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Combining dexamethasone and TNF- α siRNA within the same nanoparticles to enhance anti-inflammatory effect

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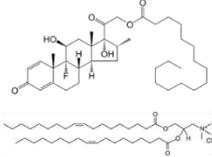
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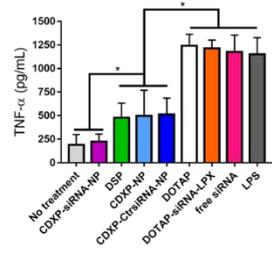
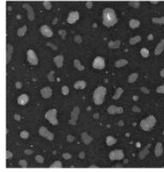
Dexamethasone palmitate (DXP)



DOTAP (cationic lipid)

Formulation
Into Nanoparticles

TNF- α siRNA
Binding



**Synergistic
Inflammation Reduction on
LPS-activated macrophages**

Abstract

We propose to combine two therapeutic anti-inflammatory approaches with different mechanisms of action in a single drug delivery system consisting of cationic dexamethasone palmitate nanoparticles (CDXP-NP) associated with TNF- α siRNA. The CDXP-NPs are obtained by the solvent emulsion evaporation technique using dexamethasone palmitate, a prodrug of dexamethasone, associated with a cationic lipid, DOTAP. Their physicochemical properties as well as their ability to bind siRNA were evaluated through gel electrophoresis and siRNA binding quantification. SiRNA cellular uptake was assessed by flow cytometry and confocal microscopy on RAW264.7 macrophages. TNF- α inhibition was determined on LPS-activated RAW264.7 macrophages. Stable and monodisperse nanoparticles around 100 nm with a positive zeta potential (+59mV) were obtained with an encapsulation efficiency of the prodrug of 95%. A nitrogen/phosphate (N/P) ratio of 10 was selected that conferred the total binding of siRNA to the nanoparticles. Using these CDXP-siRNA-NPs, the siRNA was strongly internalized by RAW264.7 macrophage cells and localized within the cytoplasm. On the LPS-induced RAW264.7 macrophages, a larger inhibition of TNF- α was observed with CDXP-siRNA-NPs compared to CDXP-NPs alone. In conclusion, from these data, it is clear that a combination of DXP and TNF- α siRNA therapy could be a novel strategy and optimized alternative approach to cure inflammatory diseases.

Keywords: corticosteroids, dexamethasone, siRNA, inflammation, macrophages

1 Introduction

Inflammation is a biological response to a sudden injury, leading to classical symptoms such as swelling, redness, pain. Physiopathology involves a variety of related cells and cytokines and a set of changes in those triggers the occurrence of inflammation (Chen et al., 2018). Among all cytokines, tumor necrosis factor-alpha (TNF- α) plays a major role such as activating immune cells during inflammation (Zelová and Hošek, 2013). TNF- α has been shown to play a pivotal role in orchestrating the cytokine cascade in many inflammatory diseases and for this reason, it has been proposed as a therapeutic target for many diseases including rheumatoid arthritis, Crohn's disease, atherosclerosis, psoriasis, sepsis, diabetes, and obesity (Parameswaran and Patial, 2010).

Glucocorticoids (GCs) are steroid hormones, with anti-inflammatory and immunosuppressive properties, that are widely used for the treatment of inflammation and autoimmune diseases (Chan et al., 2020). GCs have been applied for the therapeutic care of inflammatory and immune disorders such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and nephrotic syndrome (Hardy et al., 2020; Spies et al., 2011). The recent pandemic of COVID-19 has shown that a GC such as dexamethasone (DXM) was among the only drugs efficient in reducing the post-infection hyper inflammation resulting in a significant diminution of mortality (Johnson and Vinetz, 2020). GCs administration is however limited to short-term treatment and low doses. Indeed, it has been shown that GCs can cause the emergence of serious side effects such as diabetes, high blood pressure, or Cushing syndrome (Money, 2017; Oray et al., 2016). Besides, GCs are hydrophobic molecules with relatively poor oral bioavailability. Furthermore, GCs systemic administration leads to broad distribution throughout the body that is responsible for the observed side effects. As a result, the overall therapeutic efficacy and patient compliance with GC-based treatments can be poor (Onishi et al., 2011).

To overcome issues related to GCs administration, one approach consists of increasing GCs accumulation within the targeted tissue using nanoparticulate drug delivery platforms. Regarding Dexamethasone (DXM), it has been already encapsulated into polymeric nanoparticles (NPs) (Gomez-Gaete et al., 2008; Gómez-Gaete et al., 2007) with however a low encapsulation efficiency, a strong burst release and the presence of drug forming crystals. DXM has also been encapsulated into liposomes again with a weak encapsulation efficiency (Koning et al., 2006; Rauchhaus et al., 2009). To solve problems related to GCs encapsulation and release, we have recently designed lipid nanoparticles to deliver dexamethasone palmitate (DXP) in a collagen-induced arthritis murine model (CIA) (Lorscheider et al., 2019b). The fatty acid derivative was synthesized and used to obtain, through controlled hydrolysis, a long-term effect. Pharmacokinetics parameters were evaluated after intravenous (IV) injection of dexamethasone palmitate nanoparticles (DXP-NP) to healthy mice. The

release of DXM from DXP-NP in plasma was controlled for up to 18 hours compared with the free drug, which was rapidly eliminated from plasma after administration (Lorscheider et al., 2019a).

The physiopathology of inflammation is complex and involves a variety of related cells and cytokines. Thus, it is difficult to achieve an ideal therapeutic effect with one single small molecule drug or one anti-cytokine therapy due to the large number of targets that one of these drugs should reach. It appears more effective to combine two therapeutic approaches with different mechanisms of action. When multiple therapeutic agents target the same cellular pathway, they can cooperate to obtain a higher therapeutic effect and higher targeting selectivity. Based on these considerations, developing the co-delivery of GCs and siRNA together appears a simple, direct, and feasible method. As shown in the literature, a potent approach should be based on the co-administration of GCs with a companion nucleic acid such as TNF- α siRNA (Wang et al., 2017).

Although siRNA are effective, safe, and most importantly target-specific therapy, there are still barriers that must be taken into consideration for their delivery (Pal Singh et al., 2019). SiRNA are unstable in biological fluids which makes them poorly available in their intact form at the target site (Fattal and Bochot, 2008). Moreover, unfavorable physicochemical properties of siRNA such as high molecular weight and negative charge prevent them to pass through the cell membrane. Even though after a successful entry into cells siRNA must be protected by delivery systems in terms of endosomal escape, lysosomal degradation, and provide siRNA release (De Rosa et al., 2003, 2002; Deng et al., 2016; Lechanteur et al., 2018; Toub et al., 2006).

It is therefore a challenge to deliver dexamethasone (hydrophobic) and siRNA (hydrophilic) within the same nanoparticle while keeping the latest intact and favoring its intracellular penetration. To address this challenge, we propose a novel type of nanoparticle which composition is based on the formulation of DXP-NP described by our group (Lorscheider et al., 2019a, 2019b). These nanoparticles have been covered by a cationic lipid able to bind a TNF- α targeted siRNA through electrostatic interactions (Zhi et al., 2018). Particle characterization as well as *in vitro* cell trafficking and TNF- α inhibition were explored offering interesting perspectives for this novel nanomedicine combination.

2 Materials and Methods

2.1 Materials

Dexamethasone palmitate (DXP) was purchased from Interchim (France). 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) was obtained from Avanti Polar Lipids, Inc. N-(carbonyl-methoxypolyethylenglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, mPEG-2000-DSPE, Na-salt (DSPE-PEG) was provided by Lipoid., Dexamethasone disodium phosphate (DSP) was purchased from Fagron (The Netherlands). Milli-Q water was purified using a RIOS system from Merck-Millipore (France). High-performance Liquid chromatography (HPLC)-grade chloroform was obtained from Carlo Erba Reagents (France). HPLC-grade acetonitrile and dimethyl sulfoxide (DMSO) was purchased from VWR Chemicals (France). Duplex siRNA directed against TNF- α was provided by Integrated DNA Technologies Inc. (IDT, 100 Coralville, IA, USA) as lyophilized and purified duplexes. The TNF- α siRNA consisted of the following duplex sequences:

Sense 5'-GUCUCAGCCUCUUCUCAUCCUGT-3',

Antisense 3'-UACAGAGUCGGAGAAGAGUAAGGACGA-5', (Mw 16,807.3 Da).

A negative control sequence of siRNA (Ctr siRNA) was acquired from Eurogentec (Eurogentec, Angers, France). The exact sequence of Ctr siRNA was not disclosed by the supplier. Fluorescent label dye siRNA 6-FAM was obtained from Eurogentec (Eurogentec, Angers, France). Agarose Type-D5 DNA Grade was bought from Merck-Millipore (France). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT, Methylthiazolyldiphenyl-tetrazolium bromide was purchased from Sigma. Fluorescent stain ethidium bromide solution was obtained from Bio-Rad laboratories. TBE Buffer (Tris-borate-EDTA) was purchased from Promega. Nuclease free water was acquired from Sigma, RNaseZap™.

2.2 Preparation of cationic dexamethasone palmitate nanoparticles

Nanoparticles were prepared at five different lipid concentrations using a fixed prodrug concentration with the following DXP/DOTAP ratios: 5/2.5; 5/2; 5/1.5; 5/1; 5/0.5 in mg/mL using an emulsion solvent evaporation method derived from Lorscheider et al (Lorscheider et al., 2019a). Briefly, 10 mL of Milli-Q water was pre-chilled at 4°C. DXP was dissolved with the desired amounts of DOTAP (25; 20; 15; 10 or 5 mg) and completed to 1 mL with chloroform. Then, the 10 mL water phase was placed on ice and the organic phase was injected using a solvent-compatible syringe. The mixture was then vortexed for 30 seconds to obtain a pre-emulsion. The pre-emulsion was then ultrasonicated at 40% amplitude (300W Branson Digital Sonifier) for 2 minutes by keeping it in the ice bath. The organic phase was evaporated under reduced pressure using a rotary evaporator for 1h. Afterward, the suspension volume was completed to 10 mL with the chilled milli-Q water in a

volumetric flask, and nanoparticles were stored under an inert atmosphere either at 4°C or room temperature (RT) protected from light.

2.3 Preparation of cationic dexamethasone nanoparticle-associated-TNF α siRNA

Cationic dexamethasone palmitate nanoparticles (CDXP-NP) were mixed with anionic siRNA varying the nitrogen/phosphate (N/P) ratio (the charge molar ratio between the cationic DOTAP and the anionic siRNA). They were prepared at 1, 2, 5, 10 and 20 N/P ratios. Desired volumes of CDXP-NP were placed into Eppendorf tubes then fixed 50 μ L of 5 μ M siRNA solution were added producing (CDXP-siRNA-NP). Subsequently, the solutions were vortexed for 30 seconds and incubated at RT for 30 minutes. All the experiments were carried out under a biosafety cabinet and at RNase free conditions. N/P Ratio was calculated according to the following formula, since DOTAP bears one charge and siRNA 53 charges:

$$\frac{N}{P} = \frac{1 * \text{moles of DOTAP}}{53 * \text{moles of siRNA}}$$

2.4 Dynamic Light Scattering

Particle size distribution and zeta potential were determined by using a Zetasizer Nano-ZS from Malvern Instrument (UK), based on quasi-elastic light scattering. Measurements were performed in triplicate at an angle of 173° and a temperature of 25°C. Samples were diluted (1/10) in Milli-Q water for size measurements and in 1 mM NaCl for zeta potential measurements.

2.5 Agarose Gel Electrophoresis Assay

The binding affinity between anionic siRNA and cationic CDXP-NP was evaluated by the agarose gel electrophoresis method. To prepare 1% agarose gel, 1 g of agarose powder was dissolved in 100 mL of 1X TBE buffer. The mixture was boiled to obtain transparent agarose solution and was then left under a fume hood for 5 minutes. Later, 1.25 μ L of 10 mg/mL ethidium bromide (EtBr) solution was added to the warm agarose solution and it was decanted to the tray smoothly without the formation of bubbles allowing to form a gel by cooling. After decanting, a comb was placed on the gel to obtain wells. Meanwhile, samples were prepared by using DNA loading dye/sample at 1:3 ratio and 5 μ L from prepared samples were loaded into wells. DNA loading dye consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water. Electrophoresis was run at 120 V for 30 minutes using Apelex PS 608 electrophoresis power supply. siRNA bands were visualized using Bio Rad® ChemiDoc gel imaging system under UV light.

2.6 siRNA Quantification

Quantification siRNA associated to CDXP-NP was calculated from measuring the amount of unbound free siRNA for each N/P ratio (1, 2, 5, 10, and 20) when preparing CDXP-siRNA-NP. Samples were

diluted until reaching a concentration of 1000 ng/mL siRNA and were ultra-centrifugated at -4°C, 40.000 rpm for 15 min. Quantification of free siRNA was performed on the supernatant. QuantiFluor® RNA System kit containing RNA-binding dye (492nm excitation/540nm emission) was used and measurements were carried out using a spectrofluorimeter. A 1 mL RNA standard solution was prepared at 5 µg/mL using nuclease-free water (NFW) and diluted with NFW to obtain different samples which concentration ranges between 39 ng/mL to 2500 ng/mL. RNA-dye solution was diluted at a 1/200 ratio with 1X TE buffer in the dark and vortexed. One to one volume of RNA-dye solution was added to each RNA standard solution, to each sample, and as well as to the NFW as a negative control (Emission 0, Em_0). The calibration curve was obtained from emission values corresponding to each sample which was calculated using $Em - Em_0$. The equation of the calibration curve was the following $Y = 0.3111X - 20.82$, $R^2 = 0,9986$. Sample values were interpolated to the calibration curve to calculate the quantity of free siRNA. Afterward, entrapment efficiency was determined by comparing entrapped/associated siRNA to the initial amount of siRNA in the preparation of CDXP-siRNA-NP. Results were expressed as a percentage.

2.7 Quantification of Dexamethasone palmitate

Quantification of DXP concentration in CDXP-siRNA-NP was calculated using HPLC with a UV detection. The analysis was carried out at 240 nm using a SymmetryShield™ RP18 column (5 µm, 250×4.6 mm; Waters, France). The column temperature was maintained at RT. A gradient mobile phase system was used (Supplementary information Table S1). Eluent A was composed at 90:10 v/v with Milli-Q water: acetonitrile (ACN) while eluent B was composed of 100 % ACN. The mobile phase flow rate was set at 1mL/min with a 50 µL of injection volume and the run time was 40 min. The retention time of DXP was 22 min. The calibration curve was obtained from DXP in ACN between 62.5-1000 µg/mL and found linear, $Y = 65145X + 429882$, $R^2 = 0,9993$. DXP encapsulation efficiency was calculated as a percentage ratio between the actual encapsulated DXP amount compared to the initial DXP amount used. Drug loading of DXP and corresponding DXM was calculated as a percentage of actual encapsulated DXP amount compared to the total amount used in CDXP-siRNA-NP.

2.8 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed at I2BC facility (CNRS, Gif-sur-Yvette, France). First, 400 mesh formvar-coated copper grids were plasma treated to render them hydrophilic. Then, 5 µL volumes of 1/5 diluted samples were placed onto formvar-coated copper grids for 2 minutes. Subsequently, the grids were gently dried and negative staining was performed by adding a drop of 2% w/w phosphotungstic acid (PTA) for 30 seconds. Excess solution was removed, and grids were left to dry before observation. The observations were carried out on a JEOL

JEM-1400 microscope at an acceleration voltage of 120kV. Images were acquired using an Orius camera (Gatan Inc, USA).

2.9 Cell Culture

The murine macrophage cell line RAW 264.7 was purchased from American Type Culture Collection 165 (ATCC). The cell suspension was obtained by scraping the cells in 10 mL of fresh Dulbecco's modified Eagle's medium (DMEM, high glucose) (Sigma Aldrich, St. Louis MO, USA) supplemented with 10% fetal bovine serum (FBS), 10 000 unit/mL penicillin and 10 mg/mL streptomycin. For cell splitting, 1 mL cell suspension was added into 75 cm² flask and completed with 14 mL fresh full medium and incubated at 37°C and under 5% CO₂-95% O₂ atmosphere. The cells were sub-cultured twice a week and used at 80% confluence between 3-12 passage numbers.

2.10 Cell Viability

RAW264.7 cell viability was evaluated as a function of CDXP-NP and CDXP-siRNA-NP concentration using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Cells were seeded in 96-well plates at 8X10³ cells/100 µL density per well using full medium and incubated 24h at 37°C. The next day, samples were prepared by dilution in FBS-free medium at equivalent DXP concentrations (2-124 µg/mL concentrations). Subsequently, the full medium was removed gently from the wells and replaced with 100 µL of samples. After 4h of incubation at 37°C, 100 µL medium supplemented with 20% FBS was added into wells, and incubation was maintained for an additional 20h. Then, 20 µL from 5 mg/mL MTT/Phosphate-buffered saline (PBS) solution was added to each well and incubated 2h at 37°C to allow the transformation of formazan crystals. The medium was then carefully discarded and 200 µL of DMSO was added to dissolve formazan crystals. The 96-well plate was shaken for 5 minutes at 300 rpm then the absorbance was measured at 570 nm with a microplate reader. Cell viability was then calculated as a percentage using the formula below. All measurements were performed n=5, 3 times.

$$\% \text{ cell viability} = \frac{\text{Abs of treated cells} - \text{blank}}{\text{Abs of untreated cell} - \text{blank}} * 100$$

Where Abs is the absorbance. The same protocol was used for the cytotoxicity investigation of dexamethasone sodium phosphate (DSP), DOTAP-only, free siRNA, DOTAP/siRNA at equivalent concentration either of DOTAP or siRNA or CDXP-NP.

2.11 Quantification of Secreted TNF-α

The anti-inflammatory efficacy of both the CDXP-NP and CDXP-siRNA-NP was evaluated based on the amount of secreted TNF-α levels after activating the RAW264.7 macrophage cells by lipopolysaccharide (LPS). Cells were seeded in 96-well plates at a density of 8x10³/100 µL per well

using full medium and incubated 24h at 37°C. Samples were prepared by diluting in FBS-free medium at equivalent to 34 µg/mL DXP concentration. Also, samples were tested without using cells to check their potential interference with the MTT assay. MTT assay was performed at the end of the experiment to confirm that all experiments are carried out in subtoxic conditions. At the end of the 24h incubation, the full medium was kindly removed from wells and replaced with 90 µL of samples. Additionally, two columns were replaced with 90 µL of FBS-free medium as further controls. Incubation remained 4h at 37°C. Afterward, a 100 µL volume of 20% FBS contained medium was added on wells and incubation carried 20h more. LPS solution was prepared at 1 µg/mL in 20% FBS containing medium. 10 µL volume of LPS solution was added either to the samples or to one of the two columns as obtaining positive control (LPS-only) 3 hours before the ends of the incubation time (hour 21st). On the other hand, 10 µL volume from 20% FBS contained medium was added to the other one of the two columns to have negative control (No treatment). At the end of the experiment, supernatants were collected and stored at -20°C until performing TNF-α assay using a Cytometric Bead Array (CBA) mouse inflammation kit as described below. Meanwhile, the first 200 µL of the full medium then 20 µL MTT/PBS solution at 5 mg/mL concentration were added to cells and incubated 2 hours at 37°C. The same protocol was used for the anti-inflammatory activity investigation of DSP, DOTAP-only, free siRNA, DOTAP/siRNA, and CDXP-NP and CDXP-siRNA-NP and Ctr siRNA. For that reason, all samples were prepared in an amount equivalent or corresponding to the 34 µg/mL DXP contained in CDXP-NP or equivalent siRNA concentration.

2.12 CBA Assay

For the CBA test, BD Biosciences CBA mouse inflammation kit was used. First, all the beads were taken in an Eppendorf tube and diluted with Assay Diluent to obtain Top Standard (TS) solution as described in the CBA kit. Then, it was diluted 8 times with a 1:1 ratio using assay diluent in volumes of 300 µL each time. Finally, the assay diluent was taken into an Eppendorf tube of the same volume and incubated for 15 minutes at RT. Following this step, respectively at volumes of 50/5/5 µL from standard solution/TNF-cytokine solution/PE-detection reagent was taken in an Eppendorf tube. This process was repeated for each standard solution and one time for assay diluent to obtain a calibration curve (between 0-5000 pg/mL) then incubated at RT for 2 hours. Meanwhile, samples were defrosted then diluted at 1/10 ratio with the CBA wash buffer and vortexed at low speed. At volumes of 50/5/5 µL from diluted samples/TNF-cytokine solution/PE-detection reagent was taken in an Eppendorf tube, respectively. This process was repeated for each sample and incubated at RT for 2 hours. At the end of the incubation time, a volume of 1 mL of CBA wash buffer was added to either calibration samples or the samples and centrifuged at 200 g, 5 minutes. Following, supernatants were aspirated then a volume of 250 µL of CBA wash buffer was added to each sample to resuspend

the pellets and vortexed before running the experiment. Samples were quantified with the BD Accuri C6 Cytometer. All measurements were performed in triplicate.

The cellular siRNA uptake and its intracellular distribution with both CDXP-siRNA-NP and free siRNA in RAW264.7 cells were assessed against various incubation times by flow cytometry and confocal microscopy. For flow cytometry, 2 mL of the cell suspension at a final concentration of 15×10^4 / mL was added to the 6-well plates then left to incubate at + 37°C for 24 hours. The next day, CDXP-siRNA-NP and free siRNA were prepared using 1% fluorescent siRNA. Subsequently, the samples were diluted to 2 mL volumes with FBS-free medium at a final DXP equivalent concentration of 34 µg/mL or the corresponding siRNA concentration. Meanwhile, after 24 hours of incubation, the old cell media was gently removed from the wells with a pipette. A volume of 1 mL was added to the wells for the samples with an incubation time of 1, 2, 3, and 4 hours. Also, 1 mL volume of cell suspension alone was added to the wells to obtain negative control columns versus incubation hours. At the end of their incubation periods of 1, 2, 3, and 4h investigations were performed by flow cytometry. The following procedure was used to collect the cells: samples were first removed from the wells and replaced with 1 mL PBS and cells were scraped off and placed into an Eppendorf tube. The scrapping procedure was repeated once. Cells in Eppendorf tubes were centrifuged at 400 g for 4 minutes and the supernatant was removed. 1 mL of PBS was added to the pellets for reconstitution. Samples were vortexed for 30 seconds before being analyzed. Relative fluorescence intensity was measured against time with the help of Plot 7 region FL2-A / SSC-A filter in a flow cytometer. The analysis was carried out with 5×10^4 activity, 220 µL for 2 minutes, and in slow mode.

Confocal Laser Scanning Microscopy (CLSM) images were obtained after 4h incubation. For that purpose, cells were seeded at 5×10^4 / 300 µL per well using IBIDI 8-well plates then incubated 24h along at + 37°C. The next day, CDXP-siRNA-NP and free siRNA were prepared using 1% fluorescent siRNA in an FBS-free medium. Following the end of the incubation period, old cell media were removed from the plates, and samples in a volume of 140 µL were replaced and left for incubation at + 37°C for 4 hours. Samples were then imaged with an inverted STED-gated Leica TCS SP8 microscope (Leica, Germany) using an HC PL APO CS2 63x/1.40 oil immersion objective lens and under optimal incubation conditions provided by a heating stage. The instrument was equipped with a WLL (White Light Laser) laser. Red fluorescence emission was collected with a 560-760nm wide emission slit using a sequential mode. The pinhole was set at 1.0 Airy unit giving an optical slice thickness of 0.89µm. Twelve-bit numerical images were acquired using Leica SP8 LAS X software (Version 2.0.1; Leica, Germany). The same protocol was used for the fluorescent background investigation of cells alone (No Treatment).

3 Results and Discussion

3.1 Preparation of cationic dexamethasone palmitate nanoparticles

To produce CDXP-NP, various DOTAP concentrations with a fixed DXP content were used applying the solvent emulsion evaporation method as described (Lorscheider et al., 2019a). The lowest DOTAP concentration of 0.5 mg/mL led to nanoparticles with the highest hydrodynamic diameter (120 nm) but the lowest Pdl (0.13) and highest zeta potential (70mV) (Figure 1). When DOTAP concentration increased, although the mean diameter was reduced, the polydispersity increased from 0.17 to 0.22 with increasing DOTAP concentration. As shown by Jensen et al. (Jensen et al., 2012), DOTAP is thought to limit the enlargement of polymeric NPs due to its surfactant and condensation characteristics which affect the size. The zeta potential diminished to 57mV as DOTAP concentration increased (Figure 1). Statistical analysis was carried out using one-way ANOVA by Dunnett's multiple comparisons test and we concluded that NP with the least amount of DOTAP (0.5 mg/mL) had a significant difference in terms of hydrodynamic particle size (D_H) and zeta potential over all other CDXP-NP formulations. The zeta potential decrease can be explained by two hypotheses: i) as DOTAP concentration increases, the ionic strength increases and electrostatic screening becomes more important (Israelachvili, 2011) ii) for high concentrations, DOTAP may distribute within the nanoparticles and not only on their surface, therefore, hiding some charges. One can also imagine a combination of the two phenomena.

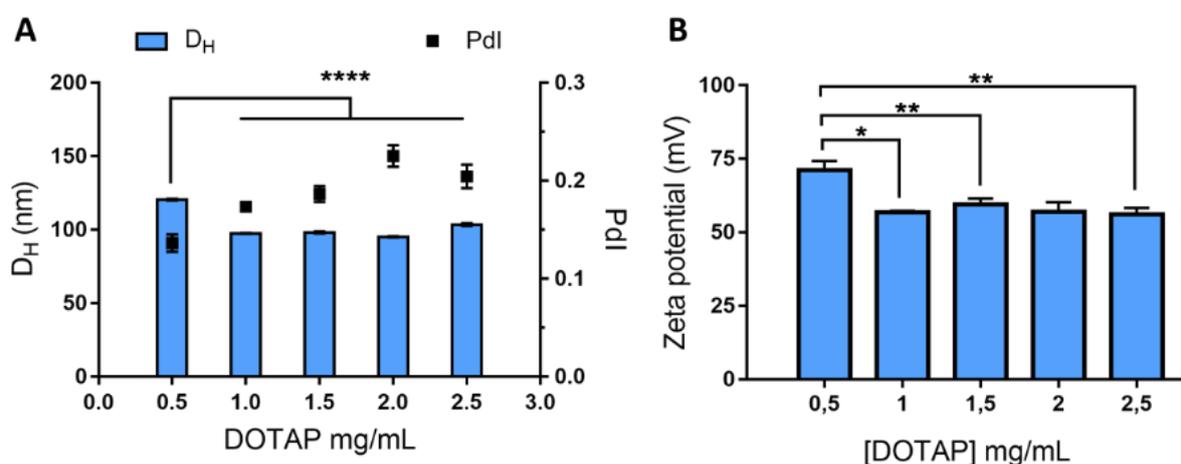


Figure 1: Effect of various DOTAP concentrations in terms of hydrodynamic diameter (D_H) and Pdl (A) and zeta potential (B).

Formulation stability was evaluated over 7 weeks at 4°C and room temperature (RT). At 4°C neither hydrodynamic diameter (102 ± 4 nm) and Pdl (0.18 ± 0.02) nor zeta potential (59 ± 4 mV) changed significantly (Supporting information Figure S1). At RT, the hydrodynamic diameter started to increase moderately after 3 weeks of storage while neither the Pdl nor the zeta potential was

drastically modified (Supporting information figure S1). Storage at 4°C was therefore preferred to favor stability.

All these results suggest that DOTAP lipophilic chains interact strongly with the lipophilic dexamethasone palmitate (Doi et al., 1989) and the cationic lipid is efficient to stabilize DXP into nanoparticles. Recent literature showed DXP could be successfully formulated with a high encapsulation efficiency (98% w/w) in anionic PEGylated NP using DSPE-PEG 2000 (Lorscheider et al., 2019b) as a stabilizer. These data support the idea that amphiphilic lipid molecules can coat the surface of lipophilic prodrug nanoparticles. This is not a peculiar property of lipid nanoparticles since DOTAP was also shown to cover the surface of poly(lactide-co-glycolide) (PLGA) nanoparticles either unloaded (Jensen et al., 2012) or loaded with budesonide (Leng et al., 2017).

3.2 Preparation of cationic dexamethasone nanoparticles associating TNF- α siRNA

From a very first fast screening, using gel electrophoresis, we looked at siRNA binding strength at two N/P ratio to select DOTAP concentrations to be used among the ones providing a low size and good monodispersity (1, 1.5, and 2 mg/mL). From all samples, at an N/P ratio of 10, siRNA migration was detected only for DOTAP concentration of 1 mg/mL but not for higher concentrations (supporting information Figure S2). Considering their good physical characteristics and their ability to tightly bind siRNA, DXP-DOTAP 5/1.5 mg/mL (CDXP-NP) were chosen as a good compromise for further studies.

The encapsulation efficiency and loading of DXP in CDXP-NP formulation were then quantified by HPLC. DXP encapsulation efficiency was found to be 95% w/w. The corresponding DXP loading and the corresponding active drug loading DXM was therefore 73% w/w leading to an equivalent loading of the parent drug DXM of 45% w/w. Our results confirmed DXP could be efficiently formulated in CDXP-NP using DOTAP as a stabilizer with similar high encapsulation efficiency as with PEGylated lipids in place of the cationic lipids (Lorscheider et al., 2019a).

Binding of siRNA to CDXP-NP was performed at various N/P ratios = 1, 2, 5, 10, and 20 and yielded complexes noted CDXP-siRNA-NP. At N/P=1, large and polydisperse aggregates were formed with a hydrodynamic diameter around 3000 nm, a Pdl of 1, and almost a neutral zeta potential. The neutral zeta potential explains the observed aggregation (Figure 2 A). As the N/P ratio increases, the size of CDXP-siRNA-NP decreases from 180 ± 2 nm (n=3) for N/P=2 down to 90 ± 1 nm for N/P=20. Pdl is reduced below 0.3 for all samples and even below 0.2 for N/P 10 and 20 (Figure 2A). The zeta potential becomes positive between 60 and 80 mV as soon as there is an excess of positive charges arising from CDXP-NPs (Figure 2B). The complexation efficiency of siRNA was then determined. At N/P=1, the percentage of complexed siRNA was around 68%. At higher N/P ratios, almost all the siRNA was complexed (95%) due to an excess of positive charges provided by CDXP-NPs (Figure 2C).

To assess the strength of complexation, gel electrophoresis was performed with CDXP-siRNA-NP and free siRNA. At N/P = 1, 2, and 5, the electric field led to some level of siRNA migration. Almost all siRNA migrated for N/P=1 and the amount migrating was reduced as N/P increased. For N/P 10 and 20, the siRNA migration could not be observed (Figure 2D). Since the strength of complexation may have a strong impact on the efficiency of siRNA delivery (Pal Singh et al., 2019), the formulation CDXP-siRNA-NP at N/P 10 was selected for further experiments as a compromise between small size (around 100 nm), positive zeta potential (67 mV), complexation efficiency (95%), and siRNA amount. The loading efficiency was of the same magnitude as PLGA cationic nanoparticles (Hasan et al., 2012), around a few percent.

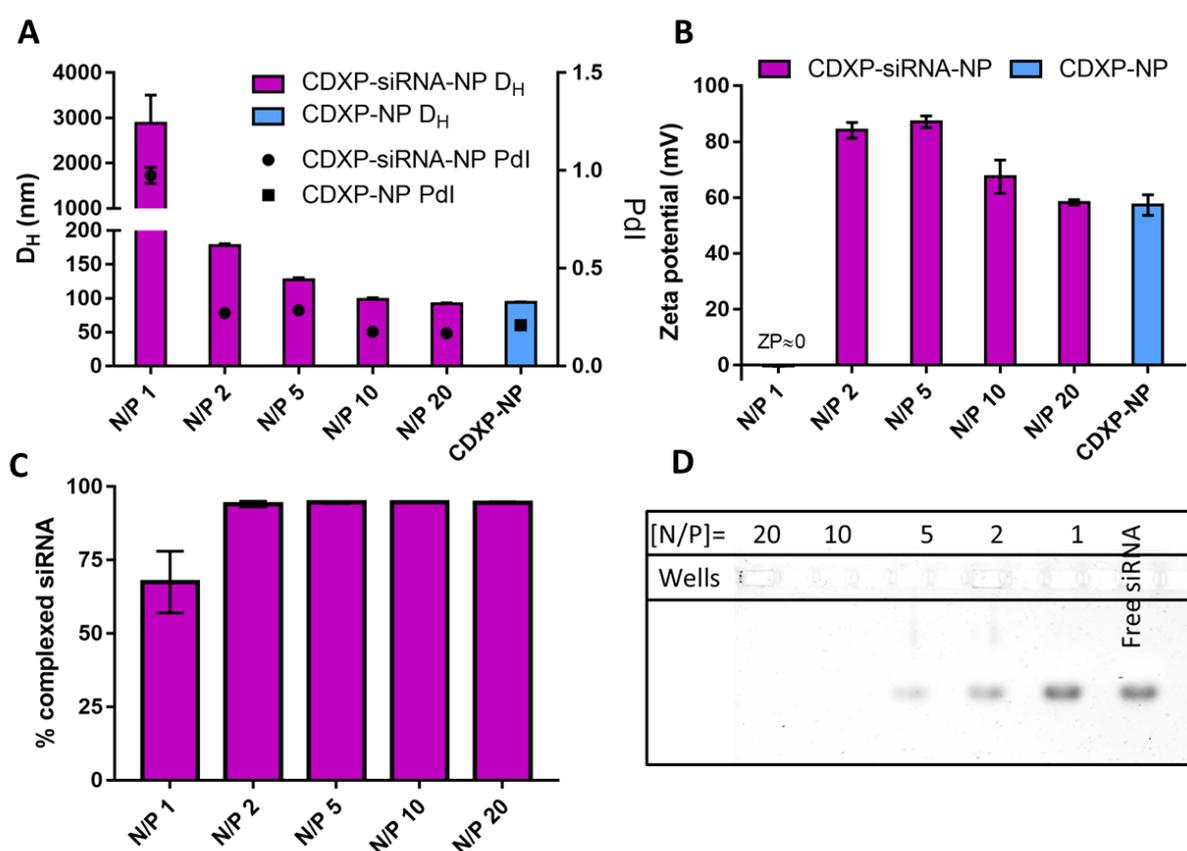


Figure 2: Physico-chemical characterization of lipoplexes (CDXP-siRNA-NP) formed by the association of siRNA and CDXP-NP at different N/P ratios in terms of size and Pdl (A), zeta potential (B), percentage of complexed siRNA (C). Agarose gel electrophoresis of free siRNA or CDXP-siRNA-NPs at different N/P ratios (D).

3.3 TEM observations

TEM was performed to observe CDXP-NP and CDXP-siRNA-NP and compare the structures obtained to those formed by DOTAP and DOTAP-siRNA-lipoplexes (LPX). Indeed, the excess of DOTAP was not

removed nor dosed due to the scarcity of methods to quantify it. CDXP-NP possess a spherical shape and some polydispersity can be observed (Figure 3A). DOTAP micelles were difficult to image properly and possess a more faceted morphology (Figure 3B). These types of structures are not visible on CDXP-NP images suggesting that all DOTAP is associated to the nanoparticles. When siRNA is complexed to CDXP-NP, spherical and non-spherical particles could be observed (Figure 3C). Similar morphologies were observed with DOTAP-siRNA-LPX. Sizes are in the same range as measured by dynamic light scattering (Figure 2A and Supporting information Table S2).

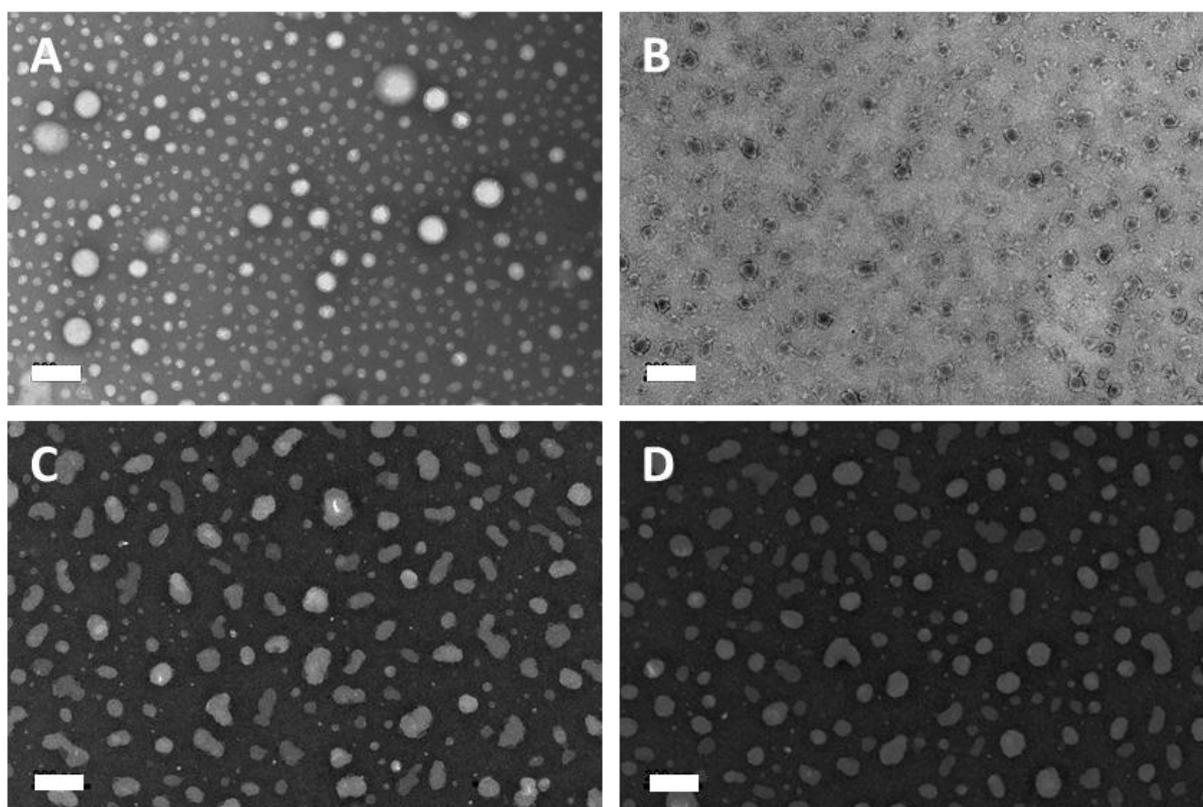


Figure 3: TEM images of CDXP-NP (A), DOTAP micelles (B), CDXP-siRNA-NP (C) and DOTAP-siRNA-LPX (D). The scale bars represent 200 nm in all images

3.4 In-vitro studies

Before evaluating the inhibition of TNF- α , the cytotoxicity of all formulations was investigated by MTT assay on RAW264.7 macrophages. Since cationic nanoparticles may aggregate in serum-supplemented cell culture medium (Doktorovova et al., 2014), formulations were first incubated in an FBS-free medium for 4h before adding FBS for the remaining time, preventing aggregation (data not shown). Interestingly, CDXP-NP and CDXP-siRNA-NP, above an equivalent DXP concentration of 34 $\mu\text{g}/\text{mL}$, have a very similar cytotoxicity profile (Figure 4). Other samples such as soluble dexamethasone (DSP), DOTAP, DOTAP-siRNA-LPX, and free siRNA did not induce a reduction of cell viability in the studied concentration range. In other words, while the components of nanoparticles

and lipoplexes did not show a toxic effect alone, their combination leads to some toxicity (Figure 4). Cytotoxicity does not arise from the cationic charges, as DOTAP and DOTAP-siRNA-LPX are also positively charged. Most probably, cytotoxicity arises from the better cellular entry of dexamethasone as a prodrug since it is formulated as cationic nanoparticles, whereas entrance is lower for soluble form DSP. Given the absence of cytotoxicity up to 34 $\mu\text{g}/\text{mL}$ (eq. DXP concentration), the inhibition of TNF- α release was performed at this concentration.

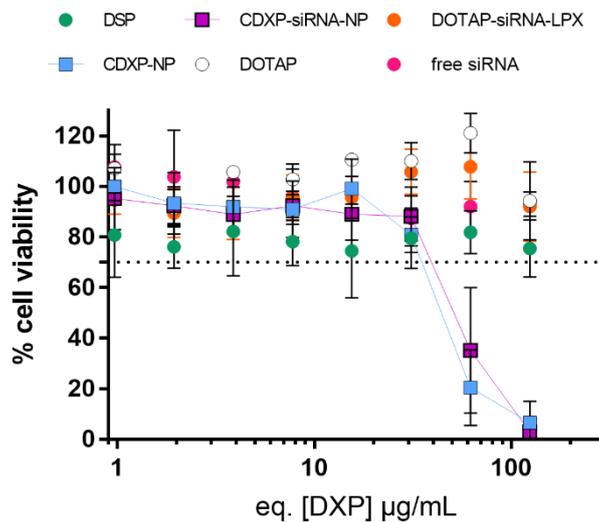


Figure 4: RAW264.7 murine macrophages viability after treating with DSP solution (green circles), CDXP-NP (blue squares), CDXP-siRNA-NP (purple squares), DOTAP (white circles), DOTAP-siRNA-LPX (orange circles), and free siRNA (pink circles) for 24 h at different concentrations. Concentrations are expressed as equivalent DXP.

The cellular siRNA uptake was then evaluated on RAW264.7 cells by flow cytometry and confocal microscopy. Confocal microscopy images show significant fluorescence in cells incubated with CDXP-siRNA-NP as compared with those incubated with free siRNA or untreated cells (Figure 5). Fluorescence in punctuated and mostly perinuclear.

The siRNA internalization kinetics was then assessed by flow cytometry. As shown in Figure 6, siRNA internalization was much more important after incubation with CDXP-siRNA-NP than with free siRNA, with stronger uptake as soon as after 1-hour incubation that kept increasing overtime until 4 hours ($p < 0.05$). After 4 hours incubation, uptake was found ~ 15 times higher with CDXP-siRNA-NP than with free siRNA. These data demonstrate cationic lipidic prodrug nanoparticles possess the same capacity as cationic polymers to induce a high siRNA uptake by RAW264.7 cells compared to free siRNA (Bohr et al., 2020, 2017).

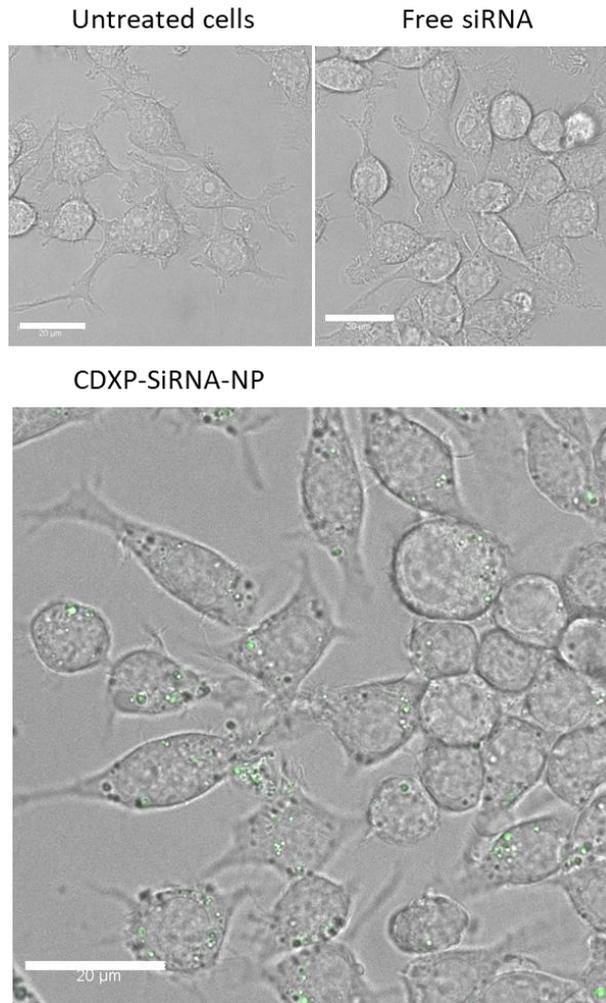


Figure 5: CLSM images of cells after 4h incubation with either free siRNA or CDXP-SiRNA-NP. The scale bar represents 20 microns.

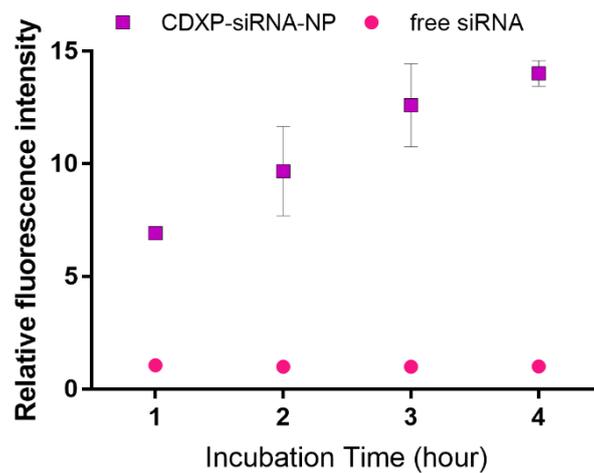


Figure 6: siRNA uptake by RAW264.7 cells versus vary incubation time for CDXP-siRNA-NP (purple) or free siRNA (pink). Uptake was significantly different between groups at all times ($p < 0.05$). Data are presented as mean \pm SD (N=3).

Finally, TNF- α release inhibition was evaluated (Figure 7). There are three main groups for the different treatments. First of all, neither treatments with DOTAP, DOTAP-SiRNA-LPX nor free siRNA led to a reduction of TNF- α secretion as compared with LPS-activated cells. In the second group, treatments were significantly different compared to LPS-activated cells for DSP, CDXP-NP, and CDXP-CtrsiRNA-NP ($p < 0.05$), leading to a strong reduction of TNF- α secretion compared to untreated cells. These effects were different than those induced by CDXP-siRNA-NP ($p < 0.05$) for which TNF- α secretion was even significantly greater. These results show that neither DOTAP-SiRNA-LPX nor free siRNA can reduce TNF- α secretion at the siRNA concentration used (28 nM). DOTAP alone displays a poor efficiency of siRNA inhibition unless it is combined with helper lipids such as dioleoylphosphatidylethanolamine (DOPE) (Zhi et al., 2018). However, in most cases where DOTAP was associated to polymer nanoparticles, no helper lipid was needed to obtain inhibition (Hasan et al., 2012).

One can notice that all treatments containing dexamethasone either soluble or as lipidic prodrug nanoparticles led to a reduction of TNF- α secretion although to a different extent. We can observe that CDXP-NP and CDXP-CtrsiRNA-NP were found equivalent to DSP probably due to the anti-inflammatory effect of dexamethasone itself either in soluble form or as lipidic prodrug nanoparticles. A clear significant difference was found for CDXP-siRNA-NP compared to DSP, CDXP-NP and CDXP-CtrsiRNA-NP ($p < 0.05$). These results prove that the further reduction of TNF- α is sequence-specific and does not only arise from dexamethasone. Indeed, the combination of CDXP-NP and TNF- α siRNA leads to a synergistic effect in reducing TNF- α secretion.

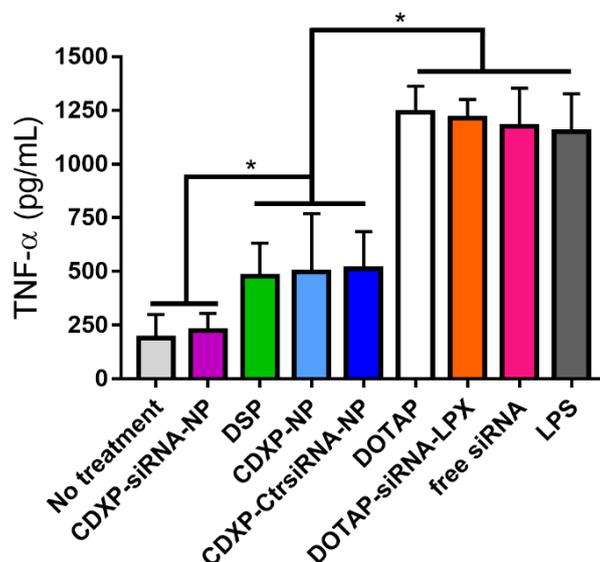


Figure 7: Quantification of TNF- α cytokine secretion as a function of treatment: No treatment (light grey), LPS (dark grey), DSP solution (green), CDXP-NP (blue), CDXP-siRNA-NP (purple), CDXP-CtrsiRNA-NP (dark blue), DOTAP (white), DOTAP-siRNA-LPX (orange), free siRNA (pink). siRNA concentration was 28 nM. Data represent mean \pm SD (N=3). Statistical analysis was performed using an unpaired nonparametric Mann-Whitney test between groups (*, $p < 0.05$).

4 CONCLUSION

Results clearly demonstrate that it is possible to combine within the same nanoparticle two different antiinflammatory drugs, the first being a lipophilic prodrug of dexamethasone, the second a TNF- α siRNA. Both drugs were loaded to a high extent through the use of a cationic lipid that interacts strongly with the DXP-NP through the acyl chains and with siRNA through the cationic head groups. The resulting nanoparticles called CDXP-siRNA-NP were stable and highly internalized in vitro by macrophages. As a result, a strong inhibition of TNF- α was observed related to the combined effect of dexamethasone and the siRNA. As a perspective, we believe that such small particles would extensively migrate to inflamed tissues in several inflammatory diseases.

5 ACKNOWLEDGMENTS

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Supporting Information

Combining dexamethasone and TNF- α siRNA within the same nanoparticles to enhance anti-inflammatory effect

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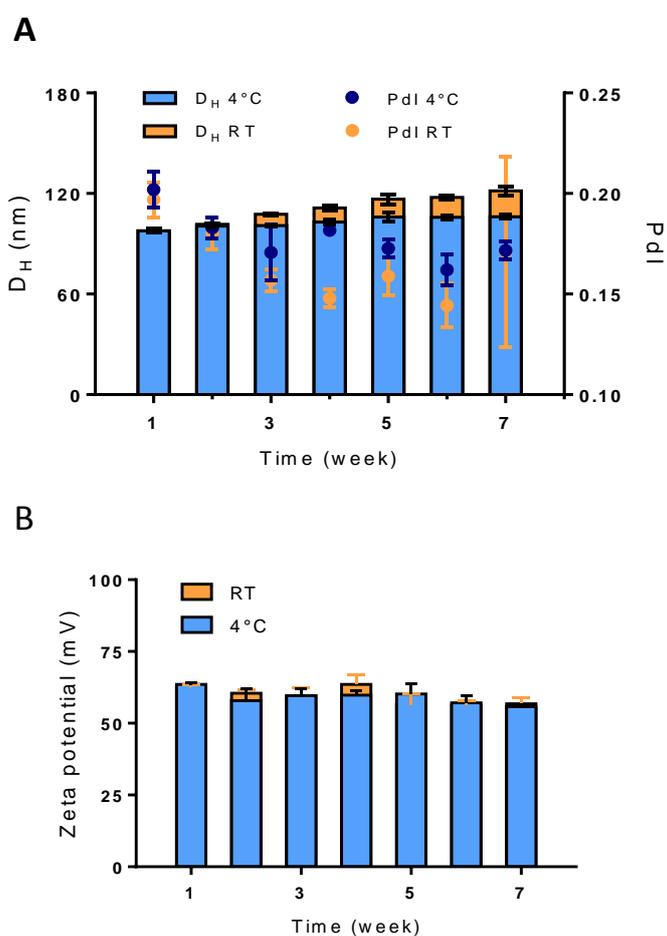


Figure S1: Stability of the CDXP-NP formulation at 4°C and room temperature (RT) over 7 weeks in terms of D_H, PDI (A) and zeta potential (B).

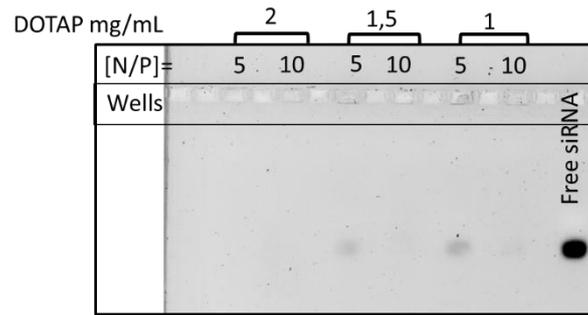


Figure S2: Agarose gel electrophoresis of CDXP-siRNA-NP at different N/P ratios and different DOTAP concentrations

Table S1: Gradient mobile phase system

Process	Time (minute)	Flow rate (mL/min)	% Eluent A	% Eluent B
		1.00	65.0	35.0
	5.00	1.00	65.0	35.0
	7.00	1.00	10.0	90.0
	30.00	1.00	10.0	90.0
	34.00	1.00	65.0	35.0
	40.00	1.00	65.0	35.0

Table S2: Hydrodynamic diameter D_H , Pdl and zeta potential of formulations imaged by TEM

	Z-Average ; Pdl ; ZP
CDXP-NP	97 nm ; 0,2 ; + 62 mV
CDXP-siRNA-NP N/P 10	104 nm ; 0,2 ; + 67 mV
DOTAP	76 nm ; 0,19 ; + 58 mV
DOTAP-siRNA-LPX N/P 10	85 nm ; 0,28 ; + 41 mV

