Introduction
The chemotherapy for the brain cancer treatment is very difficult due to the presence of blood–brain barrier (BBB) and blood–tumor barrier (BTB) (Van et al., 2015; Fortin, 2012). BBB consist of the endothelial cells, joined together by tight junctions by which transport of most of the drugs from the blood into the brain is hampered. The luminal side of the BBB also contains efflux membrane transporters like P-glycoprotein (Pgp) and multidrug resistance related proteins which recognize the anticancer drugs as substrates (Löscher and Potschka, 2005). BBB and BTB limit the amount of drugs that can be delivered to the tumor site. It causes insufficiency of drug at the target site to achieve a therapeutic concentration. Therefore, there is a need to employ a strategy which can deliver the cytotoxic drug to the brain at the therapeutic level. Ligand conjugated nano carrier bearing drug capable of recognizing the brain capillary endothelial cells and brain cancer cells have shown promising potential in brain cancer treatment (Pinto et al., 2017).

Abstract

Objective: The aim of this study was to investigate the targeting potential of transferrin anchored solid lipid nanoparticles (SLN) loaded with doxorubicin (D-SLN-T). Material and methods: Transferrin receptors (TR) are highly expressed on blood brain barrier as well as brain cancer cells. Therefore, targeting TR using transferrin (Tf) improved the anticancer activity of prepared nanoparticles. The Tf coupled SLN were characterized by fourier transform infrared spectroscopy, transmission electron microscopy, particle size, particle size distribution, zeta potential, % entrapment efficiency, vitro drug release and cell line studies. Results and conclusion: The average particle size of the D-SLN-T was found to be 210.3±1.3 nm with a negative surface charge. The cell line studies, on U87 MG brain cancer cell lines, showed that the cytotoxicity of D-SLN-T was highly increased when anchored with transferrin. Also, the presence of transferrin on the surface of the D-SLN-T enhanced the cellular uptake of drug on U87MG cell line. The results of the cytotoxicity and cellular uptake studies clearly showed the potential of D-SLN-T in brain cancer treatment.

Keywords: Transferrin, brain, solid lipid nanoparticles, doxorubicin, drug delivery

*Address for Corresponding Author:
Prof. Vandana Soni,
Department of Pharmaceutical Sciences,
Dr. Hari Singh Gour, Central University, Sagar, Madhya Pradesh, 470 003, India.
Email: drvandanasoni@gmail.com

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Materials and Methods

Materials

DOX and FA were purchased from Himedia, Mumbai, India. Transferrin, Tristearin and Cholesterol were purchased from Sigma Aldrich, Germany. HSPC and DSPE were provided as a gift sample from Lipoid, Germany. Aminocaproic acid (AMA), N-hydroxysuccinimide (NHS), Dicyclohexylcarbodiimide (DCC) were purchased from Spectrochem, Mumbai. All other chemicals and reagents were used are of analytical grade.

Preparation of the DOX loaded Solid Lipid Nanoparticles (D-SLN)

SLN were prepared using solvent diffusion method as reported by Wong et al. (2004) with slight alterations. Briefly, tristearin, HSPC, DSPE and cholesterol (1:1.5:1:1.2) were dissolved (with heating at 70 °C) in 10 mL of ethanol containing drug. The lipid phase was added into aqueous phase (containing phosphate buffer saline; PBS; pH 7.4; 0.5 % v/v, Tween-80) using syringe (2 mL/min) with constant stirring. The mixture was mechanically stirred (Remi, Mumbai, India) for 1 h at 2,000 rpm. This leads to the formation of lipid suspension which was sonicated by probe sonicator (PCI, India) for 2 min. The formed D-SLN were concentrated by centrifugation and again suspended in fresh PBS (pH 7.4). Unentrapped drug was separated by using Sephadex G-50 minicolumn. The method of preparation of D-SLN is shown in figure1.

Coupling of Tf with D-SLN

Tf was anchored with D-SLN using the method reported by Gupta et al. (2007) with slight modification. The carboxyl group of Tf was covalently coupled to the amino group of DSPE orienting outside on the surface of preformed D-SLN using EDC as the coupling agent. In short, D-SLN were suspended in PBS (pH 7.4) containing Tf (lipid:Tf ratio; 90:10 w/w). To this suspension, EDC (10 mg per mL lipid/Tf) were added with vortex followed by incubation for 2 h at room temperature. Uncoupled Tf was removed using Sephadex G-50 column and Tf coupled SLN (D-SLN-T) were analyzed by FTIR (PerkinElmer, Pyrogon 1000).

Size, size distribution and zeta potential

The average particle size, PDI and zeta potential of formulations were determined by photon correlation spectroscopy (PCS) with a Zeta sizer (Malvern Instruments, UK), equipped with the Malvern PCS software.

Drug content

The entrapment efficiency of the drug in D-SLN and D-SLN-T was estimated by the method reported by Fry et al (1978). D-SLN and D-SLN-T formulations free from any unentrapped drug were lysed by using Triton X-100 (0.1%v/v). The dispersion was filtered and absorbance of the filtrate was taken at 480 nm using UV spectrophotometer.

Figure 1. Method of preparation of SLN
Percent drug entrapment was calculated with the help of the formula:

Percent (%)drug entrapment = \frac{\text{Amount of drug released from the lysed SLN}}{\text{Amount of drug initially taken to prepare the SLN}} \times 100

Surface Morphology of D-SLN-T

The surface morphology of SLN was determined by transmission electron microscopy (TEM; Philips, Tecnai 20, Holland) at 200 kV acceleration voltage and 50,000X magnification. Sample was prepared by diluting suspension of D-SLN-T with distilled water and stained with 2% solution of phosphotungstic acid in water.

Coupling efficiency of Tf

The anchored amount of Tf on the surface of the D-SLN-T was determined by Bradford protein assay method (Carlsson et al., 2011). Briefly, 1 ml D-SLN-T dispersion and 1ml dye solution (Coomassie blue G) was taken in10 ml volumetric flask. Both were mixed and diluted up to 10 ml with distilled water. The mixture was kept for 30 min at room temperature and then at 595 nm absorbance was taken by UV-visible spectrophotometer. The observed absorbance was compared with a blank solution containing similar quantity of dye. Conjugated amount of Tf is expressed in mg/mM of phospholipids.

In vitro drug release

In vitro drug release of entrapped drug from D-SLN and D-SLN-T was determined in PBS (pH 6.8) by using dialysis bag (MW, 3500 Da) diffusion method. Both formulations (containing drug equivalent to 5mg) were filled in a dialysis bag and placed in a beaker containing 20 ml PBS (pH 6.8), separately. The buffer in the beaker was magnetically stirred at 100 rpm at 37 ± 1°C. At predetermined time intervals, samples (1 ml) were taken out and replenished with the same volume of PBS (pH 7.4). The drug content was determined in withdrawn samples using UV spectrophotometer at λmax of 480 nm.

Cytotoxicity and cellular uptake studies

The cytotoxicity of the D-SLN and D-SLN-T was determined by the Sulforhodamine B (SRB) assay method. For the cytotoxicity study, U87MG cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Then the cells were plated in 96 well microtiter plates. The inoculated cells were incubated for 24 h at 37° C, 5 % CO2, 95 % air and 100 % relative humidity. Then the cells were incubated with the formulations in concentrations, i.e. 0.01 μg/ml-100 μg/ml at standard conditions for 48 h. The cells were fixed by the addition of 50 μl of 30 % (w/v) cold TCA and again incubated at 4°C for 1 h followed by washing with water and drying. Each well was stained with 50 μl of 0.4% (w/v) SRB solution in 1 % acetic acid followed by 20 min incubation at room temperature. After staining, excess stain was removed by using 1 % acetic acid (v/v) and dried. Bound stain was removed with 10 mM trizma base, and the absorbance was recorded at a wavelength of 540 nm on microplate spectrophotometer (Model 680, Bio-Rad, Japan). The viability of cells was expressed in percentage compared to untreated cells (control).

For the cellular uptake study, brain cancer cells were cultured in petridishes containing RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine until density reached up to 80% confluence. The medium was replaced with plain coumarin 6, coumarin 6-SLN, coumarin 6-SLN-T and incubated for 2 h. After that, dispersion was removed and cells were kept at 37°C. The cells were fixed by the addition of 1 ml of 70% ethanol solution. After 20 minutes ethanol solution was removed, and cells were washed three times using PBS. Then, 10 μl of 5 mg/ml propidium iodide (PPI) was added to stain nucleus. After 30 minutes, stain cells were again washed three times with PBS (pH 7.4) and observed under fluorescence microscope.

Statistical analysis

The obtained data were analyzed by ANOVA, lack of fit tests and multiple correlation coefficients. Student's t test was used to test the statistical significance wherever applicable. Obtained data were expressed as mean±SD (n=3).

Results and discussion

In the present research, formulations D-SLN and D-SLN-T were prepared by the solvent injection method using a mixture of lipids. The mixture of lipids enhanced the stability of the nanoparticles. The free amino groups of DSPE and carboxyl groups of Tf were reacted and formed the amide bond. The formation of amide bond was confirmed by FTIR spectroscopy. The appearance of the characteristic peak at 3,447 cm−1 and at 1,656 cm−1 in the FTIR spectra of the D-SLN-T, represents –N–H and –C–O stretching of the amide group which confirmed the coupling of Tf with SLNs (Figure 2). The coupling efficiency of Tf on the surface of SLN was determined by Bradford protein assay method. The coupling efficiency is important to determine as it decides the in vivo internalization of the formulation. The percent of Tf coupled to the surface of the D-SLNs-T was found to be 28.36 %, which confirmed the good coupling efficiency and may supports in vivo internalization. The surface morphology of D-SLN-T was observed by TEM microscopy. Figure 3 shows the spherical shape and smooth surface of the D-SLN-T. The average particle size of D-SLN and D-SLN-T was found to be 202.2±0.8 nm and 210.3±0.9 nm, respectively (Table 1). The
particle size of D-SLN-T was found to be greater than D-SLN formulation may be due to the coupling of Tf on surface of D-SLN-T. The PDI results confirmed the uniformity of the particle size in the formulation as PDI was less than 0.3. The zeta potential D-SLN-T and D-SLN was found to be -15.2± 1.33mV and -14.8± 1.43mV, respectively. The almost same zeta potential of both the formulations showed that coupling of Tf not markedly affects the surface charge of the formulation. The entrapment efficiency of drug in D-SLN-T was found to be less than D-SLN. This could be due to leaching of drug during the coupling process of Tf on the surface of D-SLN-T.

*In vitro* release of drug from D-SLN and D-SLN-T was determined in PBS (pH 6.8). The cumulative % of drug release from D-SLN and D-SLN-T was found to be 58.2 ± 1.3% and 45.3 ± 2.5%, respectively in 48 h time interval (Figure 4). The decrease in drug release profile from the D-SLN-T than D-SLN could be due to presence of Tf on the surface of D-SLN-T, which formed a barrier to the diffusion of the drug.

**Table 1.** Particle size and entrapment efficiency of the optimized SLN

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulations</th>
<th>Average Particle size (nm)</th>
<th>Entrapment efficiency (%)</th>
<th>PDI</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-SLN</td>
<td>202±0.8</td>
<td>45.3±0.2</td>
<td>0.244±0.05</td>
<td>-14.8± 1.43mV</td>
</tr>
<tr>
<td>2</td>
<td>D-SLN-T</td>
<td>210±0.9</td>
<td>40.0±0.2</td>
<td>0.253±0.05</td>
<td>-15.2± 1.33mV</td>
</tr>
</tbody>
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*Figure 2.* FTIR spectrum of D-SLN-T

*Figure 3.* TEM photomicrograph of D-SLN-T

*Figure 4.* Cumulative % drug release from D-SLN and D-SLN-T

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Cytotoxicity and cellular uptake studies
The cytotoxicity of the plain drug, D-SLN and D-SLN-T was assessed by sulphorodamine blue (SRB) assay in U87MG cell line by the determination of half maximal inhibitory concentration (IC50) (Figure 5). The IC50 values of plain drug, D-SLN, D-SLN-T was found to be 6.5, 3.2 and 2.3µg/ml, respectively. The IC50 values clearly showed that the D-SLN-T was more toxic than D-SLN and plain drug for U87 MG cells. This could be due to the higher uptake of D-SLN-T through Tf receptors, which were overexpressed specifically in cancer cells. Plain drug showed least toxicity may be due may be because of the efflux of drug by Pgp present on the cancer cells, suggesting the multiple drug resistance effect of U87MG cell lines (Seebacher et al., 2016).

In all the formulations, survival fraction of cells was found to be inversely dependent on the concentration of the drug. The results obtained from the study reveals that D-SLN-T will be a better drug carrier for the treatment of brain cancer.

Figure 5. Cytotoxicity assessed by SRB assay on U87MG cells after 48 h. The figure shows % survival fraction of U87MG cells treated with plain DOX, D-SLN and D-SLN-T at 0.1 µg/ml, 1µg/ml, 10 µg/ml and 100 µg/ml dose. Data are presented as the mean±standard deviation (SD)(n=3)

Figure 6. Confocal photomicrograph shows the intracellular uptake of coumarin6, coumarin6-SLN and coumarin6-SLN-T by U87MG cells after treatment with plain 2 h. Data are presented as the mean±standard deviation (SD) (n=3).
To check the uptake of the Tf coupled and uncoupled SLNs in brain cancer cells, cellular uptake studies were performed on the U87MG cells. For this, coumarin 6 was used as a fluorescent stain to track the formulation. Higher cellular uptake was found in case of coumarin6-SLN-T than plain coumarin6 and coumarin6-SLN after 4 h. This may be due to transferrin receptor mediated endocytosis via TR overexpressed on the cancer cells. In figure 6(I), the red colour shows the localization of the nucleus by using PPI stain. In figure 6(II), blue colour localized the formulation i.e. coumarin 6, coumarin 6-SLN and coumarin 6-SLN-T. Figure 6(III) is the merged images of both 6(I) and 6(II). Figure 6 also clearly shows that the percentage of fluorescent cells were increased with time as compared to untreated control cells, suggesting the enhanced cellular uptake of coumarin 6-SLN-T with time (p ≤ 0.05) (Danhier et al., 2009). The cellular uptake studies, clearly showed the efficiency of the Tf anchored SLNs in delivering the entrapped drug in U87MG cells.

Conclusions
The present research work demonstrated the preparation and characterization of doxorubicin loaded SLN anchored with transferrin for the treatment of brain cancer. The cellular uptake and cytotoxicity studies on U87MG cell lines confirmed the uptake of D-SLN-T formulation and cytotoxicity in brain cancer cells. The results of the studies suggested the potential of the D-SLN-T

In improving the uptake and localization of drugs inside the cancer cells.

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References


