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FORMULATION AND EVALUATION OF LOPERAMIDE LOADED POLYSORBATE 80 AND PEG CONJOINED COATED NANO LIPOSOMES

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ABSTRACT

Loperamide (Lp) cannot pass blood brain barrier but if delivered to brain it produces opiatic activity. Peripherally it produces antidiarheal activity. Its central antinociceptive activity was blocked by Pglyco protein (Pgly) efflux of BBB. Thus a nano sized liposomes for Lp coated with Polysorbate80 and Polyethylene Glycol 20000 (PEG) which favours blood brain barrier (BBB) penetration would be a best carrier for delivering Lp to brain. Thus an approach was made to formulate Lp loaded nano liposomes and conjoined coating the lipid carrier with Polysorbate80 (PS80) which is a Pgly efflux inhibitor and Polyethylene Glycol 20000 (PEG). The formulation were characterised for Entrapment efficiency (EE) , Invitro release study ,zeta potential , particle size distribution ,Scanning electron microscopy (SEM) and Differenctial Scanning calorimetry (DSC) .Entrapment efficiency(EE) of S_2G_2 was found to be lower (40.51±1.44) . *Invitro* release study in Artificial Cerebrospinal fluid (ACSF) for 44 hours showed a maximum Lp release for S_0G_0 (76.8% ± 2.14) and minimum for S_0G_2 (33.2% ± 3.27) indicating PEG potential to retard drug release rate. Release kinetic study confirmed the Lp released from CCLs followed Higuchi model of diffusion . Among CCLs, the Zeta potential (ZP) for S_2G_2 were found to be high (-18.28 mV $\pm$ 0.31) , low mean particle size (196 nm ± 3.85) and low poly disperse index (PDI) (0.18± 0.31). SEM and DSC ensured the Lp present in the S_2G_2 as amorphous and as molecular dispersion state. Thus S_2G_2 CCLs was found to be suitable for BBB penetration and can be further studied for invio performances.

KEYWORDS: *loperamide , Blood brain barrier , PGlycoprotein efflux , zeta potential*

INTRODUCTION

BBB is anatomically defined as cerebral microvascular endothelium with tight endothelial cell junctions with various transport mechanisms , influx and efflux transport proteins , degrading enzymes etc which makes it more defensive than CSF barrier. Most of the macromolecules are excluded or effluxed from this barrier unless they are carried by specific transporters or receptors at the luminal side of the endothelial cells .Small hydrophilic molecules such as glucose , aminoacids , nucleosides are delivered by specific proteins expressed in the luminal surface of this BBB endothelial cells. Essential peptides and proteins (macromolecules) are carried by receptor or adsorptive mediated transcytosis of BBB.[1,2] Loperamide(Lp) whic is an opiatic drug is impemable to BBB , but if delivered to brain can produce CNS opiatic activity such as Lp transported by human serum albumin nanoparticle having monoclonal antibody produces antinociceptive effect in the tail flick test.[3] Moreover , early studies also confirmed the presence of Pgly inhibitors such as Indole alkaloids, quercitin , surfactants etc.[4] which can inhibit Pgly efflux and can allow drug or carrier through BBB. Polysorbate 80 (PS80) was studied extensively and its coating produced the most brain targeting effect via intravenous administration.[5] The mechanism of PS80 inhibition of Pgly efflux was by adsorbing apolipoprotein- E globules(ApoE) of blood over this PS80 coated drug. The adsorbed ApoE mimics the drug as LDL particle and sensitizes LDL initiated endocytosis through LDL receptor of the brain capillary endothelial cells making to pass

through BBB.[6] Thus PS80 coated or adsorbed in a lipid based drug delivery like liposomes could be one of the suitable carrier for Lp to brain as liposome could provide sustained , lipophilic , nano sized carrier for Lp favouring brain target.[7,8] But the reticulo endothelial system detection (RES) limits the successive brain target by liposomes. Therefore stealthing could be promoted for these PS80 coated liposomes by Polyethylene glycol (PEG) coating which avoids RES and potentiates circulation time , less free drug concentration and selective accumulation in target tissues and ensures its high concentration in target tissue for an extended period of time.[9,10] In our present study PS80 and PEG (Polyethylene glycol 20000) were conjointly coated over the Lp loaded liposomes (CCLs) at different ratios and characterised for Entrapment efficiency (EE) , *Invitro* release study , Zeta potential (ZP) ,poly disperse index (PDI) , SEM and DSC studies.

MATERIALS AND METHODS

Formulation of CCLs (modify it)

Lp loaded liposomes were prepared by "Reverse phase eveaporation Technique" using lecithin (Lec) and cholesterol (Ch) with modification.[11] Lp (5ml,2mg/mL) were dissolved in chloroform and were added to liposomes prepared using lecithin and cholesterol using szoka.[11].The solution is homogenised using a homogeniser and thus formed emulsion were vortexed to form suspension.sonicatior(Probe sonication) were used to form MLV vesicles.Conjoined coating of PS80 and PEG were prepared by dissolving varying concentration (0% - 2%) of Polysorbate 80 (P80) and Polyethylene glycol 20000 (PEG) with continuous magnetic stirring (9000rpm; 45 min) followed by centrifugation (75,000g; 20 min).Free excess supernatant PS80 and PEG were removed and retained MLV were lyophilised..Uncoated MLV were prepared as above but without PS80 and PEG.

Release kinetics

CCLs were studied for its *invitro* release in artificial cerebrospinal fluid (ACSF) using pH7.4 Mamallian ringer solution.[12] Conjoined coated Liposomes (CCLs) lyophilised powder (equivalent to 2 mg of Lp) were suspended in water by ultrasonication (4 KHz/s ; 5 min) to make a homogeneous suspension and tied in a dialysis bag (cellophane membrane, molecular weight cut off 10,000–12,000, Hi-Media, India).This bag was placed in 20 mL ACSF(pH 7.4) maintained at 37°C with continuous magnetic stirring. Aliquots of sample were withdrawn at selected time interval maintaining sink condition and analysed.[13] The sampling was done for every 15 min (For 1st h), every 30 min(till 5th h), every 1 h (till 10th) , every 2 h(till 15th),every 4 h (till the 30th h), and every 8 h till the end of 44th h.

Invitro release kinetics

Invitro release datas were treated with release kinetic equations.[14] viz zero order, first order ,Higuchi's model and Korsmeyer–Peppas model to determine the kinetics of drug release from CCLs as in table (Table 2).

Table 2
Release kinetic models and equation used to determine the release kinetics of CCLs

Model	Equation
Zero order	$Q_t = Q_0 + K_0t$
First order	$\log C = \log C_0 - K_1t / 2.303$
Higuchi Model	$Q = K_H X t^{1/2}$
Korsmeyer-Peppas model	$Mt / M\infty = Kt^n$

Q_t - amount of drug dissolved in time t, Q_0 - initial amount of drug in the solution ,K_0 - zero order release constant, C_0 is the initial , concentration of drug, k_1 is the first order rate constant, and t is the time, Q is the amount of drug released in time t per unit area A ,K_H –Higuchi constant , $Mt / M\infty$ is a fraction of drug released at time t, k is the release rate constant and n is the release exponent.

Entrapment efficiency

Entrapment efficiency.[15] were 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 swollen 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 CCLs 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 bottom. Distilled water was further added to the minicolumn and centrifuged. Absence of free drug was confirmed by testing the centrifugate after application of the saturated drug solution. Diethyl ether was used to lyse the CCLs followed by shaking with chloroform for 30 minute for solubilising the drug. Two immisible liquids were separated using a suitable separating funnel and the drug removed was quantified By Isocratic HPLC.[16] Entrapment efficiency of the Lp in the CCLs was calculated using the formula:

Percentage entrapment (%E) = [Entrapped drug (mg) /Total drug added (mg)] X 100

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. CCLs sample was diluted with KCl **(**0.1 M**)** and placed in the electrophoretic cell where an electric field of 15.2 V/cm was applied. Each sample was analyzed in triplicate

Mean Particle size and size distribution

The mean particle size and particle size distribution of CCLs were determined by Zetasizer Nano ZS (Malvern Instruments, Malvern,UK) at a temperature of 25 ±2°C and at an angle 90° to the incident beam applying the principle of photon correlation spectroscopy (PCS) . CCLs Dispersions were diluted with double distilled water to ensure that the light scattering intensity was within the instrument's sensitivity and analysed.

Differential Scanning Calorimetry (DSC)

Changes in endothermic peak for CCLs and Lp were studied by differential scanning calorimeter study (DSC- Q20, V4.5A ,TA Instruments) calibrated with indium. Samples of Lp , CCLs and blank CCLs (without Lp) were sealed in standard aluminium pans and analysed. Thermograms were obtained at a scanning rate of 10 °C/min. Isotonic PBS buffer (pH 7.4) was employed as reference. Each sample was scanned between 0°C and 300°C. The phase transition is defined as the maximal endothermic heat appropriate for the sample.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to verify the particle shape of all the CCLs . Freeze dried CCLs were resuspended by ultrasonication (4 KHz/s ; 5 min) in distilled water and dropped on to a silicon grid and left dried at room temperature. Then the CCLs were vaccuum coated with gold for 3 min.The image were then captured under a scanning electron microscope (JEOL-Japan) at an accelerated voltage of 15kV under argon atmosphere .

RESULTS AND DISCUSSIONS

Invitro release

Biphasic release was observed in all the formulation with an initial burst effect, followed by reduced drug release rate (Fig:1) **.** Initial burst effect of Lp from all the CCLs was attributed to the drug detachment from CCLs surface and the later slow release was due to sustained drug release from the inner lamella of the vesicles.[17,18,19] The drug release pattern varies for each formulation suggesting the diffusion of drug through lipid bilayers and polymer coating. Absence of both coating in S_0G_0 showed the higher release rate at the end of the 44th hour (76.8% ± 2.14) but S_0 G_2 (PEG 2%) and S_2G_2(PEG 2% and PS80 2%) showed lower release of 33.2% ± 3.27 and 42.11 ± 2.66 . A trend of reduction in release rate with increasing PEG coating concentration was in the following order,S_0G_0 (76.8%) > $S_{1.5}G_{0.5}$ (60.8 %) > S_2G_0 (56.6%)>S_1G_1 (50.2%) >$S_{0.5}G_{1.5}$ (46.2%) > S_2 G_2 (42.11) >S_0 G_2 (33.2%) .This reduction in Lp release was attributed to the resistance offered by the PEG random polymeric chains creating unfavourable entropy which increases the compression and stability of the coating layer retarding the drug diffusion from the liposomes.[20]

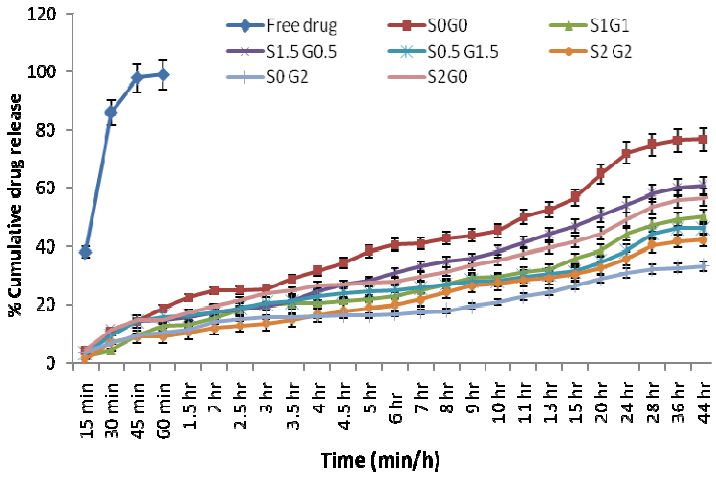

Figure 1
Invitro drug release profile of Lp in various CCLs and Free Lp in ACSF at 37 ± 0.5°C. p< 0.05 (n=3)

Release kinetics

Among the correlation coefficient(CC) R^2 for zero order ,first order ,Higuchi's model and Korsmeyer – peppas order, only the higuchi's model has the highest CC indicating the best fitting model and confirms the drug release from the CCLs were mainly driven by a diffusion controlled mechanism.[21] (Table:4)

Table 4
Correlation Coefficient for different CCLs drug release models

	Zero order	First order	Higuchi	Korsmeyer–Peppas	
	R^2	R^2	R^2	R^2	n
$S_0 G_0$	0.8634 ± 0.0021	0.9852 ± 0.0038	0.9894 ± 0.0033	0.9822 ± 0.0039	0.3321 ± 0.0044
S_1G_1	0.8822 ± 0.0034	0.9931 ± 0.0057	0.9993 ± 0.0027	0.9935 ± 0.0011	0.3451 ± 0.0016
$S_{1.5} G_{0.5}$	0.8935 ± 0.0054	0.9833 ± 0.0032	0.9958 ± 0.0036	0.9911 ± 0.0032	0.3735 ± 0.0031
$S_{0.5} G_{1.5}$	0.8425 ± 0.0041	0.9824 ± 0.0027	0.9937 ± 0.0242	0.9918 ± 0.0024	0.3642 ± 0.0052
$S_2 G_2$	0.8722 ± 0.0074	0.9834 ± 0.0036	0.9952 ± 0.0387	0.9924 ± 0.0039	0.3861 ± 0.0034
$S_0 G_2$	0.8625 ± 0.0044	0.9864 ± 0.0034	0.9927 ± 0.0011	0.9905 ± 0.0124	0.3912 ± 0.0018
S_2G_0	0.8444 ± 0.0028	0.9824 ± 0.0022	0.9963 ± 0.0034	0.9914 ± 0.0037	0.3422 ± 0.0013

R^2- Squared correlation coefficient , n - Release exponent of Korsemeyer peppas model .p< 0.005, (n= 3) ± SD

Entrapment efficiency(EE)

EE were in the range of 40% to 50 % for all CCLs and no appreciable difference in the entrapment efficiency among coated and uncoated CCLs was observed , as the coating was done after Lp loaded liposome preparation. But these EE of CCLs indicates the potential of the coat in preventing any drug leakage from vesicle damage or ostwalds ripening of CCLs.[22] (Table 5)

Characterisation study

Table 5
Zeta Potential , Mean Particle diameter, PDI and EE of different CCLs

Zeta potential (mV)	Mean particle diameter (nm)	PDI[$]	EE %
S_0G_0	-1.44 ± 0.411	314 ± 2.56	0.64	48.32 ± 0.31
S_2G_0	-4.22 ± 0.321	230 ± 2.01	0.21	43.12 ± 1.24
$S_{1.5}G_{0.5}$	-7.37 ± 0.036	246 ± 1.55	0.34	44.67 ± 0.67
S_1G_1	-10.52 ± 0.448	210 ± 2.84	0.23	49.37 ± 0.82
$S_{0.5}G_{1.5}$	-12.71 ± 0.018	272 ± 1.63	0.41	42.11 ± 1.04
S_0G_2	-15.42 ± 0.142	211 ± 1.22	0.32	46.22 ± 0.27
S_2G_2	-18.28 ± 0.312	196 ± 2.05	0.18	40.51 ± 1.44

*[$]PDI-Poly dispersity Index , *EE – Entrapment Efficiency Mean ± SD (n=3)*

Zeta potential

All CCLs exhibited negative ZP (Fig. 2,Table 5) with S_2G_2 having the higher ZP(-18.28 mV ± 0.312) and lower for S_0G_0 (-1.44 ± 0.411). The ZP order of CCLS were of S_2G_2(-18.28)>S_0G_2(-15.42)>$S_{0.5}G_{1.5}$(-12.71)>S_1G_1(-10.52)>$S_{1.5}G_{0.5}$ (-7.37)>S_2G_0(-4.22)>S_0G_0(-1.44). We observed an inverse correlation of ZP with *invitro* release such as S_0G_0 which had lower ZP (-1.44 ± 0.411) showed a maximum Lp release (76.8 ± 2.14) but S_2G_2 which had higher ZP (-18.28 ± 0.312) showed only less Lp release (42.11 ± 1.55) and this is due to the negative charge attraction over Lp drug thus, decreasing its release from CCLs.[21]

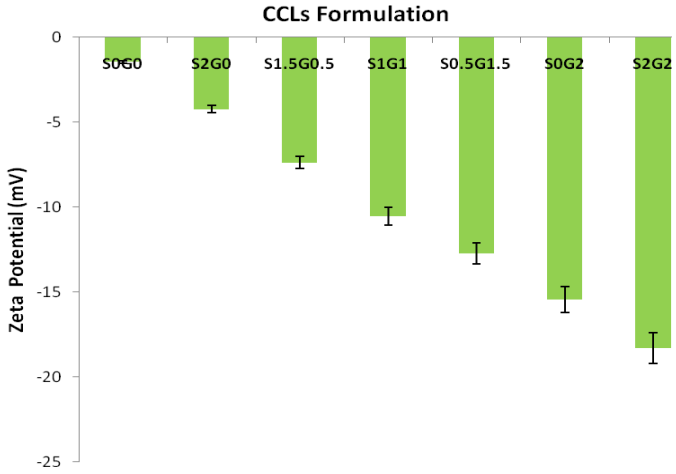

Figure 2
Zeta potential of different CCLs formulation (n=3)

Mean particle diameter

The mean particle diameter of all CCLs (Fig 3, Table 5)were found to be within the range of 100 to 300 nm and no appreciable influence by conjoined coating was observed as the coat was given after the formation of liposomes. The mean particle size of S_2G_2 were found to be in nanodimension (196 ± 3.85) (Fig 2) ensuring its potential to favour BBB penetration.[23] Presence of both PS80 and PEG at 2% in S_2G_2 might have prevented the liposome from ostwalds ripening.[24] thus keeping lower mean diameter than the other CCLs.

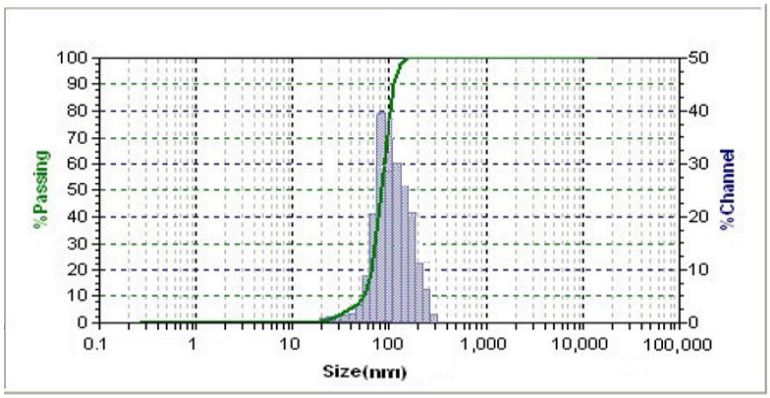

Figure 3
Particle size distribution of S_2G_2 . p<0.05 (n=3)

Poly Dispersive Index (PDI)

The homogeneous distribution of the nanosized particles was determined by PDI values (Table 5) which is a measure of particle size heterogeneity. CCLs with PDI values between 0.1 and 0.25 have a uniform size distribution and are expected to be stable. A PDI value greater than 0.5 indicates a less uniform size distribution.[25] Secondary particle growth due to vesicle fusion promoted by smaller vesicles due to its high membrane curvature results in more of larger vesicles termed ostwalds ripening.[24] Ostwald ripening increases the PDI value by promoting secondary particle or vesicle growth because of high membrane

curvature of small vesicles but PDI for S_2G_2 were found to be less than the other CCLs and this might be due to the protective effect of conjoined coat from ostwalds ripening process. Absence of coat in S_0G_0 showed higher PDI (0.64) indicating its larger deviation from homogeneous dispersion with other CCLs. We also have observed that PS80 influences PDI more than PEG does , and this was indicated by the increasing PDI value order as S_2G_2 (0.18) < S_2G_0 (0.21) <S_1G_1(0.23) <S_0G_2 (0.32) < $S_{1.5}G_{0.5}$ (0.34) < $S_{0.5}G_{1.5}$ (0.41) <S_0G_0 (0.64).

Differential Scanning Calorimetry (DSC)

Thermograms of drug Lp , empty and Lp loaded CCLs were shown in the figure (Fig:4). Lp showed an endotherm at 223.86^{0}C . Thermogram of CCLs without Lp showed a broad endotherm at 81.25°C followed by a major peak at 143.41°C corresponding to the lipid bilayer components - phospahtidyl choline and cholesterol.[26] The broad thermogram between 50°C to 100 ^{0}C was due to its water content which evaporates during the heat process.[27] In CCLs with Lp , lipid endotherm at 143.41°C was shifted to a broad endotherm at 153.21°C , but Lp endotherm (223.86^{0}C) was absent but merges to a broad endothermic peak at 143.41°C indicating the complete interaction with lipids .

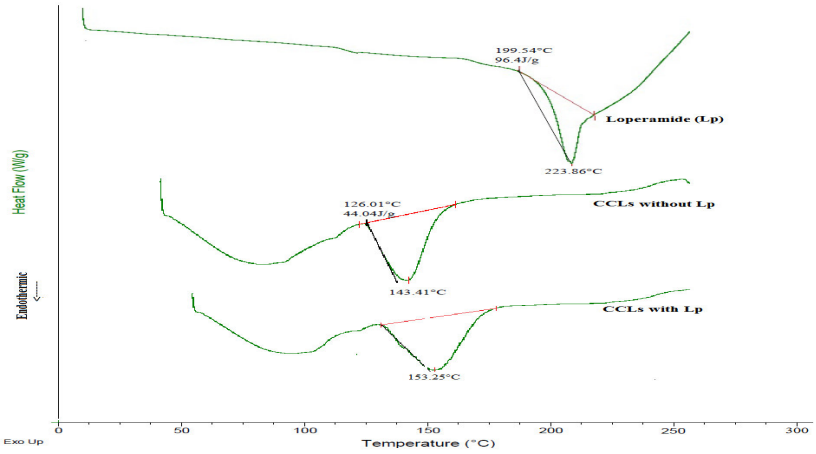

Figure 4

DSC thermogram of Drug –Lp, CCLs with and without Lp

Scanning Electron Microscopy (SEM)

In order to obtain more information about the particle size and shape, SEM analysis was performed.SEM photographs revealed the surface morphology of the CCLs. They were spherical shape and in nanometer range which was in agreement with the size data determined by PCS. Absence of any drug crystals suggests the complete entrapment of Lp in CCLs. Some particle shape deviate from the sphericity and this might be due to the lipid modification during drying process treatment (Figure: 5).

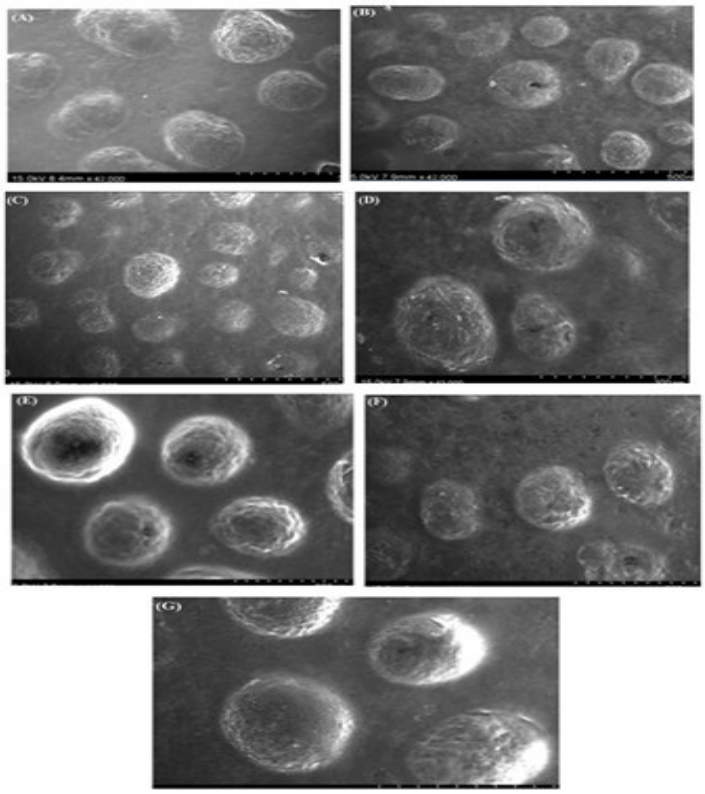

Figure 5
Scanning electron microscopy of different CCLs formulation
(A) $S_0 G_0$ (B) $S_1 G_1$ (C) $S_{1.5} G_{0.5}$ (D) $S_{0.5} G_{1.5}$ (E) $S_2 G_2$ (F) $S_0 G_2$ (G) $S_2 G_0$

CONCLUSION

In conclusion , S_2G_2 CCls conjoined coated liposomes would be a suitable carrier for brain targeting delivery system and should be further studied for its invivo performances

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CONFLICT OF INTEREST

Conflict of interest declared none

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