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Research Article

Development and Characterization of Methotrexate-bearing Emulsomes for Management of Skin Cancer

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ABSTRACT

The present study is aimed to manage skin cancer via topical route, because of conventional chemotherapy having lots of drawbacks like drugs introduced into the body through an injectable route thus drugs are distributed between normal cell and cancerous cell than its leading to unaccepted side effects and requires high dose of drug. In this paper, main focus onto overcome these drawbacks, emulsomes were developed loaded with methotrexate bio-active agent. A 3^2 factorial design approach was employed firstly for the formulation development of emulsomes for different concentration of lipid content which was phosphatidyl choline, cholesterol and solid core lipid. Developed methotrexate loaded emulsomes were characterized for particle size, polydispersity index, zeta potential and entrapment efficiency and results revealed 127.21 ± 1.11 nm, 0.216, -24.21 ± 1.09 mv, and 81.37 ± 0.8%, respectively. Thus optimized lipid content was further used for optimization of type and concentration of surfactants9 and sonication time. Hence 18 formulations of emulsomes were developed and characterized for particle size, polydispersity index, zeta potential and entrapment efficiency and result 126 ± 1.09 nm, 0.215, -24.81 ± 1.19 mv, and 85.17 ± 0.3%, respectively. These formulations were tested for their *in-vitro* drug release studies and release data show the value of $t_{½}$ for formulations EM4F11, EM4F18 and EM4F8 was more than 12 h and they were retarded drug release up to 85% till 24 h to get better-sustained drug release profile. Then stability study was performed for formulation EM4F11 at 2 ± 2°C, 25 ± 2°C and 60% RH and 40 ± 2°C and 75% RH and data identified that the formulations stored at temperature 25 ± 2°C and 60% RH and it was less than 2% degradation at the end of six months. Hence developed emulsomes formulation might represent a promising system for improving the bioavailability of lipophilic drugs. Moreover, emulsomes produce sustained drug release, which is beneficial clinically. Thus the presented system adds additional benefits to the well-known opportunities to the conventional delivery system and is helpful in minimizing the side effects of anti-cancerous drugs and well suited for the management of skin ailment.

Introduction

Skin provides the largest interface between the human body and the external environment. Most important function of the skin is to regulate what enter the body via skin and what exit. Skin exhibits barrier properties due to the presence of stratum corneum.[1] Skin cancer is the most common malignant disease found particularly in Caucasians. More than a million new cases are reported worldwide each year. The various types of skin cancer are named after the cells they originate from and their clinical behavior. The most common types are basal cell carcinoma (BCC), squamous cell carcinoma (SCC) (together referred to as non-melanocytic skin cancers (NMSC)) and malignant melanoma (MM).[2,3] Human skin is repeatedly exposed to ultraviolet radiation that influences the function and survival of many cell types and is regarded as the main causative factor in the induction of skin cancer. Ionizing radiation, pollutants, chemicals and occupational exposures are also linked with skin cancers.[4]

Cancer can be managed with anticancer drugs, currently between 2–3 million non-melanoma skin cancers and 1,

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32000 melanoma skin cancer occur globally each year. In 2021, American cancer society estimated new cases 106110 of melanoma of skin and approximately 7180 patients died of disease. Conventional therapy includes surgery and chemotherapy, but many of the therapeutic agents have undesired side effects.[5]

Methotrexate is a folic acid analogue is one of the oldest and highly efficacious antineoplastic drugs, which acts by inhibiting dihydrofolate reductase (DHFRase) - blocking the conversion of dihydrofolic acid (DHFA) to tetrahydrofolic acid (THFA). Utilizing the folate carrier it enters into cells and is transformed to more active polyglutamate form by the enzyme folypolyglutamate synthase (FPGS). Methotrexate has cell cycle specific action, i.e., kills cells in S phase. It exerts major toxicity on bone marrow; low doses (given repeatedly) cause megaloblastic anaemia, but high doses produce pancytopenia. Mucositis and diarrhea are common side effects. Desquamation and bleeding may occur in GIT.[6] To overcome the side effects of anti-cancerous drug an alternative non-parenteral route of administration can be used to manage skin cancer. Topical route of administration for therapeutic agent provides direct contact with the desired site of the skin. Dosage forms via topical routes, i.e., ointment, cream and gel are insufficient to provide medicament at target site usually in higher concentration. Novel drug delivery system like liposomes,[7] nanocapsules[8] and nanoparticles[9] etc. helps to target the tissues through skin layer and can provide better therapy for skin cancer.[10,11]

Encapsulation of anti-cancerous drugs into vesicles provides advantages such as high stability, reduction in drug dose, minimizing the side effects and significantly reducing the cost of treatment. Novel drug delivery systems are particularly important for targeted delivery of drugs because of their ability to localize the activity of drug at the cancerous cells thereby lowering its concentration at the normal cell.[12] Emulsomes is a novel lipoidal vesicular system with an internal solid fat core surrounded by a phospholipid bilayer. Emulsome is an advanced nanocarrier technology for poorly aqueous soluble drugs. It possesses both emulsion and liposome features.[13] Emulsome represents lipid-based drug delivery systems with various therapeutic applications, particularly for poor aqueous soluble drugs.[14] Besides the other vesicular formulations, emulsomes are much more stabilized and nano range vesicles. It is a new emerging delivery system and therefore could play a fundamental function in effectively treating the life-threatening ailment.[15] Emulsomal formulations composed of solid lipid core material and stabilized by cholesterol and soya lecithin. The drug is loaded followed by sonication to produce emulsomes of small size. The polymer used for core material should be solid at room temperature (25°C). The high soya lecithin concentration stabilized the emulsomes in the form of O/W emulsion.[16] Emulsomes can be used to administer lipophilic or hydrophilic compounds via parenteral, oral, ophthalmic, rectal, veginal, and intranasal routes. They can include lipophilic medicines in their cores while encapsulating hydrophilic bioactive molecules in the aqueous compartment of the outer phospholipids layer.[17,18] After IV injection, liver and spleen macrophages can passively absorb emulsomes from the bloodstream due to their colloidal structure. For the treatment of liver illness, this method of absorption is beneficial. Because of their excellent biocompatibility, biodegradability, stability, high entrapment efficiency and sustained drug release, emulsomes may therefore be effective drug delivery devices.[19]

Emulsomal entrapment of Methotrexate showed beneficial effects over the unentrapped drugs, such as decreased tumor proliferation rate and higher plasma levels accompanied by slower elimination.[20,21] The present study aims to minimize the side effect and dose of anti-cancerous drug methotrexate as well as enhance the availability of drug at skin for which emulsomes were developed due to Lipoidal nature of emulsomes also beneficial for skin cancer management through topical delivery.

Table 1: The matrix design for independent variables and their levels for optimization of lipid content.

Levels	*Cholesterol (CH) X_1*	*Phosphatidylcholine (PC) X_2*	*Tristearin (TS) X_3*
-1	30	50	50
0	40	60	100
1	50	70	150

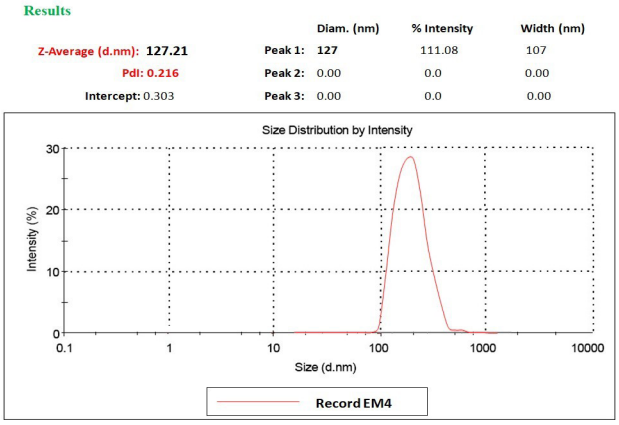

Fig. 1: Particle size distribution and polydispersity Index (PDI) of emulsomes (EM4).

Materials And Methods

Methotrexate was purchased from Neon Pharmaceutical Ltd. Mumbai India. Phospholipid (Soya Lecithin) and Tristearin was purchased from Himedia Laboratories Pvt. Ltd. Tween 80 and other chemicals and reagents were of

analytical grade and were used as they were procured. Distilled water used in all the experiments.

Development of Emulsomes

Methotrexate loaded Emulsomes were prepared by the cast film method[22] with modification. Tristearin (TS), Phosphatidylcholine (PC) and Cholesterol (CH) in different ratio were dissolved in minimum quantity of chloroform containing 3 to 4 drops of methanol in 500 mL round bottom flask. The organic solvent was evaporated under reduced pressure using a rotary flash evaporator at 40°C and kept aside for overnight flushed with nitrogen gas.

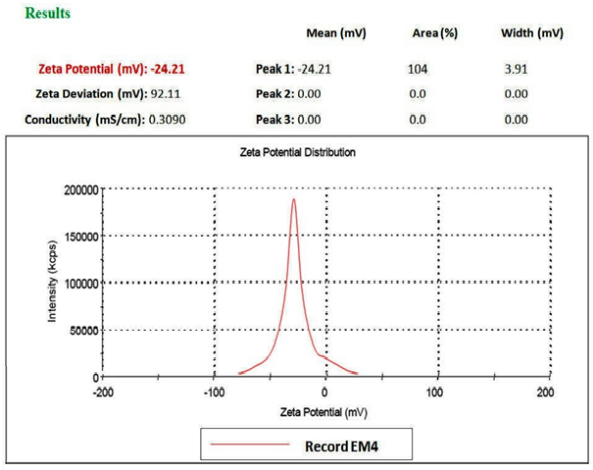

Fig. 2: Zeta potential (mV) of emulsomes (EM4).

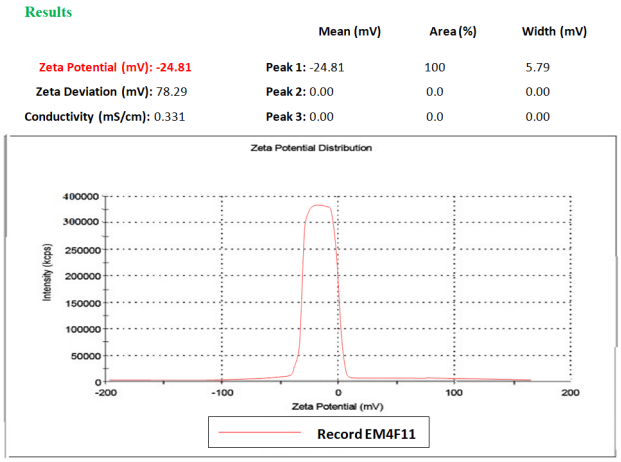

Fig. 4: Zeta potential (mV) of emulsomes (EM4F11).

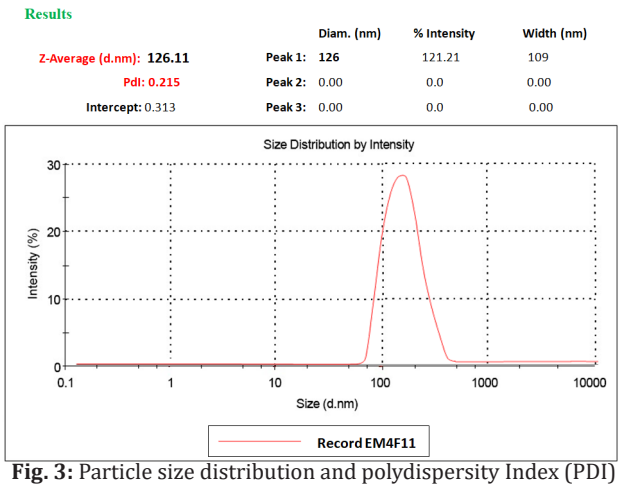

Fig. 3: Particle size distribution and polydispersity Index (PDI) of emulsomes (EM4F11).

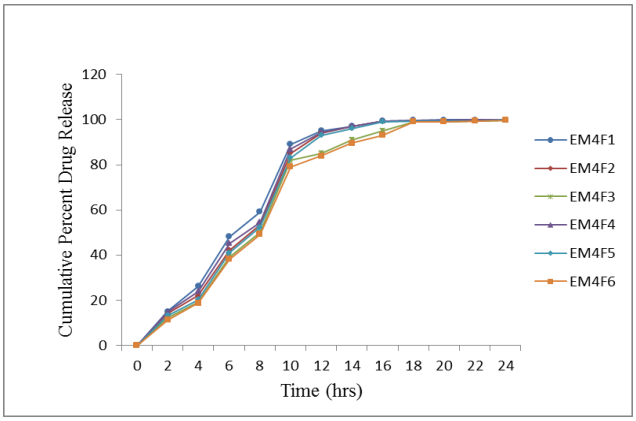

Fig. 5: Comparative drug release from emulsome formulations (EM4F1 - EM4F6).

Table 2: Matrix design to optimized lipid content for three levels and characterization parameters of emulsomes.

Formulation code	*Factors*			*Particle size (nm)*	*Zeta potential (mV)*	*PDI*	*Entrapment efficiency (%)*
	CH (mg) Xa	*PC (mg) Xb*	*TS (mg) Xc*				
EM1	50	0	150	122.01 ± 1.02	-20.12 ± 1.02	0.216 ± 0.08	76.53 ± 1.3
EM2	0	70	150	123.03 ± 1.04	-20.18 ± 1.05	0.215 ± 0.02	79.78 ± 1.1
EM3	50	50	100	129.03 ± 0.08	-22.91 ± 1.03	0.221 ± 0.27	71.86 ± 1.1
EM4	30	70	100	126.11 ± 1.11	-24.21 ± 1.09	0.216 ± 0.05	81.37 ± 0.8
EM5	50	60	50	130.12 ± 1.06	-22.12 ± 1.08	0.224 ± 0.07	68.32 ± 1.2
EM6	30	60	150	128.21 ± 1.02	-23.01 ± 1.03	0.219 ± 0.05	76.53 ± 0.9
EM7	40	70	50	131.22 ± 1.09	-22.02 ± 1.04	0.229 ± 0.04	63.05 ± 0.8
EM8	40	50	150	130.81 ± 1.08	-22.16 ± 1.05	0.228 ± 0.07	65.12 ± 1.2

values are expressed as mean ± SD (n=3).

Table 3: Experimental layout for emulsomes (EM4) for types of surfactant, concentration of surfactants and sonication time and characterization parameters.

Formulation code	Lipid content (X_1)			Surfactants (X_2)		Sonication Time (X_3) (Min.)	Particle size (nm)	Zeta potential (mV)	PDI	Drug Entrapment (%)
	CH (mg) Xa	PC (mg) Xb	TS (mg) Xc	Tween 20 (%)	Tween 80 (%)					
EM4F1	30	70	100	5	0	5	155.13 ± 1.11	-20.01 ± 0.91	0.229 ± 0.94	61.25 ± 0.6
EM4F2	30	70	100	10	0	5	154.21 ± 1.07	-20.11 ± 0.89	0.229 ± 0.09	63.05 ± 0.8
EM4F3	30	70	100	15	0	5	152.11 ± 1.05	-20.09 ± 1.01	0.228 ± 0.99	65.12 ± 1.2
EM4F4	30	70	100	0	5	5	130.12 ± 1.06	-20.03 ± 0.91	0.228 ± 0.11	62.75 ± 0.2
EM4F5	30	70	100	0	10	5	152.99 ± 1.08	-20.18 ± 0.91	0.227 ± 0.98	64.15 ± 0.3
EM4F6	30	70	100	0	15	5	150.18 ± 1.15	-21.08 ± 1.01	0.227 ± 0.05	68.32 ± 1.2
EM4F7	30	70	100	5	0	10	130.21 ± 1.12	-22.12 ± 1.08	0.224 ± 0.07	71.81 ± 0.9
EM4F8	30	70	100	10	0	10	127.21 ± 1.11	-24.21 ± 1.09	0.216 ± 0.95	81.37 ± 0.8
EM4F9	30	70	100	15	0	10	129.01 ± 0.91	-22.91 ± 1.03	0.219 ± 0.05	78.13 ± 1.1
EM4F10	30	70	100	0	5	10	129.99 ± 0.65	-22.16 ± 1.05	0.221 ± 0.27	76.03 ± 0.3
EM4F11	30	70	100	0	10	10	126.11 ± 1.09	-24.81 ± 1.19	0.215 ± 0.02	85.17 ± 0.3
EM4F12	30	70	100	0	15	10	128.11 ± 1.03	-23.01 ± 1.03	0.217 ± 0.35	79.98 ± 1.2
EM4F13	30	70	100	5	0	15	131.78 ± 0.88	-21.91 ± 0.95	0.229 ± 0.07	78.11 ± 0.9
EM4F14	30	70	100	10	0	15	130.19 ± 1.11	-22.02 ± 1.04	0.228 ± 0.05	80.17 ± 0.7
EM4F15	30	70	100	15	0	15	155.13 ± 1.11	-23.01 ± 0.96	0.226 ± 0.95	82.97 ± 0.8
EM4F16	30	70	100	0	5	15	154.21 ± 1.07	-21.98 ± 1.15	0.228 ± 0.97	79.03 ± 0.9
EM4F17	30	70	100	0	10	15	152.11 ± 1.05	-22.81 ± 1.11	0.227 ± 0.23	81.17 ± 1.1
EM4F18	30	70	100	0	15	15	130.12 ± 1.06	-23.21 ± 1.06	0.225 ± 0.05	83.07 ± 0.1

Bold value indicate the optimized value, for each formulation (mean ± SD) n=3.

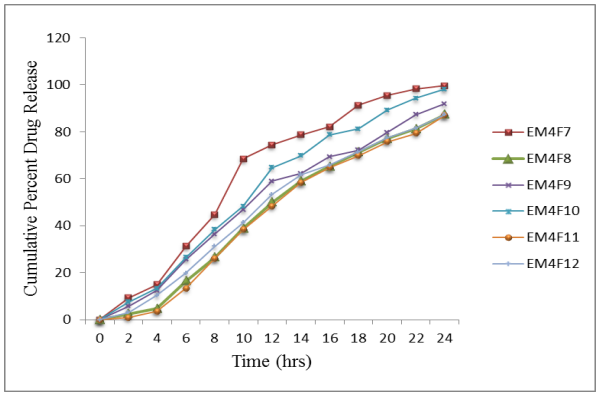

Fig. 6: Comparative drug release from emulsome formulations (EM4F7 - EM4F12).

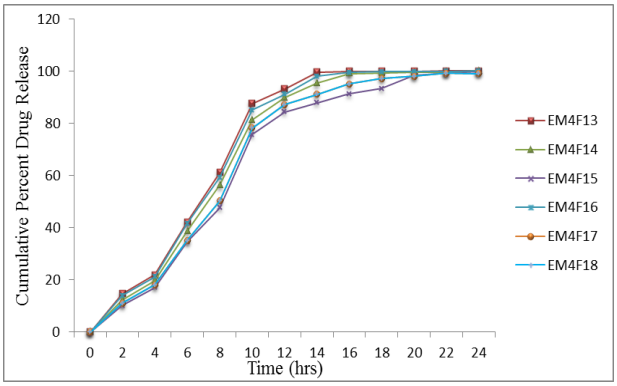

Fig. 7: Comparative drug release from emulsome formulations (EM4F13 - EM4F18).

The dried film was hydrated with 10 mL phosphate buffer solution pH 7.4 containing 10 mg of Methotrexate with slow rotation of round bottom flask at temperature 37 ± 1°C. Hydration of dry film facilitates swelling of lipids and formation of emulsomes vesicles dispersed in the aqueous phase. The mixture was sonicated by probe sonicator to obtain nano sized emulsomes vesicles. The free unentrapped drug was removed by passing the dispersion through a sephadex G-50 column using mini centrifuge column method.[23,24]

Optimization of Emulsomes

Optimization of the formulation was done based on a 3^2 factorial design method and incorporating interactive and polynomial terms to evaluate the response as dependent variables (Equation 1):

$$Y = b0 + b1X1 + b2X2 + b12X1X2 + b11X1X1 + b22X2X2 \quad (1)$$

Where Y is the dependent variable, b_0 is the arithmetic mean response of all runs, and bi (b_1, b_2, b_{12}, b_{11} and b_{22}) is the estimated coefficient for the factor Xi (X_1, X_2, X_{12}, X_{11} and X_{22}). X_1 and X_2 represent the data of each factor

Table 4: *In-vitro* anticancer activity (IC_{50}) of the optimized emulsomal gels against cancer cell lines

S. no	*Formulations*	*IC 50*
1	Methotrexate solution	58.50
2	Methotrexate loaded emulsomes (EM4F11)	41.12
3	Plain Emulsomes	50.25

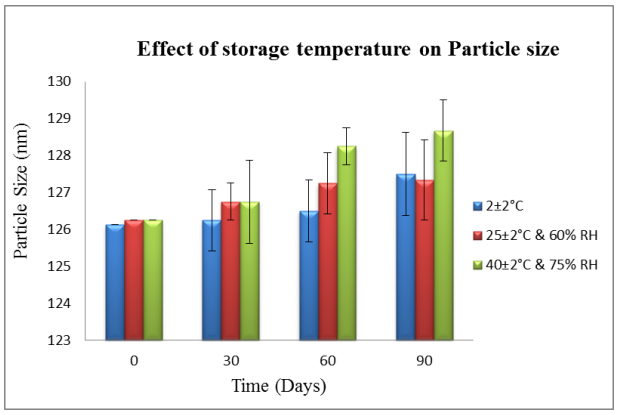

Fig. 8: Bar graph showing particle size of emulsomes (EM4F11) at temperature 2 ± 2°C, 25 ± 2°C & 60% RH and 40 ± 2°C & 75% RH.

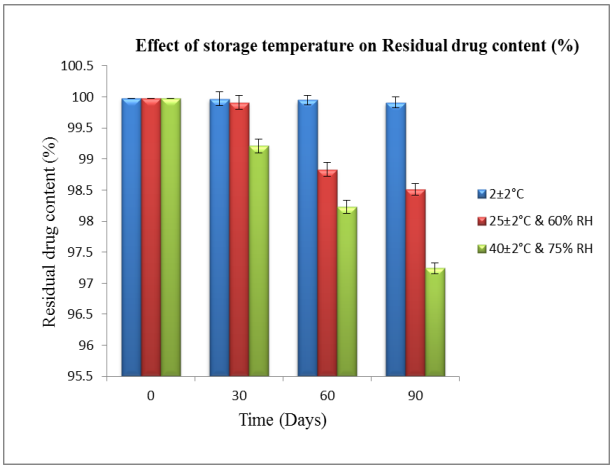

Fig. 9: Bar graph showing residual drug content of emulsomes (EM4F11) at temperature 2 ± 2°C, 25 ± 2°C & 60% RH and 40 ± 2°C & 75% RH.

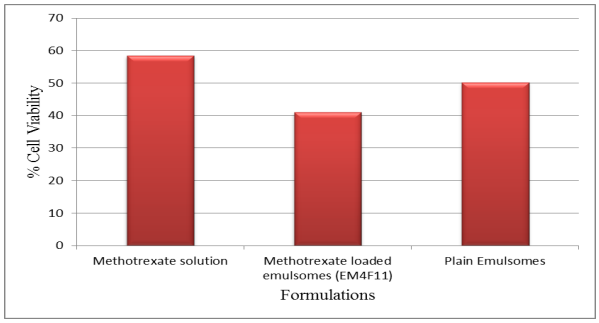

Fig. 10: *In-vitro* anticancer activity (IC_{50}) of the optimized emulsomes against cancer cell lines.

at a time from low-medium-high values. The interaction terms X_1X_2 indicate how responses vary when two factors are changed simultaneously, whereas polynomial terms (X_{12} and X_{22}) are introduced to investigate nonlinearity where b_1 is the estimated coefficient for the factor X_1, while Y_1 is the measured response. The coefficients for the linear effects (b_1 and b_2), interaction (b_{12}) and the quadratic effects (b_{11} and b_{22}) have been determined from the results of the experiments. A comparison between the experimental and predicted values of the responses is also presented in terms of percent accuracy.
Bias was calculated by the following equation:

$$\% \text{ Accuracy } = \frac{\text{Predicted value - Experimental value}}{\text{Predicted value}} * 100$$

Predicted value

Firstly three variables were selected as independent variables to optimized lipid content like, Cholesterol (CH) (X_1), Phosphatidylcholine (PC) (X_2) and Tristearin (TS) (X_3) at three levels upper, middle and lower level (-1, 0, +1) shown in Table 1. The effect of independent variables (X_1, X_2 and X_3) were studied on dependent variables i.e. Particle Size (Y_1), Zeta Potential (Y_2), Poly Dispersity Index (Y_3), percent Entrapment Efficiency (Y_4).

Characterization of Emulsomes

Optimized emulsomes formulations were characterized for size and size distribution, shape and surface morphology, percent entrapment efficiency, zeta potential and *in-vitro* drug release.

Size and Size Distribution

The average vesicle size and size distribution were determined by photon correlation spectroscopy using zeta sizer Nano-series using a flow-through cell at 25°C. The samples of dispersion were kept in polystyrene cuvettes and observations were made at 90° fixed angle every time. Maintaining constant size and size distribution for a prolonged period of time is an indication of the stability of emulsomes.

Shape and Surface Morphology

Prepared vesicles were characterized for shape and surface morphology by Transmission Electron Microscope (Tecnai G2, Hillsboro Oregon, USA) using copper grid coated with carbon film. The phosphotungstic acid (PTA 1% w/v) used as a negative stain. The sample (10 µL) was placed on the grids and allowed to stand at room temperature for 90 sec and excess of the fluid was removed by touching the edge of filter paper. All samples were examined under a Transmission Electron Microscope at an acceleration voltage of 100 kV and photomicrographs were taken at 1400X.

Percent Entrapment Efficiency

The percent entrapment efficiency was determined after separation of the unentrapped drug by the use of mini column centrifugation method.[23-24]

Preparation of Sephadex G-50 Column

Sephadex G-50 (1.2 gm) was swelled in 20 mL of 0.9% NaCl solution for 5 hours at room temperature with occasional shaking. The gel was formed and it was stored at 4°C overnight. To prepare the mini-column, the hydrated gel was filled up to top in the barrel of 1-mL disposable syringe, plugged with whatman filter pad. Then barrel was placed in the centrifuge and centrifuged at 2000 rpm for 3 min to remove saline solution. Eluted volume was removed from the centrifuged tubes and exactly 0.2 mL of emulsomal suspension (undiluted) was applied drop wise on the gel bed in the center. Columns were again centrifuged at 2000 rpm for 3 min to expel and remove void volume containing emulsomes to the centrifuge tubes. Elute was remove and 0.25 mL of saline was applied to each column and centrifuge again. The amount of drug entrapped in the vesicle was then determined by disrupting the vesicle using 1-mL of 0.1% v/v Triton-X 100, filtering it and the drug content was determined using UV-vis spectroscopy at 304 nm. The percentage efficiency was determined by following equation:

$$\text{Percent drug Entrapment Efficiency} = \frac{\text{Amount of entrapped drug}}{\text{Total amount of drug}} \times 100$$

The formulation EM4 was considered the finest formulation based on characterization, i.e., Particle size, Zeta potential, Poly dispersity index and percent entrapment efficiency tabulated in Table 2, respectively. Hence, 18 formulations of EM4 were prepared for the optimization of types of surfactant, concentration of surfactant and Sonication time by applying factorial design approach and again characterized for particle size, zeta potential, PDI and percent Entrapment Efficiency. All characterization parameters enlist in Table 3.

In-vitro Drug Release Studies

In-vitro drug release studies of methotrexate-loaded vesicles were performed using dialysis method with cellophane membrane of 10 kDa molecular weight (sigma, MO, USA). The formulation (2 mL) was placed in the donor compartment and the receiver compartment was filled with 35 mL dialysis medium (saline phosphate buffer pH 7.4) stirred continuously at 100 rpm using magnetic stirrer at 37 ± 1°C. At 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 hours time intervals, 5 mL of solution from the receptor compartment was pipette out and immediately replaced with fresh 5 mL phosphate buffer solution to maintain equal volume level each time. All samples were withdrawn in triplicate. Samples were analyzed for amount of methotrexate release by disrupting the vesicle using 1-mL of 0.1%v/v Triton-X 100, filtering it and the drug content was determined using UV-vis spectroscopy at 304 nm. The obtained values were fitted into zero order, first order, Higuchi matrix and Korsmeyer-Peppas model.[22]

Determination of Cell Viability by MTT Method

Cell Culture Procedure

Cancer cell lines murine melanoma (B16F10) was used for the testing *in-vitro* anticancer activity. Cancer cell lines were purchased from National Center for Cell Science, Pune. The target cells used in this experiment were a continuous line of murine melanoma (B16F10), The cells were maintained at 37°C under 5% CO_2 and 100% humidity in DMEM and supplemented with 10% fetal calf serum and antibiotics (200 µL/mL penicillin G, 200 µg/mL streptomycin and 2 µg/mL fungizone). The medium was changed every other day. When the cells reached confluence, they were detached using 0.2 % (w/v) trypsin and transferred to new culture flasks. After sufficient growth for experimentation, the cells were trypsinized and plated in 96-cluster well culture plates at a concentration of 1×10^4 cells/well. Each well contained 100 µL of cell suspension and the plates were incubated for 24 hours at 37°C under 5 % CO_2 to obtain a monolayer culture. After 24 hours of incubation, the old medium was removed from each well. Then, add 100 µL of methotrexate-loaded emulsomes at concentrations of 0.01, 0.1, 0.25 and 0.5%; the positive control; or negative control was inserted into a 96-cluster well culture plate (8 wells/test material). Two 96-cluster well culture plates were separately prepared to evaluate cell viability using MTT assays. The experiments were repeated in triplicate. Following a 24 hours incubation period at 37°C under 5% CO_2, the cell viability of both plates was assessed.[25]

MTT Assay

The test materials were removed from each well of the first plate. Then, 50 µL of MTT reagent (5 mg/mL) was added and incubated for 2 hours at 37°C in the CO_2 incubator. The MTT solution was then discarded and 100 µL of isopropanol was added. The plates were placed on a shaker to solubilize the formations of purple crystal formazan. The absorbance was measured using a microplate reader at a wavelength of absorbance at 304 nm. Anticancer activity of prepared optimized formulations was performed *in-vitro* by cell viable assay method.

Storage Stability Studies

The developed emulsomes (EM4F11) stored at temperature 2 ± 2, 25 ± 2°C and 60% RH and 40 ± 2°C and 75% RH for a period of 90 days as per the ICH guidelines. Instabilities in emulsomes formulations are caused by hydrolysis or oxidation of the Phospholipid molecules and are indicated by leakage of the encapsulated drug and alteration in vesicle size due to fusion and aggregation. These

formulations were examined by any alteration in particle size and residual drug content at regular intervals, i.e., 30, 60 and 90 days.

Result And Discussion

The development of emulsomes, based on A 3^2 factorial design approach was employed for lipid content, i.e., Phosphetidylcholine (PC): Cholesterol (CH): Tristearin (TS) (Table 1). The result of all independent variables was evaluated for Particle size and size distribution, shape and surface morphology, %entrapment efficiency and zeta potential. It was found that among all the formulations EM4 having Phosphetidyl choline: Cholesterol: Tristearin (70:30:100) have small particle size 127.21 ± 1.11 nm, zeta potential -24.21 ± 1.09, polydispersity index 0.216 ± 0.05 and percent entrapment efficiency 81.37 ± 0.8 (shown in Table 2) and EM4 was selected formulation. The optimal hydration temperature was 35°C, with an optimum hydration time of 30 minutes. The result concluded that as the concentration of solid lipid varies and the amount of phosphetidyl choline increase the particle size increase, thus increase the PDI and zeta potential shown in Table 2 and Figs. 1 and 2, respectively. The charge of vesicles size and drug entrapment efficiency also correlated with each other due to the thickness of wall of lipid layers. The formulation EM4 has more than 80% drug entrapment.

All the result of dependent variables concluded that the formulation EM4 was selected for the optimization of effect of surfactants in different concentration and sonication time, then 18 formulations were prepared using 33factorial design and characterized for particle size and size distribution, shape and surface morphology, %entrapment efficiency and zeta potential. Surfactant concentration and type of surfactants optimized for 5 to 15% and optimization of sonication time 5 to 15 minutes also done. Emulsomes were optimized for different types and concentrations of surfactants and tween 20 and tween 80 as the concentration increased particle size was increased and entrapment efficiency also increased, but for particular sonication time and concentration emulsomes showed maximum entrapment efficiency. The particle size may be affected by the sonication time. So, in terms of particle size and entrapment efficiency sonication time was optimized. With longer sonication times, particle size was reducing due to the significant energy input during sonication. When the sonication time was raised from 5 to 10, entrapment effectiveness also improved. Moving and packing of the PC bilayers may be the cause of the increased drug entrapment. Further increasing the sonication period to 10 and 15 minutes revealed a striking decline in the effectiveness of drug entrapment. Sonication converts multi-laminar vesicles to unilaminar vesicles and the process is also useful in overcoming the problem of agglomeration of vesicles. It was found that formulation EM4F11 has small particle size 126.11 ± 1.09 nm, zeta potential -24.81 ± 1.19, polydispersity index 0.215 ± 0.02 and percent entrapment efficiency 85.17 ± 0.3 (shown in Table 3).

In-vitro Drug Release Studies

The *in-vitro* drug release studies of all emulsomes formulations were carried out using dialysis membrane for 24 hours in phosphate buffer solution pH-7.4 and samples were analyzed using UV-vis spectroscopy at 304 nm. The release of methotrexate in phosphate buffer solution pH-7.4 from emulsomes was slow and steady and ranged from 11.23 to 99.74%. EM4F1 shows the highest percentage of drug release among in EM4F1 to EM4F6 formulations (Fig. 5). Drug release data of EM4F7 to EM4F12 formulations and for EM4F13 to EM4F18 show in Figs. 6 and 7, respectively. Out of all the formulations, EM4F11 showed the highest percentage drug release in the medium. No burst release was observed in any of the formulations. The formulations EM4F1 to EM4F18 showed the value of n>0.5, followed Fickinan release and supercase II transport mechanism. The value of $t_{½}$ of EM4F11 was indicated more than 12 hours and were retarded drug release up to 85% till 24 hours to get better sustained drug release profile. It was shown better controlled release mechanism of prepared drug delivery system.

Storage Stability Studies

The stability of a formulation is known as the power of the materials to stay on inside definite restrictions over a fixed phase of time and is known as product shelf life. The stability studies of the emulsomes were evaluated per the ICH guidelines, presented in Tables 4 and 5, and graphically in Figs. 8 and 9. The data identified that the EM4F11 stored at temperatures of 25 ± 2°C and 60 ± 5% RH, informed that it was less than 5% degradation at the end of three months. It may indicate that the formulations could provide excellent stability.

Determination of Cell Viability by MTT Method

The purchased cells were plated or incubated in 96-multiwell plates (10^4 cell/well) for 24 hours. All the synthesized compounds were dissolved in dimethyl sulphoxide before being evaluated as anti-cancerous properties. The solutions of all formulations in different concentrations were added to the cell monolayer. The viability of the cells was evaluated in two different doses of the formulation (100 and 10 µM) in triplicate for 48 h by using MTT assay. The viability of the cells was calculated by the MTT assay method. MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used in the concentration of 5 mg/mL in assay.

The optimized formulation EM4F11 was tested for its *in-vitro* anticancer activities against murine melanoma

(B16F10) using the MTT assay. The assay results expressed as IC_{50} (µM) were summarized in Table 4. Here, the IC_{50} value represents the concentration of a compound resulting in 50% inhibition of cell growth after 48 hours incubation and is the average of three independent experiments. It is observed that the results show potential for methotrexate loaded emulsomes and methotrexate solution so, they were selected for the determination of IC_{50} values, *i.e.,* concentration needed to inhibit cancer cell by 50% by the treatment of synthesized compounds (Fig. 10).

Conclusion

The incorporation of methotrexate to a lipid-based vesicular drug delivery system could be a way to improve the performance of methotrexate in a topical delivery for deep skin lesions. In the present research work, we have reported the development and evaluation of lipid based nano-sized carrier, emulsomes, as a vehicle for delivery of methotrexate to the skin for management of skin lesions. We successfully prepared methotrexate-loaded emulsomes via a film cast method using factorial design. *In-vitro* release study revealed that a developed formulation could provide sustained release of drug from emulsomes and could provide sustained local concentration of efficient drugs against cancer. The localize delivery system could minimize drug loss, maintain therapeutic drug level at tumor site for extended period, while decreasing harmful side effects and systemic toxicity.

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