In vitro release and skin permeation of tacrolimus from monoolein-based liquid crystalline nanoparticles

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The objective of the present study was to determine the effect of different ratios of monoolein and oleic acid on in vitro release and skin permeation of tacrolimus from monoolein-based liquid crystalline nanoparticles. The nanoparticles were prepared by sonicated a mixture of melted monoolein, poloxamer 407, oleic acid and tacrolimus to which distilled water was added. Formation of cubosomes and hexosomes was confirmed by transmission electron microscopy and optical microscopy, and average particle size of the formulations was about 150-200 nm. The encapsulation efficiency for tacrolimus in all the formulations was > 99 %. In vitro release of the drug was proportionally reduced by the amount of monoolein used. Addition of oleic acid further reduced the tacrolimus release. The skin permeation was also in agreement with the in vitro release. This study provides a strategy to control the release and skin permeation of tacrolimus from nanoparticles, thus expanding the area of tacrolimus usage.

Keywords: Liquid crystalline nanoparticles, Tacrolimus, Monoolein, Oleic acid, Cubosomes, Hexosomes.

I. MATERIALS AND METHODS

1. Materials

Monoolein was received as a gift from Danisco (Tokyo, Japan), and poloxamer 407 was purchased from BASF (Ludwigshafen, Germany). Tacrolimus was acquired from Shanghai Qiao Chemical Science (Shanghai, China). Oleic acid was purchased from Sigma-Aldrich (St. Louis, United States). All other chemicals were of reagent grade and used without further purification.

2. Preparation of tacrolimus-loaded liquid crystalline nanoparticle formulations

Tacrolimus-loaded liquid crystalline nanoparticle was prepared with slight modification of the method reported by previous researchers [7, 10, 21, 22]. Briefly, different amounts of monoolein ranging from 2 to 8 % w/w of the formulation were taken as described in the Table I. Variation in the formulations was brought about by addition of oleic acid (0, 25, and 50 % w/w as compared to monoolein). The amount of poloxamer 407 used was fixed to 12 % w/w of the total amount of monoolein and oleic acid.

The designated amounts of monoolein, poloxamer 407, and oleic acid were taken and melted in a water bath at 60 °C followed by tacrolimus addition with continuous stirring to complete dissolution. Required amount of distilled water was gradually added to the melted mixture with vortexing for 1 min followed by sonication for 1 h. The liquid crystalline nanoparticle dispersion prepared was stored at room temperature for further studies. The detailed composition of the liquid crystalline nanoparticle formulations is summarized in Table I.

3. Characterization of tacrolimus-loaded liquid crystalline nanoparticle formulations

3.1. Particle size analysis

The particle size analysis was performed with Zetasizer Nano ZS (Malvern, United Kingdom). The solid content was adjusted to 0.02 % w/w by dilution with distilled water.
Table I - Composition, particle size, and entrapment efficiency of liquid crystalline nanoparticles.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>MO (g)</th>
<th>OA* (g)</th>
<th>P 407** (g)</th>
<th>Water (mL)</th>
<th>Mean particle size (nm)</th>
<th>PDI</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1A</td>
<td>1.0</td>
<td>0.000</td>
<td>0.120</td>
<td>48.88</td>
<td>149.1 ± 3.64</td>
<td>0.22 ± 0.01</td>
<td>99.9 ± 0.73</td>
</tr>
<tr>
<td>T1B</td>
<td>1.0</td>
<td>0.250</td>
<td>0.150</td>
<td>48.60</td>
<td>204.3 ± 1.76</td>
<td>0.24 ± 0.01</td>
<td>99.3 ± 0.33</td>
</tr>
<tr>
<td>T1C</td>
<td>1.0</td>
<td>0.500</td>
<td>0.180</td>
<td>48.32</td>
<td>192.1 ± 6.42</td>
<td>0.17 ± 0.02</td>
<td>99.7 ± 1.26</td>
</tr>
<tr>
<td>T2A</td>
<td>1.5</td>
<td>0.000</td>
<td>0.180</td>
<td>48.32</td>
<td>151.4 ± 1.66</td>
<td>0.19 ± 0.01</td>
<td>100.6 ± 1.11</td>
</tr>
<tr>
<td>T2B</td>
<td>1.5</td>
<td>0.575</td>
<td>0.225</td>
<td>47.90</td>
<td>197.1 ± 3.62</td>
<td>0.20 ± 0.01</td>
<td>101.0 ± 0.67</td>
</tr>
<tr>
<td>T2C</td>
<td>1.5</td>
<td>0.750</td>
<td>0.270</td>
<td>47.78</td>
<td>185.0 ± 4.41</td>
<td>0.17 ± 0.01</td>
<td>99.7 ± 0.24</td>
</tr>
<tr>
<td>T3A</td>
<td>2.0</td>
<td>0.000</td>
<td>0.240</td>
<td>47.76</td>
<td>148.4 ± 2.94</td>
<td>0.19 ± 0.02</td>
<td>101.0 ± 0.44</td>
</tr>
<tr>
<td>T3B</td>
<td>2.0</td>
<td>0.500</td>
<td>0.300</td>
<td>47.20</td>
<td>193.8 ± 1.53</td>
<td>0.19 ± 0.01</td>
<td>99.5 ± 1.64</td>
</tr>
<tr>
<td>T3C</td>
<td>2.0</td>
<td>1.000</td>
<td>0.360</td>
<td>46.64</td>
<td>191.5 ± 4.03</td>
<td>0.17 ± 0.01</td>
<td>99.8 ± 0.55</td>
</tr>
<tr>
<td>T4A</td>
<td>3.0</td>
<td>0.000</td>
<td>0.360</td>
<td>46.64</td>
<td>156.9 ± 3.94</td>
<td>0.19 ± 0.02</td>
<td>100.5 ± 0.78</td>
</tr>
<tr>
<td>T4B</td>
<td>3.0</td>
<td>0.750</td>
<td>0.450</td>
<td>45.80</td>
<td>189.9 ± 2.69</td>
<td>0.18 ± 0.01</td>
<td>99.5 ± 0.40</td>
</tr>
<tr>
<td>T4C</td>
<td>3.0</td>
<td>1.500</td>
<td>0.540</td>
<td>44.96</td>
<td>201.9 ± 7.70</td>
<td>0.18 ± 0.02</td>
<td>100.0 ± 0.28</td>
</tr>
<tr>
<td>T5A</td>
<td>4.0</td>
<td>0.000</td>
<td>0.480</td>
<td>45.52</td>
<td>154.9 ± 3.97</td>
<td>0.19 ± 0.01</td>
<td>99.9 ± 0.57</td>
</tr>
<tr>
<td>T5B</td>
<td>4.0</td>
<td>1.000</td>
<td>0.600</td>
<td>44.40</td>
<td>192.8 ± 5.58</td>
<td>0.21 ± 0.02</td>
<td>100.4 ± 0.72</td>
</tr>
<tr>
<td>T5C</td>
<td>4.0</td>
<td>2.000</td>
<td>0.720</td>
<td>43.28</td>
<td>215.4 ± 6.92</td>
<td>0.28 ± 0.03</td>
<td>101.0 ± 1.02</td>
</tr>
</tbody>
</table>

In the formulation code, T refers to tacrolimus-loaded liquid crystalline nanoparticles. MO: monoolein; OA: oleic acid; P 407: Poloxamer 407; PDI: polydispersity index. *Number in the parenthesis refers to the percentage of OA compared to MO. **Amount of P 407 was fixed to 12 % w/w of the total weight of MO and OA.

3.2. Microscopy
A drop of the liquid crystalline nanoparticle formulation was placed on the slide, covered with a coverslip and observed under the optical microscope. Olympus Microscope (Japan) was used to take the pictures of liquid crystalline nanoparticles.

3.3. Transmission electron microscopy (TEM)
The liquid crystalline nanoparticle dispersion was negatively stained with 2 % phosphotungstic acid solution (pH 6.8). A drop of the stained dispersion was transferred onto the carbon-coated grid (200 mesh) and air-dried. Then, pictures were taken by transmission electron microscope (Hitachi 7600, Japan).

3.4. Entrapment efficiency
Entrapment efficiency (EE) was determined by ultracentrifugation. Liquid crystalline nanoparticle dispersion (1 mL) was ultra-centrifuged using Amikon Ultra-4 (MWCO: 10,000 g/mole, Billerica, United States), and the filtrate was analyzed for tacrolimus by HPLC system equipped with Class VP computer software, LC 10 AD VP pump, and SPD 10A UV-Vis detector at 210 nm. Total drug in the dispersion was measured using HPLC after destruction of nanoparticles by dilution with methanol (× 10). Preliminary experiments were performed for the optimization of ultracentrifugation, which proved 3500 rpm for 15 min was sufficient for the separation of the unentrapped drug from entrapped drug. EE was calculated by the following equation: 
EE (%) = 100 × (D1 - D2)/D1, where D1 and D2 are the amount of total and free (unentrapped) drug in the sample, respectively. Mobile phase consists of acetonitrile/distilled water (70/30, v/v) at a flow rate of 1.0 mL/min, and the column used was Inertsil ODS-3 (4.6 × 150 mm, GL Science, Japan).

4. In vitro release of tacrolimus from liquid crystalline nanoparticles
Since tacrolimus is a highly hydrophobic drug and the nanoparticles are liquid crystals in nature, spontaneous release of the drug was not detected for a week. Therefore, we performed in vitro release study of the tacrolimus-loaded liquid crystalline nanoparticles after a week of preparation using Spectra/por dialysis membrane. A period of one week was used for the determination of particle size and encapsulation efficiency of the formulations. Liquid crystalline nanoparticle dispersion equivalent to 500 µg of tacrolimus was taken into dialysis bag (MWCO: 10,000 g/mole) and immersed into 30 mL of release medium specified by USP 30 (0.005 % of hydroxypropyl cellulose solution in distilled water with pH adjusted to 4.5 by phosphoric acid). Shaking water bath at 37 °C was used at 25 strokes/min. The whole medium was replaced with fresh medium at each sampling time to maintain sink condition and to prevent degradation of tacrolimus during the release study. The medium replacement was performed within a few seconds to prevent drying of the dialysis membrane. Amount of drug released was determined using HPLC condition as described above. Commercially available tacrolimus capsule (Prograf) powder dissolved in distilled water was used as control.

5. Permeation of tacrolimus from liquid crystalline nanoparticles following intradermal administration
Animal care and procedures were conducted according to the guidelines for animal use in toxicology (Society of Toxicology USP 1989) and the study protocol was approved by the Animal Care and Use Committee, College of Pharmacy, Yeungnam University. Permeation study of the tacrolimus-loaded liquid crystalline nanoparticles was performed after a week of its preparation. Male hairless mice (six-week old) were sacrificed in an ether chamber right before the experiment. The dorsal skin of mouse was excised followed by the removal of adhering fat and tissues. Then, 100 µL of liquid crystalline nanoparticle formulation of tacrolimus (300 µg/mL) was intradermally injected to the skin using 25 G needle. The skin was then mounted onto the Franz diffusion cell equipped with 10 mL receptor compartment. Distilled water:ethanol (3:1) was used as the receptor phase, thermostated to 37 °C under stirring. Donor compartment was then covered with parafilm to prevent drying of the skin. Tacrolimus was allowed to permeate to the donor compartment. An aliquot (100 µL) of receptor solution was then taken and assayed for tacrolimus using HPLC at the predetermined time of 1, 2, 3, 6, 12 and 24 h. The HPLC conditions are the same as described above. Commercially available tacrolimus capsule (Prograf) powder dissolved in distilled water was injected to the mice skin and used as control.

6. Retention study of tacrolimus in the mice skin
For the retention study, effective diffusion area of the mounted skin was cut off after the permeation study. Then, it was minced with a sterile surgical scalpel. The minced skin was added to a vial containing 5 mL of ethanol:water (3:2 v/v) and homogenized at 13,500 rpm for...
10 min on ice bath. The supernatant was collected and centrifuged at 16,000 g for 5 min. An aliquot (0.5 mL) of thus obtained clear supernatant was taken and 0.05 mL of the internal standard was added. Ethanol (0.45 mL) was added to the mixture which was vortexed for 30 s to allow precipitation of protein. The sample thus obtained was centrifuged at 16,000 g for 1 min, and the supernatant was collected and filtered with Whatman nylon syringe filter (0.45 µm) and analyzed for tacrolimus using HPLC.

7. Statistical evaluation

All the data obtained were analyzed by SPSS 20.0 for Windows using Student’s t-test, and significance level of p < 0.05 was considered statistically significant between data sets.

II. RESULTS

We prepared the liquid crystalline nanoparticle formulations containing different amounts of monoolein and oleic acid using the pseudo-binary system. Compositions of liquid crystalline nanoparticle formulations are summarized in Table I. The appearance of all the formulations was milky white liquid dispersion as seen in Figure 1. Figure 2A shows typical lognormal distribution of the liquid crystalline nanoparticles prepared with monoolein concentration having the diameters ranging from 50 to 500 nm. The mean diameter of the liquid crystalline nanoparticles was about 150 nm. Figure 2B shows the particle size distribution of nanoparticles containing oleic acid in addition to monoolein, which ranged from 60 to 700 nm. The mean diameter was about 200 nm.

Optical microscopy and TEM pictures were taken to investigate the shape of the disperse nanoparticles. These images show that different types of structures were formed. Figure 3A and B showed the formation of a cube-like shape, which can be explained as cubosomes [23, 24]. On the other hand, the addition of oleic acid in the formulation revealed fan-like shape which is typical for hexosomes (Figure 3C) [25]. A fan-type texture is formed due to the formation of focal conic domains of columns as described in earlier research [26]. Figure 3D shows a hexagonal structure confirming hexosome formation as described by earlier study [27].

EE of tacrolimus into the liquid crystalline nanoparticles was very high and independent of the composition used in this study. For all liquid crystalline nanoparticle formulations, the EE was as high as 99% or above, as shown in Table I.

Figure 4A shows the effect of monoolein content on the in vitro release of tacrolimus from the liquid crystalline nanoparticles for 2 days using dialysis bag. Commercially available tacrolimus capsule (Prograf) was used as control. It was found that the release of tacrolimus from all the liquid crystalline nanoparticle formulations was significantly different from that of control (p < 0.001). The release of tacrolimus was proportionally decreased by the amount of monoolein in the formulation. Figure 4B shows the cumulative amount of tacrolimus released after two weeks of release study. Among the formulations, significant differences were found in T3A (p < 0.05), T4A (p < 0.01) and T5A (p < 0.01) when compared to T1A while there was no difference between T1A and T2A.

Figure 5 represents the in vitro release profile of tacrolimus from the liquid crystalline nanoparticles formulations using the dialysis bag. Figure 5A-E represent the release patterns for formulations containing monoolein and oleic acid. The release pattern was similar in all the formulations and was found to be steady for as long as two weeks. With an increase in the amount of monoolein, there is a reduction in the amount of tacrolimus released. Furthermore, the reduction was proportional to percentage of oleic acid compared to monoolein in the formulations.

Figure 6 represents the mice skin to which tacrolimus-loaded liquid crystalline nanoparticles were injected intradermally. The bulged skin indicated by the arrows represents intradermally injected area for the in vitro skin permeation study of the tacrolimus-loaded liquid crystalline nanoparticles.

Figure 7A represents biphasic permeation of tacrolimus following intradermal administration of the formulations containing monoolein:

\[ \text{Figure 1} \quad \text{Monoolein-based liquid crystalline nanoparticle formulation.} \]

\[ \text{Figure 2} \quad \text{Size distribution of tacrolimus-loaded liquid crystalline nanoparticles. A: without oleic acid, B: with oleic acid.} \]

\[ \text{Figure 3} \quad \text{Images for tacrolimus-loaded liquid crystalline nanoparticles. A: Optical microscopic image of formulation containing monoolein without oleic acid. B: TEM image of the formulations containing monoolein without oleic acid. C: Optical microscopic image of formulations containing monoolein and oleic acid. D: TEM image of the formulations containing monoolein and oleic acid.} \]

\[ \text{Figure 4} \quad \text{In vitro release of tacrolimus from the liquid crystalline nanoparticles. A: Optical microscopic image of formulation containing monoolein without oleic acid. B: TEM image of the formulations containing monoolein without oleic acid. C: Optical microscopic image of formulations containing monoolein and oleic acid. D: TEM image of the formulations containing monoolein and oleic acid.} \]

\[ \text{Figure 5} \quad \text{In vitro release of tacrolimus from the liquid crystalline nanoparticles. A: in vitro release profile of tacrolimus for 2 days. B: Amount of tacrolimus released at the end of two weeks' study (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.} \]
initially fast followed by slow permeation. There was a significant reduction in the permeation of the drug in T1A (p < 0.05), T3A (p < 0.05), T4A (p < 0.05) and T5A (p < 0.05) compared to control which is in consistent with the result of in vitro release study.

Figure 7B represents the total amount of tacrolimus permeated to the receptor compartment following the intradermal injection. Significant reduction was found in all liquid crystalline nanoparticle formulations compared to control. Furthermore, the extent of the reduction was proportional to the monoolein content in the formulation.

Figure 7C presents the percentage of tacrolimus retained in the mice skin after 24 h of the permeation study. Overall, permeation of tacrolimus from liquid crystalline nanoparticle was biphasic and controllable by the amount of monoolein added to the formulations.

Figure 8A represents the tacrolimus permeation from the formulations containing monoolein and oleic acid. Permeation pattern was similar to that found in liquid crystalline nanoparticle formulations prepared without oleic acid, but the amount of permeated tacrolimus was further reduced with the addition of oleic acid. Figure 8B shows cumulative amount of tacrolimus permeated from the formulations for 24 h following intradermal injection. Figure 8C represents the percentage of tacrolimus retained in the mice skin 24 h after intradermal injection of the formulations.

C-series of the liquid crystalline nanoparticles (T1C, T2C, T3C, T4C and T5C) were not suitable for skin permeation study because there was aggregation of the particles clogging the inside of the 25 G syringe needle within one week after preparation. The permeation study was conducted after a week of preparation because particle size and encapsulation efficiency analysis need to be done initially.

III. DISCUSSION

The liquid crystalline nanoparticle formulations comprising different amounts of monoolein and oleic acid were successfully prepared. The addition of oleic acid to the formulation caused a slight increase in the size of the nanoparticles which might be attributed to the hydrophobic interaction of oleic acid with monoolein. The other reason might be the formation of hexosomes which have particle size greater than cubosomes. Optical microscopic and TEM pictures confirmed the formation of cubosomes and hexosomes in different formulations. Nanoparticles were found to be cubosomes for the formulations containing monoolein [23, 24]. On the other hand, formation of hexosomes was seen for the formulations containing oleic acid in addition to monoolein [25]. The increase in the hydrocarbon

Figure 5 - In vitro release profile of tacrolimus from liquid crystalline nanoparticle formulations using the shaking water bath. A: Release profiles for T1A, T1B and T1C. B: Release profiles for T2A, T2B and T2C. C: Release profiles for T3A, T3B and T3C. D: Release profiles for T4A, T4B and T4C. E: Release profiles for T5A, T5B and T5C. Refer to Table I for formulation code.

Figure 6 - Intradermal injection of the tacrolimus-loaded liquid crystalline nanoparticle formulation for the skin permeation study.

Figure 7 - Skin permeation study for tacrolimus-loaded liquid crystalline nanoparticles containing monoolein. A: Percentage tacrolimus permeation profile. B: Total tacrolimus permeated. C: Percentage tacrolimus retained following 24 h of the skin permeation study (*p < 0.05, **p < 0.01). Each value represents mean ± standard deviation (n = 3). T2A was not included because it was almost same as T1A.

Figure 8 - Skin permeation study for tacrolimus-loaded liquid crystalline nanoparticles containing oleic acid in addition to monoolein. A: Percentage tacrolimus permeation profile. B: Total tacrolimus permeated. C: Percentage tacrolimus retained following 24 h of the skin permeation study (*p < 0.05, **p < 0.01). Each value represents mean ± standard deviation (n = 3). T2B was not included because it was almost same as T1B.
chain space, due to the oleic solubilization, might have changed the monoglyceride molecular packing and furthermore transformed the mesophases from the cubic phase into the reverse hexagonal phase [28]. The encapsulation efficiency of tacrolimus in the liquid crystalline nanoparticles was found to be very high because of the lipophilic nature of tacrolimus which lends itself to be easily incorporated in the hydrophobic core of the liquid crystalline nanoparticles [29, 30].

As observed from the in vitro release study using the dialysis bag, the amount of tacrolimus released from the formulations was inversely proportional to the amount of monoolein used. The reason behind this may be the increase in lipophilicity in the formulation which attracts the hydrophobic drug tacrolimus towards itself leading to slow release from the nanoparticles [29, 30].

The in vitro release study of different formulations showed a similar release pattern although the amount of tacrolimus released varied among the different groups containing monoolein and oleic acid. Oleic acid showed similar effect as that of amount of monoolein in the release of tacrolimus. There was an inverse relationship between their amount and the release of tacrolimus, which is due to an increase in lipophilicity [29, 30]. Oleic acid further reduced tacrolimus release which might be explained by the formation of hexosomes that are capable of further controlling the release [15].

We performed permeation and retention study using mice skin because the in vitro study results can be correlated to in vivo experiment planned for the next study using psoriasis-induced mice model. The permeation of tacrolimus from the liquid crystalline nanoparticles following intradermal administration to mice skin showed a controlled release profile which can be attributed to the formation of cubosomes and hexosomes in different formulations when compared to control. The result is supportive to in vitro tacrolimus release study. The data for the percentage of tacrolimus retained in the mice skin following intradermal delivery further suggests the ability of hexosomes and cubosomes to retain tacrolimus for a prolonged duration of time. The study thus proved that once the liquid crystalline nanoparticle is in the dermis, it can act as a depot for tacrolimus. Both hexosomes and cubosomes have this property but hexosomes are found to further slow the release of tacrolimus as seen in the study. The depot can be employed to treat the locally affecting skin diseases for a prolonged period of time.

The variations in the amount of monoolein and oleic acid can be used to control the release of tacrolimus from the liquid crystalline nanoparticles (cubosomes and hexosomes). Notably, very slow release of tacrolimus from the hexagonal-phase structure appears to be a more promising candidate than cubosomes as a novel controlled-release delivery system. The data acquired in this study further encourage us in the employment of the tacrolimus-loaded liquid crystalline nanoparticles for intradermal delivery in the treatment of various autoimmune skin diseases such as atopic dermatitis and psoriasis. Further in vivo studies are warranted to prove the efficacy of the monoolein-based liquid crystalline nanoparticles for the treatment of locally affecting autoimmune skin diseases such as psoriasis.

From the above-stated findings, it can be concluded that the variations in the amounts of monoolein and oleic acid can be used to control the release of tacrolimus from the formulations. An appropriate amount can be determined to bring about a desired release profile. Since tacrolimus is a drug with narrow therapeutic index, the formation of a controlled drug delivery system can expand its area of use. Both the cubosomes and hexosomes can be utilized for the intradermal drug delivery purposes. The data acquired during this study further encourage us in the use of the tacrolimus-loaded liquid crystalline nanoparticles for intradermal delivery for the treatment of different autoimmune skin diseases like atopic dermatitis and psoriasis.

**REFERENCES**


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