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# The exploration of endocytic mechanisms of PLA-PEG nanoparticles prepared by coaxialtri-capillary electrospray-template removal method

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The nano-sized poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) particles with core-shell structure were efficiently prepared by using coaxial tri-capillary electrospray-template removal method. The cellular uptake mechanism, intracellular distribution and exocytosis in A549 cell model of electrosprayed PLA-PEG nanoparticles were systemically studied. The drug release behavior of electrosprayed PLA-PEG nanoparticles were also investigated. Our results showed that PLA-PEG nanoparticles can be endocytosed quickly by A549 cells. The cellular uptake of PLA-PEG nanoparticles was an energy dependent endocytosis process. Caveolae-mediated endocytosis was only one of endocytosis pathways in A549 cells for PLA-PEG nanoparticles, while clathrin mediated endocytosis was not involved in the endocytosis process. The endocytosed PLA-PEG nanoparticles enriched in the head of A549 cells and only a small amount of them was transported into lysosome after 24 h incubation. These findings provided insights into the application of electrosprayed PLA-PEG nanoparticles in nano drug delivery field.

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## 1. Introduction

Nanoparticles, such as micelles and nanogels, are currently being widely explored for drug delivery applications, because these nanocarriers can deliver anti-tumor agents to target sites in the body, which improve therapeutic efficacy and minimize undesired side-effects [1–5]. A wide variety of methods have been reported for the fabrication of nanoparticles, such as emulsion-solvent evaporation, microfluidic systems, self-assembly [6,7]. However, there are some inevitable disadvantages associated with these methods. For example, the widely studied emulsion-solvent evaporation method face challenges in producing uniform-sized nanoparticles for drug delivery purposes. Moreover, the yield of particles prepared by emulsion-solvent evaporation method was very low [8–11]. The self-assembly method has limitations with respect to encapsulation of hydrophilic agents. And it was difficult to scale-up because of the batch-nature of the method [12]. Electrospray, which is based on the principle of electrospinning, is an emerging technique for the rapid and high throughput preparation of particles [13]. Compared to conventional manufacturing methods, the electrospray method has several unique advantages including enhanced encapsulation efficiency of hydrophilic and hydrophobic agents, scale-up potential, tight control over particle size and excellent particulate reproducibility [6,14]. Furthermore, core-shell structured particles can be conveniently obtained through coaxial electrospray [10,15]. Despite the aforementioned advantages, however, the size of electro-sprayed particles was always in micron to submicron range [6,11]. There have been few reports on the production of polymeric particles with sizes less than 100 nm. Particle size, no doubt, plays an important role in the therapeutic treatment. Nanoparticles with sizes below 100 nm, for instance, can accumulate into tumor through enhanced permeation and retention effect. While microparticles with size above 500 nm can be cleared rapidly by macrophages from circulation [16–18]. It means that most of electrosprayed particles are not suitable for intravascular injections, limiting the application of electrosprayed particles as carriers for the controlled delivery of drugs. In order to efficiently produce nano-sized polymericparticles, our group modified the traditional electrospray technique [6]. Coaxialtri-capillary electrospray-template removal method which not only remains aforementioned advantages of traditional electrospray technique

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but also can efficiently generate nano-sized particles was innovatively used.

The mechanism of cellular uptake is important in the field of nanomedicine, cancer diagnosis, and treatment [19–21]. It has been reported that polymeric nanoparticles enter cells via endocytic pathways [22,23]. For example, Zhang et al. found that PLA-b-PEG micelles enter cells via dynamin- and caveolin-dependent endocytosis [24]. However, the specific endocytic mechanism by which cells internalize electrosprayed PLA-PEG nanoparticles remains unknown. The mechanisms of nanocarrier cell internalization are dramatically influenced by nanoparticles' physicochemical properties such as size, shape, charge and surface coating [16,17,20,25,26]. Obviously, the structure of electrosprayed nanoparticles is more compact than micelles, so the physicochemical properties of electrosprayed nanoparticles is different than micelles. We therefore hypothesized that the endocytic mechanism of elctrosprayed PLA-PEG nanoparticles may be different from PLA-PEG micells' endocytic mechanism. It's will be interesting and meaningful to figure out which forms of endocytosis involved in the cellular uptake of electrosprayed PLA-PEG nanoparticles.

The aim of this work was to efficiently prepare nano-sized core-shell PLA-PEG particles by electrospray technique and deeply study the cellular uptake mechanism, intracellular distribution and exocytosis of these nanoparticles. Therefore, in this study, we innovatively used coaxialtri-capillary electrospray-template removal method to prepare nano-sized core–shell PLA-PEG particles. Compared with traditional electrospray method, which was used to prepare micron or submicron particles, coaxialtri-capillary electrospray-template removal method has ability to generate core-shell polymeric particles with sizes about 100 nm. The cellular uptake mechanism of electrosprayed nanoparticles was explored by using several selective endocytic inhabitors. The intracellular distribution and exocytosis of electrosprayed nanoparticles were also studied. We use Ritger-Peppas model to detailedly investigate the drug release behavior and mechanism of electrospryed nanoparticles.

## 2. Experimental section

### 2.1. Materials

Polylactide-b-polyethylene glycol (PLA-PEG, PEG 10000, Mn = 46,000) was synthesized in our lab. Polyethylene glycol (PEG, Mn = 20,000), Sodium azide ($NaN_3$) and Sucrose were purchased from Sinopharm Chemical Reagent Co. Ltd. Fluoresceine isothiocyanate (FITC), Hochest 33342, Nile Red, Phalloidine-FITC, Thiazolyl blue, and Chlorpromazine were purchased from Sigma-Aldrich. Lysotracker Green and Mitotracker Green FM were purchased from Biyuntian Biomedical Technology Co. Ltd. Trifluoroethanol (TFE) was bought from Weihai Newera Chemical Co. Ltd. Paclitaxel (PTX) was supplied by Hangzhou Haida Pharmaceutical Chemical Co. Ltd. All other chemicals and solvents were of analytical grade and used as received.

### 2.2. Preparation of nano-sized core–shell PLA-PEG particles

The nano-sized core–shell PLA-PEG particles were prepared by using a coaxialtri-capillary electrospray-template removal method, as we have reported [6]. The schematic diagram of coaxial tri-capillary electrospray system to prepare core–shell–corona microparticles is shown in Scheme 1. The setup is composed of a tri-capillaryspray head, a high-voltage power supply, three syringe pumps and a collecting plate. The spray head consists of three coaxially arranged stainless needles. A foil paper on the platform was used as a collecting plate. 10% (w/v) PEG solution in TFE was used as corona fluid, 3% (w/v) PLA-PEG solution in chloroform as shell fluid and 3% (w/v) PEG solution in chloroform as core fluid. All three fluids were fed through syringe pumps independently with different feed rate. In order to remove the template PEG on microparticles' surface, the foil papers for collecting electrosprayed particles were cut into pieces and soaked in deionization water for several hours at room temperature. Afterwards, the suspension was filtered with a low speed quantitative filter paper to remove foil papers and possible aggregates. The filtrate was stored in refrigerator at 4 °C till characterization.

### 2.3. Preparation of PTX-loaded PLA-PEG nanoparticles

PTX-loaded PLA-PEG nanoparticles were prepared by using the same method as in the previous section. The widely used anticancer drug Paclitaxel (PTX) was selected as a model drug. 10% (w/v) PTX dissolved in chloroform was used as core fluid, 3% (w/v) PLA-PEG as the shell fluid and 10% (w/v) PEG as corona fluid. The flow rates of shell and corona fluids were fixed at 0.5 and 0.8 mL/h, respectively. The flow rate of drug solution was adjusted from 0.2 to 0.3 mL/h to achieve different drug loading contents in the nanoparticles.

### 2.4. Characterization of micro- and nanoparticles

The morphologies of the particles were characterized by scanning electron microscope (SEM, Hitachi S-4800, Hitachi, Japan) and transmission electron microscopy (TEM, JEM-1230, JEOL, Japan). The particle sizes and Z-potentials of the nanoparticles were determined by nano particle potential analyzer (Malvern ZCEC, Malvern, UK). The structures of the microparticles were observed by laser scanning confocal microscope (LSCM, SP5II, Leica, Germany).

### 2.5. Cellular uptake of PLA-PEG nanoparticles

The adenocarcinomic human alveolar basal epithelial cell line A549 was kindly provided by Professor Guping Tang (Zhejiang University, Hangzhou, China). The murine macrophage cell line RAW264.7 were purchased from China Center for Type Culture Collection. A549 cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin and 100 ug/mL streptomycin at 37 °C in 5% $CO_2$ in a humidified atmosphere.

#### 2.5.1. Cellular uptake kinetics of PLA-PEG nanoparticles

*2.5.1.1. The effect of PLA-PEG nanoparticle concentration on cellular uptake.* The A549 cells were seeded into a 24-well plate (Costar, Corning Corp., NY) at a density of $8 \times 10^4$ cells per well and incubated overnight. After discard culture solution, the cells were coincubated with Nile Red labeled PLA-PEG nanoparticle solution which was diluted with culture medium containing 2% FBS for 3 h at 37 °C. The final concentration of PLA-PEG nanoparticle varied from 1 to 100 μg/mL. After the incubation, the cells were washed with PBS three times. 200 uL of tyrisin solution (0.25%, w/v) was added to each well. Three minutes later, 400 uL of FBS solution (10%, w/v) was added to each well. Then the cells suspension was collected and centrifuged at 1200 rpm for 5 min. Thereafter, the supernatant was discarded and 200 μL of PBS was added to make the cells resuspended. Flow cytometry was used to detect cellular uptake of PLA-PEG nanoparticles.

*2.5.1.2. The effect of incubation time on cellular uptake of PLA-PEG nanoparticles.* A549 cells were coincubated with Nile Red labeled PLA-PEG nanoparticle solution (10 ug/mL) for 1, 2, 3, 6, 12, 24 h, respectively. Then the A549 cells were treated with the same method as mentioned above. Flow cytometry was used to detect cellular uptake of PLA-PEG nanoparticles.

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