International Journal of Pharmaceutics 519 (2017) 128–137

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Docosahexaenoic acid loaded lipid nanoparticles with bactericidal activity against *Helicobacter pylori*

Catarina Leal Seabra[a,b,c,d], Cláudia Nunes[e], Maria Gomez-Lazaro[a,b], Marta Correia[f], José Carlos Machado[a,c,g], Inês C. Gonçalves[a,b], Celso A. Reis[a,c,d,g], Salette Reis[e], M. Cristina L. Martins[a,b,c,*]

[a] *i3S—Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal*
[b] *INEB—Instituto de Engenharia Biomédica, Universidade do Porto, Portugal*
[c] *IPATIMUP—Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal*
[d] *ICBAS—Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal*
[e] *UCIBIO, REQUIMTE, Laboratório de Química Aplicada, Faculdade de Farmácia, Universidade do Porto, Portugal*
[f] *Escola da Saúde, Universidade do Algarve, Portugal*
[g] *Faculdade de Medicina, Universidade do Porto, Portugal*

ARTICLE INFO

Article history:
Received 9 November 2016
Received in revised form 4 January 2017
Accepted 6 January 2017
Available online 11 January 2017

Keywords:
Docosahexaenoic acid (DHA)
Helicobacter pylori
Nanostructured lipid carriers (NLC)
Polyunsaturated fatty acid (PUFA)

ABSTRACT

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid present in fish oil, has been described as a promising molecule to the treatment of *Helicobacter pylori* gastric infection. However, due to its highly unsaturated structure, DHA can be easily oxidized loosing part of its bioactivity. This work aims the nanoencapsulation of DHA to improve its bactericidal efficacy against *H. pylori*.

DHA was loaded into nanostructured lipid carriers (NLC) produced by hot homogenization and ultrasonication using a blend of lipids (Precirol ATO5®, Miglyol-812®) and a surfactant (Tween 60®). Homogeneous NLC with 302 ± 14 nm diameter, −28 ± 3 mV surface charge (dynamic and electrophoretic light scattering) and containing 66 ± 7% DHA (UV/VIS spectroscopy) were successfully produced.

Bacterial growth curves, performed over 24 h in the presence of different DHA concentrations (free or loaded into NLC), demonstrated that nanoencapsulation enhanced DHA bactericidal effect, since DHA-loaded NLC were able to inhibit *H. pylori* growth in a much lower concentrations (25 μM) than free DHA (>100 μM).

Bioimaging studies, using scanning and transmission electron microscopy and also imaging flow cytometry, demonstrated that DHA-loaded NLC interact with *H. pylori* membrane, increasing their periplasmic space and disrupting membrane and allowing the leakage of cytoplasmic content. Furthermore, the developed nanoparticles are not cytotoxic to human gastric adenocarcinoma cells at bactericidal concentrations. DHA-loaded NLC should, therefore, be envisaged as an alternative to the current treatments for *H. pylori* infection.

1. Introduction

Gastric carcinoma, the third leading cause of cancer-related deaths worldwide (AmericanCancerSociety, 2015), has been associated with persistent *Helicobacter pylori* (*H. pylori*) gastric infection. This bacterium, which colonizes the gastric mucosa of over half of world population, is also responsible for the development of gastritis and peptic ulcer (Correa and Houghton, 2007; Matysiak-Budnik and Megraud, 2006; Pinho et al., 2013). It has been estimated that approximately 75% of the global gastric cancer burden is attributable to *H. pylori* infection (Amieva and Peek, 2016). Therefore, *H. pylori* eradication is the most promising strategy to avoid *H. pylori*-related gastrointestinal disorders. The present recommended treatment is a combination of antibiotics (Goncalves et al., 2014; Lopes et al., 2014; Malfertheiner et al., 2012; Parreira et al., 2016), however, this therapy may fail due to several reasons but principally by bacterial resistance to available antibiotics (Vakil, 2006).

* Corresponding author at: i3S—Instituto de Investigação e Inovação em Saúde, INEB—Instituto de Engenharia Biomédica, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal.
E-mail address: cmartins@ineb.up.pt (M. C.L. Martins).

http://dx.doi.org/10.1016/j.ijpharm.2017.01.014
0378-5173/

The use of natural compounds with *H. pylori* bactericidal effect, namely polyunsaturated fatty acids (Sun et al., 2003; Thompson et al., 1994) and phenolic compounds (Brown et al., 2009; Paulo et al., 2011; Romero et al., 2007) has been explored as an alternative to conventional antibiotics (Goncalves et al., 2014). Fatty acids are attractive compounds due to their potency, a broad spectrum of activity and lack of typical resistance mechanisms against the actions of these compounds (Desbois, 2012; Desbois and Lawlor, 2013; Desbois and Smith, 2010). Particularly, polyunsaturated fatty acids (PUFAS) found naturally at high levels in many marine organisms, have been reported as having a high potent activity against *H. pylori*, including linolenic acid (C18:3) (Sun et al., 2003), eicosapentaenoic acid (C20:5) (Meier et al., 2001), and docosahexaenoic acid (DHA) (C22:6) (Correia et al., 2012). Indeed, it was previously reported that some PUFAS, as the linolenic acid, can inhibit up to 50% of bacteria growth at 1 mM concentration (Petschow et al., 1996), whereas 2 mM kills all bacteria (Petschow et al., 1996; Thompson et al., 1994). This antibacterial activity of PUFAs is dependent on many factors, including concentration, the target microorganism and its physiological state, the physiological conditions associated with delivery (e.g., pH and temperature) and interaction with the microbial cell (Desbois, 2012).

DHA, an omega-3 polyunsaturated fatty acid present in fish oil, was able to inhibit *H. pylori* (26695, B128 and SS1) growth *in vitro* in a dose-dependent manner. Moreover, a study using gastric infected mice, demonstrated that free DHA treatment was able to decrease 50% of *H. pylori* gastric colonization (Correia et al., 2012, 2013). As a result of its highly unsaturated structure, DHA can be easily oxidized losing its bioactivity. Therefore DHA encapsulation is recommended for its protection against environmental factors (oxygen, light, humidity, gastric acid, etc.) (Correia et al., 2012; Desbois and Smith, 2010; Taneja and Singh, 2012).

The present work aims to evaluate if the encapsulation of DHA into nanostructured lipid carriers (NLC) can improve DHA activity against *H. pylori in vitro*. NLC are delivery systems suitable for the encapsulation of poorly water-soluble drugs. They are prepared by blending biocompatible and biodegradable solid and liquid lipids, stabilized in aqueous suspensions by hydrophilic surfactants. The advantage of NLC regarding other lipid nanoparticles, namely solid lipid nanoparticles (SLN), is their higher drug loading capacity, improvement of drug stability and controlled release. This is attributed to their partial-crystallized structure with imperfections in the core solid matrix that provides higher space for drug incorporation (Beloqui et al., 2016; Poonia et al., 2016; Tamjidi et al., 2013).

2. Materials & methods

2.1. DHA nanoencapsulation and characterization

2.1.1. NLC preparation

NLC were produced by hot homogenization and ultrasonication (Neves et al., 2013) using 200 mg of Precirol ATO5® (Gattefosé, France), 90 mg of Miglyol-812® (Acofarma, Spain) and 60 mg of Tween 60® (Merck, Germany). Briefly, the two lipids (Precirol ATO5® and Miglyol-812®) and Tween60® were weighted and heated together at 65 °C to promote their mixture. Ultrapure water (4.2 mL) preheated at 65 °C was added, and the mixture homogenized using an ultra-turrax (T25; Janke and Kunkel IKA-Labortechnik, Germany) under high speed stirring at 12000 rpm during 20 s. Afterward, a sonicator was used (Vibra-Cell model VCX 130 equipped with a VC 18 probe, Sonics and Materials Inc., Newtown, USA), with a tip diameter of $^1/_4$" (6 mm), at 60% amplitude for 5 min obtaining the NLC. For the production of DHA-loaded NLC, DHA (Cayman Chemical Company, USA) was added in different concentrations (1.0, 2.0 and 2.5% v/v) before addition of hot ultrapure water, obtaining three different formulations.

2.1.2. NLC size and charge measurement

The size and surface charge (ξ-potential) of the produced NLC were characterized by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) respectively, using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Diluted NLC (1:50 in ultrapure water) were placed on a disposable capillary cell and triplicate measurements were conducted at a backscattering angle of 173° at 37 °C. DLS and ELS were acquired using equipment maintained by Biointerface and Nanotechnology Unit.

2.1.3. NLC morphology assessment

The NLC morphology was evaluated by Cryo-Scanning Electron Microscopy (CryoSEM) and NanoSight®.

For CryoSEM analysis, it was used a JEOL JSM-6301F (Tokyo, Japan) with an Oxford Instruments INCA Energy 350 (Abingdon, UK) and a Gatan Alto 2500 (Pleasanton, CA, USA). A diluted sample of NLC (1:100 in ultrapure water) was placed on a grid, rapidly cooled in liquid nitrogen slush (−210 °C), and transferred under vacuum to the cold stage of the preparation chamber. A fracture and sublimation for 120 s at −90 °C was then performed and the sample coated with a gold-palladium alloy by sputtering for 40 s. Finally, the sample was transferred under vacuum into the SEM chamber and observed at −150 °C, at CEMUP (*Centro de Materiais da Universidade do Porto*).

For NanoSight® analysis, NLC were diluted (1:20000) with ultrapure water and morphology was evaluated using a NanoSight NS300 (Malvern Instruments, UK).

2.1.4. DHA entrapment efficiency

DHA nanoencapsulation was quantified by UV/VIS-spectroscopy. Freeze-dried NLC were dissolved in absolute ethanol for 15 min, centrifuged during 30 min at 4000 rpm, at 20 °C and its supernatant analyzed by measuring its absorbance using a UV/VIS spectrophotometer (Lambda 45, Perkin Elmer, USA) at 237 nm. All measurements were performed in triplicate.

The DHA loading was calculated using a calibration curve prepared with a concentration range between 0.01 and 3% v/v of DHA in absolute ethanol (0.0761–22.8 mM), with a correlation coefficient of R = 0.996. The amount of DHA in NLC and the entrapment efficiency (EE) were calculated according to the following equation (Eq. (1)).

$$EE = \frac{\text{DHA measured (mM)}}{\text{DHA added during NLC production (mM)}} \times 100 \quad (1)$$

2.1.5. NLC stability

2.1.5.1. In water. The storage of NLC was evaluated in ultrapure water at 4 °C and 20 °C during 2 months by periodic measurements of their size and surface charge using the DLS and ELS techniques as described above. Measurements were performed at 4° and 20 °C and in triplicate.

2.1.5.2. In bacteria growth medium. NLC were incubated in Brucella Broth medium (BB, Oxoid, France) supplemented with 10% of Fetal Bovine Serum (FBS, Gibco, USA) (BB + 10%FBS medium) during 3 h at 37 °C and 150 rpm. At time-point 0 and 3 h, samples were diluted (1:20000) ultrapure water and analyzed using the NanoSight NS300 (Malvern Instruments, UK).