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RESEARCH ARTICLE

Preparation and evaluation of amphipathic lipopeptide-loaded PLGA microspheres as sustained-release system for AIDS prevention

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Abstract

At present, AIDS drugs are typical inhibitors that cannot achieve permanent effects. Therefore, the research of blocking HIV infection is essential. Especially for people in the high-risk environment, long-term prevention is important, because HIV can easily infect cells once the drug is interrupted. However, there is still no long-acting AIDS prevention drug approved. Hence, the purpose of this study is to prepare a fusion inhibitor loaded poly(D, L-lactic-co-glycolic acid) (PLGA) microspheres as a sustained-release system for long-term AIDS prevention. As the HIV membrane fusion inhibitor (LP-98) used in this research is amphiphilic lipopeptide, $W_1/O/W_2$ double-emulsion method was chosen, and premix membrane emulsification technique was used for controlling the uniformity of particle size. Several process parameters that can impact drug loading efficiency were summarized: the concentration of LP-98 and PLGA, and the preparation condition of primary emulsion. Finally, the microspheres with high loading efficiency (>8%) and encapsulation efficiency (>90%) were successfully prepared under optimum conditions. Pharmacokinetic studies showed that LP-98-loaded microspheres were capable to continuously release for 24 days in rats. This research can promote the application of sustained-release microspheres in AIDS prevention, and the embedding technique used in this study can also provide references for the loading of other amphipathic drugs.

KEYWORDS
AIDS prevention, amphipathic, fusion inhibitor, PLGA microspheres, sustained-release

Abbreviations: AIDS, acquired immunodeficiency syndrome; CLSM, confocal laser scanning microscopy; EE, encapsulation efficiency; FDA, the U.S. Food and Drug Administration; HIV, human immunodeficiency virus; LE, drug loading efficiency; PLGA, poly(D,L-lactic-co-glycolic acid)

1 | INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a chronic infectious disease caused by human immunodeficiency virus (HIV) which attacks immune system cells [1,2]. According to The Joint United Nations Programme on HIV/AIDS, more than 37 million people are living with HIV around the world and the incidence rate of AIDS is still rising year by year. Currently, most of the treatment drugs for AIDS are typically inhibitors, which cannot realize the permanent cures. Besides, some of the treatments lead to severe side effects that decrease patients' living quality [3–5]. Therefore, the research of blocking human immunodeficiency virus infection from the source and preventing the occurrence of the disease is necessary. Contemporarily, antiretrovirals are the best option for maximal viral suppression, which include reverse transcriptase inhibitors, integrase strand transfer inhibitors, protease inhibitors, and entry inhibitors (CCR5 antagonists and fusion inhibitors) [6–9]. HIV fusion inhibitors can effectively block HIV from getting into and infecting CD_4^+ T cells, which stops HIV multiplication in the human body at the first stage and exerts greater preventive effect [10,11]. Enfuvirtide (T-20) was the first and only fusion inhibitor approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV infection [12,13]. AIDS prevention needs effective and continued drug concentration to reduce the risk of infection. However, the *in vivo* half-life of T-20 is only approximately 4 h. Then, it needs frequent high-dose administration, which may lead to drug resistance and poor patient compliance [13–15]. Therefore, it is necessary to develop long-acting drug delivery for effective prevention. Presently, only vaginal rings have a sustained-release effect on AIDS prevention, but the application is limited to women, and most of them are surgically implanted, which is inconvenient for use [4–6,16].

Biodegradable polymeric microspheres is one of the most widely used drug delivery system, which have the following advantages: (i) long-acting drug release, (ii) the ability to be completely degraded *in vivo*, (iii) good biocompatibility, and (iv) regulating the release rate as need. This can significantly minimize the side effects caused by repeated administration and increase the patient compliance. Moreover, microspheres with narrow size distribution do not need to be sieved, avoiding raw material waste and reducing preparation costs. Besides, uniform microspheres have the advantages of good batch reproducibility and repeatable sustained-release behavior, which have important clinical value. The overall goal of this study is to prepare an HIV fusion inhibitor loaded microspheres for injection with narrow size distribution and high encapsulation efficiency (EE), which are more convenient for use and suitable for more people. In this research, lipopeptide fusion inhibitor (LP-98) provided by Chinese Academy of Medical Sciences and Peking Union Medical College was chosen as the active pharmaceutical ingredient, because it has a potent and broad anti-HIV activity [17]. Besides, it also has been comprehensively characterized for its modes of action and durability, including low cytotoxicity, and strong binding ability to human serum albumin [18]. Although LP-98 can last three days *in vivo*, multiple injections are remain needed for AIDS prevention. Thus, in this study microspheres would be used to embed LP-98 and realize long-term release. As the solubility of LP-98 in water is relatively higher, it was dissolved in the aqueous phase to ensure the good dispersion of drug in microspheres. Meanwhile, amphiphilic lipopeptide is emulsifiable and easily distributed at the oil–water interface. Compared with single emulsion method, double emulsion method can make it distribute more at the inner oil–water interface rather than on the emulsion surface, thereby increasing drug loading efficiency and reducing the initial burst release of microspheres (Figure S1). Therefore, $W_1/O/W_2$ double emulsion method was finally chosen. Poly(D,L-lactic-co-glycolic acid) (PLGA) was employed as a drug carrier for its good biocompatibility and biodegradability, and it has been approved by the FDA [19]. Premix membrane emulsification technique combined with double emulsion-solvent evaporation method was used in this research [20–22]. The two important parameters, trans-membrane pressure, and preparing methods of pre-double emulsion were optimized

PRACTICAL APPLICATION

The major challenges of acquired immunodeficiency syndrome (AIDS) prevention are drug resistance caused by the frequent administration and high infection rate engendered by treatment interruption. Therefore, long-acting preventive drugs are necessary for people in high-risk environments. This study applies sustained-release microspheres for AIDS prevention and summarizes several key factors affecting the uniformity and drug loading efficiency of microspheres. Besides, the double-emulsion method was used to prepare microspheres in this research depending on the amphipathicity of lipopeptide. This work will provide a way for the embedding of other amphipathic drugs to solve the problems of low drug loading and encapsulation efficiency.

to obtain microspheres with a narrow size distribution. Moreover, formulation and preparation conditions of primary emulsion have been optimized for high drug loading and encapsulation efficiency. Finally, *in vitro* release and pharmacokinetic studies were performed to evaluate the sustained release behavior of microspheres.

2 | MATERIALS AND METHODS

2.1 | Materials

PLGA (5050 DLG 2.5A, MW ≈ 20 kDa) was purchased from Evonik (USA). Lipopeptide was provided by the Chinese Academy of Medical Sciences and Peking Union Medical College. PVA (degree of viscosity 20.0 mPa·S, degree of hydrolysis 88%) was purchased from Jiangxi Alpha Hi-Tech Pharmaceutical Co., Ltd. (China). Fast Membrane Emulsifier (FM0210/500 M) and microporous membranes (30 μm) were provided by Senhui (Suzhou) Microsphere Tech Co., Ltd. (China). Acetonitrile and trifluoroacetic acid (TFA) (both in HPLC grade) were purchased from Dikma Co., Ltd. (USA). All other reagents were analytical grade.

2.2 | Surface tension measurement of LP-98

LP-98 is a lipopeptide molecule with a hydrophilic polypeptide end and a hydrophobic fatty chain end. Here, a series of LP-98 aqueous solutions with different concentrations were prepared to measure their surface tension with K100 from Germany's Kruss.

2.3 | Preparation of the microspheres via membrane emulsification

Microspheres loaded with LP-98 were prepared by premix membrane emulsification technique combined with double emulsion ($W_1/O/W_2$) solvent evaporation method. First, 0.5 mL LP-98 aqueous solution was mixed with 5 mL methylene dichloride containing PLGA by homogenization for 2 min. Then, the primary emulsion was added into a 50 mL of external aqueous phase containing 1.5% w/v PVA and 1% w/v NaCl and pre-emulsified at room temperature for 30 s under magnetic stirring at 300 rpm or under homogenization at 4000 rpm. Then, pre-double emulsion was pressed into the microporous membrane (30 μm) for three times to gain relatively uniform emulsion droplets. The obtained emulsion was stirred to evaporate the organic solvent and collected by centrifugation. Finally, the prepared microspheres were washed with water three times and then lyophilized for 3 days.

2.4 | Characterization of microspheres

2.4.1 | Particle size measurement

Particle size distribution was measured by Mastersizer 2000 (Malvern, UK). It was referred to as Span value and calculated as follows:

$$Span = \frac{D_{v,\,0.9} - D_{v,\,0.1}}{D_{v,\,0.5}}$$

where $D_{v,0.9}$, $D_{v,0.5}$, and $D_{v,0.1}$ are volume size diameters at 90, 50, and 10% of the cumulative volume, respectively. The smaller Span value means the narrower size distribution of microspheres.

2.4.2 | Microscopic observation

The shape and surface morphology of microspheres were observed by a JSM-6700F (JEOL, Japan) SEM at 5.0 kV. To obtain the cross-sectional morphology, a frozen section method was adopted. Microspheres were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura, USA) and cut with a cryotome (Leica, Germany).

FITC was employed to label LP-98, and Nile red was used to label PLGA to observe the distribution of LP-98 within microspheres by confocal laser scanning microscopy (CLSM) (SP8 STED 3X, Leica, Germany).

2.5 | Measurement of encapsulation efficiency

Approximately, 20 mg of freeze-dried microspheres were dissolved in 2 mL acetonitrile, and then 3 mL of 0.03% ammonia was added. Acetonitrile was used to dissolve PLGA, while LP-98 was slightly soluble in acetonitrile. The addition of weakly alkaline ammonia solution could ensure the dissolution of LP-98, and PLGA did not precipitate again. The mixture was suspended to dissolve LP-98 completely. Next, it was filtered and then injected into a reversed-phase high-performance liquid chromatography (RP-HPLC) system to determine the concentration of LP-98. The analysis was performed at room temperature using a 300Extend-C18 (250 mm × 4.6 mm × 5 μm, Agilent) chromatographic column. Linear gradient elution was performed from 40 to 100% acetonitrile in ultrapure

water containing 0.05% TFA for 20 min. The flow rate was 1 mL/min, and UV absorbance was 220 nm. Drug loading efficiency (LE) and encapsulation efficiency of the microspheres were calculated by the following equations:

$$LE\ \left(\%, \frac{w}{w}\right) = \frac{\text{Mass of drug in microspheres}}{\text{Mass of microspheres}} \times 100\%$$

$$EE\ (\%) = \frac{\text{Loading efficiency}}{\text{Theoretical loading efficiency}} \times 100\%$$

2.6 | *In vitro* release

The 20 mg LP-98 loaded microspheres were incubated in 1.5 mL 10 mM PBS medium (pH 7.4) under agitation 37°C. Supernatants were periodically replaced with fresh buffer of equal volume by centrifugation at 8000 rpm for 5 mins. Calculate the cumulative release by freeze-drying the remaining microspheres and detecting the content of LP-98.

2.7 | Pharmacokinetic study

The animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Research Council (Eight Edition, 2011) and approved by the Experimental Animal Ethics Committee in Beijing. Male Sprague-Dawley (SD) rats (200–300 g) were used to study the *in vivo* drug release behavior of the microspheres. Eighteen rats were randomly divided into three groups (n = 6 per group): physiological saline group, LP-98 solution group, and LP-98-PLGA microsphere group. The LP-98 solution group was administrated as a single subcutaneous injection at a dose of 6.0 mg/kg. The LP-98-PLGA microspheres group was administrated as a single subcutaneous injection at an LP-98 dose of 60 mg/kg. The microsphere suspension was prepared with physiological saline. All samples were shaken before use.

Blood samples (approximately 1.0 mL) were collected after injection at 0.5, 3, 6, 24 h, and 2, 3, 4, 7, 10, 14, 17, 21, 24, and 28 days. Serum was separated via centrifugation at 12 000 rpm for 5 min, then stored at −70 to −80°C in the ultralow temperature freezer (SANYO, Japan) until assay.

The concentration of LP-98 in serum was analyzed by HPLC-MS (Jasper HPLC-ABSciex-4500MD, USA). Pharmacokinetic parameters such as maximum serum concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were determined directly from the observed data.

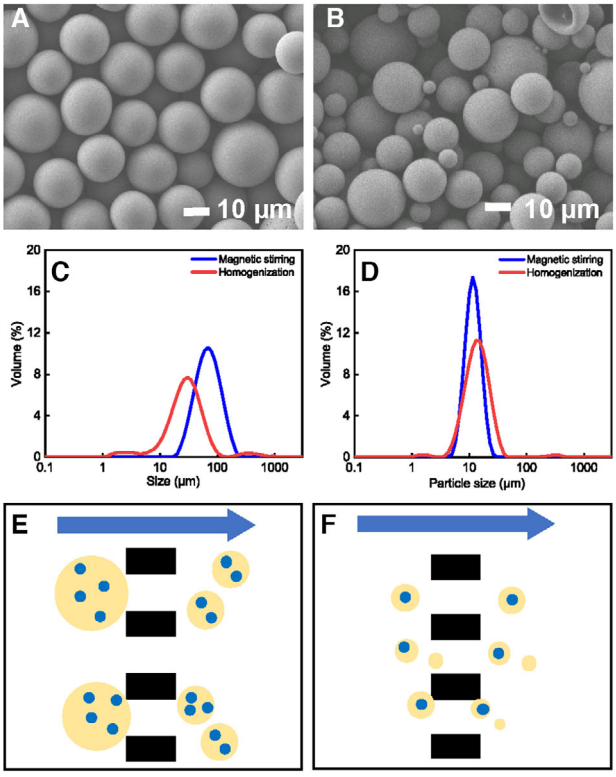

FIGURE 1 (A, B) SEM images of microspheres when pre-double emulsion prepared by magnetic stirring method and homogenization method, respectively, (C, D) Size distribution of pre-double emulsion and microspheres prepared by different pre-double emulsification methods. (E, F) Schematic diagram of premix membrane emulsification, respectively, the droplet size of pre-double emulsion was larger or smaller than membrane pore size

2.8 | Statistics analysis

All analysis was presented as means ± SD. A one-way ANOVA (GraphPad, Prism 8.0.2) was used to determine statistical significance, and the difference is considered significant when $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Preparation of microspheres

3.1.1 | Effect of pre-double emulsification methods on uniformity of mirospheres

Before membrane emulsification, it's necessary to prepare pre-emulsion with suitable droplet size, otherwise, the later membrane emulsification will be inefficient. Two preparation methods of pre-double emulsion: magnetic stirring and homogenization were compared in this study. As results shown in Figure 1A, the microspheres were

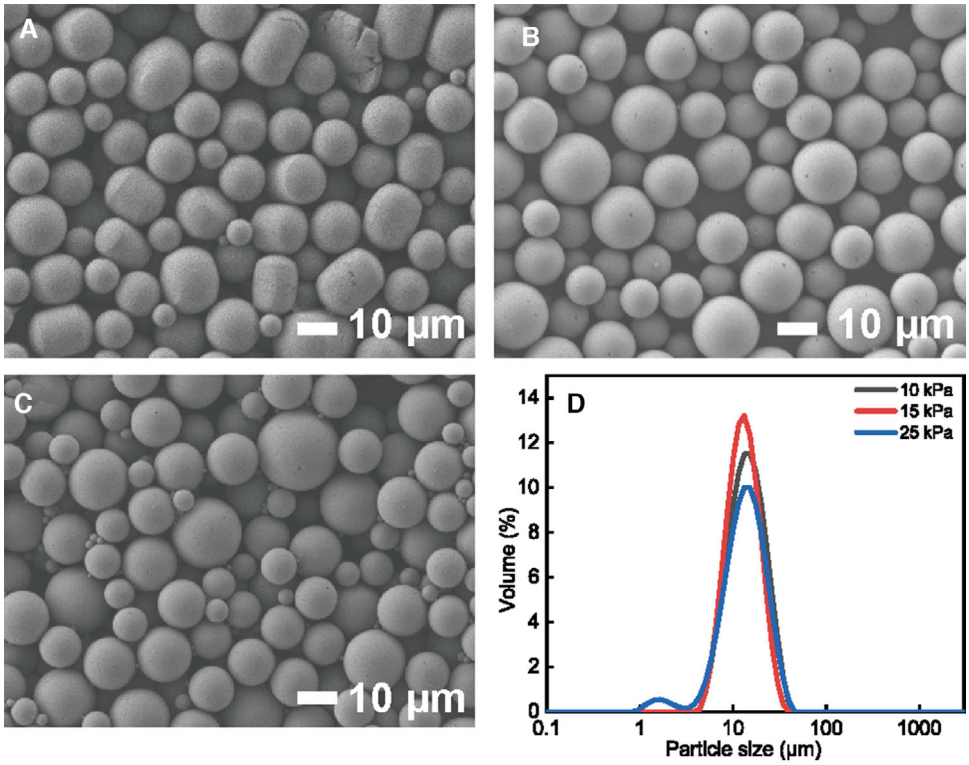

FIGURE 2 (A–C) SEM images of microspheres prepared under different trans-membrane pressures, 10, 15, and 25 kPa, respectively. (D) Size distribution of microspheres prepared under different trans-membrane pressures

more uniform when pre-double emulsion was prepared by magnetic stirring (diameter of 12.53 μm, Span of 0.95). This is because the droplet size of pre-emulsion was larger than the membrane size 30 μm, larger droplets of the pre-emulsion can be broken into smaller and more uniform droplets through membrane emulsification (Figure 1C,E). In contrast, many droplets smaller than the membrane pore generated under high-speed homogenization would pass the microporous membrane freely, leading to a broad size distribution of microspheres (diameter of 14.59 μm, Span of 1.28) (Figure 1B–F). Therefore, magnetic stirring was chosen for the preparation of pre-double emulsion. In addition, the effects of different stirring speeds on microspheres were also investigated, and 300 rpm was the optimal speed.

3.1.2 | Effect of trans-membrane pressure on uniformity of microspheres

Trans-membrane pressure is an important control parameter in the process of membrane emulsification. As shown in Figure 2, 15 kPa was the optimal membrane pressure in this study (diameter of 13.43 μm, Span of 0.85). Because low pressure allowed the droplet to deform in the pore and form microspheres with larger diameter or elliptical shape (Figure 2A, diameter of 14.39 μm, Span of 0.99). On the contrary, high trans-membrane pressure caused strong friction between the emulsion and membrane, it resulted in many droplets with extremely small size (Figure 2C, diameter of 11.37 μm, Span of 1.29). Only suitable trans-membrane pressure can ensure the uniformity of particle size.

3.2 | Loading efficiency and encapsulation efficiency

In order to obtain LP-98-loaded microspheres with high loading and encapsulation efficiency, three key factors on drug loading were investigated: PLGA concentration in the oil phase, LP-98 concentration and preparation conditions of primary emulsion.

3.2.1 | Effect PLGA concentration in the oil phase

As shown in Figure 3, EE was successfully improved as PLGA concentration increased. When PLGA concentration was up to 100 mg/mL, EE reached more than 95%. This was because PLGA was the framework of microspheres and LP-98 was distributed inside the microspheres. When PLGA concentration was low, the viscosity of the oil phase became lower, the LP-98 was easier to diffuse to the outer

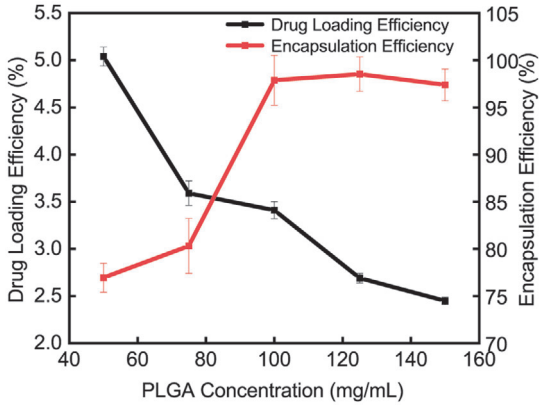

FIGURE 3 Effect of poly(D,L-lactic-co-glycolic acid) concentration on drug loading efficiency and encapsulation efficiency of microspheres (n = 3 results are mean ± SD)

water phase during emulsification and solidification. When PLGA concentration increased, higher viscosity of the oil phase retarded the diffusion of LP-98, leading to the improvement of EE. Whereas, an increase of PLGA concentration would reduce the drug loading efficiency of the microsphere, due to the increase of PLGA proportion in the microspheres. Therefore, to obtain microspheres with higher LE and EE, 100 mg/mL PLGA was selected.

3.2.2 | Effect of amphiphilicity and concentration of LP-98

In the encapsulation of hydrophilic peptides or proteins, drug leakage from the internal water phase to the external water phase is a big challenge [23–27]. Fortunately, LP-98 shows better encapsulation and drug loading efficiency, which was attributed to the amphiphilicity of lipopeptide. To confirm this conclusion, LP-98 aqueous solution in different concentrations was measured by surface tension tester (Figure 4A), and the inflection point was found at 0.01 mg/mL. This result suggested that LP-98 could reduce the surface tension to stabilize the oil–water interface and improve the stability of the primary emulsion. As shown in Figure 4B, compared with the pure water group, LP-98 group could remain stable without phase separation for 22 h. However, when LP-98 concentration was too high, the excessive drug would exceed the oil phase loading capacity, resulting in the diffusion of the drug from the inner water phase to the outer water phase, and leading to low encapsulation efficiency and high production costs [28]. From the Figure 4C, when LP-98 concentration was 150 mg/mL, the loading efficiency was the highest but the encapsulation efficiency was only 70%, so we selected 100 mg/mL as the optimal LP-98 concentration.

3.2.3 | Effect of homogenization speed

During primary emulsion preparation, the speed of homogenization possibly has an impact on the drug loading efficiency of microspheres [20]. Therefore, the influence of homogenization speed on LE was investigated, and the results showed that as the speed was accelerated, the LE of microspheres was improved (Figure 5A). This was because, under higher homogenization speed, the inner water phase could be broken into smaller and more uniform droplets, which made the emulsion more stable and the microspheres more compact. Consequently, the drug had less chance to leak out to the external water phase during membrane emulsification. To prove this assumption, SEM and CLSM were used to observe the internal structure

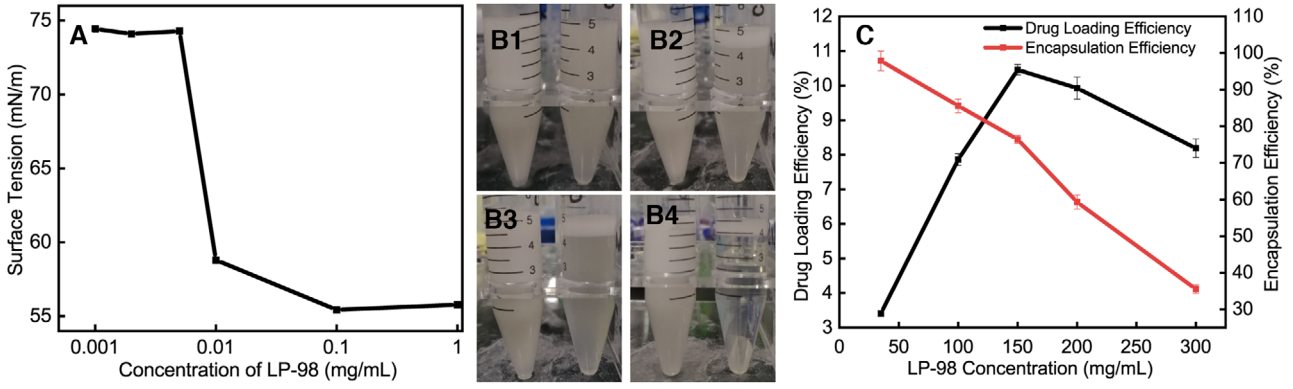

FIGURE 4 (A) Surface tension of LP-98 solutions in different concentrations; (B) Phase separation of emulsion at different times: LP-98 group (the left tube): LP-98 aqueous solution: PLGA dichloromethane solution (W:O) = 1: 10; Control group (the right tube): water: PLGA dichloromethane solution (W:O) = 1: 10; (C) Effect of LP-98 concentration on drug loading efficiency and encapsulation efficiency of microspheres (n = 3 results are mean ± SD)

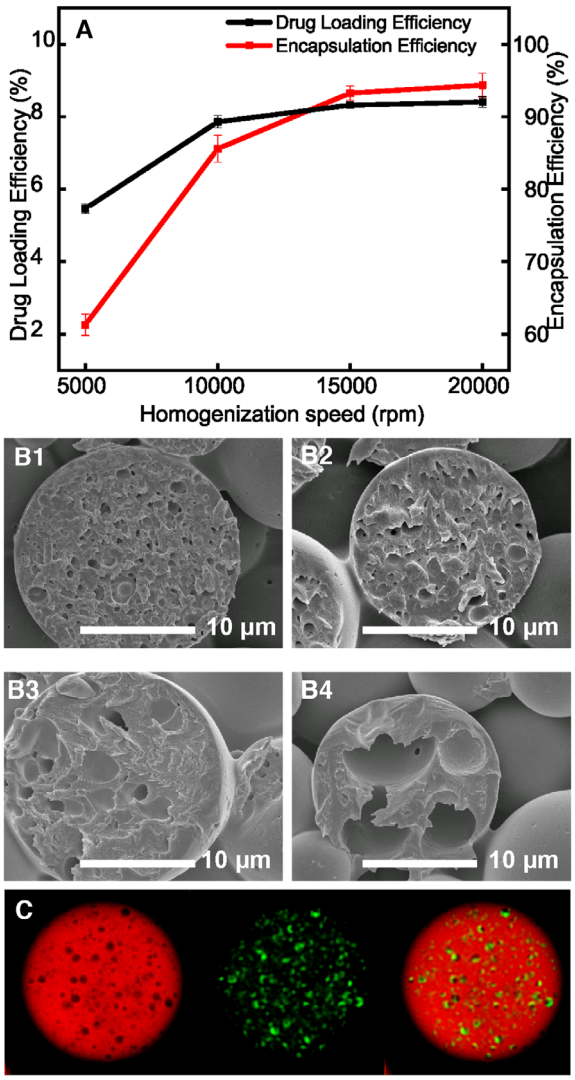

FIGURE 5 (A) Effect of homogenization speed on drug loading efficiency and encapsulation efficiency of microspheres (n = 3 results are mean ± SD). (B) SEM images of microspheres prepared at different homogenization speeds, 20 000, 15 000, 10 000, and 5000 rpm, respectively. (C) Confocal laser scanning microscopy images of drug-loaded microspheres (B1 sample)

of microspheres. As shown in Figure 5B, higher homogenization speed produced smaller internal pore, and denser microstructure and LP-98 were dispersed in the caving of the microspheres (Figure 5C). Therefore, 20 000 rpm was determined as the optimal homogenization speed.

3.3 | *In vitro* release

The cumulative release profile *in vitro* of LP-98-loaded microspheres was shown in Figure 6. It exhibited an initial burst release (about 25% within 0.5 h), which was within acceptable limits. Afterwards, it showed a steady and slow release behavior and released about 90% within 42 days.

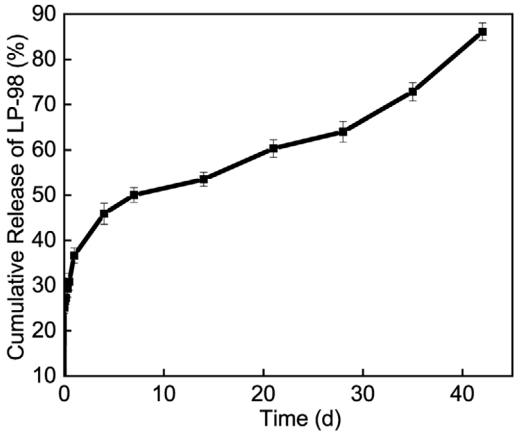

FIGURE 6 Cumulative *in vitro* release profiles of LP-98 loaded microspheres

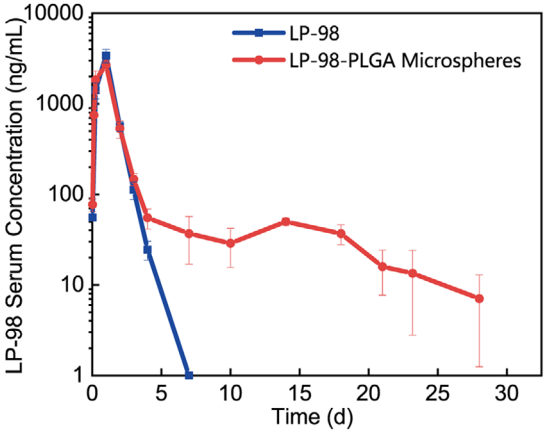

FIGURE 7 LP-98 serum concentration vs. time profiles of LP-98 and LP-98-PLGA microspheres (n = 6 results are mean ± SD)

3.4 | Pharmacokinetics of LP-98 microspheres

LP-98-loaded microspheres prepared under the optimum condition were used for *in vivo* release by detecting LP-98 concentration in serum at different times. As shown in Figure 7, the serum concentration of LP-98 solution group reached C_{max} (3413.33 ± 581.63 ng/mL) at the 24th hour after injection, and then it decreased rapidly from the first to the fourth day, and the drug was completely undetectable at the seventh day. Whereas LP-98-PLGA microsphere group showed a relatively stable and sustained release, and the C_{max}(2693.33 ± 336.68 ng/mL) was also at the 24th hour, nearly 1000 ng/mL lower than that of LP-98 group. Moreover, it fluctuated in the range of 10–50 ng/mL, higher than the minimum effective concentration of 10 ng/mL. After 28 days, the concentration became lower than 10 ng/mL. Therefore, LP-98-PLGA microspheres can realize stable release for 24 days.

4 | CONCLUDING REMARKS

In this research, LP-98-loaded microspheres with narrow size distribution have been successfully prepared by premix membrane emulsification technique combined with a double emulsion method for long-term AIDS prevention. Preparation methods of pre-double emulsion and transmembrane pressure were key parameters affecting the uniformity of microspheres. Formulation and emulsification conditions influenced the stability of primary emulsion and encapsulation efficiency of microspheres. The optimum condition was as follows: the concentration of PLGA and LP-98 are both 100 mg/mL, and the primary emulsion was prepared with homogenization at 20 000 rpm, then pre-double emulsion was prepared by magnetic stirring at 300 rpm, final membrane emulsification was carried out under 15 kPa. The microsphere with a diameter of 14 μm, Span of 0.9, EE of 95%, LE of 9% was obtained. In addition, pharmacokinetic studies were performed using prepared microspheres under the optimum conditions, and the results showed that it can be continuously released for 24 days in rats. These results revealed a promising potential of LP-98 loaded microspheres as a long-term drug delivery system for AIDS prevention.

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CONFLICT OF INTEREST

The autors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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