Research article

Preparation, characterisation and *in vitro* anti-inflammatory activity of Baicalin microsponges

Miao Li [a,b,1], Jiajie Gan [a,c,1], Xuhui Xu [a,b,1], Shuai Zhang [a,c], Yuanyuan Li [a], Le Bian [a,c], Zibo Dong [a,b,c,*]

[a] *School of Pharmacy, Jiangsu Ocean University, Lianyungang, 222005, PR China*
[b] *Jiangsu Key Laboratory of Marine Pharmaceutical Compound Screening, Lianyungang, 222005, PR China*
[c] *Jiangsu Marine Pharmaceutical Resources Development Engineering Research Center, Lianyungang, 222005, PR China*

ARTICLE INFO

ABSTRACT

Baicalin, a flavonoid extracted from traditional Chinese medicine, *Scutellaria baicalensis* has significant anti-inflammatory effects. Microsponges are drug delivery systems that improve drug stability and slow the release rate. The combination of baicalin and the microsponges produced a new and stable system for its delivery, resulting in a novel formulation of baicalin. Baicalin microsponges (BM) were prepared using the quasi-emulsion solvent diffusion method. Effects of the mass ratio of the polymer (ethylcellulose) to baicalin, the concentration of the emulsifier polyvinyl alcohol (PVA), the stirring speed on the encapsulation efficiency (EE), and yield of the microsponges were investigated by combining the one-factor test and Box-Behnken design (BBD). The preparation process was standardised using 2.61:1 mass ratio of ethyl cellulose to baicalin, 2.17% concentration of PVA, with stirring at 794 rpm. Optimised BM formulations were evaluated for the parameters of EE (54.06 ± 3.02)% and yield of (70.37 ± 2.41)%, transmission electron microscopy (TEM), and *in vitro* cell evaluation. Results of the *in vitro* anti-inflammatory assay showed that baicalin microsponges-pretreated-lipopolysaccharide (LPS)-induced RAW264.7, mouse macrophages showed reduced inflammatory response, similar to that seen in baicalin-treated macrophages.

1. Introduction

Microsponge delivery systems (MDS) are sponge-like polymer microspheres with many channels and pores on the surface and inside. They consist of porous microspheres with particle sizes below 300 μm and are used as polymer delivery systems [1]. Compared to traditional topical drug formulations, MDS have better compatibility and high drug-loading capacity. Microsponges of tens of micrometres contain tens of thousands of micropores with a large specific surface area, which can adsorb a wide range of active ingredients (e.g. volatile oils, emollients, antifungal agents, anti-infective agents, sunscreens, and perfumes), and can be prepared in different forms, such as creams, gels, soaps, and emulsions [2].

Baicalin (BAI) is a naturally occurring flavonoid compound extracted and isolated from the dried root of Scutellaria baicalensis, a dicotyledonous plant of the family Labiatae, with a variety of pharmacological activities such as anti-bacterial [3], anti-inflammatory

---

[4], anti-thrombotic [5], against diarrhoea, detoxifying action, haemostasis and anaesthetic, and anti-metamorphosis, improvement of metabolic abnormalities and intestinal flora, repair of lung damage, anti-radiationand, and anti-cancer effects [6–11]. The baicalin-mediated PI3K/AKT pathway attenuates lipopolysaccharide (LPS)-induced cardiomyocyte apoptosis and inflammatory response [12], myocardial injury by polarising M1 to M2 in macrophages, inhibits apoptosis, and myocardial inflammatory response after ischaemia/reperfusion [13]. Baicalin may also protect lung function and achieve anti-inflammatory effects by regulating the expression of the TLR4/NF-κB signalling pathway and inhibiting inflammatory responses [14,15], and may also achieve anti-inflammatory effects in experimental colitis by inhibiting the activation of the TLR4/NF-κB pathway [7]. Baicalin also ameliorates neuroinflammation-induced depressive-like behaviour [12,16,17]. Baicalin inhibits the production of inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, etc., as well as inflammatory mediators such as nitric oxide, prostaglandins, leukotrienes, and reactive oxygen species, thus achieving anti-inflammatory effects [9,18].

Baicalin is mainly used as a dispersible tablet, capsule, and soft capsule in pharmaceutical preparations. Baicalin has also been extensively studied for the construction of functional medical materials such as copper metal-organic skeleton materials for drug delivery systems [19], and lipid-based systems. Lipid-based systems include the targeted delivery of nanoliposomes. Lipid-based systems include targeted-delivery nanoliposomes, solid lipid nanoparticles, nanoemulsions, and self-microemulsifying drug delivery systems [20]. Novel formulations of baicalin have been examined, and baicalin microsponges have been found to be promising medical materials with slow-release properties for pharmaceutical applications.

As drug reservoirs, microsponges release drugs slowly and continuously for 8–24 h [21]. The globular structure of the microsponge formed many pores, inside which the drug was wrapped. The structure of a microsponge has a natural protective effect on drugs, preventing the influence of adverse external environments and improving drug stability. Microsponges also exhibit the slow release effect; however, because of the existence of pores in the sphere, the dissolution of the drug takes more time, and the drug can be released according to requirement under certain conditions (such as pressure and temperature) to play its role. Baicalin microsponges exhibit stability and slow release; therefore, they have extensive prospective uses.

In this study, the quasi-emulsion solvent diffusion method [22] was used to obtain a stable and feasible process for the preparation of baicalin microsponges (BM) by using the encapsulation rate and yield of baicalin microsponges as indicator. The process parameters for the preparation of baicalin microsponges were optimised using a one-way test and the Box-Behnken response surface design (BBD) method, and were then validated. In addition to qualitative and quantitative analyses and routine characterisation of the microsponges, an *in vitro* release test was used to examine whether the microsponges had sustained release. In addition, lipopolysaccharide (LPS) was used to induce mouse macrophage (RAW264.7) cells *in vitro* to establish an inflammatory model and examine the anti-inflammatory effect of BM, to provide data for the development of baicalin microsponge drugs. The research roadmap of this article was shown in Fig. 1.

2. Materials and methods

2.1. Materials and reagent

Baicalin active pharmaceutical ingredients (API) (Xi'an Baikang Biotechnology Co., Ltd., Xi'an, China); Baicalin Control (China Academy of Food and Drug Administration, Beijing, China); Methanol (Shanghai McLean Biotechnology Co., Ltd., Shanghai, China);
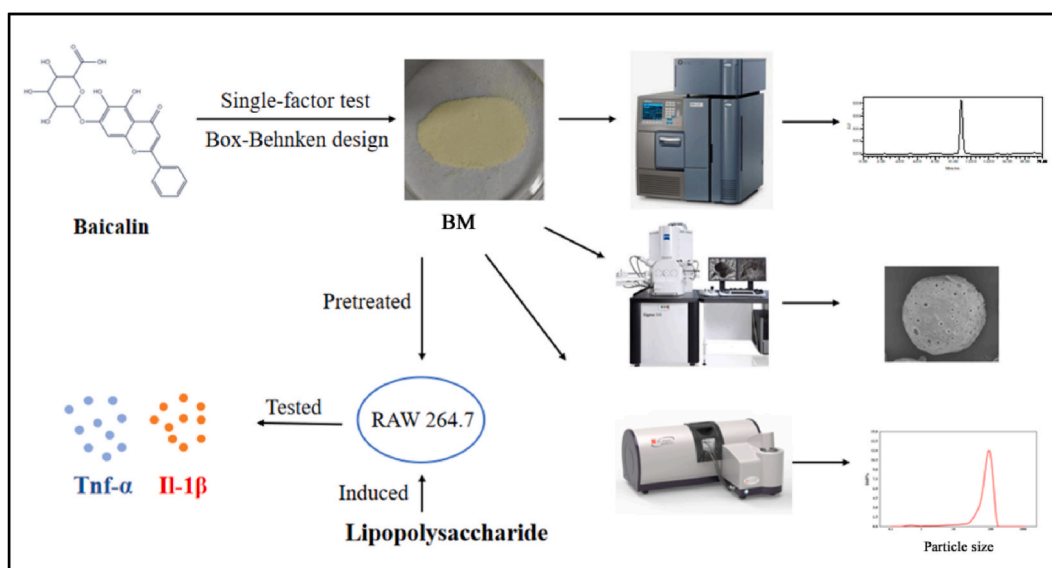
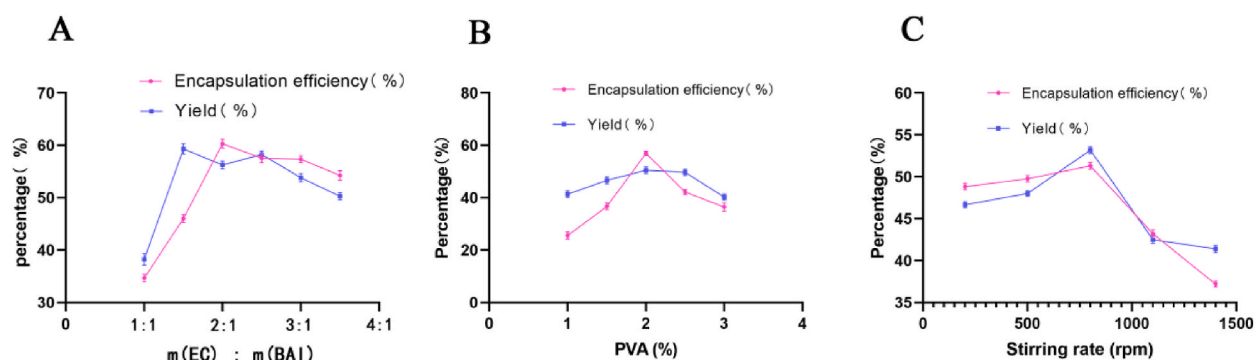

Fig. 1. Brief research roadmap.

Fig. 2. Results of the one-factor test.

Polyvinyl Alcohol (Chengdu West Asia Chemical Co., Ltd., Chengdu, China); Ethyl Cellulose (Chengdu West Asia Chemical Co., Ltd., Chengdu, China); Methylene Chloride (Sinopharm Group Chemical Reagent Co. Ltd., Shanghai, China); phosphoric acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); foetal bovine serum (Biological Industries, USA); sodium bicarbonate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); trichloromethane (Tianjin YD Chemical Reagent Co., Ltd., Tianjin, China); methanol (Tianjin YD Chemical Reagent Co. Ltd., Tianjin, China); ethanol (Tianjin Yongda Chemical Reagent Co., Ltd., Tianjin, China); iso-propanol (Tianjin Yongda Chemical Reagent Co., Ltd., Tianjin, China); IL-1β kit (Thermo Fisher Scientific, USA); TNF-α kit (Thermo Fisher Scientific, USA); ELISA reagents (SINOPACIFIC Group Chemical Reagent Co. Ltd., Shanghai, China); ELISA kit (Thermo Fisher Scientific, USA); ultrapure water (Speed Instrument Technology Co., Ltd., Shanghai, China).

2.2. Experimental equipment

BSM-120.4 Electronic Balance (Shanghai Joujing Electronic Technology Co., Ltd., Shanghai, China); MS105DU Electronic Balance (Mettler Toledo Technology Instrument Co., Ltd., Switzerland); Alliance e2695 High Performance Liquid Chromatograph 2998 PDA Detector (Waters, USA); Sigma 500 Scanning Electron Microscope (Zeiss, Germany); BT-9300S Laser Particle Sizer (Dandong Baxter Instrument Co., Ltd., Dandong, China); KQ2200DE CNC Ultrasonic Cleaner (Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China); Gradient PCR Instrument (Beijing Dongsheng Innovative Bio-technology Co., Ltd., Beijing, China); Ultra-clean Workbench (Beijing Yatai Cologne Instrument Technology Co. Ltd., Beijing, China); Small Benchtop High Speed Centrifuge (Eppendorf, Germany); CO2 Constant Temperature Incubator (Wiggens, Germany); Benchtop Constant Temperature Shaking Incubator (Tianjin Leibertechnik Instrument Co., Ltd., Tianjin, China); Multi-functional Microplate Tester (Bio-Tek, USA).

2.3. Experimental methods

2.3.1. Preparation of baicalin microsponges

This product is prepared by the quasi-emulsion solvent diffusion method and consists of two phases: internal and external. The outer phase consisted of water and polyvinyl alcohol (PVA). The internal phases comprise baicalin, dichloromethane, and ethyl cellulose. Weigh an appropriate amount of polyvinyl alcohol in a conical flask. Add a certain amount of purified water, wait until it is fully dissolved, and then stir with a magnetic stirrer until uniformly mixed, to form the external phase; add baicalin, ethyl cellulose, methylene chloride and incubate in a 60 °C water bath, stirring and heating to fully mix uniformly to form the internal phase; add the internal phase to the external phase drop by drop while it is still hot, and continue stirring for 3 h (for adequate evaporation of organic solvents), and wait until the precipitation is complete. After that, filter, separate, and wash this solution with water for microsponges, and dried in a vacuum drying oven at 40 °C for 24 h [23].

2.3.2. Particle size analysis

A BT-9300S laser particle size analyser was used to determine the particle size determination of baicalin microsponges, with water as the dispersing medium (refractive index of the medium was 1.333). The refractive index of baicalin microsponges was 1.468, the cycling speed was 1600 rpm, and the instrument could determine a range of 0.10–1200 μm. Three parallel measurements were performed and the average value was recorded.

2.3.3. Determination of baicalin microsponge yield and encapsulation efficiency (EE)

Baicalin microsponges were prepared according to the above method and the baicalin content in the microsponges was determined using HPLC. Yield and encapsulation efficiency of the baicalin microsponges were calculated according to formulae (1) and formula (2), respectively [24].

$$\text{Yield } (\%) = (M_{ms}/M_{rm}) \times 100\% \tag{1}$$

$$\text{EE } (\%) = (M_{act}/M_{the}) \times 100\% \tag{2}$$

Where, M_{ms} is the mass of the generated baicalin microsponge, M_{rm} is the sum of the masses of baicalin and ethyl cellulose initially added, M_{act} is the mass of baicalin in the microsponge as measured using by HPLC, and M_{the} is the amount of baicalin added to prepare the microsponges.

2.3.4. Determination of baicalin in baicalin microsponges

2.3.4.1. Chromatographic conditions [25,26]. The Agilent ZORBAX SB-C18 column (4.6 mm × 250 nm, 5 μm) was used with methanol-water-phosphoric acid (47:53:0.2) as the mobile phase at a flow rate of 1.0 mL·min^{-1}, the column temperature was 30 °C, and the injection volume was 10 μL with the detection wavelength of 280 nm.

2.3.4.2. Preparation of control solution. The appropriate amount of baicalin control was weighed and methanol was added to obtain a solution containing approximately 60 μg·mL^{-1} baicalin.

2.3.4.3. Preparation of test solution. Approximately 5 mg of baicalin microsponge was taken in a 25 mL measuring flask, and the appropriate amount of methanol was added and the suspension was sonicated for 30 min to allow dissolution, cooling, and condensation to scale.

2.3.4.4. Preparation of negative test solution. A negative sample lacking baicalin was prepared according to the baicalin microsponge preparation process and a negative test solution was prepared according to the test solution preparation method.

2.3.5. Observation of appearance and morphology of baicalin microsponges

The finished baicalin microsponges were placed in glassware against a black background. Their appearance was observed visually observed, and their morphology was observed using a scanning electron microscope.

2.3.6. Determination of in vitro release degree

The dialysis bag was then placed in boiling water and heated for approximately 10 min. The air inside the bag was removed and one end was clamped using a clip. Approximately 10 mg of baicalin API and 48.2 mg of BM were taken and 5 mL of purified water was added to make a suspension, which was placed in a dialysis bag, and the other end of the bag was clamped. The dialysis bag was suspended in a beaker, and 200 mL phosphate buffer (pH 3–4) was added as the release medium, and the test was performed at (37.0 ± 0.5)°C and with centrifugation at 100 rpm. At 1, 4, 8, 12, 24 and 36 h, 1 mL release solution was taken (replenished in time with an equal amount of release medium at the same temperature), filtered through a microporous membrane, and measured according to the chromatographic conditions described in section 2.3.4.

2.3.7. Anti-inflammatory activity of baicalin microsponges in mouse macrophages (RAW264.7) in vitro

2.3.7.1. Cell culture. RAW 264.7 cells (Source: mouse. Purchased from Cell Resource Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences) were cultured in high-sugar DMEM medium containing 10% foetal bovine serum at 37 °C, with 5% carbon dioxide in the incubator, and experiments were performed using cells in the logarithmic growth phase.

2.3.7.2. Cytotoxicity assay. Compound toxicity to RAW 264.7 cells was determined using the Cell Counting Kit-8 (Biosharp, BS350A) according to the manufacturer's instructions. Cultured RAW 264.7 cells (1×10^4 cells/well) were inoculated into 96-well plates. After treating the cells with different concentrations of BM (6.25, 12.5, 25, 50, 100, 200 μM) for 24 h, 100 μL medium containing 5 % CCK8 (5 μL CCK8 + 95 μL complete medium) was added to each well and cells were incubated for 4 h, of which three replicate wells were used for each group. Finally, the absorbance of each well was measured at 450 nm using an enzyme-labelled detector.

2.3.7.3. NO testing. RAW 264.7 macrophages were seeded in 96-well plates (1×10^4 cells/well). Cells were pretreated with three different concentrations of baicalin for 2 h. Then the cells were incubated with LPS (1 μg·mL^{-1}) for 24 h to induce an inflammatory response, and the supernatants were collected for analysis. The Griess method was used to measure NO production [27].

2.3.7.4. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assay [28,29]. RAW 264.7 macrophages were inoculated in 6-well plates (1×10^4 cells/well). The compound-cell mixture after 24 h of incubation was stimulated with 10 μm mL^{-1} LPS for 12 h. Each well was rinsed three times with PBS. Total RNA was extracted from each group of cells using TRIzol reagent. 1 mL Trizol (CW0580S) and 200 μL ice chloroform were added to the cells, and cells were allowed to stand at room temperature for 5 min, and then centrifuged at 12,000×*g* for 15 min at 4 °C. The supernatant was removed, then transferred to an RNase-free microcentrifuge tube, and 500 μL microcentrifuge isopropanol was added to it. The supernatant was then transferred to an RNase Free tube, mixed with 500 μL of isopropanol, and allowed to stand at 4 °C for 10 min. One millilitre 75 % ethanol was added to the precipitated RNA, which was then shaken gently for 30 s, and centrifuged at 8000×*g* at 4 °C for 5 min. The RNA from which the supernatant was discarded was air-dried at room temperature and solubilised in 10 mL DEPC H$_2$O. After determining the purity and concentration of the RNA, mRNA was reverse transcribed into cDNA. cDNA was detected using real-time quantitative PCR using the MonAmp SYBR Green qPCR Mix (Monad, MQ10101), MonScript RTIII Super Mix (excluding the genome; Monad, MR05201), RNase-free H$_2$O, and 5′ and 3′ primers

real-time quantitative PCR assay. Relative mRNA expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method [30].

2.3.7.5. Statistical analyses. Primers for inflammatory cytokines are shown in Table 1.

Data were analysed and graphs were generated using GraphPad Prism 9.5 software and data were displayed as mean standard deviation. One-way analysis of variance (ANOVA) was used for statistical analyses of comparisons between three or more groups, and the *t*-test was used for statistical analyses of comparisons between two groups. # Comparison of the control group with the LPS group is shown using #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. * Comparison of the LPS group with the compound group is shown using *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All data were tested in triplicates and averaged.

3. Results

3.1. Optimisation of preparation process parameters

3.1.1. Single-factor test

The one-way test method was used to investigate the effects of different mass ratios of ethylcellulose (EC) to baicalin on the encapsulation rate and yield of microspongesusing 2% poly (vinyl alcohol) and stirring at 700 rpm, results are shown in Fig. 2A; effects of different concentrations of polyvinyl alcohol on the encapsulation rate and yield of microsponges were investigated by using a 2:1 mass ratio of ethyl cellulose to baicalin and stirring at 700 rpm, results are shown in Fig. 2B; effects of different stirring speeds on the encapsulation rate and yield of microsponges were investigated by using a 2:1 mass ratio of ethyl cellulose to the drug and 2% concentration of polyvinyl alcohol, results are shown in Fig. 2C.

3.1.2. Box-Behnken response surface design [31–33]

Polyvinyl alcohol concentration (A), the mass ratio of ethyl cellulose to baicalin (B), and stirring speed (C) were used as the investigating factors, and a three-factor, three-level Box-Behnken response surface design was selected, which was processed using the Design-Expert 11 software. Referring to the results of the single-factor test, the ranges of the three factors were determined as follows: polyvinyl alcohol concentration of 1–3%, ethyl cellulose to drug ratio of 1:1–3:1, and stirring speed of 500–1000 rpm. The encapsulation efficiency of baicalin (Y_1) and the yield of microsponges (Y_2) were used as evaluation indices. The experimental arrangements and results are shown in Table 2.

Statistical analyses of each effect value and its influencing factors were performed using Design-Expert 11 software. Multiple regression analysis were performed on the EE (Y_1) and yield (Y_2) (dependent variables) obtained for each of the three independent variables (A, B, and C) in the model to fit the response to the experimental data and obtain the second-order polynomial model as shown in Equations (3) and (4).

$$Y_1 = 62.46 + 3.19\ A - 3.06\ B - 4.06C - 2.98\ A*B + 4.56\ A*C - 3.18\ B*C - 10.49\ A*A - 16.17\ B*B - 5.32C*C \tag{3}$$

$$Y_2 = 74.44 + 4.99\ A - 0.7237\ B + 3.67C + 11.24\ A*B - 5.36\ A*C - 4.79\ B*C - 9.97\ A*A - 8.29\ B*B - 7.62C*C \tag{4}$$

Analysis of variance (ANOVA) for each of the fitted equations was performed using the Design-Expert 11 software and is shown in Table 3. The results of ANOVA of the model obtained using this software showed that $P = 0.0002 < 0.05$ for the encapsulation rate fitted model and $P = 0.1233 > 0.05$ for the model dislocation term, indicating that the fitted equations fit the experimental results better; $P = 0.003 < 0.05$ for the yield-fitted model and $P = 0.1533 > 0.05$ for the model dislocation term, indicating that the fitted equations also fit the experimental results better. Effect is also better.

The ANOVA results showed that the effects of factors A, B, C, AC, A^2, B^2 and C^2 on the encapsulation rate of the microsponges were statistically significant, while the factors A, C, AB, AC, BC, A^2, B^2 and C^2 had statistically significant effects on the yield of baicalin microsponges. Fig. 3. shows the surface and contour plots of the responses to the selected factors to further explain the relationship between the dependent and independent variables.

These graphs are useful for identifying the interactions between two variables and understanding how a change in the amount of one variable affects the effect of the other. After identifying the effects of the dependent and independent variables on the responses, a desirability function was used to simultaneously optimise the independent variables for each response. Using mathematical calculations, multiple responses are converted into single responses using a desirability function.

Table 1
RT-qPCR primers sequences.

Genes	Forward(5′-3′)	Reverse(3′-5′)
tnf-α	GACGTGGAACTGGCAGAAGA	GGCTACAGGCTTGTCACTCG
il-1β	AGGAGAACCAAGCAACGACA	CTCTGCTTGTGAGGTGCTGA
β-actin	CCTTCCAGCAGATGTGGATTAG	TGAAGTGGTAACAGTCCGTTTAG
b2ma	CAGTTTCACCTCACCAAGAG	GGCTCCCAAGAATAGATGTTT
40sa	GAAGACCATCGTCATCAGAC	CGATGTCACCTAAAGTCACG
gapdha	GAGCACCGTTCATGCTATC	GACCATCCCTCCACAGTTTT
odc1a	CTCACTATCAGATGACAAAGATG	GACACGGGGTAACACAC
ef1αb	TATCTCCAAGAACGGACAGA	TGGTGATTTCCTCAAAACGA

Table 2
Box-Behnken design and results for baicalin microsponge preparation process (mean ± s, n = 3).

No.	A	B	C	Y_1/%	Y_2/%
1	1	1	750	35.06 ± 0.76	62.36 ± 1.28
2	3	1	750	46.61 ± 1.49	53.33 ± 0.91
3	1	3	750	30.94 ± 0.95	36.57 ± 0.92
4	3	3	750	30.57 ± 1.54	72.50 ± 1.69
5	1	2	500	53.43 ± 2.39	42.78 ± 0.69
6	3	2	500	51.49 ± 1.65	60.00 ± 0.08
7	1	2	1000	32.70 ± 2.26	64.44 ± 0.31
8	3	2	1000	48.99 ± 1.92	60.22 ± 0.57
9	2	1	500	41.19 ± 1.98	51.67 ± 0.49
10	2	3	500	45.37 ± 1.91	61.67 ± 1.24
11	2	1	1000	42.92 ± 2.13	65.00 ± 1.59
12	2	3	1000	34.39 ± 2.48	55.83 ± 0.19
13	2	2	750	63.21 ± 0.49	73.33 ± 0.47
14	2	2	750	63.65 ± 1.72	75.56 ± 0.45
15	2	2	750	65.03 ± 0.82	74.44 ± 0.90
16	2	2	750	59.15 ± 1.38	71.11 ± 1.10
17	2	2	750	61.25 ± 1.65	77.78 ± 1.17

Note: (A) Polyvinyl alcohol concentration; (B) Mass ratio of ethyl cellulose to baicalin; (C) Stirring speed; (Y_1) Encapsulation efficiency of baicalin; (Y_2) Microsponge yield.

Table 3
ANOVA for the effect of factors on effect sizes.

Source of variance	Y_1 /%			Y_2 /%		
	F value	P value	α	F value	P value	α
A	7.32	0.0304	*	17.05	0.0044	**
B	6.74	0.0356	*	0.3590	0.5679	
C	11.84	0.0108	*	9.24	0.0189	*
AB	3.19	0.1172		43.30	0.0003	**
AC	7.46	0.0293	*	9.85	0.0164	*
BC	3.63	0.0985		7.87	0.0263	*
A^2	41.61	0.0004	**	35.85	0.0005	**
B^2	98.93	<0.0001	**	24.77	0.0016	**
C^2	10.69	0.0137	*	20.92	0.0026	**

Significance: * significant difference, $P < 0.05$; ** highly significant difference, $P < 0.01$.

Among the three factors, the polyvinyl alcohol concentration had the greatest effect on the encapsulation rate and yield, followed by stirring speed and finally the mass ratio of ethyl cellulose to baicalin, that is, A > C > B.

3.2. Optimal process prediction

The factor levels were analysed using the Design-Expert 11 software and the optimal solution was evaluated using normalised values. The results showed that the optimal process conditions were polyvinyl alcohol dosage of 2.17%, ethyl cellulose-baicalin (2.61:1), stirring speed of 794 rpm. The predicted values of baicalin encapsulation rate and microsponge yield were 53.40% and 72.39%, respectively.

3.3. Determination of baicalin in baicalin microsponges by RP-HPLC

3.3.1. RP-HPLC analyses
The results of the experiments carried out according to the chromatographic conditions, preparation of test solution, control solution, and blank control test solution in the method for determination of baicalin microspongeaccording to the chromatographic conditions described in section 2.3.4, are shown in Fig. 4.

3.3.2. Establishment of standard curves
Baicalin control solution (577 µg·mL^{-1})was taken and 0.5, 0.8, 1.0, 1.2, 1.5, 2.0, 2.5 mL of solution was precisely pipetted in a 10 mL measuring flask, diluted with methanol to the scale, shaking well to obtain the series of control solution, respectively, precision pipetting of 10 µL of the respective control solution, injected into the high-performance liquid chromatography, measured under the chromatographic conditions described in section 2.3.4, peak area as the vertical coordinates and mass concentration as the horizontal coordinates, The regression equation and correlation coefficient of baicalin were calculated as $Y = 43030X-106415$ and $R^2 = 0.9999$. The results showed that baicalin showed a good linear relationship between the concentrations of 28.85–144.25 µg·mL^{-1}.
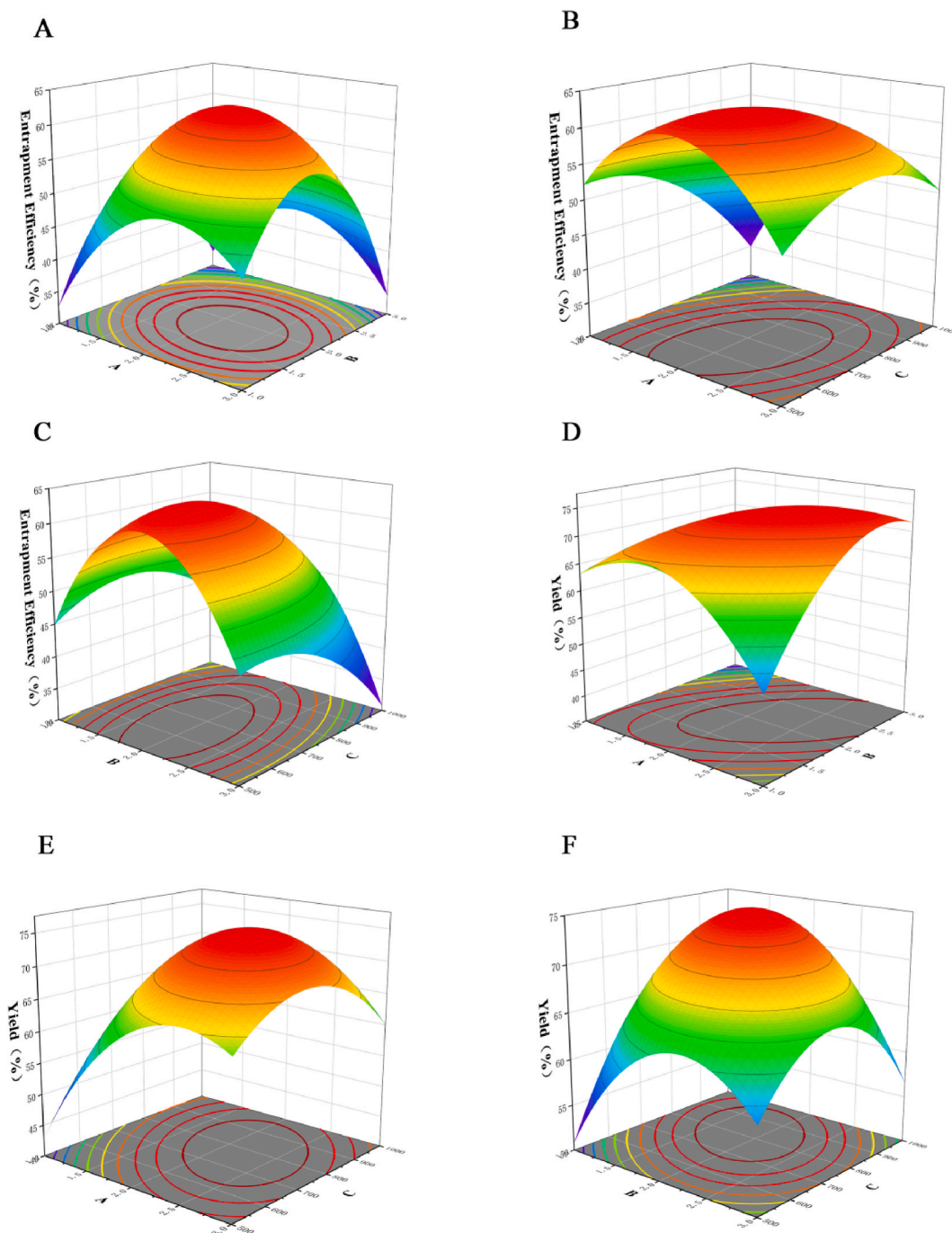
Fig. 3. Response surface plots of the interaction of the 3 factors on EE and yield. (A) Effect of Factors A and B on Y_1; (B) Effect of Factors A and C on Y_1; (C) Effect of Factors B and C on Y_1; (D) Effect of Factors A and B on Y_2; (E) Effect of Factors A and C on Y_2; (F) Effect of Factors B and C on Y_2.

Results of the proprietary test showed that the retention time of baicalin was 10.611 min with peak symmetry, and that auxiliary materials did not interfere with the detection of baicalin. The method has good reproducibility, stability and instrumental precision (RSD <2%), which can meet the requirements for quantitative analysis and detection of this product.

3.3.3. Process validation and sample determination

A certain amount of baicalin and excipients were taken, and three batches of baicalin microsponges were prepared according to the optimised process. The test solutions were prepared according to the aforementioned method, and samples were injected. The contents of the samples were calculated, and then the encapsulation rate of baicalin was calculated along with the yield of microsponges [34].
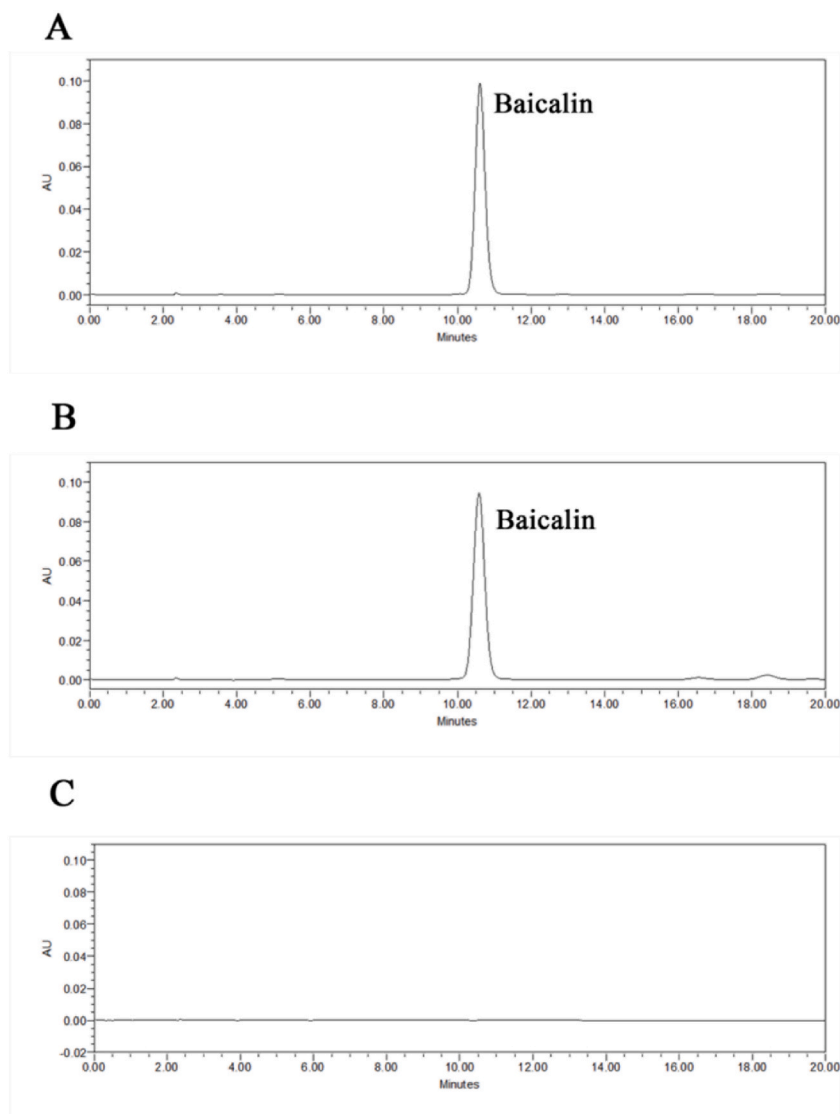
Fig. 4. Baicalin microsponge HPLC plot (A) Baicalin control solution; (B) Test solution; (C) Negative test solution.

Results are shown in Table 4 in comparison with the predicted values. From the data in the table, it can be seen that the deviation between the measured and predicted values is within acceptable limits. The BBD experimental design was suitable for process optimisation.

3.4. Morphological observation of baicalin microsponges

Baicalin microsponges prepared using the above process were yellowish (Fig. 5A). Under the scanning electron microscope, the particles were observed to be uniformly distributed, and the baicalin microsponges had a porous spherical structure (Fig. 5C–D). Particle size and particle size distribution of baicalin microsponges were determined using a BT-9300S laser particle size analyser, with the following results: average particle size of baicalin microsponges in surface area was (13.45 ± 1.62) μm, and the average particle

Table 4
Comparison of measured and predicted values of microsponges prepared under optimal process conditions (n = 3).

Response	Predicted value/%	Observed value/%	Residuals	Bias[a]/%
Y_1	53.40	54.06 ± 1.24	0.66	−0.11
Y_2	72.39	70.37 ± 2.41	−2.02	2.87

[a] Bias (%) = (predicted value − observed value)/observed value × 100.

size in volume was (58.66 ± 2.56) μm. The particle size distribution is shown in Fig. 5B.

3.5. Results of the in vitro release degree measurement

As seen in Fig. 6, the baicalin API's cumulative release rate reached (76.73 ± 0.57)% in just 6 h and was essentially released in 12 h. The average cumulative release rate of BM in 36 h was (85.92 ± 0.72)%, suggesting that BM had a somewhat delayed release effect.

3.6. Results of the anti-inflammatory activity assay of baicalin microsponges in vitro in mouse macrophage RAW264.7

3.6.1. Effects of BM on the proliferation of RAW 264.7 cells
To determine the effect of BM on cell viability, RAW264.7 cells were treated with 0 μM (as control), 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM and 200 μM BM for 24 h with reference to the concentration of baicalin [35], which had been used in previous studies on the anti-inflammatory activity of baicalin, and were assayed by using the CCK8 method. The results showed that BM was not cytotoxic to mouse macrophage RAW264.7 in the range of 0–200 μM, as shown in Fig. 7A.

3.6.2. Effect of BM pretreatment on the release of pro-inflammatory factors from RAW264.7 cells
To investigate the preventive effect of baicalin microsponges on LPS-induced cellular inflammation, RAW 264.7 cells were pre-treated with 50 μM, 100 μM, and 200 μM baicalin microsponges for 2 h, followed by exposure to LPS for 24 h [14]. The transcriptional levels of *inos*, *tnf-α*, and *il-1β* were detected in LPS-induced RAW 264.7 cells. The levels of inflammatory factors under the effect of BM were analysed, and the results showed that the transcript levels of pro-inflammatory mediators *inos*, *tnf-α* and *il-1β* were significantly increased in the LPS-induced group compared with those in Control group, and that the BM group could to a certain extent reduce the transcript levels of pro-inflammatory mediators *inos*, *tnf-α* and *il-1β*, and the anti-inflammatory effect was enhanced with the increase
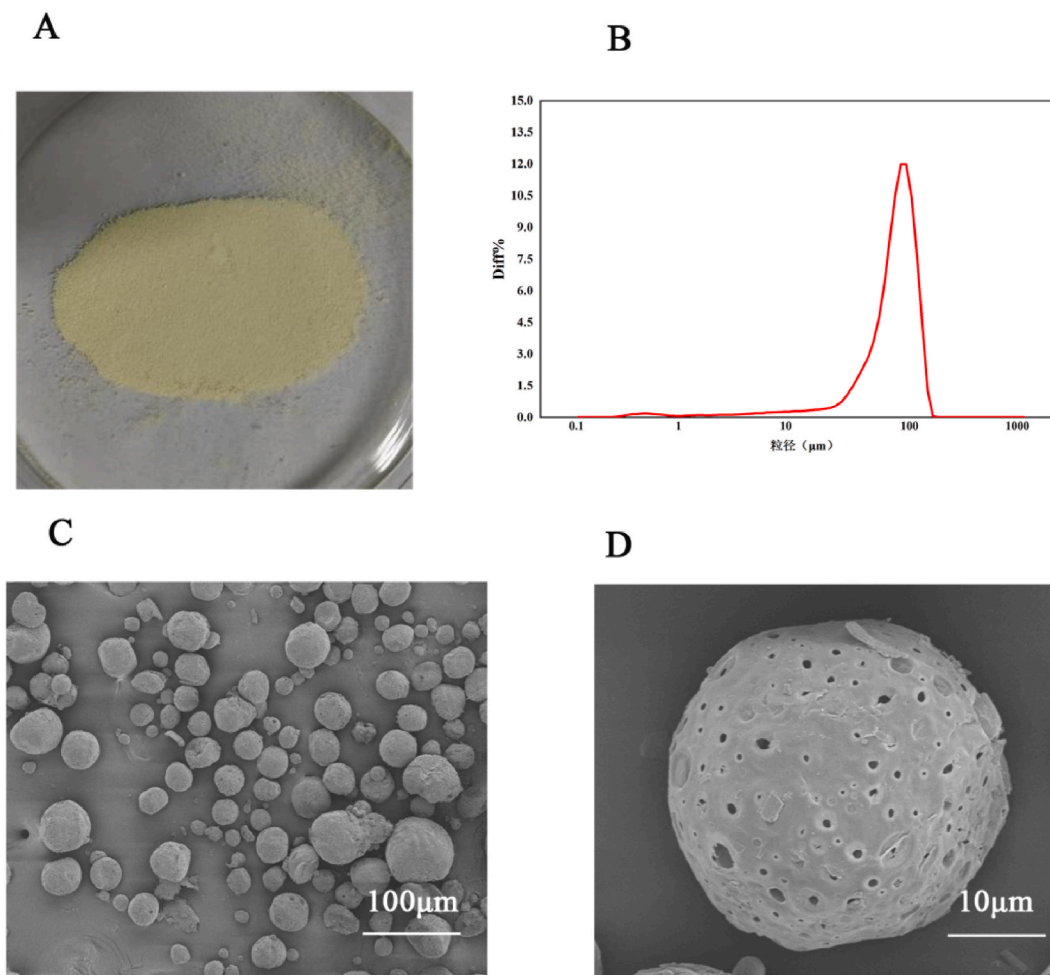

Fig. 5. Characterisation results of microsponges. (A) Baicalin microsponges; (B) Particle size distribution; (C) Magnified 100× TEM photo; (D) Magnified 1000× TEM photo.
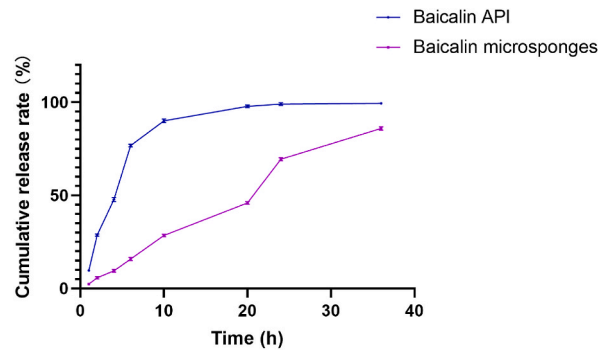
Fig. 6. *In vitro* release profiles of baicalin and baicalin microsponges (n = 3).
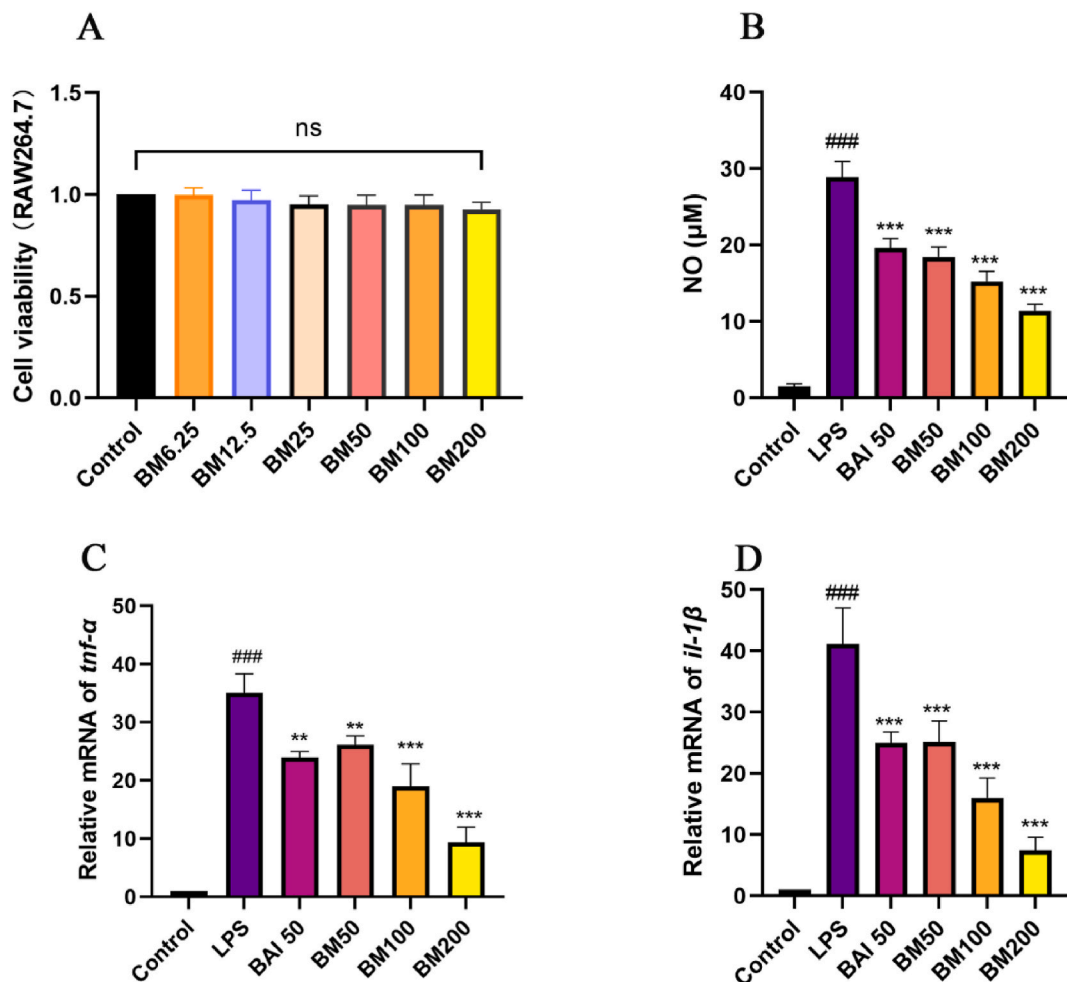

Fig. 7. Effect of BM pretreatment on LPS-induced inflammatory response in RAW264.7 cells. (A) Cytotoxicity of baicalin microsponges on RAW264.7 cells determined by CCK8 assay; (B–D) Effects of baicalin microsponges and baicalin pretreatment on LPS-induced *inos*, *tnf-α* and *il-1β* production in RAW264.7 cells. Baicalin pretreatment was used as a positive control. # Comparison of control group with LPS group, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$. * Comparison of LPS group with compound group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Values are expressed as mean ± SD, n = 3 per group.

of concentration. The results are shown in Fig. 7B–D. In this study, we pretreated LPS-induced inflammatory response of RAW264.7 cells with BM and found that BM mian pretreated LPS-induced RAW264.7 cells could reduce the levels of inflammatory factors and play an anti-inflammatory role.

4. Discussion

The common preparation methods for microsponges include liquid-liquid suspension polymerisation and quasi-emulsion solvent diffusion [36]. The liquid-liquid suspension polymerisation method uses reagents with strong toxicity, and usually also need to add catalysts or radiation are required to start the polymerisation process; the operation is cumbersome, whereas the quasi-emulsion solvent diffusion method only needs to add the inner phase into the outer phase of high-speed stirring, which is simple, with the advantages of safety, convenience and speed, so the preparation of microsponges is commonly used in this method.

The quasi-emulsion solvent diffusion method is divided into two internal and external phases. The inner phase consisted of baicalin, ethyl cellulose, and dichloromethane, while the outer phase consisted of water and polyvinyl alcohol. Polymeric ethyl cellulose acts as a plasticiser and enhances the plasticity of the formulation [1], which directly affects the formation of microsponges and drug encapsulation. When setting the ratio of ethylcellulose to baicalin in the initial pre-test prescription, a prescription with a greater amount of baicalin than ethylcellulose was tried, and it was found that the encapsulation rate of the drug was low. During filtration, it was found that the water in the conical flask was turbid, which indicated that a larger amount of baicalin was not encapsulated in the microsponge, and thus was lost with the water. Therefore, it is speculated that the amount of ethylcellulose has to be greater than that of baicalin in order to better encapsulate it, and the subsequent results further proved that ethylcellulose is a plasticising agent, and that it can directly affect the formation of the microsponge. Subsequent experimental results confirm this speculation. Poly (vinyl alcohol) is an emulsifier that cannot form microsponges if lacking; it is also related to the pore formation of microsponges, and its concentration also has a great influence on the encapsulation rate and yield of microsponges. Dichloromethane also plays an important role in the formation of the microsponges, and an appropriate dosage can bring the formed microsponges closer to a spherical shape.

It is necessary to compare multiple influencing factors and levels, and to analyse their influence on the experimental results to optimise the design of the factors and results for process optimisation. Uniform and orthogonal design methods have been commonly used in the past, however, their experimental accuracy is not high, and the mathematical model is not adequately predictable. In recent years, BBD response surface analysis has been increasingly used. This method uses multiple quadratic regression as a function-estimation tool. In multi-factor experiments, a polynomial is used to approximate the relationship between factors and indicators. Based on the response surface and contours of the function, an analysis is conducted to examine the relationship between two factors, and between factors and the response surface. It has the advantages of higher accuracy of the regression formula, shorter experimental period, and the ability to study the interaction between several factors. Therefore, it has been increasingly used in the development of new dosage forms, prescription screening of new dosage forms, and process optimisation of new preparations. In this study, a single-factor experiment was used to screen the range of influencing factors by using the encapsulation rate and yield as evaluation indices. On this basis, the Box-Behnken response surface method was used to rapidly predict the optimal preparation process through a three-factor, three-level response surface test using the envelope rate and yield as the response values. The Design-Expert 11 software was used to save time and reduce costs.

The process validation results revealed high operational reproducibility of baicalin microsponges manufacture, as projected by the BBD method. HPLC provides rapid, accurate, high-resolution, high-sensitivity, and high-accuracy baicalin measurement in baicalin microsponges. The baicalin microsponges showed good morphology, were spherical with porous channels, and were more evenly dispersed, according to measurements made using the scanning electron microscope. According to the results of *in vitro* release measurements, baicalin can be encapsulated by the porous structure of BM, which contributes to its slow-release effect [37]. Drug release is primarily dependent on diffusion and a polymer solubilisation mechanism, which extends the release time. A microsponge-based diclofenac diethylamide gel has been prepared with a release rate of only 11% after 4 h. It is expected to be an alternative to conventional therapies for safer and more effective treatment of diseases [38].

Sepsis, infectious shock, and inflammatory bowel disease are a few of the inflammatory illnesses in which lipopolysaccharide (LPS) is implicated. Baicalin has been shown in previous studies to have both preventative and inhibitory effects on inflammation s. Additionally, a different study showed that baicalin pretreatment decreased LPS-induced activation of the MyD 88/NF-κB p65 pathway in RAW 264.7 cells and inhibited LPS-induced inflammatory responses, but not post-treatment [14]. In this study, RAW 264.7 cells induced by lipopolysaccharide (LPS) were pretreated with baicalin microsponges. The experimental outcomes showed that the pre-treatment with baicalin microsponges decreased the transcript levels of inflammatory factors *inos*, *tnf-α*, and *il-1β* in the cells, suggesting that it had some protective effect on normal RAW 264.7 cells. In conclusion, encapsulating baicalin in a slow-release delivery system, such as a microsponge, has no influence on its anti-inflammatory properties.

5. Conclusion

In this study, we successfully prepared baicalin microsponges to deliver baicalin using a quasi-emulsion solvent diffusion method. The formulation was optimised using BBD experiments, and a stable and feasible preparation process for the microsponges and optimised process parameters were determined. The encapsulation rate of baicalin microsponges was $(54.06 \pm 1.24)\%$ and the yield was $(70.37 \pm 2.41)\%$. *In vitro* anti-inflammatory experiments showed that baicalin microsponges, like baicalin, reduced LPS-induced inflammatory responses in RAW264.7 cells when pretreated. These results suggested that baicalin microsponges have the potential to be used as slow-release anti-inflammatory agents. However, further in vivo studies are required to verify required.

Funding

and the National Natural Science Foundation of China (81773968).

Ethics declarations

Review and/or approval by an ethics committee was not needed for this study because this study did not involve human or animal subjects.

Data availability statement

All data generated or analysed during this study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Miao Li: Writing – original draft, Methodology, Data curation, Conceptualization. **Jiajie Gan:** Writing – original draft, Methodology, Investigation. **Xuhui Xu:** Writing – original draft, Methodology, Investigation. **Shuai Zhang:** Methodology, Investigation, Data curation. **Yuanyuan Li:** Validation, Software. **Le Bian:** Methodology, Investigation, Data curation. **Zibo Dong:** Supervision, Project administration, Funding acquisition, Conceptualization.

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.

Abbreviations

BM   Baicalin microsponges
RP-HPLC   Reverse phase high-performance liquid chromatography
LPS   Lipopolysaccharide
PBS   Phosphate belanced solution
TLR   Toll-like receptor
NF-κB   Nuclear transcription factor-κB
PI3K   Phosphatidylinositol 3-kinase
AKT   Protein kinase B
IL-1β   Interleukin-1β
IL-6   Interleukin-6
IL-8   Interleukin-8
TNF-α   Tumor necrosis factor-α
API   Active pharmaceutical ingredients
CCK-8   Cell Counting Kit-8
DEPC   Diethyl pyrocarbonate
DMEM   Dulbecco's Modified Eagle's Medium

References

[1] G. Singhvi, P. Manchanda, N. Hans, S.K. Dubey, G. Gupta, Microsponge: an emerging drug delivery strategy, Drug Dev. Res. 80 (2019) 200–208, https://doi.org/10.1002/ddr.21492.
[2] M. Bhatia, M. Saini, Formulation and evaluation of curcumin microsponges for oral and topical drug delivery, Prog Biomater 7 (2018) 239–248, https://doi.org/10.1007/s40204-018-0099-9.
[3] L.Y. Peng, M. Yuan, Z.M. Wu, K. Song, C.L. Zhang, Q. An, F. Xia, J.L. Yu, P.F. Yi, B.D. Fu, H.Q. Shen, Anti-bacterial activity of baicalin against APEC through inhibition of quorum sensing and inflammatory responses, Sci. Rep. 9 (2019) 4063, https://doi.org/10.1038/s41598-019-40684-6.
[4] X. Wang, L. Xie, J. Long, K. Liu, J. Lu, Y. Liang, Y. Cao, X. Dai, X. Li, Therapeutic effect of baicalin on inflammatory bowel disease: a review, J. Ethnopharmacol. 283 (2022) 114749, https://doi.org/10.1016/j.jep.2021.114749.
[5] Y. Huang, S.Y. Tsang, X. Yao, Z.Y. Chen, Biological properties of baicalein in cardiovascular system, Curr Drug Targets Cardiovasc Haematol Disord 5 (2005) 177–184, https://doi.org/10.2174/1568006043586206.
[6] W. Zhu, Z. Jin, J. Yu, J. Liang, Q. Yang, F. Li, X. Shi, X. Zhu, X. Zhang, Baicalin ameliorates experimental inflammatory bowel disease through polarization of macrophages to an M2 phenotype, Int. Immunopharm. 35 (2016) 119–126, https://doi.org/10.1016/j.intimp.2016.03.030.
[7] L. Cui, L. Feng, Z.H. Zhang, X.B. Jia, The anti-inflammation effect of baicalin on experimental colitis through inhibiting TLR4/NF-κB pathway activation, Int. Immunopharm. 23 (2014) 294–303, https://doi.org/10.1016/j.intimp.2014.09.005.
[8] C.L. Zhang, S. Zhang, W.X. He, J.L. Lu, Y.J. Xu, J.Y. Yang, D. Liu, Baicalin may alleviate inflammatory infiltration in dextran sodium sulfate-induced chronic ulcerative colitis via inhibiting IL-33 expression, Life Sci. 186 (2017) 125–132, https://doi.org/10.1016/j.lfs.2017.08.010.
[9] H. Liao, J. Ye, L. Gao, Y. Liu, The main bioactive compounds of Scutellaria baicalensis Georgi. for alleviation of inflammatory cytokines: a comprehensive review, Biomed. Pharmacother. 133 (2021) 110917, https://doi.org/10.1016/j.biopha.2020.110917.

[10] Y. Cui, Q. Wang, R. Sun, L. Guo, M. Wang, J. Jia, C. Xu, R. Wu, Astragalus membranaceus (Fisch.) Bunge repairs intestinal mucosal injury induced by LPS in mice, BMC Compl. Alternative Med. 18 (2018) 230, https://doi.org/10.1186/s12906-018-2298-2.

[11] M. Zeinali, S. Abbaspour-Ravasjani, M. Ghorbani, A. Babazadeh, T. Soltanfam, A.C. Santos, H. Hamishehkar, M.R. Hamblin, Nanovehicles for co-delivery of anticancer agents, Drug Discov. Today 25 (2020) 1416–1430, https://doi.org/10.1016/j.drudis.2020.06.027.

[12] L.T. Guo, S.Q. Wang, J. Su, L.X. Xu, Z.Y. Ji, R.Y. Zhang, Q.W. Zhao, Z.Q. Ma, X.Y. Deng, S.P. Ma, Baicalin ameliorates neuroinflammation-induced depressive-like behavior through inhibition of toll-like receptor 4 expression via the PI3K/AKT/FoxO1 pathway, J. Neuroinflammation 16 (2019) 95, https://doi.org/10.1186/s12974-019-1474-8.

[13] M. Xu, X. Li, L. Song, Baicalin regulates macrophages polarization and alleviates myocardial ischaemia/reperfusion injury via inhibiting JAK/STAT pathway, Pharm. Biol. 58 (2020) 655–663, https://doi.org/10.1080/13880209.2020.1779318.

[14] Y.J. Fu, B. Xu, S.W. Huang, X. Luo, D.S. Deng, C. Liu, Q. Wang, J.Y. Chen, L. Zhou, Baicalin prevents LPS-induced activation of TLR4/NF-κB p65 pathway and inflammation in mice via inhibiting the expression of CD14, Acta Pharmacol. Sin. 42 (2021) 88–96, https://doi.org/10.1038/s41401-020-0411-9.

[15] A. Płóciennikowska, A. Hromada-Judycka, K. Borzęcka, K. Kwiatkowska, Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling, Cell. Mol. Life Sci. 72 (2015) 557–581, https://doi.org/10.1007/s00018-014-1762-5.

[16] C. Wang, M.Q. Gao, Research Progress on the antidepressant effects of baicalin and its aglycone baicalein: a systematic review of the biological mechanisms, Neurochem. Res. (2023), https://doi.org/10.1007/s11064-023-04026-3.

[17] X. Jin, M.Y. Liu, D.F. Zhang, X. Zhong, K. Du, P. Qian, W.F. Yao, H. Gao, M.J. Wei, Baicalin mitigates cognitive impairment and protects neurons from microglia-mediated neuroinflammation via suppressing NLRP3 inflammasomes and TLR4/NF-κB signaling pathway, CNS Neurosci. Ther. 25 (2019) 575–590, https://doi.org/10.1111/cns.13086.

[18] X. Yin, P. Krikorian, T. Logan, V. Csizmadia, Induction of RIP-2 kinase by proinflammatory cytokines is mediated via NF-kappaB signaling pathways and involves a novel feed-forward regulatory mechanism, Mol. Cell. Biochem. 333 (2010) 251–259, https://doi.org/10.1007/s11010-009-0226-y.

[19] X. Li, K. Zou, J. Gou, Q. Du, D. Li, X. He, Z. Li, Effect of baicalin-copper on the induction of apoptosis in human hepatoblastoma cancer HepG2 cells, Med. Oncol. 32 (2015) 72, https://doi.org/10.1007/s12032-015-0527-9.

[20] J. Wang, X. Feng, Z. Li, Y. Liu, W. Yang, T. Zhang, P. Guo, Z. Liu, D. Qi, J. Pi, The flavonoid components of Scutellaria baicalensis: biopharmaceutical properties and their improvement using nanoformulation techniques, Curr. Top. Med. Chem. 23 (2023) 17–29, https://doi.org/10.2174/1568026623666221128144258.

[21] M. Rahman, W.H. Almalki, S.K. Panda, A.K. Das, S. Alghamdi, K. Soni, A. Hafeez, M. Handa, S. Beg, Z. Rahman, Therapeutic application of microsponges-based drug delivery systems, Curr. Pharmaceut. Des. 28 (2022) 595–608, https://doi.org/10.2174/1381612828666220118121536.

[22] S.K. Jain, M. Kaur, P. Kalyani, A. Mehra, N. Kaur, N. Panchal, Microsponges enriched gel for enhanced topical delivery of 5-fluorouracil, J. Microencapsul. 36 (2019) 677–691, https://doi.org/10.1080/02652048.2019.1667447.

[23] D. Dutta, N. Goyal, D. Kumar Sharma, Formulation and development of herbal microsponge sunscreen gel, J. Cosmet. Dermatol. 21 (2022) 1675–1687, https://doi.org/10.1111/jocd.14274.

[24] N. Jadhav, V. Patel, S. Mungekar, G. Bhamare, M. Karpe, V. Kadams, Microsponge Delivery System: an updated review, current status and future prospects, Journal of Scientific and Innovative Research 2 (2013) 1097–1110. www.jsirjournal.com.

[25] R.H. Xin, G. Liang, J.F. Zheng, Design and content determination of Genhuang dispersible tablet herbal formulation, Pak. J. Pharm. Sci. 30 (2017) 655–661.

[26] M. Xu, Q. Yuan, H. Zhao, F. Li, X.L. Hu, Y.Y. Fu, G.H. Shi, X.M. Wang, N. Zhang, [Determination of chlorogenic acid and baicalin in Yinhuang film-coated tablets by HPLC isocratic elution], Zhongguo Zhongyao Zazhi 38 (2013) 691–693.

[27] D. Tsikas, Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the l-arginine/nitric oxide area of research, J. Chromatogr. B 851 (2007) 51–70, https://doi.org/10.1016/j.jchromb.2006.07.054.

[28] B. Yang, B. Liu, Z. Chen, DNA extraction with TRIzol reagent using a silica column, Anal. Sci. 37 (2021) 1033–1037, https://doi.org/10.2116/analsci.20P361.

[29] D.J.P.G. Rocha, T.L.P. Castro, E.R.G.R. Aguiar, L.G.C. Pacheco, Gene Expression Analysis in Bacteria by RT-qPCR (2020) 119–137, https://doi.org/10.1007/978-1-4939-9833-3_10.

[30] W. Xia, L. Zhang, J. Mo, W. Zhang, H. Li, Z. Luo, H. Yang, Effect of static compression loads on intervertebral disc: an in vivo bent rat tail model, Orthop. Surg. 10 (2018) 134–143, https://doi.org/10.1111/os.12377.

[31] A. Singh, A. Vaish, R. Shukla, Box-Behnken design optimized silibinin loaded glycerylmonooleate nanoliquid crystal for brain targeting, Chem. Phys. Lipids 244 (2022) 105193, https://doi.org/10.1016/j.chemphyslip.2022.105193.

[32] C.T. Sengel-Turk, E. Ozkan, F. Bakar-Ates, Box-Behnken design optimization and in vitro cell based evaluation of piroxicam loaded core-shell type hybrid nanocarriers for prostate cancer, J. Pharm. Biomed. Anal. 216 (2022) 114799, https://doi.org/10.1016/j.jpba.2022.114799.

[33] B.S. Mahmoud, C. McConville, Box-behnken design of experiments of polycaprolactone nanoparticles loaded with irinotecan hydrochloride, Pharmaceutics 15 (2023), https://doi.org/10.3390/pharmaceutics15041271.

[34] P. Korucu Aktas, I. Baysal, S. Yabanoglu-Ciftci, B. Arica, Development and in vitro evaluation of crizotinib-loaded lipid-polymer hybrid nanoparticles using box-behnken design in non-small cell lung cancer, AAPS PharmSciTech 24 (2023) 178, https://doi.org/10.1208/s12249-023-02634-4.

[35] Y. Yun, C.Z. Wang, L. Gui, Z.X. Li, [Effect of baicalin on expression of TLR4 in RAW264.7 cells infected by ESBLs Escherichia coli], Zhongguo Zhongyao Zazhi 38 (2013) 1590–1594.

[36] A. Biharee, S. Bhartiya, A. Yadav, S. Thareja, A.K. Jain, Microsponges as drug delivery system: past, present, and future perspectives, Curr. Pharmaceut. Des. 29 (2023) 1026–1045, https://doi.org/10.2174/1381612829666230404082743.

[37] M. V Junqueira, M.L. Bruschi, A review about the drug delivery from microsponges, AAPS PharmSciTech 19 (2018) 1501–1511, https://doi.org/10.1208/s12249-018-0976-5.

[38] R.A. Osmani, N.H. Aloorkar, D.J. Ingale, P.K. Kulkarni, U. Hani, R.R. Bhosale, D. Jayachandra Dev, Microsponges based novel drug delivery system for augmented arthritis therapy, Saudi Pharmaceut. J. 23 (2015) 562–572, https://doi.org/10.1016/j.jsps.2015.02.020.