Preparation and in vitro release of total alkaloids from alstonia scholaris leaf-loaded mPEG-PMA microspheres

Nianfeng Jiang,1,4 Xiangyu Zheng1,3, Yan Feng,1 Hongtao Wu,1 Mingwei Yuan,1 Yi He3, Hongli Li1,∗ and Minglong Yuan1,∗

1 National and Local Joint Engineering Research Center for Green Preparation Technology of Biobased Materials, Yunnan Minzu University, Kunming 650500, People’s Republic of China
2 Jilin Institute of Chemical Technology, Jilin 132000, People’s Republic of China
3 School of Chemistry and Chemical Engineering, and Chongqing Key Laboratory of Soft-Matter Material Chemistry and Functional Manufacturing, Southwest University, Chongqing 400715, People’s Republic of China
4 These authors contributed equally to this work.

∗ Authors to whom any correspondence should be addressed.
E-mail: hongli_1982@163.com and yml@188.com

Keywords: drug loading, biocompatibility, anti-inflammatory liveness, hemolysis rate

Abstract

The total alkaloids extracted from the leaves of Alstonia scholaris (ASAs) have been reported to reduce fever, remove phlegm, and relieve coughs. However, their drug half-lives are short. Thus, to obtain sustained-release preparations of total alkaloids from ASAs, mandelic acid oxyanhydride (mandelic acid OCA) was synthesized by the reaction of L-mandelic acid (MA) with triphosgene, and subsequent copolymerization with polyethylene glycol monomethyl ether (mPEG) of different molecular weights yielded the corresponding mPEG poly-MA (mPEG-PMA) copolymers. ASAs-loaded microspheres were then prepared using the double emulsion method, and their in vitro release (15 d, 37 °C) and in vitro degradation behaviors were studied. The morphology, size, embedding efficiency, and drug loading efficiency were investigated for the prepared microspheres, and screening was carried out using the mPEG10K-PMA drug-loaded microspheres to analyze their biological characteristics. Anti-inflammatory experiments using Kunming mice and Sprague Dawley rats showed that the microspheres exhibited good anti-inflammatory properties. Moreover, the ASAs-loaded microspheres exhibited a good biocompatibility, and the hemolysis rate was <5%.

1. Introduction

Alstonia scholaris (L.) R. Br. is a tree of the Chicken Bone Changshan genus of the Oleaceae, which is mainly distributed in the tropical regions of Asia and Africa [1]. Previously, Salim et al reported the isolation of indole alkaloids from Alstonia Scholaris leaves (ASAs) [2], while Luo discovered the most effective alkaloids present in Alstonia Scholaris leaves for the treatment of respiratory diseases [3–7]. However, the half-lives of the active compounds present in ASAs are short, thereby limiting their clinical application due to the requirement for multiple administrations.

Chitosan, sodium alginate, polyflectic acid, and other biodegradable materials are widely used as drug carriers in drug delivery systems [8–14]. In addition, due to the mild conditions employed for the ring opening polymerization of oxyanhydrides, which exhibit a favorable biocompatibility, their degradation rates can be controlled. These compounds are therefore expected to be applicable in controlled drug release or carrier therapies [15–19]. Furthermore, poly(mandelic acid) (PMA) and polyactic acid (PLA) are lipophilic polymers with good biodegradabilities and biocompatibilities, thereby permitting their use in drug delivery systems [20]. However, PMA exhibits a rather low solubility in water. In contrast, polyethylene glycol monomethyl ether (mPEG) has a good water solubility, and is currently employed in the preparation of amphiphilic block copolymers.
polymers, drug-loaded micelles, and microspheres to improve the water solubilities of these lipophilic materials [21–27]. Based on previous studies, it is therefore expected that the problems associated with short drug half-lives and low drug utilization efficiencies in traditional drug delivery systems could be effectively improved by the use of sustained drug release preparations [28].

Thus, we here in report the use of mPEG-PMA as a carrier to prepare ASAs-loaded microspheres using the double emulsion method. The resulting mPEG-PMA copolymer is then characterized by proton nuclear magnetic resonance (\(^{1}\text{H NMR}\)) spectroscopy and gel permeation chromatography (GPC), and the \textit{in vitro} release and degradation behaviors of the ASAs-loaded microspheres are studied. Finally, a series of analyses and biological experiments (e.g., hemolytic, anticoagulant, and cytotoxicity experiments) are carried out on the ASAs-loaded microspheres to confirm the suitability of mPEG-PMA for preparing ASAs-loaded sustained-release materials.

2. Method

2.1. Materials

L-Mandelic acid was provided by Chengdu McCarthy Chemical Co., Ltd. The ASAs were provided by the Kunming Institute of Botany, Chinese Academy of Sciences (batch number: 20180501). DMAP (\(\text{C}_7\text{H}_{10}\text{N}_2\)) was provided by GL Biochem Ltd (Shanghai, China). Deionized water is obtained by water machine (UPH-I-20T, Chengdu, China). HL-7702 normal liver cells were provided by the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2\(^{6}\text{H}\)-tetrazolium bromide (MTT) was provided by the Kunming Cell Bank. Poly(ethylene glycol methyl ether) (Mw 2000, 5000, and 10000) and fetal bovine serum (FBS) were obtained from Adamas (Shanghai, China). An EDTA trypsin digestion solution and cyan streptomycin were provided by Solarbio. Kunming mice, Sprague-Dawley (SD) rats, and rabbits were purchased from Kunming Medical University. Shijiazhuang Kangli Pharmaceutical Co., Ltd. Provided the aspirin samples, while Yunnan Phytopharmaceutical Co., Ltd provided the Dengtaiye KeLi (DKL).

2.2. Preparation of the mPEG-PMA

Mandelic acid OCA (Mac-OCA) was synthesized as described in the literature [29, 30]. Dimethylaminopyridine (DMAP) was firstly employed as a catalyst to deprotonate the active proton of the terminal –OH group of mPEG, which initiated the ring-opening polymerization of Mac-OCA. More specifically, mPEG (0.0002 mol), Mac-OCA (0.0202 mol), and DMAP (0.00007 mol) were added to a dry round-bottomed flask at a certain mass ratio along with a certain amount of dichloromethane (DCM), and nitrogen replacement of the environment was performed three times. The resulting mixture was then allowed to stir for >24 h at 25 °C. After subsequent vacuum concentration, ethanol precipitation, and filtration, a pale-yellow viscous substance was obtained by vacuum drying over P_{2}O_{5} for 24 h to give the mPEG-PMA copolymer. The synthetic process is shown in figure 1.

2.3. Characterization of mPEG-PMA

The \(^{1}\text{H NMR}\) spectrum of mPEG-PMA was recorded at 400 MHz (Bruker 400 MHz, Germany) using CDCl\(_{3}\) as the solvent. The molecular weight of the polymer was determined by GPC (Waters 2414, Waters Inc., Milford, MA, USA) using a polystyrene standard.

2.4. Preparation of the blank microspheres and the ASAs-loaded microspheres

All microspheres were synthesized as described in the literature [31].
weights of the microspheres were accurately determined using an analytical balance, and aliquots registered with the Kunming Science and Technology Bureau.

All trials were approved by the Laboratory Animals Ethics Committee of Yunnan Minzu University and were subjected to GPC to determine their molecular weights. The microsphere weight and number average relative molecular weight Mn and pH value are then used to determine the degradation performance of the microspheres.

2.9. Hemolysis experiments and coagulation experiments of the microspheres

Table 1 shows the results for EE and LE.

| Sample            | Average diameter (μm) | EE (%)       | LE (%)  |
|-------------------|-----------------------|--------------|---------|
| mPEG5k-PMA        | 1.593 ± 0.38          | 64.27 ± 2.21 | 3.01 ± 0.13 |
| mPEG10k-PMA       | 2.260 ± 0.42          | 69.39 ± 2.04 | 3.15 ± 0.17 |
| mPEG20k-PMA       | 3.290 ± 0.39          | 72.88 ± 1.87 | 4.37 ± 0.09 |

EE: encapsulation efficiency; LE: loading efficiency.

2.5. Microsphere morphology, particle size, and particle size distribution

The morphological characteristics of the mPEG-PMA microspheres and the blank microspheres were observed using scanning electron microscopy (SEM; NOVA NANOSEM-450, FEI, Hillsboro, OR, USA). A laser particle size analyzer (Mastersizer 2000, United Kingdom) was used to determine the particle size distributions of the microspheres. For this purpose, a small amount of the microsphere suspension was diluted in a 15 ml centrifuge tube to give an almost transparent solution.

2.6. Determination of the EE and LE values of the microspheres

The lyophilized microspheres (20 mg) were accurately weighed, dissolved in DCM (0.5 ml), and added to methanol (2 ml). Following centrifugation to collect the supernatant, filtration was carried out using a 0.45 μm microporous membrane, and an aliquot (20 μl) was injected into the chromatogram. The embedding efficiency (EE) and loading efficiency (LE) values were calculated according to the area normalization method, and all experiments were repeated in triplicate to obtain average values.

\[
EE = \left( \frac{\text{weight} \; 1}{\text{weight} \; 2} \right) \times 100\%
\]

where weight 1 is the weight of the ASAs in the microspheres, and weight 2 is the weight of the added ASAs.

\[
LE = \left( \frac{\text{weight of ASAs in microspheres}}{\text{total weight of microspheres}} \right) \times 100\%
\]

Table 1 shows the results for EE and LE.

2.7. In vitro release experiments of the ASAs-loaded microspheres

Initially, a sample (50 mg) of the lyophilized microspheres was placed in a centrifuge tube with PBS (5 ml), and oscillated at 37 °C. The centrifuge tube was removed at fixed time intervals. After centrifugation for 6 min, a sample (1 ml) was taken from the centrifuge tube and an aliquot (1 ml) of PBS was added prior to restarting oscillation. Determination of the ASAs present in the solution was then carried out by HPLC (Agilent-1220, California, America, C18 column, 5 μm, 250 mm × 4.6 mm; column temperature, 40 °C; mobile phase, 20% A (HCOOH:H2O = 0.1:100) and 80%B (CH3CN), 1 ml min\(^{-1}\) flow rate), and the total release of ASAs was calculated according to the following formula. The release curve was obtained by plotting the drug release amount against time (d).

\[
Q = C_n \times V_b + V_c \sum_{n=1}^{t} C_n \cdot V_b
\]

Q: total amount of ASAs released

\(C_n\): ASAs concentration at time \(t\)

\(V_b\): bulk volume of PBS

\(V_c\): removal volume of PBS

2.8. In vitro degradation of the microspheres

As described in section 2.7, the supernatant was removed by centrifugation, and subsequently its pH value was determined. The microspheres were then rinsed with pure water and freeze-dried for 24 h at –50 °C. The weights of the microspheres were accurately determined using an analytical balance, and aliquots (3 mg) were subjected to GPC to determine their molecular weights. The microsphere weight and number average relative molecular weight Mn and pH value are then used to determine the degradation performance of the microspheres in vitro over time.

2.9. Hemolysis experiments and coagulation experiments of the microspheres

All trials were approved by the Laboratory Animals Ethics Committee of Yunnan Minzu University and were registered with the Kunming Science and Technology Bureau (SYXX (Yunnan) K2017-0001, January 16, 2017).
Sample preparation and application of the test methods for the hemolysis and coagulation experiments were carried out as described in the literature [31]

\[
\text{Hemolysis rate}(\%) = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{negative control}})}{(\text{Abs}_{\text{positive control}} - \text{Abs}_{\text{negative control}})} \times 100\%
\]

The anticoagulant properties of the samples were calculated using the following formula:

\[
\text{BCI} = \frac{(I_0/I_w)} \times 100\%
\]

where \(I_0\) is the relative absorbance after the desired contact time between the blood mixture, calcium chloride, and the sample, and \(I_w\) is the relative absorbance after mixing the blood with a desired quantity of deionized water.

2.10. Cytotoxicity of the microspheres

Pulmonary cell lines were inoculated into flasks and cultured in DMEM high-sugar medium. After incubation in an incubator containing 5% CO\(_2\) at 37 °C, the cells showed monolayer adherence growth and were sub-cultured every 3–5 d. Finally, the cells were digested and sub-cultured with 0.25% trypsin.

HL-7702 cells (5 \(\times\) 104 cells/ml), logarithmically grown at 180 \(\mu\)g l\(^{-1}\), were inoculated into 96 well plates. After overnight incubation, 20 \(\mu\)l samples (5 mg ml\(^{-1}\)) were added, and three concentration gradients were set up with three multiple holes at each concentration. An aliquot (20 \(\mu\)l) of 5 mg ml\(^{-1}\) MTT was added to each well after 24 h and incubation carried out for 4 h. After this time, the medium was removed, and DMSO (0.15 ml) was added to quench the reaction. The absorbance at 490 nm was measured using an enzyme label (Molecular Devices, Spectra Max iD3). The survival rate was calculated using the following formula [32, 33]:

\[
\text{Survival rate} = \frac{(\text{OD}_{\text{specimen}})}{(\text{OD}_{\text{control}})} \times 100\%
\]

2.11. Statistical analysis

Plots were drawn using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA 92037, USA), and all data were analyzed using one-way ANOVA. The results are expressed as mean ± standard deviation (\(\bar{X} \pm s\)). When * \(P < 0.05\), the difference between the groups is considered significant; where ** \(P < 0.01\), the difference between the groups is considered extremely significant [34].

2.12. Anti-inflammatory liveness test of the ASAs-loaded microspheres

2.12.1. Effect of p-xylene on auricle swelling in mice

According to their body weight and sex, 35 mice (20 ± 2 g) were divided into seven groups containing five mice. These groups were named Gm1 (aspirin group), Gm2 (control group), Gm3, Gm4, Gm5, Gm6, and Gm7. The Gm1 group was administered aspirin once per day on the experimental day, while the other groups were administered aspirin once per day for three consecutive days. The Gm2 group was administered 1% CMC-Na to a level of 20 ml kg\(^{-1}\). After 30 min, xylene (0.05 ml) was evenly applied to the right ear of each mouse in each group. The left ear is not painted as a control. At a time of 1 h after inflammation, the animals were sacrificed for cervical spondylolisthesis. The same area of each ear was cut using a perforator measuring 10 mm in diameter, and the difference in weight between the ears was taken as the degree of swelling.

2.12.2. Effect of egg white on paw swelling in rats

Thirty-five SD rats (200 ± 20 g) were randomly divided into seven groups according to the previous grouping method and group assignments. The Gr1 group was administered egg white by gavage only once on the experimental day, while the other groups were administered egg white once per day for three consecutive days. The Gr2 group was administered an equal volume of 1% CMC Na by gavage, wherein the gavage volume of each group was 10 ml kg\(^{-1}\). An aliquot (0.01 ml) of egg white was injected subcutaneously at the end of each test period. The foot volume of each mouse was measured before inflammation, and at times of 0.5, 1, 2, 3, 4, and 5 h after inflammation. The difference in the foot area before and after inflammation was taken as the degree of swelling, and the swelling rate was calculated using the following formula:

\[
\text{Swell} = \frac{((A_{t0} - A_{t0})/A_{t0}) \times 100\%}{A_{t0}}
\]

\(A_{t0}\): foot area before injection
\(A_{t0}\): foot area after injection
3. Results and discussion

3.1. Characterization of the mPEG-PMA copolymer

As shown in figure 2, the $^1$H NMR results were consistent with those of previous studies. The molecular weights ($M_n$ and $M_w$) of the mPEG10k-PMA copolymer determined by GPC (Waters 2414) are shown in table 2. More specifically, the $M_w$ of the copolymer was 13345 Da, the molecular weight distribution was narrow, and the polydispersity index ($PDI$) was 1.14.

### Table 2. Distribution of the $M_n$, $M_w$, and PDI of the prepared mPEG-PMA samples.

| Sample         | $M_n$  | $M_w$  | PDI  |
|----------------|--------|--------|------|
| mPEG2k-PMA     | 3321   | 4019   | 1.21 |
| mPEG5k-PMA     | 5375   | 6343   | 1.18 |
| mPEG10k-PMA    | 11706  | 13345  | 1.14 |

- Determined by GPC via a universal calibration curve and appropriate Mark-Houwink parameters for THF.
- Calculated by $^1$H NMR spectroscopy.
- $M_n$: number-average molecular weight; $M_w$: weight-average molecular weight.
- PDI: The polydispersity index of the molecular weight distribution.

3.2. Morphological characterization and particle size distribution analysis of the microspheres

Figure 3 shows the SEM images of the ASAs-loaded microspheres, where it can be seen that the spherical shape of the mPEG2k-PMA microspheres in figure 3(A) is superior, the surface is smooth, and the particle size is smaller, but there are also a small number of microspheres with a large particle size and reduced adhesion. Figure 3(B) shows the SEM images of the mPEG5k-PMA microspheres, where a suitable size, smooth surface, and good adhesion can be observed. In addition, figure 3(C) shows the SEM images of the mPEG10k-PMA microspheres, which exhibit relatively large particles of different sizes. In this case, it was expected that the presence of small amounts of residual organic solvents may have caused aggregation of the microspheres. As indicated in figure 4, the particle sizes of the mPEG2k-PMA, mPEG5k-PMA, and mPEG10k-PMA microspheres were 1.593 ± 0.38, 2.26 ± 0.42, and 3.29 ± 0.39 μm, respectively. Although Widder et al [35] reported that the intravascular
injection of microspheres measuring <1.4 μm in diameter did not result in pulmonary embolism, the diameters of the microspheres prepared herein were larger than 1.4 μm, thereby indicating their potential suitability for use in non-injection drug delivery or oral administration [36].

3.3. Encapsulation rates and drug loading properties of the microspheres
The mPEG2k-PMA, mPEG5k-PMA, and mPEG10k-PMA drug-loaded microspheres were prepared according to the double emulsion method [29] to give EEs of 64.27 ± 2.21, 69.39 ± 2.04, and 72.88 ± 1.87%, in addition to LEs of 3.01 ± 0.13, 3.15 ± 0.17, and 4.37 ± 0.09%, respectively. Furthermore, it was found that with an increase in the polymer molecular weight, chain length, diameter, and microsphere inner space, the EE and LE also increased, which is consistent with previous research results [31, 37].
3.4. In vitro release analysis of the ASAs-loaded microspheres

Figure 5 shows the in vitro release curves obtained for the ASAs-loaded microspheres. As indicated, cumulative release of the mPEG2k-PMA microspheres reached 31.55 ± 1.01% within 24 h, and could be sustained at a stable level over 15 d. After this time, the total release reached 65.09 ± 1.54%. In addition, over 24 h, the cumulative release of the mPEG5k-PMA microspheres reached 31.28 ± 1.16%, and this was sustained over 15 d, with the total release in this case reaching 62.07 ± 1.29%. Furthermore, the cumulative release of the mPEG10k-PMA microspheres reached 25.48% ± 1.21% within 24 h and could be sustained at a stable level over 15 d. After this time, the total release reached 60.1 ± 1.34%. Due to the fact that the drugs were embedded in the polymer materials via a physical method, and subsequent drug release was conducted through the degradation and dissolution of the polymer materials, it was found that upon increasing the size of the microspheres, the drug release rate initially decreased, and so this was attributed to the first degradation of the microspheres with a large specific surface area and a small drug loading [38]. Due to the slower rate of polymer degradation, the release rate subsequently slowed down.

3.5. Degradation analysis of the microspheres in vitro

Based on the drug loading, morphology, embedding efficiency, and in vitro release results, mPEG10k-PMA was chosen as the optimal microsphere for this study. Thus, figure 6 shows the degradation process of the ASAs-loaded mPEG10k-PMA microspheres, wherein the variation in the molecular number, pH value, dry mass with time and SEM image of mPEG10k-PMA microspheres after 60 days of degradation are shown. More specifically, in PBS (pH = 7.40, 10 mM), the curve showed a downward trend over time. After 60 d, the pH value of the mPEG10k-PMA microspheres decreased from 7.40 to 5.42, the dry weight loss of the microspheres was 63.61%, and the Mw decreased from 11706 to 7446 Da. During this time, the lipid bonds present in the polymer were hydrolyzed, resulting in a decrease in the molecular weight and the dry weight, in addition to the production of mandelic acid, which in turn reduced the pH. After 60 days of degradation, the surface of the microspheres is no longer smooth, and the microspheres are bonded.

3.6. Hemocompatibility of the microspheres in vitro

3.6.1. In vitro hemolysis

Figure 7 shows the hemolytic activities of the total alkaloids from the ASAs, the mPEG10k-PMA microspheres, and the ASAs-loaded mPEG10k-PMA microspheres. It can be seen from the figure that the hemolysis rates of the total alkaloids increased upon increasing the sample concentration. More specifically, the hemolysis rates of the mPEG10k-PMA microspheres loaded with the total alkaloids were 1.33, 2.48, and 3.80% at concentrations of 0.4, 4, and 40 μg ml⁻¹, respectively. These results showed that erythrocyte destruction by the microspheres was low at these concentrations, and the obtained values are in accordance with the ISO hemolysis standard [39].

3.6.2. In vitro coagulation

Figure 8 shows the anticoagulant indices of the total alkaloids from the ASAs, the mPEG10k-PMA microspheres, and the ASAs-loaded mPEG10k-PMA microspheres. As shown, the blood clotting index (BCI) values of the mPEG10k-PMA microspheres loaded with the total alkaloids at levels of 0.4, 4, and 40 μg ml⁻¹ were higher than...
those of the ASAs, while the anticoagulant capacities of the drug-loaded microspheres and the polymer materials increased upon increasing the concentration \((0.4–40 \, \mu \text{g m}^{-1})\). This result indicating that the drug-loaded microspheres do not affect the anticoagulant properties of ASAs itself.

3.7. Cytotoxicity of the microspheres in vitro

Figure 9 shows the results of cytotoxicity tests for the total alkaloids from the ASAs, the mPEG10k-PMA microspheres, and the ASAs-loaded mPEG10k-PMA microspheres. These tests were carried out on normal human liver cells (HL-7702). As can be seen, the ASAs do not exhibit any toxicity toward HL-7702 cells between concentrations of 0.4 and 40 \(\mu\text{g ml}^{-1}\), and a similar result was observed for mPEG10k-PMA between

---

**Figure 6.** Degradation process of the ASAs-loaded mPEG10k-PMA microspheres ((A): the change of Mn with time; (B): the change of pH with time; (C): the change of Dry Mass with time; (D): SEM image of mPEG10k-PMA microspheres after 60 days of degradation.)

**Figure 7.** Hemolysis test results for the ASAs-loaded mPEG10k-PMA microspheres. *P < 0.05 and **P < 0.01, compared with the control (i.e., a whole blood sample containing ASAs).
concentrations of 0.4 and $4 \mu g \cdot m l^{-1}$. However, upon increasing the concentration to $40 \mu g \cdot m l^{-1}$, the survival rate of HL-7702 cells decreased to 96.83%. In addition, for the ASAs-loaded mPEG10k-PMA microspheres, no toxicity was observed at 0.4 $\mu g \cdot m l^{-1}$, although at concentrations of 4 and $40 \mu g \cdot m l^{-1}$, the cell survival rates decreased to 97.25 and 91.31%, respectively, thereby indicating a generally low toxicity overall [40].

3.8. Analysis of the anti-inflammatory effects of the microspheres

To determine the anti-inflammatory effects of the microspheres, the effects of p-xylene on ear swelling in mice under different treatment regimens were initially examined, and the results are shown in Table 4. As indicated, the drug-loaded microspheres effectively inhibited auricle swelling in mice, with the inhibitory effect observed for 10 mg kg$^{-1}$ of the ASAs-loaded microspheres being superior to that of 10 mg kg$^{-1}$ aspirin, which was similar to that of 300 mg kg$^{-1}$ DKL. Moreover, the inhibition rates achieved using 20 and 40 mg kg$^{-1}$ of the microspheres alone were significantly higher than that achieved using 10 mg kg$^{-1}$ aspirin.

Figure 8. Anticoagulation test results for the ASAs-loaded microspheres. *$P < 0.05$ and **$P < 0.01$, compared with the control (i.e., a whole blood sample containing ASAs).

Figure 9. Cytotoxicity test results for the ASAs-loaded microspheres. *$P < 0.05$ and **$P < 0.01$, compared with the control (i.e., cells that grew in the same environment, but in the absence of ASAs).
Table 3. Anti-inflammatory activity of the ASAs on xylene-induced auricular swelling in mice.

| Groups                      | Dose (per kg) | Auricular swelling (± s, mg) | Inhibition rate (%) |
|-----------------------------|---------------|-----------------------------|---------------------|
| Control                     | —             | 50.85 ± 3.61                | —                   |
| Aspirin                     | 10 mg         | 34.65 ± 2.90                | 31.86 ± 1.90        |
| DKL                         | 0.3 g         | 37.65 ± 3.04                | 25.96 ± 1.51        |
| ASAs                        | 0.1 g         | 39.65 ± 0.78                | 22.03 ± 1.26        |
| ASAs-Loaded microspheres    | 10 mg         | 33.55 ± 5.44                | 34.02 ± 2.24        |
| 20 mg                       | 27.10 ± 5.09  | 46.71 ± 3.86                |                     |
| 40 mg                       | 21.25 ± 7.21  | 58.31 ± 4.14                |                     |

Table 4. Effect of the ASAs on egg-white-induced pedal swelling in rats.

| Groups                      | Dose (per kg) | Inhibitory rate of toe swelling (%) |
|-----------------------------|---------------|-----------------------------------|
|                             | 1 h           | 2 h                               | 3 h | 4 h | 5 h |
| Control                     | —             | —                                 | —   | —   | —   |
| Aspirin                     | 20 mg         | 66.77 ± 3.23                      | 62.07 ± 3.04 | 57.02 ± 2.84 | 52.17 ± 2.56 | 49.55 ± 2.54 |
| DKL                         | 0.24 g        | 68.81 ± 3.30                      | 65.86 ± 3.21 | 60.39 ± 2.90 | 54.31 ± 2.72 | 54.48 ± 2.74 |
| ASAs                        | 0.16 g        | 78.97 ± 4.12                      | 69.99 ± 3.55 | 68.29 ± 3.12 | 60.14 ± 2.87 | 57.87 ± 2.86 |
| 20 mg                       | 47.64 ± 1.94  | 42.85 ± 1.87                      | 38.21 ± 1.77 | 35.11 ± 1.61 | 32.87 ± 1.49 |                     |
| ASAs-loaded microspheres    | 40 mg         | 55.75 ± 2.85                      | 49.01 ± 2.56 | 45.14 ± 2.47 | 40.63 ± 2.36 | 37.59 ± 1.69 |
| 80 mg                       | 64.09 ± 3.14  | 58.94 ± 2.88                      | 53.49 ± 2.81 | 47.24 ± 2.52 | 42.18 ± 1.84 |                     |

The effects of egg white on paw swelling in SD rats were also examined, and the results are shown in table 4. As indicated, the ASAs-loaded microspheres presented an inhibitory effect on the swelling of the toes. In addition, although the effect of aspirin at a dose of 20 mg kg\(^{-1}\) was greater than that of DKL at a dose of 240 mg kg\(^{-1}\), superior results were obtained for the ASAs-loaded microspheres at doses of 20, 40, and 80 mg kg\(^{-1}\).

4. Conclusions

In this study, a series of biodegradable copolymers were prepared by the reaction between mandelic acid oxanhydrate (MA-OCA) and polyethylene glycol monomethyl ether (mPEG) with different molecular weights. These copolymers were used as carrier materials to prepare Alstonia scholaris leaf (ASAs)-loaded microspheres using the double emulsion method. The microspheres were characterized by their morphology, particle size, drug loading, encapsulation efficiency, and in vitro release performances. In addition, the in vitro degradation conditions and optimal drug loadings were determined for the mPEG\(_{10k}\)-PMA microspheres. In vitro release experiments showed that the microspheres could release the total alkaloids from the ASAs continuously for 15 d, and undergo complete degradation over ∼60 d. Furthermore, coagulation, hemolysis, and anti-inflammation experiments demonstrated that the microspheres exhibited excellent anti-inflammatory activities and biocompatibilities. More specifically, anti-inflammatory experiments showed that the ASAs-loaded microspheres showed a superior performance than aspirin or Dengtaiye KeLi (DKL) under the same dosage conditions. The results presented herein therefore point to a new research direction for the development of sustained release formulations.

Acknowledgments

National Natural Science Foundation of China (81760644), and the Yunnan Science and Technology Project: Major Science and Technology Specialties of Biological Medicine (2018ZF008), We would like to thank Editage (www.editage.cn) for English language editing.
Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Conflicts of interest

The authors declare no conflict of interest.

ORCID iDs

Hongli Li  https://orcid.org/0000-0002-8303-8615

References

[1] Baliga M 2012 Review of the phytochemical, pharmacological and toxicological properties of Alstonia Scholaris Linn. R. Br (Saptaparna) Chin. J. Integr. Med. 19 1–14
[2] Salim A, Garson M and Craik D 2004 New indole alkaloids from the bark of Alstonia scholaris J. Nat. Prod. 67 1591–4
[3] Shah J, Cai X, Feng T, Zhao Y, Wang J, Zhang L, Yan M and Luo X 2010 Pharmacological evaluation of Alstonia scholaris anti-inflammatory and analgesic effects J. Ethnopharmacol. 129 174–81
[4] Shah J, Cai X, Zhao Y, Feng T and Luo X 2010 Pharmacological evaluation of Alstonia scholaris: anti-tussive, anti-asthmatic and expectorant activities J. Ethnopharmacol. 129 293–8
[5] Cai X, Tan Q, Liu Y, Feng T, Du Z, Li W and Luo X 2008 A cage–monoperene indole alkaloid from Alstonia scholaris Org. Lett. 10 577–80
[6] Zhao Y, Shang J, Pu S, Wang H, Wang B, Liu L, Liu Y, Shen H, Luo X and Zhao Y 2016 Effect of total alkaloids from Alstonia scholaris on airway inflammation in rats Journal of Ethnopharmacology 178 258–65
[7] Cai X, Du Z and Luo X 2007 Unique monoperpenoid indole alkaloids from Alstonia scholaris Org. Lett. 9 1817–20
[8] Jie S and Burgess D 2012 Accelerated in-vitro release testing methods for extended-release parenteral dosage forms Journal of Pharmacy, Pharmacology 64 986–96
[9] Shen J, Lee K, Choi S, Qu W, Wang Y and Burgess D 2016 A reproducible accelerated in vitro release testing method for PLGA microspheres Int. J. Pharm. 498 274–82
[10] Chen M, Wong H, Lin K and Chen H 2009 The characteristics, biodistribution and bioavailability of a chitosan-based nanoparticulate system for the oral delivery of heparin Biomaterials 30 6629–37
[11] Dodane V and Vilivalam V 1998 Pharmaceutical applications of chitosan Pharm. Sci. Technol. Today 1 246–53
[12] Prego C, Fabre M, Torres D and Alonso M 2006 Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery J. Pharmaceutical Research 23 549–56
[13] Koyama Y, Hirano T, Kakizawa Y, Okano F, Takarada T and Maeda M 2014 pH-responsive release of proteins from biocompatible and biodegradable reverse polymer micelles J. Controlled Release 173 89–95
[14] Mukhopadhayy P, Sarkar K and Soam S 2013 Formulation of pH Responsive Carboxylethyl Chitosan and Alginate Beads for Oral Delivery of Insulin Journal of Applied Polymer Science 129 835–45
[15] Mohamed F and Walle C 2010 Engineering biodegradable polystyrene microspheres with specific drug targeting and drug release properties J. Pharm. Sci. 97 71–87
[16] Jain J P, Modi S, Domb A J and Kumar N 2005 Role of polyanhydrides as localized drug carriers J. Controlled Release 103 541–63
[17] Chickering E D, Jacob S J, Desai A T, Harrison M and Harris W 1997 Bioadhesive microspheres: III. An in vivo transient and bioavailability study of drug-loaded alginate and poly[fumaric-co-sebacic anhydride] microspheres J. Controlled Release 48 35–46
[18] Furtado S, Abramson D, Burrill R, Olivier G, Gourd C, Bubbers E and Mathiowitz E 2008 Oral delivery of insulin loaded poly(fumaric-co-sebacic) anhydride microspheres Int. J. Pharm. 347 149–55
[19] Thomas C G, Liu Z, Goddard M, Reineke J, Bailey N, Cross M, Burrill R and Mathiowitz E 2003 Enhancing the oral bioavailability of the poorly soluble drug dicumarol with a biodegradable polymer J. Pharm. Sci. 92 1677–89
[20] Wang Y, Wang S, Li H, Yao B, Chen H and Yuan M 2019 Preparation, characterization and biological activity of catherixin-DF-30-loaded poly(LA-co-MA) microspheres J. Nanosci. Nanotechnol. 19 2435–42
[21] Dong Y and Feng S 2004 Methoxy poly(ethylene glycol)-polylactide) (MPEG-PLA) nanoparticles for controlled delivery of anticancer drugs Biomaterials 25 2843–9
[22] Hans M, Shimoni K, Danino D, Siegel S and Lowman A 2005 Synthesis and characterization of MPEG-PLA Prodrug Micelles Biomacromolecules 6 2708–17
[23] Xiong L and Processing Z 2013 Preparation and in vitro properties of MPEG-PLA microspheres loaded 5-fluorouracil for controlled release Journal of Macromolecular Science: Part D—Reviews in Polymer Processing 52 268–72
[24] Min L, Gang Z and Tan Y 2012 Preparation of sustained release microspheres of MPEG-PLA nerve growth factor and their sustained release in vitro Journal of Third Military Medical University 34 2075–7
[25] Mu C, Balakrishnan F, Cui P, Yin Y, Lee Y, Choi H, Yong C, Chung S, Shim C and Kim D 2010 The effects of mixed MPEG-PLA/Pluronic copolymer micelles on the bioavailability and multidrug resistance of docetaxel Biomaterials 31 2371–9
[26] Yi W, Xia W, Wei W, Ho S, Feng G, Guan H M and Guo S 2012 Microcosmic mechanisms for protein incomplete release and stability of various amphiphilic MPEG-PLA microspheres Langmuir 28 13964–92
[27] Wei Y, Wang Y, Wang W, Ho S, Wei W and Ma G 2011 MPEG-PLA microspheres with narrow size distribution increase the controlled release effect of recombinant human growth hormone J. Mater. Chem. 21 12691–9
[28] Zhou J 2006 Experimental study in the treatment of hepatocellular carcinoma with epirubicin-loaded Poly(D,L)-lactic acid microspheres locally administered
[29] Colin B, Blanca M-V, Cossio F and Didier B 2008 Monomer versus alcohol activation in the 4-dimethylaminopyridine–catalyzed ring-opening polymerization of lactide and lactic C-carboxyl anhydride Chem. A Eur. J. 14 5304–12
[30] Olivier T, Marchal E, Martin-Vaca B, Cossío F and Bourissou D 2006 An activated equivalent of lactide toward organocatalytic ring-opening polymerization J. Am. Chem. Soc. 128 16442–3

[31] He Y, Li H, Zheng X, Yuan M, Yang R, Yuan M and Yang C 2019 Preparation, in vivo and in vitro release of polyethylene glycol monomethyl ether-polymandelic acid microspheres loaded panax notoginseng saponins Molecules 24 2024

[32] Van M, Kaspers G and Cloos J 2011 Cell Sensitivity Assays: The MTT Assay Methods Mol Biol 88 237–45

[33] Dariusz S, Steer S, Clothier R and Michael B 1993 An improved MTT assay J. immunol. methods 157 203

[34] Shen M, Li H, Yuan M, Jiang L, Zheng X, Zhang S and Yuan M 2018 Preparation of bergenin - Poly (lactic acid) polymers and in vitro controlled release studies International Journal of Biological Macromolecules 116 354–63

[35] Widder K, Senyei A and Ranney D 1979 Magnetically responsive microspheres and other carriers for the biophysical targeting of antitumor agents Advances in Pharmacology & Chemotherapy 16 213

[36] Wei Y, Wang Y, Kang A, Wang W, Ho S, Gao J, Ma G and Su Z 2012 A novel sustained-release formulation of recombinant human growth hormone and its pharmacokinetic, pharmacodynamic and safety profiles Mol. Pharmaceutics 9 2039

[37] Zheng X, Li H, He Y, Yuan M, Shen M, Yang R, Jiang N, Yuan M and Yang C 2019 Preparation and in vitro release of total alkaloids from alstonia scholaris leaves loaded mPEG-PLA microspheres Materials 12 1457

[38] Cory B, Martin K, Amanda C, Kyekyoon K and Pack D 2002 Precise control of PLG microsphere size provides enhanced control of drug release rate J. Controlled Release 82 137–47

[39] Wang K, Guo W, Tang J and Yu L 2011 Short carbon fiber reinforced polyetheretherketone for hip prosthetic biocompatibility and biomechanics Journal of Clinical Rehabilitative Tissue Engineering Research 15 6351–4

[40] Song X, Bo L, Xu K, Liu J, Wen J, Wang J, Liu X, Li J and Qi Y 2012 Cytotoxicity of water-soluble mPEG-SH-coated silver nanoparticles in HL-7702 cells Cell Biology and Toxicology 28 225–37