Research Article

Toxicity testing of sildenafil base-loaded liposomes in in vitro and ex vivo models for pulmonary application*

Mukta Paranjpe[1], Vanessa Neuhaus[2], Armin Braun[2] and Christel C. Mueller-Goymann[1]

[1] Institut für Pharmazeutische Technologie, TU Braunschweig, Germany
[2] Fraunhofer Institut für Toxikologie und Experimentelle Medizin, Hannover, Germany

The aim of this study was to formulate sildenafil base-loaded liposomes using a solvent-free method, followed by the investigation of the potential cytotoxicity of the prepared liposomes in in vitro and ex vivo cell culture models. The liposomes were manufactured by dispersing 5% w/v phospholipid in aqueous phase at RT. The prepared dispersion was then homogenized using an Avestin extruder. The particle size of the prepared liposomes was between 100 and 110 nm and was stable up to 6 months. For in vitro models, human alveolar epithelial cell line (A549) and mouse heart endothelium cell line (MHEC5-T) were used. For ex vivo models, rat precision cut lung slices (PCLS) and rat heart slices (PCHS) were used. All the models were treated with liposomes loaded with or without sildenafil in a concentration range of 0–2500 μg/mL of phospholipid. The toxicity was assessed in vitro and ex vivo using MTT assay. Median lethal dose (LD_{50}) values for A549 cells and PCLS were found between 800 and 1800 μg/mL while for MHEC5-T cells and PCHS they were found between 1000 and 1200 μg/mL. PCHS demonstrated higher LD_{50} values as compared to PCLS. Considering the high LD_{50} values, sildenafil-loaded liposomes may have the potential for the treatment of pulmonary hypertension via inhalation route.

Practical applications: The liposomes have a potential for administration in lungs. Toxicity study was performed to determine a safe dose for potential human administration.

Keywords: Liposomes / Lung and heart tissue / Sildenafil base / Solvent free / Toxicity testing

Received: February 25, 2014 / Revised: May 26, 2014 / Accepted: June 22, 2014

DOI: 10.1002/ejlt.201300406

1 Introduction

Pulmonary arterial hypertension (PAH) is one of the most complicated pulmonary diseases with short life expectancy and often affects infants and young people [1]. PAH is characterized by progressive elevation of pulmonary artery pressure and vascular resistance along with right ventricular failure resulting in death due to limited median survival time. PAH is often idiopathic and is also generally associated with hypoxic lung disease [2]. Currently used treatment options for PAH include endothelin receptor antagonists, calcium antagonists, anticoagulants, intravenous prostacyclins and less specific, inhaled nitric oxide therapy [3]. Most of these therapies often exhibit severe side effects. An endothelin receptor antagonist such as bosentan although considered effective, has major side effects such as liver damage, nausea, vomiting, unusual tiredness, flushing, etc. Also, prostacyclin therapy has limited use since prostacyclins are unstable at RT and have to be administered intravenously by using permanent tunnelized catheters, which on long-term use can lead to adverse effects on the veins [4]. Now, inhaled iloprost and prostacyclin analogue treprostinil have been approved for administration via inhalation [5].

Sildenafil, a phosphodiesterase 5 inhibitor is currently used for the treatment of pulmonary hypertension and hypoxic lung disease. Sildenafil citrate (SC) provides

Correspondence: Prof. Christel C. Mueller-Goymann, Institut für Pharmazeutische Technologie, TU Braunschweig, Germany
E-mail: c.mueller-goymann@tu-bs.de
Fax: +49-531-391-8108

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PAH,** pulmonary arterial hypertension; **PBS,** phosphate buffered saline tablets; **PCHS,** precision rat heart slices; **PCLS,** precision cut lung slices; **PdI,** polydispersity index; **PS,** particle size; **SB,** sildenafil base; **SC,** sildenafil citrate; **SLN,** solid lipid nanoparticles

*This paper is part of a Special Issue on phospholipids in pharmaceutics covering the International Symposium on Phospholipids in Pharmaceutical Research held in Heidelberg, Germany, September 16–17, 2013. The Special Issue can be accessed online at http://onlinelibrary.wiley.com/doi/10.1002/ejlt.v116.9/issuetoc

sufficient aqueous solubility while sildenafil base (SB) is water insoluble [6]. Currently, SC (Revatio®) is available for the treatment of PAH in peroral form [7]. The main disadvantage of the peroral route of administration is its biodegradation in the liver due to first-pass metabolism [8]. In order to circumvent the first pass effect, we have formulated SB-loaded liposomes intended for inhalation therapy.

The pulmonary route is an attractive non-invasive method of administration for local delivery of drugs. Additionally, the lungs offer many advantages like a large surface area and a rapid absorption of drugs due to high vascularization [9, 10]. Liposomes are primarily prepared from physiological components such as phospholipids, which are inherent components of lung tissue, making them especially advantageous for pulmonary drug delivery. Lipid-based systems are an ideal platform for lipophilic drugs, which have a poor solubility in aqueous systems. Increased solubility in the lipid matrix of the formulation offers an advantage by improving therapeutic efficiency as well as pharmacokinetic parameters of the formulation. Liposomes possess sustained release properties, which ensure maximum drug effect over a prolonged time period [11]. Liposomes for inhalation are currently being extensively investigated and have a huge potential in pulmonary delivery. Currently, ciprofloxacin-liposomes and amikacin-liposomes are being clinically investigated for the treatment of cystic fibrosis and lung infections [12, 13]. The key for a successful inhalation of a liposomal product is to maintain the critical physicochemical parameters like particle size (PS) and encapsulation efficiency after nebulization [11, 14]. Liposomes are generally prepared by dissolving phospholipids and cholesterol in organic solvents such as chloroform and methanol followed by the removal of the solvent under vacuum and forming a lipid film. The dried lipid film is then hydrated using aqueous buffer solution, followed by homogenization or sonication of the liposomal dispersion [15]. For studies concerning tissue culture for testing of liposomes, residual solvents can be harmful to the cell/tissue, thus interfering with the cell viability studies. Many studies have been performed for preparing liposomes without the use of any solvent [16]. Small/large volume membrane extrusion technique utilizes a membrane through, which liposomal dispersion is passed several times at low pressures. This solvent-free extrusion method has been established for preparation of unilamellar liposomes [17–19].

The main purpose of this study was to formulate SB-loaded liposomes without any organic solvents. These liposomes were analyzed for PS measurements over 6-month time period. Toxicity of the SB-loaded and plain liposomes was assessed using in vitro and ex vivo tissue culture models. For in vitro studies, human alveolar epithelium cell line (A549) and mouse heart endothelial cell line (MHEC5-T) was used. For ex vivo studies, rat precision cut lung slices (PCLS) and heart slices (PCHS) were used. As sildenafil affects mainly vascular cells, effects of sildenafil on both heart and lung tissues were studied. Toxicity study is an important phase of formulation development and was performed to determine a safe dose for potential human administration.

2 Materials and methods

2.1 Materials

Lipoid® S100 (PL) was kindly provided by Lipoid GmbH (Ludwigshafen, Germany). It is deoiled, granulated, and purified form of soya lecithin containing ~100% phosphatidyl choline. For liposomal formulations, double distilled water was used. SB was purchased from Sequoia Research Products (Pangbourne, United Kingdom); solutions of sildenafil in culture medium were abbreviated as SB SOL. Phosphate buffered saline tablets (PBS) were purchased from MP Biomedicals (Illkirsch, France). MHEC5-T cells were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). A549 cells were a kind gift from Fraunhofer ITEM (Hannover, Germany). Adult Wistar rats were purchased from Charles River (Sulzfeld, Germany) (for ex vivo studies, see Section 2.5).

2.2 Preparation of liposomes

Plain liposomes were prepared by first hydrating 5% w/w PL with aqueous phase, i.e., sterilized PBS solution by stirring overnight using a magnetic stirrer at RT. The hydrated dispersion was then homogenized using an extruder (Avestin® Emulsiflex C5) with a polycarbonate filter with a pore size of 100 nm for 11 cycles. The homogenized dispersion was then analyzed for PS and polydispersity index. SB was used in the concentration of 1% w/w. Drug loading was performed passively. Accurately weighed SB was added to the homogenized dispersion and again stirred overnight for allowing SB to incorporate within the lipid. Final concentration of the liposomal dispersion was 4.95% PL, 0.05% SB, and 95% aqueous PBS (all w/w), with the final ratio of PL: aqueous PBS as to 5:95. The SB-loaded liposomes were optically analyzed using a polarization microscope for undissolved drug crystals. No crystals were detected, yet the liposomal dispersions were filtered using a sterile filter of a membrane size of 200 nm. The prepared liposomes were sterilized by gamma radiation (Caesium 137, 125 Gy, 59 min) before using for cell culture and ex vivo animal studies. Use of solvent was avoided in order to prevent the effects of residual solvents on the live cell/tissue. The prepared liposomes were stored at 20°C in temperature-controlled room.

2.3 Particle size measurements

Z-average PS, i.e., the hydrodynamic diameter and the polydispersity index (PdI) was analyzed using dynamic laser light scattering by Zetasizer® Nano ZS (Malvern Instruments, Herrenberg, Germany). To achieve an appropriate concentration in order to avoid multiscattering, the samples were diluted 1:100 with demineralized water and then measured using polycarbonate cuvettes (Sarstedt AG & Co, Nümbrecht, Germany). Each measurement was performed for 300 s at a scattering angle of 173° in triplicate.

2.4 In vitro studies

For the in vitro cell studies, MHEC5-T cell line (mouse heart endothelial cell line) and A549 (human alveolar epithelium) cells were used as described previously [20]. In brief, cells were seeded in 24 well plates (TPP, Trasadingen, Switzerland) at a density of 3×10^4 cells per well in 1000 μL Dulbecco's modified Eagle's medium (DMEM, Biochrom, Berlin, Germany) with 10% v/v fetal bovine serum (FBS Biochrom, Berlin, Germany), 2% v/v L-glutamine (Biochrom, Berlin, Germany) and 1% v/v penicillin–streptomycin (PAA, Cölbe, Germany). The cells were allowed to attach to the plate surface overnight. The cell culture medium was changed the next day and cells were exposed to 0–2500 μg/mL (phospholipid concentration) liposomal dispersion in 1000 μL of fresh DMEM. Cells were treated with plain and SB liposomes and SB SOL for 24 h. Afterwards, cells were washed with PBS (MP Biomedicals, Illkirsch, France) and cell viability was assessed. 1% v/v Triton-X (ICN Biomedicals, OH, USA) was used as negative control for 0% viability, i.e., 100% toxicity.

2.5 Ex vivo studies

The ex vivo studies were performed as described previously [20]. In brief, female adult Wistar rats were kept under conventional housing conditions (22°C, 55% humidity, and 12 h day–night cycle). This study was approved according to the local animal ethics requirements. The rats were maintained on laboratory food and tap water ad libitum. For acclimatization of the animals, minimum two weeks period was maintained. For testing liposomes ex vivo, rats were euthanized with i.p. overdose of phenobarbital–sodium (Merial, Hallbergmoos, Germany). Dissection was performed immediately to preserve the vitality of the tissue. The lungs were filled in situ with 1.5% w/v low melting agarose in medium solution (Sigma–Aldrich, Germany). The heart was not filled with agarose. The organs were placed immediately in a sterile glass beaker containing cold PBS (Lonza, Walkersville, USA) and the beaker was placed on ice. Subsequent to gelation of the agarose, lobes of the lungs were separated. The heart and lung tissue was cut into approx. 300–350 and 200–300 μm slices, respectively, using a Krumdieck tissue slicer (Alabama Research and Development, AL, USA) in Earl's balanced salt solution (EBSS) (Sigma–Aldrich). The slices were washed thrice with DMEM-F12 (Invitrogen, Darmstadt, Germany) to remove traces of agarose. They were then treated with different concentrations of plain and SB-liposomes as well as SB SOL for 24 h under tissue culture conditions at 37°C, 5% CO_2, and 100% air humidity. All the experiments were performed in triplicate, each well containing two slices. 1% Triton-X was used as negative control for 0% viability, i.e., 100% toxicity. Cell/tissue viability was analyzed using MTT assay.

2.6 Cell viability assay

The cell viability of plain and SB liposomes in A549 cells, MHEC5-T cells, PLCS and PCHS was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma–Aldrich, Seelze, Germany) as described previously [21, 22]. Mitochondria in viable cells convert the yellow water soluble MTT to an insoluble formazan salt, which is solubilized by lysis solution containing 0.01% v/v hydrochloric acid in 90% v/v isopropyl alcohol. 500 μL of 0.5 mg/mL MTT solution was added to the cells/slices and plates were incubated for 3 h at 37°C. After 3 h, medium was removed carefully and replaced with 300 μL of lysis solution and mixed thoroughly by shaking slowly on a plate shaker (ELMI Skyline, Progen Scientific, London, UK) for 15–20 min. Optical density (OD) was then read using a plate reader (Power wave-XS, Bio-tek Instruments, Vermont, USA) at a wavelength of 570 nm. The results were analyzed with respect to untreated controls.

2.7 Statistical analysis

The normal distribution of the samples was analyzed using normality tests, i.e., Shapiro–Wilk and Kolmogorov–Smirnov tests. Due to non-normal distribution the Kruskal–Wallis test as a non-parametric test was performed for the comparison between the groups, i.e., the effect of plain liposomes vs. SB liposomes on cell viability. Significance within the same group but in different concentrations and between the groups, i.e., in vitro vs. ex vivo was also tested. Results were considered statistically significant with $p < 0.05$.

3 Results

3.1 Particle size distribution over time

For both the prepared liposomes, PS measurements were performed at different time points for stability analysis: 1 day, 1 week, 2 weeks, 1 month, 3 months, and 6 months. As shown in Fig. 1, PS was observed to be stable throughout 6-month time period for both the liposomes. Neither PS nor PdI increased with increasing shelf life. The addition of SB in liposomes did not affect the PS in comparison with plain liposomes.

3.2 In vitro studies

3.2.1 Effect of liposomes on A549 cells

The monolayers of A549 cells were treated with plain and SB liposomal formulations in concentrations ranging between 0 and 2500 μg/mL of the phospholipid and 0 and 25 μg/mL of SB (see upper and lower abscissa in Fig. 2). Median lethal dose 50% (LD_{50}) was observed for plain liposomes between 1250 and 1300 μg/mL and for SB liposomes between 1800

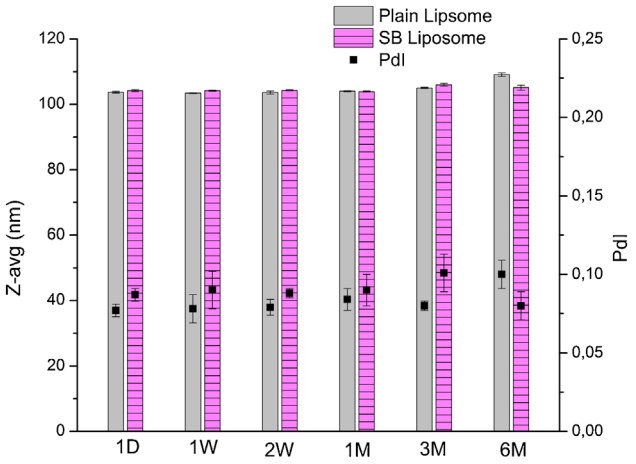

Figure 1. Time based PS (nm) and PdI values for plain liposomes and SB liposomes ($n = 3$, mean ± SD)

and 1900 μg/mL. From the higher LD_{50} values for SB liposomes, due to a small concentration of SB in the phospholipid, only a slight protective effect of SB liposomes in comparison to plain liposomes can be inferred. Cell viabilities decreased to less than 40% above a concentration of 2000 μg/mL of the phospholipid base (Fig. 2). SB SOL in the same concentration was not found to be toxic to the cells and cell viability was observed above 85% for both in vitro as well as ex vivo models (see Fig. 6). Also, in plain liposomes for all the cell models, in vitro and ex vivo, SDs were found to be extremely small and could not be plotted in the graphs, as shown by absence of error bars for plain liposomes.

3.2.2 Effect of liposomes on MHEC5-T cells

Similar to A549 cells, the monolayers of MHEC5-T cells were treated with plain and SB liposomes in concentrations ranging between 0 and 2500 μg/mL. For MHEC5-T cell, LD_{50} values were observed to be lower in comparison to A549 cells. For plain liposomes values were observed between 500 and 600 μg/mL, while for SB liposomes values were between 1100 and 1150 μg/mL. It was found that MHEC5-T cells were more sensitive than A549 cells. Also, from Fig. 3 it can be observed that SB liposomes had higher LD_{50} values as compared to plain liposomes, suggesting a protective effect of sildenafil.

3.3 Ex vivo studies

3.3.1 Effect of liposomes on PCLS

For ex vivo studies, PCLS were treated with plain and SB liposomes as well as only SB solution in medium. From Fig. 4, it can be observed that cell viability decreased to 50% at concentrations between 750 and 800 μg/mL for both plain and SB liposomes. For PCLS, addition of SB in liposomes did not affect the cell viability. For both the plain and SB-loaded liposomes, the cell viability decreased to less than 30% at concentrations from 1200 μg/mL onwards. Higher SDs were observed for both the liposomes due to inter-animal variation. As the multiple experiments were performed in parallel, PCLS and PCHS from more than one animal were utilized as a result, higher SDs were observed for both ex vivo models.

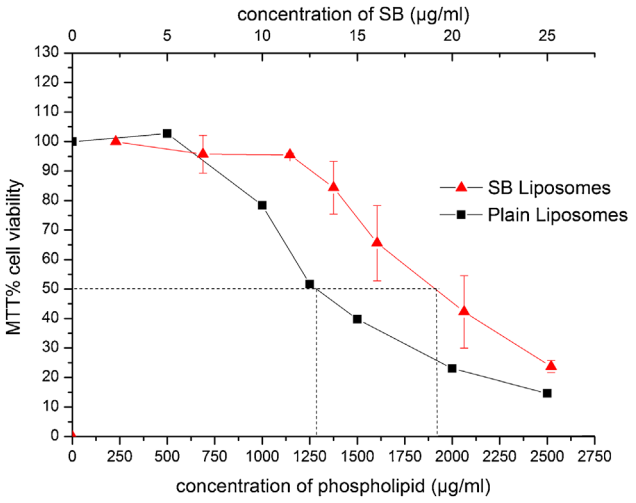

Figure 2. Effect of liposomes on the metabolic activity of human A549 cells. The metabolic activity of A549 cells treated with plain and SB liposomes was assessed by the MTT assay. Values represent mean of four independent experiments with triplicates of each concentration (±SD).

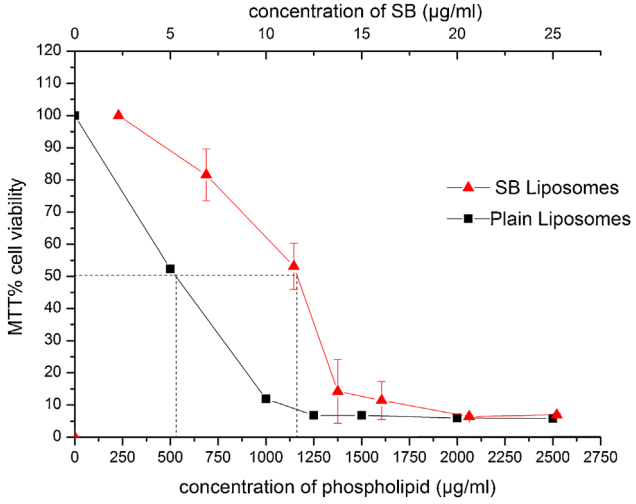

Figure 3. Effect of liposomes on the metabolic activity of murine MHEC5-T. The metabolic activity of MHEC5-T cells treated with plain and SB liposomes was assessed by the MTT assay. Values represent mean of four independent experiments with triplicates of each concentration (±SD).

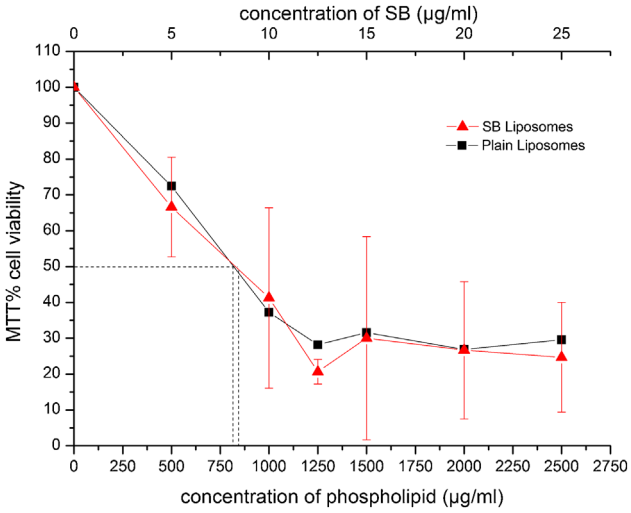

Figure 4. Effect of liposomes on the metabolic activity of PCLS. The metabolic activity of PCLS treated with plain and SB liposomes was assessed by the MTT assay. Values represent mean of four independent experiments with triplicates of each concentration (±SD).

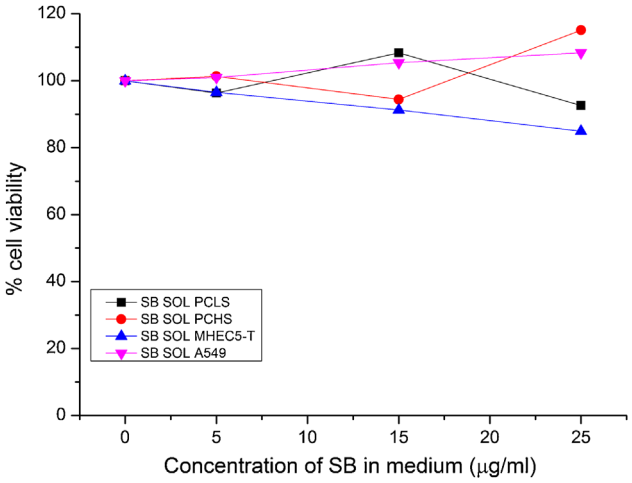

Figure 6. Effect of SB-solution (SB-SOL) on the metabolic activity of in vitro and ex vivo models. The metabolic activity of both in vitro and ex vivo models treated with SB-SOL was assessed by the MTT assay. Values represent mean of four independent experiments with triplicates of each concentration (±SD).

3.3.2 Effect of liposomes on PCHS

PCHS showed slightly higher tolerance in comparison with PCLS. LD_{50} value for plain liposomes as well as for SB liposomes was observed between 900 and 1000 μg/mL. Addition of SB did not increase the cell viability as compared to plain liposomes. A gradual decrease in cell viability was observed at concentrations above 1000 μg/mL for both liposomes as seen in Fig. 5.

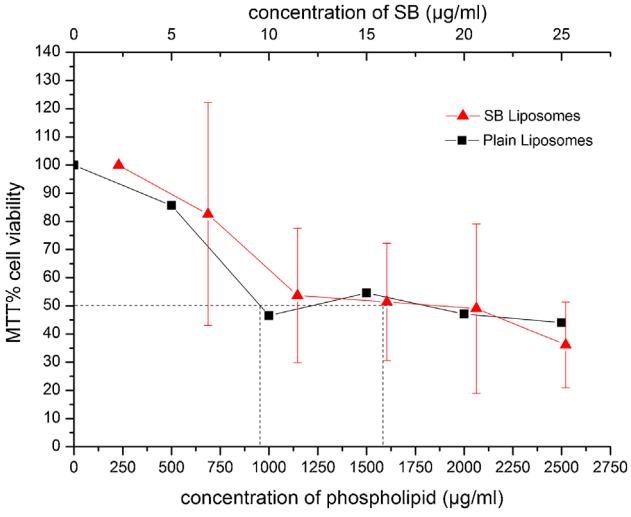

Figure 5. Effect of liposomes on the metabolic activity of PCHS. The metabolic activity of PCHS treated with plain and SB liposomes was assessed by the MTT assay. Values represent mean of four independent experiments with triplicates of each concentration (±SD).

3.4 Effect of sildenafil base solution in medium on in vitro and ex vivo models

Apart from plain and SB liposomes, SB solution, i.e., same concentration of SB in cell culture medium was also tested on both models. From Fig. 6, it can be observed that for in vitro models, the cell viability was between 85 and 110%. For ex vivo models, it was observed between 90 and 110%. Hence, from the cell viability values it can be said that SB alone did not have toxic effect on the cells within the respective concentration range.

4 Discussion

Liposomes have been investigated using multiple types of manufacturing methods. The most commonly used method for the preparation of liposomes is the lipid film method, which utilizes organic solvents to dissolve lipids to form the lipid film. The main disadvantage of this method is the residual solvents in the liposomal dispersion; this may affect the physicochemical stability along with toxic effects for cell culture studies. The liposomes prepared using solvents are generally polydisperse and have high PSs [19]. In order to avoid all the obstacles, in this study liposomes were prepared using a solvent-free large volume extrusion method. An extruder with a polycarbonate membrane and with a low energy input (0–350 bar pressure) was able to produce liposomal particles with low polydispersity indices and PSs stable over a long shelf life. The minimum volume to be handled with the large volume extruder is about 15–20 mL.

This is advantageous for preparing liposomes loaded with expensive drugs, as well as low dead volume (~1 mL) makes this method economical, avoiding wastage of the final product [18, 19]. Also, the incorporation of only phospholipids reduces the toxicity and enhances acceptability in the lungs. The first liposomal product for acute respiratory distress syndrome (ARDS) was Alveofact®, a purified bovine lung surfactant for administration by pulmonary instillation in pediatric patients [23]. Later, liposomal amphotericin-B was developed for treatment of lung infections, although for intravenous administration. Inhaled liposomes are still a challenge. Maintaining critical physiological parameters is the key for a successful liposomal product [14]. Physicochemical properties of liposomes have been investigated in multiple studies. In a study performed by Manca et al. [24], the authors investigated the influence of increasing concentration of phospholipid in rifampicin-loaded liposomal formulations. They prepared rifampicin-loaded liposomes in different compositions with soy phosphatidylcholine and hydrogenated soy phosphatidylcholine, with and without the addition of cholesterol and oleic acid. The liposomes were prepared using the film method followed by sonication. They observed a PS of about 100 nm for both the grades of phospholipids. An increase in PS and PdI was observed after addition of cholesterol and oleic acid. Furthermore, the authors observed no increase in PS after dialysis of the liposomal formulations [24]. On the similar lines, for SB liposomes of the present contribution, PS was observed in the range of about 100 nm and no significant change in PS was seen over 6 months, indicating stable formulations. In another study performed by Chimote and Banerjee [25], isoniazid-loaded liposomes were prepared using phosphatidylcholine and cholesterol by utilizing the organic solvent mediated film method. The prepared lipid film was hydrated using buffer followed by extrusion using a polycarbonate membrane of 100 nm pore size [25]. The final liposomal dispersion was observed to have a PS of 755 nm, which is significantly large for liposomes extruded via a 100 nm membrane. The increase in PS can be attributed to residual solvents followed by interference in stability and shelf life. Hence, it is certainly an advantage to prepare liposomes using a low energy input method like extrusion and if possible avoiding use of solvent at all and preferably using only phospholipids as the base.

For toxicity studies in lung tissue, many models have been established. In a previous study performed in our group, SC and SB-loaded solid lipid nanoparticles (SLN) were tested for toxicity in lung and heart cell models. In studies performed in A549 cells as an in vitro model, LD_{50} values for plain SLN as well as sildenafil-SLNs were observed between 1500 and 2000 μg/mL [19]. In the present study, plain liposomes showed LD_{50} values between 1250 and 1300 μg/mL, while SB liposomes had LD_{50} values between 1800 and 1900 μg/mL. From in vitro experiments, it can be observed that SLN and liposomes with or without sildenafil have LD_{50} values in the similar range. For ex vivo studies in PCLS, values for LD_{50} in SLN were higher as compared to the liposomes. For sildenafil SLN LD_{50} values were observed between 1200 and 1500 μg/mL, while for liposomes LD_{50} were observed between 750 and 800 μg/mL. Higher LD_{50} values in SLNs can be attributed to the presence of triglycerides in the formulation along with phospholipids. It has to be noted that for both systems, i.e., SLNs as well as liposomes, a higher sensitivity was found in the ex vivo model as compared to the in vitro model. Lung slices being a whole tissue possess multiple cell types such as pulmonary epithelial and endothelial cells, dendritic cells, fibroblasts, macrophages, lymphatic cells, etc. [20, 26, 27]. As a result, each cell may react differently and hence PCLS exhibit a higher sensitivity in comparison to a monolayer of immortalized A549 cells. Statistical analysis between the groups, i.e., in vitro vs. ex vivo also showed a significant difference ($p < 0.05$), suggesting higher sensitivity in PCLS compared to A549 cells. Due to multiple cell types present in both the ex vivo models, each cell type may react differently with variable sensitivity towards plain and SB liposomes. Also, owing to the use of more than one animal in ex vivo experiments, inter-animal variation in the slices might be the reason for high SDs and large error bars.

For in vitro heart cell models, several cell lines have been established such as human-derived primary cardiomyocytes, fetal cardiomyocytes, and murine-derived mouse heart endothelial cell line and rat ventricular cardiomyocytes [28, 29]. Although there are cell culture models available, there is relatively poor information available with respect to the toxicity of liposomes in heart tissue. Studies analyzing the effect of metallic nanoparticles have been performed by Jawad et al. The authors demonstrated an effect of titanium dioxide nanoparticles on human embryonic stem cell-derived cardiomyocytes and fibroblasts [30]. However, metallic nanoparticles have a completely different composition and properties as compared to liposomal formulations. For ex vivo models, heart tissue slices and aortic rings are another attractive option for testing cardiovascular drugs [31]. Kaneko and Coppen established an ex vivo model consisting of adult and embryonic rat heart slices for testing cardiovascular drugs. They found that the adult rat heart slices could be viable up to 3 days [32]. In spite of the information available in the literature on cardiovascular drugs and metallic nanoparticles, there is still a lack of data on heart tissue models and toxicity testing of lipid-based systems. Hence, we have established a new ex vivo model of heart slices for toxicity testing of lipid-based systems along with the immortalized MHEC5-T cell line.

5 Conclusion

Liposomes loaded with SB were efficiently manufactured using a low energy input extrusion method. These

formulations proved stable with respect to shelf life for 6 months. From the stability and uniformity in PS, it can be concluded that the use of solvents for the preparation of liposomes using the conventional dried lipid film method can be avoided. From the in vitro and ex vivo studies, both the tissue slices PCLS and PCHS had higher sensitivity than the immortalized A549 and MHEC5-T cell lines. In in vitro studies, in both cell lines, SB liposomes showed higher LD_{50} values, suggesting a protective effect in comparison with plain liposomes. A novel model for testing drugs in heart tissue, the PCHS can provide valuable information in toxicity testing of lipid-based drug delivery systems. In terms of applicability, nebulization studies will be performed with reference to PS measurements before and after nebulization along with visual interpretation using transmission electron microscopy. From the preliminary physicochemical characterization and in vitro and ex vivo results, SB liposomes have a potential for possible administration in human lungs. Further experiments with respect to cytokine inhibition and assessment of the movement of pulmonary vessels with videomicroscopy using a vasoconstrictor in presence of sildenafil will be performed in the future.

M. Paranjpe is thankful to the German Academic Exchange Service (DAAD) for scholarship.

The authors have declared no conflict of interest.

References

[1] Jaafar-Maalej, C., Elaissari, A., Fessi, H., Lipid-based carriers: Manufacturing and applications for pulmonary route. *Expert Opin. Drug Deliv.* 2012, *9*, 1111–1127.

[2] Rang, H. P., Dale, M. M., Ritter, J. M., Flower, R.J., *Pharmacology*, Sixth Edition, Churchill Livingstone Elsevier Publication, 2007, ISBN: 0-8089-2354-4.

[3] Dupuis, J., Hoeper, M. M., Endothelin receptor antagonists in pulmonary arterial hypertension. *Eur. Respir. J.* 2008, *31*, 407–415.

[4] Galiè, N., Manes, A., Branzi, A., Prostanoids for pulmonary arterial hypertension. *Am. J. Respir. Med.* 2003, *2*, 123–137.

[5] Bourge, R. C., Tapson, V. F., Safdar, Z., Benza, R. L., et al., Rapid transition from inhaled iloprost to inhaled treprostinil in patients with pulmonary arterial hypertension. *Cardiovasc. Ther.* 2013, *31*, 38–44.

[6] Jung, S.-Y., Seo, Y.-G., Kim, G. K., Woo, J. S., et al., Comparison of the solubility and pharmacokinetics of sildenafil salts. *Arch. Pharm. Res.* 2011, *34*, 451–454.

[7] Antoniu, S. A., Sildenafil for pulmonary arterial hypertension: When blue turns into white. *Expert Opin. Pharmacother.* 2006, *7*, 1801–1810.

[8] Malgorzata, S., Thiery, V., Adam, S., Nanocarriers as pulmonary drug delivery systems to treat and to diagnose respiratory and non respiratory diseases. *Int. J. Nanomedicine* 2008, *3*, 1–19.

[9] Azarmi, S., Roa, W. H., Löbenberg, R., Targeted delivery of nanoparticles for the treatment of lung diseases. *Adv. Drug Deliv. Rev.* 2008, *60*, 863–875.

[10] Sakagami, M., In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. *Adv. Drug Deliv. Rev.* 2006, *58*, 1030–1060.

[11] Gaspar, M. M., Gobbo, O., Ehrhardt, C., Generation of liposome aerosols with the Aeroneb Pro and the AeroProbe nebulizers. *J. Liposome Res.* 2010, *20*, 55–61.

[12] Clancy, J. P., Dupont, L., Konstan, M. W., Billings, J., et al., Phase II studies of nebulised Arikace in CF patients with Pseudomonas aeruginosa infection. *Thorax* 2013, *68*, 818–825.

[13] Serisier, D. J., Bilton, D., de Soyza, A., Thompson, P. J., et al., Inhaled, dual release liposomal ciprofloxacin in non-cystic fibrosis bronchiectasis (ORBIT-2): A randomised, double-blind, placebo-controlled trial. *Thorax* 2013, *68*, 812–817.

[14] Cipolla, D., Gonda, I., Chan, H.-K., Liposomal formulations for inhalation. *Ther. Deliv.* 2013, *4*, 1047–1072.

[15] Torchilin, V. P., Weissig, V., *Liposomes—A Practical Approach*, Oxford University Press, New York 2003.

[16] Zheng, S., Alkan-Onyuksel, H., Beissinger, R. L., Wasan, D. T., Liposome microencapsulations without using any organic solvent. *J. Dispers. Sci. Technol.* 1999, *20*, 1189–1203.

[17] MacDonald, R. C., MacDonald, R. I., Menco, B. P., Takeshita, K., et al., Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* 1991, *1061*, 297–303.

[18] Joseph, S., Bunjes, H., Influence of membrane structure on the preparation of colloidal lipid dispersions by premix membrane emulsification. *Int. J. Pharm.* 2013, *446*, 59–62.

[19] Joseph, S., Bunjes, H., Preparation of nanoemulsions and solid lipid nanoparticles by premix membrane emulsification. *J. Pharm. Sci.* 2012, *101*, 2479–2489.

[20] Paranjpe, M., Neuhaus, V., Finke, J. H., Richter, C., et al., In vitro and ex vivo toxicological testing of sildenafil-loaded solid lipid nanoparticles. *Inhal. Toxicol.* 2013, *25*, 536–543.

[21] Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 1983, *65*, 55–63.

[22] Scherließ, R., The MTT assay as tool to evaluate and compare excipient toxicity in vitro on respiratory epithelial cells. *Int. J. Pharm.* 2011, *411*, 98–105.

[23] Rudiger, M., Naturally derived commercial surfactants differ in composition of surfactant lipids and in surface viscosity. *AJP: Lung Cell. Mol. Physiol.* 2004, *288*, L379–L383.

[24] Manca, M., Sinico, C., Maccioni, A., Diez, O., et al, Composition influence on pulmonary delivery of rifampicin liposomes. *Pharmaceutics* 2012, *4*, 590–606.

[25] Chimote, G., Banerjee, R., In vitro evaluation of inhalable isoniazid-loaded surfactant liposomes as an adjunct therapy in pulmonary tuberculosis. *J. Biomed. Mater. Res.* 2010, *94B*, 1–10.

[26] Nassimi, M., Schleh, C., Lauenstein, H.-D., Hussein, R., et al., Low cytotoxicity of solid lipid nanoparticles in in vitro and ex vivo lung models. *Inhal. Toxicol.* 2009, *21*, 104–109.

[27] Nassimi, M., Schleh, C., Lauenstein, H., Hussein, R., et al., A toxicological evaluation of inhaled solid lipid nanoparticles used as a potential drug delivery system for the lung. *Eur. J. Pharm. Biopharm.* 2010, *75*, 107–116.

[28] Parameswaran, S., Kumar, S., Verma, R. S., Sharma, R. K., Cardiomyocyte culture—An update on the in vitro cardiovascular model and future challenges. *Can. J. Physiol. Pharmacol.* 2013, *91*, 985–998.

[29] Plendl, J., Sinowatz, F., Auerbach, R., A transformed murine myocardial vascular endothelial cell clone: Characterization of cells in vitro and of tumours derived from the clone in situ. *Virchows Arch.* 1995, *426*, 619–628.

[30] Jawad, H., Boccaccini, A. R., Ali, N. N., Harding, S. E., Assessment of cellular toxicity of TiO_2 nanoparticles for cardiac tissue engineering applications. *Nanotoxicology* 2011, *5*, 372–380.

[31] Bussek, A., Wettwer, E., Christ, T., Lohmann, H., et al., Tissue slices from adult mammalian hearts as a model for pharmacological drug testing. *Cell. Physiol. Biochem.* 2009, *24*, 527–536.

[32] Kaneko, M., Coppen, S.R., Histological validation of heart slices as a model in cardiac research. *J. Cell. Sci. Ther.* 2012, *3*, doi: 10.4172/2157-7013.1000126.

