Nanostructured lipid carriers as a delivery system of tobramycin and ciprofloxacin: preparation, testing and validation in the *Caenorhabditis elegans* model

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Abstract: In this work, a nanostructured lipid carrier (NLC) formulation was developed, optimized and characterized, followed by the encapsulation of two antibiotics currently used in cystic fibrosis therapy, ciprofloxacin and tobramycin.
Average size of nanoparticles ranged from 668.1±232.6 to 202.4±2.7nm while their polydispersity index (PdI) ranged from 0.840±0.08 to 0.208±0.03 and zeta potential (ZP) ranged from -20.3±0.52 to -56.9±3.72mV. Nanoparticles with ciprofloxacin and tobramycin exhibited a similar average size compared to empty nanoparticles. Zeta potential of nanoparticles with tobramycin was much lower than that of empty nanoparticles, suggesting that these nanoparticles are long-term instable. Thermal analysis showed that these particles are solid at room temperature as well as at body temperature. Empty nanoparticles and nanoparticles with ciprofloxacin were stable up two months in opposition to nanoparticles with tobramycin. Nanoparticles with ciprofloxacin had an encapsulation efficiency of 68.16±4.9% and a burst release where all the drug was released in the first 7 hours. Transmission electron microscopy showed images of nanoparticles with spherical shape and with a size of approximately 200 nm. The efficacy of encapsulated antibiotics was assessed using *Caenorhabditis elegans* as an animal model of infection and bacterial pathogens such as *Burkholderia contaminans* IST408 and *Burkholderia cenocepacia* k56-2, as model pathogens. The significantly difference was observed in the survival of *C. elegans* infected with *B. contaminans* IST408 upon exposure between nanoparticles with ciprofloxacin or without nanoparticles (p-value<0.05) and between empty nanoparticles or nanoparticles with antibiotic (p-value<0.01). For *C. elegans* infected with *B. cenocepacia* k56-2 no significant difference in survival was observed when worms were fed with nanoparticles with antibiotic.
Key words: Nanostructured lipid carriers, Ciprofloxacin, Tobramycin, *C. elegans*

Introduction

Lipid drug carriers have been investigated for years and include oil-in-water (o/w) emulsions, liposomes, microparticles and nanoparticles[1].

Solid lipid nanoparticles are colloidal dispersions with a solid matrix composed of biodegradable lipids, emulsifiers (to stabilize the lipid dispersion) and water, with a mean diameter ranging from 50 to 1000nm. These lipids include triglycerides, fatty acids, steroids and waxes. They are prepared from lipids which are solid at room temperature as well as at body temperature[2–4].

Compared to liposomes and emulsions, solid nanoparticles combine the advantage of physical stability, protection of the incorporated drug from degradation, and controlled release. They can be applied in various applications such as parenteral, oral, dermal, ocular, pulmonary and rectal[5,6]. Solid lipid nanoparticles have some limitations. The main disadvantages of this system include poor drug loading capacity, drug expulsion after polymeric transition during storage and relative high water content of the dispersion (70-99%)[4].

Nanostructured lipid carriers, are systems that minimise or avoid some potential problems associated with SLN. For the NLCs production different solid lipid molecules at room temperature are mixed with liquid lipid (oil). The resulting nanoparticle shows a lower melting point compared to SLNs but is still solid at body temperature. NLCs have higher loading capacity and controlled drug released due the dissolution of the drug into oil and encapsulation into solid lipid phase[7].

The development of a colloidal delivery system such as nanoparticles allowed the improvement of drug delivery. Generally, the drug or a biological active material are dissolved, entrapped, adsorbed or attached. The advantages of nanoparticles as drug delivery systems rely on their biodegradability, non-toxicity and capability of being stored over long periods[3].

In the present work, the two antibiotics that will be used for the encapsulation in lipid nanoparticles are ciprofloxacin and tobramycin, which are presently used in cystic fibrosis therapy.

Oral administration is the most preferred route for drug administration due to greater convenience, less pain, high patient compliance, reduced risk of cross-infection, and needle stick injuries[2].The aim of oral administration is the improvement of oral bioavailability either by increasing gastrointestinal absorption or by bypassing the first-pass metabolism[2].

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic that inhibits bacterial DNA gyrase, an enzyme essential for DNA replication[8–10].

Tobramycin is an amiglycoside antibiotic that binds irreversible to a specific aminoglycoside receptor on the bacterial 30S small ribosomal subunit, interfering with the initiation complex between messenger RNA and the ribosomal subunit. This leads to an inhibition of protein synthesis initiation, leading to bacterial cell death. Tobramycin also induces misreading of the mRNA template, causing incorrect amino acids to be incorporated into the polypeptide chain during the elongation process[11].

Cystic fibrosis is an autosomal recessive disorder that affects approximately 70 000 individuals worldwide[12,13]. In Portugal the incidence of CF is estimated to be about 1:6000[14]. Respiratory failure caused by infection and inflammation is the cause of about 80% of mortality among CF patients[15,16]. CF airways are not infected at birth and bacterial opportunists enter the upper and lower respiratory tract by inhalation or aspiration. These bacteria growths and establish themselves in the lungs, leading to a local inflammation and the establishment of a chronical inflammatory response. This triad of chronic

obstruction, infection and inflammation leads to a lifelong degradation of the lung anatomy and function, contributing to the premature death of persons with CF. The *Burkholderia cepacia complex* (Bcc) is a heterogeneous group of gram-negative comprising at least 20 genetically related bacterial species. These bacteria are important opportunistic pathogens, especially in cystic fibrosis patients, and are associated with a worse prognosis and decreased life expectancy. One of the most striking features of Bcc infections is the unpredictable clinical outcome, ranging from asymptomatic carriage to the cepacia syndrome. The large majority of CF patients infected with Bcc develop a chronic infection that can last for years, leading to progressive loss of lung function[17]. CF individuals are highly susceptible to bacterial respiratory infections, thus intensive antibiotic therapy is in use to maintain lung function and reduce inflammation in infected patients[16].The eradication of infections caused by bacteria is very difficult and often unpredictable, due to their intrinsic resistance to the vast majority of clinically available antimicrobials[18].

Caenorhabditis elegans is a small, free-living soil hermaphroditic nematode, that feeds on microbes. The nematode is a widely used model due to the ability to grow hundreds of animals on a single Petri dish (each adult can lay between 250 and 300 eggs), feeding on bacteria (usually *Escherichia coli*), small size, transparency, rapid life cycle, short lifespan (2-3 weeks), easy and inexpensive growth in the laboratory[19,20]. The worm can be easily maintained in the laboratory, where it grows on agar plates or liquid cultures with *E.coli* as food source. The main path of uptake of nanoparticles in *C. elegans* is the alimentary system where the worms ingest nanoparticles actively during feeding or passively diffuse through the cuticle during exposure or the vulva, anus, and excretory pore, because these openings connect the body of the worm to its environment[19]. The particle size should be smaller than 1000nm to be ingested, that is the dimensions of the nematode mouth. This ingestion could occur through two different mechanisms: voluntary ingestion or non-voluntary ingestion of the nanoparticles simultaneously with ingestion of *E.coli* OP50 bacteria due to continuous pumping action of the pharynx[21].

The first aim of this work was the development, optimization and characterization of a nanostructured lipid carrier. Different fatty acids (lauric acid, myristic acid, palmitic acid and stearic acid), oils (coconut oil and sunflower oil) and surfactants (Tween 80 and Span 80) were used. Second objective of this work was the encapsulation of two antibiotics, ciprofloxacin and tobramycin, which are currently used in CF therapy[16] and test their efficacy in *C.elegans*. Nanoparticles with encapsulated ciprofloxacin were fed to infected *C. elegans* that was used as an animal model of infection. Pathogens belonging to the Bcc group (*Burkholderia contaminans* IST408 and *Burkholderia cepacia* k56-2) were used as model pathogens to infect the *C. elegans* nematode. As the worm feeds mainly of *E. coli*, the food source can be easily replaced by the pathogen under study following the disease progress. After the nematode infection, the efficacy of encapsulated antibiotics in nanoparticles can be assessed by comparing the survival of nematode in presence or absence of nanoparticles.

Materials

Lipids used were lauric acid (≥99%) and palmitic acid purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA), myristic acid (99%), coconut oil and stearic acid from Acros Organics (Belgium). Sunflower oil is a commercial alimentary product and were purchase from Fula (Portugal). Surfactants were Tween 80 (Merck-Schuchardt, Germany) and Span 80 (Thermo Fisher Scientific, USA). Co-surfactant was Hexadecane (≥99%) from Sigma-Aldrich (St. Louis, MO, USA). Tobramycin, Ciprofloxacin and Fluorescamine were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). DiOC18(3) were purchased from invitrogen by Thermo Fisher Scientific (Carlsbad, California, USA).

Water was from a Millipore Milli-Q® ultrapure water purification unit.

Caernorhabditis elegans (strain Bristol N2) was obtained from Caernorhabditis Genetics Center - University of Minnesota, Minneapolis, USA.

E. coli OP50 (uracil-requiring mutant of *E.coli*) was obtained from[22].

B. contaminans isolate IST408 were obtained from bronchial secretions of a patient with CF in January 1995 from the HSM CF Center, Lisbon, Portugal[23,17].

B. cenocepacia K56-2 was obtained from the sputa of CF patient and were originally obtained from Hospital for Sick Children, Toronto, Ontario, Canada[24].

Methods

Preparation of lipid nanoparticles

Two different methodologies were used for lipid nanoparticle production. In the first methodology nanostructured lipid carriers were prepared by a simple magnetic stirring method based on the microemulsion technique[25,26]. (Formulation NLC_1).

In the second methodology, sonication was used instead of gradual cooling. After the heating step, samples were sonicated for 5 minutes (48% amplitude; pulse 10/5 seconds ON/OFF) in a probe-type sonicator Sonoplus (Bandelin, Germany). For this methodology, different lipids were used (lauric acid, myristic acid, palmitic acid and stearic acid) as well as different surfactants (Span 80 and Tween 80) and oils (sunflower oil and coconut oil) with the proportions of 0.8%w/w, 1%w/w and 1.2%w/w, respectively. Span 80 was added to the lipid phase instead of aqueous phase due to its high lipophilicity. For the loaded nanoparticles, the antibiotics ciprofloxacin and tobramycin were added to the aqueous phase 15 min before the mixing step. The different formulations of lipid nanoparticles used in this study and respective composition are represented in table 1 (Formulation NLC_1 – NLC_TOB).

Table 1: Different formulations of lipid nanoparticles.

Formulation	Surfactant	Cosurfactant	Fatty acid	Oil
NLC_1	Tween 80	Hexadecane	Lauric acid + Myristic acid	Coconut oil
NLC_2	Span 80	-	Stearic acid	Sunflower oil
NLC_3	Tween 80			
NLC_4	Span 80			
NLC_5			Lauric acid	
NLC_6			Myristic acid	
NLC_7			Palmitic acid	
NLC_CIP			Stearic acid	
NLC_TOB				

Characterization of lipid nanoparticles

Size and zeta potential

The mean particle size (in nm), polydispersity index and zeta potential (in mV) were determined by photon correlation spectroscopy in a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK).

Transmission electron microscopy observation

The morphological study of NLCs was performed by transmission electron microscopy (TEM) in a (Hiatchi H8100, Tokyo, Japan) with incorporated LaB6 filaments and a CCD camera (Olympus-Keenview), operated at an acceleration voltage of 200kV. For sample preparation, a drop of the nanoparticle suspension was deposited in a carbon covered copper grid and dried at room temperature. Samples analysed were NLC-4, NLC_CIP and NLC_TOB.

Differential Scanning calorimetry

DSC thermal analysis were obtained using DSC 200 F3 Maia® (Netzsch, Germany). For DSC analysis, 5 mg of dried nanoparticle were crimped in a standard aluminium pan and heated from 25ºC to 120ºC (for remove residual water) and then cooled to 25ºC under constant purging of nitrogen. This set of temperatures was repeated twice.

Washing of lipid nanoparticles by ultrafiltration

Lipid nanoparticles washing was performed by ultrafiltration using Spin-X® UF centrifugal filter device with a cut-off of 10,000 Da (Corning, USA). Nanoparticles were centrifuged at 5000 rpm during 15 min in a centrifuge (Labofuge 200, Heraeus Sepatech). This step was performed tree times and the filtrate was collected for quantification of free drug.

Encapsulation efficiency and drug loading

The encapsulation efficiency (EE) and drug loading capacity (DL) of the antibiotic in NLCs were determined indirectly by measuring the concentration of free antibiotic in aqueous phase using reverse-phase high performance liquid chromatography (RP-HPLC) for ciprofloxacin. Percentages of EE and DL were estimated according to equations 1 and 2, respectively.

$$EE\% = \frac{W_{total} - W_{free}}{W_{total}} \times 100$$

Equation 1

$$DL(\%) = \frac{W_{total} - W_{free}}{W_{lipid} + (W_{total} - W_{free})} \times 100$$

Equation 2

Where W_{total} is the total weight of drug added, W_{free} is the weight of free drug dissolved in dispersion medium, W_{lipid} is the total lipids weight in the formulation.

Quantification of tobramycin

The absence of chromophore or fluorophore groups in the tobramycin, makes direct UV or fluorometric detection inapplicable and chemical derivatization of primary amino groups is often carried out[27]. In this work, fluorescamine was used for derivatization of tobramycin for UV-VIS spectrophotometric (Sampath and Robinson, 1990 and María Moreno-Sastre et al, 2016) [28,34,35] and spectrofluorometric detection (Tekkeli et al, 2013)[27].

Quantification of ciprofloxacin

Ciprofloxacin was quantified with RP-HPLC (reverse phase-high performance liquid chromatography. The HPLC method used was adapted from Wu, Shihn-Sheng, et al[8] with slightly modifications. The isocratic mobile phase used was 88:12 (v/v) 2% aqueous solution of acetic acid – acetonitrile. The flow rate was fixed at 0.5 mL/min with an injection volume of 10 µL. Detector was set at 280 nm.

Drug release

Preliminary release studies of ciprofloxacin from nanoparticles were performed using regenerated cellulose dialysis tubing (Orange scientific, Belgium) with a cut-off between 12,000 and 14,000 Da. A 5 ml nanoparticle sample suspension was added to the dialysis tubing for 45 mL of phosphate buffer (pH=7.4) at 37ºC, protected from the light and under stirring. 1 mL aliquot of the reaction medium was taken at t=0, t=30 min, t=1h-t=8h, t=10h, t=24h, t=36h, t=48h. Samples were quantified with RP-HPLC.

Determination of nanoparticle ingestion by *C. elegans*

C. elegans cultures were prepared according to standardized methods.

A total of 1 mg per 20mL of fluorescent dye DioC18(3) was incorporated into nanoparticles of formulation NLC_4, NLC_TOB and NLC_CIP. 50 µl of each nanoparticle formulation were dispersed over the surface of individual agar plate containing *E. coli* OP50 and were air-dried for one hour. L4 stage *C. elegans* were then transferred to these plates. After 3h adult worms were transferred with 1 ml of M9 buffer to tubes and centrifuged (1 minute, 3000 rpm). Supernatant was discarded and 1ml of NaN_3 1mM was added. After 2 minutes, suspension was centrifuged once again (1 minute, 3000 rpm). 10 µl of the pellet was visualized in a fluorescence microscope Zeiss axioplan with 10x of magnification.

Nanoparticles with encapsulated ciprofloxacin efficacy assessment

50 µL aliquots of suspension of pathogens *B. contaminans* IST408, *B. cenocepacia* K56-2 and non-pathogenic *E. coli* OP50 were prepared from overnight growth cultures. These bacterial suspensions were plated onto the surface of 35 mm diameter petri plates containing 4ml of NGM II and then incubated for 24h. Aliquots of 50µL of nanoparticles with the antibiotic ciprofloxacin (diluted for the concentration of 32µg/mL) were dispersed over the surface of individual plates containing *B. contaminans* IST408, *B. cenocepacia* K56-2 and a negative control of non-pathogenic *E. coli* OP50. Approximately 20 hypochlorite-synchronized *C. elegans* BN2 larvae at the L2 development stage were pipetted per plate. The actual number of worms were determined visually with the aid of a stereomicroscope. Controls of empty nanoparticles and plates without nanoparticles were also tested. Each condition was performed in multiples of five and all the assay was performed in triplicate.

Plates were incubated during 3 days at 20°C. The morphological appearance, the ability to generate descendants and the percentage of live worms were checked daily.

Results and Discussion

Characterization of lipid nanoparticles

Size and zeta potential

The different nanoparticles prepared were analysed with dynamic light scattering to determine the average size, PdI and zeta potential. The pH of the nanoparticles suspension prepared with different formulations was also measured.

The results obtained for the determination of the average size and PdI is shown in figure 1.

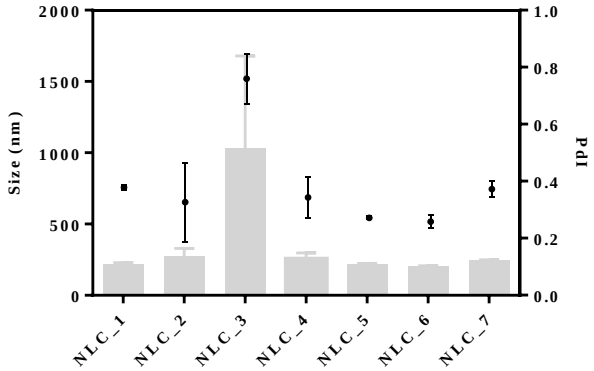

Figure 1: Characterization of the average size (vertical bars) and PdI (black dots) of the empty nanoparticles prepared with formulations NLC_1 to NLC_7.

The initial formulation used in this work (NLC_1)[25,26] was produced without recurring to sonication. These nanoparticles presented an average size of 219.0±11.0nm and PdI of 0.400±0.04. This formulation was excluded due to lower melting point of the nanoparticles and the extreme toxicity towards *C. elegans* (data not shown).

Experiments with nanoparticles containing a single fatty acid were performed without sonication and the resulting suspension formed visible aggregates; thus, the sonication step was added to the preparation of these nanoparticles (formulation NLC_2 to NLC_7).

Nanoparticles containing Tween 80 and stearic acid (formulation NLC_3) presented a large size (668.1±232.6nm) and a high value of polydispersity index (0.840±0.08). PdI measure the size distribution of the nanoparticles. Higher PdI values indicate a higher polydispersity of the dispersion which is not desirable due to the different sizes of nanoparticles in the medium.

Results obtained for the determination of the zeta potential of the formulations NLC_1 to NLC_7 as well as the pH are shown in figure 2.

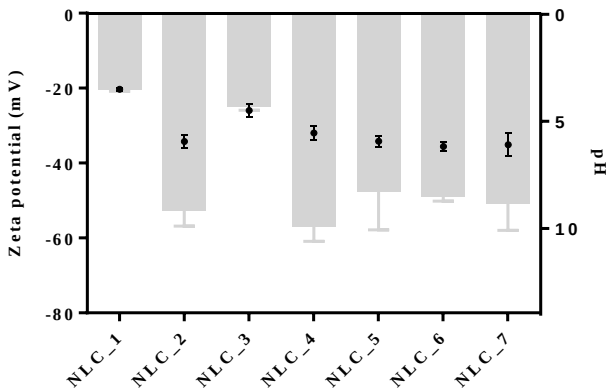

Figure 2: Zeta potential (vertical bars) and pH (black dots) of empty nanoparticles NLC_1 – NLC_7.

The zeta potential (ZP) indicates the overall charge that a particle acquires in a specific medium.

Nanoparticles containing Tween 80 (Formulations NLC_1 and NLC_3) presented a low zeta potential value, -20.3 ±0.52mV and -24.1± 0.65mV, respectively. These values predict a long term instable nanoparticles. Low ZP values (negative or positive) predict the attraction of the nanoparticles and they can flocculate or coagulate[29]. In opposition, values ranging from -47,5±0.90 mV to -56,9±3.72 mV were determined for nanoparticles formulated with surfactant Span 80, suggesting higher stability. High ZP values predict the prevention of aggregation of the nanoparticles due to electric repulsion. Generally, nanoparticles whose ZP values are not comprehended between -30mV and +30mV are considered stable[29]. Differences of the zeta potential between nanoparticles containing different surfactants could be due to the different pH of the final nanoparticles solution. Zeta potential is strong dependent of the pH of the solution. The pH of the suspension of nanoparticles with Tween 80 ranging from 3.5±0.08 to 4.48±0.32 and for suspension of nanoparticles with Span 80 ranging from 5.93±0.29 to 6.16±0.21. The medium with Span 80 is less acidic, therefore, the nanoparticles tend to acquire a more negative charge.

No significant differences were observed in average size, PdI or ZP in nanoparticles formulated with the different oils (sunflower oil-NLC_4 and coconut oil-NLC_2).

Taking into account the considerations above, the formulation NLC_4 was chosen, due to good size and polydispersity index and high zeta potential which predict a long-term stability.

Antibiotics ciprofloxacin and tobramycin were encapsulated in nanoparticles obtained with formulation NLC_4. The average size, PdI and ZP obtained for these nanoparticles are presented in figure 3.

A 5 mg of antibiotic was added in each formulation (0.25 mg/mL). The size and the polydispersity index of the nanoparticles containing the antibiotics ciprofloxacin (258.5±50.7nm;0.399±0.08) and tobramycin (255,2±63.5nm;0.324±0.09) was similar compared to empty nanoparticles (255.9±40.8nm; 0.342±0.06).

The ZP of ciprofloxacin loaded nanoparticles (-48.9±4.09mV) is slightly lower that the empty ones (-56,9±3.72mV).

However, nanoparticles loaded with tobramycin exhibited a significant decrease of the zeta potential (-22.0±3.62mV). The pH of the tobramycin loaded nanoparticles increase (pH=7.58±0.13) in comparison with empty nanoparticles (5.53±0.34). Considering the basic character of tobramycin, and the increasing of pH in the suspension of nanoparticles loaded with tobramycin was expected the observation of the increase of zeta potential due to the more negative charged medium. We hypothesize that tobramycin may be binding to the surface of the particle, conjugated with the stearic acid. Tobramycin is composed by 5 primary amino groups that could be binding to the carboxyl groups of fatty acids.

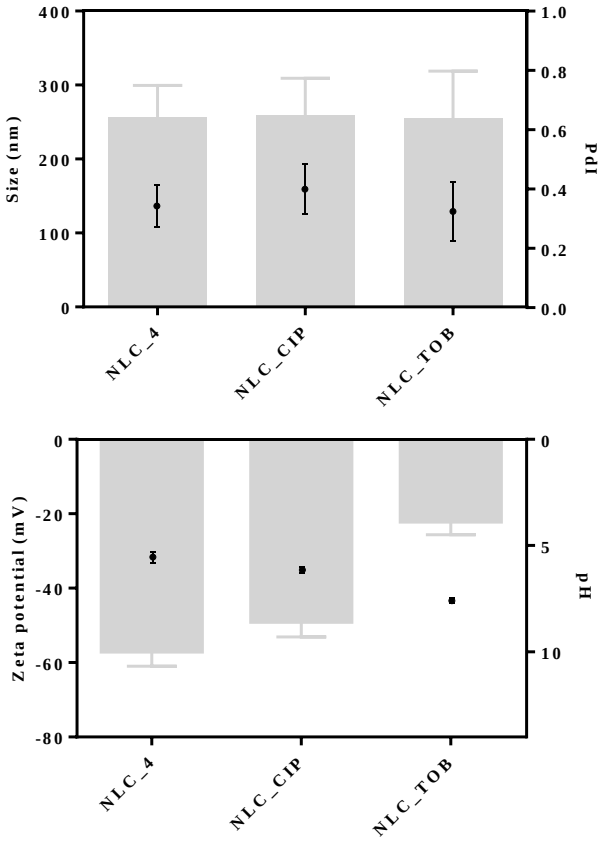

Figure 3: Average size (vertical bars), PdI (black dots), zeta potential (vertical bars) and pH (black dots) of nanoparticles loaded with ciprofloxacin (formulations NLC_CIP), tobramycin (formulation NLC_TOB) and empty nanoparticles (formulation NLC_4).

Different concentrations of each antibiotic (0.25 mg/mL, 0.5 mg/mL and 1 mg/mL) were also incorporated in the nanoparticles. No significant variations were observed in the average size, PdI and zeta potential of nanoparticles, except the increase of the size of nanoparticles with 1 mg/mL of ciprofloxacin (data not shown).

Nanoparticles prepared from formulations NLC_4, NLC_CIP and NLC_TOB were stored at room temperature for two months and their size, PdI and zeta potential was assessed after one and two months of preparation. Results indicate that, in both formulations, nanoparticles are stable for at least 60 days. The nanoparticles average size slightly decreases in both nanoparticles formulations, due to the deposition of the nanoparticles. Zeta potential also decrease with time (data not shown).

For the nanoparticles loaded with tobramycin, a phase separation in the suspension was detected after one month (data not shown). Therefore, we conclude that these nanoparticles are not long-term stable, which is in agreement with the zeta potential measurements previously obtained (Figure 3).

Differential scanning calorimetry

Nanoparticles prepared without the sonication step (formulation NLC_1) were composed of a mixture of fatty acids (lauric acid and myristic acid). No melting event was observed for nanoparticles of this formulation. When the individual components were tested, the temperature of this mixture of fatty acids significantly decrease (data not shown). Myristic acid and Lauric acid have melting temperatures of 56.3ºC and 46.5ºC, respectively. When mixed, the melting temperature drops to 37.2ºC. Based on differential scanning calorimetry, Keles S. et al (2005), reported that the lauric acid and myristic acid are phase change materials that have a high melting point. However, their melting point can change when mixed, forming a eutectic mixture[30]. This phenomenon was also observed for other mixtures of fatty acids such as lauric acid and palmitic acid[31], stearic acid and lauric acid[32]. Thus, this formulation it's closer to a nanoemulsion than a nanostructured lipid carrier because is not solid nor at room temperature nor at body temperature.

Empty nanoparticles prepared using a step of sonication (formulations NLC_2 to NLC_7) as well as loaded nanoparticles (formulations NLC_CIP and NLC_TOB) were analysed by differential scanning calorimetry. The melting points obtained for these nanoparticles are summarized in table 2. No significant differences were observed for nanoparticles prepared with different surfactants (NLC_3 with Tween 80 and NLC_4 with Span 80) and with different oils (NLC_2 with coconut oil and NLC_4 with sunflower oil). Nanoparticles containing different fatty acids exhibited a trend towards the increase of melting temperatures with the length of the fatty acid chain, what is the expected due to the increase of melting temperature of the individual fatty acid. Nanoparticles of formulations NLC_5 and NL_6 are not solid at body temperature. A solution to this problem could be the increase of the amount of the solid lipid in the formulation and the decrease of oil content. Nanoparticles with both antibiotics (NLC_CIP and NLC_TOB) present a melting point similar to that of empty nanoparticles (formulation NLC_4).

Table 2: Melting point of nanoparticles in the formulations NLC_2 to NLC_7.

Formulation	Temperature (ºC)	SD
NLC_2	58.1	0.95
NLC_3	59.9	0.50
NLC_4	58.9	2.25
NLC_5	32.2	1.30
NLC_6	36.5	1.00
NLC_7	55.3	0.60
NLC_CIP	59.5	0.85
NLC_TOB	56.9	0.30

Transmission electron microscopy observation

The first TEM visualization of nanoparticles prepared with formulations NLC_4, NLC_CIP or NLC_TOB was not clear. The presence of some sort of film was observed masking the image and obstructing nanoparticles visualization (data not shown). This was presumably caused by the high lipophilicity of Span 80. To avoid this problem the nanoparticles were filtered and clearer images were obtained. Figure 4 presents the TEM images obtained for empty nanoparticles (A) and tobramycin loaded nanoparticles.

Empty nanoparticles (A) and nanoparticles with tobramycin (B) presented a spherical shape and an average size of 200 nm, this is consistent with the dynamic light scattering data (255.9±40.8nm and 255.3±63.5nm, respectively).

Nanoparticles loaded with ciprofloxacin (C) were also filtered. Despite, the obtained images being less clear that the previous ones, it is still possible to observe the nanoparticles with sizes ranging from 100 nm to 200 nm (data not shown). Again, these values are consistent with the DLS data (258±50.7nm).

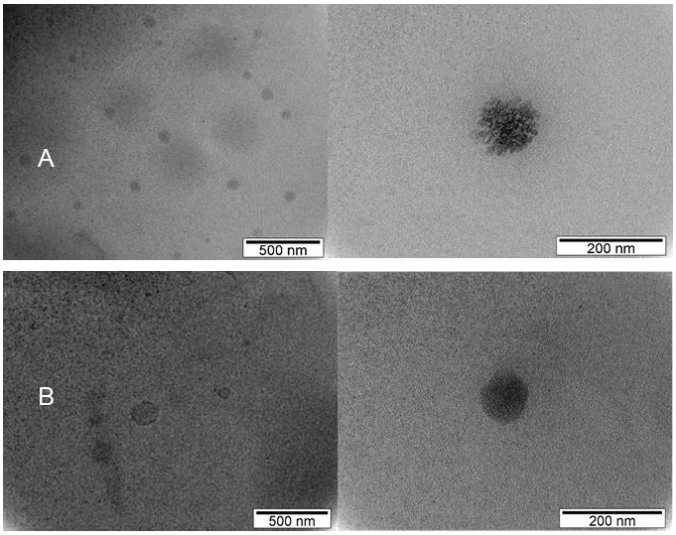

Figure 4: Transmission electron microscopy images of empty nanoparticles (A) and nanoparticles loaded with tobramycin (B).

Encapsulation efficiency and drug loading

Filtrates resulting from the filtration of tobramycin loaded nanoparticles had no absorbance (with a blank of water + fluorescamine) neither intensity of fluorescence (with a blank of phosphate buffer + fluorescamine), suggesting that there was no antibiotic in the filtrate. Results seems to show an unexpectedly high encapsulation efficiency of 100%. This may be possible due to the 5 primary amino groups that tobramycin presents, these can associate to carboxylic group of the stearic acid, improving the encapsulation yield. However more studies should be performed to determine what occur with this antibiotic and its quantification.

Encapsulation efficiency of ciprofloxacin loaded nanoparticles was calculated by the quantification of ciprofloxacin presented in the filtrate that was obtained with filtration of the nanoparticle suspension.

Encapsulation efficiencies of nanoparticles loaded with 0.25, 0.5 and 1mg/mL of ciprofloxacin (formulation NLC_CIP) was 68.16±4.9%, 67.26±8.27% and 64.11±9.99%, respectively while drug loading capacity was 0.79±0.10%, 1.84±0.03% and 4.46±0.44%, respectively. The amount of antibiotic added to the formulation was 5mg for 400 mg carrier to 20mg for 400mg carrier.

Dharmendra Jain et al.[10], obtained an EE% of 38.71%±2.38% to 8.66%±1.64% for SLNs formulations with microemulsion technique (25mg/100mg carrier to 100mg/100mg carrier). Ghaffari et all[33], obtained an EE% of 88±4.5% with emulsification-sonication method. Gamal A. Shazly[34] obtained for SLNs with stearic acid an EE of 73.94% with emulsification-sonication method.

The lipid type used for the preparation of lipid nanoparticles has a significative impact on the encapsulation efficiency and drug loading capacity of the nanoparticles. Other type of lipids could be used to optimize the maximum percentage of encapsulated drug. Also, additional studies should be performed to determine the maximum drug loading capacity of the lipid nanoparticles of these formulations, this is, the maximum amount of antibiotic that can be incorporated in nanoparticles.

Drug release profile of ciprofloxacin

Release studies were performed with dialysis tubing, that is a semi-permeable membrane which facilitated the exchange of molecules in solution. Nanoparticle suspension was added to the dialysis tubing and the exterior medium was composed of phosphate buffer. Samples of the medium were taken at different times and ciprofloxacin in the medium was quantified by HPLC. Release of free antibiotic was also measured as control. The release profile of ciprofloxacin from nanoparticles and free ciprofloxacin over time is represented in figure 5. The profile shows a burst release were all the drug is released in the first 7 hours (96% release at t=7h). Antibiotic in nanoparticles had a similar release profile compared to free antibiotic (95% release at t=3h). This result could suggest that the antibiotic is at surface of the nanoparticle.

Drug could be associated to the nanoparticles in three different states: at the nanoparticle surface, in the core as a reversible complex, or in the core as irreversible complex. Generally, drug release follows more than one type of mechanism. In case of release from the surface, drug adsorbed on the surface of nanoparticles dissolves instantaneously when in contact with the release medium. The early phase of the release corresponds to the release of drugs physically bound to the surface of the nanoparticles and the delayed phase of the release of entrapped drug due to diffusion of drug from the rigid matrix structure[10]. The formulation of this assay had an encapsulation efficiency of 69.62% and free antibiotic was not separated from the nanoparticles. Other possibility could be the washing of nanoparticles for more accurate results.

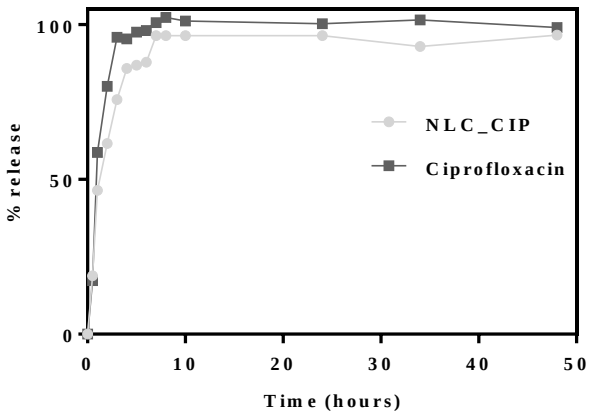

Figure 5: Release profile of ciprofloxacin from nanoparticles (Formulation NLC_CIP) and free ciprofloxacin over 48h.

Dharmendra Jain et al.[10], produced five different types of nanoparticles of albumin, gelatine, chitosan, and solid lipid nanoparticles with ciprofloxacin. In their results chitosan and gelatin nanoparticles can release the drug for as long as 96 h, whereas drug release through SLN was observed for up to 80 h. On the other hand, free ciprofloxacin showed a burst release with almost 50% of free drug release in 30 min and more than 90% drug diffusing in 70 min[10]. Their results suggest that SLNs can act promising carriers for sustained ciprofloxacin release. Gamal A. Shazly[34], produced SLNs with different lipids and nanoparticles formulated with only stearic acid as lipid component displayed the strongest burst effect and the most rapid released. They proposed that this could be due to the fast dissolution of ciprofloxacin molecules existing in the surface layer of the SLNs. Ghaffari et al.[33] also obtained a similar release of ciprofloxacin encapsulated in SNL with a significant burst effect.

Assessment of nanoparticle ingestion by *C. elegans*

To determine if worms did ingest or not the nanoparticles, a fluorescent dye was incorporated into the nanoparticles. The dye was encapsulated together with each antibiotic, ciprofloxacin or tobramycin. Figure 6 shows the microscopic images of *C.*

elegans after 3h of nanoparticle exposure. Green Light corresponds to nanoparticles loaded with the fluorescent Dye. Is possible observe that worms ingested the nanoparticles and the fluorescence is observed along the digestive system of the worms. Remarkable we have observed that only a few worms were fluorescent when tobramycin-containing nanoparticles were used, opposite to the observed for ciprofloxacin.

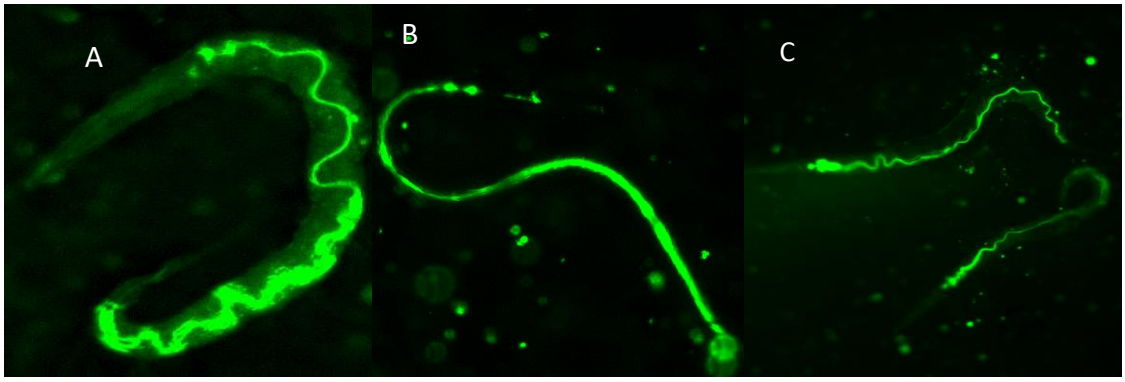

Figure 6: Microscopy images of *C. elegans* fed with nanoparticles containing fluorescent dye and the antibiotics ciprofloxacin or tobramycin. A- nanoparticles loaded with Dio; B- nanoparticles loaded with ciprofloxacin and Dio; C- nanoparticles loaded with tobramycin and Dio.

Nanoparticle efficacy assessment

We have compared the survival percentage of worms in non-pathogenic *E. coli* OP50 in the presence of nanoparticles with ciprofloxacin and empty nanoparticles. Control experiments with no nanoparticles were also carried out. Results are shown in figure 7.

Concerning the survival of worms in *E. coli* OP50 (Figure 7), some mortality occurred among the worms exposed to empty nanoparticles (29.7%) and nanoparticles with ciprofloxacin (28.92%). The mortality of worms exposed to empty nanoparticles or to nanoparticles containing ciprofloxacin is not significantly distinct. However, results obtained with each formulation tested is significant different for those obtained with no nanoparticles (P<0.0001). Altogether these results indicate that lipid nanoparticles present some toxicity to the worms.

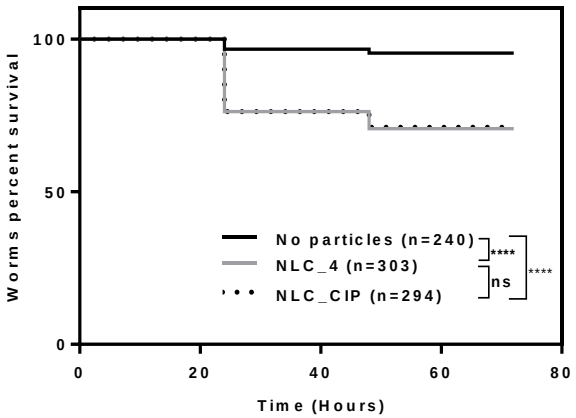

Figure 7: Percentage survival of worms in the absence of nanoparticles or empty nanoparticles (formulation NLC_4) and nanoparticles with ciprofloxacin (formulation NLC_CIP) when fed with the non-pathogenic *E. coli* OP50. The survival curves were compared using the log-rank (Mantel-Cox) test and p-value is represented by * when P<0.05, **when P<0.01*** when P<0.001, **** when P<0.0001 or ns (not significant).

We have also assessed the survival percentage of infected worms with *B. contaminans* IST408 or *B. cenocepacia* K56-2 using the same conditions as those described above for *E. coli* OP50. Infected worms with *B. cenocepacia* strain K56-2 presented a mortality of 45.24% for the control with no nanoparticles, 38.71% in presence of empty nanoparticles and 36.87% in presence of nanoparticles with ciprofloxacin (data not shown). This small difference observed is not significant according to the statistic model using the log-rank (Mantel-Cox). In a study published by Cardona *et al*, in 2005, the percentage of survival of worms infected with *B. cenocepacia* k56-2 was 22% at day 2[35]. The difference of percentage compared with this assay is possibly due to different stages of the *C. elegans* used (L2 larval stage worms were used in this study while Cardona et al used L4 stage worms). After 3 days a visual inspection of the worms revelled that the infected worms were visibly smaller compared to those fed with *E. coli* OP50. Infected worms with *B. cenocepacia* k56-2 laid eggs after this period.

Similar experiments were carried out using worms infected with *B. contaminans* 408. Results obtained show that infected worms presented a mortality of 84.5% for control without nanoparticles, 87.8% for control with empty nanoparticles, and 77.5% for nanoparticles with the antibiotic ciprofloxacin, after 3 days. The difference observed in worms mortality in absence of nanoparticles or nanoparticles with ciprofloxacin is significantly different according to the statistic model using the log-rank (Mantel-Cox) (p-value=0.0410). The difference is more evident comparing the survival of worms in presence of empty nanoparticles or nanoparticles with the antibiotic (p-value=0.0018) (Figure 8).

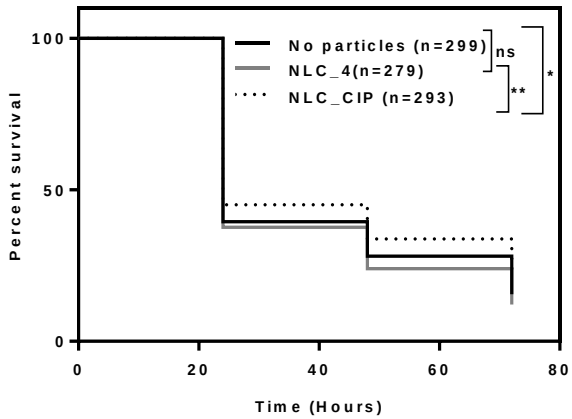

Figure 8: Percentage survival of *B. contaminans* IST408-ifected worms in the absence of nanoparticles or presence of empty nanoparticles (formulation NLC_4) and nanoparticles with ciprofloxacin (NLC_CIP). The survival curves were compared using the log-rank (Mantel-Cox) test and p-value is represented by * when P<0.05, **when P<0.01*** when P<0.001, **** when P<0.0001 or ns (not significant).

The percentage of mortality of *C. elegans* by *B. contaminans* strain 408 was previous reported by Sousa *et al* (2010) (80%); The differences in the survival rates might be due to the fact that the strain of *C. elegans* used by those authors, DH2, was different from this study, BN2[36].

Worms were visibly infected in the 3 conditions, being considerable smaller than those fed with *E. coli* OP50.

C. elegans as animal model, presents many advantages, among them, oral absorption is the main route of drug administration in worms. Thus, in this study, worms were used as *in vivo* animal model for evaluate the oral absorption as well as toxicity and efficacy of lipid nanoparticles. Despite nanoparticles presented some toxicity for *C. elegans*, components used such as fatty acids (lauric acid, myristic acid, palmitic acid and stearic acid) according to FDA are direct food substances affirmed as generally recognized as safe. Span 80 and Tween 80 are considered a food additive permitted for direct addition to food for human consumption[37].

Conclusions and Future work

The first part of this work consisted on the design and optimization of a nanostructured lipid carrier formulation. A final formulation (NLC_4) was successfully prepared by an emulsification-sonication technique. These formulations were composed of stearic acid, sunflower oil, Span 80 and milli-Q water and the nanoparticles obtained exhibited an average size of 255.9±40.8nm, PdI of 0.342±0.06 and zeta potential of -56.0±3.72 mV. The sonication procedure results in some disadvantages such as difficulty in scale up and metal contamination coming from the tip.

Nanoparticles with ciprofloxacin and tobramycin presented an average size and PdI similar to those of empty nanoparticles. However, the zeta potential of nanoparticles with tobramycin was much lower suggesting, that these nanoparticles are less long-term stable. Stability studies proved that the nanoparticles with ciprofloxacin were stable up to two months in opposition to the observed for tobramycin loaded nanoparticles where the emulsion had break and two phases were visible after one month. Lyophilization and spray drying are good examples of alternatives for lipid nanoparticles storage for long periods of time, preventing degradation reactions such as hydrolysis, allowing the maintenance of the initial nanoparticle size.

Transmission electron microscopy provided images of nanoparticles with spherical shape and with a size of approximately 200 nm.

A burst release of ciprofloxacin from the nanoparticles was verified (all the drug was released in the first 7hours), similar to free antibiotic. This suggests that antibiotic is at the surface of the lipid nanoparticle. Encapsulation efficiency of these nanoparticles was between 68.16±4.9% and 64.11±9.99% and drug loading between 0.79±0.10% to 4.46±0.44%. The type of lipids, oils and surfactants used for the preparation of lipid nanoparticles has a significative impact on the encapsulation efficiency and drug loading capacity of the formulations. Other type of formulations could be used to optimize the maximum percentage of encapsulated drug.

Thermal analysis showed a melting temperature of 58.9ºC±2.25ºC, 59.5±0.05ºC and 56.9±0.30ºC for the nanoparticles of formulations NLC_4, NLC_CIP and NLC_TOB, respectively. These nanoparticles are solid at room temperature as well as at body temperature.

Caenorhabditis elegans was used as an animal model of infection and pathogens belonging to the Bcc group were used as model pathogens (*Burkholderia contaminans* IST408 and *Burkholderia cepacia* K56-2). In the first strain, significant differences were observed between nanoparticles with ciprofloxacin or the control without nanoparticles (p-value<0.05) and between empty nanoparticles or nanoparticles with the antibiotic (p-value<0.01). In the case of *B. cenocepacia* K56-2 no significant difference was observed in the rescue experiments without nanoparticles and antibiotic loaded nanoparticles.

Fluorescence microscopy confirmed the uptake of the lipid nanoparticles by the nematode.

Sterilization of the nanoparticles is necessary since these nanoparticles were designed for medical application, more specifically for oral administration. Some processes that could be tested include filtration, autoclaving or γ-radiation. Further studies need to be performed to ensure that nor the antibiotic or lipid nanoparticle integrity are affected by the sterilization.

Antibacterial activity of the nanoparticles is another procedure that should be assessed.

Formulations also could be tested in other microorganisms such as *Pseudomonas aeruginosa*.

In conclusion, ciprofloxacin was successfully encapsulated in nanostructured lipid carriers and their efficacy was successfully tested in *C. elegans.*

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