Preparation of curcumin-loaded pluronic F127/chitosan nanoparticles for cancer therapy

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Abstract
Nanoparticles (NPs) have been proven to be an effective delivery system with few side effects for anticancer drugs. In this study, curcumin-loaded NPs have been prepared by an ionic gelation method using chitosan (Chi) and pluronic® F-127 (PF) as carriers to deliver curcumin to the target cancer cells. Prepared NPs were characterized using Zetasizer, fluorescence microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Our results showed that the encapsulation efficiency of curcumin was approximately 50%. The average size of curcumin-loaded PF/Chi NPs was 150.9 nm, while the zeta potential was 5.09 mV. Cellular uptake of curcumin-loaded NPs into HEK293 cells was confirmed by fluorescence microscopy.

Keywords: chitosan, pluronic, curcumin, ionic gelation

Classification numbers: 2.04, 2.05

1. Introduction
Curcumin (figure 1) is a phenolic compound isolated from the turmeric plant and primarily used as a natural yellow pigment, which can absorb the visible light at a wavelength between 420–425 nm. Curcumin has a variety of biological activities and pharmacological actions, such as anti-inflammatory, anti-carcinogenic and anti-virus properties, as well as promising clinical applications due to its low toxicity [1, 2]. In recent years, curcumin has been shown to inhibit cell proliferation in a variety of human cancer-cell lines in vitro [3, 4] and has been used both to prevent and to treat various cancers in vivo [5]. However, it has extremely low aqueous-solubility and rapid intestinal and hepatic metabolism, which result in poor systemic bioavailability and restrict its oral use [6].

In order to deliver curcumin to targeted organs, its hydrophobic property needs to be modified. A wide variety of drug carriers has been studied as a means of improving the therapeutic efficacy of drugs. Recently, many studies have been published on the production of nanoparticles (NPs) to incorporate curcumin. These studies have focused on polymeric NPs based on amphiphilic block copolymer PF.

Pluronic® F-127 is a hydrophilic nontoxic copolymer widely used as a pharmaceutical excipient for its stabilizing properties and capability to increase the solubility of drugs. PF is an A–B–A-type triblock copolymer consisting of polyoxyethylene (PEO) units (A) and polyoxypropylene (PPO) units (B) with a thermoreversible gelation property [7]. With the increase in the temperature of PF aqueous solution, the PPO block tends to dehydrate and to form a core with an outer shell of hydrated PEO chains that aggregate into spherical micelles [8]. The micellar structure of this copolymer in an aqueous environment can be used for incorporation of hydrophilic and hydrophobic drugs and prolongation of drug release [9, 10]. Unfortunately, the use of PF micelles as prolonged drug carrier is not practical because of the particles’ aggregation and structural changes when exposed to varying concentrations or temperatures. Several studies have shown that the particle’ stability can
be greatly improved after being coated by another polymeric layer with materials such as polyacrylic-co-glycolic acid, polyvinylchloride or chitosan [11]. Chitosan (Chi) is a natural linear polycation polysaccharide obtained by partial \( N \)-deacetylation of chitin. Chi has many advantages as a carrier in nanoparticulate drug delivery system. It is nontoxic, biocompatible and biodegradable and has been proven to control the release of drugs, proteins and peptides. It is soluble in aqueous media, avoids the use of organic solvents and does not require further purification of NPs [12]. With the presence of free amine group in its line structure, Chi has a cationic nature and can interact with various crosslinkers to form NPs. The positive charge of Chi caused by the primary amino groups in its structure is responsible for its mucoadhesive properties and therefore prolongs the residual time at the absorption site. Chi NPs are expected to be appropriate carriers for oral absorption of drugs [13].

In this study, we describe the preparation of PF/Chi NPs for curcumin delivery to cancer cells. The drug-carrying combinations of Chi and PF offer promising combinations by modifying the controlled drug release profile using PF with protection and transfection-enhancing effects using Chi.

### 2. Materials and methods

#### 2.1. Materials

Medium-molecular-weight Chi with a degree of deacetylation of about 89% was purchased from Sigma-Aldrich. Curcumin, sodium nitrite (NaNO\(_2\)), PF, sodium tripolyphosphate (TPP), sodium hydroxide, hydrochloric acid, acetic acid were all purchased from Merck (Germany). All chemicals were of analytical grade.

#### 2.2. Methods

##### 2.2.1. Preparation of low-molecular-weight chitosan

A low-molecular-weight Chi was prepared by depolymerization medium molecular weight Chi according to the method described by Moghaddam et al [14]. Briefly, Chi was dissolved in acetic acid (6% \( v/v \)) to obtain a solution of 2% \( v/v \) Chi in acetic acid. Low-molecular-weight Chi was obtained after addition of 10 ml of NaNO\(_2\) (7 mg ml\(^{-1}\)) to the dissolved Chi at room temperature under magnetic stirring. After 1 h, the depolymerized Chi was precipitated by raising the pH to 9.0 by adding NaOH. The white-yellowish solid was filtered, washed with acetone three times, and dissolved in 0.1 N acetic acid. Purification was carried out by subsequent dialysis against purified water (Sigma dialysis tubes, 12 kDa). The dialyzed product was lyophilized using a freeze dryer and stored at 4 \(^\circ\)C until further use.

#### 2.2.2. Preparation of curcumin-loaded NPs

After Chi NPs preparation process was completed, PF was incorporated into NPs by adding 0.25% \( \text{(w/v)} \) TPP solution to Chi NPs in 1% \( \text{(v/v)} \) acetic acid solution was obtained by dissolving low-molecular-weight Chi in 1% \( \text{(v/v)} \) acetic acid. Chi NPs were prepared spontaneously upon addition of 0.25% \( \text{(w/v)} \) TPP solution to Chi solution under gentle magnetic stirring at room temperature for 1 h. The volume ratio of Chi solution 0.1% \( \text{(w/v)} \):TPP solution 0.25% \( \text{(w/v)} \) was 2:1 and the opaque suspension was assigned as NPs.

#### 2.2.3. Size measurement and determination of zeta potential

The mean diameter and size distribution of the NPs were measured by dynamic light scattering using Zetasizer (Nano-ZS; Malvern Instruments, Malvern, UK). The zeta potential of freshly prepared NPs was determined by laser Doppler electrophoresis using Zetasizer (Malvern Instruments).

#### 2.2.4. Measurement of drug encapsulation efficiency

The encapsulation efficiencies of curcumin were calculated indirectly by measuring the amount of remaining curcumin in the medium collected at the bottom of the falcon. The absorbance was measured on a Synergy HT (Biotek) at 420 nm wavelength. Each sample was measured in triplicate. The percentage of curcumin encapsulation was determined using the following equation:

\[
EE = \frac{\text{Total curcumin} - \text{Free curcumin}}{\text{Total curcumin}} \times 100\%.
\]

#### 2.2.5. Particle characterization: scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The surface morphology of the fresh prepared NPs was observed using a SEM (S-4800, Hitachi). NPs were dried on an aluminum disk at room temperature. The fixed NPs were coated with gold using a sputter coater.

TEM (JEM1010-JEOL) was used to examine and compare the topography of the NPs. Freeze-dried NPs were suspended in deionized water before observation.

### Table 1. OD values and concentrations of blank and free curcumin samples.

|         | OD value | Concentration (mg ml\(^{-1}\)) |
|---------|----------|-------------------------------|
| Blank   | 0.044    | 0.00                          |
| Free curcumin | 3.921 | 11.5                          |

The preparation of Chi NPs was achieved via the ionic gelation method by Calvo et al [15]. A 0.1% \( \text{(w/v)} \) Chi solution was obtained by dissolving low-molecular-weight Chi in 1% \( \text{(v/v)} \) acetic acid. Chi NPs were prepared spontaneously upon addition of 0.25% \( \text{(w/v)} \) TPP solution to Chi solution under gentle magnetic stirring at room temperature for 1 h. The volume ratio of Chi solution 0.1% \( \text{(w/v)} \):TPP solution 0.25% \( \text{(w/v)} \) was 2:1 and the opaque suspension was assigned as NPs.
Figure 2. SEM (a) and TEM (b) image of Chi-PF NPs loaded with curcumin.

Figure 3. Cellular uptake of curcumin-loaded PF/Chi NPs by HEK293. (a) Phase-contrast image, (b) fluorescence image and (c) merged phase-contrast and fluorescence image.

3. Results and discussion

In the curcumin-loaded PF/Chi NPs structures, the core consists of a spherical PF formed via self-assembly, which is a spontaneous process at or above the critical micelle concentration (CMC) [16–19]. Then, a layer of Chi was coated on the surface of these NPs using electrostatic interactions between the positively charged Chi and the negatively charged PF. The Chi shell can protect the enclosed bioactive compounds from natural degradation, such as hydrolysis, thereby prolonging its activity in the systemic circulation [20, 21].

Once at target cells, the uptake of the NPs can occur through an endocytosis process [22]. The Chi shell is then degraded by lysozyme, which is normally present inside cellular lysosomes, allowing the breakdown of the micelles and the release of curcumin to nucleus [23, 24]. This method is advantageous because it is simple, mild and straightforward, and it can be applicable to a wide range of bioactive compounds.

3.1. Mean diameter, size distribution and zeta potential of NPs

Particle size and zeta potential of both micelles and curcumin-loaded micelles were measured using a Zetasizer. The average micelle size remained the same after curcumin encapsulation, these sizes are 150.9 and 146 nm for pluronic micelles with and without curcumin, respectively. The zeta potential of curcumin-loaded pluronic micelles in the absence and presence of Chi were −2.19 and 5.09 mV. These zeta potentials showed that these micelles were not stable and could easily coagulate, which can be problematic for drug delivery applications. However, after adding Chi, the zeta potential of curcumin-loaded PF/Chi complex has significantly increased. So, Chi can be able to improve stability and decrease aggregation of curcumin-loaded NPs complex. This is useful for their drug delivery applications.

3.2. Measurement of drug encapsulation efficiency

The study of curcumin encapsulation efficiency was carried out to determine an appropriate concentration of PF and Chi. Based on previous studies of Hosniyeh et al [7] and Chawan et al [25] we chose PF and Chi concentrations to be 15% in 0.25% (w/v) TPP and 0.1% in 1% (v/v) CH₃COOH, respectively. After the gelation step, curcumin was physically entrapped in the hydrophobic interior of the micelles. Excess curcumin in the solution was removed by centrifugation and used to calculate the encapsulation efficiency according to equation (1). Optical density (OD) values and concentrations of blank and free curcumin samples are shown in table 1.

Applying the measured values into equation (1), we obtained the following result:

$$EE = \frac{30 - 11.5}{30} \times 100\% = 61.67\%.$$  

So the encapsulation efficiency of curcumin was approximately 61.67%. Our result is compatible with the result of Hosniyeh et al [7].
3.3. Morphology of NPs

An SEM image (figure 2(a)) of PF/Chi NPs loaded with curcumin shows that particles are spherical and uniform. The TEM image of freshly prepared curcumin-micelles (figure 2(b)) indicates that the self-assembled micelles are well dispersed as individual particles with spherical shape. In Chi-PF NPs, darker spots were observed inside the spherical matrix of the NPs. These darker spots are responsible for the contrast observed between Chi and PF. This micrograph proves the incorporation of PF inside the NPs matrix. Our results are consistent with those of other research groups.

3.4. Cellular uptake of curcumin-loaded PF/Chi NPs by HEK293 cells

The study of intracellular uptake of curcumin by HEK293 cells was conducted to demonstrate that the cellular uptake of the NPs was possible. The amount of curcumin taken up by HEK293 cells for up to 24 h was analyzed by fluorescence microscopy (figure 3). Figure 3(a) shows that phase-contrast image of HEK293 cells and the green fluorescence displayed by curcumin (figure 3(b)) appear on the image, and the merged fluorescent images (figure 3(c)) show the penetration of curcumin into a HEK293 cell. However, we realized that only few cells displayed green fluorescence after incubation with curcumin-loaded PF/Chi NPs, so cellular uptake efficiency is not high. Our results are consistent with previous studies of Chawan et al. [25], which suggested that only 15% anticancer drug had accumulated in the cells at 24 h.

The cellular uptake of the Chi NPs, which have no targeting functional group on the surface, was probably mediated by nonspecific adsorptive endocytosis [26]. The positive charge of the Chi NPs could be easily attracted by negatively charged cell membrane [27], allowing the particles to be taken into the cells.

4. Conclusion

Curcumin-loaded PF/Chi NPs were successfully prepared by a mild process without using organic solvents. These NPs were formed using a self-assembled PF micelle as a core coated with Chi via an electrostatic interaction. The presence of a Chi layer on the NPs was confirmed by the change in its zeta potential and morphologically characterized by TEM images. Cellular uptake of curcumin-loaded PF/Chi by HEK293 cells was shown in fluorescence microscopy images. Mechanism for the cellular uptake of curcumin was believed to be due to charge-dipole attraction between the positive charge of the chitosan NPs and negative charge of the cell membrane, which allows the curcumin to be taken into the cells.

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