

2018

Development and In Vitro Release of Isoniazid and Rifampicin-Loaded Bovine Serum Albumin Nanoparticles

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Ge, Zhaohui; Ma, Rong; Xu, Guangxian; Chen, Zhen; Zhang, Dangfeng; Wang, Qian; Hei, Long; and Ma, Wei, "Development and In Vitro Release of Isoniazid and Rifampicin-Loaded Bovine Serum Albumin Nanoparticles" (2018). *School of Information Systems and Management Faculty Publications*. 26. https://digitalcommons.usf.edu/qmb_facpub/26

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Received: 2017.05.31
Accepted: 2017.07.19
Published: 2018.01.24

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Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support:

National Natural Science Foundation of China (30960391) and the Natural Science Foundation of Ningxia Hui Autonomous Region (NZ13144)

Background: Bovine serum albumin nanoparticles loaded with isoniazid and rifampicin (INH-RFP-BSA-NPs) were prepared and their release characteristics were studied *in vitro*.





Material/Methods: The INH-RFP-BSA-NPs were prepared by a modified self-emulsion solvent diffusion method, with albumin and polylactic acid used as carriers and to form the nanoparticles structure. Transmission electron microscopy was used to observe the morphology of the INH-RFP-BSA-NPs. The size distribution of the INH-RFP-BSA-NPs were assessed using a submicron particle-size analyzer for drug loadings, and the coating rate of the INH-RFP-BSA-NPs was measured by high-performance liquid chromatography. A dynamic membrane dialysis method was used to study the *in vitro* release characteristics of the INH-RFP-BSA-NPs.

Results: The INH-RFP-BSA-NPs were smooth, sphere-like, relatively uniform in size, and well-dispersed, and the average diameter was 60.5 ± 4.6 nm. Drug loading and entrapment efficiencies were high, at 19.8% and 87.8% for isoniazid, respectively, and 20.1% and 98.0% for rifampicin, respectively. Drug release was slow and sustained with 97.02% INH cumulative release at 6 days, and full release of RFP requiring 5 days.

Conclusions: INH-RFP-BSA-NPs exhibit uniform NP diameter, good dispersion, high drug loading and encapsulation rates, and have sustained release properties.

MeSH Keywords: **Albumins • In Vitro • Isoniazid • Nanoparticles**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/905581>

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Background

Isoniazid (INH) and rifampicin (RFP) are primary first-line drugs for treating tuberculosis. The structure and pharmacokinetic characteristics of INH and RFP in their conventional formulation means they are rarely used in regions with endemic disease as it is difficult to maintain an effective bactericidal concentration of these drugs *in vivo*, which is the main factor leading to ineffective chemotherapy, delayed recovery, and relapse of extrapulmonary tuberculosis diseases, especially spinal tuberculosis [1,2]. In addition, both INH and RFP in their conventional formulations are associated with hepatic and renal toxicity, which further contributes to poor compliance and poor therapeutic effects. Developing novel anti-tuberculosis drug formulations that have sustained-release action, targeted delivery, high efficiency, and low toxicity will help increase the use of these simple drugs to rapidly treat tuberculosis, with high efficacy and reduced toxic side effects, and will potentially increase patient compliance. In this study, we used bovine serum albumin (BSA) and polylactic acid (PLA) as co-carriers to prepare novel INH- and RFP-loaded nanoparticles (NPs). The pharmaceutical characteristics and the *in vitro* drug release properties of these NPs were investigated to provide evidence for the pharmacokinetics *in vivo* in animal models of tuberculosis.

Material and Methods

Primary reagents and materials

The following reagents and materials were used: bovine serum albumin (BSA) (Amresco, Solon, OH, USA); polylactic acid (PLA, MW20000, Shandong Institute of Medical Instrument, China); INH, RFP (Wuhan Fuchi Biotechnology Co., Ltd., China); Tween 80 (Amresco); INH and RFP standard preparation (National Institutes for Food and Drug Control, approval No. 100578-200401; 110757-200206); bag filter (MD77,8000-14000, Beijing Solarbio Science and Technology Co., Ltd., China); trehalose (Amresco); mannitol (Shanghai Kaiyang Biotechnology Co., Ltd., China); and chromatographically pure methanol and methylene cyanide. Other reagents were analytically pure.

Primary instruments

The primary instruments used were: a constant-temperature magnetic stirring apparatus (Jiangsu Jintan Kexie Instrument Co., Ltd., China); H-600-4 (Hitachi, Japan); a NICOMP™380 sub-micron particle-size analyzer (Amresco, Solon, OH, USA); a high-performance liquid chromatography system (Agilent1100, Agilent Technologies, USA) equipped with quaternary gradient pump, diode array detector, automatic degasser, automatic sampler, column temperature compartment, and Agilent 1100 chromatographic work station; an XH-B laboratory digital vortex mixer;

an LL-3300 lyophilizer (Thermo Electron Corporation, USA); an HPD-25 diaphragm vacuum pump (Tianjin Hengao Science and Technology Co., Ltd., China); a pH5-3B precise pH meter (Shanghai Leichi Instrument, China); a 1/100000 electronic analytical balance (Mettler Toledo, Swiss); a thermostatic shaker incubator (Jiangsu Jintan Yitong Co., Ltd., China); and 1-ml disposable syringes (Shandong Pharmaceutical Glass Co., Ltd., China).

Methods

Preparation of INH-RFP-BSA-NPs

An aqueous phase was prepared by thoroughly dissolving 0.4 g of INH (Wuhan Fuchi Biotechnology Co. Ltd., Wuhan, China) and 1.0 g of BSA (Amresco, Solon, OH, USA) in 200 ml of purified water at room temperature using ultrasound. This aqueous phase mixture was divided equally into four 50-ml portions. The organic phase was prepared by first fully dissolving (with agitation) 0.16 g of PLA (MW20000, Shandong Institute of Medical Instruments, Shandong, China) in 34 ml of acetone and then adding 0.42 g of RFP (Wuhan Fuchi Biotechnology Co. Ltd.). We dissolved 400 µl of Tween-80 (Amresco) in 34 ml of dehydrated alcohol at room temperature, and added it to the PLA/RFP solution.

Magnetic stirring for longer than 10 min was used to mix 17 ml of the organic-phase mixture into 50 ml of the aqueous-phase mixture. This mixture was placed in the dark at room temperature for 24 h to allow complete volatilization of the organic solvent until a semi-transparent red NP solution resulted. To this, 3% (w/v) trehalose (Amresco) and 2% (w/v) mannitol (Shanghai Kaiyang Biotechnology Co. Ltd.) were added and the mixture was freeze-dried at between -45°C and -5°C. This produced a BSA NP powder with good dispersion performance.

Chromatographic analysis of INH-RFP-BSA-NPs

INH was analyzed using a ZORBAX SB-C18 chromatographic column (5 µm, 4.6×250 mm; Agilent1100, Agilent Technologies, Palo Alto, CA, USA) with a SB-C18 (5 µm, 4.6×12.5 mm) precolumn. The column temperature was 25°C. The mobile phase was 0.02 mol/l of potassium dihydrogen phosphate buffer solution (pH 6.0): methanol: acetonitrile at a ratio of 90: 5: 5. The flow rate was 0.9 ml/min, the wavelength was 264 nm, and the sample volume was 20 µl.

For RFP chromatography, a ZORBAX XDB-C18 chromatographic column (5 µm, 4.6×150 mm) and a XDB-C18 precolumn (5 µm, 4.6 mm × 12.5 mm) were used. The column temperature was 35°C, the mobile phase was 0.02 mol/l of sodium dihydrogen phosphate buffer solution (pH 7.0): methanol (30: 70), the flow rate was 1.0 ml/min, the wavelength was 330 nm, and the sample volume was 20 µl.

Establishment of standard curves and the regression equation

INH control solution was diluted using water to give a dilution series between 2 and 200 µg/ml. Taking the peak area of INH as the x-axis and the mass concentration of INH as the y-axis, a linear regression equation was acquired: $Y=20.504X-0.310$, $r=0.9999$, with regression rates of 100.03%, 98.90%, and 99.25% corresponding to a high, middle, and low concentrations of INH, respectively. The precision (intra-day/inter-day) expressed as the percentage relative standard deviation (RSD) was <1.0%.

RFP control solution was diluted using methanol to give a series of solutions between 1.44 and 144 µg/ml. Taking the peak area of RFP as the x-axis and the mass concentration of RFP as the y-axis, a linear regression equation was acquired: $Y=8.498X+0.307$, $r=0.9999$, with regression rates of 100.10%, 100.03%, and 100.33% corresponding to high, middle, and low concentrations of RFP, respectively. The RSD was <1.0%.

Using the same method as described above, blank NPs (without INH or RFP) were prepared. The freeze-dried powder was dissolved in the chromatography mobile phase and analyzed under the conditions described above. The blank NPs did not produce a peak on the test chromatogram. Test and control samples were all 10 µl. The time of the main peak on the chromatogram for the tested sample was consistent with that on the chromatogram for the control sample; the blank adjuvant did not interfere with the main peak under the chromatographic conditions used.

Stability testing

BSA NPs were stored in the refrigerator at 4°C in the dark for 1, 3, 5, 7, 10, 15, and 20 days. Samples were tested for stability using the chromatographic conditions detailed above, and the peak area score was calculated. The RSD for both INH and RFP was <1.5%. These findings suggest that BSA NPs preserved at 4°C in the dark for 20 days were stable.

INH-RFP-BSA-NPs morphology

A transmission electron microscope was used to observe the morphology (Hitachi, Tokyo, Japan) and analyze the diameter of the INH-RFP-BSA-NPs. These particles were photographed and their data recorded by a laser diffraction NICOMP™380 submicron particle-size analyzer (Amresco).

The amount of drug loading and the encapsulation rate was determined using a previously described dialysis method [3,4]. As only a small amount of RFP dissolves in water, once the organic solvent in the NP solution had volatilized completely, the NP solution was filtered using a 0.22-µm microporous membrane

(Xiboshi, CA, USA). The nonencapsulated RFP was separated from the NP carrier and then eluted with methanol. The RFP content was determined using high-performance liquid chromatography and the concentration of dissociated drug was calculated and introduced into the following equation [4] to calculate the RFP loading amount and the encapsulation rate:

$$\text{RFP loading amount} = \frac{Wt - CsVs}{Wc} \times 100\%,$$

$$\text{RFP encapsulation rate} = \frac{Wt - CsVs}{Wa} \times 100\%,$$

where Wt indicates total drug amount, Cs is the drug concentration in the supernatant (or dialysate, eluent, including washing solution), Vs is the volume of supernatant, CsVs is the amount of dissociated drug, Wc is the mass of drug-loaded NPs (total mass of drugs and carriers), and Wa is the total drug amount.

In vitro drug release: observation and parameter determination

A 3-ml aliquot of prepared BSA was transferred to a 10-ml bag filter pre-treated by boiling. Both ends of the bag filter were fastened, and then the whole bag filter was placed in a conical flask with 150-ml phosphate-buffered saline (pH 7.4) and shaken at 100 r/min at 37±1°C in a thermostatic shaking incubator for 120 h until metabolism was complete. A 3-ml sample was withdrawn at 1, 2, 3, 5, 7, 9, 10, 12, 24, 36, 48, 72, 96, and 120 h, and 3 ml of phosphate-buffered saline was added to replace the volume removed at each sampling point. The mass concentration of drug at each time point was calculated using high-performance liquid chromatography, and the amount and percentage of drugs released was calculated. *In vitro* drug release curves were created with time as the x-axis and the percentage of drugs released as the y-axis.

Results

Morphology and diameter of INH-RFP-BSA-NPs

BSA NPs were red and the freeze-dried powder had good dispersion performance (Figure 1). Viewed through a transmission electron microscope, the BSA NPs had a smooth round surface and each NP was independent and uniform in size (Figure 2). The diameters of the BSA NPs, as determined by laser diffraction particle size analyzer, ranged between 30 and 110 nm (60.5±4.6 nm).

Determination of drug loading in INH-RFP-BSA-NPs

The INH and RFP loading in the INH-RFP-BSA-NPs was 19.8% and 20.1%, respectively, with an encapsulation rate of 87.8%

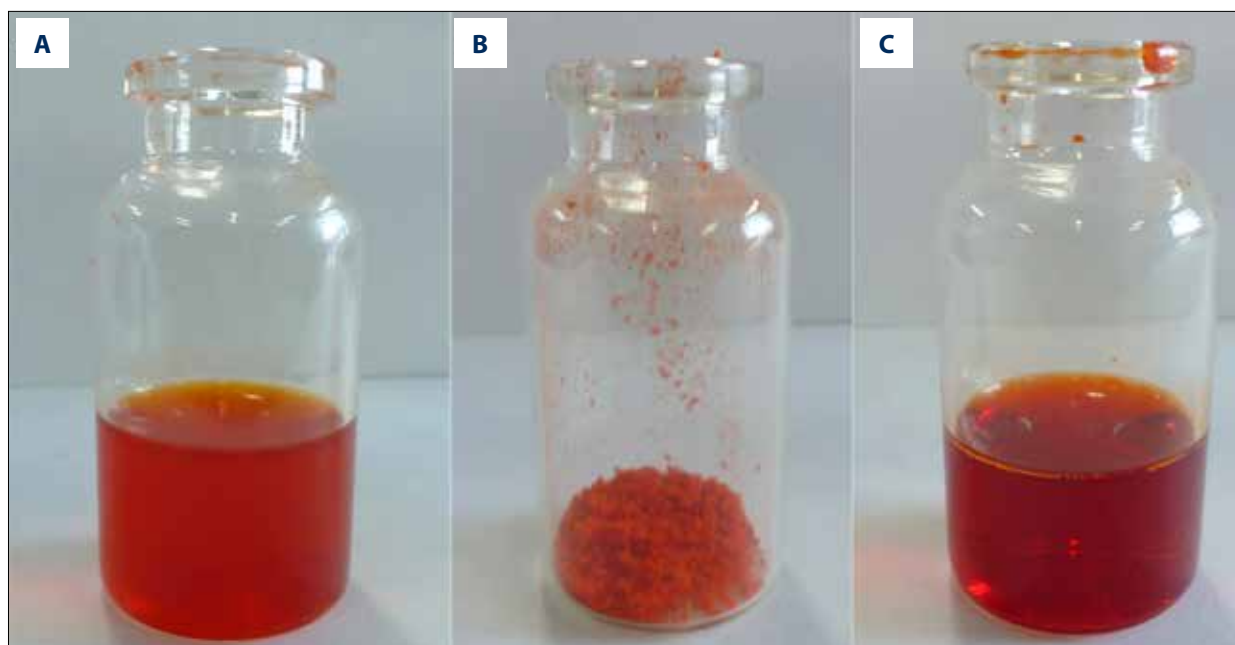


Figure 1. Appearance of bovine serum albumin nanoparticles. (A) Synthesized bovine serum albumin nanoparticle solution. (B) Freeze-dried bovine serum albumin nanoparticle powder. (C) Re-dissolved freeze-dried powder.

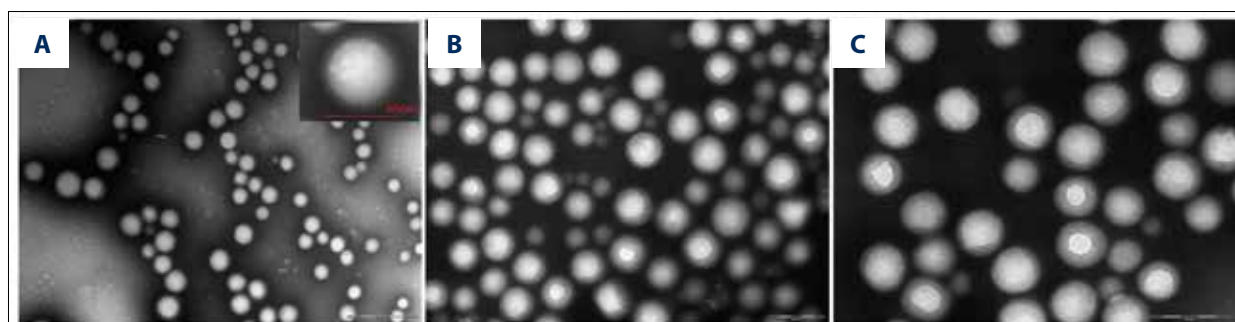


Figure 2. Ultrastructure of bovine serum albumin nanoparticles viewed with a transmission electron microscope at different magnifications. (A) $\times 10,000$. (B) $\times 20,000$. (C) $\times 30,000$.

and 98.0%, respectively. The chromatograms of standard INH and RFP solution samples are shown in Figure 3.

Detection of *in vitro* cumulative release of INH and RFP from NPs

By day 6, 97.02% of INH had been released from the INH-RFP-BSA-NPs and by day 5, 100% of RFP was released, showing that the INH-RFP-BSA-NPs had good *in vitro* sustained release effects (Figure 4).

Discussion

Conventional-formulation anti-tuberculosis drugs have limited effectiveness therapeutically due to their structure and pharmacokinetic characteristics. Studies to change the formulation of

conventional anti-tuberculosis drugs to improve their therapeutic effects have been performed [5,6]. Rat studies have shown that anti-tuberculosis drugs with microspheres and liposomes as carriers can selectively concentrate in affected tissue, the lung and the brain, with effective mycobacterium tuberculosis-killing effects coupled with low toxicity [7,8]. Nanoparticles, with diameters less than those of either microspheres or liposomes, can protect the loaded drugs from degradation and deactivation. They can also provide sustained-release, controlled-release, and targeted-release of drugs, thus greatly increasing the therapeutic effects of the drugs and decreasing toxic and adverse reactions [9,10]. Sharma et al. [11] found that wheat germ agglutinin poly(lactic-co-glycolic acid; PLGA) NPs can attach to the pulmonary alveolar membrane of the respiratory tract. The ability of wheat germ agglutinin PLGA NPs administered once every other week to kill *Mycobacterium tuberculosis* was equivalent to taking the original nanosphere preparation

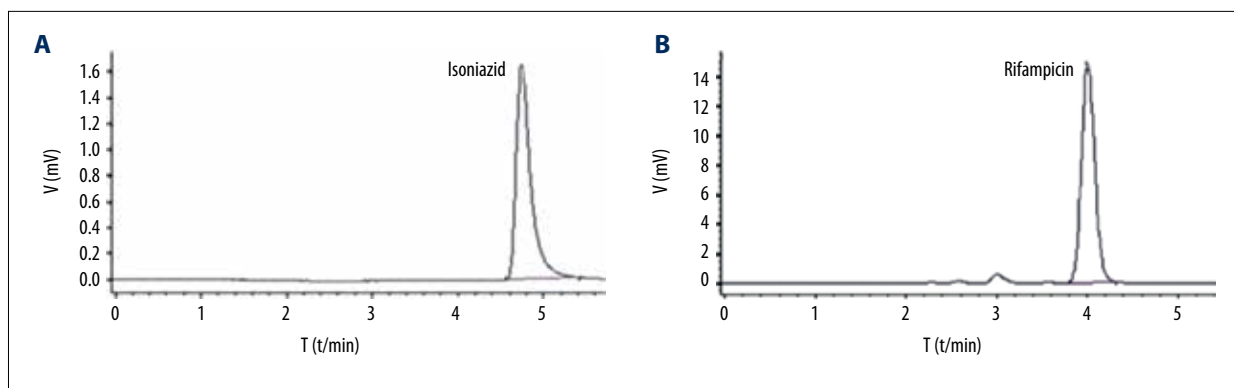


Figure 3. High-performance liquid chromatograms of standard isoniazid (INH) and rifampicin (RFP) solutions, (A) standard INH solution; (B) standard RFP solution.

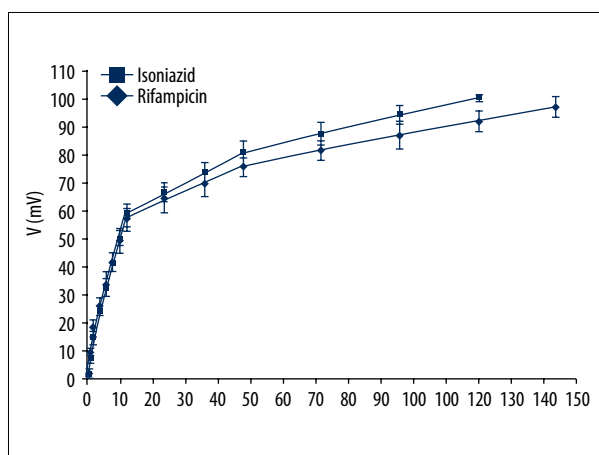


Figure 4. *In vitro* release of isoniazid (INH: ■) and rifampicin (RFP: ◆) from INH and RFP-loaded bovine serum albumin nanoparticles (INH-RFP-BSA-NPs) into culture medium.

of the drugs orally for 45 days. Ahmad et al. [12] reported that INH and RFP-loaded alginate-chitosan NPs administered once via atomized inhalation maintained the concentration of INH and RFP above the lowest bacteria-killing concentration in the lung, liver, and spleen of guinea pigs for 15 days. The mean diameter of the drug-loaded NPs prepared in this study was less than 150 nm, which meets the requirement of *in vivo* intravenous administration. *In vitro* studies have shown that the time for *in vitro* cumulative release of INF was more than 144 h, and for RFP it was over 120 h, which were greatly superior to the *in vitro* release performance of the oral formulation. This confirms that NPs prepared from tuberculosis-treatment drugs have sustained release properties.

The diameter of drug-loaded NPs can vary greatly because of different carriers and preparation methods. Chuan et al. [13] prepared RFP-loaded solid liposome NPs with a mean diameter of 812.2 nm using a film dispersion method. Pandey et al. [14] prepared RFP, INH, and PLA pyrazinamide-glycolic acid (PLG)

NPs using multiple-emulsion embedding technology, with 80% of the NPs having a diameter of 186–290 nm. Particles with diameters less than 5 μm are easily captured by the pulmonary capillary bed and phagocytosed by reticuloendothelial cells, while NPs with diameters less than 150 nm can be target-distributed in the bone marrow, exhibiting the ability to pass through the bone marrow-blood barrier [15,16]. The NPs referenced above had a high encapsulation rate, but no ability to pass through the bone marrow-blood barrier because of their relatively large diameter, and so were not suitable for treating tuberculosis of the bones and joints. In the present study, using a modified self-emulsification-solvent diffusion method [17], we prepared INF and RFP-loaded BSA NPs with particle diameters of 60.5 ± 4.6 nm, specifically to pass through the bone marrow-blood barrier, to allow targeted treatment of spinal tuberculosis using drug-loaded NPs in the future.

A successful drug-loaded NP should contain a high drug load and encapsulation rate and the preparation technique should be optimized. Cai et al. [18] used a modified self-emulsification-solvent diffusion method to prepare RFP-loaded PLGA NPs, with a diameter of 128 nm, an encapsulation rate of 65.84%, and RFP loading of 3.78%. Although RFP had been well-encapsulated, the NP had a low drug loading and therefore could not meet the requirements of treatment. Based on our previous study [19], we used BSA and PLA as co-carriers, acetone (mixed with water) and dehydrated alcohol as the organic phase, and adjusted the order of addition of acetone and dehydrated alcohol. Polylactic acid was first dissolved in acetone, then the RFP was added according to the solubility (less than 14 mg/ml) of RFP in acetone. In addition, to improve the surface property of the NPs and to increase the loading of undissolved RFP, 0.1% (v/v) Tween-80 was added to dehydrated alcohol, and the final mixture of dehydrated alcohol and Tween-80 was added to acetone. The INH-RFP-BSA-NPs prepared by this technique have diameters of 60.5 ± 4.6 nm, with INH and RFP loading rates of 19.8% and 20.1%, respectively and INH and RFP encapsulation rates of 87.8% and

98%, respectively, which were superior to those reported in other studies [20,21].

In the present study, we used BSA and PLA as co-carriers because: (1) the amino acids in the BSA connect together via peptide bonds, which twist together and provide ramified spacing, creating beneficial conditions for setting and carrying drugs [22]; (2) BSA exhibits great loading capacity for hydrophilic drugs [23]; and (3) PLA is suitable as a carrier material due to its low toxicity, low immunogenicity, good biodegradation, and biocompatibility. Our findings confirm that a combination of BSA and PLA enables loading and embedding of 2 first-line anti-tuberculosis drugs with completely different polarity.

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Conclusions

INH-RFP-BSA-NPs, prepared using this simple method exhibit uniform NP diameter, good dispersion, and high drug loading and encapsulation rates. This provides the basic conditions for *in vitro* sustained release of small-diameter particles, laying the foundation for further study of pharmacokinetic data and targeted treatment in experimental animals with tuberculosis.

Conflicts of interest

None.