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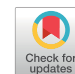
# Intracellular delivery, accumulation, and discrepancy in antibacterial activity of four enrofloxacin-loaded fatty acid solid lipid nanoparticles



Kuiyu Meng[a], Dongmei Chen[a,b], Fei Yang[a], Aoxue Zhang[a], Yanfei Tao[b], Wei Qu[a], Yuanhu Pan[a], Haihong Hao[a], Shuyu Xie[a,*]

[a] National Reference Laboratory of Veterinary Drug Residues (HZAU), Huazhong Agricultural University, Wuhan, Hubei 430070, China
[b] MAO Key Laboratory for Detection of Veterinary Drug Residues, Huazhong Agricultural University, Wuhan, Hubei 430070, China

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ABSTRACT

Four fatty acid-solid lipid nanoparticles (SLNs) were formulated and evaluated for intracellular delivery, accumulation, as well as discrepancy in antimicrobial efficacy of their loaded enrofloxacin by using RAW 264.7 cells. The delivery efficacy of enrofloxacin into the macrophages by docosanoic acid SLNs (DAS), octadecanoic acid SLNs (OAS), hexadecanoic acid SLNs (HAS), and tetradecanoic acid SLNs (TAS) were 26.1–29.0, 9.3–10.3, 4.7–5.3 and 4.5–5.0 folds, respectively, compared to free drug when co-incubation for 0.25 − 4 h. The longer fatty acid prepared nanoparticles loaded enrofloxacin eliminated more slowly and accumulated in the cells for a longer time.The confocal microscopy also demonstrated that higher amount of fatty acid SLNs entered the cells with stronger accumulation performance and less amount SLNs absorbed on the cytomembrane as the carbon chain of fatty acids increased. The bactericidal activity of the four fatty acid SLNs against intracellular *Salmonella CVCC541* significantly enhanced compared to the free enrofloxacin. These results revealed that fatty acid SLNs, especially docosanoic acid nanoparticles, might be effective nanocarriers to ferry enrofloxacin or other lipid soluble drugs into cells for intracellular bacterial infection treatment.

## 1. Introduction

The treatment of infections caused by facultative or obligate intracellular bacteria, e.g., *Salmonella* ssp., *Staphylococcus aureus* (*S. aureus*), *Brucella* ssp., *Listerial monocytogenes, Legionella pneumophilia, Klebsiella* ssp., *Corynebacterium* ssp., and *Mycobacterium tuberculosis,* are of great important in global medical and veterinary community [1,2]. These microorganisms fight against host defense through several special mechanisms to survive and spread in host cells [1,2]. The long-lasting survival of bacteria in macrophages makes ineffective antibiotics treatments that are less able to penetrate cell membranes [2]. Most of the antibiotics (β-lactam) have poor permeability across the cell membrane and intracellular accumulation properties, leading to negligible effects against intracellular bacteria *in vivo,* although they have been proved to be effective *in vitro* [2,3].

Fluoroquinolones, being known for their potent broad-spectrum bactericidal activity and efficient diffusion in the cells, is an effective approach to prevent and treat various intracellular bacterial infections for humans and animals [4]. Enrofloxacin is intensively used against various intracellular agents (e.g., *Salmonellosis* and S. *aureus* mastitis) in veterinary field [4–6]. However, its intracellular retention is low due to its rapid efflux from macrophages via organic anion carriers when the extracellular concentration decreases. The cells loaded with enrofloxacin released 80%–90% enrofloxacin from macrophages and polymorphonuclear leukocytes, respectively, within 10 min when they are placed into free medium without enrofloxacin [4]. Moreover, repeated and relative high doses often be given to get better therapeutic efficacy, which enhances the treatment cost, animal inconvenience, side effects, drug residues in animal edible tissues, and drug resistance. Therefore, there is dire need of effective therapies for intracellular bacterial infection diseases.

Some studies demonstrated that encapsulating enrofloxacin into liposomes not only increased its intracellular retention time but also its therapeutics efficacy increased by many folds [7]. The retention of Liposomal enrofloxacin (3.1 folds) in monocytes was higher than that of pure enrofloxacin (1.6 folds) following incubation for 4 h [7], superiorly reducing intracellular located *S. aureus* [8]. Multilamellar liposomes composed of cholesterol and dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) encapsulated enrofloxacin showed higher drug concentration in Kangal dog blood cells [9]. Liposomes composed of

* Corresponding author.
*E-mail address:* snxsy1@126.com (S. Xie).

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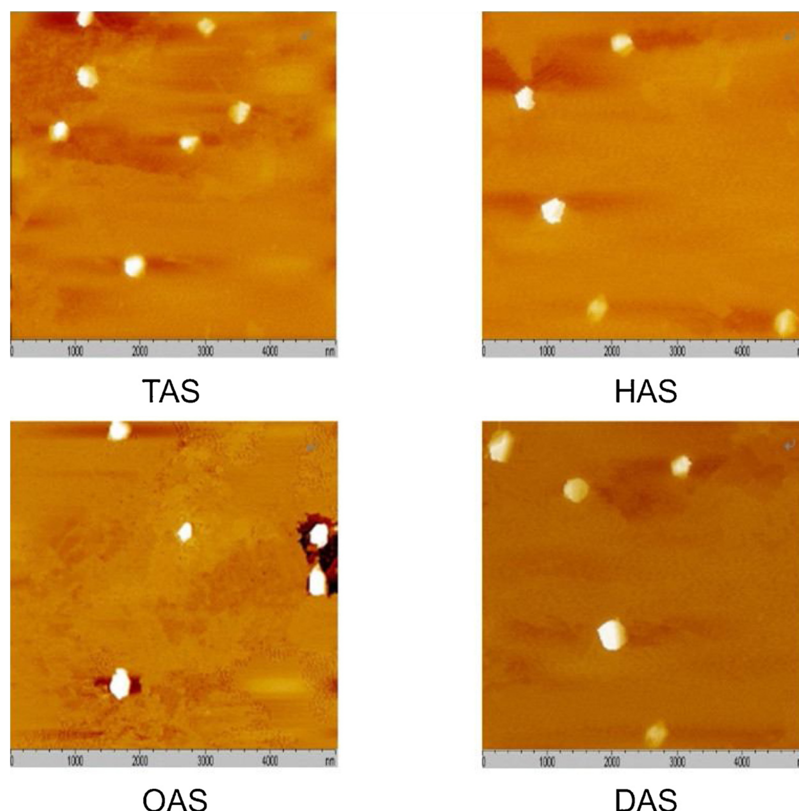


**Fig. 1.** Photographs of atomic force microscopy (AFM) of various fatty acid SLNs.

**Table 1**
Physicochemical characteristics of various fatty acid-SLNs (mean ± S.D., n = 3).

| Formulation | EE (%) | LC (%) | MD (nm) | PDI | ZP (mV) |
|---|---|---|---|---|---|
| TAS | 65.26 ± 1.76 | 6.44 ± 0.23 | 341.4 ± 4.9 | 0.241 ± 0.014 | −19.9 ± 0.4 |
| FITC-TAS | | | 342.6 ± 6.3 | 0.225 ± 0.013 | −19.6 ± 0.3 |
| HAS | 67.53 ± 2.25 | 6.65 ± 0.22 | 348.8 ± 3.5 | 0.264 ± 0.013 | −20.6 ± 0.9 |
| FITC-HAS | | | 360 ± 5.7 | 0.221 ± 0.012 | −19.9 ± 0.4 |
| OAS | 72.57 ± 2.90[a] | 7.12 ± 0.28[a] | 408.5 ± 6.3[a,b] | 0.352 ± 0.015[a,b] | −21.3 ± 0.6[a] |
| FITC-OAS | | | 398.1 ± 7.1 | 0.278 ± 0.014 | −20.1 ± 0.4 |
| DAS | 86.56 ± 1.67[a,b,c] | 8.37 ± 0.15 [a,b,c] | 414.5 ± 3.8[a,b] | 0.265 ± 0.019[c] | −22.1 ± 0.1[a,b] |
| FITC-DAS | | | 420.1 ± 5.1 | 0.223 ± 0.012 | −20.3 ± 0.4 |

EE: Encapsulation efficiency; LC: Loading capacity; MD: Mean diameter; PDI: Polydispersity index; ZP: Zeta potential.
[a] Statistical significances compared with tetradecanoic acid SLNs are $p < 0.05$.
[b] Statistical significances compared with palmitic acid SLNs are $p < 0.05$.
[c] Statistical significances compared with stearic acid SLNs are $p < 0.05$.

lecithin PC and cholesterol encapsulated enrofloxacin had enhanced killing activity against S. *aureus* resided in neutrophils of dogs [10]. Unfortunately, the instability and low loading capacity of liposomes restrict its wide usages in veterinary clinic [1].

Solid lipid nanoparticles (SLN), due to their good biocompatibility, may be effective carriers to ferry antimicrobial agents into cells for enhancing intracellular efficacy. The saturated long carbon chain fatty acids, solid at room temperature, are available for pharmaceutical use and thus commonly used to prepare SLNs [11]. Our previous work has shown that docosanoic acid SLNs (DAS) can efficiently accumulate in cells and exhibit a more effective effect on intracellular Salmonella *CVCC541* than free drug [12]. Due to their discrepancy in carbon chain length, the fatty acids affect the *in vitro* release and pharmacokinetics of SLNs using various fatty acid as lipid matrix entrapped enrofloxacin in mice [11]. Probably because of the difference in hydrophobicity, the long-chain fatty acid SLN showed better results [26,27]. Therefore, it is speculated that the fatty acids may affect the intracellular delivery, accumulation, and antibacterial activity of the SLNs by using fatty acids as lipid matrix encapsulated drugs.

In this study, four fatty acid SLNs were prepared and evaluated for intracellular delivery, accumulation, and bactericidal activity of their encapsulated enrofloxacin to select the best one.

## 2. Materials and methods

### 2.1. Materials

Docosanoic acid (DAS), octadecanoic acid (OAS), hexadecanoic acid (HAS), and tetradecanoic acid (TAS)were purchased from Shanghai Aladdin Biochemical Polytron Technologies Inc. (Shanghai, China). Poly vinyl alcohol (PVA) and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-di-Phenyl-2H-tetrazolium bromide (MTT) were bought from Sigma Chemical Co., Ltd. (St Louis, MO, USA). RAW 264.7 cell line was offered from National Veterinary Drug Residues Reference Laboratory of Huazhong Agricultural University (Wuhan, China). Bisbenzimide H 33342 trihydrochloride (Hoechst 33342), and 1,1′-dioctadecyl-

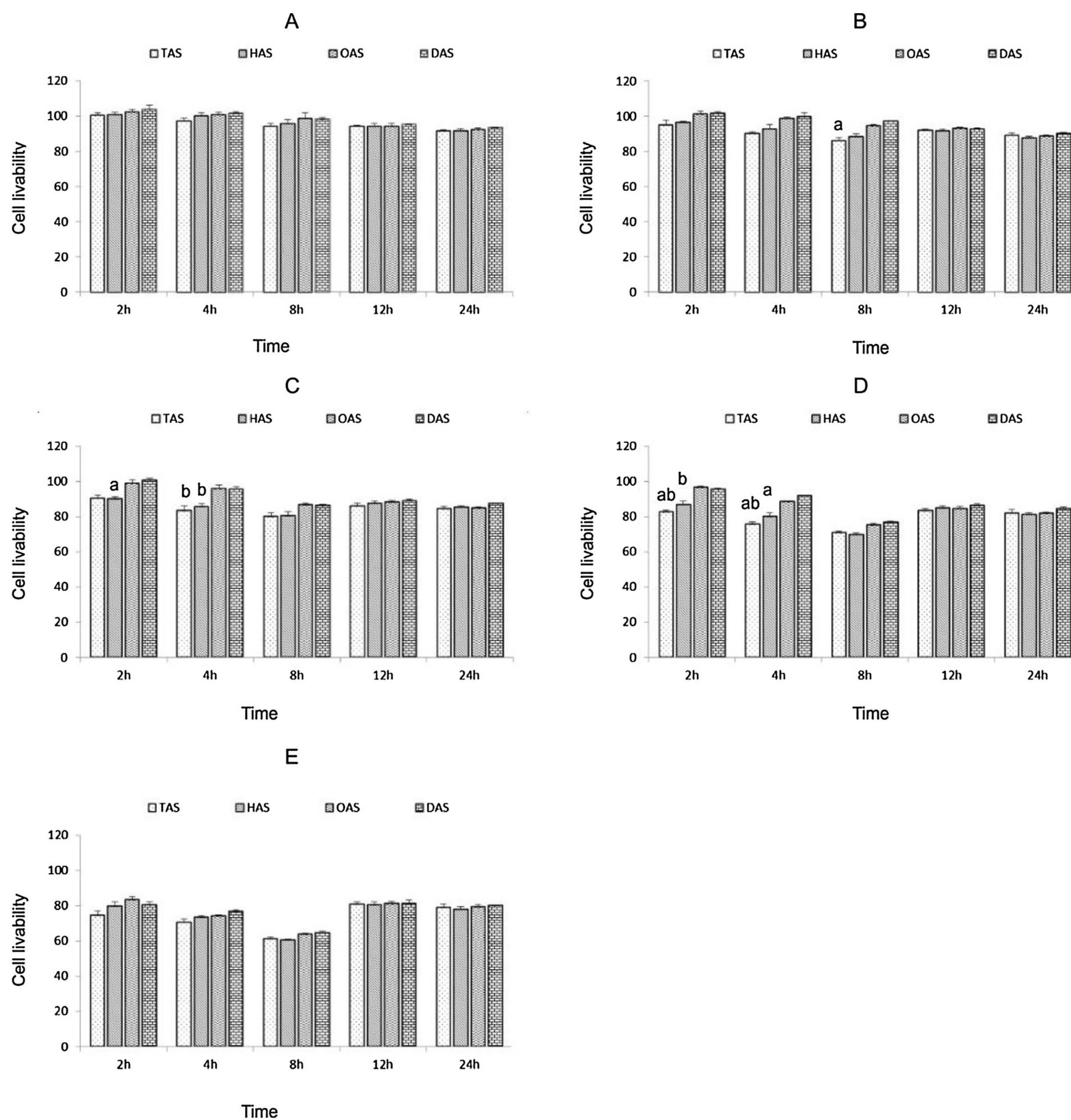


**Fig. 2.** Cytotoxicity of various fatty acid SLNs on the RAW264.7 cells.
A: 2.5 μg/mL SLNs encapsulated drug; B: 5 μg/mL SLNs encapsulated drug; C: 10 μg/mL SLNs encapsulated drug; D: 20 μg/mL SLNs encapsulated drug; E: 40 μg/mL SLNs encapsulated drug.
[a]Statistical significances compared with DAS are $p < 0.05$. [b]Statistical significances compared with OAS are $p < 0.05$.

3,3,3′,3′- tetramethylindocarbocyanine perchlorate (DiI) were obtained from Wuhan Merris Biotechnology Co., Ltd (Wuhan, China). Fluorecein isothiocyanate isomer I (FITC, molecular weight: 389.39) was obtained from Wuhan Saituo Baiao Biological Engineering Co., Ltd (Wuhan, China). Methyl alcohol and acetonitrile were purchased from Tedia (Ohio, USA). The water was prepared with a Milli-Q system (Millipore, Bedford, MA). Other chemicals and reagents used in this study were of highly analytical grade.

### *2.2. Formulation of enrofloxacin-loaded fatty acid SLNs*

The SLNs were prepared according to our previous work [11,12]. Briefly, 0.2 g of enrofloxacin weighted into a 50 mL centrifuge tube containing 1.8 g of 100 °C melted fatty acid. After the enrofloxacin was completely dissolved in the molten fatty acid, 20 mL of 2 % boiling PVA solution was poured and sonicated for 8 min using 3 mm microprobes with 78 W (VCX, Newtown, CT, USA) to prepare a nanosized emulsion. SLNs were collected by centrifugation at 27,700*g* (Hitachi Centrifugation CR21GIII; Hitachi Koki Co., Ltd., Japan) for 60 min and lyophilized for 48 h (Freeze Dry System; Labconco, America). The control nanoparticles were formulated in the same method without enrofloxacin.

The FITC-labeled nanoparticles were prepared in the same manner by adding 5 mg of fluorescein isothiocyanate (FITC) -labled octadecylamine (ODA-FITC) in 90 mg melted fatty acids. The ODA-FITC was prepared with reference to the preparation method of Yuan et al. [14]. Briefly, 10 mg of octadecylamine (ODA) and 20 mg of FITC were completely dissolved in 60 mL N,Ndimethylformamide (DMF) and shaked in water bath at 50 °C for 48 h. After complete reaction, 20 mL of distilled water was added to the mixture for precipitation of ODA-FITC, and then the precipitate was collected by filtration through a

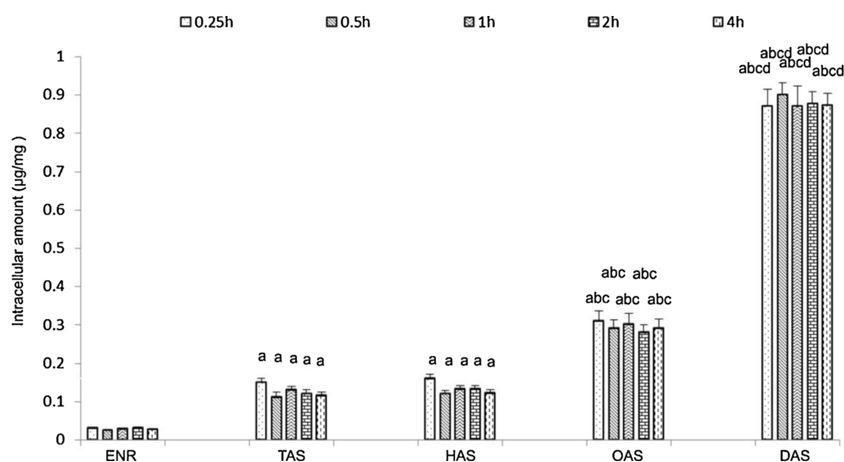


**Fig. 3.** The uptake of enrofloxacin in RAW264.7 cells. ENR: Free enrofloxacin; TAS: Tetradecanoic acid SLNs; HAS: Hexadecanoic acid SLNs; OAS: Octadecanoic acid SLNs; DAS: Docosanoic acid SLNs.
[a]Statistical significances compared with enrofloxacin are $p < 0.05$. [b]Statistical significances compared with TAS are $p < 0.05$. [c]Statistical significances compared with HAS are $p < 0.05$. [d]Statistical significances compared with OAS are $p < 0.05$. ($n = 3$).

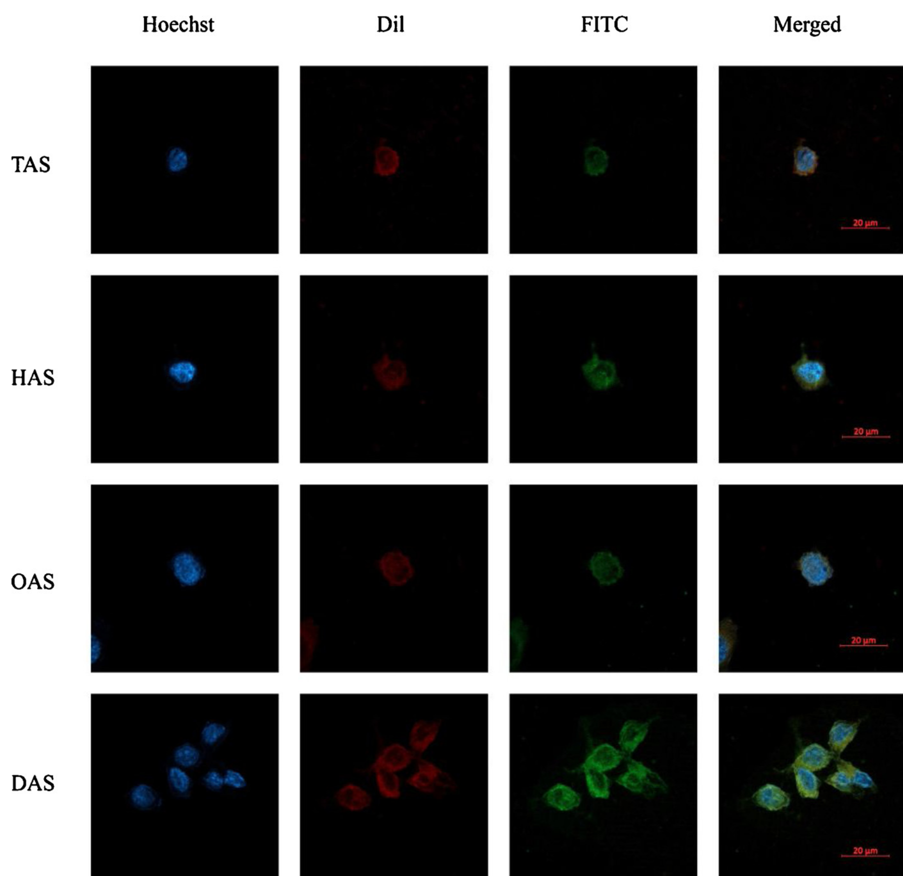


**Fig. 4.** Confocal fluorescence images of RAW 264.7 cells treated with FITC-labeled SLNs (green), where the Hochest nuclear stain was shown in blue and cell membrane was shown in red (DiI). TAS: Tetradecanoic acid SLNs; HAS: Hexadecanoic acid SLNs; OAS: Octadecanoic acid SLNs; DAS: Docosanoic acid SLNs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

0.45 m micropore filter.

### 2.3. Characterizations of SLNs

The encapsulation efficiency (EE) and loading capacity (LC) were detected using Waters 2695 series High Performance Liquid Chromatography (HPLC, Waters Corp., Milford, MA). The chromatographic column used was a ZORBAX SB-2 C18 column (250 × 4.6 mm, inner diameter: 5 μm; Agilent Technologies, USA). The mobile phase was acetonitrile and 0.1 % formic acid solution in a ratio of 14: 86. The zeta potential, polydispersity index (PDI), and size were determined by ZetasizerZX3600 (Malvern Instruments, UK). The samples were diluted 25 times in distilled water for obtaining the optimum kilo counts per second (kcps) of 20–400 for measurements as recommended by the manufacturer, to determine the size, PDI, and zeta potential. The morphology of nanoparticles prepared with various fatty acids was measured using an Agilent 5500 AFM (Agilent Technologies, AZ, USA) [12,13].

### 2.4. Cytotoxicity

Cytotoxicity of various fatty acid SLNs was evaluated by methyl thiazolyl tetrazolium (MTT) assay using RAW 264.7 cells. The $1 \times 10^4$ RAW 264.7cells were seeded onto a 96-well microplate. After incubation for 24 h, the medium containing various concentrations (2.5, 5, 10, 20, and 40 μg/mL) of the four-fatty acid SLNs encapsulated enrofloxacin was used to replace the primary medium. After co-incubation for fixed time, MTT solution was added into each well and then continued to incubate for 4 h. The formazan product, was dissolved in 150 μL dimethyl sulfoxide (DMSO) under constant shaking for 10 min

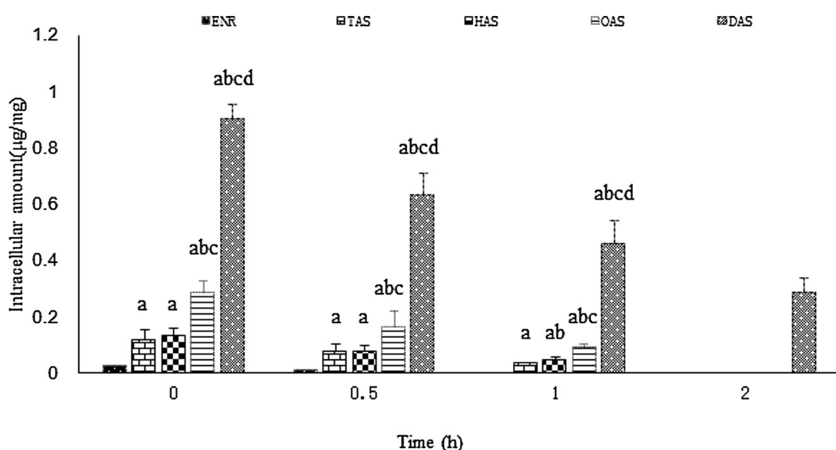


**Fig. 5.** The retention of enrofloxacin in RAW264.7 cells. ENR: Free enrofloxacin; TAS: Tetradecanoic acid SLNs; HAS: Hexadecanoic acid SLNs; OAS: Octadecanoic acid SLNs; DAS: Docosanoic acid SLNs. [a]Statistical significances compared with enrofloxacin are $p < 0.05$. [b]Statistical significances compared with TAS are $p < 0.05$. [c]Statistical significances compared with HAS are $p < 0.05$. [d]Statistical significances compared with OAS are $p < 0.05$.

after removing the culture medium and the absorbance was determined by a multiskan spectrum microplate reader (Elx800; Bio-tek instrument, Inc., USA) for the livability assay. The relative cell livability was calculated according to following equation [15].

$$\text{Relative cell livability}(\%)\frac{(\text{OD490, sample} - \text{OD490,blank})}{(\text{OD490, control} - \text{OD490,blank})} \times 100$$

### 2.5. Internalization and distribution of nanoparticles in cells

The cells were seeded at the density of $1 \times 10^5$ cells/cm$^2$ onto a 6-well cell culture plate. After 24 h, the cells were incubated with the same concentration of free enrofloxacin or four fatty acid SLNs encapsulated enrofloxacin (10 μg/mL) for 0.25, 0.5, 1, 2 and 4 h. After washing with cold PBS for three times, the adherent cells were lysed using 150 μL of RIPA cell lysate. The protein content and the concentration of enrofloxacin in cells were detected by the bicinchoninic acid (BCA) method and HPLC, respectively, as described in our previous study [12]. Briefly, the collected cells were sonicated for 30 s. Then, 5 μL cell lysate was taken for protein content determination by BCA method. The remainder of the cell lysate was deproteinized using 1 mL methanol under vortex mixing for 2 min and centrifugation at 23,700*g* for 15 min at 4℃. The supernatant was evaporated to dryness under a nitrogen evaporator. The concentrates were dissolved with 500 μL mobile phase and injected into HPLC vials for analysis.

Simultaneously, the cells of $1 \times 10^4$ were transferred into each dish of a confocal dish. After incubation for 24 h, the FITC-labeled fatty acid SLNs with same fluorescence intensity were transferred to the adherent cells and continued to incubate for 0.5 h. After washing twice with PBS, the cells were then stained with DiI and Hoechst 33342 for membrane and nuclear staining. The cells were visualized with a confocal laser scanning microscopy (CLSM, Zeiss, Germany).

### 2.6. Intracellular retention of fatty acid SLNs

The adherent RAW 264.7 cells in the 6-well culture plates were exposed to free and four fatty acid SLNs encapsulated enrofloxacin with equal concentration of 10 μg/mL for 30 min. The medium containing enrofloxacin was replaced with fresh blank medium, and incubation was done for 0.5, 1 and 2 h. Finally, concentration of enrofloxacin in the cells was determined by HPLC as described above.

Simultaneously, the cells of $1 \times 10^4$ were transferred into each dish of a confocal dish. After incubation for 24 h, the FITC-labeled fatty acid SLNs with same fluorescence intensity were transferred to the cells and incubate for 0.5 h. The old medium containing FITC-labeled fatty acid SLNs was replaced with fresh blank medium, and incubation was done for 0.25, 0.5, 1 and 2 h. After washing twice with PBS at each collection time point, the cells were then stained with DiI and Hoechst 33342 for membrane and nuclear staining. The cells were visualized with a confocal laser scanning microscopy (CLSM, Zeiss, Germany).

### 2.7. Determination of activity against Salmonella resided in RAW 264.7 cells

After incubation of $10^7$ CFU/mL *Salmonella CVCC541* with the adherent cells in 24-well culture plates for 1 h, the extracellular bacteria were removed and incubated with gentamicin at the concentration of 100 μg/mL for 0.5 h for killing the extracellular *Salmonella* [25]. After washing with 4 °C PBS, the cells were added with 1 mL 5 × MIC (0.3 μg/mL) free or four various fatty acid SLNs encapsulated enrofloxacin in each well, respectively. After 0, 4, 12, 24, and 48 h of incubation, the washed cells were lysed and spread on LB agar plates to determine the number of viable *Salmonella CVCC541* by colony counting. The time-kill curves were drawn by plotting average counts versus time.

### 2.8. Statistical analysis

The Data were showed in the form of mean ± S.D. The physicochemical properties and intracellular delivery differences of the four-fatty acid SLNs were analyzed by one-way analysis of variance (ANOVA). The *p*-value of 0.05 was considered as significant difference.

## 3. Results

### 3.1. The properties of fatty acid SLNs

Atomic force microscopy showed that all the four fatty acid SLNs were well dispersed with uniform quasi-spherical shape (Fig. 1). The order of EE and LC of the four fatty acid SLNs were DAS > OAS > HAS > TAS in the same preparation process (Table 1). Long carbon chain fatty acid tended to form larger size, whereas, the size of DAS had no significant difference as compared to the OAS (Table 1). The PDI of these four SLNs were not significantly different except the OAS. The zeta potential of SLNs was slightly enhanced with increase of length of carbon chain (Table 1). The properties of FITC-labeled nanoparticles were not significantly different as compared with unlabeled nanoparticles in terms of size, PDI and Zeta potential.

### 3.2. Cytotoxicity

The RAW 264.7 cells were used to determine the cytotoxic potential of four enrofloxacin-loaded fatty acid SLNs by co-incubating with 2.5–40 μg/mL SLNs encapsulated drug for 2 ~ 24 h (Fig. 2). The four fatty acid SLNs showed low cytotoxicity. The livability of RAW 264.7 cells treated with the four fatty acid SLNs was higher than 90 % at <

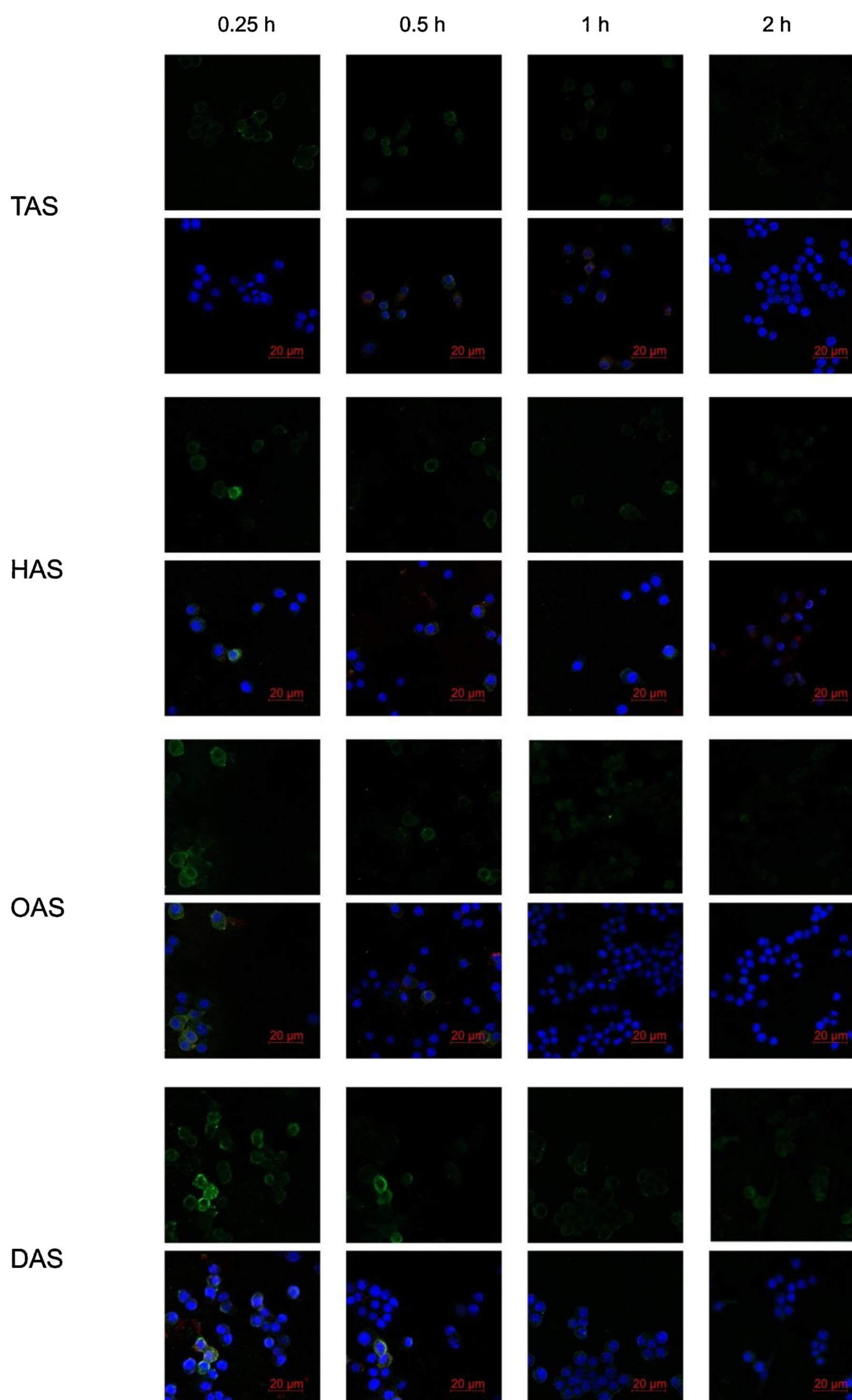


**Fig. 6.** After removing nanoparticles for 0.25,0.5, 1, 2 h, the confocal images of nanoparticles staying inside the cells,where the Hochest nuclear stain was shown in blue and FITC-labeled nanoparticle was shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

20 μg/mL concentrations during 12 h of incubation. However, concentration and time-dependent cytotoxicity was observed as the incubation doses and time increased continuously. The four fatty acid SLNs showed no significant differences in cytotoxicity.

### 3.3. Cellular uptake of four fatty acid SLNs in RAW 264.7 cells

The drug uptake in RAW 264.7 cells was evaluated by incubating with 10 μg/mL free or SLNs loaded enrofloxacin for 0.25, 0.5, 1, 2, and 4 h. The cellular uptake of enrofloxacin was enhanced by all the fatty acid SLNs (Fig. 3). Free, TAS, HAS, and OAS encapsulated enrofloxacin reached to maximum intracellular concentrations of 0.03, 0.15, 0.16, and 0.31 μg/mg protein within the first 0.25 h, while DAS encapsulated enrofloxacin continued to enhance, reaching the maximum intracellular amount of 0.87 μg/mg proteins at 0.5 h. After 4 h of incubation, TAS, HAS, OAS, and DAS incorporated enrofloxacin accumulated in macrophages approximately 5.0, 5.3, 10.3 and 29.0-folds higher than the free drug,

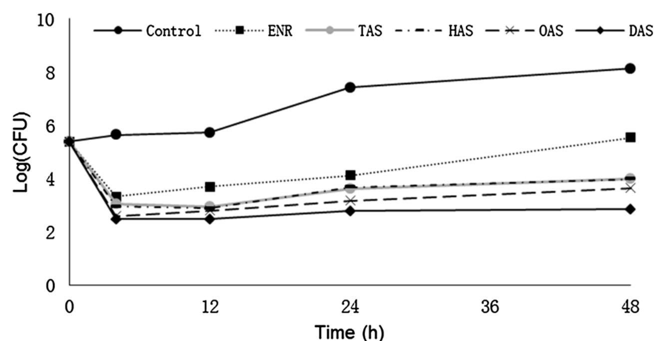


**Fig. 7.** Sterilization curve of enrofloxacin and four fatty acid SLNs entrapped enrofloxacin against intracellular *salmonella CVCC541*. Control: DMEM; ENR: Free enrofloxacin; TAS: Tetradecanoic acid SLNs; HAS: Hexadecanoic acid SLNs; OAS: Octadecanoic acid SLNs; DAS: Docosanoic acid SLNs.

respectively. These results demonstrated that the longer fatty acid formulated nanoparticles could enhance the intracellular enrofloxacin accumulation more significantly.

The confocal microscopy observation demonstrated that higher amount of longer fatty acid FITC-labeled SLNs were entered in the cells after 0.5 h of incubation (Fig. 4). The labeled green fluorescence of longer fatty acid SLNs mainly distributed around the perinucleus, which indicated that the SLNs with longer fatty acid are more easily internalized into RAW 264.7 cells, especially for DAS. Particularly, yellow dots overlaps of green fluorescence of nanoparticles and the red fluorescence of RAW 264.7cell membrane were observed for TAS prepared by the shortest carbon chain of fatty acid, suggesting large TAS absorbed on the cell surface.

### *3.4. Intracellular retention of fatty acid SLNs*

The intracellular retention performance of SLNs loaded enrofloxacin in RAW 264.7 cells was stronger than free drug and influenced by fatty acid (Fig. 5). After removing extracellular drug for 0.5 h, the concentrations of free, TAS, HAS, OAS and DAS encapsulated enrofloxacin in RAW 264.7 cells were decreased from 0.03, 0.12, 0.14, 0.30, and 0.93 μg/mg proteins to 0.01, 0.09, 0.08, 0.17, and 0.64 μg/mg proteins, respectively. After continuous incubation for 1 h, the intracellular concentration of free, TAS, HAS, OAS, and DAS loaded enrofloxacin were decreased by 80.66 %, 68.03 %, 61.79 %, 66.49 % and 50.03 %, respectively. Following 2 h of clearing extracellular drug, the intracellular drug in the DAS treatment was still maintained at 0.2897 μg/mg, whereas the other three fatty acid nanoparticles encapsulated enrofloxacin were not detectable in cells. The results indicate that the nanoparticles loaded enrofloxacin accumulated in the cells for a longer time, especially for the DAS loaded enrofloxacin.

Confocal microscopy observations showed that when extracellular nanoparticles were removed, the nanoparticles in cells were gradually decreased during 2 h (Fig. 6). After 2 h of removing extracellular drug, the intracellular green fluorescence of DAS and OAS were still observed, while the green fluorescence of TAS and HAS in the cells were not founded. Moreover, the intracellular green fluorescence was much stronger when incubating with DAS as compared with OAS at all the determined time after removing extracellular SLNs. Consequently, these results showed more persistent resident time of longer fatty acid prepared SLN, while DAS have highest resident time in cells.

### *3.5. Activity of fatty acid SLNs loaded enrofloxacin against intracellular Salmonella*

All four enrofloxacin-loaded fatty acid SLNs exhibited significantly stronger bactericidal activity against *Salmonella CVCC541* resided in cells than free enrofloxacin at all the determined time points (Fig. 7). The intracellular antimicrobial efficacy of the fatty acid nanoparticles loaded enrofloxacin was enhanced significantly than free enrofloxacin with respect to increase of carbon chain length and treatment time. After incubation of 4 h, the viable colonies of *Salmonella CVCC541* obtained from the control, free enrofloxacin, and TAS, HAS, OAS, and DAS treated cultures were $4.47 \times 10^5$, $2.03 \times 10^3$, $1.10 \times 10^3$, $8.91 \times 10^2$, $3.98 \times 10^2$, and $3.01 \times 10^2$ CFU/mL, respectively. As the incubation time increased, the bacteria in RAW 264.7 cells were hardly recovered when treated with DAS, while slowly regrew when treated with TAS, HAS, and OAS as compared with free enrofloxacin where growth was more significantly rapid. After 48 h, the viable bacterial colonies in cells treated with TAS, HAS, OAS and DAS were reduced by 95.83 %, 96.04 %, 98.12 % and 99.71 %, respectively, while the bacteria treated with free enrofloxacin were increased by 145.83 %.

## 4. Discussions

In order to reduce the efflux and increase intracellular retention time, four enrofloxacin-loaded fatty acid SLNs were prepared and then their intracellular delivery performance was compared. The LC and EE of SLN were increased with increase in fatty acid carbon chain length due to stronger hydrophobicity of fatty acids that leads to enhance containment of lipophilic drugs [16,17]. Therefore, because of the principle of similarity and compatibility, enrofloxacin is easily soluble in molten longer chain fatty acids. The cytotoxicity of fatty acid SLNs was assessed by MTT assay before cellular uptake and intracellular accumulation experiment for the selection of rational incubation dosage and time. All four fatty acid formulated nanoparticles showed good cytocompatibility when incubating for 12 h at the concentration below 20 μg/mL, which indicated that the incubation concentration of 10 μg/mL will not affect the normal growth of RAW 264.7 cells. All the four fatty acid SLNs could more efficiently delivery their loaded drug into RAW 264.7 cells than native drug and reached to maximum level after incubation for 0.25 or 0.5 h. The intracellular delivery rate was significantly faster than that of glycerin monostearate SLNs, which reached a plateau after 4 h of incubation [14]. This phenomenon suggests that fatty acid SLNs might be more effective delivery system for intracellular drug delivery. In order to prove that SLNs loaded drugs enter in the cell rather than adsorb on the surface, the FITC-labeled four fatty acid SLNs were prepared. The cellular uptake and intracellular retention performance of SLNs were improved as the carbon chain length of fatty acids increased. It was interesting that the enhanced intracellular delivery and retention of loaded enrofloxacin was more significant in case of DAS as compared to other three fatty acid nanoparticles. All SLNs except DAS eliminated after 2 h by removing extracellular drug which showed high intracellular retention capability of DAS. Reix et al. Found that poly(lactide-co-glycolic acid) nanoparticles in Caco-2 cells were almost completely exocytosed after 2 h [30]. In a study, when polylactic acid-glycolic acid nanoparticles incubated with mesothelial cells, the intracellular nanoparticles decreased upto 80 % after removing the nanoparticles for 3 h [31]. The intracellular delivery of DAS is stronger than those of liposomal enrofloxacin [7]. Apart from enhanced intracellular delivery, FITC-labeled longer fatty acid formulated SLNs mainly distributed around the perinucleus, while shorter fatty acid formulated SLNs mostly adsorbed on the cell surface. These results indicated that the length of fatty acids also influence their intracellular delivery, distribution, and accumulation. This might be primarily due to the higher lipophilicity of long chain fatty acids. Higher lipophilicity of long-chain fatty acids might enhance the interaction of SLNs with cell membrane phospholipids or the adhesion of the SLNs to the cell membrane and thus enhance the phagocytosis of nanoparticles by macrophages [18,29].

In addition, due to the strong hydrophobicity, the relatively long hydrocarbon chain results in stronger affinity of enrofloxacin with nanoparticles which lead to prolong drug release (Reference?). Our results also demonstrated that DAS has highest drug loading, which may result

in more drug accumulation in the cells. In most of the cases, the phagocytic rate of polymer nanoparticles and liposomes increases with the particle size (200–1000 nm) [1,19–21]. Studies have found that the surface charge of nanoparticles affects their cellular uptake [22,23]. Our previous studies have also shown that larger size SLNs have greater cellular uptake [12]. Although size and zeta potential are important factors influencing the uptake of nanoparticles, the difference in size and zeta potential of SLNs formed by fatty acids is insignificant. Therefore, the cellular uptake efficacy of various SLNs might be influence by the length of carbon chain fatty acids. The significant intracellular delivery and accumulation discrepancy of all four fatty acid SLNs might be due to different cellular internalized routes. The exact mechanism and decisive factors of longer fatty acid nanoparticles, especially for DAS, in mediating higher cellular delivery efficacy of encapsulated enrofloxacin will be studied in future.

Although, the enhanced cellular uptake of loaded antibiotics might lead to more efficient intracellular bactericidal activity in most condition, but it is not true if drug has poor release or distribution of the nanoparticles in the cells. For example, the chloramphenicol-loaded chondroitin sulfate nanoparticles were less than the free drug for killing of *S. Paratyphi* resided in human whole blood and RAW 264.7 macrophage cells [24]. Therefore, antimicrobial efficacy of these fatty acid SLNs against intracellular bacteria was determined. The RAW 264.7 macrophage cells infected with *Salmonella CVCC541* were treated with free and four fatty acid SLNs encapsulated enrofloxacin. After 4 h, all the four SLNs proved to be more efficient than free enrofloxacin against *Salmonella CVCC541*. The amount of Salmonella in RAW 264.7 cells when incubated with chloramphenicol loaded chondroitin sulfate nanoparticles for 6 h in the cells was decreased significantly [24]. In a previous study, fucoidan coated ciprofloxacin loaded chitosan nanoparticles started to decrease intracellular Salmonella after 6 h of incubation with RAW 264.7 cells [28]. The bactericidal activity of SLNs encapsulated drug was more significant as the carbon chain of fatty acid and the incubation time were increased. The enhanced bactericidal activity was probably due to the enough release and intracellular retention of fatty acid SLNs loaded drug into the *Salmonella*-containing vacuole. It is more interesting that DAS could completely inhibit the growth of bacteria resided in the intracellular compartments of the macrophages. These results suggested that the enrofloxacin-loaded fatty acid SLNs, especially DAS, could be a promising effective approach to overcome the treatment difficulty in intracellular bacterial infections.

## 5. Conclusion

Fatty acid SLNs can significantly enhance the cellular uptake, intracellular distribution and accumulation of encapsulated enrofloxacin, thus exhibited more therapeutic effect against intracellular *Salmonella CVCC541*. The DAS, HAS, and OAS mainly distributed in cytoplasm, while TAS mostly adsorbed on the cytomembrane. The intracellular delivery and antibacterial efficacy significantly improved with increase of fatty acid chain length. The enrofloxacin-loaded DAS might be one of the most attractive formulation for intracellular bacterial infection treatment due to the best loading capacity, intracellular delivery, accumulation and antibacterial efficacy.

## CRediT authorship contribution statement

**Kuiyu Meng:** Conceptualization, Methodology, Software. **Dongmei Chen:** Supervision. **Fei Yang:** Data curation, Writing - original draft, Software, Validation. **Aoxue Zhang:** Investigation. **Yanfei Tao:** Supervision. **Wei Qu:** Supervision. **Yuanhu Pan:** Supervision. **Haihong Hao:** Supervision. **Shuyu Xie:** Writing - review & editing.

## Declaration of Competing Interest

The authors have declared no conflict of interest.

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