Preparation of Polymeric Micelles of Curcumin with Pluronic P123 and Assessment of Efficacy against B16 Cells In vitro

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Abstract
Polymeric micelles of curcumin were obtained using Pluronic P123 to increase the solubility and bioavailability of curcumin. The curcumin-P123 micelles were prepared by a thin-film dispersion method and the content of curcumin was determined by high-performance liquid chromatography. The entrapment efficiency was optimized by an orthogonal design to determine the best preparation method. The microscopic morphology, diameter, and drug-loaded amount of the micelles were determined by transmission electron microscopy, particle size distribution, Fourier transform, and X-ray diffraction. The in vitro drug release rates were measured by a dialysis method. The entrapment efficiency of curcumin was 94.7% and the loading capacity was 3.06% under optimized conditions (curcumin 5 mg, Pluronic P123 150 mg, water 10 mL, and methanol 10 mL). The average size and zeta potential of the round or ellipse polymeric micelles were 117.23 ± 2.57 nm and 7.87 ± 2.50 mV, respectively, with uniform particle size distribution. The polymeric micelles were dispersed into block copolymers in molecular and amorphous forms based on X-ray diffraction and FTIR analysis. The polymeric micelles of curcumin showed significant sustained drug release in vitro compared with a pharmaceutical solution. The anti-tumor efficacy of curcumin-P123 on B16 cells was assessed in vitro by a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The antitumor effects of the polymeric micelles was higher than that of curcumin. The prepared curcumin-P123 micelles increased the drug solubility in the aqueous phase. The polymeric micelles of curcumin have an important effect and excellent research value because of their sustained drug release and excellent inhibitory effect on tumor cells.

Keywords: Curcumin; Pluronic P123; Polymeric micelles; Orthogonal design; X-ray diffraction; (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Introduction
Curcumin is a low-molecular-weight hydrophobic polyphenol extracted from the rhizomes of Curcuma longa. Curcumin has been broadly applied as a cancer treatment because of its remarkable pharmacological anti-carcinogenic, anti-inflammatory, anti-human immunodeficiency virus, and anti-oxidant activities as well as its apoptosis induction effect, and has been used in traditional therapies for a long time. Curcumin caused no side effects in clinical trials, even at doses as high as 12 g oral daily administration [1-3] and it has been listed as one of the most promising anticancer agents by the US National Cancer Institute. However, therapeutic application of curcumin is limited because of its limited water solubility, rapid degradation at physiological pH, and low bioavailability. Various approaches have been proposed to circumvent these problems, such as modifying curcumin molecules [4], encapsulating the curcumin into liposomes [5], polymeric micelles [6], inclusion complex formation [6], formatting polymeric prodrugs [7], and other types of conjugates [8,9].

Recent studies have shown polymeric micelles act as hydrophobic drug carriers and are one of the most exciting drug-delivery strategies, with potential application for water-insoluble drugs such as curcumin [10]. Polymeric micelles are a novel drug-delivery system that are formed from an amphiphilic polymer by spontaneous self-assembly of a nonpolar core and hydrophilic shell [10-12]. Drugs can be encapsulated and protected in the core and uptake by the mononuclear phagocyte system can be avoided by minimizing the interaction between the hydrophilic shell and bio-components [13]. Drug molecules can be loaded into the polymeric micelles, which function as nanosized containers, by physical encapsulation, chemical conjugation, or electrostatic interactions. Pluronic block copolymers are prepared from hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) to form the triplex PEO-PPO-PEO [14]. These block copolymers have an inhibitory effect on P-glycoprotein-mediated drug efflux action and sensitize multidrug resistant cancer cell lines, resulting in enhanced cellular drug uptake, nuclear translocation, and transcriptional activation of gene expression [15]. Pluronic P123 (PEO09-b-PPO70-b-PEO020), where 40 and 70 designate the total average number of the PEO and PPO repeat units and b stands for ”block”, respectively, possesses a high proportion of the hydrophobic block, promising high drug loading. The PPO blocks are hydrophilic and can contribute to aggregation against water and offer a local hydrophobic microenvironment where the hydrophobic drug can be loaded, while the hydrophilic PEO part can maintain the dispersion stability of the micelles. In this study, curcumin was loaded into polymeric micelles to increase solubility and bioavailability. Curcumin was loaded into the hydrophobic core of Pluronic P123 polymeric micelles through physical encapsulation.
Materials and Methods

Materials

Curcumin was purchased from Nanjing Wide Embellish Biological Co., Ltd. (Nanjing, China). P123 and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Spectra/Por dialysis tubing (MWCO 3500) was supplied by Spectrum Labs, Inc., (New York, NY, USA). Acetonitrile and methanol were chromatographic grade and other chemicals were analytical reagents.

Preparation of curcumin-loaded micelles

The micelles were prepared using a conventional thin film-dispersed method [9]. Curcumin (5 mg) and P123 (100 mg) were dissolved into a suitable amount of methanol in a 500 mL flask and mixed for 5 min. The methanol was removed by vacuum-evaporation to obtain a thin film. The film was dried overnight at room temperature and rehydrated with 10 mL of double-distilled water. The suspension was sonicated using an ultrasonic cell disruptor in an ice bath at 400 W for 120 cycles (2s on, 3s off) to obtain a homogeneous solution. The volume of the solution was decreased by filtration through a microfilter with a membrane pore size of 0.22 μm to obtain a clear yellow solution of curcumin-P123, which was freeze-dried.

The preparation conditions (amounts of curcumin (A) and p123 (B), volumes of water (C), and methanol (D)) of the curcumin micelles were optimized by a Taguchi L9 (34) orthogonal design to maximize encapsulation efficiency (Table 1). Preliminary tests were based on literature results.

| Level | Factors |
|-------|---------|
|       | A The usage of curcumin (mg) | B The usage of P123 (mg) | C The volume of methanol (mL) | D The volume of water (mL) |
| 1     | 3       | 50      | 10       | 8       |
| 2     | 5       | 100     | 15       | 10      |
| 3     | 7       | 150     | 20       | 12      |

Table 1: Factors and levels of a Taguchi L9 (34) orthogonal experiment.

Determination of curcumin micelle characteristics

Measurements of zeta potential, size, and morphology observation: Particle size, size distribution and zeta potential of the prepared curcumin micelles were measured on a Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). The particle size distribution was reported as the polydispersity index (PDI).

The morphology of the micelles was observed by TEM (JEOL2010F, Jeol, Tokyo, Japan). Micelles were diluted in phosphate-buffered saline (PBS) (pH 7.2) and a drop of the dilute sample was placed on the surface of a 200-mesh carbon-coated copper grid. The excess aqueous solution was removed by blotting with filter paper after 2 min. Samples were dried appropriately and examined by TEM.

X-ray diffraction analysis: Complete incorporation of curcumin in P123 was determined by X-ray diffraction analysis(Miniflex 600RigakuJapan). The diffractometer was operated in reflection mode at 40 V and 25 mA. The samples of isolated curcumin, physical mixture of P123 and curcumin, curcumin micelles, and empty micelles were placed in special sample holders. Measurements were taken between 3 and 50° (2θ) with a step and a scan speed of 0.9° s⁻¹.

Entrapment efficiency of curcumin and loading capacity: Purified micelles (1 mL) were mixed with methanol (20 mL) by vortex. The amounts of entrapped curcumin were measured by high-performance liquid chromatography (HPLC) at 437 nm (absorbance maximum of curcumin) and quantified by comparison to a standard curve. The experiments were performed in triplicate. The encapsulation efficiency (EE%) and the loading capacity (LC%) were calculated according to the following Equations (1) and (2), respectively.

\[
EE\% = \left( \frac{\text{total amount of determined curcumin}}{\text{initial amount of curcumin loading}} \right) \times 100\% \quad (1)
\]

\[
LC\% = \left( \frac{\text{total amount of determined curcumin}}{\text{total amount of dried micelles}} \right) \times 100\% \quad (2)
\]

In vitro drug release

The release of curcumin from the micelles was measured using equilibrium dialysis in PBS (0.01 M, pH 7.4) containing 1% Tween 80 (w/v). Curcumin micelle solution (5 mL) was placed into a dialysis bag and then exposed to release medium (15 mL) at 37°C. A 1-mL sample was withdrawn from the dialysis bag at predetermined time intervals and replaced with fresh release medium. The content of curcumin was measured by HPLC and calculated as cumulative percent released.

The free curcumin was used as a control using a 35% ethanol solution as the release medium. The release experiments were repeated in triplicate. The cell viability was calculated using the following equation (3):

\[
\text{Cell viability} = \left( \frac{\text{Abs sample}}{\text{Abs control}} \right) \times 100\% \quad (3)
\]

Where Abs control is the absorbance value for the control cells, which contain all reagents except the drug, and Abs sample, is the absorbance value of the drug.

In vitro evaluation of curcumin micelles anti-cancer activity

A mice melanoma (B16) cell line was provided by Kunming Cell Bank, Chinese Academy of Sciences. The cytotoxicity of curcumin micelles was examined by a standard MTT assay [16]. Briefly, cells (5000/well) were seeded in 96-well plates and allowed to attach for 24 h. The medium was replaced with fresh medium (100 µL) containing various concentrations of free curcumin or curcumin micelles. After 24 h, MTT solution (10 µL, 5 mg/mL in PBS) was added to the cells and incubated for 4 h. The medium was removed and replaced with dimethyl sulfoxide (150 µL) to dissolve the formazan crystals, which were transformed by live cells. Absorbance of the wells were measured using a microplate reader at a wavelength of 570 nm. Triplicate samples were analyzed for each experiment.

Results and Discussion

Optimization of micellar preparation

The influence of the amount of curcumin (A) and P123 (B) and the volume of methanol (C) and water (D) on encapsulation efficiency were compared by orthogonal experiments. The sequence of the four parameters affecting the encapsulation efficiency was A>D>B=C (Table 2) by comparing the extremum (48.43±32.93±23.96±16.43), and the single-factor analysis showed that A: 1>2>3, B: 3>2>1, C: 1>2>3,
and D: 1>2>3 by comparing the value1, value 2 and value 3. The optimum preparation procedure was A2B3C2D2: curcumin 5 mg, P123 150 mg, methanol 10 mL, and water 10 mL.

Table 2: Result of orthogonal experiments.

| Group | Factors | EE (%) |
|-------|---------|--------|
|       | A       | B      | C     | D | |
| 1     | 3       | 50     | 10    | 8 | 92.89 |
| 2     | 3       | 100    | 15    | 10| 90.8 |
| 3     | 3       | 150    | 20    | 12| 67.5 |
| 4     | 5       | 50     | 15    | 12| 46.9 |
| 5     | 5       | 100    | 20    | 8 | 89.27 |
| 6     | 5       | 150    | 10    | 10| 94.27 |
| 7     | 7       | 50     | 20    | 10| 12.37 |
| 8     | 7       | 100    | 10    | 12| 31.26 |
| 9     | 7       | 150    | 15    | 8 | 62.28 |
| Value 1 | 83.73 | 50.72 | 72.81 | 81.48 | - |
| Value 2 | 76.81 | 70.44 | 66.66 | 65.81 | - |
| Value 3 | 35.3 | 74.68 | 56.38 | 48.55 | - |
| Extremum | 48.43 | 23.96 | 16.43 | 32.93 | - |

Characterization of curcumin micelles

The curcumin-P123 micelles morphology was determined by TEM. The micelles were spherical or ellipsoidal in shape with a smooth surface. The average diameter distribution (Figure 1) and polydispersity index demonstrated a narrow size distribution of the curcumin-P123 micelles. The mean size of the curcumin-P123 micelles was 117.23 ± 2.57 nm with a polydispersity index of 0.223 ± 0.027 and the positive zeta potential was 7.87 ± 2.50 mV (Figure 1). Studies have demonstrated that nanoparticles smaller than 200 nm are distributed in tumors more efficiently than larger ones [17].

FTIR study and X-ray diffraction analysis: FTIR spectroscopy was used to analyze the chemical adsorption of curcumin-P123 micelles [8]. The spectra of blank micelles, curcumin-P123 micelles, and the physical mixture of curcumin and P123 were similar (Figure 2). A strong adsorption peak at 1510 cm \(^{-1}\) was present in the spectrum of curcumin, attributed to the phenyl ring stretching mode [18]. This peak is not visible in the FTIR spectrum of curcumin-P123. It can be concluded that the curcumin was loaded in the micelles and had not adhered to the surface of the P123.

X-ray diffraction was also used to ascertain the physicochemical status of the drug in the preparation. The patterns of curcumin-P123 micelles and blank micelles were similar and did not show the peaks of crystalline curcumin compared with the pattern of curcumin (Figure 2), providing further evidence that the curcumin had been entrapped into the curcumin-P123 micelles.

Drug loading, encapsulation efficiency, and release

The ability of P123 to carry curcumin was analyzed in vitro by HPLC. The LC% and EE% of curcumin-P123 micelles were 94.73 ± 2.94% and 22.23 ± 2.13%, respectively. The EE% and LC% of curcumin in our curcumin-P123 micelles were substantially improved compared with those of other reported preparations, which were 71.74% and 1.62%, respectively [19].

The in vitro drug release of the curcumin-P123 micelles exhibited a sustained-release profile, which was similar to that seen in a previous study [20]. Only 5.88% of curcumin was released from curcumin-P123 micelles within the first 3 h, while 58.25% of Cur was released from the ethanol solution during the same time period (Figure 3). After 12 h, 85.11% of the free curcumin was dissolved in the release medium, and 98.75% was released after 48 h. By contrast, 30.27% of curcumin was released to the medium from curcumin-P123 micelles within 12 h, and 74.95% was released after 48 h. A potential reason for this phenomenon may be that a small amount of curcumin that had been adsorbed on the micelle surface or in the hydrophilic layer coated by Van der Waals’ force was quickly desorbing during the early stage. The slow release from the hydrophobic core may be caused by erosions or swelling of the polymer material [21]. These release mechanisms cause the release curve of curcumin-P123 micelles to rise rapidly at first and then give stable release. These drug release characteristics are consistent with administration principles of anti-cancer drugs, where the initial release makes a higher drug concentration reaches the tumor area, and then slow release of the remaining drug maintains drug efficacy for a longer time.

In vitro evaluation of the anti-cancer activity of curcumin micelles

The results of a cell growth inhibition assay of cell exposed to different concentrations of curcumin formulations for 24 h are showed in Figure 3. The proliferation of B16 cells were inhibited significantly by curcumin-P123 micelles when compared with curcumin formulated in 1% Tween 80, and the inhibitory effects were concentration dependent. The inhibitory effect of curcumin micelles on the B16 cells was much higher than that of free curcumin solubilized with 1% Tween 80 (p<0.05) at high concentrations of the drugs. Blank micelles had no inhibitory effect on cell proliferation.
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