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Multivesicular liposomes for the sustained release of thymopentin: stability, pharmacokinetics and pharmacodynamics

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The objective of the present study was to investigate the storage stability of thymopentin multivesicular liposomes (TP5-MVLs) prepared with different emulsifiers, and to study the pharmacokinetics and pharmacodynamics of the produced TP5-MVLs *in vivo*. The stability studies of TP5-MVLs indicated that MVLs particles prepared with mixed emulsifiers (Myrj52:solutolHS15 = 2:3) were stable at the storage temperature of $4 \pm 2^{\circ}$C within 3 months. In addition, FITC-TP5-loaded MVLs was prepared for pharmacokinetic studies that after subcutaneous administration, the fluorescence signal lasted for about 5 days in plasma demonstrating that the rate of drug release from MVLs was very slow. The pharmacodynamic studies indicated that the therapeutic efficacy of TP5-MVLs after subcutaneous administration once every four days was the same as free TP5 solution after intravenous or subcutaneous administration once daily. In conclusion, MVLs, which possessed great storage stability, can be utilized to reduce the administration frequency of TP5, and therefore, served as a promising sustained release delivery system for polypeptide.

1. Introduction

Nowadays, development of immunomodulatory agents, natural or synthetic substances, becomes increasingly important and valuable due to their interaction with immune system. They can stimulate the natural defense mechanisms of the body, and consequently restore or enhance the original immunological functions (Panico et al. 1997). However, these immunomodulatory agents, which belong to therapeutic proteins or peptides, will be cleared rapidly from the blood circulation. As a result, frequent injection is necessary in order to maintain therapeutic blood levels. Therefore, a sustained release system has to be utilized to increase drug circulation time.

Thymopentin (TP5) is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr) corresponding to the active site of the 49-amino acid human hormone thymopoietin (Tischio et al. 1979). As an immunomodulatory agent, TP5 has been clinically used in the treatment of rheumatoid arthritis (Ambrogi et al. 1992; Sundal et al. 1994) acquired immunodeficiency syndrome (AIDS) (Coppola et al. 1996; Merigan et al. 1996), severe acute respiratory syndrome (SARS) (Zhang et al. 2003), cutaneous T-cell lymphoma (Bernengo et al. 1992) and cancer immunodeficiency (Bodey et al. 2000). It rectifies imbalances in the immune system without observable side effects even at very high doses (Fan et al. 2006). However, the half-time of TP5 in plasma is only 30 s, which limits its efficient application (Tischio 1979). In order to prolong the duration of drug in the circulation, maintain the therapeutic level in the blood for a long time, reduce the frequency of drug administration and improve patient compliance, multivesicular liposomes (MVLs) is served as a sustained release system for TP5 in this study.

MVL, a unique lipid-based depot-delivery system, has been demonstrated to be an effective sustained delivery system with the release duration from days to several weeks (Ye et al. 2000). MVL, which comprises a set of closed packed non-concentric internal aqueous chambers separated by a network of lipid layers, is structurally different from unilamellar vesicles (ULV) and multilamellar vesicles (MLV) (Mantripragada 2002). Therefore, the degeneration of a single or several breaches of lipid layers in MVLs will not lead to a total internal drug release. As such, the unique structure of MVLs confers an increased level of stability and longer duration of drug release. Meanwhile, because of the numerous internal aqueous chambers, MVLs can be prepared with high encapsulation efficiency for water-soluble drugs and even for therapeutic proteins or peptides. In addition, the particle size of MVLs, approximately 5–50 μm, is about 10 times larger than that of ULV and MLV. Due to the large size, these MVLs particles will not be rapidly cleared by tissue macrophages and can act as a drug-depot (Ramprasad et al. 2003).

In a previous work (Zuo et al. 2009), TP5 loaded MVLs (TP5-MVLs) prepared by a double emulsion method exhibited a spherical shape with mean particle size and encapsulation efficiency of 13.2 μm and 80.15%, respectively. Meanwhile, the *in vitro* studies showed that the release kinetics of TP5-MVLs incubating in phosphate buffered saline (pH 7.4) at 37 °C was fitted to a zero-order equation: $Y = 0.5355\,t + 20.431$, ($r^2 = 0.9814$), and the cumulative release amount of TP5 from MVLs was 92.01% within 132 h. Importantly, the much cheaper and more readily available soybean phospholipid was utilized rather than the completely synthetic and more expensive dipalmitoylphosphatidylglycerol (DPPG) or dioleoylphatidylcholine (DOPC). With the cheaper phospholipid the produced MVL particles also possessed high encapsulation efficiency with sustained release properties *in vitro*. For the purpose of further investigating the *in vivo* sustained release effect and therapeutic efficacy of TP5-MVLs prepared with soybean phospholipid, in the present

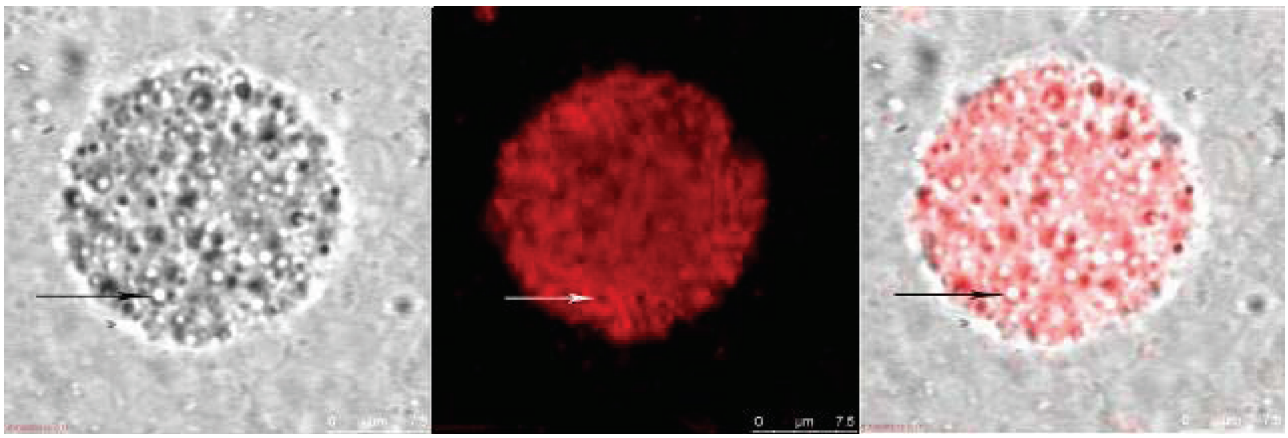

Fig. 1: Confocal micrographs of DepoFoam particles. The arrow indicates the aqueous chamber

study, pharmacokinetic and pharmacodynamic studies were performed. First, the stability of MVLs prepared with various emulsifiers was investigated at the storage temperature of 4 °C. Then, FITC-TP5-loaded MVLs was prepared for the pharmacokinetic studies. Finally, the therapeutic efficacy of TP5-MVLs after subcutaneous administration once every four days was compared with that of free TP5 solution after intravenous or subcutaneous administration once daily in pharmacodynamic studies.

2. Investigations results

2.1. Construction features of MVLs

As shown in Fig. 1, the left image shows the inner aqueous chambers under natural light and the middle one reveals the outer lipid layer labeled with red fluorescence. In the merged image, the aqueous chambers are surrounded by red fluorescence labeled lipid, indicating that discrete single non-concentric aqueous chambers exist inside the lipid vesicles. As a result, the TP5 loaded MVLs was prepared successfully.

2.2. The stability studies of TP5-MVLs

TP5-MVLs prepared with the mixed emulsifiers (Myrj52:SolutolHS15 = 2:3) was stable under the storage temperature of 4 ± 2 °C within 3 months. As shown in Table 1 the amount of free TP5 in TP5-MVLs suspension did not increase significantly within 3 months (only 3% of drug was released from MVLs).

However, when poloxamer188 (F68), Tween 80, Myrj52 or Solutol HS15 was used as the emulsifier individually, the results of stability studies were substantially different. The morphology of MVL particles prepared with various emulsifiers was observed under a microscope and is shown in Fig. 2. When only F68 was used as the emulsifier, each chamber of MVLs particles seemed too loose (Fig. 2A). Meanwhile, if Tween 80

Table 1: Stability of TP5-MVLs prepared with mixed emulsifiers (Myrj52:SolutolHS15 = 2:3) at the storage temperature of 4 ± 2 °C

Time (months)	Free TP5 (%) in supernatant of the suspension
1	1.5 ± 0.53
2	2.4 ± 0.82
3	3.1 ± 0.68

Values are mean ± S.D. (n = 3)

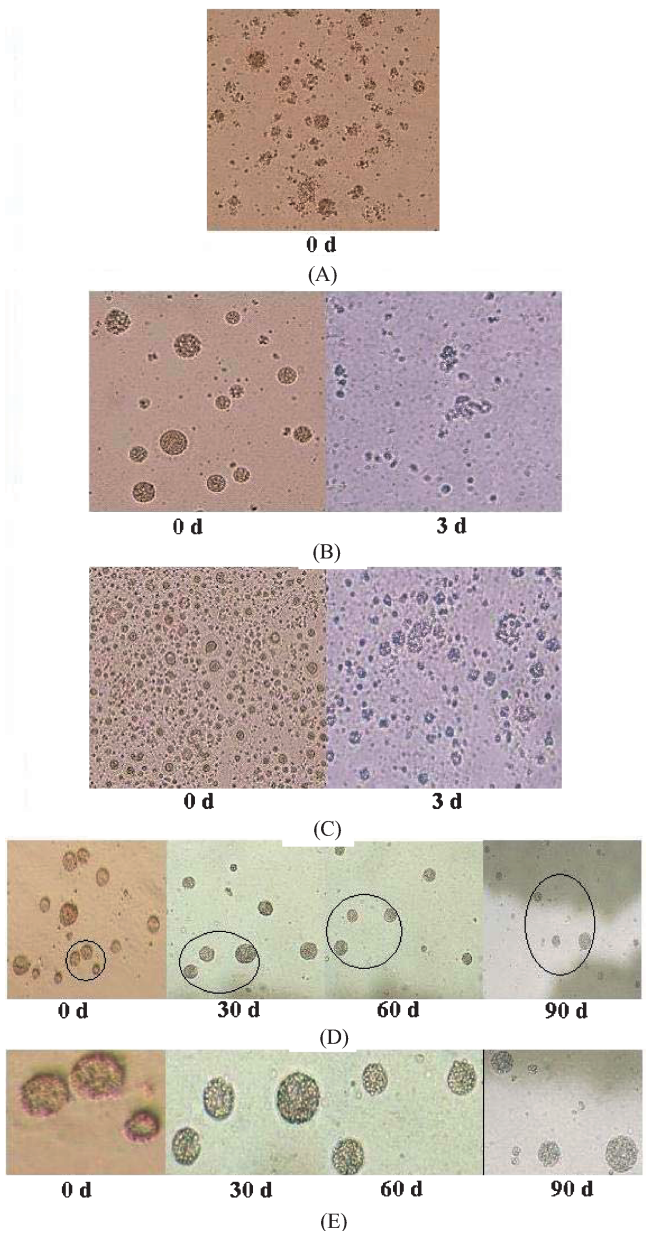

Fig. 2: Photomicrographs of TP5-MVLs with different emulsifiers at the storage temperature of 4 ± 2 °C at 400 × magnification taken by a light microscope. Morphology of TP5-MVLs prepared with F68 (A), with Tween 80 (B), with Myrj52 (C), with mixed emulsifiers (Myrj52:SolutolHS15 = 2:3) (D). (E) is the amplified edition of (D)

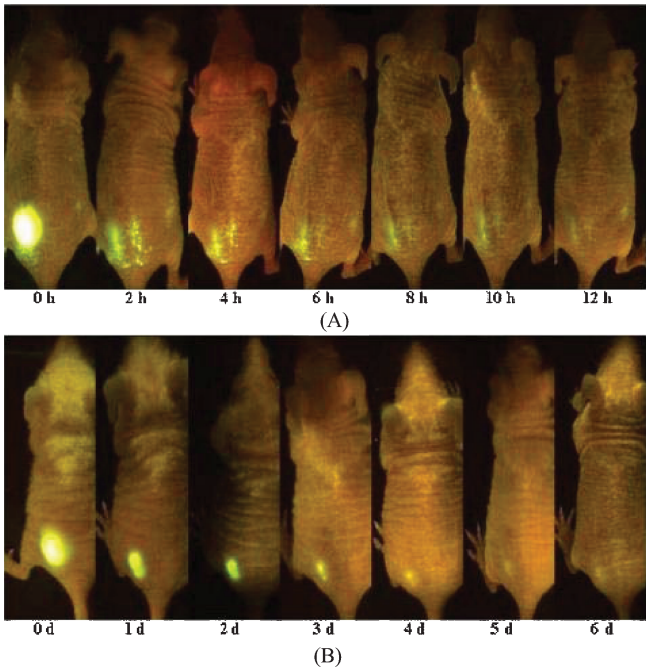

Fig. 3: Fluorescent images of FITC-TP5 solution (A) and FITC-TP5-loaded MVLs (B) via subcutaneous injection at the back of the rats

served as the emulsifier, the particles were smooth and unifrm at the very beginning. However, only 3 days later, the particles started to disrupt and many little chips were observed (Fig. 2B). When Myrj52 was used, MVLs particles were confused and disorderly in day zero, and 3 days later, only chips were left (Fig. 2C). In addition, MVLs could not be formed when only Solutol HS15 served as the emulsifier, because the particles would aggregate at the moment of removing the organic solvent by rotary evaporation. Figure. 2D shows the changes of appearance of MVLs prepared with the mixed emulsifiers (Myrj52:SolutolHS15 = 2:3). During the storage period, the shape and size of particles changed slightly and even at the end of 3 months, the particles still seemed round and smooth. For a clearer observation, particles in the circle in Fig. 2D were amplified (Fig. 2E). Although the chambers in particles became a little larger and were not as compact as before after 90 days, the integrity of particles was not destroyed.

2.3. In vivo qualitative and quantitative kinetic studies

After subcutaneous administration of FITC-TP5 solution and FITC-TP5-loaded MVLs, respectively, the change of fluorescence signal was observed using fluorescence imaging technique. As shown in Fig. 3, the fluorescence signal in the control group decreased very quickly after subcutaneous injection of FITC-TP5 solution, and 12 h later the fluorescence was hardly to see (Fig. 3A). Compared with FITC-TP5 solution, however, when FITC-TP5-loaded MVLs was injected at the same dose, the fluorescence signal decreased so slowly that it was still visible for about 6 days (Fig. 3B), indicating that MVLs showed a significant sustained release property. Therefore, according to the results mentioned above, MVLs may be considered as an efficient sustained release delivery system.

Although the fluorescent images provided valuable evidence for the efficacy of the sustained release system of MVLs, quantitative kinetic studies were necessary to further demonstrate the pharmacokinetic features in detail. Pharmacokinetic studies of FITC-TP5 solution and FITC-TP5-loaded MVLs were performed in rats via a single subcutaneous injection at the same dose of 5 mg/kg. Figure 4 shows the profile of FITC-TP5

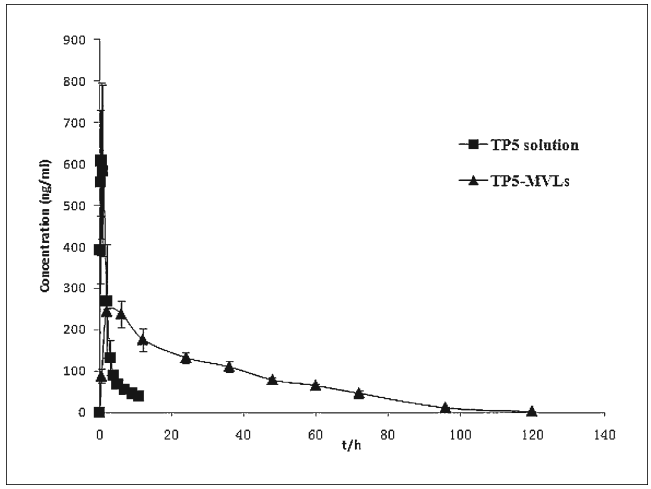

Fig. 4: Mean FITC-TP5 plasma concentration-time profile of FITC-TP5 solution (■) and FITC-TP5-loaded MVLs (▲) following subcutaneous administration at a dose of 5 mg/kg. Values were expressed as mean ± S.D. (n = 6)

blood concentration versus time for the two formulations of FITC-TP5. In addition, the main pharmacokinetic parameters calculated by DAS are listed in Table 2. After administration of FITC-TP5 solution, plasma concentration reached the maximal value within 1 h, which indicated the rapid absorption of the free drug in rats. Then, the concentration decreased quickly that 11 h after administration, drug could not be detected. When FITC-TP5-loaded MVLs was administered, however, the maximal concentration was obtained more slowly with lower value, and the drug could stay in the blood for a much longer time.

As shown in Table 2, the maximum plasma concentration (C_{max}) of FITC-TP5-loaded MVLs is about 2.7-fold lower than FITC-TP5 solution, but the value of T_{max} of MVLs is about 5.22-fold higher than that of FITC-TP5 solution. In addition the mean residence time (MRT) of MVLs is much longer than that of FITC-TP5 solution.

2.4. Pharmacodynamic studies of TP5-MVLs

The changes of the peripheral blood $CD3^+$, $CD4^+$, $CD8^+$ T-lymphocyte subsets in immunosuppression rats following various administrations are shown in Table 3. The results indicated that in the peripheral blood of immunosuppression rats, the percentage of $CD4^+$ was remarkably increased and the $CD8^+$ value significantly reduced as compared with those of normal control rats, respectively. Therefore, $CD4^+/CD8^+$ ratio in immunosuppression rats was obviously increased, indicating that the T-lymphocytes subsets were significantly affected by immunosuppression and the immunosuppression model was successfully built to the further use.

Table 2: Pharmacokinetic parameters of FITC-TP5 solution and FITC-TP5-loaded MVLs following subcutaneous administration at a dose of 5 mg/kg

Parameters	FITC-TP5 solution	FITC-TP5-loaded MVLs
C_{max} (ng/l)	686.606 ± 152.5	254.156 ± 20.315
T_{max} (h)	0.639 ± 0.2	3.333 ± 2.1
$AUC_{0\to t}$ (ng/l*h)	1607.011 ± 378.8	9334.972 ± 697.1
$AUC_{0\to\infty}$ (ng/l*h)	1865.895 ± 343.5	9390.656 ± 692.871
$MRT_{0\to t}$ (h)	2.639 ± 0.4	31.969 ± 0.661
$MRT_{0\to\infty}$ (h)	4.83 ± 1.3	32.604 ± 0.732

Values are mean ± S.D. (n = 6)

Table 3: The changes of $CD3^+$, $CD4^+$, $CD8^+$ T-lymphocyte subsets in various groups ($n = 5$). $CD4^+$ % represent the percentage of $CD4^+$ among $CD3^+$ cells. $CD8^+$ % represent the percentage of $CD8^+$ among $CD3^+$ cells

Group	$CD4^+$ %	$CD8^+$ %	$CD4^+/CD8^+$
Normal control	61.6 ± 4.6	37.0 ± 4.7	1.69 ± 0.3^a
Immunosuppression control	81.0 ± 6.6	16.2 ± 3.5	$5.26 \pm 1.6^{b,c}$
TP5 solution, iv	58.7 ± 4.8	34.2 ± 13.4	2.09 ± 1.3^a
TP5 solution, sc	56.3 ± 12.9	39.9 ± 12.6	1.63 ± 0.9^a
TP5-MVLs, sc	58.1 ± 5.3	26.0 ± 5.5	2.33 ± 0.6^a

Values are mean ± S.D. (n = 6), [a] $p < 0.05$ vs. Immunodepression control, [b] $p < 0.05$ vs. TP5 solution, i.v., [c] $p < 0.05$ vs. TP5-MVLs, sc

3. Discussion

The unique structure of MVLs particles shown in Fig. 1 may be an important factor contributing to the sustained release. Unlike the unilamellar liposomes, a rapid release of total loaded drug from MVLs is impossible as the non-concentric aqueous chambers in MVLs will be broken one by one, not at the same time, which is also the reason why MVLs can serve as an efficient sustained release system.

In the stability studies of TP5-MVLs, the emulsifiers used in the second aqueous phase may play an important part in maintaining the formulation stable. Emulsifier, consisting of a hydrophilic head and a hydrophobic tail and locating at the oil/water interface, can reduce the surface tension and therefore enhance the emulsion stability.

As we know, TP5 is degraded fast in plasma and thus it is hard to measure its plasma content accurately using the conventional methods (such as HPLC). Therefore, in the present study, FITC was covalently conjugated to TP5 with the molecular ratio of 3:1, and FITC-TP5-loaded MVLs was utilized in *in vivo* qualitative and quantitative kinetic studies. The changes of fluorescent signal intensity, determined by a fluorescent spectrophotometer, could represent the sustained release property of TP5. The results of *in vivo* qualitative and quantitative kinetic studies indicate that after administration at the same dose, MVLs could provide a much more stable plasma concentration for a long time than FITC-TP5 solution. Meanwhile, the $AUC_{0\rightarrow t}$ of FITC-TP5-loaded MVLs was much larger than that of FITC-TP5 solution, indicating that the bioavailability of TP5 could be significantly enhanced by MVLs system. It is presumed that after administration of MVLs, a lipid reservoir would be formed at the injection site from which the drug could be slowly released and absorbed (Mantripragada 2002). Therefore MVLs system could provide not only a more stable plasma concentration but also a significantly enhanced bioavailability.

The $CD4^+$ and $CD8^+$ represent the maturation of T-lymphocytes (Wang et al. 2006). In the immunological suppression or deficient patients, the peripheral blood $CD3^+$, $CD4^+$, $CD8^+$ values will be diminished and the $CD4^+/CD8^+$ ratio will be irregularly changed. As an immunomodulator, TP5 could regulate the $CD4^+/CD8^+$ ratio to a normal level. Therefore, the ratio was used to evaluate the pharmacodynamic effect of TP5 with various formulations. In pharmacodynamic studies, after administration of TP5 of various formulations in immunosuppression rats, all the $CD8^+$ values increased and the percentage of $CD4^+$ was regulated to a normal level. Consequently, the increased $CD4^+/CD8^+$ ratio in immunosuppression rats was evidently reduced following administration. In view of the therapeutic efficacy for modulating the irregular $CD4^+/CD8^+$ ratio, TP5-MVLs after subcutaneous administration was the same as that of TP5 solution after subcutaneous or intravenous administration. Based on these results, subcutaneous administration of TP5-MVLs once every four days may be applied as an efficient sustained release system instead of intravenous or subcutaneous administration of TP5 solution once daily. In another word, the application of MVLs could substantially decrease the frequency of injection and improve the patient compliance.

In the present study, a method of multiparameter flow cytometry with three-color analysis was utilized, in which $CD3^+$, $CD4^+$, $CD8^+$ T-lymphocyte subsets were labeled with fluorescence of different color. It was reported that T-lymphocytes were divided into cytotoxic T cells (Tc), suppressor T cells (Ts), as well as inducer-helper T cells (Ti/Th) and so on. CD3 cells express in all T-lymphocytes while CD4 express on the surface of Ti/Th cells, and CD8 only express on the surface of Ts/Tc cells (Wang et al. 2006). $CD4^+/CD8^+$ ratio was irregularly changed due to the inhibition of the proliferation and differentiation of T-lymphocyte after administration of immunosuppression agent cyclophosphamide.

It is concluded that using the cheaper and readily available phospholipids TP5-MVLs could also be prepared with high quality, including high encapsulation efficiency, remarkable sustained release property both *in vitro* and *in vivo*, superior particle stability, enhanced bioavailability as well as better compliance with the same therapeutic efficacy. Furthermore, it was reported that the MVLs particles did not show any local or systemic toxicity in humans or animals, and there was no foreign body response at the injection site after subcutaneous administration (Ye et al. 2000; Katre et al. 1998). Such MVLs, with advantages in compliance and no biotoxicity, can serve as a promising sustained release delivery system in human beings.

4. Experimental

4.1. Materials

TP5 was purchased from Kaijie (Chengdu, China); TP5-MVLs was prepared completely based on our previous work; Myrj52 and FITC were obtained from sigma (St Louis, US); SolutolHS15 was supplied by Basf chemical company, (Ludwigshafen, Germany); rhodamine-DHPE was from Biotium, inc.(Hayward, CA); cyclophosphamide was purchased from Beyotime biotechnology company, (Jiangsu, China); CD3-FITC, CD4-PE, CD8-PerCP, IgM-FITC, IgG2a-PE, IgG1-PerCP, hemolysin were purchased from Biolegend, (San Diego, CA, USA). Ultrapure water was used for all solutions and dilution. All the other reagents were of analytical grade.

4.2. Preparation of TP5-MVLs

TP5-MVLs were prepared by a double emulsification method (Kim et al. 1983; Katre et al. 1998). Briefly, a lipid solution containing 5 mg of soybean oil (Tieling Beiya Medical Oil Company, China), 40 mg of soybean phospholipid (Shanghai Taiwei medicine limited company, China), 20 mg of cholesterol (Chengdu Kelong Chemical Plant, China) in 0.5 ml of chloroform-diethyl ether (1:1) was emulsified with 0.4 ml of an aqueous solution (the first aqueous solution) to prepare a water-in-oil emulsion (the first emulsion). The first aqueous solution was 30 mg/ml of TP5, gelatin (3%, w/v) and sucrose (5%, w/v) in 50 mM arginine-containing buffers (pH = 7). And the emulsification condition was 16000 rmp for 1 min with a liquid emulsor (Shanghai Fluko Electromechanical Equipment Co. Ltd.). A subsequent emulsification with the second aqueous solution of 3.4% glucose containing 40 mM lysine and 0.5% emulsifying agent (Myrj52:SolutolHS15 = 2:3), yielded a water-in-oil-in-water double emulsion (the second emulsion). The second emulsion was transferred to a 10 ml round bottom flask, and chloroform and diethyl ether were removed by rotary evaporation at approximately 4–8 °C for about 30 min, to form the TP5-MVLs.

4.3. Construction feature of MVLs

The construction feature of MVLs was observed by a confocal microscope (Leica TCS SP5, Mannheim, Germany) (Mantripragada 2002). The lipids are labeled by fluorescent probes, rhodamine-DHPE (red fluorescence).

4.4. The stability studies of TP5-MVLs

Storage stability of the produced TP5-MVLs suspension within three months was studied. At predetermined time intervals, 0.1 ml of TP5-MVLs suspension stored at $4 \pm 2\,^{\circ}C$ was withdrawn and diluted with 0.2 ml of normal saline. The supernatants and pellets were separated by centrifugation at $621 \times g$ for 10 min. The amount of free drug was determined by HPLC analysis (Zuo et al. 2009).

4.5. In vivo studies

4.5.1. Fluorescence labeling of TP5 with FITC

The labeling of TP5 was conducted according to a reported method with some modifications (Li et al. 2007). Briefly, TP5 was dissolved in ultrapure water, FITC solution (5 mg/ml) in acetone was added dropwise under magnetic stirring. Then the mixture solution was stirred at $4\,^{\circ}C$ for 20 h at dark. After then the solution was filtered by C_{18} column (10 cm × 1.0 cm) and lyophilized, the obtained FITC-TP5 was kept at $-20\,^{\circ}C$ away from light until further use.

4.5.2. Preparation of FITC-TP5- loaded MVLs

The preparation procedure of FITC-TP5-loaded MVLs was almost the same as that of TP5-MVLs, except for small modifications. 30 mg of FITC-TP5 instead of TP5 was dissolved in the first aqueous solution.

4.5.3. Animals

Sprague-Dawley rats (male, 180–240 g) and hairless mice (male, 20–22 g) were purchased from the Experimental Animal Center of Sichuan University (protocol number for animal study: CSDGZ-10). The animals were allowed food and water *ad libitum*. Temperature and relative humidity were maintained at $25 \pm 2\,^{\circ}C$ and 50%. All the animals were used and treated as prescribed in the 'Guide for the care and the use of the laboratory animals' (NIH Publication No. 92–93, revised 1985) and all the animal protocols and experiments were approved and supervised by Animal Ethics Committee of Sichuan University.

4.5.4. Fluorescence imaging of FITC-TP5 solution and FITC-TP5-loaded MVLs in vivo

One hairless mouse was given FITC-TP5 solution (10 mg/kg) *via* subcutaneous injection at the back as a control. The other one was processed following the same procedure, but FITC-TP5-loaded MVLs were treated instead. Fluorescence imaging was performed in macro imaging system LT-9 equipped with illumatool dual light system LT-99D2 (Lightools Research, Encinitas, CA, USA).

4.5.5. Pharmacokinetics studies of FITC-TP5-loaded MVLs

4.5.5.1. Drug administration and blood sampling. Two groups of six rats each were treated with FITC-TP5 solution and FITC-TP5-loaded MVLs at a single dose of 5 mg/kg via subcutaneous injection at the back, respectively (Wang et al. 2006). Blood samples (more than 0.3 ml for each sample) were collected from the ophthalmic veins at predetermined time intervals after administration. The time point of FITC-TP5 solution group was 0.167, 0.333, 0.5, 1, 2, 3, 4, 5, 7, 9, 11 h while that of FITC-TP5-loaded MVLs group was 0.5, 2, 6, 12, 24, 36, 48, 60, 72, 96, 120 h. The heparinized blood was immediately centrifuged at $1125 \times g$ for 5 min. Rat plasma was obtained and stored at $4\,^{\circ}C$ until analysis.

4.5.5.2. Sample preparation, measurements and data analysis. 0.4 ml dehydrated alcohol was added to 0.1 ml plasma for protein precipitation. The mixture was vortexed for 1 min and then centrifuged at $5878 \times g$ for 5 min. 0.4 ml of supernatant was obtained for content determination using fluorescent spectrophotometer. The determination conditions were as follows: excitation wavelength, 502 nm; emission wavelength, 526 nm; slit for excitation, 5 nm; slit for emission, 5 nm. Under those conditions the assay was linear in the range of 2–20 ng/ml ($r^2 = 0.9956$) with the lowest limit of detection (LOD) of 0.1 ng/ml.
Blood drug level versus time data were analyzed by a non-compartmental model using Drug and Statistic software (DAS, Anhui, China).

4.5.6. Pharmacodynamics studies of TP5-MVLs

4.5.6.1. Drug administration. Twenty-five healthy rats were equally divided into five groups (five each). Group 1 was given physiological saline once daily for 10 days as a normal control. The rats in group 2 to 5 were administered cyclophosphamide solution intraperitoneally at a dose of 35 mg/kg once daily for three consecutive days to construct immunodepression models. The rats in group 2 without further treatment were used as an immunodepression control. The rats in group 3 were given TP5 solution (0.09 mg/kg) once daily for seven days by tail vein injection and the rats in group 4 were subcutaneously administered of TP5 solution (0.9 mg/kg) once daily for seven days, respectively. Additionally, the rats in group 5 were subcutaneously administered of TP5-MVLs (0.9 mg/kg) once every four days for seven times.
Blood samples were collected from each rat after different administrations and put into anticoagulant tubes for T-lymphocyte subsets analysis.

4.5.6.2. Sample preparation, measurements and data analysis. Lymphocyte subsets were determined by multiparameter flow cytometry with three-color analysis (Wang et al. 2006). The blood specimens were treated as follows: First, as an isotype control, 100 μl of anticoagulated blood was mixed with 20 μl FITC-IgM, 20 μl PE-IgG2a and 5 μl PerCP-IgG. Then, another 100 μl of blood was mixed with 2 μl FITC-CD3, 2 μl PE-CD4 and 4 μl PerCP-CD8 as a sample. After mixing by vortex for 30 s and incubation for 20 min at $25\,^{\circ}C$ at dark, red blood cells in control and sample tubes were lysed by hemolysin following the manufacturer's instruction. Subsequently, the specimens were centrifuged at $350 \times g$ for 5 min in order to collect the cells in sediment. Then the cells were washed with PBS twice and finally resuspended in 0.5 ml of PBS. The samples were kept at $4\,^{\circ}C$ at dark and the T-lymphocyte subsets analysis was performed within 4 h using BD FACS Canto™ II flow cytometer (USA). A total of 10,000 events per sample tube were collected.
Statistical analysis was carried out using the Student's *t*-test, in all cases, $P < 0.05$ was considered significant and the data were expressed as mean ± S.D.

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