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FK506-loaded solid lipid nanoparticles: Preparation, characterization and *in vitro* transdermal drug delivery

Ruihua Wang[1][#], Longnian Li[1][#], Bing Wang[2], Tangde Zhang[1] and Ledong Sun[1]*

[1]Department of Dermatology, Zhujiang Hospital, No. 253, Gong Ye Da Dao Zhong, Haizhu District, Guangzhou 510515, China.
[2]Department of Dermatology, Nanfang Hospital, No. 1838, North Guangzhou Dadao, Guangzhou 510515, China.

FK506-loaded solid lipid nanoparticles (FK506-SLNs) were prepared by a modified emulsification and low temperature solidification method and characterized by size analysis and entrapment efficiency. The influence of experimental factors, such as lipid carriers, surfactant concentration, emulsification temperature and stirring velocity on particle size and entrapment efficiency were investigated to optimize the formulation. The feasibility of fabricating FK506-SLNs by this method was successfully demonstrated in this investigation. The SLNs were characterized for appearance, morphology, particle size, entrapment efficiency, zeta potential and *in vitro* release, and then they were incorporated in a carbopol hydrogel to determine the influence on the *in vitro* drug transdermal delivery. Commercial tacrolimus ointment served as a comparison. Excised full thickness rat skins were mounted on Franz diffusion cells and the formulations were applied for 48 h. Greater skin permeation of FK506 was detected from the SLNs hydrogel than from the commercial ointment. A higher retention of FK506 was detected in the skins following SLNs hydrogel application than from the commercial ointment, which suggested a drug localizing effect.

Key words: Tacrolimus, entrapment efficiency, drug retention, targeting.

INTRODUCTION

Tacrolimus (FK506) is an immunosuppressant of macrolides with the same mechanism of action as cyclosporine. However, the relative molecular weight of FK506 is smaller, which allows it to penetrate the skin more easily (Lauerma and Maibach, 1994). In the 1990s, FK506 was incorporated into an ointment to treat skin diseases. It acted primarily on T cells in affected skin, blocking T cell activation by inhibiting the activity of the enzyme calcineurin (Liu et al., 1992). FK506 could affect other immune cells, such as mast cells, eosinophils and basophils, by preventing cytokine production and release in/from these cells (Rustin, 2007). FK506 does not have corticosteroids-like side effects, which is significant; its

formulation is considered to be safe. Even so, the currently available commercial tacrolimus ointment is not ideal, which has attracted public attention to the issues of latent systemic exposure and the safety of long-term usage about which the carcinogenic potential of tacrolimus 0.1% ointment was once reported (Becker et al., 2006). Because of this, the US FDA has urged a 'black box' warning for topical calcineurin inhibitors (Ring et al., 2008).

Solid lipid nanoparticles (SLNs), introduced in the 1990s, are promising drug carriers on the nanometer scale (Gulati et al., 1998). They are produced from biocompatible and biodegradable lipids (Padois et al., 2011) that encapsulate the drug and protect it from unnecessary damage, such as oxidation. Because the lipids are nontoxic, the skin irritation is minor when SLNs are used externally, as has been shown with many drugs, such as imiquimod (Ni et al., 2006) and podophyllotoxin (Zhang et al., 2008). As a topical application preparation, SLNs has other advantages besides reduced skin

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*Corresponding author. E-mail: sunledong126@126.com or tdgz@medmail.com.cn. Tel: +620-61641984, +620-62782080.

[#]These authors contributed equally to this work.

irritation. For example, the controlled release can maintain a high drug concentration over a prolonged time on the skin surface and within the skin tissue (Jenning et al., 2000), the film formation can form an "occlusive effect" on the skin surface (De Vringer and De Ronde, 1995) that promotes drug permeation and the nanoscale structure can enhance drug intake through skin (Müller et al., 2000) through intimate contact with the epidermal cells. Finally, SLNs have targeting capability (Harivardhan Reddy et al., 2006), which makes it extremely attractive.

In this study, FK506-SLNs were prepared by an emulsification and low temperature solidification method. The influence of experimental factors on particle size and entrapment efficiency was investigated to optimize the formulation.

The developed SLNs were characterized for appearance, morphology, particle size, entrapment efficiency, zeta potential and *in vitro* release. FK506-SLNs were then incorporated in a carbopol hydrogel to study the skin penetration *in vitro* using Franz diffusion cells.

MATERIALS AND METHODS

Stearic acid was purchased from Shanghai chemical reagent production, purchase and supply wulian chemical plant, China. Soy lecithin, stearamide, poloxamer188, Brij78 and carbopol 940 were from Sigma-Aldrich Inc., USA. Water used in all the studies was distilled before use. All other chemicals used in the study were of analytical grade. Standard tacrolimus was purchased from Tongtian biometric technologies Ltd., Shanghai, China. FK506 was from Fuchi Company Ltd., Wuhan, China.

Preparation of solid lipid nanoparticles

FK506-SLNs were prepared by a modified emulsification and low temperature solidification method reported previously (Trotta et al., 2003). Briefly, stearic acid, FK506 and stearamide, which regulated the surface charge, were dissolved in methylene chloride to obtain a drug-lipid mixture. Soy lecithin, which was melted in absolute alcohol, was mixed with the drug-lipid mixture to obtain an oil phase. The oil phase was injected into the aqueous phase, which was made of dissolved poloxamer188 and Brij78 (1:1, M:M, mass ratio) in distilled water at the same temperature at 72ºC with stirring at 1200 rpm using a mechanical stirrer (JB-3A, Shanghai leici instrument Inc. China). The mixture was stirred continuously for 2 to 3 h until 5 ml of a crude emulsion was obtained. The FK506-SLN emulsion was formed when the crude emulsion was rapidly poured into 5 ml of an ice-water mixture. It was homogenized at 0°C in an ultrasonic cleaner to obtain a stable FK506-SLN suspension. The blank SLN suspension was obtained using the same method, but FK506 was not added in the "drug-lipid mixture".

Size analysis and Zeta potential

The correct amount of FK506-SLN suspension was diluted with distilled water. The particle size, polydipersity index and Zeta potential were detected using a Zeta-sizer 3000 HS (Malvern Co. Ltd., Britain).

Morphology observation

The FK506-SLN suspension and the blank SLN suspension were individually dripped on copper nets with supporting film. They were then negatively stained with 2% (vol. %) phosphotungstic acid. The surface morphology of the FK506-SLNs and the blank SLNs were studied by transmission electron microscopy (HiTachi H-7650).

Entrapment efficiency

The entrapment efficiencies were determined by a microcolumn centrifuge-HPLC method. The mini centrifuge was prepared as follows. Fully swelled Sephadex G-50 was loaded into the syringe (5 ml) to a height of approximately 3.5 cm. The syringe had the piston rod taken off and the bottom was cushioned with double-deck filter paper. PBS, as a balance solution, was added to the top of syringe. The syringe was placed in a 50 ml centrifuge tube and centrifuged at 2000 rpm for 2 min to obtain a microcolumn of Sephadex G-50. Next, we isolated FK506 from FK506-SLNs. The prepared FK506-SLN suspension was added to the top of the microcolumn and centrifuged at 1500 rpm for 3 min. The solution produced by centrifugation was collected, and the microcolumn was washed three times with 0.5 ml of distilled water. The eluent and the solution produced by centrifugation were merged in a 5 ml brown volumetric flask that was filled to capacity with acetonitrile, which was used as a demulsifier for the SLNs. The solution was filtered through a 0.45 µm micropore filter membrane, and the filtrate was analyzed by HPLC. The same volume of a FK506-SLN suspension that went straight through demulsification processing without microcolumn processing was analyzed by HPLC as well. The entrapment efficiencies (EE) were calculated as follows: EE=$(\rho 1/\rho 2)\times 100\%$. $\rho 1$ and $\rho 2$ are the mass concentrations of FK506 before and after isolation, respectively.

Differential scanning calorimetry (DSC) and X-ray diffraction (XRD)

A certain amount of FK506, a mixture of FK506 and blank SLNs, blank SLNs and FK506-SLNs were weighed. The four groups were studied using differential scanning calorimetry (DSC204FI, NETZSCH Co. Ltd. Germany) by setting the scanning range at 20 to 200°C and the scanning frequency at 10°C/min. The results are presented in Figure 4. Additionally, the four groups were studied by x-ray diffraction with a D/max-3A (Japanese) with a scanning range of 22 to 60°C and Cu Target Ka (35 Kv, 25 mA) as the X ray source. The results are shown in Figure 5.

In vitro release experiment

Ten milliliters of a FK506-SLN suspension and FK506 solution of the same concentration were put in individual dialysis bags with a molecular weight cut-off of 12000 to 14000 D. Both ends of the dialysis bags were clamped and put separately in 200 ml conical flasks that contained 100 ml of release medium (PBS, pH 7.4). The flasks were incubated at 37°C in the dark with shaking at an oscillation frequency of 80 /min. Two milliliters of sample was taken from the release medium at 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72 h and was replaced with 2 ml of PBS after each sample was taken. After filtering through a 0.45 µm micropore filter, the samples were studied by HPLC. The accumulative release quantity of FK506-SLN suspension and FK506 solution was calculated follows: $Qn=Cn*V0+(C1+C2+C3+……+Cn-1)*V$. In this formula, Qn is the accumulative release quantity at time (n); Cn is the drug concentration at time (n); V0 is the volume of the release medium; V represents the volume of every sample (2 ml). The release curves

as of the FK506-SLN suspension and FK506 solution are shown in Figure 6.

Preparation and characterization of FK506-SLN hydrogel

Preparation of FK506-SLN hydrogel

The hydrogel agent Carbopol 940 was used to form the FK506-SLN hydrogel as described by Liu et al. (2008). In brief, 1% Carbopol 940 was mixed with 5% glycerol and left overnight. The solution was mixed with the FK506-SLN suspension followed by continuous stirring for 30 min. The Carbopol-based FK506-SLN hydrogel (10 g:10 mg) was obtained by adding 1% triethanolamine to the stirring hydrogel.

Determination of drug content and the leak rate

Half a gram of the FK506-SLN hydrogel was dissolved into alcohol in a 25 ml volumetric flask to determine the drug content. The mixture was shaken with an ultrasonic cleaner to complete the demulsification process. The mixture was filtered through a 0.45 µm micropore filter membrane. The filtrate was analyzed for drug content by HPLC.

To determine the drug releasing from hydogel, 0.5 g of the hydrogel was placed in distilled water for 4 h. The mixture was then vortexed for 10 s and centrifuged at 20000 rpm at 4°C for 30 min. The free drug was obtained by diluting the supernatant with alcohol in a 25 ml volumetric flask. The free drug (Wy) and the initial free drug (Wcy) were analyzed by HPLC. The leak rate was calculated according to the following formula: $L\% = (Wy-Wcy)/Wb \times 100\%$, where L stands for the leak rate and Wb represents the encapsulated drug.

Temperature test

The amount of hydrogel sample (batch number:20101106) was weighed and placed in a refrigerator set at 4, 25 or 40°C for 30 days. Samples were taken at various intervals, and the appearance of the hydrogel, the leak rate and the drug content were evaluated.

In vitro transdermal drug delivery

The full-thickness abdomen skins from SD rats (weight range: 280 to 320 g, purchased from Northern Medical University, Guangzhou, China) were used for all the permeation experiments. After the hair was removed with a razor, the skin was excised and examined for integrity using a lamp-inspection method (Liu et al., 2007). The preparation of the skin samples were finished after the fat tissue below the skin was carefully removed to make sure the thickness of each skin was similar.

The permeation experiments were processed using a transdermal diffusion test instrument (TK128B, Shanghai Kaikai Lt.). The skin samples were dried with filter paper and mounted on modified Franz diffusion cells such that the dermal side of the skins was in contact with the receiver fluid and the corneum faced the donor chambers. One gram of the formulation (either FK506-SLN hydrogel or commercial tacrolimus ointment) was gently placed in the donor chambers, enabling a film to cover the entire exposed skin surface. Subsequently, the Franz diffusion cells were filled with the receiver fluid that consisted of absolute ethyl alcohol and saline (3:7, v/v) in the manner described previously (Liu et al., 2007; Hu et al., 2010). Absolute ethyl alcohol was used to solubilize FK506. Then the receiver chambers was stirred continuously at 400 rpm.

Samples were taken at 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h. Each sample was 1 ml, and the volume was replaced with fresh receiver fluid. All samples were filtered through a 0.45 µm micropore filter. The concentration of FK506 in the filtrate was analyzed by reverse-phase HPLC. The accumulated permeation quantity (Q) per unit of skin area was calculated according to the following formula:

$$Q= [Cn \times Vo+ \sum_{i=1}^{n-1} Ci \times V]/S$$

In this formula, Cn is the drug concentration of samples taken at time (n); Ci is the drug concentration of samples taken at time (i(n-1)); Vo represents the volume of the diffusion cell (6.5 ml); V is the sample volume (1 ml), and S is the diffusion area (2.5434 cm^2).

The amount of drug stagnation in the skin

At the end of the permeation experiments, the area of the skin in which the drug had diffused was excised, rinsed with distilled water and ground to homogeneity with the addition of adequate ethanol. FK506 was extracted from the homogenate using a digital sonifier (DIGITAL SONIFIER 250, BRANSON, America) at 0°C. Thereafter, the homogenate was vortexed, followed by centrifugal separation at 4000 rpm for 5 min. The retention in the skin, namely the total amount of FK506 extracted from the skin at the end of the permeation experiment, was determined from the supernatant. All samples were filtered through a 0.45 µm micropore filter. The concentration of FK506 extracted from the skin was analyzed by HPLC. The sonication had no effect on the stability of FK506. No HPLC peak signal of FK506 in the control group was observed nor was it found in the residual extracted skins.

HPLC analysis

The FK506 concentration was determined by HPLC using an Agilent G1310A pump (Agilent 1100 Series, USA) and a Hypersil C18 column (5 µm, 250 mm×4.6 mm). The mobile phases consisted of acetonitrile/water/phosphoric acid at a ratio of 70:30:1 (v/v/v). UV absorption was read at 220 nm, and the drug was identified via its UV spectrum using a diode array detector. The HPLC analysis was performed at 50°C with a mobile phase flow rate of 1 ml/min. When the mass concentration of FK506 was between 1.6 and 80 µg/ml, there was a linear relation between the peak area and concentration of FK506 (r=0.9998). The minimum limit of detection was 0.5 µg/ml.

Statistical analysis

All experiments were carried out in triplicate. Statistical analysis was performed with the SPSS17.0 software package. The results are expressed as the mean ± standard deviation ($\bar{x} \pm s$). Statistically significant differences were determined using Student's t-test and analysis of variance (ANOVA), with $p<0.05$ as the minimal level of significance.

RESULTS AND DISCUSSION

Choose recipe and preparation of FK506-SLNs

The particle size, entrapment efficiency and stability were

Table 1. Characteristics of FK506-SLN with different solid lipids.

Solid lipid	Particle size (nm)	Entrapment efficiency EE (%)	Stability (4°C)
Stearic acid	134±18.0	88.74±0.87	No sediment for 3 months
Compritol 888 ATO	230.1±13.6	62.10±1.22	Deposit after 2 weeks
Glycerol monostearate	103.4±15.4	None detected.	No sediment for 3 months

Table 2. Characteristics of FK506-SLNs with different surfactants.

Surfactant	Particle size (nm)	Entrapment efficiency, EE (%)	Stability (4°C)
Brij78	178.4±13.6	45.67±0.97	Deposit after 1 month
Poloxamer188	302.6±10.50	38.43±0.65	Deposit after 2 weeks
Brij78:Poloxamer188 (1:1)	134.0±8.56	88.74±0.76	No sediment for3 months

evaluated to determine the optimum prescription and preparation technique for FK506-SLNs.

Influence of solid lipid

Stearic acid, glycerol monostearate and Compritol 888 ATO were chosen as solid lipids to test in the preparation of a batch of FK506-SLN suspensions according to the method described previously. The results are analyzed in Table 1. Comparing the characteristics of each solid lipid, it is clear that stearic acid is the best choice. For Compritol 888 ATO, the high melting point led to an enhanced viscosity of the SLN suspension, which resulted in a larger particle size compared to the other two (Mehnert and Mäder, 2001). Though the particle size of SLNs prepared from glycerol monostearate is the smallest of the three, after these SLNs underwent demulsification, FK506 was not detected by HPLC. It is possible that ester exchange and condensation reactions took place between the carboxyl in glycerol monostearate and the diol units in tacrolimus upon heating, which resulted in the destruction of FK506. Overall, only the FK506-SLNs prepared from stearic acid resulted in particles with good features, such as small particle size, high entrapment efficiency and good stability. Moreover, stearic acid is a native physiologic molecule and can easily be metabolized in the human body (Zhong-hong et al., 2008).

Influence of the surfactant

Brij78, poloxamer188 and a mixture of the two (1:1, W:W) were used separately to prepare FK506-SLNs. Table 2 shows that the mixture of surfactants is better suited for the preparation of FK506-SLNs. Improved results through the use of a mixture of surfactants have been seen previously (Zhou, 2007).

Screening the optimum prescription and preparing techniques

We evaluated four factors that were seen to have obvious effects on the stability of FK506-SLNs in previous tests. The four factors are as follows: The ratio of the drug to stearic acid (A), the ratio of the drug to soy lecithin (B), the ratio of the oil phase to the aqueous phase (C) and the rotation speed (D) We evaluated the particle size and the entrapment efficiency by orthogonal experiment design (Table 3).

From the statistics given in Table 3, it can be seen that the four factors have different effects on the particle size and EE. The degree of effect, in order, is C>A>D>B, but there is no obvious difference between any two of the four factors. The optimum preparation procedure was determined to be A2B2C3D2; that is, the ratio of drug to stearic acid is 1:4, the ratio of drug to soy lecithin is 1:3, the ratio of the oil phase to the aqueous phase is 3:5 and the rotation speed is 1500 rpm/min.

Using the parameters previously chosen, the FK506-SLNs had a mean particle size of 134 nm (Figure 1), a polydipersity index of 0.35 and a narrow particle size distribution. The zeta potential was -25.2±10.3 mV, and the EE was 88.74%. Transmission electron microscopy revealed that the SLNs were round and homogeneous and that no large aggregate remained in the hydrogel (Figure 2). There was no drug detected in the blank SLNs (Figure 3), whose particle sizes were smaller than the FK506-SLNs.

Entrapment efficiency

The entrapment efficiency (EE) is an important index reflecting whether the prepared SLNs meet clinical requirements. In our experiments, the microcolumn method, which could easily isolate the lipid-soluble drug from the solid lipid (Hong et al., 2005), was used to assay

Table 3. The orthogonal test and its results.

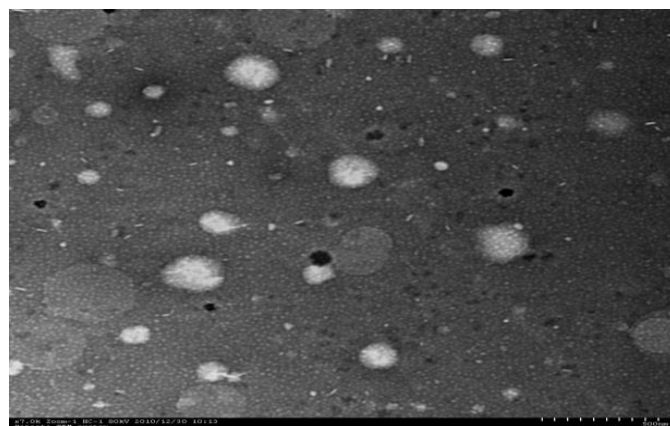| No.	A	B	C	D	Particle size (nm)	EE (%)	Comprehensive score
1	1	1	1	1	201.6	76.92	11.69
2	1	2	2	2	147.8	59.38	29.82
3	1	3	3	3	139.2	88.54	48.70
4	2	1	2	3	128.3	53.54	36.65
5	2	2	3	1	123.9	79.91	52.03
6	2	3	1	2	149.9	78.58	38.37
7	3	1	3	2	111.8	59.13	47.69
8	3	2	1	3	165.8	87.83	35.04
9	3	3	2	1	157.3	73.03	31.89
i	90.21	96.00	85.11	95.62			
ii	127.05	116.9	98.36	115.88			
iii	114.63	118.96	148.43	120.39			
iv	30.07	32.01	28.37	31.87			
v	42.35	38.97	32.79	38.63			
vi	38.21	39.66	49.48	40.13			
R	12.28	7.65	21.11	8.20			

Comprehensive score (Zhang and Yang, 2001) = (the highest value of the particle size - the tested particle size) × 0. 5 + (the tested EE - the smallest value of EE) × 0.5. In this formula, 0.5 is the metric of the particle size and EE.
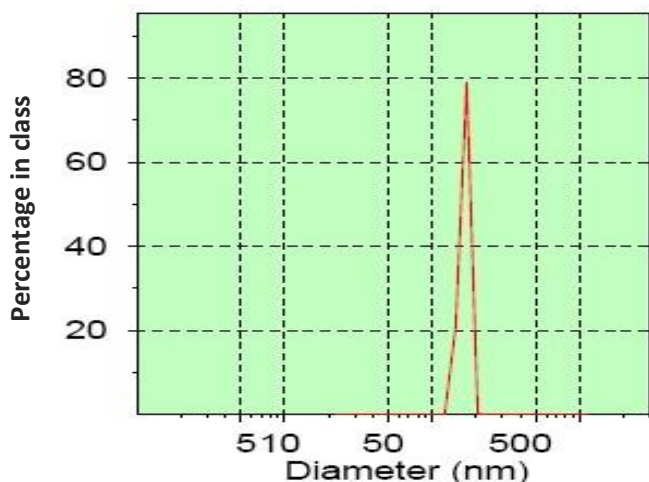
Figure 1. The particle sizes of FK506-SLNs.

Figure 2. TEM photograph of FK506-SLNs.

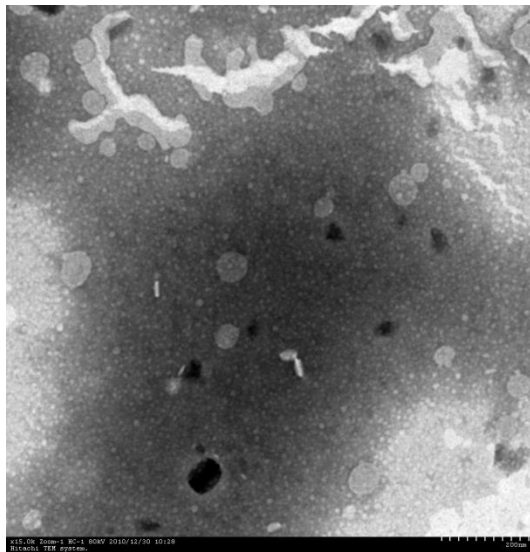EE. Other methods, such as dialysis, ultrafiltration and ultracentrifugation, were not able to easily isolate the drug from the solid lipid.

Differential scanning calorimetry and X-ray diffraction

Differential scanning calorimetry (DSC) is a tool that provides insight into the melting and recrystallization behavior of SLNs (Jenning et al., 2000). In our experiments, the DSC thermograms of the pure drug (FK506) (a), the blank SLNs (b), a mixture of blank SLNs and FK506 (c), and FK506-SLNs (d) showed marked variations in their thermal profiles (Figure 4). FK506-SLNs did not show the melting peak of FK506 at 122.40°C, while the physical mixture showed a melting peak at 122.40°C, indicating that FK506 was dispersed homogeneously into the SLNs. Although the thermograms of the blank SLNs and FK506-SLNs were analogous, there was a significant difference in their melting enthalpies. The melting enthalpies of the blank SLNs and FK506-SLNs were -114.50 and -92.65 J/g, respectively. These results demonstrated that there was
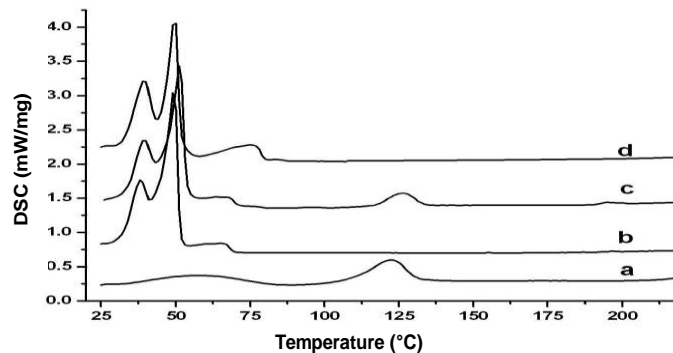
Figure 3. TEM photograph of the blank SLNs.

Figure 4. Differential scanning calorimetry (DSC) patterns. a, FK506; b, blank SLNs; c, the physical mixture of blank SLNs and FK506; d, FK506-SLNs.

an interaction between FK506 and lipid molecules in FK506-SLNs that was different from the physical mixture.

The X-ray diffraction data shown in Figure 5 are in good agreement with the results obtained by DSC measurements. The diffraction pattern of the pure drug showed remarkable difference from that of the SLNs, while the pure drug had sharp peaks that were almost absent in the diffractograms of the SLNs. This indicates a less ordered crystal arrangement in the SLN formulations compared to the pure drug. From these results, it can be concluded that the drug entrapped in SLNs is in an amorphous or disordered crystalline phase of molecular dispersion (Mu and Feng, 2003). The amorphous form is thought to have higher energy, with an increased surface area and subsequently higher solubility, dissolution rates and bioavailability (Corrigan et al., 2003; Morissette et al., 2004).

In vitro release studies

In this study, dynamic dialysis was chosen to separate free FK506 from FK506-SLNs. The drug release from the FK506 solution and FK506-SLNs suspension at 37°C are shown in Figure 6. The results indicated that the release from the dissolution medium solution was much faster, with approximately 96% of the FK506 released within 8 h. In contrast, only 55% of FK506 released from SLNs was within 8 h. The burst release at the beginning indicates that a certain amount of FK506 was incorporated at the surface of the SLNs. Subsequently, the FK506-SLN suspension exhibited sustained release; the accumulated drug release percentage at 72 h was only about 86%. The reduced FK506 release from the SLNs in the latter stage may be attributed to lipid matrices, where the drug

dispersed slowly (Grassi et al., 2003). This controlled release pattern has been reported in large volumes (Solmaz et al., 2010; Yonezawa et al., 2001).

Burst release as well as sustained release has been reported for SLNs dispersions. For transdermal application, both features are of interest. Burst release can be useful in improving the penetration of drugs, while sustained release becomes important for active ingredients to be supplied to the skin over a prolonged period of time (Puri, 2010).

Stability study

At room temperature, sedimentation was discovered after a week. However, FK506-SLNs exhibited good stability over 60 days at 4°C. No obvious changes in clarity or degradation were found. The mean diameter of FK506-SLNs was 146±12.8 nm and the entrapment efficiency was decreased by 2.6%. After high-speed centrifugation, the FK506-SLN suspension was still homogeneous, with no separation or crystallization phenomena observed. These results imply that the transition of stearic acid in SLNs in storage from metastable forms to stable forms occurs slowly, due to the small particle size and the presence of an emulsifier, and this transition leads to drug expulsion from SLNs (Chen et al., 2006; Venkateswarlu and Manjunath, 2004).

Characterization of the FK506-SLN hydrogel

The FK506-SLN hydrogel obtained by the method described previously in "preparation of FK506-SLN hydrogel" was slightly blue and exhibited fine consistency and moderate viscosity, which made this hydrogel easy to spread for topical use. The pH value of the FK506-SLN hydrogel was determined to be between 6.0 and 7.5. In this pH range, a carbopol solution can become a stable
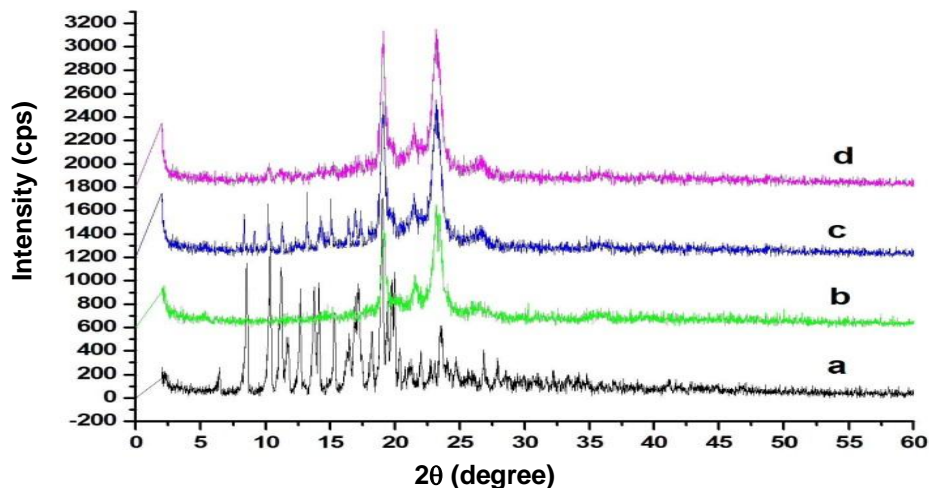
Figure 5. X-ray diffraction (XRD) patterns. A, FK506; b, blank SLNs; c, the physical mixture of blank SLNs and FK506; d, FK506-SLNs.
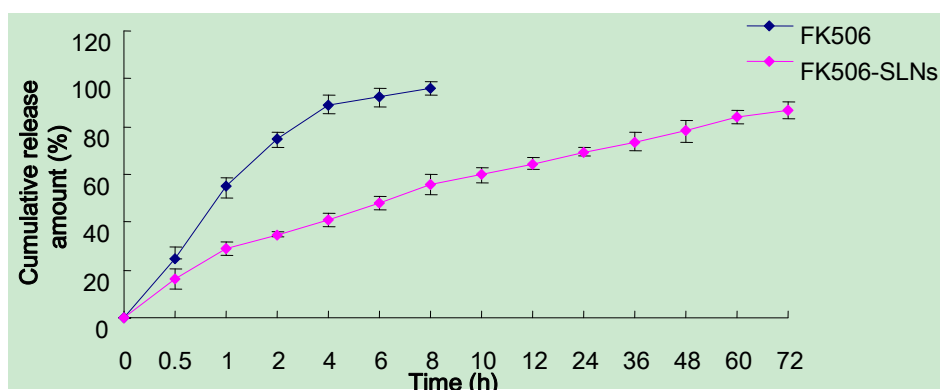
Figure 6. The *in vitro* release profile of FK506 from FK506-SLNs.

hydrogel, which has many advantages, such as fast drug release, easy spreading, no irritation of skin and mucosa and film formation (Mourtas et al., 2007).

Three batches of FK506-SLN hydrogel prepared at different times were tested for drug content (Table 4). The mean content of FK506 in this carbopol hydrogel is 0.096% (0.1%, w/w), which meets the standards (Chinese pharmacopoeia (edition 2005)) required for a hydrogel.

The temperature tests revealed that the FK506-SLN hydrogel was stable when it was placed at a low temperature. For at least 30 days, there were no obvious changes in the appearance, the leak rate and drug content when this hydrogel was kept at 4°C. In contrast, at 25°C, a small amount of milk-white sediment was seen on the thirtieth day. A great quantity of sediment appeared on the fifth day when the hydrogel was stored at 40°C, and the leak rate (0 to 9.3%) and the drug content (0.968 to 0.947%) were significantly increased and reduced,

respectively. Therefore, the FK506-SLN hydrogel should be kept in a cool place.

Permeability test in vitro

To assess the skin penetration of FK506 from the SLN hydrogel, the *in vitro* ability to permeate the skin was assessed using Franz diffusion cells. The commercial tacrolimus ointment (0.1%, w/w) was used as a control. *In vitro* permeation studies were performed for 48 h. The permeation of FK506 through the rat skin from the SLN hydrogel and the commercial ointment was calculated in terms of the mean cumulative amount diffused at each sampling time point, as shown in Table 5. To more easily understand the release behavior, the curve of the accumulated permeation quantity (Q) vs. time was plotted in Figure 7. It is evident that the SLN hydrogel results in greater permeation of FK506 compared to the

Table 4. The drug content of the FK506-SLN hydrogel.

Batch number	Tested drug content (%)	Mean drug content (%)
20101103	0.0948	
20101104	0.0962	0.0960
20101105	0.0968	

Table 5. The mean accumulated amount of each formulation ($\mu g/cm^2$, $\bar{X}$ ±s, n=3).

Permeation time (h)	FK506-SLN hydrogel ($\mu g/cm^2$)	Commercial ointment ($\mu g/cm^2$)
0.5	2.97±0.15	1.14±0.33
1	6.92±0.64	4.13±0.73
2	11.56±1.20	8.55±1.84
4	20.29±2.45	15.48±1.84
6	27.55±3.30	21.92±3.71
8	34.02±3.62	28.52±4.29
12	42.36±5.90	36.52±3.91
24	65.23±4.97	51.34±4.22
48	109.02±8.88	83.71±8.09
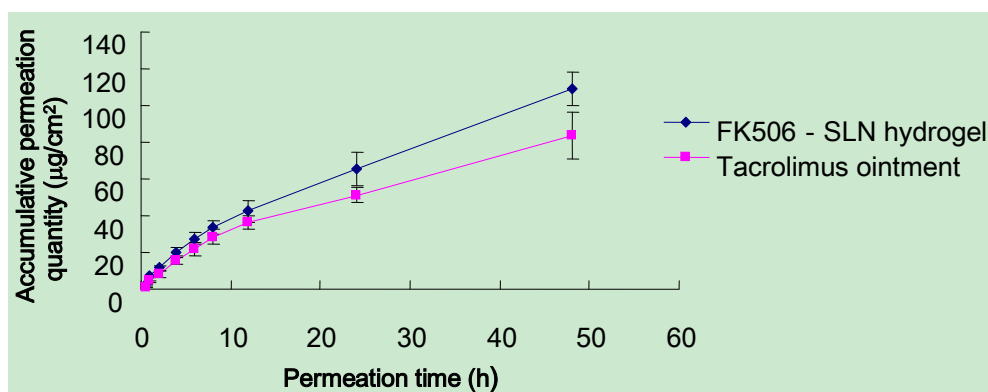
Figure 7. Cumulative transdermal quantity of FK506 during 48 h ($\mu g/cm^2$, $\bar{X}$ ±s, n=3).

commercial ointment. This observation is in accordance with previously published literature (Kim et al., 2009). The high permeation of FK506 from the SLN hydrogel may be related to the inherent characteristics of SLNs, such as the compatibility of the SLN lipid matrix with skin lipids (Olbrich et al., 2002), the "occlusive effect" (Lv et al., 2009), and the small size of the particles (134 nm in our study), which all enhance the ability of FK506 to penetrate the skin. In addition, the ointment is denser than the hydrogel, which may be another factor explaining the slower FK506 permeation from the commercial ointment than from the SLN hydrogel.

The experimental values were regressed based on the accumulated permeation quantity (Q) per unit of skin area as a function of permeation time (t), and two *in vitro*

transdermal kinetic equations were obtained as follows: FK506-SLN hydrogel: Q=2.152t+10.323, r=0.986; commercial tacrolimus ointment: Q=1.669t+8.354, r=0.976. These values indicate that pseudo-first order kinetics are followed by the two formulations.

The *in vitro* skin permeation of FK506 was assessed by the amount of FK506 deposited in the skin. Figure 8 shows that the drug retention of the SLN hydrogel is significantly greater than that of the commercial ointment (p<0.05). This result agrees with previous studies (Chen et al., 2006; Liu et al., 2007; Lopes et al., 2006).

In our study, the mechanism of FK506 permeation through skin from SLN hydrogel was not examined. However, some studies (Jain et al., 2005; Shah et al., 2007) have postulated that the drug is transported in a
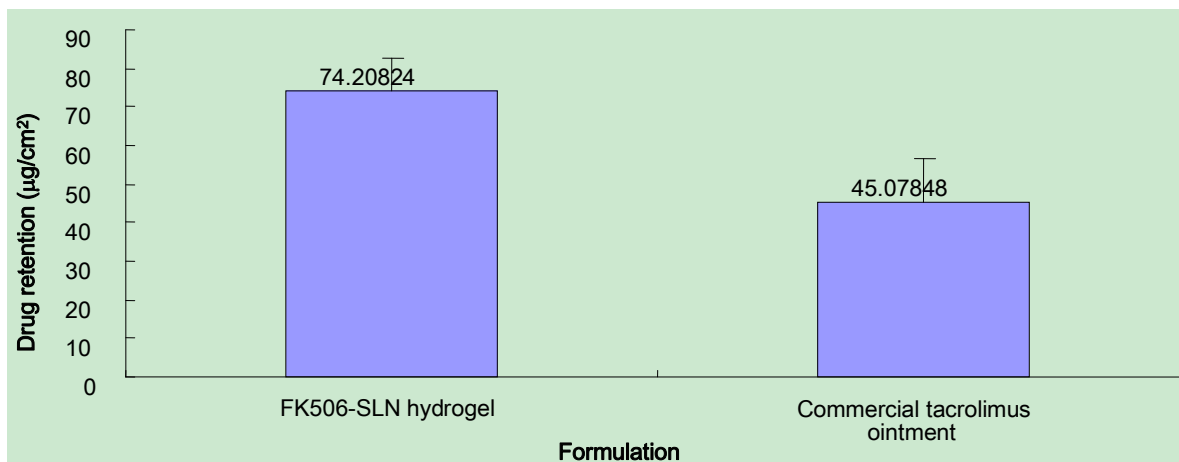
Figure 8. The retention of FK506 in the skin after 48 h (µg/cm^2, $\overline{X}$ ±s, n=3).

form that is encapsulated in the SLN matrix. That is, FK506 permeates through the skin by being encapsulated in SLNs. This hypothesis was supported by our previous trials. Physiological saline cannot lead to the demulsification of SLNs. In our previous trials, when physiological saline alone was used as receiver fluid, the drug was still encapsulated in SLNs and no FK506 was detected by HPLC. This is why we chose to use ethanol as part of the receiver fluid used to demulsify FK506-SLNs and release FK506 from SLNs. We speculate that after being transported across the skin, the SLNs expelled FK506 from the SLN matrix because of the consequence of multiplex transitions occurring in the solid lipid (Sharma et al., 2009), which caused the drug to be released from SLNs within the skin.

Topical application of FK506-SLN hydrogel may deliver the drug directly to the site of action and thus produce high tissue concentrations of FK506 (Liu et al., 2007; Lopes et al., 2006), which are highly desirable characteristics for enhancing the treatment of skin diseases such as atopic dermatitis, psoriasis and vitiligo.

Conclusion

In conclusion, we have successfully incorporated FK506, a poorly water-soluble drug, into SLNs through an emulsification and low-temperature solidification method. The drug EE was approximately 88.74% under optimal conditions. The results of DSC and X-ray diffraction experiments indicated that the FK506 molecules were dispersed mainly into the lipid to form a homogeneous matrix structure. The formulated FK506-SLNs exhibited a nanometer range spherical structure with a sustained release profile *in vitro*. The FK506-SLNs were successfully incorporated into a carbopol hydrogel. Through the *in vitro* experiments, the FK506-SLN hydrogel exhibited greater permeation and better skin

targeting effects and skin localization compared to ointment formulations.

The FK506-SLN hydrogel may be a promising carrier for the topical delivery of FK506, although further studies are required for confirmation. Future studies should focus on the mechanism of FK506 permeation through and into human cadaver skin from SLN hydrogels and a comparative evaluation of the clinical efficacy.

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