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INTRODUCTION

Receptor mediated transcytosis (RMT) is the most common transport mechanism to transport drugs into the brain due to its capability to selectively uptake the macromolecules by binding the selective ligands to its selective receptor presents on the brain capillary endothelial cells. Transferrin receptors (TfR) are mostly abundant on the brain capillaries and the transferrin (Tf) is used as a targeting ligand binds to its receptor (TfR) and transport drugs by RMT is becoming the interesting strategy in this study. Asiatic Acid (AA) has been chosen as a model drug in this study due to its well known neuroprotective properties by preventing and decreasing the amyloid β level in neurofibrillary tangles, reduce oxidative stress and increase ACh synthesis in ACh deficits in cerebral cortex. Solid lipid nanoparticles (SLNs) has been chosen as novel drug carrier in this study due to its size dependent properties, wide applications, higher biocompatibility, higher drug loading, higher stability, low cost exipients and several other advantages. Taken together, this study designed to focus on AA loaded SLNs conjugated with Tf targeting to the TfR on brain capillary endothelial cells, characterize its physicochemical properties and to study its drug release behavior in vitro.

MATERIALS AND METHODS

Drugs and chemicals Asiatic Acid and human holo-transferrin, and the membrane phospholipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) were purchased from Sigma-Aldrich, UK while the triglycerides, trimyristin and tripalmitin were purchased from TCI, Japan. The lipophillic surfactant Tween 80 and Span 80 were purchased from VWR International Ltd., UK. The hydrophilic surfactant, Pluronic F127 was purchased from BASF, Mount Olive, NJ, and 1-ethyl-3-(3-dimethylaminopropyl carbondiimide hydrochloride) (EDC) (Sigma-Aldrich, Germany) was used as zero length crosslinker. All aqueous solution was prepared Ultrapure water (Maxima Ultra Pure Water, Elga-Prima Corp, UK) with a resistivity greater than 18 MΩ/cm and phosphate buffered saline (PBS; pH 7.4). In addition, acetonitrile (Sigma) and PBS (pH 7.7) were used as HPLC mobile phase.

Preparation of AA loaded SLNs AA loaded SLNs were prepared by high pressure homogenization technique according to Soni, V., *et al* (2005) with the optimization results of each component by full factorial design. The DSPE, tripalmitin and trimyristin were used as lipid phase and Span 80 was used as lipophilic surfactatnt melted together with AA. Tween 80 and pluronic F 127 was used as hydrophilic surfactants.

Conjugation of transferrin with AA loaded SLNs The conjugation of AA SLNs and Tf was performed by the reported by Soni, V., *et al.* (2005) with slight modification. EDC was used as a zero length crosslinking agent by crosslinking the carboxyl group of Tf with amino group presents on DSPE lipid on the preformed SLNs via forming carbonyl amide linkage. The unbound drugs and excessive products were removed by passing the dispersion through the sephadex G50 column.

Particle size and zetapotential measurement To measure the average size, PDI, and zetapotential of both AA SLNs and AA Tf-SLNs, Nano-ZS zetasizer (Malvern Instruments, Malvern, UK) at 25 ^{0}C was used. Each measurement was made in five replicates (Luo, *et al.*, 2010).

Fourier transform infrared spectroscopy The conjugation between carboxyl group of Tf and amino group presence on the preformed surface of SLNs were comfirmed by FT-IR using KBr pellet technique and scanned in the range of 400 – 4000 cm^{-1} (Florey, 1979).

Drug Entrapment Efficiency The amount of entrapped AA in Tf conjugated and unconjugated SLNs were determined by using the method Fry, *et al.* (1978). The unentrapped drug from formulations was removed by passing the formulation through a Sephadex G-50 minicolumn and centrifuged followed by lysing the SLNs with Triton X-100 and content of AA will be analyzed by HPLC. The encapsulation efficiency was calculated by the following equation,

$$EE\ (\%) = \frac{W\ entrapped\ drug}{W\ initial\ drug} \times 100\ \%$$

Where $W_{entrapped\ drug}$ and $W_{initial\ drug}$ are the weight of entrapped drug in SLNs after G-50 column separation and the initial weight of drug that added in the system, respectively.

In Vitro Drug Release Study The release of AA from the conjugated and unconjugated formulations were studied in-vitro using dialysis membrane (Sigma Aldrich, molecular weight cut off: 12,400 Dalton, average flat width 25 mm (1 in) in diameter, porosity 0.45 μm) with the method reported by Gupta, *et al.* (2007) with slight modification. Samples were withdrawn periodically at 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 24, 36 hours and replaced with same amount of fresh buffer. The amount of drug were quantified by HPLC (Gonther, *et al.* 1996). The following formula was used to calculate the percentage of drug release from the SLNs system.

$$\text{Drug released}\ (\%) = \frac{Cr \times Vr}{A} \times 100$$

Where C_r is concentration of drug in receptor compartment, V_r is volume of receptor compartment, and A is the amount of drug in donar compartment at time zero and the release mechanism was analyzed by DD solver 1.0 add in program in Microsoft Excel (Gupta, *et al.* 2005).

Statistical Analysis All the mentioned experiments were repeated at least three times. Statistical analysis was performed using One-way ANOVA. A *P*-value of less than 0.05 would be considered as statistically significant.

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RESULTS

Particle size and zeta potential measurement The AA SLNs were prepared by high pressure homogenization method and conjugated with Tf. The result obtained from Nanozetasizer showed that the Tf conjugated nanoparticles produced the average particle size of <200 nm and the non conjugated nanoparticles produced <180 nm in diameter. To measure the size difference upon the addition of drug molecules, the measurement of plain nanoparticles conjugated and nonconjugated with Tf without loading drug showed <180 nm and <160 nm respectively. Zeta potential value was found to be less negativity after conjugation with Tf and there was no significant difference in poly dispersity index.

Fourier transform infrared spectroscopy The spectra obtained from AA loaded Tf-SLNs explained that the intensities of lipids of CH_2 bending at 1465 cm^{-1} and NH_2 bending at approximately around 3310 cm^{-2} after Tf conjugation and the intensities at 1729, 1736, 2916~ cm^{-1} IR spectra indicated the presence of C=C, C=O and C-H bend and stretch presents in lipids and AA. The peak level at 1654.44 at AA TF-SLNs revealed the bond formation of cabonyl group C=N and N-H bending at 1557.40 with increased and prominent peaks after conjugation with transferrin in drug loaded SLNs while the physical mixture showed no prominent bonding interaction when compared with AA Tf-SLNs as shown in Figure 1 (Florey, 1979).

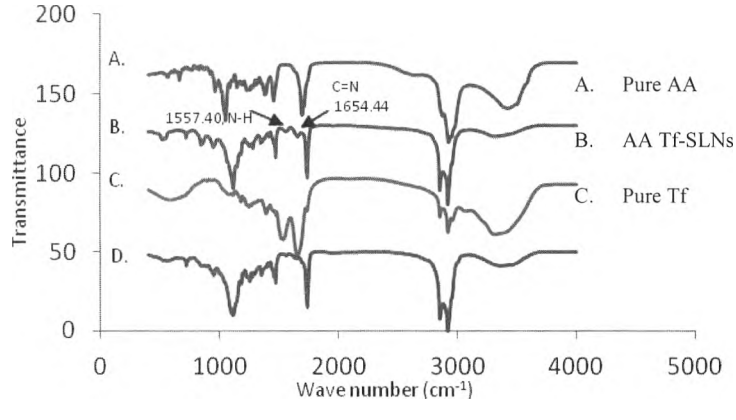

Figure 1 The IR spectra of A. Pure AA, B. AA Tf-SLNs, C. Pure Tf, D. Physical mixture

Drug Entrapment Efficiency The percent of AA loaded into the plain SLNs and Tf-SLNs were analyzed by HPLC and the result found out that the unconjugated SLNs entrapped ≤ 81% while the conjugated nanoparticles entrapment was reduced to ≤ 70% was observed.

In Vitro Release Study The percent of drug content was analyzed by HPLC at various time interval in vitro release study determined that the Tf conjugated AA loaded SLNs has higher drug released and showed sustained drug release behavior when compared with unconjugated nanoparticles, as ≤ 70% and ≤ 55.3 respectively as shown in Table 1 and the drug was released by Hopfenberg with Tlag model.

Table 1 The percentage of drug release at 4 different time points.

Time (hours)	AA SLNs (%)	AA Tf-SLNs (%)
4	≤ 3	≤ 5
12	≤ 9.5	≤ 10
24	≤ 18.4	≤ 22.5
36	≤ 55.3	≤ 70

DISSCUSSIONS

In the present study, the Tf conjugated AA loaded SLNs were prepared, characterized its physicochemical properties and evaluated its drug release properties. The plain and AA loaded Tf conjugated and unconjugated nanopartilces were prepared and measured its size, polydispersity and zetapotential by nanozetasizer as shown in. The Tf conjugated nanoparticles showed slightly bigger particle size than non-conjugated nanoparticles in both plain and drug loaded SLNs, this could explained the conjugation of Tf molecules on the surface of preformed SLNs and formation of Tf layer could make the particles slightly bigger in size and zeta potential values was decreased due to the slightly negative charge on Tf layer shield the surface of SLNs after conjugation. There was no significant change in polydispersity index value revealed that there was no excess Tf, unentrapped drugs or others excess components dispersed in the formulation.

From FT-IR spectrum, the intensities level in the spectra of AA Tf-SLNs at 1654.44 and 1557.40 showed (C=N) bond bending and N-H bond stretching with the formation of peaks after conjugation with Tf respectively when compared with physical mixture. This could confirm that the Tf was successfully conjugated by formation of carbonyl amide linkage between Tf and amino group presents in SLNs without interfering the physiological interactions in the preformed SLNs. Moreover, the C-H stretch and esters bends present in AA was still maintained in the spectra revealed that the drug was successfully loaded in the nanoparticles as shown in Figure 1.

The drug entrapment efficiency was analyzed by HPLC and the percent entrapment of Tf conjugated SLNs was 70% which was slightly decreased then non-conjugated SLNs and this could be due to the leaching of drug molecules on the surface of preformed SLNs when incubation during conjugation to anchor the Tf molecules on the surface of SLNs.

The drug release of Tf conjugated SLNs were significantly higher than that of non-conjugated SLNs after 36 hour period and showed the sustained drug release behavior in vitro. The release model explained that the drug was released by the mechanism of erosion of the erodible matrix on the surface of the molecules. This could indicate that the peptide had dramatic impact on erosion of the polymer matrix of the drug release profile.

CONCLUSION

In conclusion, Tf as a targeting ligand was successfully conjugated to the AA loaded SLNs due to the formation of carbonyl amide bonding without disturbing the physicochemical properties of the SLNs. AA was loaded in the nanoparticles without having distortion or interaction to the bonds between polymer matrix and surfactant. High drug entrapment efficiency and sustained drug release profile were observed in Tf conjugated SLNs with very small size with low polydispersity index. This proposed carrier could be used as targeted and enhanced drug delivery system in order to overcome BBB limitations with Tf targeting ability to selectively bind the Tf receptors present on brain endothelial cells.

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