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Poly (lactide-co-glycolide) microspheres with good biocompatibility

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Abstract

Purpose: Curcumin and Icariin have multiple pharmacological effects and are widely used in various fields, but their short half-life, poor bioavailability, and low water solubility greatly limit their application in clinical medicine. Poly (lactide-co-glycolide) (PLGA) loaded microspheres not only solve these problems but also have no toxicity in degradation. Methods: To verify whether PLGA drug-loaded microspheres have good biocompatibility, the present experiments used the emulsification-solvent evaporation method to prepare PLGA drug-loaded microspheres and successfully performed the loading of curcumin and icariin. Results: The scanning electron microscopy showed that the particle sizes of the PLGA microspheres were 2–15 μm, icariin/PLGA microspheres were 3–22 μm, and curcumin/PLGA microspheres were 5–30 μm. Moreover, the surface of the microspheres was smooth and spherical. Furthermore, the drug loading and encapsulation rate were good. In vitro experiments revealed that the prepared PLGA microspheres were safe and nontoxic, and that they could release drugs stably and slowly. Moreover, their proliferation ability was unaffected after inoculation into bone marrow mesenchymal stem cells (BMSCs), and Alcian blue Staining was performed at last, demonstrating their biocompatibility and important applications in tissue engineering.

Keywords: Curcumin, Icariin, PLGA microspheres, BMSCs

1. Introduction

Curcumin is the main component of the turmeric plant and is approved by the US Food and Drug Administration (FDA) for its wide range of pharmacological effects, including antiinflammatory and antitumor effects [1], [23]. Curcumin prevents the progression of osteoarthritis in animal models by inhibiting inflammatory factors [27]. Moreover, its antiinflammatory and antioxidant properties inhibit cardiomyocyte apoptosis in myocardial injury [15]. Curcumin can also inhibit tumor cell proliferation and thus achieve antitumor effects. Some studies have shown that it can prevent tumor development [10]. The main component of the traditional Chinese medicine Epimedium is icariin, which has antiinflammatory, antiapoptotic, antitumor, and neuroprotective effects [21], [25], [30]. Icariin can further alleviate the symptoms of osteoarthritis by inhibiting the action of tumor necrosis factor and other pro-inflammatory factors, thus preventing chondrocyte apoptosis [12].

Although curcumin and goat weed glycosides have many beneficial effects, they are limited in clinical application due to their low water solubility, poor bioavailability, and short half-life [28], [22]. A solution has emerged in the form of drug delivery systems to overcome these problems [3].

Drug delivery systems are technologies that fully control the distribution of drug presence in the body in terms of space, time, and dose [8]. Such systems include microspheres, hydrogels, and liposomes [2]. Microspheres are currently used in various applications, including medicine, materials, and food safety. In medicine, they are primarily used in the targeted treatment of tumors [4] and repair of articular cartilage in osteoarthritis [11], among others. These advantages of microspheres include good biomechanical properties, controllable drug release, and precise targeting to achieve the therapeutic purpose [19]. Meanwhile, the commonly used polymer materials for microsphere synthesis are chitosan, collagen, starch, PLLA- poly-L-lactic acid [26] PEG- Polyethylene glycol, PVP- poly(vinyl pyrrolidone) and poly(vinyl alcohol) [16] and PLGA- poly (lactide-co-glycolide) [7], which is the most widely used in the synthesis of microspheres. PLGA is composed of lactide and glycolide [17], [20]. It is one of the polymers approved by the US FDA for human use because it reduces drug toxicity while also reducing drug-induced irritation [18]. The drug-loaded microspheres were created using an emulsification-solvent evaporation method. As synthetic polymers, PLGA drug-loaded microspheres have good biomechanical properties, controlled biodegradation, and nontoxicity of their own degradation products [14], which can not only improve bioavailability but also reduce the number of drug administration times [24].

In this paper, PLGA microspheres were prepared by emulsification-solvent evaporation method. Moreover, the microscopic morphology of the microspheres was investigated, and their drug loading and encapsulation rate were measured. Their ability to be released in vitro was examined, and the PLGA microspheres were fabricated into an extract and then added to BMSCs to determine whether they were biocompatible.

2. Materials and methods

2.1 Main raw materials and reagents

Curcumin, Icariin, trypsin (Shanghai Aladdin Biochemical Technology Co., Ltd), DMSO -dimethyl sulfoxide (AR, Tianjin Damao Chemical Reagent Factory), Dichloromethane (Shanghai McLean Biochemical Technology Co., Ltd), PVA -polyethylene alcohol (Type 1788, Shanghai Aladdin Biochemical Technology Co., Ltd), anhydrous ethanol (AR, Sinopharm Chemical Reagent Co., Ltd), PBS -Phosphate Buffer Solution (Shanghai Dianrui Biotechnology Co., Ltd), Alcian, Paraformaldehyde (Sigma, USA), PLGA -Poly (lactide-co-glycolide) (Guangzhou Weihua Biotechnology Co., Ltd), DMEM medium (Sigma, USA), and BMSCs -Bone marrow mesenchymal stem cells (provided by Institute of Metals, Chinese Academy of Sciences). The above raw materials and reagents were used in the following experiments.

2.2 Preparation of curcumin, icariin/PLGA microspheres, and blank PLGA microspheres

PLGA microspheres were prepared by the emulsification-solvent evaporation method. The specific process is as follows: 100 mg of PLGA was dissolved in 5 ml of methylene chloride as oil phase at room temperature. Then, 0.5 g of polyvinyl alcohol (PVA) was added to 99.5 g of deionized water to prepare 0.5% PVA solution as external aqueous phase. Subsequently, the above-prepared oil phase was added drop-by-drop to the external aqueous phase and emulsified for 2 min with the probe of German IKAT 25 tissue disperser at 14,000 r/min in an ice bath. It was then placed in a magnetic stirrer at room temperature for 12 h for evaporation to remove dichloromethane and thus obtain the microsphere solution. Then, the microsphere solution is put in a centrifuge with 10000 r/min centrifugation for 10 min. Subsequently, we discard the supernatant to obtain the initial microspheres, deionize water washing, and conduct centrifugation again. This process was repeated three times until the excess PVA solution was removed, and then it was frozen in the storage for 24 h. A microsphere powder was obtained for use. After freeze-drying the samples, we obtained the PLGA microspheres and stored in the freezer.

Meanwhile, the PLGA drug-loaded microspheres were prepared according to the following method: curcumin and icariin were prepared by adding 50 mg each of curcumin and icariin to 0.2 ml of DMSO, respectively, in an EP tube, which was dissolved by oscillation and added to the oil phase of the above method. Then, it was added to the external aqueous phase to prepare curcumin/PLGA microspheres and icariin/PLGA microspheres [6], [29]. The

curcumin/PLGA microspheres and epimedoside/PLGA microspheres were prepared by adding the solution to the external aqueous phase.

2.3 Scanning Electron Microscope (SEM) observation

Curcumin/PLGA-loaded, Icariin/PLGA-loaded, and blank PLGA microspheres were attached to silicon wafers adhered with conductive adhesive tapes. Then, the unadhered powder was blown off, given a gold spraying treatment, and placed under the SEM for observation.

2.4 Determination of drug loading capacity (DLC) and encapsulation efficiency (EE)

The curcumin/PLGA and icariin/PLGA microspheres were accurately weighed at 5 mg each. Moreover, 5 mL of 0.1 mol/L NaOH solution was added to a clean test tube and shaken in a constant temperature oscillator for 12 h, Subsequently, 0.2 mL of the solution was taken to another clean test tube, and PBS was added to 5 mL. The OD was measured using a UV-spectrophotometer, and the concentration of the drug was plotted on the standard curve. The concentration of each drug was determined based on the calculation formula. The drug loading capacity (DLC) and encapsulation efficiency of curcumin/PLGA and icariin/PLGA microspheres were obtained:

$$DLC(\%) = \frac{Weight\ of\ drug\ in\ microspheres}{Weight\ of\ microspheres} \times 100\%$$

$$EE(\%) = \frac{Actual\ drug\ loading\ content}{Theoretical\ drug\ loading\ content} \times 100\%$$

2.5 Plotting of standard curves for pharmaceuticals

Curcumin was prepared into solutions with mass concentrations of 5, 10, 20, 40, and 80 μg/mL in DMSO and PBS as solvents. Meanwhile, icariin was prepared into solutions with mass concentrations of 10, 20, 40, 80, and 160 μg/mL in the same solvents [13]. The prepared curcumin and icariin solutions were scanned at 300–800 nm wavelengths using an ultraviolet (UV)–visible spectrophotometer (Shanghai Meppan Instrument Co., Ltd.). The maximum absorption peaks of curcumin and icariin were determined.

2.6 In vitro release

Curcumin/PLGA microspheres and icariin/PLGA microspheres were accurately weighed 10 mg each, put into a centrifuge tube, and added with 10 mL of PBS buffer solution. The microspheres were then shaken at 100 r/min in a constant temperature shaking chamber at 37°C, and 0.2 mL of the samples were taken at 0.2, 0.5, 1, 2, 4, 10, 20, 40, 60, 80, and 100 h,

147 respectively. They were then diluted and analyzed by UV-spectrophotometer. A photometer was
148 used to determine the amount and release rate, which was then supplemented with 0.2 mL of
149 PBS buffer solution. Finally, the drug release curve was plotted.

150 *2.7 Determination of cytotoxicity of drug-loaded microspheres by CCK-8 assay*

151 The freeze-dried microspheres were divided into three groups: PLGA, icariin/PLGA, and
152 curcumin/PLGA slow-release microspheres. To obtain the sample extracts, we took 50 mg of
153 each of the three samples and added them to a clean test tube for 24 h of UV irradiation to
154 remove the bacteria. Then, 3 ml of medium was added to the sample to soak it for 24 h and then
155 filtered through a filter membrane to obtain the extracts.

156 The BMSCs were digested with trypsin to make a cell suspension, diluted to a
157 concentration of 1×10^4 cells/mL, inoculated into 96-well plates. The experiments were divided
158 into five groups, namely, blank, control, and experimental groups. In particular, the blank group
159 was not inoculated with BMSCs; it was placed in the incubator for 12 h. The medium was
160 sucked out, in which the blank group (without BMSCs) was added to the medium, and the
161 control group (with BMSCs) was added to the aforementioned three kinds of extracts. Moreover,
162 the experimental group was placed in the CO_2 incubator at 37°C with a concentration of 5%.
163 Then, we discard the medium and extracts in the 96-well plate and add 150 μL of medium and
164 15 μL of CCK-8 working solution and mix. The solution was then incubated for 3 h in an
165 enzyme labeling instrument to detect absorbance at 450 nm (OD), according to the above steps,
166 respectively. It was used in the first, third, and fifth day. Then, the OD was measured.

167 *2.8 Alcian blue Staining*

168 Curcumin/PLGA microspheres, icariin/PLGA microspheres and PLGA microspheres were
169 formulated into extracts with concentrations of 0.6 μg/mL. Then added into 6-well plates
170 containing BMSCs for culture. Fixed with 4% paraformaldehyde for 1 hour at room temperature
171 after 2 weeks, and stained with Alcian blue (Sigma, USA) at a concentration of 1% for 2 hours.
172 Optical microscope was undertaken and photomicrographic images were taken.

173 ## 3. Results

174 *3.1 Morphology and particle size distribution of PLGA, curcumin/PLGA, and*
175 *icariin/PLGA microspheres*

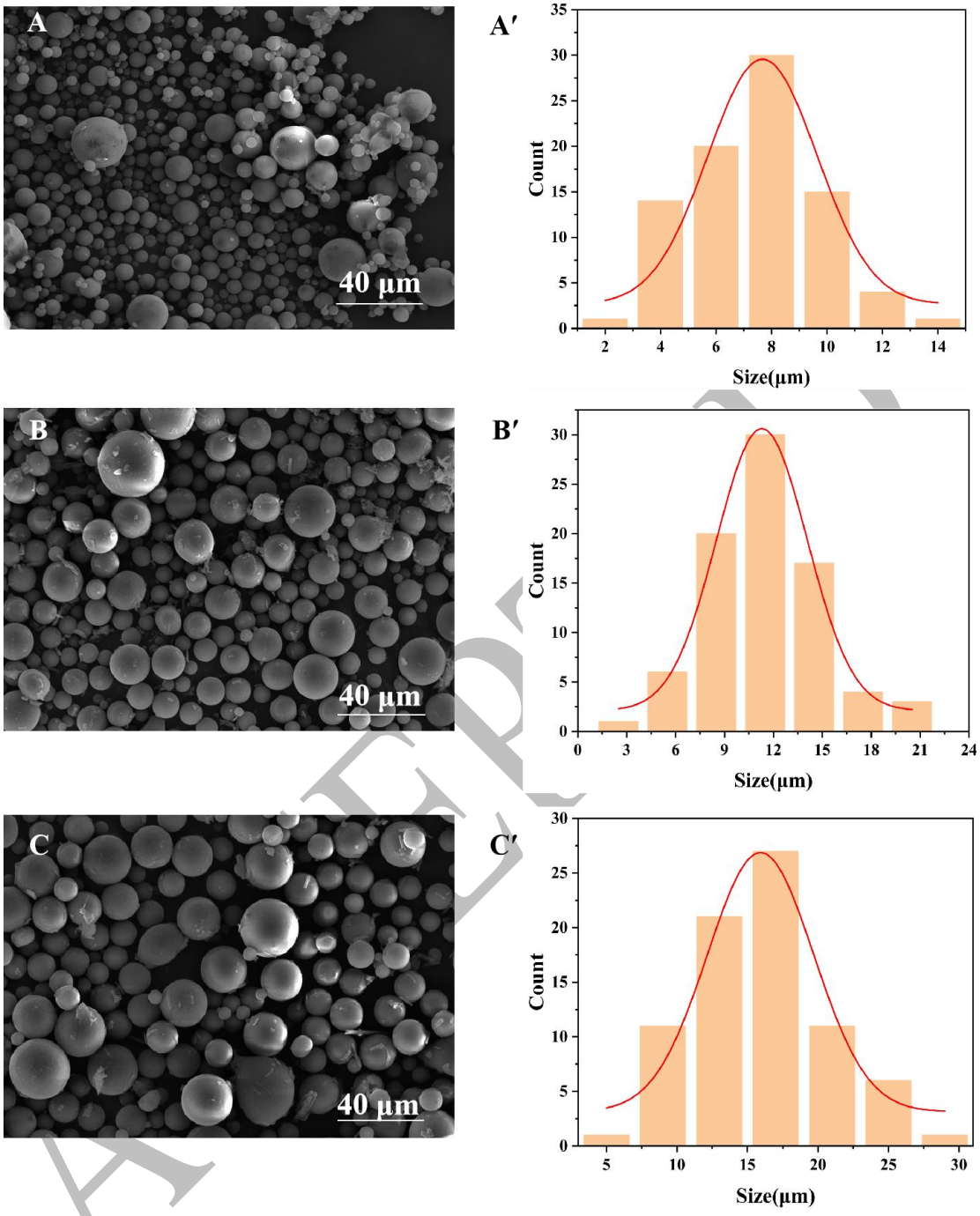

Figure 1. SEM images of microspheres: (A) PLGA microspheres; (B) Icariin microspheres; and (C) Curcumin microspheres. Microsphere particle size distribution:(A′) PLGA microspheres; (B′) Icariin microspheres; (C′) Curcumin microspheres. Scale bar: 40 μm.

Curcumin and Icariin PLGA microspheres were prepared using an emulsification-solvent evaporation method with PLGA and curcumin and icariin, respectively. “A” in Figure 1 is the microspheres synthesized by PLGA observed by SEM, and A′ represents the histogram of the

particle size distribution of the PLGA microspheres with diameters ranging from 2 to 15 μm. Meanwhile, B in Figure 1 is the PLGA-synthesized patchouli glycoside microspheres, and B′ is the histogram of the particle size distribution of such microsphere with diameters of 3–22 μm. C is the PLGA-synthesized curcumin microspheres, whereas C′ is the histogram of the particle size distribution of curcumin/PLGA microspheres with diameters ranging from 5 to 30 μm. The Curcumin and epimedoside prodrugs were crystalline or striated under the electron microscope, and Curcumin and Icariin were loaded into PLGA microspheres. It is presented as a sphere under SEM. (Figure 1 B and C).

3.2 Curcumin/PLGA and Icariin/PLGA microsphere standard curve plotting

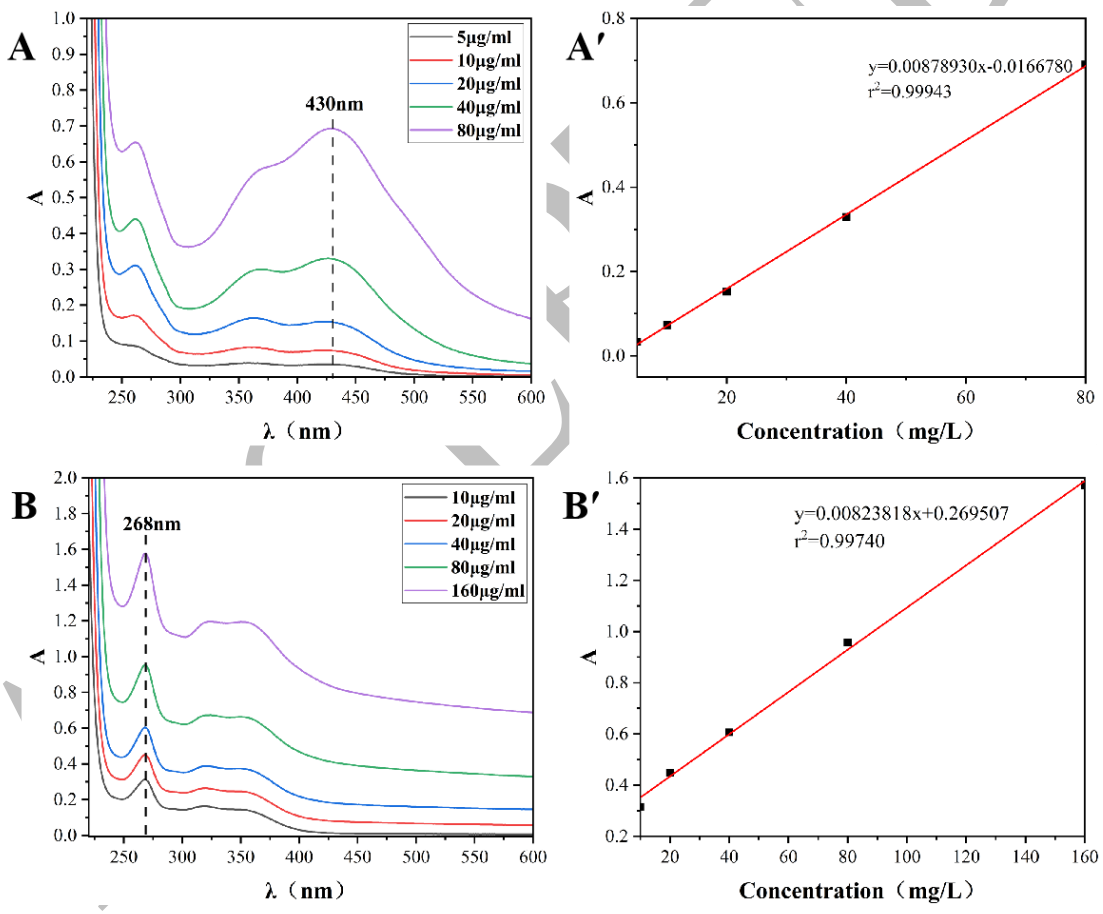

Figure 2. (A) Ultraviolet scanning spectrum of curcumin; (B) Ultraviolet scanning spectrum of icariin; (A′) Standard curve of curcumin absorbance and concentration; (B′) Standard curve of icariin absorbance and concentration.

Curcumin solution and icariin solution prepared with mixed solvents of DMSO and PBS were measured by UV–visible spectrophotometer at the wavelengths of the maximum

absorption peaks of curcumin and icariin, respectively. Regression curves were made with the absorbance (Abs) and the mass concentrations of curcumin and icariin, respectively. Figure 2 shows the results of scanning curcumin and icariin solutions between 300 and 800 nm, in which DMSO and PBS have no absorption at this position. Therefore, the wavelengths of the maximum absorption peak of the curcumin and icariin solutions were 430 and 268 nm, respectively.

After determining the wavelengths of the two drugs, regression of the mass concentrations of the respective solutions was carried out at 430 and 268 nm. According to the concentrations of the prepared solutions, The regression equations of the standard curves were obtained as $y = 0.00878930x - 0.0166780$ and $y = 0.00823818x + 0.269507$, respectively. curcumin had a correlation coefficient of $r^2 = 0.99943$, whereas icariin had a correlation coefficient of $r^2 = 0.99740$, indicating that the mass concentrations of curcumin and icariin were linearly correlated with their respective absorbances.

3.3 Drug loading capacity (DLC) and encapsulation efficiency (EE) of curcumin/PLGA and icariin/PLGA microspheres

Table 3. Drug loading capacity (DLC) and encapsulation efficiency (EE) of two microspheres

	DLC（%）	**EE（%）**
Curcumin/PLGA microspheres	37.23%±2.4%	74.47%±4.9%
Icariin/PLGA microspheres	11.83%±2.7%	23.67%±5.5%

The size of DLC and EE directly determine the performance of drug-loaded microspheres: the larger the value, the more it indicates high utilization of raw materials and the easier it is to meet the experimental needs in the organisms' body. The present experiment solved the problems of curcumin and Epimedium glycosides with short half-life and poor bioavailability.

Table 3 presents the data that were obtained according to the calculation formula. The DLC and EE of curcumin/PLGA microspheres were 37.23% ± 2.4% and 74.47% ± 4.9%, respectively. Meanwhile, the DLC and EE of the icariin/PLGA microspheres were 11.83% ± 2.7% and 23.67% ± 5.5%, respectively.

Obviously, the porous structure of the microspheres improves the specific surface area and adsorption capacity of the carrier. This result indicates that the two drug-loaded microspheres have good Drug loading capacity (DLC) and encapsulation efficiency (EE), which lays the foundation for application in tissue engineering or clinical experiments.

3.4 Bone marrow mesenchymal stem cell morphology

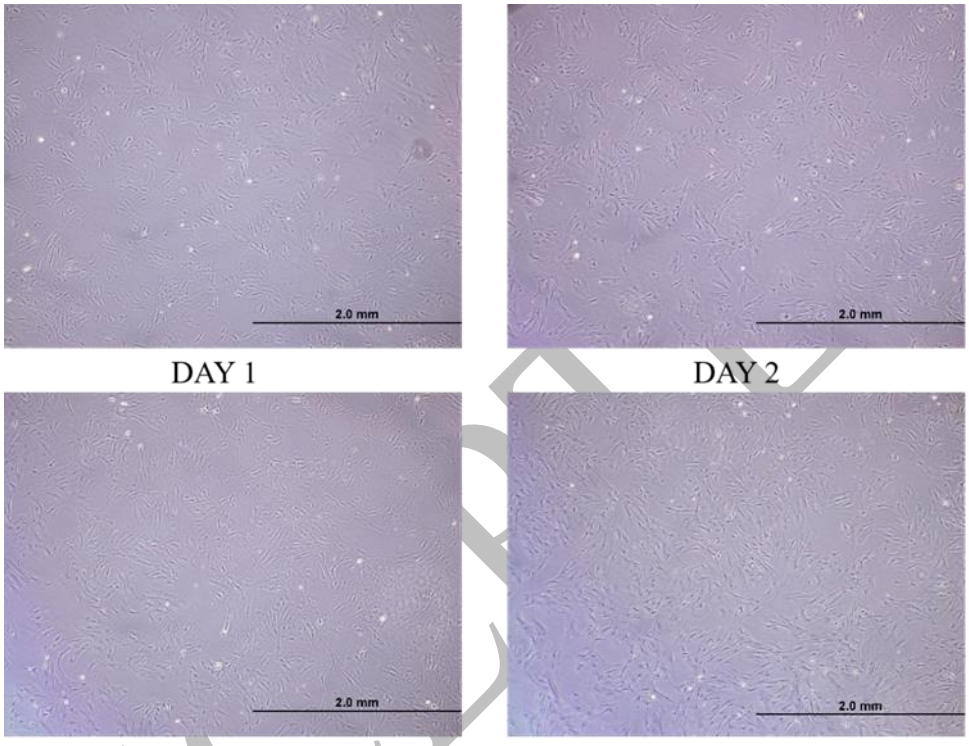

Figure 4. Optical microscope images of BMSCs cultured in an incubator after 1, 2, 3, and 4 days (magnification ×4).

BMSCs are progenitor cells with proliferation and multidirectional differentiation potential, which can differentiate toward bone, cartilage, myocytes, and other directions. Figure 4 shows the morphology of BMSCs cultured in culture flasks for 1, 2, 3, and 4 days, the number of which gradually increased. Moreover, the morphology was shuttle or spindle, which was consistent with the cellular morphology of BMSCs, indicating that this group of cells can proliferate.

3.5 In vitro drug release profile

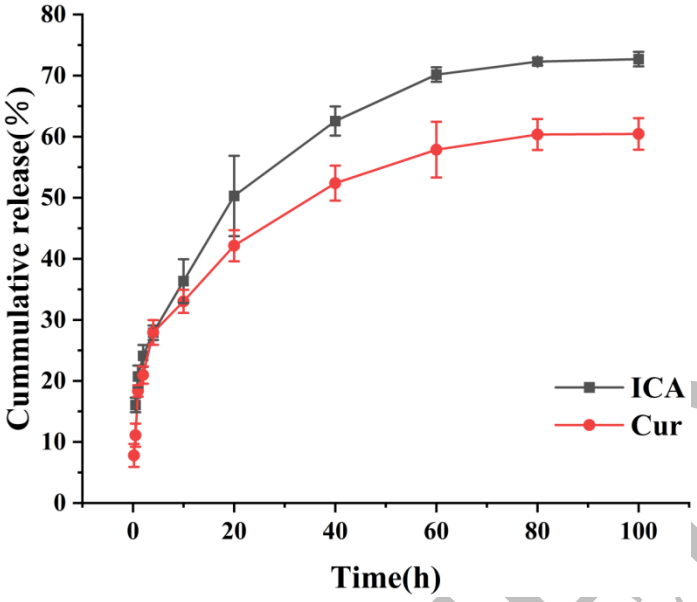

Figure 5. In vitro release profiles of curcumin and icariin from PLGA microspheres.

Fig. 5 shows the in vitro drug release curves of curcumin and patchouli glycoside PLGA microspheres. As shown in Figure 5, the release of the two types of drug-loaded microspheres is a slow and sustained process, The slope of the curve gradually decreases, which means that the drug releases more slowly over time. In the early phase (0–20 h), the release was sudden and became gradual and slow with the progress of time. In addition, the release of the drug is divided into two phases (Fig. 5), The first phase is burst release, which is often unencapsulated drug particles or drugs adhered to the surface of microspheres. The second phase is a slow release phase the drug diffuses outward through the polymer core, and the speed is slow. In the 100th h, the release rates of curcumin and patchouli glycoside drug-loaded microspheres reached 60.4% ± 2.58% and 72.7% ± 1.19. At 100 h, the release rates of curcumin and icariin were 60.4% ± 2.58% and 72.7% ± 1.19%, respectively.

3.6. The effect of drug-loaded microspheres on cell proliferation and their safety and nontoxicity (CCK-8)

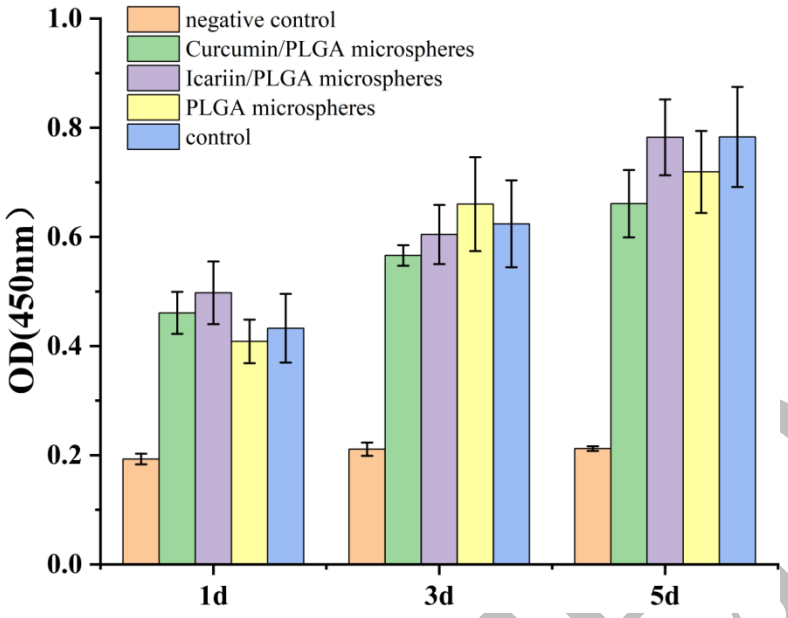

Figure 6. CCK-8 results of BMSCs cultured in microsphere extracts at 1, 3, and 5 days (n = 3; $P < 0.05$).

Cytotoxicity is an important indicator of an organism's ability to use drug-loaded microspheres. Figure 6 shows the CCK-8 results of the experimental groups cultured in their respective microsphere extracts and the control and blank groups for 1, 3, and 5 days. The CCK-8 values of drug-loaded microspheres and blank PLGA microspheres increased in the experimental group, indicating that the drug was safe and nontoxic. Meanwhile, the control group, which contained only BMSCs and medium, showed a steady increase in CCK-8 values, indicating that the BMSCs could proliferate. Lastly, the blank group, which had no BMSCs and was not loaded with drug-loaded microspheres and contained only medium, did not show a significant change in the CCK-8 values. The results of these data show that PLGA drug-loaded microspheres have good biocompatibility, and they are also a prospective study for subsequent application in biological experiments.

3.6. Alcian blue Staining

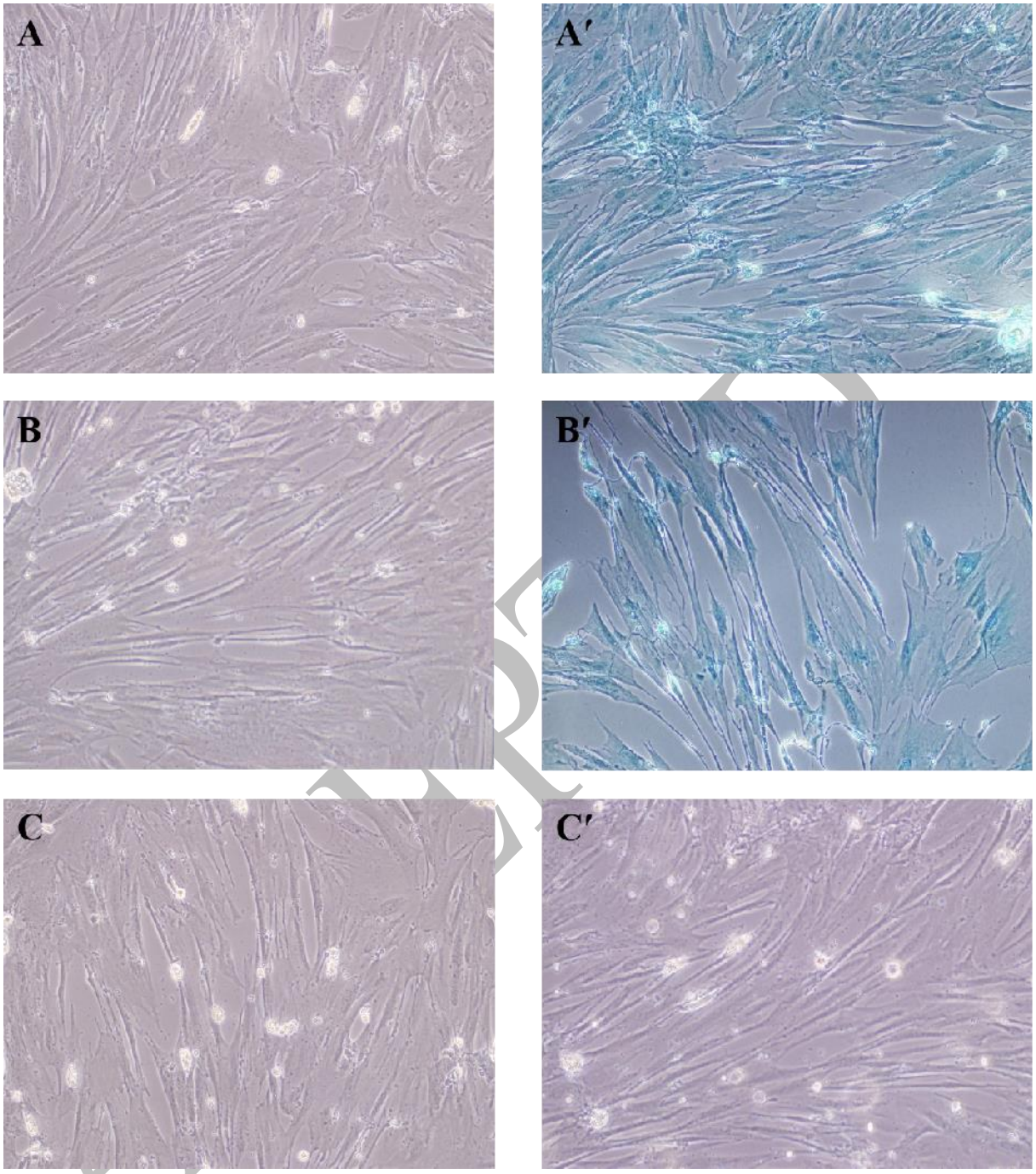

Figure. 7. (A) Optical microscope images of BMSCs and Curcumin/PLGA microspheres cultured in an incubator; (B) Optical microscope images of BMSCs and icariin/PLGA microspheres cultured in an incubator; (C) Optical microscope images of BMSCs and PLGA microspheres cultured in an incubator; (A′) Alcian blue Staining of BMSCs and Curcumin/PLGA microspheres; (B′) Alcian blue Staining of BMSCs and icariin/PLGA microspheres; (C′) Alcian blue Staining of BMSCs and PLGA microspheres. (magnification ×20)

The experiments were divided into two groups (Fig. 7), namely, blank and experimental

groups, The corresponding drug-loaded microspheres or blank PLGA microspheres were added to each well plate. After 2 weeks of culture, it was found that the morphology of the cells began to change after adding the drug-loaded microspheres, showing obvious spindle shape and spindle shape. Positive Alisin Blue staining, BMSCs with a tendency to differentiate. In addition, it also indirectly verified that the drug-loaded microspheres were safe and non-toxic.

4. DISCUSSION

In this study, curcumin and icariin were loaded into PLGA microspheres to determine their characteristics and evaluate the biocompatibility of the prepared PLGA drug-loaded microspheres. Biocompatibility not only requires that biomaterials have low toxicity, but also requires that biomaterials can stimulate the corresponding functions of the body in specific applications. Synthetic polymer materials have developed rapidly in the fields of tissue engineering and biomedicine, such as drug delivery systems, osteochondral proliferation, and tumor treatment. Among them, PLGA drug-loaded microspheres have developed rapidly. In theory, Curcumin and Icariin have anti-inflammatory and anti-tumor effects, but their application is limited due to short half-life and poor bioavailability. Synthetic polymer materials have the advantages of longer half-life in the human body and suitable biodegradation kinetics, which can not only solve the problem of poor bioavailability, but also help to increase the release cycle of drugs. According to the in vitro release experiment (Fig. 5), the release of the two types of drug-loaded microspheres is a slow and sustained process. In the initial release of the drug in the time period of 0-20 h for the burst release phase. The drug adsorbed in the surface layer of the carrier was rapidly dissolved. Which caused the release rate to rise sharply; The second phase is a slow release phase, the amount of drug release decreases and the release rate slows down, This is equivalent to improving bioavailability. The release rate is affected by many factors, such as the morphology of the microspheres, the particle size of the microspheres, the solubility of the drug in the solvent, etc. We can change the release rate by changing these factors, and can explore other methods to improve the release rate in further research. We also observed the microstructure of PLGA drug-loaded microspheres, which showed spheres with rounded appearance. And through further determination of DLC and EE, we proved that we

311 successfully prepared two kinds of PLGA drug-loaded microspheres. However, we found that
312 this is not the same size as the microspheres obtained by others [9]. The three microspheres of
313 varying sizes could be linked to the following reasons: 1) different aqueous/oil phase ratios; 2)
314 emulsifier selection and stability after emulsification; 3) different rotational speed sizes and
315 times; and 4) drug-loading-related factors. However, according to the in vitro release
316 experiment and the encapsulation efficiency and drug loading of the two drug-loaded
317 microspheres, the successful preparation of these two PLGA drug-loaded microspheres solved
318 the problems of short drug half-life and poor bioavailability.

319 Although the CCK-8 experiment can verify that the two PLGA drug-loaded microspheres
320 are safe and non-toxic, it does not determine the differentiation direction of BMSCs. If
321 necessary, we need further research. It has been reported [5] that BMSCs can differentiate into
322 cartilage, osteogenesis and adipogenesis after adding some growth factors or drugs. We added
323 two kinds of drug-loaded microspheres into BMSCs for culture and found that the results of the
324 staining experiment were positive by Alisin blue staining experiment (Fig. 7), and it is necessary
325 to do further experiments to prove the type of cell differentiation, but we have proved that the
326 two kinds of PLGA-loaded microspheres can promote the proliferation and differentiation of
327 BMSCs. In summary, PLGA drug-loaded microspheres have good biocompatibility, which
328 proves that the material is suitable for application in the biomedical field, and has made a
329 prospective study for further clinical application.

330 Although the results of this study demonstrated that the two PLGA-loaded microspheres
331 have good biocompatibility, the experiment was mainly conducted in vitro, and cannot be
332 compared with the complex environment in living organisms, such as immune rejection or
333 whether the phenomenon of burst release can cause serious adverse reactions, etc. So it is crucial
334 to evaluate whether the biomaterials can be used in a clinical environment in the long term.

335 ## 5.Conclusion

336 In this experiment, PLGA microspheres, namely, PLGA drug-loaded microspheres of
337 curcumin and icariin, were prepared by the emulsification–volatilization method, with particle
338 sizes of 2–15, 5–30, and 3–22 μm, respectively. Among them, the drug-carrying capacity and
339 encapsulation rate of curcumin/PLGA microspheres were 37.23% ± 2.4% and 74.47% ± 4.9%,

and that of icariin/PLGA microspheres were 11.83% ± 2.7% and 23.67% ± 5.5%,respectively. In vitro release experiments showed that the two kinds of drug-loaded microspheres had a good slow-release effect. Then, they were added to BMSCs and detected with CCK-8 reagent after making them into an extract, respectively. Moreover, The results showed that the two drugs loaded on PLGA microspheres were safe and non-toxic, and had good biocompatibility. This experiment solved the problems of curcumin and epimedoside's short half-life and poor bioavailability, and it is a prospective study for the future use of drug-loaded microspheres in the clinic.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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