Comparison of Two Methods of Conjugation of Tranferrin to Nanostructured Lipid Carriers

Jaleh Varshosaz¹, Abbas Jafarian Dehkordy², and Amir Khajavinia³

Abstract—The aim of the present study was comparison of two coupling methods of transferrin (Tf) to nanostructured lipid carriers (NLCs) and its effect on physicochemical properties and cytotoxicity on acute myelogenous leukemia cells. NLCs were prepared by emulsion-solvent evaporation method. Chemical coupling was mediated by an amide linkage between the surface-exposed amine group of the fatty amine of NLCs and the carboxylic group of the protein. Etoposide was loaded in NLCs as a model drug. NLCs were characterized for their particle size, zeta potential, polydispersity index, drug entrapment percentage, drug release profiles and Tf-coupling efficiency. The cytotoxicity of NLCs on K562 cells was studied by MTT assay. The percentage of drug entrapment and zeta potential decreased after Tf-coupling but the average particle size increased. Non-conjugated NLCs released moderately more drug than Tf-NLCs. The highest cytotoxic activity was observed in the chemically coupled NLCs with 15-fold lower IC₅₀ than free etoposide.

Keywords—Tranferrin targeted nanoparticles, nanostructured lipid carriers, chemical or physical conjugation.

I. INTRODUCTION

TRANFERRIN (Tf) receptor is over expressed in many malignant cells, including breast, pancreatic, prostate, colon, lung and leukemia [1-3]. Drug resistance has been shown to be associated with elevated levels of Tf receptor [4], consequently it has been investigated in a number of studies [5].

Tf-bound liposomes containing doxorubicin have been shown to enhance cellular uptake of the drug in glioma, through a receptor-mediated mechanism [6]. Chitosan vesicles decorated with Tf resulted in the rapid uptake of the doxorubicin encapsulated vesicles [7]. Lipopolyplexes targeted with Tf, are also used as carriers of antisense oligodeoxyribonucleotide for Bcl-2 protein which makes leukemia cells resistant [8] and lipid nanoparticles containing olygonucleotide are other examples of targeted delivery systems using Tf [9]. In general, two different procedures have been incorporated for the post insertion of proteins on the surface of prepared nanoparticles, including chemical binding [10] and physical coating [11]. Due to the lack of studies comparing these two methods this study aimed to compare their effectiveness in physicochemical properties of carrier NLCs and their ability in targeting Tf receptors.

II. METHODS

A. Preparation of drug loaded NLCs

NLCs were prepared by an emulsion-solvent diffusion and evaporation method followed by probe sonication. The hot lipid phase containing etoposide, cetyl palmitate (328 mg), octyldodecanol (83.8 mg), soy lecithin (100 mg) and the fatty stearyl amine were dissolved in 10 ml of a mixture of acetone and absolute ethanol (aceton: ethanol volume ratio 4:1) on a water bath at 60°C. After that the lipid phase was gently dispersed in a 1 w/v% poloxamer 188 solution at 50°C, in several portions (oil/aqueous phase volume ratio 1:5) and premixed by magnetic stirrer for 5 min at 2000 rpm. The obtained pre-emulsion was then ultrasonicated using a probe sonicator (Bandelin, Germany). Finally the temperature of the nanoemulsion was returned to room temperature. The free drug was removed using dialysis bags with molecular weight cut-off of 12400 Da by diafiltration against PBS (pH 7.4).

B. Drug loading efficiency

1 ml of NLCs dispersion was centrifuged at 10000 rpm for 20 min by an ultra centrifuge. The supernatant was diluted with de-ionized water and drug concentration was assayed by capillary electrophoresis system (Agilent 7100 capillary electrophoresis system, Agilent Technologies, Germany) with UV detector at 286 nm. The difference between the total and free drug showed the amount of the encapsulated drug. The encapsulation efficiency (EE) of etoposide in NLCs was determined as the ratio between the actual and theoretical loading.

C. Particle size and zeta potential measurements

The mean particle size and zeta potential of NLCs were measured by photon correlation spectroscopy (PCS) at a fixed angle of 90° (Zetasizer 3000HS, Malvern Instrument, UK). Nanodispersion was suitably diluted to measure mean particle size and polydispersity index.
D. In vitro drug release

3 ml of NLCs suspension was placed in dialysis bags (12400 Da cut-off) and suspended in a beaker containing 20 ml of PBS on a magnetic stirrer with the speed of 200 rpm at 37°C±0.5°C. The drug released at predetermined time intervals was determined by capillary electrophoresis at 286 nm.

E. Conjugation of NLCs with transferring

Chemical conjugation was done using 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide that was added per each ml of the mixture of lipid/Tf of NLCs suspension (lipid : Tf ratio was 90:10 w/w). the mixture was vortexed and incubated for 2 h at room temperature, in the dark. The physical coating was done by dissolving Tf in Ringer-Hepes buffer (pH 7.4) at a concentration of 1 mg/ml. NLCs suspension was added at the ratio of 1:1(w/w). The mixture was left for 3 h with moderate stirring at room temperature. In both methods the unconjugated Tf was removed by passing the dispersion through a Sephadex G-50 column.

F. Coupling efficiency of Tf to NLCs

The suspension of conjugated NLCs was centrifuged using centrifugal filter tubes with a 100 KDa cut-off. The filtrate was analyzed to determine the protein content using Bradford protein assay.

G. Cytotoxicity Assessment- MTT assay

K562 cell line was used in this study. The cells were cultured on RPMI1640 containing 10% FBS (Fetal Bovine Serum) and 1% antibiotics mixture of penicillin (10000 U/mL) and streptomycine (10000 µg/mL) at 37°C and in 5% CO₂. First 180 µl of the suspension of cells at a density of 5×10⁴ cells/ml were seeded into each well of a 96-well culture plate (SPL Lifescience, Korea) and incubated for 24 hr at 37°C in 5% CO₂ and 100% humidity before cell viability test. After cells were seeded on 96 well plate each raw was treated with: 20 µl of diverse concentrations of etoposide either as solution (in 1% DMSO) or encapsulated in nanoparticles, with blank (without drug) nanoparticles, medium or DMSO serving as respective controls and doxorubicin as positive control. Then the plate was incubated for 72 hr and after that 20 µl of MTT was added. After 3 hr incubation the cell medium was removed cautiously while not allowing the produced Formazan crystals to pour. Then 180 µL of DMSO was added to the crystals until they were dissolved. Immediately after pipetting each raw was separately analyzed by ELISA method.

III. RESULTS AND DISCUSSION

Fig. 1 obviously shows that z-average values of NLCs have increased after Tf-conjugation process due to large peptide molecule decorating the NLCs. The second explanation for the increase in particle diameter is that both chemical and physical reactions for Tf-conjugation were performed in buffer solutions for 2 and 3 hours, respectively. These conditions can potentially lead to partial particle aggregation and increase the mean particle size of the nanoparticles. It is particularly noticeable for physically coated nanoparticles due to the fact that the reaction had been carried out under magnetic stirring and that the adsorption reaction had lasted for 3 hours which is more than the time for chemical reaction (2 h).

Fig. 2 shows that all prepared formulations have positive zeta potential values as a result of the projection of positively charged amine groups of the fatty amines on the outer surface of the NLCs which is exposed to the water phase. Tf-binding has decreased the zeta potential for all formulations which could be due to masking the cationic charges on the surface of NLCs. Data analyzing by independent t-test revealed that the difference between chemically and physically conjugated NLCs are statistically significant (P<0.05) which might be a result of the difference in their Tf-coupling efficiencies.
Table 1 shows the entrapment efficiency (EE%) and loading percent of NLCs, it is obvious that plain NLCs have marked higher amount of loaded drug. This phenomenon might be explained by the higher tendency of the etoposide molecules, which have electronegative oxygen atoms, to enter nanoparticles which encompass positively charged particles. It is also clear that both EE% and loading percent values decline for the Tf-anchored NLCs which may be due to drug leaking during the conjugation reaction [10]. Physically bond nanoparticles have lower amounts of loaded drug because of longer duration of adsorption reaction (3 h) compared to the covalent reaction (2 h).

Fig. 3 illustrates different conjugation efficiency values in terms of mg Tf per mmol stearyl amine. Noticeably, chemical conjugation bonded more Tf on the surface of nanoparticles than the physical coating (p<0.005). This might be due to the fact that both physical and chemical coatings take place simultaneously during the chemical reaction time.

The drug release profiles from the Tf-coupled and plain NLCs in PBS (pH 7.4) are presented in Fig. 4. Table 1 also shows the release efficiency (RE24%) values. As can be seen in this figure and table the percentage of drug release was suppressed in the case of Tf coupled NLCs regardless of the conjugation method. Differences between the particle sizes of the nanoparticles (Fig. 1) can be considered as the second reason for the variation of release profiles which can explain the observed differences between chemically and physically coupled nanoparticles. Since smaller particle sizes have shorter drug diffusion distance and larger surface area, they show faster drug release [12].

![Table 1](attachment:image1.png)

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>PDI</th>
<th>Entrapment efficiency (%)</th>
<th>Loading (%)</th>
<th>Release efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain NLC</td>
<td>0.185±0.020</td>
<td>47.48±0.13</td>
<td>0.99±0.00</td>
<td>61.40±1.11</td>
</tr>
<tr>
<td>Tf-C*NLC</td>
<td>0.257±0.023</td>
<td>30.51±1.06</td>
<td>0.64±0.02</td>
<td>54.78±0.75</td>
</tr>
<tr>
<td>Tf-P**-NLC</td>
<td>0.296±0.035</td>
<td>18.82±1.30</td>
<td>0.39±0.03</td>
<td>51.55±0.74</td>
</tr>
</tbody>
</table>

*C=Chemically conjugated, **P=Physically decorated

![Fig. 3](attachment:image2.png)

Fig. 3. The coupling efficiency values in terms of mg transferrin per mmol stearyl amine.

![Fig. 4](attachment:image3.png)

Fig. 4. Etoposide release profiles from plain (unconjugated), chemically conjugated (C) and physically decorated (P) NLCs in phosphate buffer saline (pH 7.4) and 1% Tween 20 (n=3).

![Fig. 5](attachment:image4.png)

Fig. 5 summarizes the results obtained from MTT assay. As this figure shows, all drug loaded nanoparticles have caused higher cytotoxicity compared the free etoposide at the same concentration as the loaded drug in NLCs and their respective blank NLCs. Furthermore, all Tf targeted NLCs have dramatically higher cytotoxicity compared to their relative drug solution concentrations.

![Fig. 6](attachment:image5.png)

Fig. 6. Percent of viable cells of K562 determined by MTT assay after treatment with blank NLCs, plain (without Tf decoration), chemically conjugated (C-NLC) and physically decorated (P-NLC) nanoparticles in comparison with free etoposide at different concentrations of a) 0.375 μM, b) 0.75 μM and c) 1.5 μM, doxorubicin 580 μg/ml (positive control) and culture medium (negative control).

The percent of viable cells is more than 80% for blank NLCs at the concentrations of 0.1875 μM (equals to ⅛×IC50 of...
free etoposide solution) and more (data not shown). For all blank NLCs at the concentration of 0.375 µM (equals with ¼×IC₅₀ of free etoposide solution) the viable cells are more than 70% (Fig. 5). The cell survival at the 0.375 µM dose was 20.2% and 26.72% for chemically and physically bond Tf-NLC, respectively; which show more than 3-fold cytotoxicity compared to the free drug solution with 76.74% cell viability.

IV. CONCLUSION

This study illustrates that Tf-conjugation with NLCs results in enhanced antiproliferative effect of the encapsulated anticancer drug in AML cells. Consequently, this potential tool can be applied for further improving of the desired properties and lessen the unwanted properties of the lipid nanoparticles in the future. NLCs tagged with Tf have the potential to kill AML cancer cells more specifically and effectively while sparing normal cells. The highest cytotoxic activity was observed in the chemically coupled nanoparticles with an IC₅₀ value of 15-fold lower than free etoposide. Further studies are needed to be checked in vivo to confirm the promising results on the cell culture.

ACKNOWLEDGMENT

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REFERENCES


