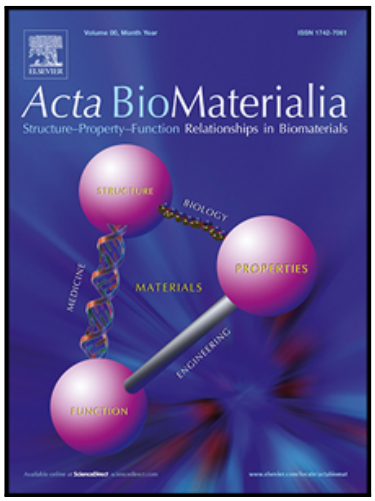

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Preparation of Cetyl Palmitate-based PEGylated Solid Lipid Nanoparticles by Microfluidic technique

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Graphycal abstract

Keywords: solid lipid nanoparticles, microfluidics, industrial scale up, 3D cell culture, drug delivery

Abstract

In recent years, several studies have shown that the use of solid lipid nanoparticles (SLN) as a colloidal drug delivery system was more advantageous than lipid emulsions, liposomes and polymeric nanoparticles. SLNs have numerous advantages of different nanosystems and rule out many of their drawbacks. Despite the numerous advantages of SLNs, translation from the preclinical formulation to the industrial scale-up is limited. In order to provide a reproducible and reliable method of producing nanoparticles, and thus, obtain an industrial scale-up, several methods of synthesis of nanoparticles by microfluidic have been developed. Microfluidic technique allows a good control and a continuous online synthesis of nanosystems compared to synthesis in bulk, leading to a narrow size distribution, high batch-to-batch reproducibility, as well as to the industrial scale-up feasibility. This work described the optimization process to produce SLNs by microfluidics. The SLNs produced by microfluidics were characterized by complementary optical and morphological techniques and compared with those produced by bulk method. SLNs were loaded with paclitaxel and sorafenib, used as model drugs. The anti-cancer efficiency of the SLNs formulation was estimated with 2D and 3D tumour models of two different cell lines, and the cellular uptake was also established with fluorescence assisted.

Statement of significance:

In this work, we describe the production of a single step continuous production for solid lipid nanoparticles (SLNs) via glass capillary-based microfluidic-chip. Comparing to conventional bulk methods, the current synthesis method showed several advantages, including a continuous production with high yield, good reproducibility and precise control over the properties of SLNs, which are critical pre-conditions for its successful industrialization. The superiority of this microfluidic-based method was confirmed by an overall physicochemical characterization of the produced SLNs. The size of the SLNs was controlled by altering the microfluidic parameters, and SLNs with dimensions ca. 100 nm were feasibly fabricated through parameters optimization. The microfluidics production of SLNs offered a good encapsulation efficiency and drug loading degree for a sustained release manner.

1. Introduction

The use of nanoparticulate systems for the treatment of diseases is in continuous progress. As a result of their ability to overcome the disadvantages of the drugs currently used, including controlled and extended drug release, increased delivery to tumor site, and reduced systemic toxicity of cytotoxic agents, they are rapidly developing [1] [2]. An ideal candidate for successful clinical translation should have the following features: (1) a simple and biocompatible component; (2) a facile large-scale production method; (3) a satisfied drug loading; and (4) and releasing behaviour and good stability.

Among all nanoparticulate systems, solid lipid nanoparticles (SLNs), have extensively been evaluated as an alternative drug delivery system [3, 4]. The main advantages of SLNs, comparing to conventional nanoparticle-based drug delivery systems, include the inherent biocompatible nature of the starting materials, possibility of obtaining a controlled release of the incorporated drug for several weeks, stability of the nanoparticle system up to 3 years, and more importantly, besides of its hydrophobic backbone, which endow the potent capability for loading hydrophobic drugs, they are also shown to effectively encapsulate hydrophilic drugs [5-7]. Although there are several advantages of SLNs, clinical use is very limited. The main reasons lie in the absence of a method that allows industrial scale-up, batch-to-batch reproducibility and control of the chemical-physical properties of nanomaterials [8].

The production of SLNs by bulk method involves a very long time of preparation, high concentrations of lipids and surfactants, and a large variability between different batches in terms of size and polydispersity of nanoparticles. The great variability of the synthesis conditions and problems associated with limited mixing process represent significant obstacles for SLNs production by conventional bulk method. The difficulties mentioned above became limiting in obtaining nanoparticles with sizes under 200 nm, which are desirable due to their ability to cross spontaneously different biological barriers [9]. Therefore, research in this field is moving in order to identify a new attractive approach capable of finely controlling a mixing process that allows to generate homogeneous nanosystem of elevated quality [10].

Recently, the focus has been on nanosystems synthetic feasibility using microfluidic based method. This new technique ensures SLN production in a reproducible and reliable way [10-20]. Compared to batch-type bulk methods, microfluidics guarantees both a high control of the synthesis conditions and continuous flow production leading to a narrow size distribution (low polydispersity index, PdI), high batch-to-batch reproducibility, as well as to the industrial scale-up feasibility [10]. However, despite the recent works using microfluidics to synthesize liposomes or lipid

nanoparticles [21-26] to our knowledge, there are currently no works concerning the production of SLNs by microfluidic techniques. Polydimethylsiloxane (PDMS) based microfluidics chip is highly sensitive to organic solvents, which is inevitable for SLNs synthesis [10, 27]. In addition, PDMS based microfluidic chips are not resistant to heating, which is usually required for SLNs synthesis. Meanwhile, the hydrophobic nature of PDMS results in the propensity of the lipid aggregation and adsorption on the walls of microfluidic channel. Whereas for glass or silicon based microfluidics chips, despite their chemical inertness to overcome these limitations, the producing methods usually involve harsh and strict conditions, which may further limit the wide application [28]. As an alternative choice, microfluidic chips composed by glass capillary are relative cheap and easy for fabricating, and therefore draw increasing attentions for synthesizing nanoparticles [13, 14, 16-20].

Here, we described a single step continuous production of SLNs via glass capillary based microfluidic chip. Comparing to the conventional bulk methods, which are usually restrained by multiple and complicated preparation steps, low production rate and poor reproducibility, the current synthesis method showed several advantages, including a continuous production with high yield, good reproducibility and precise control over the physical properties of SLNs, which are critical pre-conditions for its successful industrialization. Furthermore, the superiority of this microfluidic-based method, comparing to the conventional bulk method, was confirmed by an overall promoted physicochemical property of the produced SLNs. The size of the produced SLNs was controlled via altering the microfluidic parameters, and herein, SLNs with dimensions around 100 nm were feasibly fabricated through parameters optimization. Sorafenib (SFN) and paclitaxel (PTX) were used as model drugs, in order to test the feasibility of applying SLNs-based nanoformulation in cancer treatment. The microfluidics production of SLNs offered a good encapsulation efficiency and loading degree of the drugs for a sustained release manner. The anti-cancer efficiency of the drug-loaded SLNs formulations was further evaluated with both 2D and 3D tumour models of two different cell lines, and the tumor penetration and cellular uptake was also confirmed with fluorescence assisted imaging.

2.0. Materials and Methods

2.1. Materials and cell culturing

All chemicals were of the highest purity available and were used as received without further purification or distillation. Distearoyl phosphoethanolamine- polyethylene glycol (DSPE-PEG) and 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol)-2000]c (16:0 PEG-2-PE) was purchased from Avanti Polar Lipids. Cetyl palmitate was purchased from Farmalabor. Paclitaxel (PTX) and sorafenib (SFN) were purchased from Sigma Aldrich. All solvents used were of analytical grade and purchased from Aldrich. 4-[4-(Dihexadecylamino)styryl]-N-methylpyridinium iodide (DiA) was acquired from Thermo Scientific, USA. All aqueous solutions were prepared using water obtained from a Milli- Q gradient A- 10 system (Millipore, 18.2 MΩ·cm, organic carbon content ≥ 4 μg/L. Dulbecco's Modified Eagle's medium (DMEM), heat inactivated fetal bovine serum (FBS), L-glutamine (200 mM), non-essential amino acids (NEAA), penicillin (100 IU/mL), streptomycin (100 mg/mL) and trypsin (2.5%) were acquired from HyClone Waltham, USA. Phosphate buffer saline (10XPBS) and Hank's balanced salt solution (10× HBSS) were purchased from Hyclone. Disposable culture flasks and Petri dishes were from Corning (Glassworks).

2.2. Production of SLNs with bulk method

SLNs were prepared using an oil-in-water homogenization process at high temperature, according to a procedure reported in literature [29]. In particular, 60 mg of cetyl palmitate and 12 mg of 16:0 PEG- 2- PE were co- dissolved in chloroform (1 mL). Afterwards, a homogeneous mixture was obtained by stirring gently and then the chloroform solution was added drop-by-drop into the aqueous solution (ultrapure water, 3 mL), containing Pluronic F68 (2% p/V) at 65 °C and sonicated for 15 min by using an ultrasound probe-tip (0.27 W). The organic phase was rapidly evaporated at 65 °C by means of a rotary evaporator. The aqueous solution was left at room temperature for 2 h to promote the complete evaporation of the organic solvents, and then, it was kept at 4 °C for 15 min to allow the SLNs formation. In order to remove the surfactant and solvent residuals the produced SLNs were carefully purified by using ultrapure water and centrifugal concentrators (Centricon Centriplus YM100) at 800 g for 1 h at 4 °C. The nanoformulation was kept in ultrapure water at 4°C.

2.3. Fabrication of the microfluidic devices

The microfluidic devices were assembled from borosilicate glass capillaries and glass rods. In this work, two different devices were developed and identified as device 1 (conventional microfluidic chip) and device 2. For device 1, one end of the cylindrical glass capillary (World Precision Instruments, Inc.), with inner and outer diameters of 580 and 1000 μm, respectively, was tapered using a micropipette puller (P-97, Sutter Instrument Co., USA) to a diameter of 20 μm; this diameter was further enlarged to approximately 80 μm by using sand paper (RHYNOWET P-2500, Indasa, USA). This cylindrical tapered capillary was inserted and coaxially aligned into the left end of the cylindrical capillary with inner dimension of 1100 μm (Vitrocom, USA).[30, 31] In the case of device 2, a three-port valve is connected after the device 1. Two miscible (functioning as outer and inner phase for nanoparticle production) liquids were injected separately into the microfluidic device through polyethylene tubes attached to syringes at constant flow rates, and the air was introduced to the solution through the three-port valve to further enhance the mixing efficiency. The flow rate of the different liquids was controlled by pumps (PHD 2000, Harvard Apparatus, USA). A schematic comparison of device 1 and 2 is shown in **Figure S1**.

During the production of SLNs, it was fundamental to keep the temperature above 60 ºC. In this regard, a supporting heating facility was designed and constructed by wrapping the lipid containing syringe with electric wire, which connected with a pressure regulator; the temperature of the electric wire wrapped syringe was controlled by altering the voltage, and the temperature was set to 60 ºC. The microfluidic chip was immersed in water containing heating bath, and the temperature was also maintained at 60 ºC.

2.4. Optimization of SLNs production by microfluidics

The SLNs were prepared by nanoprecipitation in a glass capillary microfluidics device, as mentioned above [14, 16, 17, 19, 32]. In the co-flow geometry, the inner and the outer fluids flow in the same directions. During the nanoprecipitation method, the internal and external solution are pumped into the microfluidic device with a constant flow rate. These solutions are miscible, and two pumps kept the flow rate of the two phases under control and the liquids were transported from the syringes to the capillaries thanks to the use of polyethylene tubes. The lipid matrix was dissolved in an 95% ethanol solution and served as the inner fluid. In addition, an aqueous solution containing stabilizers was selected as the outer continuous fluid. The SLNs synthesis process was optimized through the variation of different parameters, including flow rate, flow speed, type of surfactants (Pluronic F68 (F68), Pluronic F127 (F127), Polyvinyl Alcohol (PVA) at the

concentrations of 2%, 3% and 4% (w/v) and Tween 80 (T80) at the concentrations 1% and 2% (w/v)).

The lipid component of the nanoparticle preparation consisted of cetyl palmitate and DSPE–PEG (3 mg/mL). Different concentrations were tested using cetyl palmitate (10, 50 and 100 mg/mL). Moreover, different inner and outer fluid flow rates were used in order to identify the one that allowed to obtain small SLNs. Both the fluids were injected into microfluidic device from separate inlets at flow rate of 5:10 to 50:100 mL/min. The cooling phase (4°C for 15 min) occurs after the complete evaporation of the organic phase. When SLNs loaded PTX or SFN were produced, the drug was added to the ethanol solution along with the lipids. Specifically, for PTX, concentrations were tested in a range from 0.5 to 1.5 mg/mL and for SFN in a range from 0.2 to 2 mg/mL. The purification of SLNs took place through the dialysis bags, in order to eliminate the surfactant and the unloaded drug. (Spectra/Por 1 Standard RC Dry Dialysis Tubing, 12-14 kDa, Spectrum Labs, USA) for 24 h at 25 °C. All the optimization experiments, i.e., the variation of the different parameters, for the production of the NPs were carried out using the device 1. After identifying the best parameters, device 2 was used in order to compare the results.

2.5. Encapsulation efficiency and drug loading determination

To obtain drug loaded SLNs, PTX and SFN were mixed into the inner fluid. The percentage of encapsulation efficacy (EE%) of PTX and SFN in the SLNs and the drug loading (DL%) was estimated using high-performance liquid chromatography (HPLC, Agilent 1200 Infinity Series). Briefly, 2 mL of DMSO and hexane 1:1 was added to disrupt the SLNs and 20 µL of the resulting transparent solution were injected into HPLC. For PTX a Discovery C18 (150 × 4.6 mm, 5 µm) column and a mobile phase consisting of water and acetonitrile (ACN) (53:47%, v/v), with detection wavelength at 227 nm, flow 1 mL/min, injection volume 20µl, while for SFN a Gemini (3 µm, NX-C18, 110Å) column and a mobile phase consisting of water and 0.1% of trifluoroacetic acid (TFA) and ACN (48:52%, v/v), with detection wavelength at 255 nm, flow 1 ml/min, injection volume 20µl, were used to quantify the targeted drugs. The EE% and DL% were calculated using Eqs. (1) and (2), respectively:

( ) ——————————— (1)

( ) ———————————

(2)

2.6. Characterization of the produced SLNs

The zeta (ζ)-potential and size distribution of the samples were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). About 1 mL of a 1:50 dilution of each sample with demineralized water was poured out into a disposable polystyrene cuvette (Sarstedt AG & Co., Germany) and the measurements were carried out at 25 ± 0.1 ºC. The surface potential of the nanoparticles was measured by pipetting 750 µL of each particle suspension into a disposable folder capillary cell (DTS1070, Malvern, UK); the sample was redispersed in MilliQ-water (pH 7.4) before assessing its ζ-potential. The resulting data are indicated as numeric average and standard deviation of the measurements of three different samples, each sample measured three times.

2.7. Transmission electron microscope imaging

The morphology and the size distribution of the SLNs were analysed by a transmission electron microscope (TEM, Jeol JEM-1400, Jeol Ltd., Japan). In order to achieve this, 10 µL of particles suspension were applied to a carbon-coated copper grid (300 mesh; Electron Microscopy Sciences, USA) for 5 min. Then, the samples were negatively stained with uranyl acetate by adding 2 µL of a 2.1% uranyl acetate solution to the grids for ~2 min. The grids were then washed once with 5 µL of Milli-Q water for 5 min to remove the excess of uranyl acetate. Finally, the grids were dried in open air overnight before imaging.

2.8. Stability studies

To evaluate the SLNs short-term stability, the size distribution of SLNs was measured in PBS (pH 7.4), in DMEM supplemented with 10% (v/v) of FBS and in fresh frozen plasma (provided by the Finnish Red Cross). Briefly, 200 µL of SLNs were incubated in 1.5 mL of physiological relevant media at 37 ºC; at defined time points (5, 15, 30, 60, 90, and 120 min), a certain amount of sample was taken, diluted in water in order to analyse the change in size over time. Triplicates of each experiments were performed.