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Surface Charge Modification and Antibacterial Study on Tilmicosin-Loaded Solid Lipid Nanoparticles against Avian Pasteurella

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Abstract

Surface charge (zeta potential) is one of the most important factors of drug nanocarriers. In this study, Tilmicosin-loaded Solid Lipid Nanoparticles (TMS-SLNs) were prepared by a hot homogenization and ultra-sonication technique using castor oil as lipid matrix and polyvinyl alcohol as surfactant. Dimethyl Dioctadecyl Ammonium Chloride (DDAC, 2%, w/w) was used as a cationic surfactant to modify the zeta potential of the SLNs for the purpose of improving the formulation. The obtained TMS-SLNs were characterized and the antibacterial ability was evaluated *in vitro* and *in vivo* against avian pasteurella. The results showed that addition of DDAC switched the zeta potential from - 11.7 ± 1.5 mv to 38.9 ± 1.5 mv. DDAC significantly increased the drug loading and enhanced the sustained release property of the TMS-SLNs. Scanning electron microscopy showed the SLNs were spherical and DDAC made no change of the morphology. Fluorescent microscopy revealed that DDAC enhanced the nanoparticle binding to the bacteria. The modified TMS-SLNs showed enhanced *in vitro* and *in vivo* antibacterial efficacy and good stability similar to the unmodified formulation. These results suggest that DDAC modification facilitated drug entrapment and sustained release property of the TMS-SLNs. This study presents useful information towards the design of TMS-SLN formulations.

Keywords: Tilmicosin; Solid lipid nanoparticle; Surface charge; Avian Pasteurella; Antibacterial efficacy

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Introduction

Antibiotics play an important role in the prevention and control of animal diseases. Tilmicosin is a relatively new semi-synthetic macrolide antibiotic which is widely used in veterinary medicine for respiratory disease treatment in cattle, swine and chicken [1,2]. However, the major tilmicosin formulations used in clinic are in the form of phosphates which display drawbacks such as low water solubility, susceptibility to acid damage after oral administration, incomplete absorption, and low bioavailability [3]. In order to enhance its clinical efficacy, repeated administrations and high doses are required, leading to acute cardiac toxicity, allergic reaction and transient swelling at administration area [4]. Novel delivery system of tilmicosin is needed for promoting its efficacy and reducing the defects.

Over decades' studies, Solid Lipid Nanoparticle (SLN) has been emerged as a potential nanotechnology-based drug delivery system for delivery of DNA, vaccines, small molecules, recombinant proteins, and antibacterial drugs, especially as the hydrophobic antibiotics carriers [5,6]. Our previous studies showed that castor oil formulated tilmicosin incorporated SLN (TMS-SLN) was a promising formulation to enhance the pharmacological activity and therapeutic efficacy of tilmicosin, meanwhile decreased reactions at injection sites [7-9]. The TMS-SLN prepared in our previous studies was negatively charged with relatively low zeta potential [9].

Surface charge is one of the most important physical characteristics of particle samples and a key parameter of nanodrug carriers. Surface potential has a great influence on the antibacterial ability of nanoparticles. When encountering nanoparticles with a large number of cationic groups on the surface, the negative charge of the bacterial cell membrane is neutralized, thereby inhibiting the respiratory function of bacteria, causing "contact death", or changing the surface charge

number producing "bacterial dissolution" to achieve the purpose of sterilization [10-13]. In addition, positively charged nanoparticles are more likely to combine with negatively charged cells and enter cells [14], so nanocarriers can deliver drugs directly to bacterial or somatic cells, enhancing bactericidal effects.

Negatively charged nanoparticles can be switched to positive by addition of cationic surfactants [15,16]. Quaternary Ammonium Compounds (QACs) are cationic surfactant which have been widely applied in pharmaceutical engineering, biological products, chemical engineering and cosmetics [17,18]. The addition of QACs can ameliorate the physicochemical properties of SLNs including the zeta potential [19-21].

In the present work, Dimethyl Dioctadecyl Ammonium Chloride (DDAC), one kind of quaternary ammonium salts, was used to modify the TMS-SLN and the effects of DDAC on the characteristics of the SLN was studied. The impact of surface charge modification on the antibacterial efficacy of the SLN was evaluated both *in vitro* and *in vivo* to provide a reference for the development of TMS-SLN formulations.

Materials and Methods

Materials

Hydrogenated Castor Oil (HCO) was obtained from Tongliao Tonghua Castor Chemical Co, Ltd (Inner Mongolia, China). Tilmicosin was purchased from Jiulong Fine Chemical Co., Ltd (Shandong, China). Polyvinyl Alcohol (PVA) was obtained from Sigma (St. Louis, MO, USA). Dimethyl Dioctadecyl Ammonium Chloride (DDAC) was bought from Aladdin industrial Inc. (Shanghai, China). Rhodamine B was bought from American Amresco Corporation. Hoechst 33342 was obtained from Hoechst AG of Germany. Other chemicals and reagents not specified in the text were of analytical grade or equivalent.

Animals and bacteria

Commercial broiler chickens of each sex (1 day old) were obtained from Beijing Huadu Broiler Corporation. The animals were housed at room temperature under natural day and night cycles with free access to water and food. They were kept for twelve days before treatment. After experiments, all the survived chickens were sacrificed. All experimental protocols concerning the handling of chickens were in accordance with the requirements of the Institutional Animal Care and Use Committee at China Agricultural University and were approved by the Committee.

Avian Pasteurella was a clinical isolate. Bacteria were streaked from glycerol-frozen stocks onto Trypticase Soy Agar (TSA) plates with 5% new born calf serum and incubated overnight at 37°C. A single bacterial colony from the fresh plates was inoculated in the Trypticase Soy Broth (TSB) with 5% new born calf serum and grown at 37°C in a shaking incubator at 180 rpm to an OD600 of 0.4. For *in vitro* antibacterial study, the bacteria were diluted in broth. For *in vivo* study, the bacteria were collected by centrifugation at 3500 rpm for 10 min at 4°C and resuspended in sterile saline at different concentrations.

Preparation of TMS-SLNs

Tilmicosin-loaded solid lipid nanoparticles were formulated by a hot homogenization and ultra-sonication method as described previously [8]. Briefly, 75 mg tilmicosin and 425 mg HCO was mixed in a 50 mL tube and heated in a boiling water bath. For surface charge modification, 10 mg DDAC was added in the lipid mixture. For fluorescence labeling of TMS-SLN, 2 mg Rhodamine B was added in the melted lipid. The melted mixture was dispersed into 10 mL, 2% PVA solution which was preheated in a boiling water bath under magnetic stirring to form an o/w emulsion. The emulsion was sonicated for 5 min (VC × 750 Vibra-Cell™, Sonics and Materials, Inc., Newtown, CT, USA, using the 13 mm microprobe with amplitude 35%) to form a nanoemulsion. The nanoemulsion was quickly poured into 30 mL cold water to obtain a nanoparticle suspension. The blank SLNs of each formulation were prepared in the same way without adding the tilmicosin. To get SLN powders, the nanoparticles were collected by centrifugation at 12,000 rpm (Centrifuge 5810 R; Eppendorf, Germany) for 30 min at 4°C, washed three times with distilled water and lyophilized for 48 h in a freeze dryer (LGj-12, Beijing Songyuan Huaxing Science Technology Development Co. Ltd., China).

Determination of mean diameter, polydispersity index, and zeta potential

The Mean Diameter (MD), Polydispersity Index (PDI), and Zeta Potential (ZP) of the SLN were determined by photon correlation spectroscopy using Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) at 25°C. The samples of SLN suspensions were diluted with distilled water by 10 to 20 times for the particle size and PDI analysis, and by 100 to 200 times for ZP determination to get optimum kilo counts per second of 20 to 400 for measurements.

Determination of drug loading

To determine the drug content in the nanoparticles, a weighed amount of freeze-dried SLN was dissolved in chloroform and the solution was analyzed directly at 292 nm using a UV spectrophotometer (U-1800, Hitachi Tech Co., Japan). The blank nanoparticles were treated similarly as blanks for the measurements. The assay was repeated three times using different samples from independent preparations. Drug loading (DL) is defined as follows:

$$DL = [(\text{Weight of tilmicosin in SLN}) / (\text{Weight of SLN})] \times 100\%$$

Scanning electron microscopy (SEM)

The morphology of TMS-SLNs was investigated by scanning electron microscopy (Quanta, 200; FEI, America). Briefly, 1 mg freeze dried samples was suspended in 1 mL distilled water and 2 μL of the suspension were placed on a glass slide. After oven drying at 45°C for 10 min, the samples were coated with gold using an Ion Sputter and examined at an accelerating voltage of 20 kV.

Fluorescent microscopy

The combination of TMS-SLNs with Avian Pasteurella was evaluated by fluorescence microscopy. The bacteria (0.1 mL suspension, OD600=0.4) were collected by centrifugation in a micro centrifuge at 3500 rpm for 5 min, washed three times with saline, and then suspend in 0.5 mL saline. The bacteria were labeled by adding 40 μg Hoechst 33342 and incubating at 37°C for 5 min. Equal volume of Rhodamine B labeled TMS-SLN suspension were mixed with the bacteria and incubate at 37°C in a shaking incubator at 180 rpm for 30 min. The sample was centrifuged at 3500 rpm for 5 min, washed three times with saline, and suspended in 0.1 mL saline. One microliter of the mixture was placed on a slide and observed under a fluorescence microscope.

In vitro release

TMS-SLNs dry powder (containing 2 mg tilmicosin) was suspended in 2mL 0.9% (w/v) NaCl solution (donor solution) in a

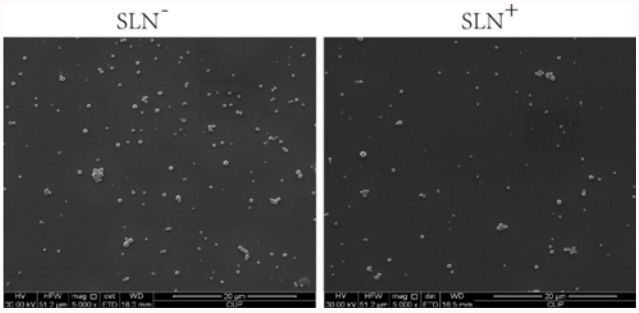

Figure 1: Scanning Electron Microscopy (SEM) of Tilmicosin-SLN. SLN⁻: 0% DDAC; SLN⁺: 2% DDAC.

dialysis bag (molecular weight: 8,000 to 14,400) and dialyzed against 38 mL 0.9% (w/v) NaCl solution (receiver solution) in a 50 mL tube at 37°C in an incubator with shaking at 110 rpm. At fixed time points, the samples (2 mL) were taken from the receiver solution for TMS quantitation by UV spectrophotometer at 291 nm, while the same amount of fresh 0.9% (w/v) NaCl solution was added to keep a constant volume. The control nanoparticles without TMS was treated the same way and used as blanks for the measurements. The experiments were carried out in triplicate.

In vitro antibacterial activity

The Minimum Inhibitory Concentration (MIC) was determined by micro dilution method with MHB using an inoculum of 5×10^5 cfu/mL. Briefly, serial dilutions of TMS-SLN suspension were made with MHB. One milliliter of the SLN dilution was mixed with 1 mL MHB containing 1×10^6 cfu/mL Pasteurella in a 4 mL sterile centrifuge tube (the final TMS concentrations: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10 µg/mL) and the mixture was incubated at 37°C in an incubator (Jiangsu Taicang Experimental Equipment Company, China) with shaking at 130 rpm. The MIC was defined as the lowest concentration inhibiting visible growth after incubation at 37°C for 18 h. The MIC of native TMS and released TMS was measured by the same way. All experiments were carried out in triplicates.

Sustained antibacterial studies were conducted by broth dilution technique. One milliliter bacterial culture containing 1×10^6 c fu/mL of organisms were added to 1 mL MHB medium in a 4 mL tube containing TMS or TMS-SLN suspension with a drug concentration of 0.2 µg/mL. The control nanoparticle formulation without TMS and bacteria were used as control. The mixtures were incubated at 37°C in an incubator with shaking at 130 rpm. At fixed time points (12, 24, 36, 48 h), the serial dilutions of the mixtures were cultured on TSA plates at 37°C. The colonies were counted when they could be observed by naked eyes. Growth graphs were plotted by calculating the number of colonies in 1 mL of the mixture *vs.* the incubating time (hour). All experiments were carried out in triplicates.

Determination of lethal dose

A preliminary experiment was performed to determine the lethal dose. Thirty chickens (12 days old) were randomly divided into 3 groups with 10 animals in each group. Bacteria (0.1 mL at concentrations of 400, 200, 100 c fu/mL) were intramuscular injected in the pectoral muscle of each chick of 3 groups. The chickens were observed every 12 h over a 72-h period. At the concentration of 400 and 200 c fu/mL, all chickens died within 72 h. At the concentration of 100 c fu/mL, 20% of the chickens survived by 72 h. Thus, the inoculation of 200 c fu/mL was used as the lethal dose for protection studies.

Mortality protection

Eighty-five chickens (12 days old) were randomly divided into 5 groups with 8 males and 7 females in each group. Four groups were inoculated with the lethal dose of bacteria while another group was set as negative control. Right after inoculation, the chickens were orally administrated with a single dose of different formulations of TMS-SLNs and native TMS (suspended or dissolved in 0.5 mL sterile distilled water) with a TMS dose of 15 mg/kg. One infected group was administrated with 0.5 mL sterile distilled water as positive control. Animals were observed every 24 h and deaths were recorded over a 10-day period.

Stability studies

Stability studies of SLN were performed after the samples were stored at 4°C and at RT for 3 months and 6 months. The values of the MD, PDI, ZP, and DL were measured for the evaluation of the physical stability of the nanoparticles.

Results

Physicochemical characteristics of TMS-SLN

SEM images showed that the nanoparticles were spherical and well dispersed (Figure 1). DDAC made no visible changes of the morphology of the SLNs. The zeta potential, mean diameter, polydispersity index and drug loading are shown in Table 1. Addition of DDAC increased the zeta potential, and also increased the drug loading and particle size.

In vitro release

The *in vitro* release behaviors of TMS-SLNs with different surface charges are summarized as the cumulative percentage release of TMS (Figure 2). The release trends of the nanoparticle formulations are very similar. The positively charged TMS-SLN (SLN⁺) showed slower releasing speed than the negatively charged SLN (SLN⁻). The total tilmicosin released from SLN⁻ and SLN⁺ at 10th day was 34.18% and 14.66% respectively. DDAC significantly decreased the release speed of the TMS-SLNs. As a control, the TMS solution exhibited a rapid drug release of 55.63% ± 1.01% within 2 h. The release is almost complete by 12 h.

The combination of TMS-SLNs with avian pasteurella

The results of fluorescence microscopy are shown in Figure 3. The TMS-SLNs were labeled with Rhodamine B (red), the bacteria

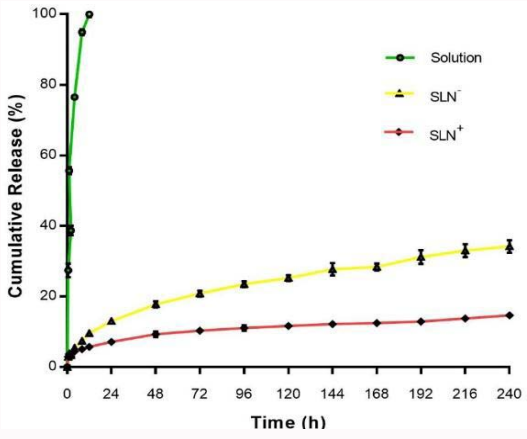

Figure 2: *In vitro* release of TMS-SLNs (mean ± SD, n=3).

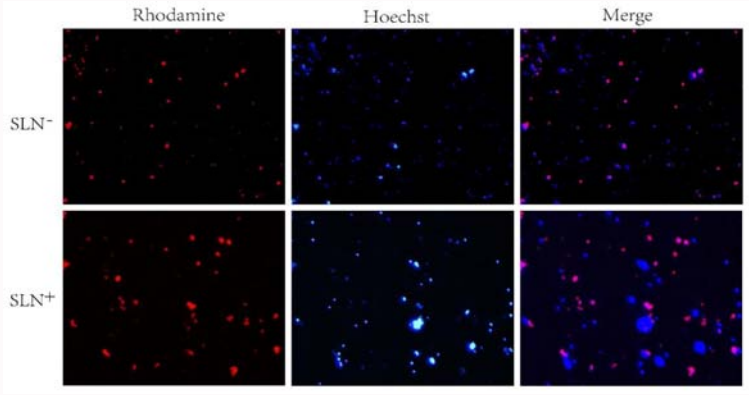

Figure 3: Fluorescence microscopy of TMS-SLNs (red), bacteria (blue) and the combination (pink).

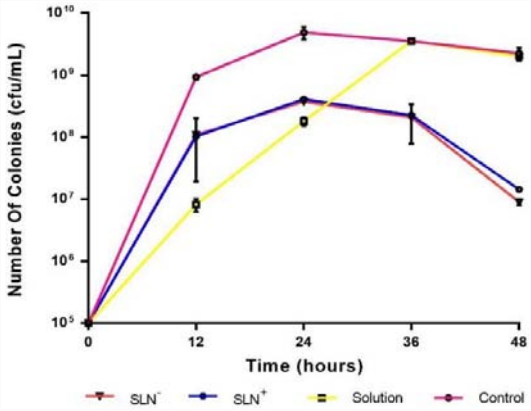

Figure 4: Sustained antibacterial effect of TMS-SLNs with a final drug concentration of 0.2 μg/mL.

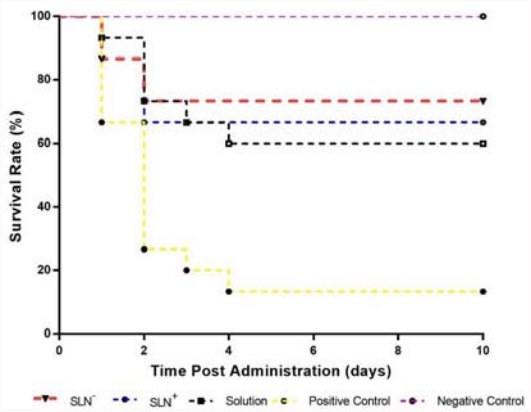

Figure 5: Percent survival of lethal dose infected chickens treated with a single oral administration of TMS at a dose of 15 mg/kg body weight.

were stained with Hoechst 33342 (blue). The pink spots are the merge of the red and blue fluorescence, indicating the combination of the SLNs with the bacteria. Both the negatively charged and the positively charged SLNs showed binding ability to the bacteria, while the pink sports in the TMS-SLN$^+$ picture were obviously bigger.

In vitro antibacterial activity

The results of *in vitro* antibacterial activity assessments are shown in Table 2. The MIC of native tilmicosin was 1.0 μg/mL. The control SLN had no antimicrobial effect. The tilmicosin released from the TMS-SLNs had the same MIC values as that of the native tilmicosin. The MIC of the SLN formulations were higher than that of the native drug, and TMS-SLN$^+$ had increased MIC value compared with TMS-SLN.

Table 1: Physicochemical characteristic of TMS-SLNs (means ± SD, n=3).

Formulation	ZP (mV)	MD (nm)	PDI	DL (%)
SLN-	-11.7 ± 1.5	452 ± 10	0.278 ± 0.079	6.41 ± 0.20
SLN$^+$	38.9 ± 1.5	499 ± 6	0.309 ± 0.012	7.87 ± 0.48

Table 2: The MIC of TMS-SLNs.

Formulation	MIC (μg/mL)
Native TMS	1
Released TMS	1
TMS-SLN$^-$	1.5
TMS-SLN$^+$	2

Next, the time-kill curves for the two TMS-SLN formulations and native tilmicosin were compared (Figure 4). Within the initial 24 h, the TMS-SLNs were less effective. The increase of bacteria colony numbers in the cultures with the two TMS-SLN formulations was faster than that of TMS solution. However, after 24 h the SLNs were much more effective than native TMS solution. Colony numbers of native TMS continually increased after 24 h and reached almost the same levels as that of the positive controls by 36 h, while the colony number of The TMS-SLNs started to decrease and dropped to significantly lower levels. The trends of antibacterial activity of the two SLN formulations exhibited very similar sustained antibacterial effect with no significant difference.

Mortality protection

The survival curves showed that the TMS-SLN groups were superior to TMS solution group for protection (Figure 5), and the SLN$^+$ with positive charges showed no significant difference as compared with the negatively charged SLN$^-$. The proportion of survivors over the observed period in the groups treated with the SLN$^-$ and SLN$^+$ were 11/15 (73.33%) and 10/15 (66.67%), respectively. The proportion of survivors in the TMS solution group and the infection control (negative control) group were 9/15 (60%) and 2/15 (13.33%).

Stability

Both the negatively charged and positively charged SLNs exhibited good stability at 4°C and Room Temperature (RT) during 6 months of storage. Slight increases in the MDs and PDIs and slight

Table 3: Characteristic of fresh and stored TMS-SLNs (means ± SD, n=3).

Formulation	Storage time	Temp	ZP (mv)	MD (nm)	PDI	DL (%)
TMS-SLN-	Fresh		-11.7 ± 1.5	452 ± 10	0.278 ± 0.079	6.41 ± 0.20
	3 months	4°C	-10.2 ± 0.3	463 ± 6	0.345 ± 0.084	6.38 ± 0.18
		RT	-10.4 ± 0.8	473 ± 5	0.303 ± 0.010	5.46 ± 0.06
	6 months	4°C	-8.0 ± 0.5	477 ± 19	0.361 ± 0.029	6.25 ± 0.11
		RT	-11.5 ± 0.8	489 ± 7	0.335 ± 0.092	5.12 ± 0.19
TMS-SLN+	Fresh		38.9 ± 1.5	463 ± 6	0.309 ± 0.012	7.87 ± 0.48
	3 months	4°C	40.2 ± 0.1	461 ± 21	0.271 ± 0.033	7.11 ± 0.30
		RT	38.6 ± 0.4	481 ± 29	0.258 ± 0.077	6.63 ± 0.78
	6 months	4°C	37.0 ± 1.1	495 ± 13	0.367 ± 0.034	6.70 ± 0.45
		RT	33.4 ± 0.5	493 ± 6	0.399 ± 0.009	6.54 ± 0.94

Temp: Temperature; ZP: Zeta Potential; MD: Mean Diameter; PDI: Polydispersity Index; DL: Drug Loading

decreases in the DL were observed (Table 3). The zeta potential of positively charged SLN decreased with the increase of storage time.

Discussion

The results of this work demonstrate that DDAC was an effective modifier to switch the zeta potential of TMS-SLNs from negative to positive. With the addition of DDAC, the drug loading and mean diameter also increased, which might be due to the increased stability of drug-loaded nanoparticles with Quaternary ammonium salt [22]. Both the negatively charged and positively charged SLNs exhibited good stability at 4°C and at Room Temperature (RT). During the storage time, slight increases in the MDs and PDIs might be due to the aggregation of the SLNs. Slight decreases in the drug loading and zeta potential could be caused by the release of the drug and DDAC from the LSNs.

Combination test showed that pink spots have corresponding spots in the other two photos, indicating that the pink spots are the combination of the SLNs with the bacteria, not the combination of free Rhodamine B with the bacteria. SLNs with either negative or positive surface charge could combine with the bacteria. The bigger pink sports observed in the TMS-SLN^+ picture could be due to more SLNs combined to the bacteria, suggesting that the surface charge modification enhanced the combination ability of the TMS-SLN with the bacteria.

In vitro release study exhibited an initial burst release followed by a period of slow sustained release. The two SLN formulations showed similar initial burst release within 2 h (SLN^-: 3.85%, SLN^+: 3.65%). This initial release may due to the drug just beneath the surface of the nanoparticles. After 2 h, positively charged formulations (SLN^+) showed slower release speed, although they had higher drug loading compared with negatively charged SLN^-. The slow release of the SLNs could mainly due to the slow diffusion of the drug molecules through the nanoparticles. Slower release of SLN^+ could be due to the increased stability and relatively larger nanoparticle sizes [9].

The same MIC of released TMS as the native TMS indicates that the drug had no change during preparation and release studies. The MIC of the two SLN formulations were higher than that of the native drug, which could be explained by the sustained release of the TMS-SLNs that resulted in lower drug concentrations in the medium.

The time-kill curve study revealed that colony number of native TMS sharply increased from 24 h and reached almost the same levels as that of the blank MHB control. The control blank-SLN with DDAC had no antibacterial effect. The two TMS-SLN formulations exhibited very similar sustained antibacterial activity without significant difference. Considering the slower drug lease of the modified TMS-SLN which could result in lower drug concentrations in the culture medium, it can be inferred that DDAC modification enhanced the *in vitro* antibacterial efficacy of the TMS-SLN.

The higher proportion of survivors in the mortality protection studies indicates that the TMS-SLNs enhanced the *in vivo* antibacterial efficacy of TMS. The enhanced efficacy was probably related to enhanced bioavailability of TMS-SLNs [8]. However, the increase of survivors in the two SLNs groups was not dramatic compared with that of the TMS solution. It could be explained by the sustained release of the SLNs. Sustained release resulted in a lower TMS concentration, while enhanced bioavailability increased the TMS concentration. As a result, TMS-SLNs showed little higher survivors compared with TMS solution. The therapeutic activities of TMS are related to their pharmacokinetic behavior [8]. Moreover, these were no significant difference between the two formulations. Taking account of the slower drug release of the positively charged TMS-SLN, surface charge modification improved the *in vivo* antibacterial efficacy of TMS-SLNs. In this lethal dose infection model, the peak of morbidity and mortality of the chickens was 2 days and by 3 days almost all the infected chickens died if not treated with TMS. The sustained release of drugs from castor oil fabricated SLNs could last for more than 5 days [8,23]. In case of natural infection, the morbidity and mortality time should be longer than that of the lethal dose infection; TMS-SLNs would have better prophylactic and therapeutic efficacy, and DDAC modification could further improve the efficacy.

Conclusion

This work demonstrates that modification with DDAC significantly enhanced the drug entrapment efficiency and sustained release property of TMS-SLNs. Surface charge modification can be a simple technique to improve the property and antibacterial efficacy of TMS-SLN formulations.

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