Design and processing of drug delivery formulations of therapeutic deep eutectic systems for tuberculosis

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HIGHLIGHTS

• The encapsulation of a liquid THEDES using supercritical CO2 was studied.
• Phase equilibria studies allowed to select PGSS for THEDES encapsulation.
• For the first time, THEDES were encapsulated through PGSS with an average encapsulation efficiency of 75%.
• The water content of THEDES did not affect significantly their encapsulation.
• The THEDES-lipid particles produced were not cytotoxic.

ARTICLE INFO

Article history:
Received 9 September 2019
Received in revised form 11 March 2020
Accepted 13 March 2020
Available online 18 March 2020

Keywords:
Therapeutic deep eutectic systems
Supercritical fluid technology
Carbon dioxide
Particles from Gas Saturated Solutions
Tuberculosis
L-arginine

ABSTRACT

Therapeutic deep eutectic systems (THEDES) emerged as alternative therapeutic agents that can enhance the bioavailability of the currently used active pharmaceutical ingredients. The use of THEDES in combination with supercritical CO2 has started to be explored to formulate drug delivery systems through green technology. This work aimed to develop THEDES-delivery systems for tuberculosis therapy, by encapsulating L-arginine-based THEDES in a lipid matrix, through supercritical CO2 technology. From the phase equilibrium study of THEDES and CO2, PGSS was selected for THEDES encapsulation. Herein, THEDES encapsulation through PGSS was accomplished for the first time, with an average encapsulation efficiency of 75%. The influence of the THEDES water-content in PGSS processing was also studied, suggesting no interference of the THEDES–water content in the formulation of the THEDES particles. Furthermore, the cell viability of the THEDES and the particles with THEDES encapsulated was measured in L929 fibroblasts and the systems prepared were non-cytotoxic.

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1. Introduction

Deep eutectic systems (DES) were first defined by Abbott, as a mixture of salts whose melting point is decreased up to ambient temperature [1]. Their potential to be used as green and sustainable solvents was rapidly highlighted by the possibility of producing liquid systems, with 100% atom economy, where no chemical reaction is involved. Moreover, they are typically non-toxic and biodegrad-
able [2–4] and they can be designed to fully comply with the green chemistry metrics. The DES definition was expanded by the scientific community to all the compounds whose combination has a lower melting temperature than the pure components. Despite not being consensual [4–6], this definition embraces a larger number of combinations, designed and tailored to specific applications. This type of system has been applied to several applications, including extraction, electrochemistry and biocatalysis [2,7]. More recently, the formulation of therapeutic DES (THEDES) has been a target of investigation, as the liquid state of the active pharmaceutical ingredients (APIs) confers them enhanced bioavailability and thus, better efficacy [8]. Moreover, the formulation of delivery systems for THEDES would allow to maintain THEDES bioavailability, improve their stability and eventually promote a controlled release to achieve the therapeutic effect [9,10].

In a previously published work, our group developed THEDES based on l-arginine and ethambutol bioactives, combined with citric acid and water, focusing tuberculosis therapy [11]. The THEDES systems produced showed to improve the solubility and permeability of the pharmaceutical components in comparison to its isolated solid forms. These characteristics can be promising to reduce the dose needed for effective tuberculosis treatment. Since tuberculosis affects mainly the pulmonary tract, a local administration would be more efficient. For that reason, we designed the encapsulation of the developed THEDES for the formulation of a THEDES-delivery system suitable for local administration, for example by spray inhalation. Glycerol monostearate was selected as model carrier for the THEDES since it is biocompatible [12] and considered safe by the FDA [13]. Moreover, since the final application is pharma, it is important to use green technology for THEDES encapsulation. Supercritical CO2 (sc-CO2) technology is a very attractive platform, widely used for the formulation of pharmaceutical products as CO2 is a green, cheap, abundant and recyclable solvent. Most importantly, it allows to produce solvent-free final products, avoiding the need for further purification steps [9]. The formulation of delivery systems for THEDES is quite recent [7,10,14–16] and the use of sc-CO2 technology for this purpose is not extensively explored [10,16].

Particles from Gas Saturated Solution (PGSS) and Rapid Expansion of Supercritical Solutions (RESS) are two processes based on sc-CO2 technology for particle formulation, with different principles. In RESS, sc-CO2 is used as solvent, to solubilize the bioactive and the carrier. During depressurization, the CO2 solvation power decreases, causing the solute (bioactive + carrier) to supersaturate and precipitate. In turn, PGSS uses CO2 as a solute, being solubilized in the carrier while decreasing its melting temperature. In this technique, the CO2 can also be dissolved into the bioactive or act as dispersive agent of the bioactive into the melted carrier. When depressurizing the mixture, the expansion and cooling of CO2 by the Joule-Thomson effect causes the precipitation of the carrier, which solidifies and forms the particles [17,18]. Both processes are easily scalable and allow to obtain a final product, which does not require further processing steps [19].

In a global perspective, this work represents a proof of concept of the ability to encapsulate a liquid THEDES system in a lipidic carrier through PGSS using a simple and green procedure.

2. Experimental

2.1. Chemicals

Citric acid monohydrate (CAS [5949-29-1], ≥99.5 %) was purchased from PanReac AppliChem (Barcelona, Spain); l-arginine (CAS [74-79-3], ≥98 %) from Sigma Aldrich (St. Louis, MO, USA); glycerol monostearate (GMS, CAS [31566-31-1]) from Gatefossé (Saint-Priest, France) and carbon dioxide (CAS [124-38-9], 99.95 %) from Air Liquide (Lisbon, Portugal). The reagents were used without additional purification. Phosphate buffer saline (PBS) solution was prepared in deionized water (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, 25 °C) with PBS tablets from Fisher BioReagents (Hampton, NH, USA). The solution was stored at 4 °C.

2.2. Preparation of THEDES

THEDES of citric acid:l-arginine:H2O were prepared at molar ratios of 1:1:7, 1:1:6, 1:1:5 and 1:1:4. The components were mixed at the respective molar ratios and stirred at 50/60 °C, until a translucid solution was formed.

2.3. Phase equilibrium of THEDES and CO2

The solubility of CO2 and THEDES was studied through visual observation of the phase equilibrium of the pseudo-binary mixture. A variable-volume view cell apparatus (hand-made) was used for this purpose. The equipment is composed by a cylindric equilibrium cell of stainless steel with an internal volume between 35 to 70 cm3, regulated by a piston coupled to a hydraulic pump (HP145, HI-force). It can operate up to a pressure of 60 MPa. Two sapphire windows are oppositely positioned, for observation of the cell interior. The cell is heated through two electrical band heaters coupled to a LMS temperature controller (EroElectronics) and the internal temperature measured by a sensor (type K) in contact with the fluid mixture. The pressure was controlled by a Digibar PE300 transducer (HMB, European Community) with an accuracy of 0.05 MPa. Stirring of the mixture inside the cell was performed by a mechanical stirrer coupled device electronically controlled by a power supply (VLP-1303 PRO, Voltcraft) at 6 V, 16 mA.

Known amounts of THEDES and CO2 were injected into the cell. The mixture was stirred continuously, and the temperature stabilized at 40 °C. At different CO2:THEDES molar ratios and constant temperature, the mixture was compressed to higher pressures and the phase behavior of the mixture (one or two phases) was observed.

2.4. THEDES encapsulation through PGSS

The PGSS apparatus (Recipient de pulverization A30, Sepharex, France) is described elsewhere [19]. Briefly, THEDES and GMS were loaded into the stirring vessel at a ratio of 9:1 GMS:THEDES (w/w) and mixed with CO2 at 8.5 MPa and 65 °C. The mixture was stirred for 15 min and further depressurized through a 250 μm nozzle to a cyclone flushed with compressed air. The particles accumulated in the collector vessel were collected and stored.

2.5. Particles physicochemical characterization

2.5.1. Morphological characterization

The particle size, size distribution and morphology of the produced particles were analyzed through scanning electron microscopy (SEM) (JEOL, model JSM6010-LV, Japan). The samples were prepared for observation by covering with gold (Au), in a sputter coater (Cressington, model 108 auto). Micrographs of the prepared aliquots were taken at an acceleration voltage of 10 kV. The median size of the particles and their size distribution were obtained from ImageJ (version 1.52a, USA) by measuring the particles diameter (500 particles per sample). The polydispersity index (PDI) was calculated by dividing the standard deviation of 500 diameter measurements by the respective mean particle size.
2.5.2. Energy dispersive x-ray spectroscopy

Energy dispersive x-ray spectroscopy (EDS) was used to perform a chemical analysis of the particles surface and evaluate the presence or absence of nitrogen, an arginine component. The analysis was performed in five sample regions, using a Link eXL-II spectroscopy (Oxford Instruments, United Kingdom), at slow vacuum mode (50 Pa) with an acceleration voltage of 15 kV, coupled to SEM.

2.6. Encapsulation efficiency

The particles were dispersed in deionized water at 15 mg/mL and destroyed in an ultrasound bath (XUBS, Grant, UK). THEDES contained in the particles remained solubilized in the water whereas the lipid component was separated through centrifugation at 14,000 rpm for 10 min and further filtration of the supernatant (0.22 μm hydrophilic PTFE filter). The filtered collected was analyzed by UV–vis spectrophotometry (VICTOR Nivo™, PerkinElmer, USA). The amount of THEDES was determined at 220 nm, using a calibration curve of THEDES linear within the range of 0.05–1.5 mg/mL.

The encapsulation efficiency was determined as the amount of encapsulated THEDES relative to the initial THEDES added, as follows:

\[
EE(\%) = \frac{\text{Encapsulated THEDES}}{\text{Total THEDES added}} \times 100
\]

2.7. Drug release profile

Particles were dispersed in PBS (10 mg/mL) and agitated at 60 rpm and 37 °C. Aliquots of 0.5 mL were collected at different time points, filtered (0.22 μm) and analyzed by UV–vis spectrophotometry. The volume was replaced by fresh PBS. The amount of THEDES was determined by UV-spectroscopy at 220 nm, using a calibration curve of THEDES linear within the range of 0.05–3 mg/mL.

2.8. In vitro cytotoxicity assay

The biological performance of the THEDES and particles was evaluated in L929 cell line, mouse fibroblasts. This cell line was provided by DSMZ Culture of Human and Animal Cell Lines (Germany). The cells were cultured in MEM media supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and 1% of antibiotic (penicillin) at maintained at 37 °C in a humidified incubator with 5% of CO₂. Cell culture medium and supplements were obtained from Corning, USA.
precisely the LCEP, analytical methods would be required. Still, this phenomenon is relevant and must be considered for processing as it may change the THEDES composition under this P, T conditions. After these observations and since there is no significant solubilization of THEDES in CO₂, the possibility of using RESS as a method for encapsulation was excluded.

Interestingly, during depressurization of the high-pressure device with the THEDES + CO₂ mixtures, foaming phenomenon was observed (Fig. 1), indicating that CO₂ can be dissolved in the THEDES liquid phase. Upon depressurization, there is a decrease in the carbon dioxide solubility which causes its release, expansion and consequent foaming of the sample. To determine precisely the CO₂ solubility in the THEDES system, an analytic method would be required. Nevertheless, the data collected using the visual method allowed to select PGSS for THEDES encapsulation since in this technique, CO₂ can either be solubilized in the THEDES or act as dispersing agent of the THEDES into the melted carrier.

3.2. THEDES encapsulation through PGSS

From the phase equilibria studies of THEDES and CO₂, it was identified the possibility of water separation from THEDES during processing. Despite not being possible to identify the minimum conditions at which this phenomenon is initiated (LCEP), it was more pronounced for higher pressures and CO₂ contents. For this reason, the lowest conditions of pressure and temperature necessary to melt the carrier should be chosen for PGSS processing. In the literature, GMS has been reported to be processed by PGSS at pressures between 6 and 23 MPa and temperatures from 57 to 80 °C [20,23,24]. However, the minimum conditions reported (6 MPa, 70 °C) [23], are below the supercritical phase of CO₂. Within the supercritical region, full lipid melting was observed at 8.5 MPa and 65 °C, which were set as the processing conditions for PGSS in this work. Additionally, THEDES of citric acid:L-arginine:H₂O with decrescent amounts of water, from 1:1:7 up to 1:1:4 M ratio were prepared and their encapsulation through PGSS was compared to evaluate the water influence in the process.

3.3. Particles characterization

The morphology of the particles produced through PGSS was analyzed by SEM images, from which is possible to observe a spongy-spherical structure (Fig. 2). This kind of structures are normally porous, which is characteristic from PGSS, due to the rapid
and cooling expansion of CO₂, which can form surface holes, foams or sponge-like structures [25,26].

The THEDES is a viscous liquid, which, if present at the particles surface would cause them to be sticky and agglomerated. The particles herein obtained through PGSS presented a free-flowing powder consistency suggesting that the liquid THEDES has been successfully incorporated within the core of the lipidic particles. Moreover, as observed, the particles containing THEDES (Fig. 2a-d)) and the empty lipidic particles (Fig. 2e)) presented a similar morphology. The similarity between their surface supports our assumption that the external part is only composed by the GMS while the THEDES may be entrapped internally in the particles. This hypothesis was corroborated by EDS analysis (Table 2). The nitrogen element, characteristic from the arginine compound of THEDES, was not identified in the particles surface. The only chemical elements detected were carbon and oxygen, the main components of GMS.

In terms of particle size, all the produced particles presented similar mean sizes, between 14 and 23 μm (Table 2). Regarding the particle size distribution, all the particulate systems were slightly polydisperse (PDI > 0.2, Table 2), presenting a gaussian distribution within the same size range (Fig. 3). The encapsulation of THEDES showed no significant influence in the particle size.

Regarding the literature studies reporting GMS particles produced through PGSS, Sousa et al. reported an average particle size of 10 μm for empty GMS particles when processed at 13 MPa and 61 °C [23]. In turn, Mandžuka & Knez published particle sizes from 13 to 27 μm, with no significant variation within the pressures (6, 11.5, 16.5 or 21 MPa) or temperatures (70 or 80 °C) studied [24]. Taking the empty GMS particles as a reference, the average size for the GMS particles obtained in this work showed an equivalent size range, of 17 ± 8 μm.

Considering the eventual application of the produced particles as inhalable formulations for tuberculosis therapy, it is important to analyze the influence of their particle size for that purpose. Since the Mycobacterium tuberculosis accumulates in the alveoli, an aerodynamic particle size lower than 5 μm would be preferential to reach that region, as it has been reported in literature [27,28]. Despite the large mean geometric sizes of the produced particles (14–23 μm), their aerodynamic size might be lower due to their porosity. Porous particles may present lower densities than non-porous particles, which confer them an adequate aerodynamic diameter for deposition in alveoli. This might be an advantage when reaching the alveoli since particles with geometric sizes lower than 5 μm can be phagocytosed by the alveoli macrophages. In turn, particles with larger geometric sizes but suitable aerodynamic sizes can reach the alveoli while avoiding being phagocytosed [29–32]. Thus, for the production of an inhalation device targeting the alveoli, further studies would have to be performed regarding the aerodynamic size of the produced particles and their deposition in the lungs.

3.4. Encapsulation efficiency (EE)

Having confirmed that the liquid THEDES was encapsulated within the core of the particles, the encapsulation efficiency can be determined simply after destruction of the lipidic matrix without the need for a rising step. As observed in Fig. 4, the mean encapsulation efficiency of THEDES in the GMS particles varied from 60 to 92 %.

Although this difference is quite large, no statistically significant differences are observed due to the intrinsic heterogeneity within each particulate system. From the results obtained, there is no evident pattern related to the water content in the THEDES, so it may be concluded that the water content did not influence the encapsulation process. The observed differences and intrinsic variability might be due to the manual depressurization of the system during processing. Slight changes during depressurization may cause high sample heterogeneity. However, in a scale-up perspective this could be easily overcome by incorporating an automatic depressurization control or by continuous PGSS processing [19].

3.5. Drug release profile

The objective of formulating the THEDES is the administration of the powder particles as an inhalable spray, hence it is expected to have a fast and local release of the drug. These systems are expected to be more efficient reaching bacteria, while avoiding systemic toxicity [28]. From the 24 h release profile (Fig. 5) it is observed a burst
release at the time point of 0 h, followed by a successive increase of THEDES in solution, up to 5 h, after which a plateau is reached.
This fast release might be explained by the porosity of the particles prepared. This behavior is compliant with the nasal administration as it is a non-invasive therapy. It further allows to control the dose administrated and the direct absorption of therapeutic agents without passing through the acidic environment of stomach and hepatic first-pass metabolism [33].

3.6. Cytotoxicity

From the biological assay performed in L929 cells it was observed that the cell viability was not compromised by any of the THEDES, GMS and formulated particles, since it presented a mean value above 80% in all cases (Fig. 6). Although it presents some variability between the systems and the particles, this might be due to the heterogeneity of the particles tested, as previously explained. The THEDES 1:1:4 and its respective particle formulation presented the lowest viability and the highest variability (STD). This might be due to its higher viscosity (lower water content). In literature, it is reported that the higher viscosity of eutectic systems can decrease the cell viability and influence intracellular activities [34,35]. However, no statistical significant differences were encountered between the systems tested.

4. Conclusions

The presented work consisted on the design and development of drug formulations for THEDES using a supercritical CO2 green technology. Having tuberculosis as the target disease, we designed a new drug delivery system with therapeutic DES encapsulated for inhalable administration. Phase equilibrium studies involving THEDES and CO2 allowed the selection of PGSS as a suitable technique for THEDES encapsulation. Citric acid:L-arginine:H2O THEDES with different water contents were effectively encapsulated in GMS particles through PGSS. The produced particles presented a geometric mean size between 14 and 23 μm, an average encapsulation efficiency of 75% and a fast THEDES release (up to 5 h), promoted by the particle’s porosity. Moreover, the THEDES and the particles synthesized did not present cytotoxicity towards L929 cell line. For application in tuberculosis, further studies would have to be made regarding the particle’s aerodynamic diameter and THEDES pharmacokinetics. Overall, this work represents a proof of concept for the development of new, green, safe and more efficient drug delivery systems, by combining THEDES and PGSS formulation. Herein it is reported for the first time the encapsulation of liquid therapeutic deep eutectic systems by supercritical fluid technology.

Declaration of Competing Interest

There are no conflicts to declare.

Acknowledgements

This project has received funding from the European Union’s Horizon 2020 (European Research Council) under grant agreement No ERC-2016-CoG 725034. This work was supported by the Associate Laboratory for Green Chemistry–LAQV which is financed by national funds from FCT/MCTES (UID/QUI/50006/2019). iNOVA4Health-UID/Multi/04462/2013 is also acknowledged. A.A. Matias thanks for the IF Starting Grant – GRAPHYT/IF/00723/2014.

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