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# Design and *in vitro* evaluation of biocompatible dexamethasone-loaded nanoparticle dispersions, obtained from nano-emulsions, for inhalatory therapy

Cristina Fornaguera, Meritxell Llinàs, Conxita Solans*, Gabriela Calderó

*Institute of Advanced Chemistry of Catalonia (IQAC-CSIC) and CIBER of Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), C/Jordi Girona, 18-26, Barcelona 08034, Spain*

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This paper is dedicated to the memory of Dr. Nuria Azemar who passed away suddenly on March 23rd, 2014.

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ABSTRACT

Polymeric nanoparticle dispersions containing dexamethasone (DXM) have been prepared from O/W nano-emulsions of the water/polysorbate 80/[4 wt% poly(lactide-co-glycolide) acid + 0.18 wt% DXM in ethyl acetate] system by a low-energy method at 25 °C. Nano-emulsions were formed at O/S ratios between 45/55 and 72/25 and water contents above 70 wt% by the phase inversion composition (PIC) method. The mean hydrodynamic diameter of nano-emulsions with a constant water content of 90 wt% and O/S ratios from 50/50 to 70/30 was below 350 nm as assessed by dynamic light scattering. The nanoparticles obtained from these nano-emulsions (by solvent evaporation) showed mean diameters of around 130 nm, as determined by transmission electron microscopy image analysis. Therapeutic concentrations of DXM were encapsulated in the nano-emulsions prior to nanoparticle preparation. DXM entrapment efficiency of the nanoparticle dispersion (above 74 wt%) decreased at increasing O/S ratios of the precursor nano-emulsion while DXM loading, which was around 10 mg/100 mL, showed the reverse tendency. DXM release from nanoparticle dispersions was about an order of magnitude slower than from an aqueous solution. *In vitro* studies performed in a lung carcinoma cell line and *in vitro* haemolysis studies performed in red blood cells revealed a dose-dependent toxicity and haemolytic response, respectively. The as-prepared nanoparticle dispersions were non-toxic up to a concentration of 40 μg/mL and non-haemolytic up to a concentration of 1 mg/mL. After purification, nanoparticle dispersions were non-toxic up to a concentration of 90 μg/mL. These results allow concluding that these polymeric nanoparticle dispersions are good candidates for inhalatory therapy.



## 1. Introduction

Nanoparticles are solid colloidal materials with diameters between 20 and 200 nm [1–3]. They are particularly interesting for biomedical applications due to their nanometric size that enables intracellular uptake. Nanoparticles can be functionalized for a specific delivery to the target tissue, thus reducing side effects [4], as well as provide a sustained release of an encapsulated drug, leading to an extended duration of the therapeutic effect, reduced dosage frequency and improvement of treatment compliance [4,5]. An interesting approach for nanoparticle preparation is by nano-emulsion templating [6].

Nano-emulsions are emulsions with droplet sizes typically between 20 and 500 nm, showing high kinetic stability and translucent aspect [1,3,7]. As they are non-equilibrium systems, an energy input is required for their formation. The source of energy is generally external (high-energy methods), but it can be from the intrinsic chemical energy of its components (low-energy methods). One of the most common low-energy emulsification methods is the phase inversion composition (PIC) method, in which emulsification is triggered by phase transitions produced changing the composition of the system at constant temperature [3,8] whereby phase inversion (from water-in-oil (W/O) to oil-in-water (O/W) structures or *vice versa*) takes place. Polymeric nanoparticles can be prepared from nano-emulsions by solvent evaporation [3,9]. This approach requires the use of a preformed polymer dissolved in a volatile solvent as the dispersed phase. Important issues are the use of appropriate solvents for biomedical applications and the process conditions, which should be mild and safe [10]. Nano-emulsion templating is a versatile technology especially suitable to prepare

* Corresponding author at: C/Jordi Girona, 18-26, 08034 Barcelona, Spain. Tel.: +34 934006159; fax: +34 932045904.
*E-mail address:* conxita.solans@iqac.csic.es (C. Solans).

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formulations for biomedical applications since it can be performed at mild temperatures allowing the preservation of pharmacological properties of drug molecules [6]. Moreover, this technology allows the incorporation of drugs in the polymer–solvent mixture, prior to nano-emulsion formation, yielding high encapsulation efficiencies.

Nano-emulsion templating is especially appropriate for the design of polymeric nanoparticles for pulmonary applications to treat respiratory diseases. The most commonly used anti-inflammatory drugs are glucocorticosteroids, such as dexamethasone (DXM) [11,12]. They show an outstanding therapeutic efficacy against airway and bronchiolar inflammation. The use of nanoparticles as advanced drug delivery systems is promising, as their inhalatory administration is a non-invasive way of delivering drugs directly to the lungs [13,14]. This study aims at preparing DXM-loaded polymeric nano-emulsions by a low-energy emulsification method, employing low toxicity components under mild processing conditions and using them as templates to obtain polymeric nanoparticles for pulmonary disease treatments. The polymer poly(lactic-co-glycolic acid) was chosen because it is biocompatible, biodegradable, it has been clinically used and it is a FDA approved compound [14–19]. To stabilize nano-emulsions, polysorbate 80 was chosen as it is a non-toxic and non-haemolytic surfactant at the concentrations used in this work [20]. DXM was selected as a model glucocorticosteroid, a proof-of-concept of the methodology to be used for further applications with other drugs. To assess biocompatibility of the system, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) toxicity test [15,21], which was first described by Spielmann et al. [22] and haemolysis tests, which can be performed *in vitro* by isolating erythrocyteswere performed. To our best knowledge, none of the studies reported so far make use of a low-energy emulsification method to prepare nanoparticles for inhalatory uses. These methods allow in addition to good size control and high drug entrapment efficiency, the use of mild process conditions and energy savings.

## 2. Materials and methods

### 2.1. Materials

Poly(lactic-co-glycolic acid), Resomer 752H (PLGA) (polystyrene equivalent molecular weight PSE – MW ~ 10,000 g/mol, determined by Gel Permeation Chromathography) was purchased from Boehringer Ingelheim. The lactic to glycolic acid ratio of the PLGA was 75/25 and the end groups were free carboxylic acids. Ethyl acetate used as the organic volatile solvent was purchased from Merck. Polysorbate 80 is a nonionic surfactant kindly provided by Crodawih an HLB value of 15 [20]. DXM, an halogenated glucocorticosteroid, freely soluble in ethyl acetate [23] was chosen as a "gold standard" for the inhalatory treatment of respiratory diseases (MW = 392.5 g/mol, $\lambda \sim 240$ nm) [17,24]. Water was MilliQ filtered.

### 2.2. Methods

#### 2.2.1. Nano-emulsion preparation

The region of O/W nano-emulsion formation in the pseudoternary water/surfactant/oil diagram was assessed visually. Samples with transparent, translucent or opaque aspect, having a bluish or reddish shine were considered nano-emulsions if prepared by mixing all components were turbid (to make sure they were not microemulsions). Nano-emulsions were produced by stepwise addition of water to mixtures of surfactant and oil (ethyl acetate) containing 4 wt% of PLGA and 0.18 wt% of DXM at 25 °C.

#### 2.2.2. Droplet size characterization

The mean droplet size and size distribution of nano-emulsions were determined by dynamic light scattering (DLS) using a spectrometer (LS Instruments, 3D cross correlation multiple-scattering) equipped with a He—Ne laser (632.8 nm) at a scattering angle of 90°, and at a constant temperature of 25 °C in triplicates. Data were treated by cumulant analysis [25]. The characterization was performed by the Nanostructured Liquid Characterization Unit of the Spanish National Research Council (CSIC) and the Biomedical Networking Center (CIBER-BBN), located at IQAC-CSIC.

#### 2.2.3. Nano-emulsion stability

Nano-emulsion stability was assessed by visual examination and light transmission along the sample height as a function of time using a Turbiscan™ Lab Instruments (Formulaction), at 25 °C. The latter measurements were carried out with 15 g of sample placed in a glass cell for 24 h with measurements at 1 h intervals.

#### 2.2.4. Nanoparticle dispersion preparation

Nanoparticle dispersions were prepared from nano-emulsions by the solvent evaporation method under reduced pressure [9], using a Büchi R-215V Rotavapor. The conditions for ethyl acetate evaporation from about 4 g of nano-emulsion at 25 °C temperature were: vacuum of 43 mbars with a rotation speed of 150 rpm during 45 min [23].

#### 2.2.5. Nanoparticle characterization

The nanoparticle size was characterized by DLS, as reported for nano-emulsions, and also by transmition electron microscopy (TEM) with a JEOL1010 TEM (Jeol Korea Ltd.). Prior to TEM characterization, nanoparticle dispersions were concentrated by centrifugation (4500 × *g*, 25 °C, 1 h). The supernatant was removed and nanoparticle pellets were washed once with water and centrifuged again after vigorous stirring. One drop of the concentrated nanoparticle dispersion resuspended in fresh water was placed on a copper grid. After 1 min, the excess of the sample was blotted with filter paper and the grid containing the sample was stained negatively with phosphotungstic acid (PTA) solution (2 wt% in MilliQ water) for another minute. Excess solution was then removed to obtain a proper negative staining. Finally, grids were placed in a Petri dish and let dry at room temperature. The mean size and size distribution of the nanoparticles were determined by image analysis using the ImageJ software. Around 1000 nanoparticles were measured for each sample. Results are presented as mean and standard deviation.

#### 2.2.6. Quantification of the nanoparticles

The number of nanoparticles was calculated using the equation given by Némati et al. [26]:

$$n = \frac{\text{mass of the polymer in themedium}}{\text{mass of one particle}} = \frac{M \cdot 10^{12}}{(4/3)\pi r^3 \cdot \rho} \quad (1)$$

where $M$ is the molecular mass of the polymer in the sample; $r$ is the hydrodynamic radius of the nanoparticles, determined by DLS; and $\rho$ is the density of the polymer, (1.26 for PLGA), reported by Vauthier et al. [27].

#### 2.2.7. Zeta potential determination

The surface charge of the nanoparticles was assessed by Electrophoretic mobility measurements with Zetasizer NanoZS instrument (Malvern Co. Ltd., UK), equipped with a He–Ne laser ($\lambda = 633$ nm). The zeta potential ($\zeta$) was calculated from the electrophoretic mobility applying the Smoluchowski equation (Eq. (2)):

$$\mu \frac{\zeta \cdot \varepsilon_r \cdot \in_0}{\eta} \quad (2)$$

where $\varepsilon_r$ represents the relative dielectric constant of water, $\varepsilon_0$ is the vacuum permittivity and $\eta$ is the viscosity of the liquid. As this