Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(7):46-53
(http://www.derpharmachemica.com/archive.html)

Preparation, Optimization and Evaluation of Diallyl Disulphide Loaded Liposomes

Rajashree H*, Sneha G

Department of Pharmaceutics, Vivekanand Education Society's College of Pharmacy, Chembur, Mumbai-400074, India

ABSTRACT

Aims: *The aim of this study was to develop liposome loaded Diallyl disulphide for topical delivery and evaluate the efficacy of liposome for the treatment of skin cancer.* ***Method:*** *Phosphatidylcholine, cholesterol and diallyl disulphide were dissolved in chloroform and subsequently transferred into round bottom flask connected to a Rotavapor. Thin film hydration method was used for the formulation of liposomes. Various factors affecting the entrapment of diallyl disulphide into the lipid bilayers were evaluated using experimental techniques. Factors like Cholesterol: Phospholipid ratio and amount of diallyl disulphide were fine tuned to obtain optimized batches.* ***Results:*** *Liposome prepared was evaluated for vesicle size, zeta potential, percent drug entrapment, diffusion studies and in vitro cytotoxicity studies. Optimized batch was found to have vesicle size of 208.1 nm, %drug entrapment 91.7 and %drug permeation was 61.1%.* ***Conclusion:*** *Results of the present studies indicate that liposomes of diallyl disulphide can provide sustained drug delivery over an extended period of time.*

Keywords: Diallyl disulphide, Liposomes, Skin cancer, Permeation, Sustained release

INTRODUCTION

Garlic (*Allium sativum*) possess promising health benefits such as anti-atherosclerotic, antifungal, antimicrobial, antithrombotic, cardiovascular disease treatment and stimulating immune system since the origin of history and is probably one of the most widely studied medicinal plants [1,2].

Allyl constituents of garlic oil have been showed to have benefits for their anticancer, antimicrobial and other pharmacologic activities. Among these, Diallyl Disulphide (DADS), a member of allyl sulfides family (diallyl sulfide, diallyl disulfide and diallyl trisulfide) has been reported to possess anti-cancer activity [3,4]. The incidence of skin cancer in people has been increasing day by day. The main reason for skin cancer is due to UV-exposure because very high amount of UV-radiation reach earth's surface due to ozone layer depletion. Skin cancer can be divided into two types: Malignant melanoma and Non-melanoma; Non melanoma was further divided into basal cell carcinoma and squamous cell carcinoma; they occur mainly due to chronic exposure to UV-sunlight. Melanoma skin cancers are an aggressive type that can metastasize and cause death. These cancers arise from melanocytes, which are pigment-producing cells, and are correlated with chronic exposure to sunlight [5-8].

Stratum Corneum (SC) is a major barrier of many compounds passing through the skin. Several approaches have been developed to weaken this barrier. One possibility for enhancing the penetration of drugs and many cosmetic chemicals is the use of vesicular systems, such as liposomes [9]. Interestingly, liposomal formulations, when employed for topical delivery, have been shown to be extremely promising for enhancing drug penetration, improving pharmacological effects, decreasing side effects, controlling drug release, has ability to entrap hydrophilic and lipophilic drugs, protecting encapsulated agents from metabolic processes. Additional advantage is that these formulations are non-toxic and biodegradable [10]. Sustained release is required for accumulation of drug in skin cancer providing the drug to stay longer in the tumour and to increase its therapeutic efficacy.

The objective of the present study was to sustain the release of the drug for longer period of time avoiding systemic drug absorption and increase the deposition of drugs within the skin. Restricting drug in skin which will allow it to act on melanocyte cells at basal layers of epidermis and will provide anti-cancer effects. In the present study, liposomes were prepared by thin film hydration method and characterized for different parameters.

MATERIALS AND METHODS

Diallyl disulphide was the gift sample obtained from Synthite Industries Ltd (Mumbai). Leciva S 70 (Phosphatidylcholine) was the gift sample from VAV Life sciences; Cholesterol was purchased from Loba Chemie. Rotary Evaporator Equipment from JSGW was used for preparation of formulations. All the other chemicals and solvents used in the experiments were of analytical grade.

Preparation of liposomes

Conventional lipid film hydration method is one of the oldest methods, described by Bangham et al.. The method involves formation of thin lipid film which is then hydrated by suitable aqueous medium which leads to the formation of vesicles. Phospholipids, cholesterol and DADS were dissolved in chloroform containing suitable concentration of Butyl Hydroxy toluene. Chloroform was evaporated under vacuum on a rotary evaporator at 60°C (above the gel-to-liquid crystalline phase transition temperature of the phospholipids) to obtain thin film. The dry lipid film was hydrated with addition of distilled water with handshaking until a homogenous dispersion was obtained. The dispersion thus obtained was Probe sonicated for further size reduction. Liposomal dispersion thus formed was stored at 4°C for its characterization and till further studies. In case of blank liposome, the same procedure was performed without the addition of DADS [11-14].

Optimization of formulation parameters for liposomes

The effect of formulation variables on the responses were statistically evaluated by applying one-way ANOVA using the software package Design Expert® version 9.0.3. A 3^2 factorial design was utilised to arrive at optimization of diallyl disulphide liposomes. Among the various contributing factors, cholesterol concentration and the drug concentration were selected for optimization of vesicular formulations while other potential variables viz. volume of hydration medium, temperature and cycle time of sonication etc. were kept constant. DADS liposomes were optimized for percentage entrapment efficiency and particle size with the help of 3^2 factorial design [15,16].

The independent variables used were Chol: PL molar ratio and amount of diallyl disulphide. Each independent variable was kept at three levels viz. low level (-1), medium level (0) and high level (+1). Experimental trials were carried out at all nine possible combinations as shown in the Table 2 with detail of variables and their levels mentioned in Table 1.

Table 1: Variables and their levels in full factorial design

Levels (Code value)	Actual values		Response	
	X1 molar ratio of Chol:PL	X2 Amount of drug (mg)	Y1	Y2
Low (-1)	1:6	50	Vesicle size	% Entrapment efficiency
Intermediate (0)	1:4	70		
High (+1)	1:2	90		

Table 2: 3^2 full factorial design layout for optimization

Runs	Batch code	X1		X2	
		Molar ratio of Chol:PL		Amount of drug (mg)	
	Leciva S70	Coded	Actual	Coded	Actual
1	F1	-1	01:06	-1	50
2	F2	-1	01:06	0	70
3	F3	-1	01:06	1	90
4	F4	0	01:04	-1	50
5	F5	0	01:04	0	70
6	F6	0	01:04	1	90
7	F7	1	01:02	-1	50
8	F8	1	01:02	0	70
9	F9	1	01:02	1	90

Statistical analysis of data was achieved using Analysis of Variance (ANOVA). The statistical analysis was conducted using Design-Expert® version 9.0.3 trial (Stat-Ease Inc., Minneapolis, MN, USA). The software performs Response Surface Methodology (RSM) which contains Multiple Regression Analysis (MRA), ANOVA and statistical optimization.

Statistical relationship in the form of equations was obtained which displays the effect of varying A and B on the dependent variables, Y1 and Y2. In addition, contour and 3D surface plots were obtained by Design-Expert, to represent the effect of the independent variables graphically.

To determine the optimal batch, mathematical model was put in use. The two equations derived i.e., for percentage entrapment efficiency (Y2) and Vesicle size (Y1) in terms of X1 (Molar ratio of Chol: PL) and X2 (Amount of Drug) were sequentially employed. Experimental trials were carried out in triplicates. Specific desired values of both the responses were assigned. The formulation with the desirability value close to unity is chosen as the optimized formulation. For the present study, the criteria for optimum batch were decided as those which will show minimum vesicle size and maximum entrapment efficiency.

Characterization of liposomal dispersion

Determination of vesicle entrapment efficiency and drug loading

The entrapment efficiency of diallyl disulphide liposomes was determined by indirect method wherein the amount of unincorporated drug was

determined. Aliquot of DADS liposomes was subjected to centrifugation. Supernatant from the mixture after appropriate dilution was analyzed using U.V. Spectrophotometer. Following formula was used for calculating EE and DL [17]:

$$\% \text{ Entrapment Efficiency } = \frac{(Wt-Wa)}{Wt} \times 100$$

$$\% \text{ Drug Loading} = \frac{(Wt-Wa)}{Wt-Wa+Wl} \times 100$$

Where, Wt-stands for total amount of drug added to the system, Wa-stands for amount of drug quantified by indirect method, Wl-stands for amount of lipid.

Vesicle size and size distribution

Liposomes were measured for vesicle size and extent of size distribution using Malvern Zetasizer ZS90 at a 90 degree scattering angle using dynamic light scattering. This technique measures the diffusion of particles moving under Brownian motion and converts this to size and a size distribution using the Stokes-Einstein relationship.

Zeta potential

Zeta potential was measured using Malvern Zetasizer ZS90 which was used as an indicator of the stability of vesicles formed by thin film hydration method.

***In vitro* drug permeation studies**

In vitro drug permeation studies were carried out for both liposomal dispersion and plain drug which was performed using Modified Franz diffusion cells. Dialysis membrane having molecular cut-off of 12-14 k Da was used. These membranes were previously soaked in the receptor medium for 24 h for hydration. The receptor medium used for the study was mixture of phosphate buffer pH 6.8 and co-solvent methanol. The diffusion cells were maintained at 37 ± 0.1°C using a re-circulating water bath and the medium in the receptor chambers were stirred continuously using magnetic stirrer. The membranes were initially left in the diffusion cells in order to facilitate hydration. Subsequently, aliquot of liposome dispersion was gently placed onto each membrane surface at the donor compartment. An aliquot of samples were withdrawn at definite time intervals (1, 2, 3, 4, 5, 6, 7, 8 and 24 h). Concentration of drug permeated was monitored using UV-spectrophotometer.

Scanning electron microscopy (SEM)

Surface morphology of the liposomes was characterized by SEM. The samples were mounted on alumina stubs using double adhesive tape, coated with gold in HUS–5GB vacuum evaporator, and then the sample was observed in Quanta 200 ESEM at an acceleration voltage of 10 KV and at various magnifications [18].

***In vitro* cytotoxicity studies**

An *in vitro* cytotoxicity study of the developed DADS liposomes was carried out using SK-MEL 2 cell line. This cell line is a representative cell line of Melanoma (Skin) cancer. It was used to evaluate the efficiency of the drug against skin cancer. These studies were performed to compare the effectiveness of DADS loaded Liposome with blank liposome [19-21].

Sulforhodamine B (SRB) Assay procedure was followed. The cell line was maintained in ideal laboratory conditions. Analysis was carried out of three different samples. The samples constituted DADS loaded liposome, blank liposome and DADS in Dimethyl Sulfoxide (DMSO) with positive control sample. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µl at appropriate plating densities, depending on the doubling time of individual cell lines, followed by incubation at 37°C, in 5% CO_2, 95% air and 100% relative humidity for 24 h, prior to addition of the formulations. After 24 h, the cells from one well, from the cell line were fixed *in situ* with Trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of drug addiction (Tz). Formulations were solubilized in DMSO as 10 mg/ml concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10 times, to make it 1000 µg/ml with complete RPMI medium. This complete RPMI medium containing test extract was further diluted with complete RPMI medium to adjust the concentration at 100, 200, 400 and 800 µg/ml. Aliquots of 10 µl of these different dilutions were added to the appropriate microtiter wells already containing 90 µl of cell suspension, resulting in the required final drug concentrations of 10, 20, 40 and 80 µg/ml. For each of the microtitre plate the well-known anticancer drug, Adriamycin (ADR) was used as a positive control.

After compound addition, the plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried.

Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an Enzyme-linked Immunosorbent Assay (ELISA) Plate Reader at a wavelength of 540 nm with 690 nm reference wavelength.

RESULTS AND DISCUSSION

Optimization plays a critical role in preparation of liposomes by changing various parameters and process variables that would affect entrapment efficiency and vesicle size. Factors such as lipid film thickness, volume of hydration medium, lipid composition, cholesterol concentration and drug concentration will affect entrapment efficiency and vesicle size. Amount of phospholipid and cholesterol were found to be crucial in preparation and stabilization of DADS liposomes. Cholesterol provides rigidity to the liposome membrane and hence variation in its concentration will ultimately result in drug leakage.

Design Expert software performs RSM which helps to quantify the relationship between one or more measured responses and the vital input factors. It helps in determination of desirable location in the design space. This space could be maximum, minimum or an area where the response is constant over a range of factor.

Optimization of formulation parameters for liposomes

The selected factors such as drug concentration and molar ratio of cholesterol : Phospholipid influences the responses vesicle size and % entrapment efficiency.

For response 1

$$\text{Vesicle size}=285.23+20.28 \times A+9.11 \times B-2.60 \times A \times B-40.65 \times A^2-36.45 \times B^2$$

The equation suggested that the factor A (Molar ratio of Chol:PL) and factor B (Amount of drug) have positive effects on the vesicle.

Positive coefficient of A term indicate that the vesicle size decreases with decrease in molar ratio of Chol:PL. Positive coefficient of B term indicate that the vesicle size decrease with decrease in drug concentration. Hence at low level of drug concentration and low level in molar ratio of Chol: PL, optimum vesicle size could be obtained. When the coefficient values of two independent factors are compared, the value of coefficient of Molar ratio of Chol: PL was found to be higher than that of Drug Concentration and hence, Molar ratio of Chol: PL was considered to be a major contributing variable for vesicle size of Liposomes. Vesicle size of DADS Liposome was found to be from 198.7 to 315 nm range.

Effect of cholesterol: Phospholipid ratio on vesicle size

Response plots as shown in Figure 1 indicate that an optimum ratio of 1:6 generates particles of optimum size. Increasing or decreasing this ratio causes changes in Particle Size. The formation of the lipid bilayer and its fluidity is accounted by the amount of cholesterol inserted between the phosphatidylcholine molecules. Presence of cholesterol is advantageous as it makes the bilayer sufficiently flexible and also contributes to proper release of the entrapped moiety. Decrease vesicle size was obtained with the batches having cholesterol: Phospholipid ratio as 1:6. This could be because, the addition of cholesterol in this ratio provides optimum rigidity to the bilayer. Decrease in vesicle size might be because increased amount of phospholipids, providing space for drug molecules to get entrapped.

Effect of drug concentration on vesicle size

The amount of drug concentration will influence the vesicle size. This is attributed to the lipophilicity of the drug. When the concentration of the drug was increasing, vesicle size also increased irrespective of the ratio of the Chol: PL.

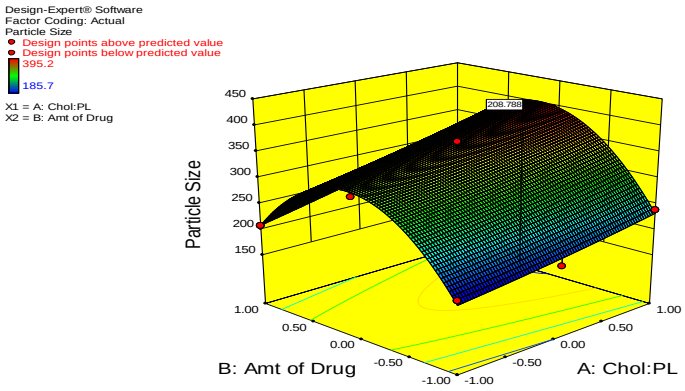

Figure 1: 3D surface response plot for the response vesicle size

The relationship between the factors and the responses was further elucidated using response surface plots. 3D response surface plots in Figure 1 gives a representation of the variations in each response when the two factors are simultaneously changed from lower to higher level. It gives a three dimensional curvature of the change in response at different factor levels. It also gives the variation in design points from the predicted response value where the desired working region is represented with blue colour (minimum particle size).

For response 2: Percent entrapment efficiency

$$\%\ \text{Entrapment efficiency}=74.54-6.80 \times A+3.50 \times B-1.89 \times A \times B+3.14 \times A^2+0.64 \times B^2$$

The equation suggested that the factor A have a negative effect on the percent entrapment efficiency and factor B have a positive effect on the percent entrapment efficiency.

Negative coefficient of A term indicate that the % entrapment efficiency increase with decrease in molar ratio of Chol:PL. Positive coefficient of B term indicate that the % entrapment efficiency increases with increase in drug concentration. Hence at high level of drug concentration and low level of molar ratio of Chol:PL, increased percent entrapment efficiency could be obtained. When the coefficient of two independent factors are compared, the value of coefficient of molar ratio of Chol:PL was found to be higher than that of drug concentration and hence, molar ratio of Chol:PL was considered to be a major contributing variable for percent entrapment efficiency of liposomes. % entrapment efficiency of DADS liposome was found to be from 68.9-91.7%.

Effect of cholesterol: Phospholipid ratio on percent entrapment efficiency

Variation in the concentration of Cholesterol:Phospholipid ratio extremely affects the entrapment efficiency, as illustrated in Figure 2. Increase in entrapment efficiency at low concentration of cholesterol shows that cholesterol act as the “vesicular cement” in the molecular cavities of phospholipid bilayer and abolishes the gel to sol transition, forming less leaky vesicles. Therefore, increase in the rigidity decreases the permeability of the entrapped drug and hence improves the entrapment efficiency.

Effect of drug amount on percent entrapment efficiency

Figure 2 suggested, increase in concentration of drug increases entrapment efficiency irrespective of the ratio of the Chol: PL. This is attributed to the lipophilicity of the drug. Therefore, entrapment efficiency can be increased by increasing the amount of drug added at the optimal cholesterol:Phospholipid ratio.

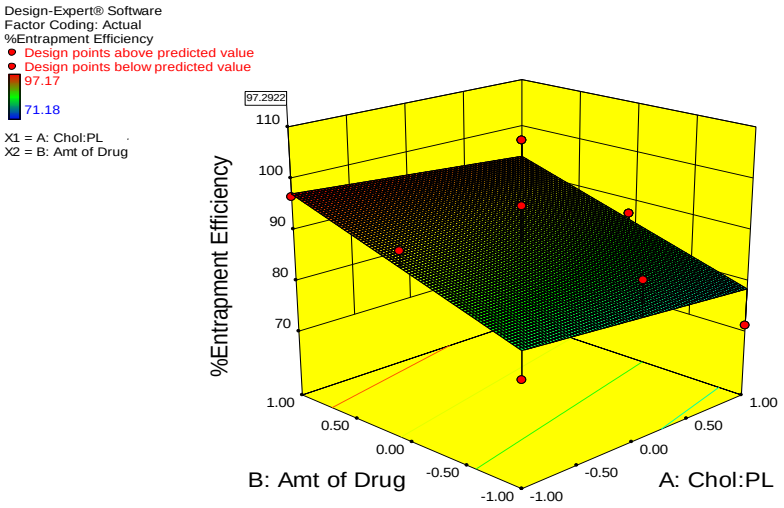

Figure 2: 3D Surface response plot for % drug entrapment efficiency

Desirability and overlay plot

These are obtained as a result of the optimization of the formulation (Figure 3). The desirability plot from Figure 4 suggests the region (working space) from which any formulation prepared would have results in desired range of the responses. Figure 3 representing an overlay plot obtained by the Design Expert® Software is used to determine ratio of Chol: PL and amount of drug, so as to have minimum vesicle size and maximum percent drug entrapment efficiency. From overlay plot (Figure 3 and Table 3), solution 1 has desirability value closer to unity and hence was the best solution. The variables associated with this solution had same values as that of batch F3 i.e., –1 or low level of Chol: PL and +1 or high level of drug concentration showing vesicle size 208.1 nm and % EE as 91.7. Hence, formulation F3 was considered as the optimized formulation.

Table 3: Solutions for optimized batch suggested by Design Expert

Solution No.	Chol:PL	Drug concentration	Vesicle size	%Entrapment efficiency	Desirability
1	-1	1	240.135	90.51	0.986
2	-1	0.85	248.49	89.52	0.958
3	-1	-1	216.7	79.72	0.927
4	-1	-0.65	241.85	81.24	0.899
5	0.96	1	198.701	73.22	0.687
6	0.92	1	202.171	73.31	0.614

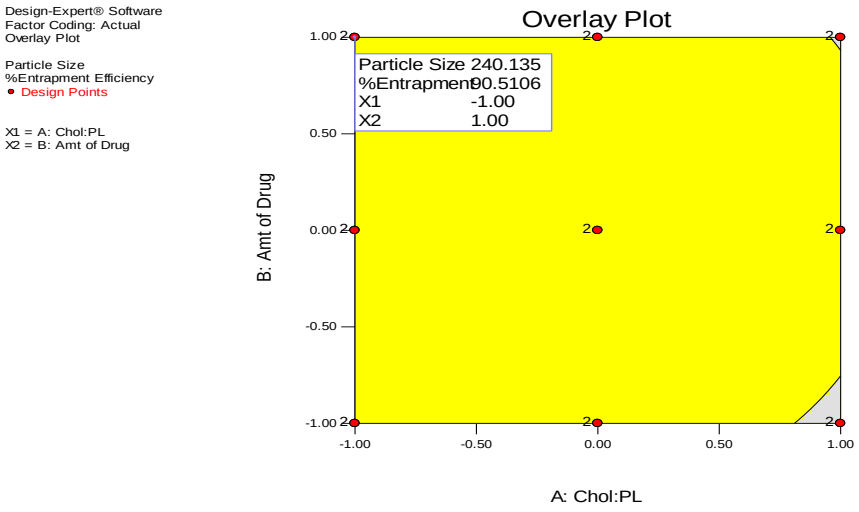

Figure 3: Overlay plot

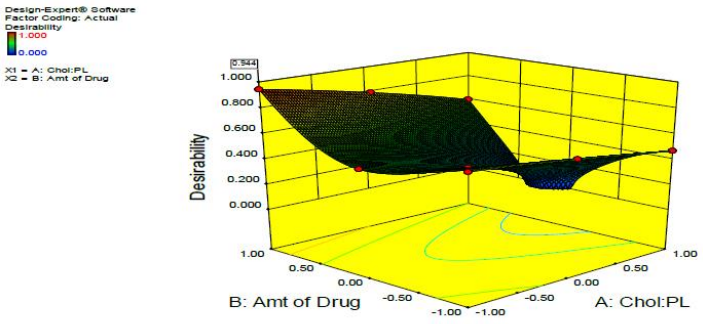

Figure 4: Desirability plot

Characterization and evaluation of liposomal dispersion

Entrapment efficiency and drug loading

Entrapment efficiency is the fraction of total drug entrapped within vesicular carrier and it is an important parameter to evaluate the potential of the vesicular drug delivery system. DADS, being a lipophilic moiety tend to have good affinity towards the lipid bilayers which was evident from the percent entrapment of 91.7 ± 1.16%. The drug loading was found to be 11.12 ± 0.23% for the optimized batch.

Determination of vesicle size and polydispersity index

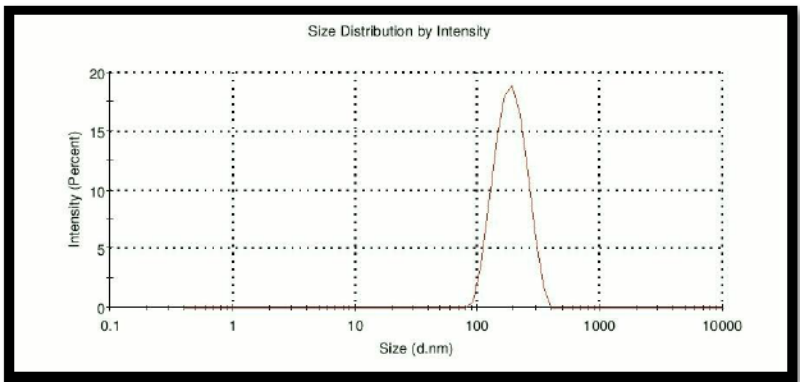

Figure 5: Size distribution of liposomes

The average size was observed to be 208.6 nm. The liposomal dispersion showed polydispersity index of 0.329 which was not substantially high (Figure 5), indicating that most of the vesicles were within the size range.

Zeta potential measurement

Liposomes in the dispersion exhibited zeta potential of -22.4 mV as seen from Figure 6, indicating their moderate stability state.

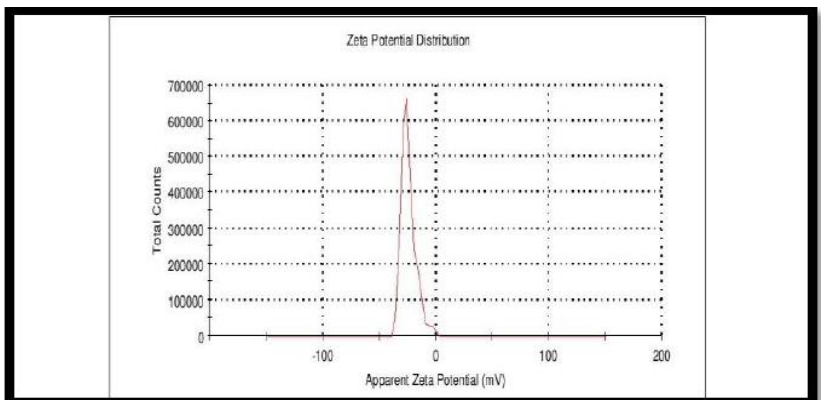

Figure 6: Zeta potential distribution

***In vitro* drug diffusion studies**

The Permeation of DADS and DADS encapsulated in the liposomes were evaluated using vertical Franz diffusion cells with a synthetic (Cellophane) membrane between the donor and receptor compartments. The use of Franz diffusion cells provides an accurate and reliable method for evaluating active compound permeated from topical formulations. Figure 7 compares *in vitro* permeation profile of plain drug and drug loaded liposomes using mixture of pH 6.8 phosphate buffers and methanol in the ratio 60:40 as receptor medium.

It was observed that 99.5% of plain drug was permeated at the end of 24 h whereas only 61.1% of the drug was permeated from the liposome dispersion. Rate and amount of drug permeated from liposomal dispersion was lesser with initial burst release followed by sustained release. This can be due to slow diffusion of the drug through lipid matrix in the latter stage which may be advantageous, causing prolonged exposure of tumour cells to drug, increasing its clinical efficacy.

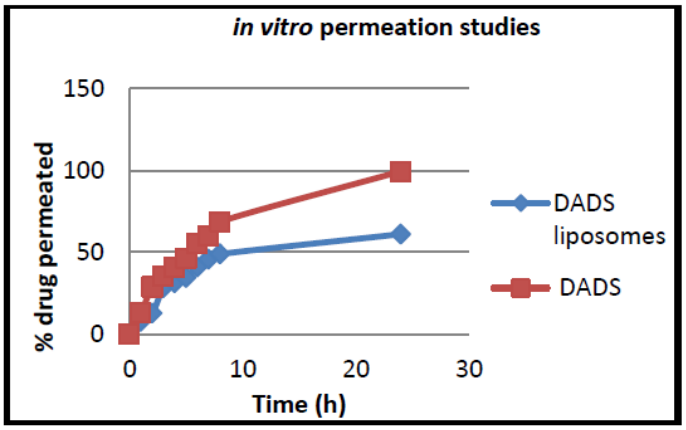

Figure 7: % drug permeated from DADS liposomes and plain drug

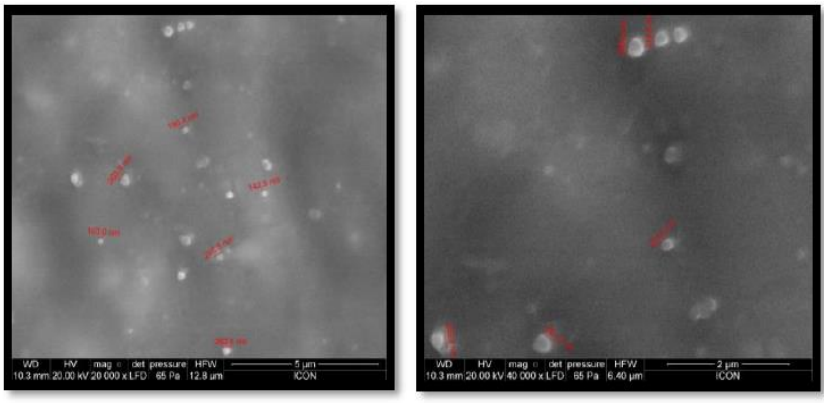

Figure 8: SEM imaging of diallyl disulphide liposome

SEM images are indicative of structure of drug loaded liposomes. Figure 8 indicates that developed liposomes are spherical in shape.

***In vitro* cytotoxicity studies**

In vitro cytotoxicity studies were carried out against SKMEL-2 cell line - a representative cell line of Melanoma type Skin Cancer. From Figure 9, it could be observed that DADS liposomes showed decrease in percent growth of the cells compared to blank liposomes. Drug in DMSO was almost as effective as the positive control.

As the concentration of drug and liposome loaded drug increases, % cell viability decreases gradually, this explains the concentration dependent cytotoxic effect of DADS. Taking into consideration growth curve, (Figure 9) it can be concluded that DADS liposomes showed improved effectiveness in preventing cell growth as compared to blank liposomes.

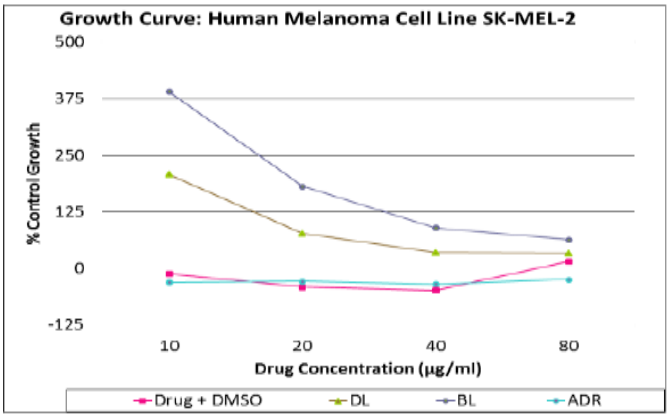

Figure 9: Growth curve of the samples against SK-MEL-2

Where, — indicates Blank Liposomes; — indicates Drug loaded Liposomes; — indicates Adriamycin; — indicates Drug + DMSO

CONCLUSION

Thin film hydration technique was employed for the preparation of liposomes. Leciva S 70 was used as a vesicle forming component while cholesterol was chosen as the rigidity modulator. Butyl hydroxyl toluene (BHT) was used as antioxidant.

A statistical optimization design (3^2 full factorial) was applied to evaluate precisely the effect of independent variables (Level of ratio of vesicle forming component–Chl:PL and drug) on the dependent variables (Vesicle size and %EE). A polynomial based equation model was generated to characterize the design space. A desirability plot was generated for optimized formulations based on the criteria selected for minimum vesicle size and maximum percent entrapment efficiency. From the optimization studies, it was seen that the Chol:PL ratio of 1:6 and drug concentration of 90 mg showed maximum entrapment efficiency and minimum vesicle size. This batch of liposome was further evaluated for various parameters. Optimized formulation showed vesicle size of 207 nm, PDI 0.329 and zeta potential -22.4 millivolts with acceptable entrapment efficiency 91.5%.

Permeability studies showed that permeation from the liposomes was retarded as compared to plain drug with maximum amount being 61.1% after 24 h. This may result in retention of drug in skin layer leading to regression of tumor. At the same time, it will not permeate to greater extent through the skin thus avoiding systemic side effects. *In vitro* cytotoxicity studies were carried out against SKMEL-2 cell line- a representative cell line of melanoma type skin cancer. The results showed that drug loaded liposomes had anticancer activity. Thus, it can be concluded that vesicular carrier systems approach can be successfully employed to improve effectiveness of DAS by improved retention at the target epidermal and dermal region of the skin and thereby reducing the frequency of administration and treatment duration.

ACKNOWLEDGEMENT

Authors are thankful to All India Council for Technical Education, New Delhi (Ref. No (REF NO:8-159/RIFD/RPS/Policy-4/2013-14) for funding this work, Synthite Industries Ltd for providing gift sample of Diallyl disulphide and VAV Life science for phospholipids. Authors are grateful for Icon Analytical Equipment Pvt Ltd, Mumbai for SEM studies. *In vitro* SRB assay for anti-cancer activity evaluation of drugs was done at Anti-Cancer Drug Screening facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai and authors will like to put on records their support.

REFERENCES

[1] E. Block, *Sci. Am*., **1985**, 252, 114-119.
[2] K.C. Agarwal, *Med. Res. Rev.*, **1996**, 16, 111-124.
[3] T.M. Tsao, M.C. Yin, *J. Med. Microbiol*., **2001**, 50, 646-649.
[4] Calvo-Gomez, O.J. Morales-Lopez, M.G. Lopez, *J. Chromatogr.* A., **2004**, 1036, 91-93.
[5] T.M. Johnson, O.M. Dolan, T.A. Hamilton, M.C. Lu, N.A. Swanson, L. Lowe., *J. Am. Acad. Dermatol*., **1998**, 38, 681-686.
[6] Janine G Einspahr, Steven P Stratton, G. Timothy Bowden, David S Alberts, *Crit. Rev. Oncol. Hematol*., **2002**, 41, 269-285.
[7] The International Agency for Research on Cancer Working Group on Artificial Ultraviolet (UV) light and skin cancer, *Int. J. Cancer*., **2007**, 120(5), 1116-1122.
[8] S. Chummun, Neil R McLean, *Surgery*., **2011**, 29(10), 529-533.
[9] Suraj R Wasankar, *Int. J. Pharm. Sci. Res*., **2012**, 3(11), 4461-4474.
[10] M.D. Owais, M. Alam, A. Khan, *Mol. Med*., **2007**, 13, 443-451.
[11] Z. Shan-Shan, Q. Du, C. De-Ying, *J. Chi. Pharma. Sci*., **2007**, 16, 116-118.
[12] A.D. Bangham, Liposomes, Marcel Dekker, NY, USA, **1983**, 1-26.
[13] B. Malaekeh-Nikouei, S. Golmohammadzadeh, M. Hosseini, H. Nassirli, *Int. J. Comp. Pharma.*, **2011**, 6(04).
[14] M. Paola, F. Maestrlli, M.L. Gonzalez-Rodriguez, *Eur. J. Pharm. Biopharm.*, **2007**, 6786-6795.
[15] R. New, Liposomes a practical approach, Oxford University Press, **1990**.
[16] X. Liu, Q. Yang, N. Kamo, J. Miyake, *J. Chromatogr*., **2001**, 913, 123-131.
[17] B. Ruozi, G. Tosi, F. Forni, A.M. Vandelli, *J. Liposome. Res*., **2005**, 15, 175-185.
[18] K. Adler, J. Schiemann, *Micron Microscopica Acta*., **1985**, 16(2), 109-113.
[19] V.A. Rane, B.B. Patel, *Int. J. Pharm. Sci. Res*., **2015**, 6(1), 294-299.
[20] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, *J. Natl. Cancer Inst*., **1990**, 82, 1107.
[21] V. Vichai, K. Kirtikara, *Nat. Protocols*., **2006**, 1, 1112-1116.