Elsevier Editorial System(tm) for European Journal of Pharmaceutics and Biopharmaceutics
Manuscript Draft

Manuscript Number:

Title: Encapsulation of cannabinoid drugs in nanostructured lipid carriers

Article Type: Note

Keywords: Nanostructured lipid carriers; cannabinoid drugs; cryo-TEM

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Manuscript Region of Origin: ITALY

Abstract: This study describes the development and optimization of a method to encapsulate the potent and expensive cannabinoids drugs in nanostructured lipid carriers; namely, URB597, AM251 and rimonabant have been considered. NLC production by melt and ultrasonication protocol has been specifically designed to optimize nanoparticle recovery and drug encapsulation efficiency. Special care has been devoted to the modality of oil and water phase emulsification and the entire production has been studied and discussed. NLC recovery, morphology, dimensional distribution and encapsulation efficiency are presented.

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Cover Letter

Ferrara, 9 december 2015

Dear Prof A. Goepferich,

Enclosed please find a manuscript to be submitted for possible publication on “European Journal of Pharmaceutics and Biopharmaceutics” as a note.
The manuscript is entitled:
"Encapsulation of cannabinoid drugs in nanostructured lipid carriers"
by Elisabetta Esposito, Markus Drechsler, Rita Cortesi and Claudio Nastruzzi.
The manuscript regards the development and optimization of a method to encapsulate the potent and expensive cannabinoids drugs in nanostructured lipid carriers.
The manuscript is original, unpublished, it is not under consideration for publication elsewhere, and all authors have read and approved the text and consent to its publication.

I thank you in advance for consideration.
Best regards
Prof Claudio Nastruzzi

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Abstract

This study describes the development and optimization of a method to encapsulate the potent and expensive cannabinoids drugs in nanostructured lipid carriers; namely, URB597, AM251 and rimonabant have been considered. NLC production by melt and ultrasonication protocol has been specifically designed to optimize nanoparticle recovery and drug encapsulation efficiency. Special care has been devoted to the modality of oil and water phase emulsification and the entire production has been studied and discussed. NLC recovery, morphology, dimensional distribution and encapsulation efficiency are presented.

Encapsulation of cannabinoid drugs in nanostructured lipid carriers

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Abstract

This study describes the development and optimization of a method to encapsulate the potent and expensive cannabinoids drugs in nanostructured lipid carriers; namely, URB597, AM251 and rimonabant have been considered. NLC production by melt and ultrasonication protocol has been specifically designed to optimize nanoparticle recovery and drug encapsulation efficiency. Special care has been devoted to the modality of oil and water phase emulsification and the entire production has been studied and discussed. NLC recovery, morphology, dimensional distribution and encapsulation efficiency are presented.

Keywords: Nanostructured lipid carriers; cannabinoid drugs; cryo-TEM.

Abbreviations: Nanostructured lipid carriers: NLC; cannabinoid drugs: CD; rimonabant: RMN; cryogenic transmission electron microscopy: cryo-TEM; photon correlation spectroscopy: PCS; oil phase: OP; water phase: WP.

1. Introduction

Cannabinoid drugs (CD) are usefully employed for the treatment of neurological disorders, spanning from anxiety, depression and post-traumatic stress, to neurodegenerative disorders such as multiple sclerosis [1]. Indeed, the cannabinoid system regulates synaptic neurotransmission and tonically controls clinical signs [1]. In addition to symptom management, CD offer the potential to slow the progression of the diseases that as yet have no satisfactory treatment. For instance, the fatty acid amide hydrolase inhibitor URB597 elicits significant, anxiolytic-like, antidepressant-like and analgesic effects [2], the inverse agonists AM251 and rimonabant (RMN) decrease appetite and body weight, while AM251 in addition to these effects, can also modulate tumour cell growth [3-6] (Table 1).

Despite these important pharmacological activities, the preclinical (in animals) and clinical use of CD remains difficult, due to unfavorable physico-chemical characteristics (i.e. low solubility in aqueous media) and important side effects (Table 1). For instance, for the administration to experimental animals, CD are usually dissolved in non-aqueous solvents or used as unstable suspensions. Therefore, the development of new formulations solving or alleviating the above reported drawbacks would be of great impact on the development of new administration protocols for CD, possibly combining the improvement of CD targeting/biodistribution to the brain.

Lipid based nanoparticle have been recently proposed as biocompatible and biodegradable nanocarrier for the solubilisation and delivery of a large number of molecules with different physico- chemical properties [7].

For the development of nanocarriers for CD, it should be considered that these drugs are often very expensive [2-6]; for this reason, the preformulatory stage would require a

production strategy in small-scale (typically 1-10 ml) avoiding as much as possible waste of CD.

In this respect, the current investigation describes a method specifically designed and validated for the encapsulation of CD in nanostructured lipid carrier (NLC). In the attempt to optimize NLC recovery and CD encapsulation efficiency (EE), the entire production process, including the modality of lipid and aqueous phase mixing have been thoroughly investigated.

2. Materials and methods

2.1. Materials

Poloxamer 188 (poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) was from BASF ChemTrade GmbH (Burgbernheim, Germany); tristearin and polysorbate 80 (polyoxyethylenesorbitan monooleate) were provided by Sigma-Aldrich (Milano, Italy); miglyol 812 N, (caprylic/capric triglycerides) was from Cremer Oleo Division (Witten, Germany). URB597 ([3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate) and AM251 ([1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide]) were from Sigma-Aldrich; rimonabant (RMN) SR1417165 (4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide was a kind gift of RTI International, Durham, NC, USA. All other chemicals were from Sigma-Aldrich.

2.2. Preparation of NLC

NLC were prepared by a method based on lipid melting and ultrasonication following two alternative procedures, namely: the direct method and the inverse method. In the first case, the oil phase (OP) was added to the water phase (WP), while in the other, the WP was added to the OP. The OP was constituted of a lipid mixture of tristearin/miglyol 2:1 (w/w); the WP was constituted of a 2.5 % (w/w) aqueous poloxamer 188 solution.

Direct method. In the direct method, 0.25 or 1.00 g of OP was melted at 80°C in a beaker and then poured in a vial containing the WP at 80°C. The OP and WP were emulsified at 15000 rpm for 1 min, using a high-speed stirrer (Ultra Turrax T25, IKA-Werke GmbH & Co. KG, Staufen, Germany); the formed emulsion was then subjected to ultrasonication (Microson TM, Ultrasonic cell Disruptor) at 6.75 kHz for 15 min and then cooled at 25 °C

[8]. NLC were then filtered through a mixed esters cellulose membrane (1.2 μm pore size) in order to remove large lipid aggregates. In order to determine the NLC recovery, all glassware and filter employed for the production were accurately weighted before and after preparation. NLC dispersions were stored at room temperature.

Reverse method. In the reverse method, 4.75 or 19.00 g of WP were heated at 80°C, thereafter the WP was poured into in a vial containing 0.25 or 1.00 g of OP previously melted at 80°C.

The mixture was emulsified with the above reported modality. In the case of NLC containing drugs, URB597 4%, AM251 4% or RMN 0.4% (the drug amounts are expressed as %, w/w, with respect to the OP) the drug powders were added to the melted OP, resulting in a homogeneous fine dispersion. Thereafter the preparation followed the above reported procedure.

2.3. Characterization of NLC

Cryo-TEM analysis. Samples were vitrified as described in a previous study by Esposito et al. [9]. The vitrified specimen was transferred to a Zeiss EM922Omega transmission electron microscope for imaging using a cryoholder (CT3500, Gatan). The temperature of the sample was kept below -175 °C throughout the examination. Specimens were examined with doses of about 1000-2000 e/nm^2 at 200 kV. Images were recorded digitally by a CCD camera (Ultrascan 1000, Gatan) using an image processing system (GMS 1.9 software, Gatan).

Photon Correlation Spectroscopy (PCS). Submicron particle size analysis was performed using a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by

washing with detergent and rinsing twice with water for injections. Measurements were made at 25 °C at an angle of 90°. Data were interpreted using the “method of cumulants” [9].

2.4. Cannabinoid drug content of NLC

The encapsulation efficiency (EE) of CD was determined by centrifugation followed by dissolution of NLC in methanol, as previously described [9]. One hundred microliter of each NLC batch was loaded in a centrifugal filter (Microcon centrifugal filter unit YM-10 membrane, NMWCO 10 kDa, Sigma Aldrich, St Louis, MO, USA) and centrifuged (Spectrafuge™ 24D Digital Microcentrifuge, Woodbridge NJ, USA) at 8,000 rpm for 20 min. The amount of CD in the lipid was determined by high performance liquid chromatography (HPLC), as below reported. The encapsulation parameters were determined as follows.

$$EE = L_{CD} / T_{CD} \times 100$$

where L_{CD} is the amount of drug encapsulated in NLC; T_{CD} and T_{LIPID} are the total weight of drug and of lipid used for the NLC preparation, respectively.

2.5. HPLC procedures

The HPLC apparatus consisted of a two-plungers alternative pump (Jasco, Japan), an UV-detector and a 7125 Rheodyne injection valve. RP-HPLC analysis was performed using a stainless steel C-18 reverse-phase column (15×0.46 cm) packed with 5 µm particles (Grace® - Alltima, Alltech, USA). A pre-column filter Alltima C18 5µm (7.5x0.46 cm) was mounted above the column. Samples of 50 µl were injected through the rheodyne injector system fitted with 50 µl fixed loop. The mobile phase flow rate was 0.8 ml/min and the

composition was: water and methanol (20:80, v/v), water and methanol (5:95, v/v) and water and methanol (10:90, v/v) for URB597, AM251 and RMN, respectively. Chromatograms were obtained with a UV detector set at 280 nm (for URB597 and AM251) and 260 nm (for RMN).

3. Results

3.1. Preparation of NLC

This study was undertaken in order to provide a facile and effective encapsulation strategy for expensive drugs. For instance, as reported in Table 1, many CD currently employed in clinical and preclinical studies are characterized by high prices. Typically, URB597 costs about 10,000 USD/g.

Since the therapeutic target of CD for the treatment of post-traumatic stress disorder is amigdala, along the years we developed a delivery strategy for CD based on lipid nanoparticles such as solid lipid nanoparticles (SLN) and NLC [8-10].

Unfortunately, the preparation strategy employed successfully by our group in previous studies resulted unsatisfactory in the case of CD, mainly in reason of low recovery.

For instance, when the direct method is used for low amount of lipids (0.25 g) the NLC recovery was substantially reduced at 73.1 % w/w with respect to 95% obtained with 1.00 g of lipids (see Table 2). The amount of lipids was reduced to 0.25 g from 1.00 g with the intention to scale down the procedure resulting in 5 ml of NLC dispersion, in order to sparingly use drugs and consequently cutting costs.

The reduction in NLC recovery was attributed to adhesion phenomena of the molten lipids to the glassware walls (ascribable to the cooling of the molten lipid phase during its pouring into the vial containing the WP) and to the formation of a large floating lipid aggregate on the surface of the WP [8-10]. This phenomenon was due to the partial coalescence of a number of OP droplets while they were still in the molten, liquid state.

In order to solve or alleviate the problem related to poor recovery, we propose here the alternative reverse modality, which is based on the pouring of the WP into the OP (Fig. 1). By the reverse method, the loss of lipid phase was indeed limited to 6.3% w/w, due to the

floating aggregate. The reverse method resulted in a final recovery of 93.7% w/w (R5-e, Table 2).

After testing the reverse method for the production of empty NLC, the procedure was applied to the preparation of CD containing NLC. Initially, NLC containing URB 597 (R5-URB597) and AM251 (R-AM251) at the concentration of 4.00 μg for mg of OP, were produced. In both cases, the recovery resulted highly satisfactory and further increased with respect to the empty NLC, reaching up to ~ 98% (Table 4). In the case of RMN, we tested also the production of NLC containing a 10-fold higher amount of drug (40.00 μg for mg of OP); the recovery resulted slightly reduced at 92.5 % w/w; this behavior was due to an increase of the losses caused by the formation of a lipid aggregate, even if it was limited to 7.5 %.

3.2. Characterization of NLC

The influence of the NLC preparation method has been investigated with respect to the effect on morphology, dimensions and encapsulation efficiency.

Specifically, Cryo-TEM analyses were performed with the aim to investigate the NLC morphology and inner structure. Fig. 1 shows cryo-TEM images of empty NLC (Fig. 1A) or containing different drugs (Fig: 1B-D), produced by the reverse method. Accordingly to the previous results published by our group [9, 10], the NLC produced by the reverse method display the typical ellipsoidal shape; notably NLC particles appear as dark “needles“ when edge-on viewed. In addition, NLC present an inner structure partially disordered (with respect to the lamellar phases typically present in SLN constituted of pure tristearin); this feature is attributed to the presence of caprylic/capric triglycerides, which are liquid at room temperature, therefore conferring a partially liquid state to nanoparticle core [8].

No significant differences on the structure are appreciable between empty and CD containing NLC, indicating that the presence of the drugs do not affect the NLC morphology.

The dimensional distribution data, obtained by PCS and reported in Table 4, indicate that NLC produced by reverse method, irrespectively of the type and amount of drug employed, display a Z Average comprised between 200 and 250 nm and a dispersity comprised between 0.20-0.25.

Therefore, the size analysis confirmed that the reverse method resulted in the production of NLC dimensionally and morphologically very similar (if not identical) to those produced by the direct method, described in previous studies [8, 10]. Typically, NLC by direct method have indeed a Z Average in the range 190-220 nm and a dispersity 0.18-0.24.

Notably, the EE was extremely satisfactory for all tested drugs, reaching a value of 99.9% w/w in the case of NLC containing AM251. Particularly, in the case of RMN encapsulation, the reverse method appeared superior to the direct one, with an EE of 98 % w/w (Table 4), with a 30% increase with respect to the direct protocol [9].

3.3. Concluding remarks

The present study demonstrated that a simple adaptation of NLC production protocol enabled to efficiently nano-encapsulate CD, obtaining a formulation suitable for in vitro and in vivo clinical and preclinical studies.

The reverse protocol allowed to prevent loss of lipid phase and consequently to improve EE with respect to previously published protocols [9].

In this respect nanoparticle based formulation for CD could represent an important clinical improvement for this class of therapeutic agents. Nanoparticle were indeed recently

proposed as tools to increase solubility and to sustain release of incorporated drugs, as well as to prevent drug chemical, photochemical or oxidative degradation [7].

Acknowledgements: This work was funded by "FIRB 2010. Fondo per gli Investimenti della Ricerca di Base" from the Ministry of the University and Research of Italy (code RBFR10XKHS).

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Figure legends

Figure 1. Scheme depicting the production strategy for NLC by "reverse method".

Figure 2. Cryo-TEM images of NLC produced by the "reverse method", namely R5-e (A), R5-URB597 (B), R5-AM251 (C) and R5-RMN (D). For the details on the composition of the depicted NLC see Tables 2 and 3. Bar corresponds to 200 nm.

Table(s)

Table 1: Characteristics of the cannabinoid drugs employed in the present study

Parameters	URB597	AM251	Rimonabant
Molecular weight (g/mol)	338.40	555.24	463.79
Solubility* (mg/ml)	EtOH (1) DMF[a], DMSO[b] (10)	EtOH, DMF[a], DMSO[b] (10)	EtOH, DMF[a], DMSO[b] (20) Water (0.002)
Pharmacological activity	potent, fatty acid amide hydrolase inhibitor, no activity on other cannabinoid-related targets[2]	inverse agonist of the cannabinoid receptor CB1[3]	inverse agonist of the cannabinoid receptor CB1[6]
use	inflammatory pain states[2], phase 1	inflammatory pain states, tumour cell growth modulation[3] pre-clinical trials	obesity and related conditions[4] (Acomplia tablets)
side effects	no undesirable side effects	sustained weight loss, transient reduction in daily food intake[5]	in 2009 officially withdrawn due to adverse psychiatric events*
Price° (50 mg)	USD 640	USD 629	USD 270

*as reported in: https://www.caymanchem.com/pdfs/9000484.pdf; http://www.drugbank.ca/drugs/DB06155

°as reported in: http://www.selleckchem.com/products/rimonabant-sr141716.html and http://www.tocris.com/dispprod.php?ItemId=2200

a: dimethylformamide;

b: dimethylsulphoxide

Table 2 Effect of preparation method on the recovery of NLC

nanoparticle acronym	preparation method	WP[a] (g)	OP[b] (g)	recovery[c] (%)	loss by adhesion[d] (%)	loss by lipid aggregate[e] (%)
D20-e	direct	19.00	1.00	95.0±0.8	4.0±0.1	1.0±0.2
D5-e	direct	4.75	0.25	73.1±0.2	20.5±0.2	16.4±0.4
R5-e	reverse	4.75	0.25	93.7±0.1	n.p.	6.3±0.1

a: Water Phase poloxamer 188 2.5% w/w; b: Lipid Phase tristearin/ tricaprilin 2:1 w/w ratio

c: Percent of recovery was calculated as follows:

% recovery = amount of NLC recovered (g) / amount of lipid used (g) x 100. Data represent the mean ± SD of 6 independent experiments

d: loss of lipids (OP) due to the adhesion (literally, "sticking") between the melted OP and the walls of the glassware employed for its melting and pouring into the WP. Data represent the mean ± SD of 6 independent experiments

e: loss of lipids (OP) due to the partial coalescence of the OP during the formation of the O/W emulsion. After cooling the coalesced OP appeared as a small flake floating on the surface of the NLC dispersion. Data represent the mean ± SD of 6 independent experiments

n.p.= not present

Table 3 Effect of preparation method on the recovery of NLC

Drug/ Nanoparticle acronym	preparation method	WP[a] (g)	OP[b] (g)	loaded drug*	recovery[c] (%)	loss by adhesion[d] (%)	loss by lipid aggregate[e] (%)
URB/ R5-URB597	reverse	4.75	0.25	4.00	98.0±0.3	n.p.	2.0±0.1
AM251/ R5-AM251	reverse	4.75	0.25	4.00	98.8±0.2	n.p.	1.2±0.1
RMN/ R5-RMN	reverse	4.75	0.25	40.00	92.5±0.2	n.p.	7.5±0.2

a: Water Phase poloxamer 188 2.5% w/w; b: Lipid Phase tristearin/ tricaprilin 2:1 w/w ratio

c: Percent of recovery was calculated as follows:

% recovery = amount of NLC recovered (g) / amount of lipid used (g) x 100. Data represent the mean ± SD of 6 independent experiments

d: loss of lipids (OP) due to the adhesion (literally, "sticking") between the melted OP and the walls of the glassware employed for its melting and pouring into the WP. Data represent the mean ± SD of 6 independent experiments

e: loss of lipids (OP) due to the partial coalescence of the OP during the formation of the O/W emulsion. After cooling the coalesced OP appeared as a small flake floating on the surface of the NLC dispersion. Data represent the mean ± SD of 6 independent experiments

n.p.= not present

*the amount of drug initially included in the OP, expressed as μg of drug for mg of OP.

Table 4: Dimensional characteristics and encapsulation parameters of the indicated NLC

Nanoparticle acronym	Z average (nm)	Mean diameter by number (nm)	Dispersity	Encapsulation efficiency[a] (%)
R5-URB597	242.4±4.5	104.0±1.8	0.25±0.02	92.8±0.8
R5-AM251	231.0±3.3	110.3±2.2	0.24±0.01	99.9±0.1
R5-RMN	204.0±2.3	92.5±1.5	0.20±0.03	98.0±1.4

a: percentage (w/w) of drug in the whole dispersion with respect to the total amount used for the preparation.
b: percentage (w/w) of drug in the whole dispersion with respect to the amount of lipid used for the preparation. Data are the means ± SD of 6 independent determinations.

Figure 1

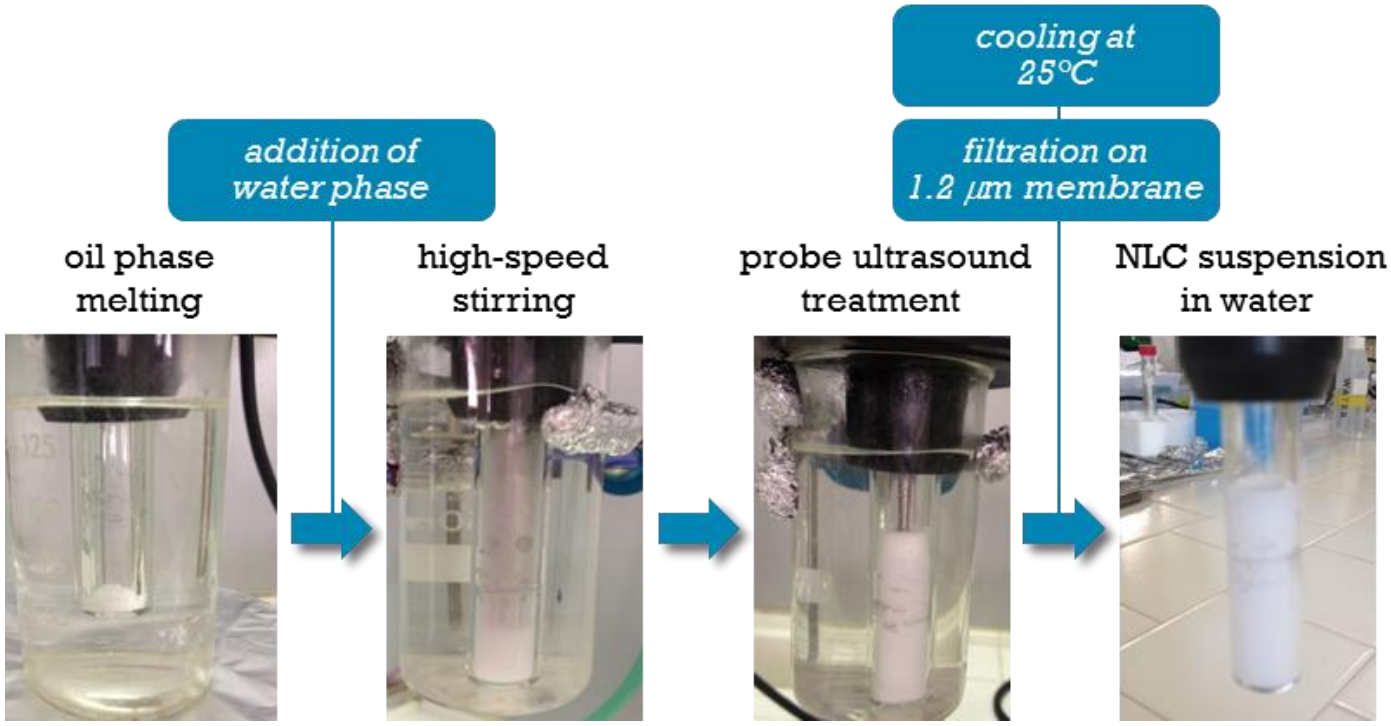
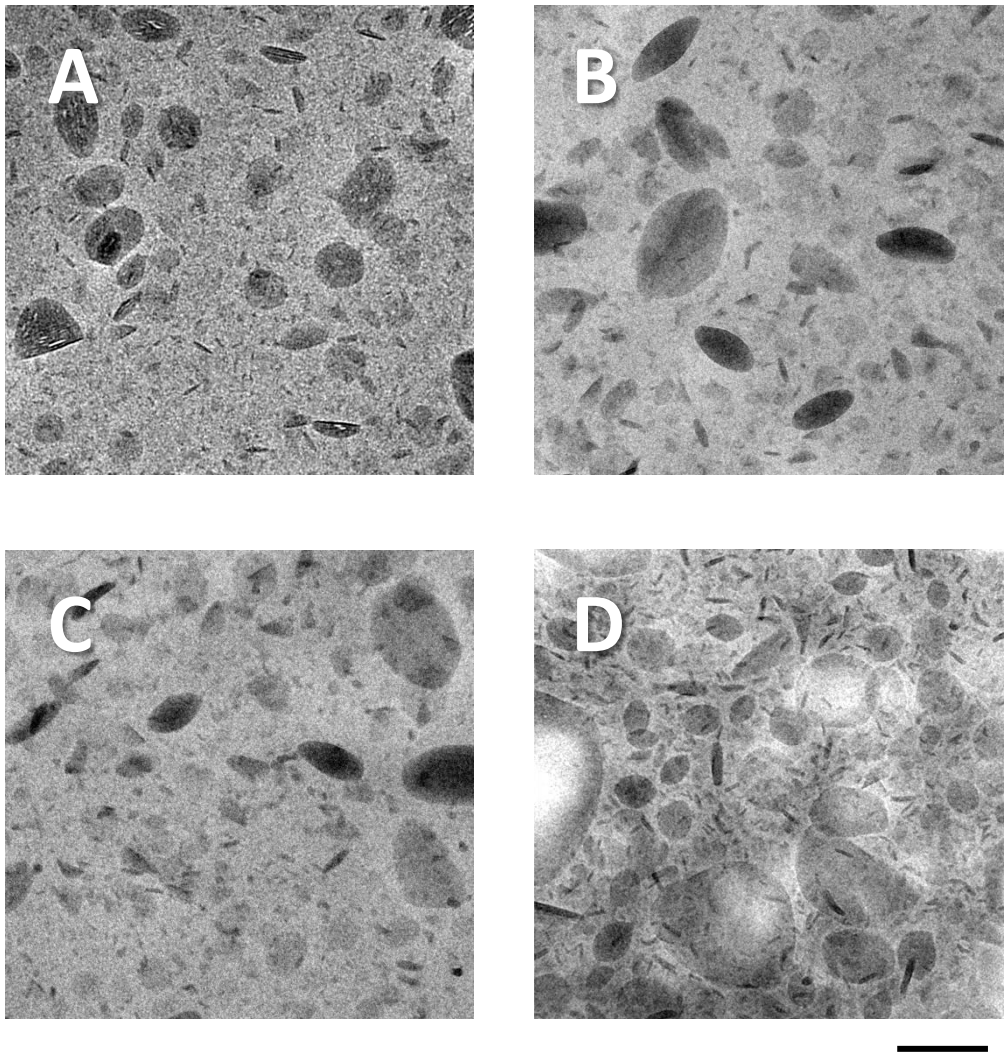

addition of water phase
cooling at 25°C
filtration on 1.2 µm membrane
oil phase melting
high-speed stirring
probe ultrasound treatment
NLC suspension in water

Figure 2



Abbreviations: Nanostructured lipid carriers: NLC; cannabinoid drugs: CD; rimonabant: RMN; cryogenic transmission electron microscopy: cryo-TEM; photon correlation spectroscopy: PCS; oil phase: OP; water phase: WP.