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Simple and effective preparation of nano-pulverized curcumin by femtosecond laser ablation and the cytotoxic effect on C6 rat glioma cells *in vitro*

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ABSTRACT

The pulverization of poorly water-soluble drugs and drug candidates into nanoscale particles is a simple and effective means of increasing their pharmacological effect. Consequently, efficient methods for pulverizing compounds are being developed. Femtosecond lasers, which emit ultrashort laser pulses, can be used to generate nanoscale particles without heating and are finding in various fields, including pharmaceutical science. Laser ablation holds promise as a novel top-down pulverization method for obtaining drug nanoparticles. We used a poorly water-soluble compound, curcumin (diferuloyl methane), to understand the characteristics of femtosecond laser pulverization. Various factors such as laser strength, laser scan speed, and the buffer solution affected the size of the curcumin particles. The minimum curcumin particle size was approximately 500 nm; the particle size was stable after 30 days. *In vitro* studies suggested that curcumin nanoparticles exhibited a cytotoxic effect on C6 rat glioma cells, and remarkable intracellular uptake of the curcumin nanoparticles was observed. The results suggest that femtosecond laser ablation is a useful approach for preparing curcumin nanoparticles that exhibit remarkable therapeutic effects.

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1. Introduction

The low bioavailability of drug candidates is a major problem for pharmaceutical researchers and pharmaceutical companies. Almost 40% of drug candidates are poorly water-soluble; consequently, various methods for improving their dissolution behavior and bioavailability have been reported (Keck and Muller, 2006; Krishnaiah 2010; Nagarwal et al., 2011; Ozeki and Tagami, 2013; Shegokar and Muller, 2010). One simple and effective method is to decrease the particle size of drugs into nanoscale particles to increase the surface area, since increased surface area increases the dissolution rate, as explained by the Noyes–Whitney equation. In addition, decreasing drug particle size increases saturation solubility, as explained by the Ostwald–Freundlich equation. The methods for decreasing the particle size of drugs are mainly categorized into two methods: the top-down method and the bottom-up method. For example, the nanocrystal technology owned by Elan Corporation is a wet milling method which is categorized as a top-down method (Merisko-Liversidge and Liversidge, 2008) (Merisko-Liversidge and Liversidge, 2011). Although wet milling methods that use media mills sometimes have problems due to impurities derived from the media, this technology is in practical use and several drugs milled using this approach are approved by the FDA.

Laser technologies are widely used in engineering and their application has been extended to other fields, including medical and pharmaceutical sciences. Femtosecond lasers, which emit an ultrashort laser pulse (1 fs = 10^{-15} of a second), has the advantage that it can be used for nano-scale fabrication without heating the target, in contrast to relatively long laser pulses (such as nanosecond lasers) which do heat the target (Chichkov et al., 1996).

Femtosecond lasers have been used in medical and pharmaceutical fields such LASIK surgery for eyes (Ozulken et al., 2013), cell surgery (Ronchi et al., 2012), transfection (Brown et al., 2008), and multiphoton laser microscopy (Mojzisova and Vermot, 2011). Recently, laser ablation has emerged as a new top-down method for decreasing the particle size of drugs. This method does not require use of a medium and thus does not generate impurities. However, only a few articles have reported that organic compounds and poorly water-soluble drug crystals can be pulverized to prepare

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nanoparticles using a femtosecond laser (Kenth et al., 2010; Sylvestre et al., 2011). Consequently, we do not have a detailed understanding of the characteristics of femtosecond laser ablation.

In this study, the pulverization of curcumin by femtosecond laser ablation is demonstrated. Curcumin is a main ingredient of turmeric and has many pharmacological effects (Noorafshan and Ashkani-Esfahani, 2013) such as an anti-inflammatory disease effect (Ali et al., 2012; Jurenka, 2009), neuro- and hepato-protective effects (Abu-Rizq et al., 2008; Reeta et al., 2009; Vera-Ramirez et al., 2013), as a treatment for heart failure (Kapakos et al., 2012; Katanasaka et al., 2013) (Morimoto et al., 2008) and anti-tumor effects (Ji et al., 2012; Odot et al., 2004). Additionally, curcumin exhibits no severe toxicity (a dose of 8 g can be tolerated for 3 months (Fan et al., 2013)). However, its low bioavailability is a major problem (Anand et al., 2007). We speculated that the size reduction of curcumin by femtosecond laser ablation, used as a new technology in the pharmaceutical field, can improve the therapeutic effect of curcumin. In this study, femtosecond ablation was demonstrated and the original curcumin powder was pulverized using various experimental conditions. The cytotoxic effect of the resulting curcumin nanoparticles was also tested on glioma cells, which are obtained from one of the most malignant types of tumor.

2. Materials and methods

2.1. Materials

Curcumin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Poloxamer 188 was kindly donated by BASF Japan (Tokyo, Japan). Sodium acetate and ethanol were purchased from Wako Pure Chemical (Osaka, Japan). PBS tablets were purchased from Takara Bio Inc. (Shiga, Japan).

2.2. Femtosecond laser ablation

A scheme outlining the laser ablation technique is shown in Fig. 1. Curcumin tablets (150 mg curcumin/tablet) were first prepared using a compact machine, then individual tablets were placed on the bottom of a 10 cm diameter glass dish and secured by a plastic ring. The glass dish was carefully filled with 50 mL of pure water, sodium acetate buffer (0.5 M, pH 5.0) or PBS (9.57 mM, pH 7.4).

An IMRA fiber laser femtosecond laser device (FCPA μ-Jewel D-1000; IMRA America, MI, USA) was used, providing 800 fs pulses centered at 1045 nm with a repetition rate of 100 kHz. The laser was trained onto the surface of the submerged curcumin tablet for 10 min. The laser was scanned continuously both longitudinally and transversely. The sample solution in the glass dish was continuously pipetted during exposure to the laser to prevent the resulting curcumin nanoparticles from shielding the curcumin tablet. Various experimental conditions were tested by changing the laser strength (0.33 W and 1 W) and scan speed (100–30,000 mm/s).

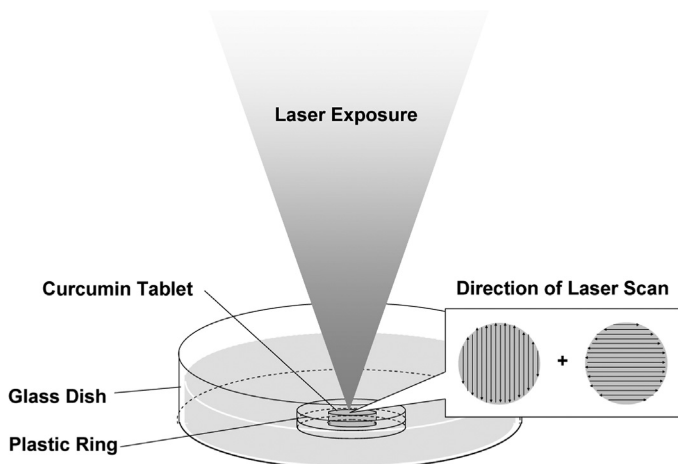

Fig. 1. Scheme showing the femtosecond laser ablation method.

After laser exposure, the sample solution was collected and the mean diameter of the curcumin particles was determined by dynamic light scattering using a particle sizer (ZetaSizer Nano-ZS; Malvern Instrument Ltd., Malvern, UK).

The samples were dried and observed by SEM (S-4300, Hitachi; Tokyo, Japan), the dried samples were dissolved in ethanol (water/ethanol = 1/9, v/v), and the fluorescence of the dissolved curcumin (excitation/emission = 485 nm/590 nm) was measured using a plate reader (Wallac 4000 ARVO multi-label counter; PerkinElmer, MA, USA) and the curcumin concentration was calculated.

2.3. Cells

C6 rat glioma cells were purchased from ATCC (VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Wako). The cells were incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO_2/95% air.

2.4. Cytotoxicity assay (WST assay)

The cytotoxicity of the curcumin samples was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) following the manufacturer's protocol with minor modifications. C6 cells were seeded into a 96-well plate at a density of 5.0×10^3 cells/well. After pre-culture of the cells for 24 h, the cells were treated with various concentrations (1, 3, 10, 30, 100 μM) of curcumin nanoparticles, original curcumin powder, or curcumin ethanol solution as a positive control as described in previous reports (Milacic et al., 2008). The curucumin nanoparticles were prepared using femtosecond laser ablation (laser scan speed, 10,000 mm/s; laser strength intensity, 1 W). For the preparation of higher concentration of curcumin nanoparticles (30 μM, 100 μM), resulting curcumin nanoparticles was concentrated by filter centrifugation (Millipore, MA, USA). Original curcumin powder (3 mM) as stock solution was dispersed in pure water. The curcumin ethanol solution (3 mM) as stock solution was prepared by dissolving curcumin in ethanol. The original curcumin powder and curcumin ethanol solution were diluted with medium before use. After exposure to curcumin for 4 h, the cells were washed with PBS, then fresh medium was added and the cells were further incubated for 44 h. The medium was aspirated, then 110 μl of the solution mixture (100 μl medium plus 10 μl of CCK-8 solution) was added and the cells were incubated for 1 h. The absorbance of the medium at 450 nm was measured using a plate reader (Wallac 4000 ARVO multi-label counter, PerkinElmer). The background absorbance of the medium was subtracted from the total absorbance and then cell viability was calculated.

2.5. Cellular uptake

Cells were seeded into a 24-well plate at a density of 2.5×10^4 cells/well. After pre-culturing the cells for 24 h, the cells were treated with 10 μM of curcumin nanoparticles, original curcumin powder, or curcumin ethanol solution. The preparation

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