth Prassl, Leigh M. Marsh

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Pulmonary hypertension (PH) is a severe disease with poor prognosis. Inhaled iloprost, prostacyclin analogue, is an approved therapy for PH. Since iloprost possesses short plasma half-life, patients require frequent inhalations. Liposomes have been reported for their ability to prolong the pharmacologic effects of drugs like anti-asthamatic, salbutamol. Therefore, we used liposomes which can serve as depot to prolong the vasodilation of iloprost.

Liposomes were characterized using bio-physical techniques. The encapsulation efficiency of iloprost in liposomes was between 45-65%. Cationic lipids used for liposome preparation promoted iloprost encapsulation. At applied concentrations 8.57µg lipid/mL, cell viability remained unaffected as shown by MTS cell viability assay. Liposomes can also be tailored for drug encapsulations using model water soluble fluorophore-quencher system, ANTS/DPX. A positive co-relation was observed between structural changes in liposomes and ANTS/DPX release.

Stability of iloprost loaded liposomes using three different nebulizers (air-jet, vibrating mesh and ultrasonic) was determined. Mesh nebulizer demonstrated highest nebulization efficiency of liposomes. Aerosol particle size was influenced by salt concentration. Nebulization data revealed that liposomes containing polymer polyvinylpyrrolidone are stable morphologically while liposomes containing polymer polyethylene glycol are stable

with respect to iloprost encapsulation. An inverse co-relation was observed between structural changes in liposomes and iloprost release.

Optimized formulations were tested for their cytotoxicity and pharmacologic efficacy using *ex vivo* (mouse) and *in vivo* (rat) models. Four out of six liposomal formulations exhibited significantly enhanced vasodilation (~30%) on isolated mouse pulmonary arteries when compared to free iloprost (~15%) as shown by wire myograph. Isolated perfused lung in combination with mesh nebulizer showed that nebulized liposomes depict similar pharmacokinetics as free iloprost.

American Thoracic Society, USA, 2014

Development Of Inhalable Liposomal Formulations For The Treatment Of Pulmonary Hypertension, [Publication Number: A4808]

L.M. Marsh, Dr., P.P. Jain, MSc, R. Leber, PhD, B. Lehofer, BSc, F. Bloder, BSc, C. Nagaraj, PhD, B. Egemnazarov, Dr, A. Olschewski, Dr, H. Olschewski, Dr, R. Prassl, Dr
Graz/AT

Rational: Pulmonary hypertension (PH) is a severe disease with poor prognosis. Current therapy includes the inhalable prostacyclin analogue, iloprost. However, as iloprost possesses a short plasma half-life, patients require frequent inhalations. Liposomes have been reported for their ability to prolong the pharmacologic effects of anti-asthma drugs such as, salbutamol. We therefore investigated whether liposomes could serve as a depot and prolong the pharmacological half-life of iloprost. Furthermore, we analyzed how liposomal carriers can be affected by formulation and nebulization device.

Methods and Results: Iloprost was encapsulated in liposomes with an efficiency of 45 to 65%. Cationic lipids used for liposome preparation promoted iloprost encapsulation. Liposomes exhibited no effects on cell viability. Iloprost formulations were tested for their pharmacologic efficacy using a wire myograph and isolated perfused rat lung. Next, the effect of nebulization on liposome integrity was determined using three different nebulizer technologies (air-jet, vibrating mesh and ultrasonic). The mesh nebulizer demonstrated highest nebulization efficiency. Liposomes containing the polymer polyvinylpyrrolidone were most morphologically stable, while liposomes containing the polymer polyethylene glycol retained more iloprost. Conversely, nebulization of model liposomes containing the water

soluble fluorophore-quencher system, ANTS/DPX, revealed a positive correlation between structural changes and drug release. Aerosol particle size could be influenced by salt concentration but not by liposome formulation.

Conclusions: Here we showed that iloprost can be encapsulated at high efficiencies and nebulized without loss of activity. Further studies are required to demonstrate efficacy in vivo.

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