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In vitro Investigation of Conventional, Chitosan Coated and Electrosteric Stealth Liposomes of Rivastigmine Tartrate for the treatment of Alzheimer's Disease

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ABSTRACT

Objectives: The main objective of the present investigation was to develop and compared conventional, chitosan-coated and electrosteric stealth liposomes of Rivastigmine Tartrate for the treatment of Alzheimer's Disease. **Methods:** The solvent evaporation method was employed to prepare liposomes and optimized by the Design of Experiment approach. The effect of various process parameters was investigated and optimized on for particle size and percentage entrapment efficiency. The compatibility studies were carried out using Fourier Transform Infrared Spectroscopy. The optimized formulations were also characterized by Transmission Electron Microscopy and Atomic Force Microscopy for their surface morphology and *in vitro* percentage drug release study by comparing it with a standard solution of Rivastigmine Tartrate. The compatibility of drug and excipient mixtures was confirmed by Fourier Transform Infrared Spectroscopy. **Results:** The optimized formulation of conventional, chitosan-coated, stealth liposome of Rivastigmine Tartrate showed vesicle size of 111.8, 153.3, 136.3 nm respectively and entrapment efficiency of 75.27±0.8 %, 80.33±0.4 % 78.2±0.2 respectively. Clear surface morphology was observed through surface morphological images. The *in vitro* drug release studies showed a significant difference in percentage cumulative drug release pattern of chitosan-coated and stealth liposomes (81±0.3 % and 76±1.2 %) when compared with the conventional liposomes (69±0.8 %), after 24 h. On subjecting it to stability studies, the liposome preparations did not show any significant changes in particle size and entrapment efficiency. **Conclusion:** The developed formulations (chitosan-coated and stealth liposomes) can deliver the active moiety on the target site for the treatment of AD.

Key words: Alzheimer's disease, Rivastigmine tartrate, Liposome, Chitosan, Stealth.

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INTRODUCTION

Alzheimer's disease (AD) is one of the 6th significant causes of death among people around the age of 65 and more. Approximately 50 million people worldwide are believed to be affected with AD or other form of dementias. AD is a progressive neurodegenerative disorder characterized by its amyloid - β clinical signs, tangles and increased oxidizing stress rates, endothelial dysfunction and considerably reduced acetylcholine levels. This leads to damage in memory, personality and other functions that finally lead to brain failure and death.[1]

AD is triggered by decreased neurotransmitter acetylcholine (ACh) synthesis. Acetylcholinesterase inhibitors (AChEIs) enhance the supply of Ach by inhibiting its degradation, thereby facilitating cholinergic communication in the brain and improving AD symptoms. Rivastigmine belongs to a category of acetylcholinesterase inhibitor drug that is used to treat mild to moderate dementia. Rivastigmine tartrate (RT) is distinguished from other known cholinesterase inhibitors (donepezil and galantamine) because it inhibits both acetylcholinesterase and butyryl cholinesterase enzyme by coupling covalently to the active sites of the enzyme.[2] RT undergoes first-pass metabolism resulting in decreased oral bioavailability even though it is totally absorbed. Patient compliance is poor owing to its short half-life (1.5 h). Current drug therapies that are available for the treatment of AD are inefficient in long term medication. To overcome the above problems, a targeted drug delivery system would enhance efficacy, safety and patient compliance.

Liposomes have been thus emerged as an encouraging carrier for the treatment of neurological disorders by acting as a drug reservoir which could play a vital role in crossing blood-brain barrier (BBB) through lipophilic endothelial cells. It reduces the dosage level, enhances the efficacy and lowers the toxicity of drug.[3] Apart from its several advantages it has got major disadvantages like destabilization during its circulation time due to the activity of the phagocytic enzymatic reaction leads to poor bioavailability. In order to improve the brain specific delivery and blood circulation time, conventional liposomes can be further modified by coating with chitosan. Chitosan coated liposomes are mainly aimed for non-parenteral delivery of the drugs. Chitosan is a biocompatible, biodegradable polymer that is used as a polymeric carrier for the nanoparticles via various routes of administrations. Chitosan will retain a typical positive surface charge along with the mucoadhesive properties so that it can adhere to the mucous membranes and release the drug in sustained manner.[4] To achieve stability of the liposomal dispersion is a challenge; it requires a repulsive interaction proportional to range and magnitude of the vander waals force. Steric stabilization can be achieved by covering the surface liposomes with adsorbed layer of bulky molecules like Poly Ethylene Glycol (PEG). The surface modification of the liposomes with the attachment of PEG shows several advantages such as it reduces the uptake by the reticulo endothelial system leads to the improved distribution, prolonged blood circulation and it also helps in reducing the aggregation of the vesicles and improve stability.[5]

Hence the aim of the present work was to formulate and characterize liposomal suspensions of RT in the form of conventional liposomes, chitosan-coated and stealth liposomes.

MATERIALS AND METHODS

Materials

RT was procured from Yarrow Chemicals Products Ltd, Mumbai, India. Soybean phosphatidylcholine phospholipid 'Phospholipon® 90 G' was a gift sample from Lipoid®, GmbH, Germany. Cholesterol was procured from HiMedia Laboratory Pvt. Ltd., Mumbai, India. PEG-DSPE from Nice Chemicals Private Limited, Kochi, India. Chitosan 50 kDa, 75-85 % deacetylated were obtained from Sigma-Aldrich, USA. All other chemicals utilized were of analytical grade.

Methods

Compatibility studies

Fourier Transform Infrared (FTIR) spectrophotometer probe (Alpha Bruker, Japan) was used to inspect and predict any physiochemical interactions between various excipients.[6] A small amount of the sample was placed and analysed at a wavelength region of 4000 to 500 cm^{-1}. The IR spectrum of the physical mixture was compared with the pure RT, cholesterol, SPC, PEG-DSPE, chitosan and formulations.

Preparation of Conventional Liposomes of RT (CLR)

Liposomes were prepared by employing thin film hydration method as per the method described by Bangham *et al.* 1965.[7] The ratios of drug, cholesterol and SPC were weighed accurately and then dissolved in 20 ml of dichloromethane in 100 ml round bottom flask and attached to a rotary evaporator [Superfit Rotavap (series 6-BU) Continental Pvt Ltd, R/185., India]. Temperature was maintained at 55°C and evaporator was rotated at 60 rpm to get thin film. The thin film was then hydrated by phosphate buffer pH 6.5 at room temperature to obtain liposomal suspension of RT.

Design of Experiments (DOE)

The software Design-Expert ® (Version 11.0.3.0 64-bit, Stat Ease Inc. Minneapolis, USA) was used to optimize the method of preparation of liposomes with less number of experiment runs to determine specific process variables shows the highest impact on the prepared liposomes.[8] In this study a full factorial design (3^2) was applied. The ratios of drug: SPC (A) and SPC: cholesterol (B) were used as independent variables whereas particle size and percentage entrapment efficiency were selected as dependent variables. The data of full factorial experimental design is given in Table 1. Optimized formulation was then formulated and used to validate the obtained polynomial equation model. For all the response variables statistical second order model including interaction and polynomial relations were produced using Multiple Linear Regression Analysis (MLRA). The general form of the model is represented as:

$$Y = \beta 0 + \beta 1A + \beta 2B + \beta 3AB + \beta 4A^2 + \beta 5B^2 + \beta 6AB^2 + \beta 7A^2B + \beta 8A^2B^2 \quad \text{(Eq. 1)}$$

Y is the measured response associated with each factor level combination, β0 is the sum of average of all quantities of outcomes for 9 runs, β1 to β8 are the regression coefficients determined from the observed experimental values of Y. A and B are the coded levels of independent variables. AB is the interaction between the main effects. A^2 and B^2 are the quadratic terms of the independent variables that were used to pretend the contour of the designed sample space. The 3-dimensional graphs were created using regression analysis. The effect of independent variables on each response parameter was visualized from the contour plots. The actual values of independent variables are mentioned in Table 1.

Preparation of Chitosan-Coated Liposomes of RT (CCLR)

The liposomal vesicles were coated by mixing with the chitosan solution in 0.5 % v/v of glacial acetic acid. The chitosan solution (0.5 % w/v) was added dropwise into the liposome suspension placed on the magnetic stirrer under controlled stirring rate of 50 rpm at room temperature. After the coating of liposomes, it was kept undisturbed for 3-4 h to get proper swelling of liposome. The prepared liposomes was sonicated upto 18 min with the interval of 3 min each at 80 % amplitude 0.5 s pulse using ultra-probe sonicator (CV-18, Sonics and Materials Inc., USA).[9]

Preparation of Electro Steric Stealth Liposomes of RT (SLR)

Thin film hydration method as stated by (Dong *et al.*) was employed to formulate the liposomes.[10] RT and 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine with conjugated methoxyl poly(ethylene glycol (DSPE-mPEG) was added to SPC, Tween 80 and DDAB in a round bottom flask and mixture was dissolved in 10 ml of chloroform at 40°C. The chloroform was evaporated under the influence of vacuum, at the rpm of 90 in the rotary flash evaporator to obtain thin film. It was then hydrated using 5 ml of phosphate buffer (pH 7.4) and was subjected to sonication upto 18 mins using Ultra probe sonicator.

Evaluation of CLR, CCLR, SLR

Average particle size, zeta potential and polydispersity index

The average Particle Size (PS), Zeta Potential (ZP) and Polydispersity Index (PDI) of all the formulations were determined by Malvern nano zeta sizer instrument (Malvern, UK).[11]

% Entrapment Efficiency

The sonicated formulations (1 ml) were centrifuged at 500 rpm for 1 h at cold centrifuge in a temperature of 4°C (Remi Elektrotechnik Ltd. Vasai, India) to get a white pellet. The pellet was treated with few drops of 0.1 NaOH vortexes for 3 min to obtain a white suspension. 5 ml of Triton X-100 was added to discharge the drug, vortexed again for 2 min to clear

Table 1: Full factorial experimental design layout.

Batch (runs)	Factor 1 Drug:SPC (A) (% w/w)	Factor 2 SPC:Cholesterol (B) (% w/w)
1	0	0
2	1	1
3	-1	1
4	0	1
5	1	-1
6	0	0
7	0	-1
8	-1	0
9	-1	-1
Code	Drug:SPC (A) (% w/w)	SPC:Cholesterol (B) (% w/w)
-1	1:5	80:20
0	1:10	70:30
1	1:15	60:40

the lysed vesicles. The Percentage Entrapment Efficiency (% EE) was calculated using the following formula.[11]

$$\% EE = \frac{\text{Drug in pellet (entrapped drug)}}{\text{Total drug added}} \times 100$$

Drug content analysis

The amounts of RT in the three formulations were calculated.[12] The sample was diluted in 10 ml water and methanol separately and filtered using 0.2 µm size membrane filter. The filtered solution was diluted and subjected to UV spectrophotometer analysis at 262 nm.

Surface morphology

Transmission electron microscopy (TEM)

The transmission electron microscopic method was carried out to define the accurate shape and morphology of the optimized liposomes.[13] A drop of diluted samples was separately injected into a carbon coated copper gritoform liquid film. By adding one drop of 2 % w/w ammonium molybdate in an ammonium acetate buffer of 2 % w/v (pH 6.8), the film on the grid was negatively stained, dried and observed using CM 120 Bio Twin TEM (Philips Electron optics BV, Netherland).

Atomic Force Microscopy

The 3D structural characterization of the optimized liposomes was illustrated utilizing atomic force microscope (Innova SPM atomic force microscope, Bruker, Santa Barbara, CA, USA). The optimized liposome was applied onto the Mica Disca as a thin smear and the sample was observed in contact mode using AFM tips at a 267-328 KHz frequency response at a scanning rate of 1.2 Hz.[14]

In vitro drug release study

In vitro drug release study was carried using vertical Franz diffusion cell. *In vitro* drug release pattern of the formulation were compared with the 2 % standard solution of RT. 1 ml liposomal samples (~0.5 mg) such as CLR, CCLR and SLR was placed in one side of the sigma dialysis membrane. Other side of the membrane was in contact with 100 ml of dissolution medium i.e. phosphate buffer pH 7.4. For initial 2 h, 12 ml of phosphate buffer pH 2.6 was placed to simulate stomach condition. The dissolution unit was kept on a magnetic stirrer at 37°C to mimic body temperature. Aliquots 5 ml of dissolution medium was withdrawn at the intervals of 5 min, 15 min, 30 min, 45 min, 60 min, 2 h, 4 h and 8 h and equal volume of medium was replaced to retain a constant medium volume. The drug concentrations were calculated by UV spectrophotometric method at 262 nm. The experiments were conducted in triplicates and the results were exhibited as mean ± standard deviation.[15]

In vitro kinetic study

The kinetic study was conducted by using different models such as zero order, first order, Higuchi model and Korsmeyer-Peppas model to understand the mechanism of drug release. Depending upon R and K values obtained from different model, the best fit model was selected to interpret the drug release.[15]

Stability study

Short term stability of optimized formulations was carried out for 3 months.[16] The optimized liposomal formulations were placed in a chamber. The optimized formulations was stored at two different storage conditions (5 ± 2°C and 25°C ± 5 % 60 % RH) in a chamber. Sampling was done every month upto three months and parameters like % EE, particle size (nm) and PDI (mV) was determined.

RESULTS

The FTIR spectrum of pure drug i.e. RT showed characteristic peaks at 1488 cm^{-1}, 1231 cm^{-1}, 3043 cm^{-1}, 1071 cm^{-1} and 1899 cm^{-1} due to C=C, C_2-H_5, N-CH_3 and C=O stretching respectively. C-H stretching in RT appeared at 3043 cm^{-1} which appear to remain same in the formulations. As per DoE total nine formulations as shown in Table 2 generated optimized variables, with a goal to validate the developed method. The responses observed were fit to nine runs and it has been noted that best fit model was quadratic polynomial model for the two dependent variables. The values from the experimental trails showed a substantial variation in % EE (53.08 % - 91.33 %) and vesicle size (105.2-122.7 nm). All the response variables were observed experimentally by the design expert software and were fitted to run design plot. After elimination of non-significant coefficients, following correlations were obtained for response variables in terms of coded factors:

Vesicle Size: +113.59–2.37*A+1.95*B..... (2)

% EE: +71.54+4.89*A+10.61*B..... (3)

In the polynomial equations, A and B are coded values for drug: SPC and SPC: Cholesterol ratio. The above equations are useful to quantify response values. A negative sign indicates antagonistic effect whereas positive sign of coefficient indicates synergistic effect. All the polynomial equations were found to be statistically significant ($p<0.05$), as determined using ANOVA. Equation (2) suggests that the factor A

Table 2: 3^2 level factorial randomized quadratic design experimental trial batches with obtained particle size (nm) and drug content (% w/w).

		Factor 1	Factor 2	Response1	Response 2
Std	Run	A:Drug:SPC	B:SPC:Cholesterol	Particle Size	Entrapment Efficiency
		(% w/w)	(% w/w)	(nm)	(%)
10	1	0	0	115.2	68
9	2	1	1	109.9	81.33
7	3	-1	1	114.8	66.33
8	4	0	1	122.7	77
3	5	1	-1	105.3	55.33
5	6	0	0	107.4	78.8
2	7	0	-1	118.2	43.66
4	8	-1	0	116.3	48.3
1	9	-1	-1	112.2	52

have a negative effect whereas the factor B has a positive effect on the particle size of the liposomes. The impact of independent variables were studied using 3D response surface plot, in Figure 1 it was found that as the concentration of drug:SPC increased there is a significant decrease in the particle size of the liposomes whereas the particle size increased when the amount of SCP:cholesterol was increased. Equation (3) suggests that the factor A&B showed a positive effect on the % EE of the liposomes. The Figure 1 showed the impact of independent variables and was found that as the concentration of drug:SPC and SCP:cholesterol increased there is a significant increase in the % EE of the liposomes. Later, optimized liposomes were evaluated and the particle size was found to be 111.8 nm and the % EE was 75.27 %. Table 3 lists the comparison of the experimental responses with that of the predicted responses, the mean of percentage error was found to be 0.93. Thus, the low magnitudes of error indicated excellent fit of model. The formulated particle size of the CLR, CCLR and SLR was found to be 111.8 nm, 153 nm, 136.3 nm, respectively. CCLR formulation showed the highest % EE value of 80.33±0.4 % as compared to the other formulations followed by CLR, SLR which showed the entrapment efficiency of 75.27±0.8 % and 78.2±0.2 % respectively. The RT existing in optimized CLR, CCLR and SLR formulations was found to be 63±0.02 % w/w, 70±0.4 % w/w and 67±0.21 % w/w, respectively. TEM studies (CLR, CCLR and SLR) showed that the liposomes possessed a good morphological character with respect to size and shape (Figure 2 A, B and C). The AFM images of the optimized liposomes CLR, CCLR and SLR were displayed in Figure

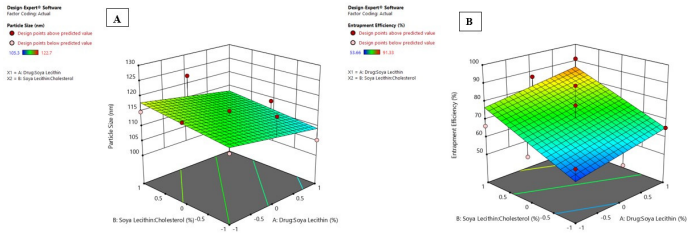

Figure 1: 3D Response surface plot.

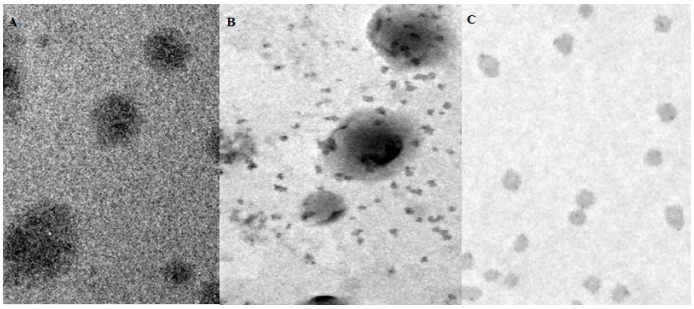

Figure 2: Transmission Electron Microscopy (A-CLR, B-CCLR and C-SLR).

Table 3: Comparison of experimental results with predicted responses of optimized formulation.

Batch code	Response	Predicted value	Experimental value	Percentage error
Optimized formulation	Particle Size (nm)	112.2	111.8	-0.4
	Entrapment efficiency (%)	76.72	75.27	-1.45
	Mean of percentage error			0.93

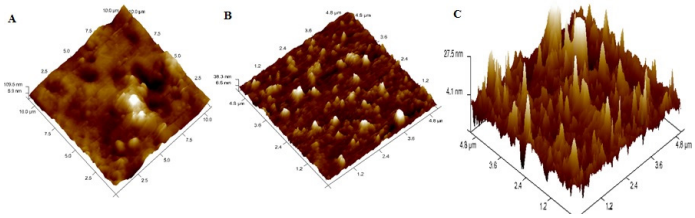

Figure 3: Atomic Force Microscopy (A-CLR, B-CCLR and C-SLR).

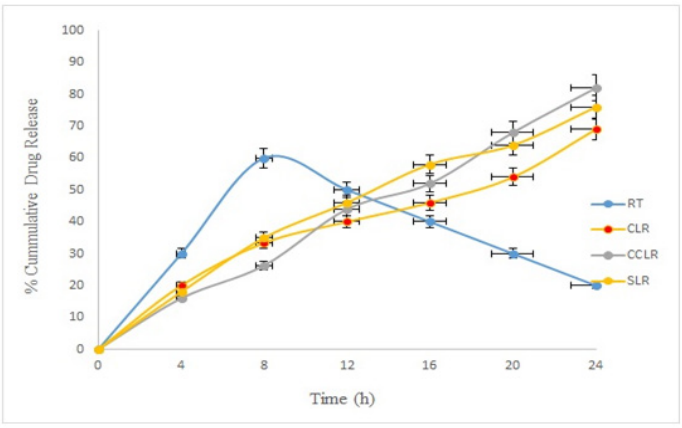

Figure 4: *In vitro* drug release study.

3 A, B and C. The *in vitro* release behaviour of CLR, CCLR and SLR is summarised in Figure 4. Cumulative drug release after 24 h was found to be 69±0.8 %, 81±0.3 % and 76 ±1.2 % respectively. The drug release kinetics from the optimized liposome was evaluated considering four drug release kinetics models including zero order, first order, Higuchi model and Korsmeyer Peppas model. Results of stability showed that CCLR have better entrapment efficiency 80.3±0.02 % compared to CLR 61.1±0.02 % and SLR 72.1±0.02 %. There was no such variation observed in SLR, CCLR particle size 136.8 nm, 152.3 nm whereas CLR showed slight variation in its size after 2nd month of storage (157.1 nm), this could be due to the fusion of adjacent particles. Zeta potential of the SLR, CCLR showed no changes over the period of 3rd month (-14.8 mV and 22.4 mV) in the context of CLR -18.3 mV.

DISCUSSION

The FTIR of drug shows slight alteration in its characteristic peaks in the formulations due to inter molecular interactions during the formulation. The result showed there was no significance presence of new peaks or disappearance of characteristic peaks which indicates that drug and the other excipients were compatible in the formulations. The desirable ranges of responses were restricted to particle size at 110 nm and % EE at 80 % as shown in Table 4 as per the software. On analysing various response variables the following combination of variables were suggested, drug: SPC ratio = 0.33 (% w/w) and SCP: cholesterol ratio = 0.061 (% w/w). The obtained range of particle size is sufficient to cross blood brain barrier.[17] The optimised conventional formula denoted as CLR. CLR was employed to prepare CCLR and SLR by coating chitosan on negatively charged phospholipid layer and incorporating PEG-DSPE coat to the conventional lipid layer respectively.[18] This alteration improves the mucoadhesive property and reduces the rate of uptake by macrophages respectively. Thereby it will remain in the circulation for a prolong time as a therapeutic reservoir.[19] CCLR showed slightly bigger size due to compact coat of polysaccharide chitosan. The PDI of CLR, CCLR and SLR was found to be 0.274,

Table 4: Criterion for numerical optimization.

Parameters	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
A: Drug:SPC (% w/v)	Is in range	-1	1	1	1	1
B: SPC:Cholesterol (% w/v)	Is in range	-1	1	1	1	1
Y1: Particle Size (nm)	Target=110	105	118	1	1	1
Y2: Entrapment efficiency (%)	Target=80	43	81	1	1	1
Optimized formulation	Drug:SPC (% w/v) 0.33			SPC:Cholesterol (% w/v) 0.061		

0.535 and 0.478 respectively, indicated that the prepared liposome has a relatively good size distribution. The PDI of these formulations indicated the narrow distribution width of the liposomal particles in the complex.[20] Zeta potential is a measure of the surface charge on the particles. If the Zeta potential is below 30 mV it can be related to the particle attraction followed by flocculation or repulsive forces. The zeta potential of the CLR, CCLR and SLR was found to be -15.8 mV, 22.3 mV and -14.8 mV respectively. The Zeta potential value depends on the type and composition of phospholipids. The negative value indicates that the particles have no charge and as a whole system is stable.[21] This indicated that RT showed higher affinity in chitosan coated lipid bilayer compared to other CLR and SLR. Unentrapped drug remained in the formulation to avoid drug loss. TEM study revealed that liposomes are spherical in shape with smooth round edges. The study suggested nanometric size range and even size distribution. The AFM image of the optimized liposomes showed well formed, discrete vesicles, when mounted in glass slide. It designed in to a vesicular nano structures with no indication of decomposition or aggregation. *In vitro* drug release study the formulations were compared with the standard solution of RT. It demonstrated burst release in the first 4 h and probably it was due to the free drug availability in the solution. As compared to the CLR, RT release from the SLR, CCLR was found to be sustained and this is probably due to the polymer attached on to the surface of the liposomes. In case of CCLR further slow release of drug was observed 26.3±0.3 % at 8 h compared to CLR 53.4±0.9 %, SLR 33.2±0.5 %. This could be due to the physico-chemical alteration of liposomes after coated with the chitosan. Moreover lipid bilayer coat in CLR, SLR and CCLR increases the signalled controlled prolong release of RT when compared to pure drug. The release of drug from the CLR followed zero order kinetics with the R^2 value of 0.8086. *In vitro* drug release kinetics of the SLR followed partial Higuchi model R^2 value 0.884 indicated the release of drug from the insoluble matrix with the time depended. Chitosan coated liposomes followed the Korsmeyer- Peppas model with the n value (0.97) indicated the super case II (relaxation) transport which is associated with the stresses and state transition in hydrophilic glassy polymers, that will swell in water or biological fluids. It generally refers to the erosion of polymeric chain.[22,23] The formulations stability influences a key role in the therapeutic activity of the drug. The main problem associated with the liposomal formulation is vesicles fusion. They tend to fuse each other when it stored for a longer time. The stability test above room temperature could not be possible, because lipids will degrade faster when it subjected to extreme temperature. The formulations were evaluated for its entrapment efficiency, vesicle size and zeta potential over the period of 3 months. The formulation stored at refrigerator temperature showed good entrapment efficiency compared to the formulation stored at room temperature.[24] The stability test result was found to be encouraging.

CONCLUSION

The study was aimed to design and compare the liposomal formulations for the treatment of AD. The *in vitro* characterization indicated the proper development of the formulations (conventional, chitosan-coated and stealth liposomes. *In vitro* release, studies showed prolonged and better release pattern of stealth and chitosan-coated liposomes when compared with the conventional liposomes. From the stability studies, the optimized formulation remained stable for four weeks and there were no significant changes observed. The study was an attempt to develop the best possible liposomal formulation of RT for the treatment of AD. However, further extension *in vivo* study is required to confirm the better product development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

ABBREVIATIONS

RT: Rivastigmine Tartrate; **AD:** Alzheimer's disease; **DOE:** Design of Experiment; **TEM:** Transmission Electron Microscopy; **AFM:** Atomic Force Microscopy; **AChEIs:** Acetylcholinesterase inhibitors; **CCLR:** Chitosan-Coated Liposomes of RT; **CLR:** Conventional Liposomes of RT; **SLR:** Electro Steric Stealth Liposomes of RT; **PS:** Particle Size; **ZP:** Zeta Potential; **PDI:** Polydispersity Index; **% EE:** Percentage Entrapment Efficiency.

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