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Research Article

PREPARATION AND CHARACTERIZATION OF pH-RESPONSIVE TRANSFEROSOMES FOR TRANSDERMAL DELIVERY OF PACLITAXEL

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ABSTRACT

Transferosomes are phospholipids vesicles as transdermal drug carriers that enhance the therapeutic efficacy of anti-cancer agents with high efficiency. In present study, Paclitaxel (PTX) was derivatized at its 2′-hydroxy function by esterification with amino acids using thin film hydration technique accompanied by sonication. Prepared pH-responsive transferosomes (pRTs) were optimized with drug conjugate: lipid ratio and characterized with respect to size, shape, surface charge, entrapment efficacy pH responsiveness, and drug release. Results of the studies indicated that optimized parameters of selected pRTs (Formulation code: F2S4C2S4) with respect to vesicle size, PDI, zeta potential, percent entrapment efficiency and turbidity were as 147.5nm, 0.187, -13mV, 71.04% and 460 NTU, respectively. In conclusion, the optimized transferosomes has demonstrated prolonged drug release effect at different pH level. The developed and optimized transferosomes containing PTX can be administered transdermally for the treatment of cancer disease with maintaining lower side effect improved patient compliance.

Keywords: Paclitaxel, Trasferosomes, pH-responsive transferosomes

1. INTRODUCTION

Transferosomes are self-optimized aggregates with the ultra-flexible membrane capable of delivery of the drug reproducibly either into or through the skin, depending on the choice of administration or application, with high efficiency. Drug delivery systems based on transferosome enhance the therapeutic efficacy of anti-cancer agents, either by increasing the exposure of tumor cells to the drug, decreasing the normal tissues damage, employing the enhanced permeability and retention effect (EPR) phenomenon or by utilizing the targeting principles. These vesicular transferosomes are several orders of magnitudes more elastic than the standard liposomes and thus well suited for skin penetration [1]. Transferosomes overcome the difficulty of skin penetration by squeezing themselves along the intracellular sealing lipid of the SC for providing selective and effective localization of pharmacological active moiety at pre-identified (eg. overexpressed receptors in cancer) target in therapeutic concentration while restricting its access to non-targets it thus reducing toxicity, maximizing therapeutic index as well as improves the Bio distribution of drug.

The transferosomes components that sustain strong membrane deformation preferentially accumulate, while the less adaptable molecules are diluted at sites of great stress. This dramatically lowers the energetic cost of membrane deformation and permits the resulting, highly flexible particles, first to enter and then to pass through the pores rapidly and efficiently. This behavior is not limited to one type of pore and has been observed in natural barriers such as in intact skin [2]. Also they possess a unique ability to get accommodated with a wide range of solubility and act as efficient carriers for both low as well as high molecular weight drugs, e.g. analgesic, corticosteroids, hormones, anticancer drugs, insulin, protein sets with high entrapment efficiency and a unique advantage of protection of the encapsulated drug, from metabolic degradation [3].

In present study, transferosomes were developed in order to take the advantage of phospholipids vesicles as transdermal drug carrier. These self-optimized aggregates, with the ultra-flexible membrane, are able to deliver the drug reproducibly either into or through the skin, depending on the choice of administration or application, with high efficiency. These vesicular transferosomes are several orders of magnitudes more elastic than the standard liposomes and thus well suited for the skin penetration.

There is provision for this, because of the high vesicle deformability, which permits the entry due to the mechanical stress of surrounding, in a self-adapting manner [4]. Flexibility of transferosomes membrane is achieved by mixing suitable surface-active components in the proper ratios. The resulting flexibility of transferosome membrane minimizes the risk of complete vesicle rupture in the skin and allows transferosomes to follow the natural water gradient across the epidermis, when applied under nonocclusive condition. Transferosomes can penetrate the intact stratum corneum spontaneously along two routes in the intracellular lipid that differ in their bilayers properties [5].

Paclitaxel (PTX) is a potential chemotherapeutic agent used in the treatment of a variety of cancers. It has major side effects related to low water solubility [6]. To improve its water solubility, decrease its toxicity, increase its bioavailability, and achieve a better therapeutic effect, nanobased delivery systems have been developed for PTX, using liposomes, micelles, polymers, inorganic nanoparticles, human serum albumin, and polyethylene glycol-polylactic acid [7, 8]. Further, an albumin-bound PTX nanoparticle formulation (Abraxane®) has been approved by the US Food and Drug Administration to treat metastatic breast cancer and non-small-cell lung cancer [9, 10]. Some previously developed protein-carrier prodrug formulations to enhance the solubility and targeting ability of PTX, have been reported with some limitations, such as the low PTX content and high immunotoxicity [11]. Therefore, there is still a pressing need to develop alternative PTX formulations.

In this work, prepared transferosomes of previously derivatized PTX at its 2′-hydroxy function by esterification with amino acidsare characterized for various parameters. Compared to conventional controlled release formulations; *in situ* forming drug delivery systems possess potential advantages likes imply manufacturing processes and ease of administration.

2. MATERIAL AND METHODS

2.1. Material

DOPE (Dioleylphosphotidylethanolamine), CHEMS (CholesterylHemisuccinate) were kindly offered by Lipoid (Germany), FA-Glu(OtBu)-PTX conjugate. All other solvents and reagents were of analytical grade.

2.2. Formulation of Transferosomes

pH-responsivetransferosomes were prepared using thin film hydration technique accompanied by sonication (24; 5; 25; 26) as per given following steps.

Step I: Formation of lipid film

The round bottom flask was loaded with 20 mg lipids, DOPE and CHEMS in separate molar proportions from 5:5 to 9:1. Tween-80 was selected as the edge activator in the amount of 10 parts of the total lipid weight, i.e. 2 mg in all cases. All of them were dissolved in a mixture of 10 ml of chloroform: methanol (2:1). The resulting solution was evaporated at a temperature of 40°C with a rotation velocity of 50 rpm to place the lipid combination on the flask's internal walls. The film was dried in vacuum oven for an hour, to remove traces of solvents, if present.

Step II: Hydration of lipid film and formation of Transferosomes

The deposited lipid film was hydrated with a solution of (10ml) HEPES buffer (pH 7.4) while rotating the flask at room temperature for 1 hour using rotary evaporator with rotation velocity as outlined in Step I. Required FA-Glu(OtBu)-PTX conjugate has been prepared in previous study already reported [12]. The resulting opaque suspension was permitted to stand at room temperature for 30 minutes.

Step III: Purification of Transferosomes

Opaque suspension of transferosomes was then carried through the Sephadex G-50 column at room temperature to separate synthetic lipid vesicles encapsulating paclitaxel conjugate solution from the non-encapsulated paclitaxel conjugate.

2.3. Optimization of formulations and process variables

2.3.1. Optimization of lipid (DOPE: CHEMS) ratio

Transferosomes optimization without drug and conjugate was the basic selection of best formulations based on optimized lipid ratios considering that pH sensitivity depends on it. Thereforevarious formulations were prepared and evaluated for vesicle size and Polydispersity Index (PDI) parameters.

2.3.2. Optimization of surfactant concentration

Optimization of surfactant concentration was performed on the basis of average vesicle size, percent entrapment efficiency and turbidity. The optimum concentration of surfactants in the lipid parts of the vesicles offers

flexibility of the vesicles (ultra vesicle deformability). The results were as represented in Table 2.

2.3.3. Optimization of PTX conjugate: lipid ratio

To optimize the PTX conjugate to lipid (DOPE: CHEMS) ratio, Tween-80 as an edge activator was take in 2 mg and sonication moment (120 second) were held constant while the drug content was changed at distinct percentage weight ratio, i.e. (5, 10, 15, 20 and 25 %w/w) in distinct formulations, and optimized for vesicle size, zeta potential and percentage drug entrapment.

2.3.4. Optimization of sonication time

Transferosomes with optimum Drug: Lipid proportion was optimized in terms of average vesicle size and trap effectiveness for optimum sonication moment. DOPE: CHEMS: FA-Glu(OtBu)-PTX conjugate optimized ratio was held constant while Sonication time was varied (i.e. 30, 60, 90, 120 and 150 seconds) for distinct formulations. Sonication time was optimized by the formulation's average vesicle size and percent EE.

2.4. Characterization of pRTs

2.4.1. Determination of vesicle size and shape

Transfer vesicles were visualized using a Tecani G2 S-TWIN-T30, Transmission Electron Microscope (TEM), at Punjab University, Chandigarh (Sophisticated Analytical Instrumentation Facility). Transmission Electron Microscope (TEM) with 100 kV accelerating voltage. A drop of the sample was put on a carbon-coated copper grid to leave a thin film on the grid. It was badly colored with 1 percent phosphotungic acid (PTA) before the film dried on the grid. A drop of the staining solution was added onto the film, and the excess of the solution was drained off with a filter paper. The grid was permitted to air dry carefully and samples were viewed on a Transmission Electron Microscope.

2.4.2. Determination of size distribution of transferosomes

The size and size distribution of the vesicles was determined by Dynamic Light Scattering (DLS) using Photon Correlation Spectroscopy (PCS) (DelsaTM Nano) from the Indian Institute of Science Education and Research (IISER), Bhopal. For measurement of vesicle size, vesicular suspension was diluted three times with HEPES buffer (pH 7.4) and measurements were conducted in triplicate.

2.4.3. Determination of surface charge

Transferosomes surface charge was determined by measuring zeta potential. It is the general charge that a lipid vesicle acquires in a specific medium. It is a measure of the extent of repulsion or attraction between particles in general and lipid vesicles in specific. Evaluation of formulations zeta potential can assist predict the stability and *in-vivo* destiny of transferosomes.

Laser Doppler anemometry used a Nanoplus, also called Doppler Electrophoretic Light Scatter Analyzer (DelsaTM Nano) from the Indian Institute of Science Education and Research (IISER), Bhopal, to determine the zeta potential of the vesicles. Nanoplus software calculates the quantity of Doppler shift followed by electrophoretic mobility and zeta potential by combining heterodyne structure and photon correlation process to conduct Fourier transforms (FFT) Slipping rate of the significant portion of the acquired correlation function medium.

2.4.4. Determination of entrapment efficiency

The entrapment efficiency was determined after separation of the unentrapped drug by the mini column centrifugation method. Sephadex G-50 was swollen in deionized water at room temperature with occasional shaking, for at least 5 hr, after which gel was formed and stored at 4°C. To prepare the mini column, Whatman paper pads were placed at the bottom of the 2.5ml syringes barrels filled with gel. Excess water was removed for one minute by centrifugation at 2000 rpm. 1 ml of the product was introduced to the top of the column followed by centrifugation as before and the vesicles were gathered from the filtrate solution. No free drug remained (tested by its lack in the centrifugation after the implementation of the saturated drug solution instead of vesicles) when a saturated drug solution was used instead of the transfers suspension, the entire drug stayed attached to the gel. This verified that after recovering the vesicles, there would be no free drug present. The amount of drug trapped in the vesicles was then determined by disrupting the vesicles by using 1ml of 1 percent Triton X-100 for 1 ml of the sample and analyzing it spectrophotomectrically at 227 nm.

2.4.5. Determination of turbidity of transferosomes

Transferosome turbidity was evaluated using Digital Nephelo–Turbidity Meter (PCI Electronics, India) from the Department of Pharmaceutical Science, Dr. H.S. Gour Central University, Sagar, M. P. The tool was calibrated using the renowned turbidity solution of

Formazine. HEPES buffer (pH 7.4) was taken as blank and the turbidity of pRTs was measured against the blank.

2.4.6. Preparation of Formazine Solution

Hydrazine sulphate (1gm) was dissolved in 100 ml deionized water. Similarly, 10 gm of hexamethylteramine was dissolved in 100 ml water. Both solutions were mixed which gives solution of Formazine having turbidity 4000 NTU (Nephelometric Turbidity Unit). Thus obtained Formazine solution can be suitably diluted to get the solution of desired turbidity. Two ml of the standard formazine solution was diluted up to 20 ml to yield Formazine solution of turbidity 100 NTU which was used for subsequent calibration of the instrument.

2.4.7. Study of pH responsiveness of transferosome membrane

The pH responsiveness of the transferosome membrane was determined by following method. Briefly, 1 ml of the transferosome suspension was combined individually with 4 ml of the solution having different pH i.e. pH 3, pH 5 and pH 7. They were then incubated at a temperature of 37°C and UV spectrophotometer was used to measure the absorbance at 227 nm at the time intervals stated.

2.4.8. Determination of degree of deformability of pRTs

Degree of deformability is a significant and distinctive parameter of transfersomal formulations that distinguishes transferosomes from other vesicular carriers such as liposomes that are unable to cross intact the stratum corneum. The survey of deformability was conducted for transfersomal formulation using the home-built machine [13, 14]. In this research, the flux of vesicles suspensions through a big amount of pores of known size (through a sandwich of various polycarbonate filters with pore diameter between 400 and 100 nm) was motivated by an internal stress. The amount of vesicle suspension, which was extruded during 5 minutes was measured, and the vesicle size and size distribution was monitored by Dynamic Light Scattering (DLS) measurement before and after filtration. The experiment was performed in triplicate, and each sample was analyzed twice. The degree of deformation was calculated using the following formula as reported by Berge Vanden et al [15].

$$D = J \times (R_V / R_p)^2$$

Where,

D = Deformability of vesicle membrane; J = amount of suspension, which was extruded during 5 min; R_V= size of the vesicle (after passes); R_p = pore size of the barrier

2.5. In-vitro drug release study

The in-*vitro* release of pRTs was researched at separate time periods using dialysis bag technique. The dialysis membrane / tube (molecular cut-off point 12000-14000 Da) was immersed in distilled water for 30 minutes, which was then centrifuged at 2000 RPM using sephadex G-50 minicolumn to extract untrapped drug from transferosomes. Transfersomal suspension (1ml) free of any untrapped drug was then taken into a dialysis tube (one end of the tube was tightly tied to thread) and suspended in a beaker containing 20ml of HEPES buffer (pH 7.4 at 37±0.5°C). The beaker's contents were shaken using a magnetic stirrer. Samples were removed periodically and replaced with the same volume of fresh HEPES buffer (pH 7.4) and the amounts of drugs were quantified using a 227 nm UV spectrophotometer.

2.6. Statistical Analysis

All data were statistical analyses by using One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison of the results in the all observations.

3. RESULTS AND DISCUSSION

Transferosome carrier is an artificial vesicle designed to exhibit the characteristics of a cell vesicle or a cell engaged in exocytosis. Transferosomes are optimized ultra deformable lipid supra molecular aggregate able to penetrate mammalian skin due to incorporation of edge activators such as Sodium cholate, Sodium Deoxycholate, Span 80, Tween 80. They consist of at least one inner aqueous compartment surrounded by lipid bilayer (Table 1 and table 2).

Table 1: Optimization of DOPE:CHEMS ratio based on the vesicle sizeand PDI

Formulation Code	Molar ratio of DOPE:CHEMS	Vesicle size (nm)	PDI
F1	5:5	201.2±10.2	0.317±0.098
F2	6:4	142.3±8.6	0.187±0.026
F3	7:3	167.1±9.4	0.324±0.034
F4	8:2	201.5±10.2	0.254±0.088
F5	9:1	248.2±11.8	0.284±0.023

(n=3, data represent Mean±SD)

Table 2: Optimization of surfactant concentration

Formulation Code	Tween-80 (mg)	Vesicle size (nm)	Entrapment Efficiency (%)	Turbidity (NTU)
F2S1	0.5	232.6 ± 11.94	64.32%	395 ± 35.27
F2S2	1.0	207.9 ± 10.84	66.04%	414± 36.82
F2S3	1.5	194.3 ± 9.75	67.09%	416 ± 38.76
F2S4	2.0	160.5 ±8.23	71.05%	460 ± 39.28
F2S5	2.5	190.2 ± 9.14	65.08%	420 ± 38.49

(n=3), data showed mean±SD; S= surfactant

Table 3: Optimization of PTX conjugate: Lipid ratio based on vesicle size, zeta potential and Entrapment efficiency

Formulation Code	PTX conjugate (%w/w)	Vesicle size (nm)	Zeta Potential	Entrapment efficiency (%)
F2S4C1	5	125.6±7.5	-9.92±2.6	62.50
F2S4C2	10	158.4±8.8	-13.07±3.4	69.80
F2S4C3	15	177.2±8.7	-18.01±2.8	68.80
F2S4C4	20	193.9±9.5	-22.3±1.8	67.20
F2S4C5	25	225.6±10.2	-26.12±2.2	66.50

(n=3, Mean±S.D.) C= Drug conjugate

Table 4: Optimization of Sonication time based on vesicle size andentrapment efficiency

Formulation code	Sonication time (seconds)	Vesicle size (nm)	% Entrapment efficiency
F2S4C1	30	210.2±10.3	59.1%
F2S4C2	60	192.8±9.8	61.9%
F2S4C3	90	168.1±8.5	63.5%
F2S4C4	120	147.4±7.5	69.8%
F2S4C5	150	125.4±6.2	55.0%

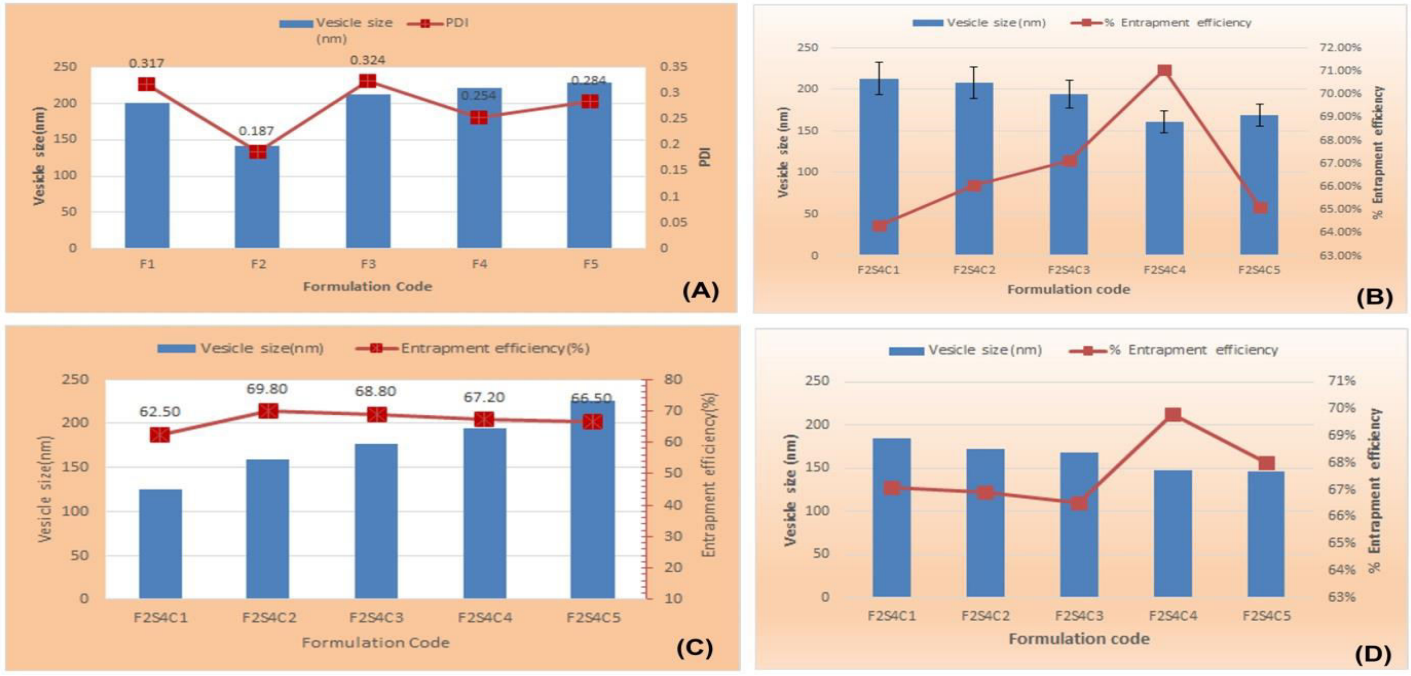

Fig. 1: (A) Size and PDI of various formulations; (B) Size, and % entrapment efficiency of various formulations; (C) Effect of Drug conjugate:Lipid ratio on vesicle size and % drug entrapment; (D) Effect of sonication time on vesicle size and % entrapment efficiency

Transferosome were prepared using a thin movie hydration technique followed by slightly modified sonication. Formulations were prepared using DOPE and CHEMS to impart pH-sensitive property to the Transferosomes. Tween-80 supplied the Transferosomes wall with an elastic nature [16]. All formulations have been optimized in terms of preparation size and PDI. The size of optimized formulation was 142 nm which was below among others and its PDI was best having lowest value 0.187. Similarly, the Entrapment Efficiency was found to be highest for F2S4 indicating highest amount of paclitaxel conjugate encapsulation (Table 3 and table 4; Fig 1). The turbidity profile, which shows the density of vesicles in the formulation, was also found to be maximum in case of F2S4. Hence, it was selected as the best formulation for further characterization. The optimized formulation was then selected to form pH-responsive transferosomes (pRTs). The observed optimized parameters of selected pRTs (Formulation code: F2S4C2S4) with respect to vesicle size, PDI, zeta potential, percent entrapment efficiency and turbidity were as 147.5nm, 0.187, -13mV, 71.04% and 460 NTU, respectively (Table 5; Fig 2).

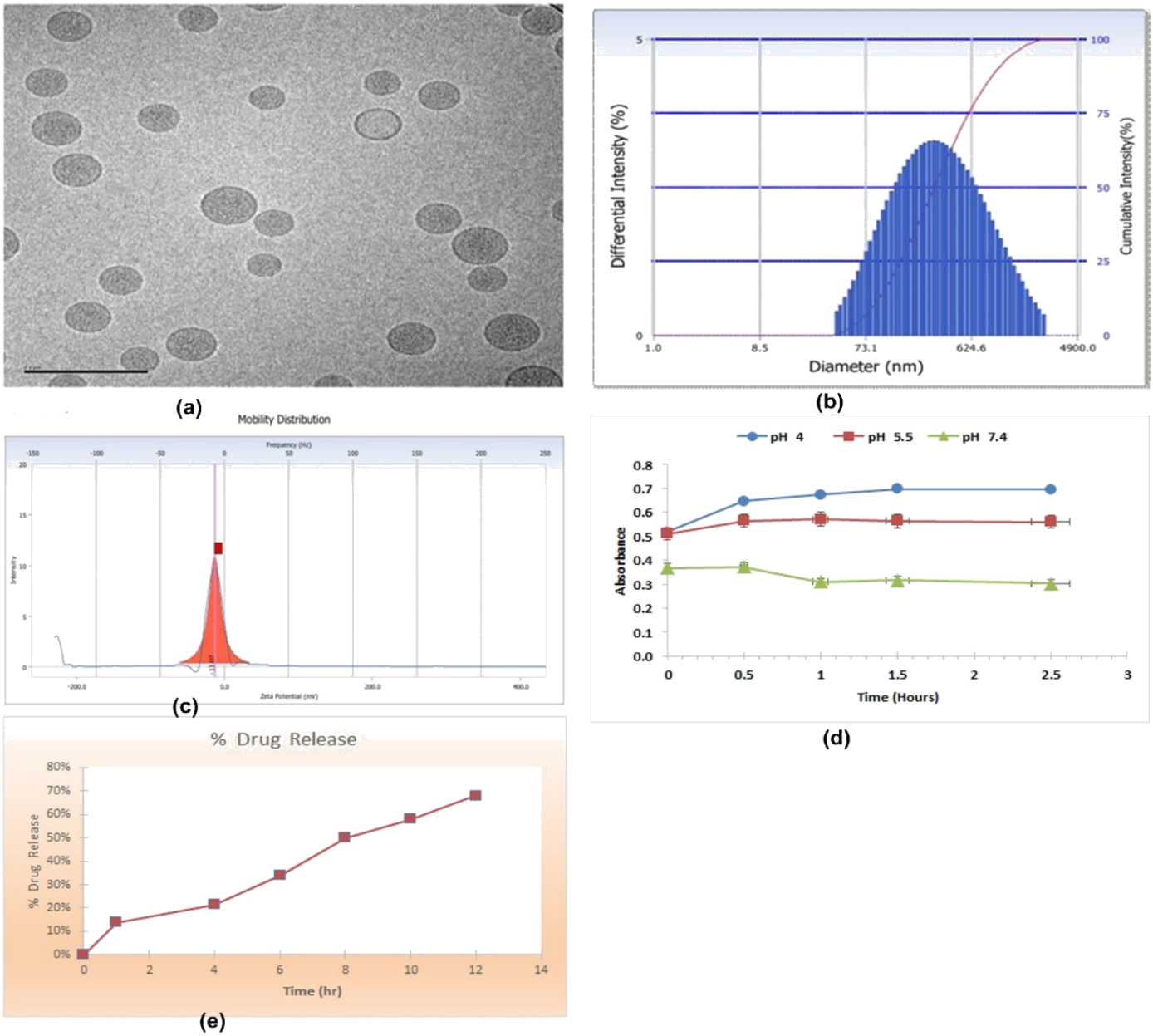

Fig. 2: (a) Transmission Electron Microscope (TEM) of pRTs (b) Vesicle size and size distribution of pRTs (c) Zeta Potential of pRTs (d) pH-responsiveness profile of Transferosome membrane at different pH, (e) Percent Cumulative drug release from optimized formulation

Table 5: Optimized variables for pRTs formulation

Parameters	Optimized values
DOPE:CHEMS	6:4
Amount of Tween	2 mg
PTX conjugate:lipid	10%w/w
Sonication Time	120 second

Results showed that formulation pRTs showed zeta potential was discovered to be -13.07mV (Fig 2). The negative sign shows that there are no aggregates in the formulation and is a monodispersed suspension. Transmission electron microscopy was used for the original characterization of the transferosomes, where samples were negatively colored using 1 percent PTA. Transferosomes emerged as spherical vesicular structures displaying a thin, sensitive membrane that encapsulates the internal aqueous compartment as shown in figure 2. Entrapment efficiency is the percentage fraction of the total drug incorporated in the Transferosomes, acquired for pRTs was 71.04 percent. High flexibility is the key characteristic of all Transferosomal drug formulations compared to the conventional liposome.

Table 6: pH responsiveness of Transferosome membrane

Time (hr)	Absorbance measured at 227nm		
	pH 4	pH 5.5	pH 7.4
0	0.5165 ± 0.0457	0.5107 ± 0.0215	0.3690 ± 0.0552
0.5	0.6466 ± 0.0225	0.5652 ± 0.0236	0.3730 ± 0.0176
1.0	0.6745 ± 0.0387	0.5711 ± 0.0271	0.3097 ± 0.0204
1.5	0.6973 ± 0.0328	0.5633 ± 0.0283	0.3178 ± 0.0173
2.5	0.6961 ± 0.0516	0.5609 ± 0.0252	0.3035 ± 0.0241

The membrane's incredibly elevated flexibility allows Transferosomes to squeeze themselves much smaller than their own diameters even through pores. This is due to the elevated flexibility of the Transferosomes membrane and is accomplished by judiciously merging at least two lipophilic/ amphiphilic parts (phospholipid plus biosurfactant) with adequately distinct packaging features into a single bilayer. The resulting deformability allows Transferosomes to spontaneously pierce the skin and minimizes the danger of full skin vesicle rupture. The negligible size distinction after passing through a sandwich of polycarbonate membranes shows that these vesicles could deform or alter their shape. The rupture of these vesicles during the passage is therefore minimal. Degree of deformability was observed as 24.28 for formulated pRTs in agreement with the studies reported. To assess acid-responsiveness in the vesicles, a Transferosome vesicle was incubated under different pH conditions at 37°C. The absorbance of the Transferosome suspension at different pH (4, 5.5 and 7.4) was monitored at 227 nm over varied time intervals (Table 6).

Table 7: Degree of deformability of Transferosomes

Amount of Transferosomes passed during 5min(J)	Initial size of the vesicle (nm)	Size of the vesicle (after passes) (nm)	Degree of Deformability
8.6 ± 0.5	147.5±1.8	141.6 ± 1.5	24.28

The absorbance rapidly increased in case of pH 4 and pH 5.5. In contrast, no noticeable change in absorbance was seen at pH 7.4. The increase in absorbance at lower pH of 4 and 5.5 is due to the released contents from the vesicles after the change in integrity of vesicle wall under the influence of pH. There was no change in vesicle integrity at pH 7.4, which therefore does not allow the release of encapsulated constituents retaining uniform absorbance.

Table 8: *In-vitro* release profile of pRTs

Time (hr)	% Drug Release
0	0
1	13.9±0.95
4	21.5±1.26
6	33.9±1.93
8	49.9±2.48
10	57.9±2.94
12	68.0±3.72

Mean ± S.D. (n=3)

The *in-vitro* paclitaxel conjugates vesicle release profile in reaction to distinct time periods showed a fast release of paclitaxel conjugate during the first hour and gradually increased up to 12hrs (Table 7 and 8). The *in-vitro* release of pRTs upto 12hr offers a peak release of 67.9 percent.

4. CONCLUSION

The optimized formulation of transferosomes has demonstrated prolonged drug release effect at different pH level. The developed and optimized transferosomes containing PTX conjugate can be used to assess further stability and *in-vivo* estimations for the treatment of cancer disease with maintaining lower side effect improved patient compliance.

5. REFERENCES

1. Schatzlein A, Cevc G. *Champaign AOCS Press.* 1995; 191-209.
2. Mathur M. *Int. Res J Pharm. App Sci*., 2013; **3(4):**40-50.
3. Ghai I, Chaudhary H, Ghai S, Kohli K, Kumar V. *Recent Pat Nanomed.,* 2012; **2**: 164.
4. Bhardwaj V, Shukla V, Singh A, Malviya R, Sharma PK. *Int J PharmaSci Res.,* 2010; **1(3):**12-20.
5. Pandey S, Manish G, Viral D, Jarina F. *Der Pharm. Lett*., 2009; **1:**143-150.
6. Paál K, Müller J, Hegedûs L. *Eur J Biochem*., 2001; **268:**2187-2191.
7. Shan L, Liu M, Wu C, Zhao L, Li S, Xu L, et al. *Int J Nanomedicine*., 2015; 5571.
8. Wang H, Zhao Y, Wu Y, et al. *Biomaterials.* 2011; **32**: 8281–8290.
9. Gupta N, Hatoum H, Dy GK. *Int J Nanomedicine*. 2014; **9**:209-221.
10. Wohl AR, Michel AR, Kalscheuer S, Macosko CW, Panyam J, Hoye TR. *J Med Chem*. 2014; **57:** 2368–2379.
11. Jain S, Sapre R, Jain NK. *Proceeding of 25th Conference of CRS*, U.S.A., 1998; 32 pp.
12. Saxena A, Kori ML. *J. AdvSci Res*, 2019; **10(4) Suppl. 2:**362-369.
13. Berge vanden BAI, Wertz PW, Junginger HE, Bouwstra JA. *Int. J. Pharm.,* 2001; **217**:13-24.
14. Gupta NV, Shivakumar HG. *Iran J Pharm Res.,* 2012; **11(2):**481-493.
15. Patel R, Singh SK, Singh S, Sheth NR, Gendle R. *J Pharma Res Sci.,* 2009; **1(4):**71-80.
16. Garg V, Singh H, Bhatia A. *AAPS PharmSciTech.* 2017; **18**:58.