Research Article

Polysorbate80 Coated Liposomes for Loperamide Delivery to Brain for its Antinociceptive Activity through Central Opiate Receptors

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ABSTRACT

Blood Brain Barrier (BBB) is a potential barrier and a challenging process for designing of new drug delivery. Loperamide (Lp) though an opiatic drug cannot elicit CNS effect due to the efflux of P glycoprotein (Pgly) present in the endothelials of BBB. But Polysorbate 80 has blocks P glycoprotein efflux thus enhancing the delivery of drug to brain. In our study we have formulated polysorbate 80 coated liposomes (PCL) and evaluated for the actinociceptive response (Eddys hot plate and Tail Flick). Zetapotential (ZP) and Poly Dispersity Index (PDI) by zetasizer, mean particle diameter by Photon Correlation Spectroscopy (PCS), particle size distribution by Transmission Electron Microscopy (TEM) and brain quantification study by LC-MS/MS for PCls were done. We found in actinociceptive study, animal showed appreciable rise in analgesic activity for PCL from control in Eddys hot plate (55.35%) ($p < 0.01$) and in Tail Flick test (68.8 %) ($p < 0.01$). Particle size analysis confirmed the size of PCL to be in nanometer size (128 ± 3.85nm) which supports permeability features of PCL through Blood Brain Barrier (BBB) than the uncoated liposomes (UL) (360 ± 2.65 µm). Zetapotential of PCL was found to be -26.4mV and UL of -14.6mV. PDI of PCL showed uniform particle size distribution (0.21) than UL (1.64). TEM revealed nanosized vesicles in PCL (128 nm, $p < 0.01$). Brain quantification study quantified the amount of Lp delivered to brain by PCL (1.04 µmol, $p < 0.01$) but showed absence of Lp in brain for UL. This study showed that PCL is a suitable carrier for loperamide to permeate brain barrier producing CNS effect.

Keywords: Polysorbate 80, Liposomes, Pglycoprotein (Pglp), Loperamide, Blood Brain Barrier.

INTRODUCTION

Among the various Blood Brain Barriers (BBB), PGlycoproetin (Pgly) Efflux system provides an efficient defence for brain for many drugs including anticancer drugs, analgesics, and antibiotics[1]. It is a poypeptide having 1280 long amino acid glycoprotein and has two analogous fractions of same length in one line, both comprising of six trans-membrane domains and with a flexible linker polypepdide region separating two ATP binding region[2].

Liposome showed limited use for brain targeting as they were preferentially effluxed out by Pgly protein making the entrapped drug unavailable for brain[3]. Polysorbate 80 has potential Pgly protein inhibition by sensitising LDL-mediated endocytosis via LDL receptors of the brain capillary endothelial cells which is triggered by the adsorption of Apolipoprotein -E globules. This mimiks LDL particles and interacts with the LDL receptors making the drug transport in to the brain by transcytosis[4]. Drugs like Loperamide which is a poorly water soluble drug and has an opiate like structure similar to morphine cannot not cross BBB, but if crosses it would be pumped back to blood by Pgly protein. It can produce only peripheral opiate effects on µ and δ opiate receptors in gastro intestinal tract[5] but does not produce any opiate agonistic activity in CNS due to its BBB impermeability[6]. Many studies revealed the CNS opiate activity of loperamide if delivered to brain such as its transport by human serum albumin nanoparticle having monoclonal antibody producing antinoceceptive effect in the tail flick test[7]. Cereport (RMP7) was used to enhance delivery of the peripherally acting opiate agonist, loperamide, to brain, inducing centrally mediated analgesic effect[8]. Sadeque demonstrated loperamide induced opiatic central mediated respiratory depression[9]. Thus polysorbate 80 coated liposome could be a good choice for drugs like loperamide for CNS delivery.

In the present study, loperamide (Lp) was entrapped in a Polysorbate 80 coated Nanosized Liposomes (PCL) and evaluated for its ability to transport to CNS. They were also characterised for the particle size and its distribution, brain quantification studies and actinociceptive studies.

MATERIALS AND METHODS

Preparation of Polysorbate80 coated Liposomes (PCL)

Lecithin (Lec) and cholesterol (Ch) were used for liposome preparation with some modification of "Reverse Phase Evaporation Technique" as described by Szoka and Papahadjoupoulos[10]. In brief, lecithin and cholesterol (9:1) were dissolved in diethyl ether. A 5 ml Lp drug (2 mg/ml) in phosphate buffer saline (PBS) solution of pH 7. 4 were added. Vitamin E at 0.6 mol % were used to prevent lipid constituent's oxidation. The formulation was emulsified using a homogenizer (Tenbroeck tissue grinder) at 5000 rpm for 20 minutes at a temperature of 50°C. The weight of the dispersion was later adjusted before the swelling resulting in a reverse type (w/o) emulsion which forms a semi solid gel like consistency. The residual diethyl ether was evaporated using a vacuum evaporator (BUCHI EL 131 Rotavapor, Germany)

under a reduced pressure (260 - 400 mm Hg) at 60 °C. The lipid gel so formed was collapsed and transformed to reach a fluid consistency by agitation using a vortex mixer. A 5 ml of warm PBS (pH7.4) was added to produces a suspension of multi lamellar vesicle liposomes (MLV) later sonicated using a microtip probe sonicator (Vibracell, sonics and materials, Inc, Danbury, CT) for 30 minutes at 40 % frequency producing a homogeneous dispersion. Nitrogen gas was flushed for 1 minute to remove trace oxygen. The liposome dispersion samples were kept at 4 °C and protected from light. Before use, they were filtered through Whatman filter paper No 42 (pore size 2.5Mm). For coating of surfactant, 1 % w/v of polysorbate 80[11] was added to the liposomal suspensions and incubated for 30 min with constant stirring. Thus polysorbate 80 coated liposomes (PCL) with drug were prepared. Uncoated liposome of loperamide (UL) were prepared as above but without Polysorbate 80 coating Blank liposome PCL without loperamide were prepared using the same protocol of PCL described above, but omitting the presence of the drug.

Entrapment Efficiency (EE)

EE[12] was done using Mini column centrifugation. Sephadex® G50 solution (10 %, w/v) was soaked in water and kept aside for 48 hours for complete swelling. This Hydrated and swelled Sephadex G -50 was packed in a column having whatmann paper pads at the bottom. Using centrifugation (3000 rev min^{-1}) excess of water was removed. A 100 µl of liposomal suspension was applied as drops at the top center of the column followed by centrifugation. The free drug bounds to the column and the vesicles pass through the column and collected at the bottom. Distilled water was further added to the mini column and the centrifugation was repeated. Absence of free drug was confirmed by testing the centrifugate after application of the saturated drug solution. Diethyl ether were used to lyse the PCL followed by shaking with PBS for 30 minute. Two immiscible liquids were separated using a suitable separating funnel and the drug removed along with PBS and quantified BY RPHPLC[13]. Entrapment efficiency of the Loperamide drug in the liposomal formulation was calculated using the formula:

$$Percentage\ Entrapment\ (\%\ E) = \frac{Entrapped\ drug\ (mg)}{Total\ drug\ added\ (mg)} \times 100$$

Actinociceptive Study

The agonistic activity of loperamide similar to morphine over opiate receptor in CNS produces actinociceptive effect. The experimental protocol was approved by the IAEC (Institutional Animal Ethical Committee) which was formed in accordance with norms of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and complied with the National Institutes of Health (NIH) guidelines on handling of experimental animals (Approval ref no 290/CPCSEA/PHARMCEUT-10/06)[14]. The animals (wistar rats) were divided in to 5 groups of 6 animal each. Control group recieved pyrogen free water by IV. The standard group received intrathecal administration of loperamide (3 µg/ml) in a volume of 10 µL through the intrathecal catheter, flushing with physiological saline (0.9 %) which was proved for its CNS effect[6]. Test1 group received loperamide PCL. Test 2 group received pure lopermaide drug and Test 3 received UL with the drug. All test groups received the respective formulation/drug through tail vein equivalent to loperamide 8mg/ml.

Eddys Hot Plate

Hot plate method (Columbus Instruments, Columbus, OH) at 52 °C ± 0. 5 , was used to evaluate thermal nociception using the hind paw withdrawal response for the thermal stimulus. The "Maximum Possible Reaction Time" (MPR) is the measure of hind paw withdrawal from a 52 °C ± 0.5 hot plate (thermal withdrawal latency). Animals with an average baseline latency less than 25 seconds were used in this study. A cut-off latency of 30 seconds was followed to avoid tissue damage or thermal hyperalgesia[15]. The degree of antinociception, expressed as percent maximal possible response (%MPR), was calculated as follows:

$$Maximal\ Possible\ Response\ (\%\ MPR) = \frac{Latency\ (Test) - Latency\ (Control)}{Latency\ (Test)} \times 100$$

Tail Flick Test

In the tail flick test, response time for the sudden withdrawal of the tail dipped in up to 5cm in water maintained at 58 °C ± 0.5 was measured. Cut off time of 10 sec was maintained to avoid damage to the tail. The time required for tail flick after administration of drug/formulation after 0, 30, 60 and 90 minutes was recorded to assess response to the noxious stimulus [16] and MPR was calculated.

Zeta Potential

Zeta potential (ZP) and Polydispersity Index measurements (PDI) were done using a Zeta Potential Analyzer (Brookhaven Instruments Ltd., Brookhaven, USA) at 25 °C. PCL or UL sample was diluted with KCl (0. 1 M) and placed in the electrophoretic cell and analyzed in triplicate.

Photon Correlation Spectroscopy (PRS)

The mean particle diameter of PCL or UL was determined by Dynamic light scattering (Brookhaven Instruments Ltd, Brookhaven, USA) at 25°C. Samples were scattered at 657 nm at an angle of 90 degrees.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) has been used for characterizing the size distribution. A drop of PCL or UL was loaded onto the copper halftone sampler and dyed by 1% (wt) phosphotungstic acid solution. The sample was then characterized by H-600 TEM (Hitachi, Japan) at a 75kV electron beam accelerating voltage.

Brain Quantification Study

Loperamide concentration in brain was measured using LC-MS/MS methods with slight variation. In brief, the brain was removed surgically and a coronal section (6mm, bregma ± 3mm) containing the cerebral cortex, striatum, etc. was cut and divided into smaller pieces. Surface blood vessels and the choroid plexus were not removed to prevent contamination of loperamide in systemic circulation. The brain sample (350 to 375 mg) was transferred into an Eppendorf tube and homogenized in three volumes of deionized water using an ultrasonic probe. An aliquot (25 µl) of homogenate was transferred to an HPLC vial, and protein was precipitated with 4 to 125 volumes of methanol.

The sample was vortex-mixed and centrifuged, and the supernatant was analyzed by Reversed-Phase Liquid Chromatography and Multiple reaction Monitoring Mass Spectrometry (LC-MS/MS)[17]. The LC-MS/MS system consists of an LC-10AD pump (Schimadzu, Columbia, MA, USA), an Ascentis Express C18 column (3cm, 2. 1mm, 2.7 mm, Supelco Analytical, Bellefonte, PA, USA), and a Micromass Ultima Platinum detector (Waters, Manchester, UK). The gradient elution from 5 % to 95 % mobile phase B was performed at a flow rate of 0. 8 mL/min over 1.4 mins using mobile phases consisting of 0.2% formic acid in deionized water (A) and 0.2% formic acid in acetonitrile (B), respectively. Retention times and mass transitions were 0.77 min and m/z 477. 2-266. 0 for loperamide.

RESULTS

Entrapment Efficiency (EE)

Though Polysorbate 80 is a hydrophilic surfactant with HLB 15 favouring o/w, we found no influence over entrapment efficiency as Polysorbate 80 was not used during liposomal preparation or during emulsification but only for coating the liposomes. The coated liposome PLC showed EE not much difference than the uncoated one (Table 1). EE of PLC showed 44% which was nearer to the UL (41%).

Actinoceceptive Study (Eddys Hot Plate and Tail Flick Study)

Hot plate and Tail withdrawal latency (Table 2, Fig 1) uses the dependent variable based on a phasic stimulus through opiod δ and µ receptor producing analgesia in acute pain models[18]. In eddys hot plate, Test1 showed significant rise of actinociceptive activity of 50.06 % ($p < 0.01$) within 30 min, 55.35 % ($p < 0.01$) within 60 min ($p < 0.01$) and 52.81 % ($p < 0.01$) within 90 min. In tail flick test, it also showed significant rise of actinociceptive activity of 62.75 % ($p < 0.01$), 64.63 % ($p < 0.01$) and 68.81% ($p < 0.01$) within 30 min, 60 min and 90 min respectively and this is similar to the effect of standard intrathecally administered Lp in actinoceciptive study.

However there is no significant rise in latency to the thermal analgesia for control, test 2 and 3 in both eddys hot plate and tail flick test.

Table 1: Particle size distribution, Polydispersity and zeta potential of PCL and UL

Formulation	Mean diameter* (nm/µm)	Zeta Potential (mV)*	Entrapment Efficiency**	Polydispersity Index (PDI)*
PCL	128 ± 3.85nm	-26.4 ± 0.42	44.44 ± 2.31	0.21 ± 0.04
UL	360 ± 2.65µm	-14.6 ± 0.64	41.53 ± 1.52	1.64 ± 0.31

All data are presented as mean ± SD (n = 6), *$p < 0.001$,**$p < 0.01$, n=6

Table 2: Effect of Drug/Formulation on hind paw removal (secs) and tail withdrawal (secs) determined by Eddys Hot Plate and Tail Flick Test respectively within the time interval of 30 min, 60, 90 min.

Group	Drug/ Formulation	Maximum Possible Response (%MPR) (sec)					
		Eddy Hot plate Test (± SD) (% MPR)			Tail Flick Test (± SD) (% MPR)		
		30min	60min	90min	30min	60min	90min
Control	Water[1]	8.04 ± 0.62	8.22 ± 1.44	7.21 ± 1.27	2.16 ± 0.33	2.2 ± 0.65	2.32 ± 0.83
Standard[2]	Loperamide[2]	17.61 ± 3.54 (54.34 %)	19.44 ± 2.16 (57.71 %)	16.7 ± 2.33 (56.8 %)	6.4 ± 2.37 (66.25 %)	7.54 ± 3.42 (70.82 %)	7.26 ± 2.33 (68.04 %)
Test 1[1]	PLC[1]	16.11 ± 2.11 (50.06 %)	18.41 ± 2.39 (55.35 %)	15.28 ± 1.88 (52.81 %)	5.8 ± 1.96 (62.75 %)	6.22 ± 2.66 (64.63 %)	7.44 ± 2.33 (68.81 %)
Test 2[1]	Loperamide[1]	9.4 ± 3.41 (14.46 %)	9.5 ± 1.52 (13.4 %)	8.34 ± 1.32 (13.54 %)	2.22 ± 1.38 (2.7 %)	2.2 ± 1.91 (2.29 %)	2.44 ± 2.54 (4.91 %)
Test 3[1]	UL	9.56 ± 1.81 (15.89 %)	9.4 ± 2.04 (12.5 %)	8.43 ± 0.33 (14.47 %)	2.23 ± 1.84 (3.13 %)	2.33 ± 2.04 (5.57 %)	2.42 ± 1.85 (4.13 %)

[1]Intravenous, [2]Intrathecal, $p<0.01$, level of significance is 0.05, n=6. Data represent mean ± SD (n = 6)

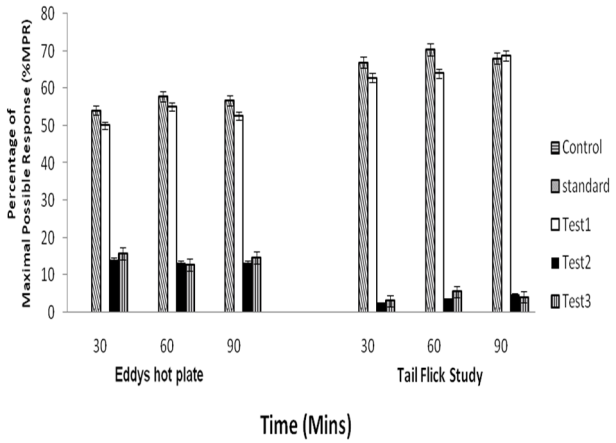
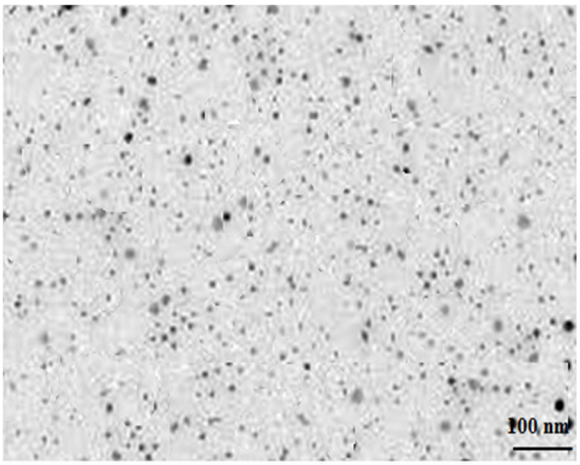


Figure 1: Actinociceptive Activity (Eddys hot plate and Tail flick study) of control, Standard, Test1 (PLC), Test2 and Test3 (UL). Control shows negligible or absence of enhanced actinociceptive activity but standard and Test 1 showed an appreciable increase in actinociceptive activity. Test 2 and 3 showed very less actinociceptive activity. The response were statistically different with $p<0.01$ (n=6).



(a)

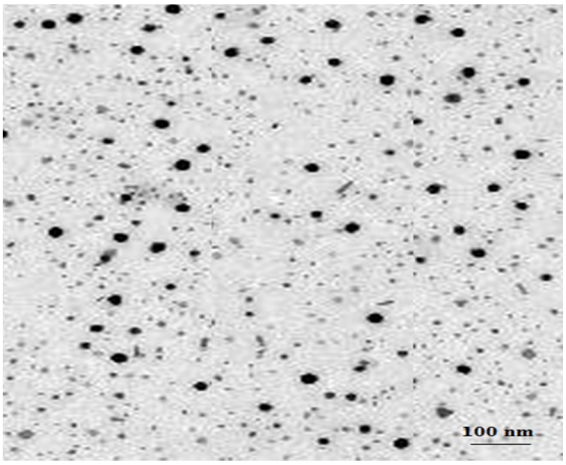
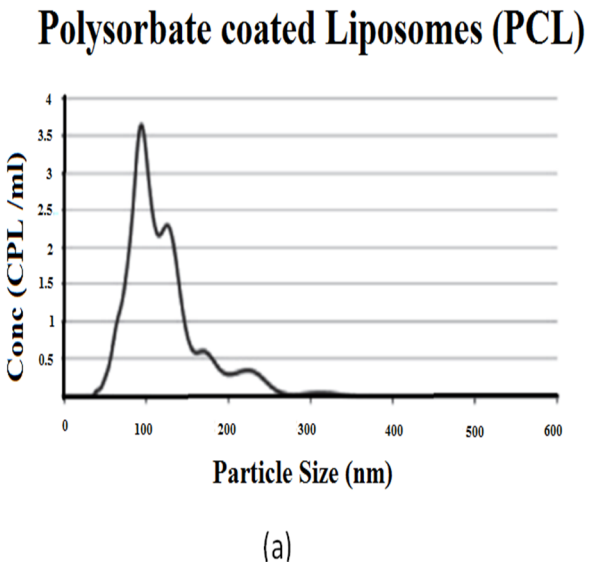
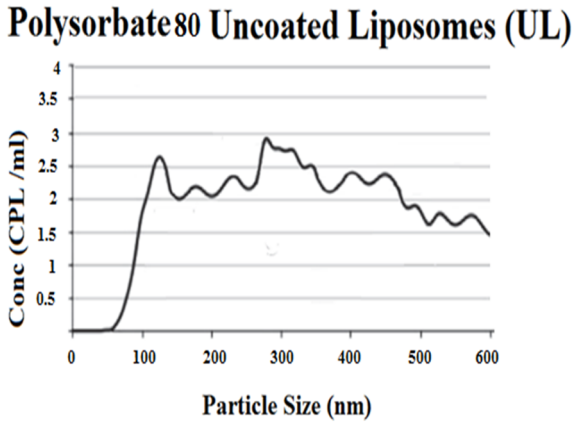


Figure 2: TEM images of Polysorbate 80 coated (PCL)/uncoated Liposomes (UL) taken at 75 kV (a) PCL showing vesicles in the range of 100 - 200nm (b) UL showing vesicles in the range of >100 µm.



(a)



(b)

Figure 3: Size distribution of Polysorbate 80 coated and uncoated Liposomes measured by TEM technique for (a) PCL (b) UL.

Zeta Potential

PCL has ZP -26.4 ($p < 0.01$) and uncoated liposomes has ZP -14.6 ($p < 0.01$). The polydispersity index (PDI) is a measure of particle size heterogenicity. Liposomes with PDI value between 0.1 and 0.25 has uniform size distribution and expected to be stable and if PDI is more that 0.5, it represents less uniform distribution of sizes[19]. For PLC, the PDI was found to be 0.21 $\pm$ 0.04 ensuring the homogeneous distribution of vesicles but in the case of uncoated liposomes the PDI was found to be 1.64 $\pm$ 0.31 indicating the deviation from the homogeneous size distribution (Table 1).

Photon Correlation Spectroscopy (PRS)

Particle size (Mean diameter) of PCL was found to be lesser (128nm) than the uncoated liposomes (360µm) (Table 1). Lesser size of PCL substantiates the preferable properties of nanoparticle for BBB permeation and has higher relative intracellular uptake compared to microparticles[20].

Transmission Electron Microscopy (TEM)

The result of TEM analysis confirms most of the particle size are in the nanoscale for PLC than UL and revealed not much aggregates of vesicles were observed (Fig 2).

In PLC, 85 % of particle size was less than 200nm. The smallest particles was in the range of 95-120 nm and constitute about more than 60 % of the total population. But in UL, the particle distribution spreads more than the range of 600 nm indicating failure of more vesicles within the range of 200 nm. Less than 8 % were in the range of 100nm. TEM allows measuring the particle size distribution projected two-dimensional images of the sample. The particle size distribution (Fig 3) is well differentiated and defines the particle concentration peaks in the range of 95 to 98 nm for PLC.

Brain Quantification Study

Interathecal Lp (standard) and PCL Lp (Test 1) showed significant rise of Lp in brain with 3.42 µmol ($p < 0.01$) and 1.04 µmol ($p < 0.01$) respectively. But Control, Test 2 and 3 showed no significant or absence of Lp in (Table 3).

Table 3: Quantitative Estimation of Lp in Brain Homogenate

Group	Drug/Formulation	Concentration of Loperamide (µmol) (± SD)
Control	Water[1]	--
Standard[2]	Loperamide[2]	3.42 ± 1.21
Test 1[1]	PLC[1]	1.04 ± 0.34
Test 2[1]	Loperamide[1]	--
Test 3[1]	UL	--

[1]Intravenous, [2]Intrathecal, SD (standard Deviation), p<0.01, n=6--Absence of loperamide

Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using analysis of variance (ANOVA) on statistical analysing software SAS (version 9.1.3 sp4 portable). Level of significance was performed between $p < 0.01$ or $p < 0.001$.

DISCUSSION

In the present investigation, polysorbate 80 coating showed promising results in Lp delivery to brain. The mechanism would be the rapid endocytic uptake of polysorbate 80 coating over the nanoliposomes as studied in cultured mouse, rat bovine and human brain capillary endothelial cells[21].

The PCL mimics lipoprotein and interacts with the LDL receptor followed by their endocytotic uptake. As the drug have already crossed the luminal membrane convincing Pglycoprotein, the drug would be less prone to another barrier[21].

The magnitude of the ZP gives an indication of the colloid stability. If all the particles in suspension have a large negative or positive zeta potential, then they will tend to repel each other and there will be no tendency for the particles to come together[22] and this supports PCL (-26.4 mV, $p< 0.01$) to be more stable than UL (-14.6 mV, $p < 0.01$). PLC showed mean nano sized particle (128 nm, $p < 0.001$) and this favours BBB penetration.

Desai found that nanosized particles showed a 2.5 fold greater uptake than 1µm microparticles, and 6 fold greater uptakes than 10µm microparticles[23].

Particle size distribution studies form TEM revealed PLC has more frequency of nanosized particles than UL and supports the formation of liposomes.

Quantification of loperamide in brain is an invasive proof for positive brain target supporting the PCL ability for BBB permeability.

CONCLUSION

PCL successfully delivered loperamide to brain thus increasing loperamide's therapeutic efficacy and can be explored further for its possible brain treatment. Thus Polysorbate80 coated liposomes is a potential alternative drug delivery and can be explored further for any other brain ailments.

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of the Author

We declare that this work was done by I. Sarath Chandran (designed the study) and Pichandy Muthuprasanna (Collected and analysed the data) and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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