Original Article

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A facile microfluidic method for production of amphotericin B lipid complex

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Abstract

Amphotericin B (AmB) is a large amphiphilic molecule, which is a drug of choice in therapy of systemic fungal infection because of its board-spectrum antifungal activity. However, conventional formulation of AmB is micelles, which is unstable in the bloodstream and releases drug to form toxic aggregates. Lipid complex of AmB has been developed to reduce toxicity while retaining activity. Microfluidic method is mixing technique, which permits millisecond mixing at the nano/microliter scale, can reproducibly generate limit size of particles of drug delivery system. In this study, microfluidic hydrodynamic focusing method was used to produce AmB lipid complex. It was obtained of two synthetic phospholipids: hydrogenated soy phosphatidylcholine (HSPC), distearoylphosphatidylglycerol (DSPG) and AmB in the molar ratio of 1:1:2.The lipid complex of AmB was characterized using differential scanning calorimetry (DSC), X - ray diffraction, Fourier-transform infrared spectroscopy (FTIR) and Field Emission Scanning Electron Microscopy(FESEM).The results showed that AmB lipid complex, prepared by microfluidic method, had smallest size at the flow rate ratio (FRR) of 5:1. The distinct phase transition temperature, the crystal peak of phospholipid, the characteristic band of-P-O- group of phospholipid were not observed in the DSC curve, X-ray diffraction diagram and FTIR spectra, respectively, of AmB lipid complex.

Keyword: Amphotericin B; Lipid complex; Microfluidic method; Hydrodynamic flow focusing method

1. INTRODUCTION

Amphotericin B (AmB) is a large amphiphilic molecule, which is a drug of choice in therapy of systemic fungal infection because of its board-spectrum antifungal activity[1]. In addition, studies have shown that it is also effective in the treatment of visceral leishmaniasis[2]. Since AmB is insoluble in water, conventionally, it was solubilized and formulated in deoxycholate forming micelles. However micelles are unstable in the bloodstream, releasing drug to form toxic aggregates, that may cause side effects including fever, nausea, vomiting, lysis of red blood cells and nephrotoxicity[3]. To reduce the toxic effects of AmB, several lipid-based formulations were developed and compared with the conventional form. In general, lipid complex of AmB were shown to be less toxic than deoxycholate formulation while retain activity[4].

Microfluidic method is a mixing technique, which permits millisecond mixing at the nano/microliter scale, can reproducibly generate limit size of particles of drug delivery system[5]. Thus, microfluidic systems have been increasingly used for fabrication of drug carriers such as liposomes, polymer nanoparticles, emulsion etc.[6–11]. In microfluidic technology, continuous and multiphase fluidic systems are usually used for production of drug carrier[12]. However, among these, the microfluidic hydrodynamic focusing (MHF) approach presents the typical physical

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characteristics of microfluidic systems (i.e., including low Reynolds number and diffusion dominated mass transfer) and represents the suitable microfluidic method for producing lipid-based systems with potential for clinical application[13]. This hydrodynamic flow focusing method squeezes a solvent–lipid solution and aqueous phase into a very narrow central. The narrow width of the focused stream and laminar flow in the channel enables rapid mixing through molecular diffusion at the liquid–liquid interface where the lipids self-assemble into vesicles or lipid complexes [14,15].

In this study, we prepared lipid complex of AmBby microfluidic hydrodynamic focusing flow method. AmB lipid complex was obtained of two synthetic phospholipids: hydrogenated soy phosphatidylcholine (HSPC), distearoyl-phosphatidylglycerol (DSPG) and AmB in the molar ratio of 1:1:2. Influence of the flow rate ratio (FRR) of aqueous to solvent-lipid phases on particle size and size distribution was evaluated. The physicochemical characterization of AmB lipid complex was systematically investigated by Fourier transform infrared spectrometry (FTIR), differential scanningcalorimetry (DSC), powder X-ray diffraction (PXRD) and field emission scanning electron microscopy (FESEM).

2. MATERIALS AND METHODS

2.1 Materials

Amphotericin B was purchased from Ningbo Honor Chemtech Co.,Ltd (China); hydrogenated soy phosphatidyl choline and distearoylphosphatidyl glycerol - Avanti Polar Lipid (US); chloroform was purchased from RCI Labscan Limited (Thailand); ethanol was purchased from DG chemical (Vietnam); acetonitril and methanol for HPLC were purchased from J.T. Baker Avantor (US).

2.2 Method preparation of AmB lipid complex

Microfluidic hydrodynamic focusing (MHF) method: AmB was dissolved in the ethanol, acidified by HCl solution of 2.5 N to pH of 0.5-1.0 (solution 1). HSPC and DSPG in the 1:1 molar ratio were dissolved in the solvent mixture of chloroform and ethanol (1:1, v/v) to form solution 2. Solution 1 was-mixed with solution 2 to form solvent-lipid phase[16]. Aqueouse phase was 0.9% sodium clorid solution. MHF device used in the study-isshown in the Figure 1, which is similar to the device in the reference[17]. Solvent and aqueous phases were heated to 65^{o}C using a water bath before mixing. Solvent-lipid phase was injected through 23G needle (internal diameter was 0.6414 mm) into the connector of MHF device using a peristaltic pump. Aqueous phase was injected into the conector through the tubing with internal diameter of 1mmby peristaltic pump. The internal diameter of the outlet of connector was 2mm. The mixture was evaporated under reduced presure to remove ethanolfor AmB lipid complex suspension production.

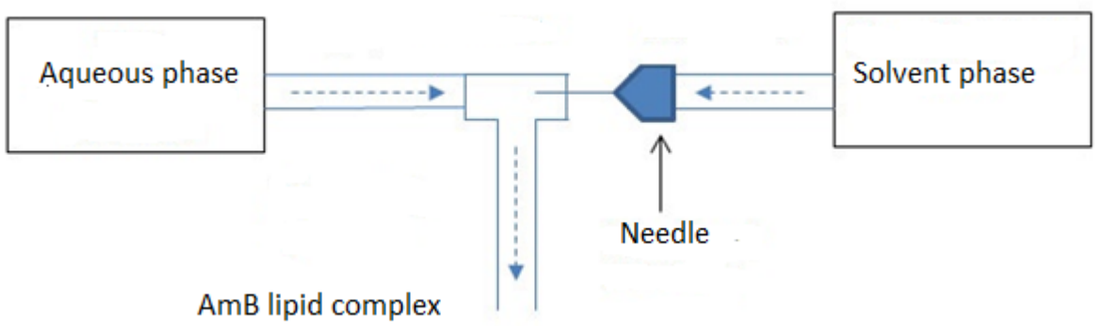

Figure 1. MHF device for producing AmB lipid complex

Purification: prepared suspension of AmB lipid complex was purified and concentrated by crossflow system (SARTOFLOW® Slice 200 Benchtop, Germany) until determined concentration of AmB (5mg/ml) using 30 kDa polyethersulfone membrane (Satorius Stedium biotech GmbH, Germany).

Drying: Suspension of AmB lipid complex was dried in the vacuum oven at 40° C for 48 h for DSC, XRD and FTIR characterization. Dry samples were stored in the refrigerator at 2-8°C.

2.3. Preparation of physical mixture and placebo sample

physical mixture was prepared by mixing AmB, HSPC and DSPG in molar ratio of 1:1:2 thoroughly for 10 min in a mortar until a homogenous mixture was obtained. The sample was passed through 40# mesh.

Placebo sample was prepared by MHF methods using device in "Method preparation of AmB lipid complex" using only HSPC and DSPG. Suspension of placebo sample was dried in the vacuum oven at 40°C for 48 h to use in the XRD and FTIR investigations.

2.4. AmB lipid complex characterization

2.4.1. Size analysis

Particle size and distribution of particle size were evaluated by laser diffraction using Mastersizer 3000 E (Malvern, UK). Each sample was diluted with purified water, filtered through membrane with pore size 0.22 µm, until laserobscuration is in the range 3 – 20%. The parameter Span is an indicator of the particle size distribution of the sample. The smaller Span shows narrower range of distribution.

2.4.2. Determination of AMB content

Quanlitative and quantitative analysis of AmB in lipid complex were performed by High-perfomance liquid chomatography (HPLC). The analytical column was RP 5C18-MS-II 250x4.6 mm (Cosmosil, Japan), operation was carried out at ambient temperature. The mobile phase was a mixture of acetonitril and 0.002M solution of disodium edetat (pH 5.0) in a ratio of 40:60 (v/v), the flow rate was 0.8 ml/min, detector was UV visible at 405 nm. Lipid complex suspension was dissolved with mixtured solvent methanol:chloroform (3:1, v/v), then diluted with solution of acetonitril : 0.002M disodiumedetat (pH 5.0) in a ratio 60:40 (v/v). The sample was filtered through 0.45 µm membrane prior to injection to the column. Under the HPLC condition, retention time of AMB was 6.3 min. HPLC linearity was determined from 6 working standard solutions of AmB. The HPLC method for quantitation of AmB in samples was validated. The calibration curve showed good linearity relationship in the range of 10-100 µg/ml (r^2=0.999). The LOD and LOQ were 3.24 and 9.82 µg/ml, respectively. The evaluation of system suitability, precision parameters were also found to produce satisfactory results.

2.4.3. Differential scanning calorimetry (DSC)

Thermal behavior of selected AmB lipid complex samples was characterized through Mettler Toledo calorimeter (Switzerland). Approximately, 5 mg of solid sample were sealed in an aluminum pan and transferred in the calorimeter. Data were recorded as formulations which were heated at 10°C/min from 40°C to a final temperature of 200°C.

2.4.4. X-ray diffraction (XRD)

The crystalline state of drug in the different samples was evaluated with X-ray powder diffraction. Diffraction patterns were obtained on a Bruker Axs-D8 Discover Powder X-raydiffractometer (Germany). The scanning angle ranged from 10 to 50° of 2θ in step scan mode (step width 1°/min).

2.4.5. Fourier transform infrared spectroscopy

Infra-red spectra were prepared using a Nicolet Magna spectrometer (USA). Thin tablets were produced through pressing approximately 2 mg dry sample 200 mg KBr under pressure of 6-7 T. The dry samples were calculated applying Jasco Spectra Manager® software (USA). The scale range of wave number was adjusted in 4000-400 cm^{-1} with resolution 0.4 cm^{-1}.

2.4.6. Morphology

Morphology of AmB lipid complex was observed by Field Emission Scanning Electron Microscope (FESEM S4800, Hitachi, Japan). The sample was put on a plate, continued drying with vacuum oven at 40°C for 48 h. The dried sample was coated with gold and observed in the FESEM S4800 (Hitachi, Japan).

2.5. Stability of suspension of AmB lipid complex

The concentrated suspension of AmB lipid complex (5mg/ml) and non-concentrated suspension was stored in the refrigerator (at 2-8°C). After determined periods of time, the size of lipid particles was evaluated.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of the mean, and statistical analysis was based on the p-value, p-value < 0.05 was considered statistically significant difference.

3. RESULTS AND DISCUSSION

3.1 Evaluation of flow rate ratio

Table 1 shows the relationship of particle size vs. flow rate ratio (ratio between aqueous and solvent streams). It is clearthatwhen FRR increased, average size (D[4,3]) of particles decreased. The largest mean size of AmB lipid complexwas 33.1 µmatFRR of1:3.2. In contrast, the smallest lipid complex particles(mean diameter =3.4µm) was producedat FRR of 7:1. The combination between the decrease of particle size due to increase of FRR confirms previous studies in the literature[18–20]. However, increasing flow rate ratio increased polydispersity. The impact of flow rate ratio on distribution are in agreement with previous work showing that the increase in FRR widen the distribution range of the particles[19]. When the flow rate ratio was higher than 5, polydispersity strongly increased; at the highest FRR (6:1- formulations F8 and 7:1 – formulation F9) Span value were highest (10.1 and 10.3, respectively). This phenomena should be explained that, at very high flow rate ratio, velocity of aqueous flow was higher than solvent phase by 6 or 7 fold, that push back the solvent flow, leading to the not well mixing phases in the connector. As the data showed, the FRR of 5:1 (Formulation F7) produced small lipid complex particle with narrow distribution; 6.5 µm and 2.25, respectively.

Table 1. Particle size and Span of AmB lipid complex (n=3)

Formulations	FRR	D[4,3] (µm)	Span
F1	1:3.2	33.1±1.2	1.476±0.120
F2	1:1	21.6±1.1	2.065±0.211
F3	1.5:1	10.3±0.8	2.065±0.17
F4	2:1	11±0.9	0.831±0.071
F5	3:1	15.3±0.7	1.481±0.110
F6	4:1	12.3±0.7	1.03±0.091
F7	**5:1**	**6.5±0.4**	**2.27±0.213**
F8	6:1	3.7±0.3	10.1±0.521
F9	7:1	3.4±0.3	10.3±0.523

3.2. Stability of suspension of AmB lipid complex

In this experiment, size of AmB lipid complex particle was reduced from 6.2 µm to 0.8 µm after purification (see Table 2). The polyethersulfone membrane (30 kDa) using in the crossflow system permits particles or molecules, smaller than 30 kDa, pass through. The waste liquid was yellow and transparent, indicated present of AmB. In addition, the processing yield before purification was 89.6±3.5% and after purification – 78.9±2.5%. Therefore, about 10% drug was filtrated from complex suspension after purification, filtrated AmB should be micellar form (because of transparent waste liquid). Thus, decreasing size of complex in the crossflow system should be explained by shortening the ribbon like structure, composed of cylinders of AmB and phospholipid in the molar ration of 1:1[21].

Table 2. Size stability of AmB lipid complex in the suspension

Time (months)		0	1	2
Before purification	D[4,3] (µm)	**6.2±0.9**	7.1±1.1	17.6±1.2
and concentration	Span	2.28±0.15	3.64±0.23	3.95±0.13
After purification	D[4,3] (µm)	**0.833±0.052**	0.868±0.061	0.901±0.063
and concentration	Span	4.01±0.31	4.62±0.32	4.67±0.28

Particle size rapidly increased in the diluted solution (p-value < 0.05), from 6.2 µm at start moment increased to17.6 µm after 2 months storage. By contrast, the changed of particles size and Span of concentrated suspension after 2 months storage was not statistically significant difference with p-value >0.05, particle size changed from 0.833 to 0.901 µm, Span value increased from 4.01 to 4.67. In this study, AmB lipid complex in the concentrated suspension (5 mg/ml of drug concentration) was more stable than in the more diluted suspensions. This phenomena should be explained by forming flocculation, which stabilized particle size of complex in the concentrated suspension.

3.3. DSC

Figure 2 presents the DSC thermograms of AmB, phospholipids (HSPC and DSPG) and AmB lipid complex. AmB exhibits three-characteristicendotherm peak at 129.82°C; 143.54°C and 183.14°C. The HSPC displays a broad endotherm at 70.44°C corresponding to its phase transition from gel state to liquid crystal state. The DSPG exhibits two endothermic peaks; the first peak appears at 49.57°C and the second at 73.67°C corresponding to pre-transition and phase transition temperature, respectively. The DSC curve of AmB lipid complex showed that original peaks of phospholipids and drug have disappeared. The significant difference of DSC graphs among four samples indicated that the AmB and phospholipids in the complex were amorphous. All the results suggested that some weak interactions, such as the hydrogen bonds or Vander Waals force are formed between AmB and phospholipid molecules[22–26].

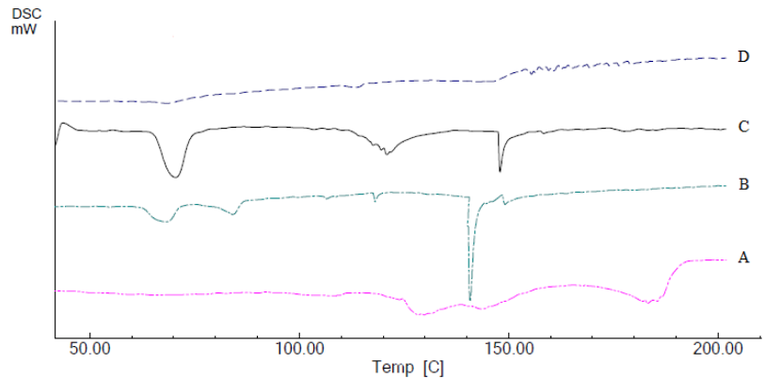

Figure 2. DSC thermograms of AmB (A), DSPG (B), HSPC (C) and AmB lipid complex (D)

3.4. X-ray diffraction

Figure 3 exhibits the XRD patterns of AmB, phospholipid mixture, physical mixture, placebo sample and AmB lipid complex. AmB displays characteristic peaks at 2θ angles of 14°, 17.4°, 20.5°, 21.8°, attributed to its crystalline nature. These peaks were evident in physical mixture and AmBsubstance. However, they disappeared in AmB lipid complex, indicated that drug dispersed in a lipid complex. Mix phospholipids showed obvious diffraction peaks at 21.5° indicating its crystalline characteristics. There is still an apparent diffraction peak at 21.5° in the sample Physical mixture and placebo. By the contrast, the crystalline peaks had disappeared in the complex compared with that of the physical mixture and placebo. It is presumed that a directional combination happened between AmB and phospholipids during the complex forming process. Therefore AmB and phospholipids actually stay in a highly dispersed state, resulting in the masking of their own crystalline characteristics.

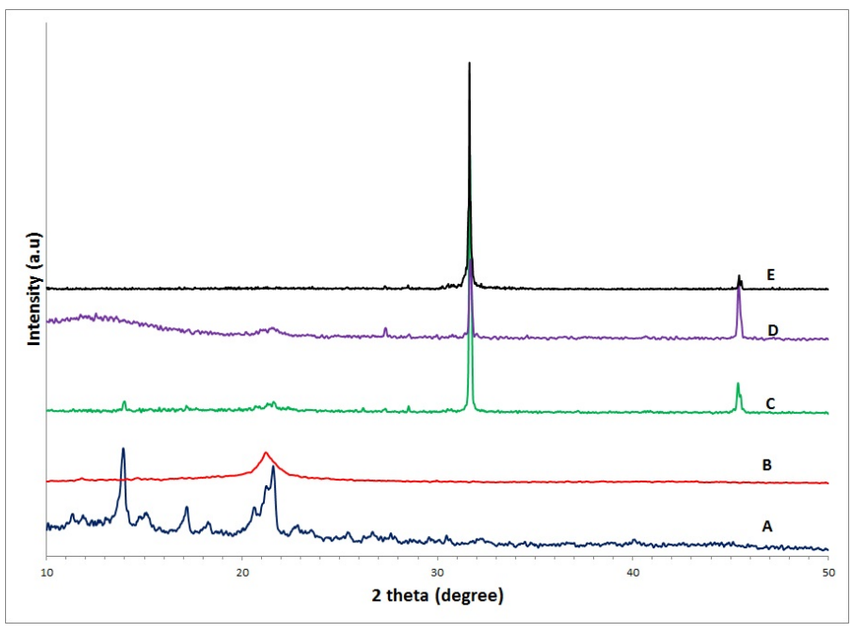

Figure 3. X-ray diffractionspectra of AmB (A), phospholipid mixture (B), physical mixture (C), placebo sample (D) and AmB lipid complex (E).

3.5 FTIR

Figure 4 presents the IR spectra of DSPG, HSPC, AmB, Physical mixture, placebo sampleand AmB lipid complex. The AmB spectrum (Figure 4F) exhibits board OH peaks at 3472.20 cm^{-1}, characteristic peak of C=O at 1691.25 cm^{-1}. The board peak at 3472.20 cm-1 show inter-molecular hydrogen bond[27]. The DSPG spectrum (Figure 4A) exhibits peaks at 1732.08 cm^{-1} (C=O stretching), 1253.73 cm^{-1} (P=O stretching), and 1093.64 cm^{-1} (P-Ostretching). The HSPC spectrum (Figure 4B) exhibits peaks at 1728.22 cm^{-1} (C=O stretching), 1253.73 cm^{-1} (P=O stretching), and 1091.71 cm^{-1} (P-O-stretching). On the contrary, the characteristic peaks at 1093.64 and 1091.71 cm^{-1} of phospholipids (P-O stretching) disappeared in AmB lipid complex (Figure 4E) probably due to weakening or removing. This phenomenon could be due to electronic bond between group NH_3^+ of AmB and P-O- of phospholipids. The peak at 1735.65 and 1718.22cm-1 (C=O stretching) were retained in the physical mixture but changed in the lipid complex, indicating their involvement in the formation of the complex. This could be due to hydrogen bond between C=O of AmB and O-H groups of phospholipid. The peaks at 1238.51 cm^{-1} (P=O stretching) of the phospholipids were retained in the physical mixture, placebo sample and in the complex, indicating their non-involvement in the formation of the complex. The board peak at 3472.20 cm^{-1} of O-H bond in AmB molecule disappeared in the lipid complex and physical mixture showed that quantity of C-H bonds is more than the O-H bonds and intensity of C-H bond overwhelms intensity of O-H bond.

3.6 FESEM

The FESEM images of AmB lipid complex were shown in the Figure 5.

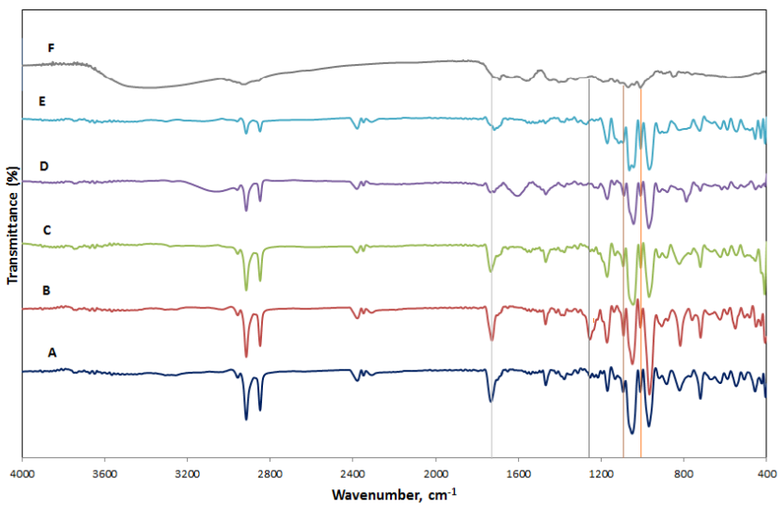

Figure 4. FTIR spectra of DSPG (A), HSPC (B), physical mixture (C), placebo mixture (D), AmB phospholipid complex (E) and AmB substance (F).

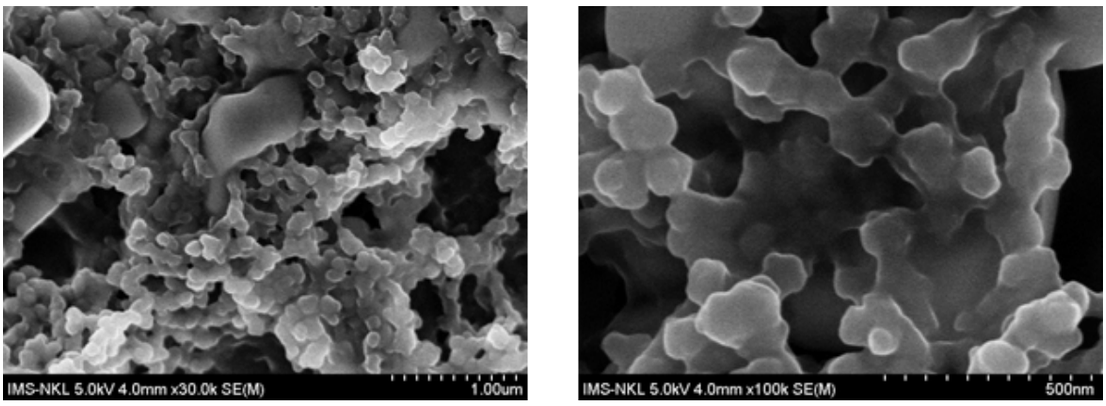

Figure 5. Morphology of AmB lipid complex

The FESEM images of AmB lipid complex are given in Figure 5. As observed in Figure 5, AmB lipid complex existed as ribbon like structure. This morphology of AmB lipid complex coincides with the morphology of the lipid complex, prepared by bulk method[28]. Although AmB lipid complex in our study obtained of HSPC and DSPC, differed from excipients of commercial AmB lipid complex (dimyristoylphosphatidylcholine - DMPC and dimyristoylphosphatidylglycerol - DMPG), but both lipid complexes had similar morphology [21].

4. CONCLUSION

Our present work might provide some new insights and paradigms for production of phospholipid complex using microfluidic method. We have demonstrated a high-throughput, robust method of preparing size-controlled lipid complex using microfluidic simple and reproducible hydrodynamic flow focusing method. The complex formation of AmB lipid complex was proven by FTIR, DSC, PXRD, and FESEM. All studies showed that drug and phospholipid combined and formed complex.

5. ACKNOWLDGEMENTS

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Declaration of interest statement

The authors report no conflicts of interest

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