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Integrated Master in Bioengineering

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Lipid-based nanocarriers for food applications: strategies to maximize bioactive compounds' bioavailability and their safety evaluation

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Resumo

A vitamina A desempenha um papel importante na biologia humana, nomeadamente na visão, diferenciação celular e proteção da pele e mucosas. Logo, o objetivo deste trabalho foi desenvolver nanopartículas lipídicas sólidas (SLNs) e transportadores lipídicos nanoestruturados (NLCs) - contendo vitamina A para fortificação alimentar.

Inicialmente, dois lípidos sólidos (ácido esteárico e Gelucire® 43/01) e dois líquidos (ácido oleico e miglyol® 812) foram testados como componentes das nanopartículas (NPs). As melhores taxas de encapsulação (cerca de 80%) obtidas para SLNs de Gelucire® 43/01 e NLCs de Gelucire® 43/01 e miglyol levaram à escolha desses lípidos. As SLNs apresentaram um tamanho aproximado de 190 nm, enquanto os NLCs de 270 nm. Ambos apresentaram índices de polidispersão entre 0,1 e 0,2 e potenciais zeta de -20 a -30 mV. Por microscopia eletrónica de transmissão confirmou-se a forma esférica das NPs e que a adição de vitamina não induziu alterações. Para melhorar a estabilidade da vitamina A nestas NPs em suspensão aquosa, adicionou-se α-tocoferol, obtendo-se então NPs estáveis por um mês.

As NPs também foram testadas sob várias condições presentes em produtos alimentícios: presença de NaCl, sacarose, pH 5 e temperaturas de 60 e 70 °C. Após 24 h, as NPs não sofrem alterações em termos de tamanho ou conteúdo vitamínico. A suplementação de limonada, escolhida como matriz alimentar modelo, com NPs também foi testada; o tamanho permaneceu estável e 70% (1.7 mg) da vitamina adicionada é preservada.

Estudos de viabilidade celular em linhas de fibroblastos mostraram que a vitamina A não encapsulada é tóxica a 50 µg/mL, mas nas NPs não apresenta toxicidade até 100 µg/mL. Ensaios preliminares de permeação indicam uma possível taxa de permeação intestinal de 25% (equivalente a 38 de 152 µg de vitamina A) para as SLNs ao fim de 4 h.

Em ensaios de digestão gastrointestinal *in vitro*, verificou-se que as NPs não sofrem alterações no estômago, sendo apenas digeridas no intestino, onde poderá ocorrer libertação da vitamina A permitindo a sua absorção.

Em conclusão, produziram-se NPs lipídicas capazes de encapsular vitamina A, estáveis sob diferentes condições e não citotóxicas. Deste modo, parecem ser adequadas para aplicação em produtos alimentares para fortificação com esta vitamina.

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Abstract

Vitamin A plays a major role in human biology, namely in vision, cell differentiation and skin/mucosa protection. Thus, the goal of this work was to develop solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) – containing vitamin A for food fortification.

Firstly, two solid lipids (stearic acid and Gelucire® 43/01) and two liquid lipids (oleic acid and miglyol) were tested as possible components of these nanoparticles (NPs). The better encapsulation rates (approximately 80%) for SLNs made of Gelucire® 43/01 and NLCs made of Gelucire® 43/01 and miglyol led to the selection of these lipids for subsequent studies. SLNs revealed an approximate size of 190 nm, while for NLCs a diameter of 270 nm was found. Both types of NPs showed polydispersity indexes in the 0.1-0.2 range and zeta potentials of -20 to -30 mV. Transmission electron microscopy confirmed the spherical shape of SLNs and NLCs and that the addition of vitamin A did not induce any morphological changes. To improve the stability of NPs in aqueous suspension, α-tocopherol was added, resulting in stable NPs both at room temperature and at 4 °C for a month.

SLNs and NLCs were also added to different media in order to simulate several conditions used in the preparation/processing of food products: solutions of NaCl, sucrose, pH 5 and temperatures of 60 and 70 °C. After 24 h, the particles suffered no changes in their size or vitamin content. Supplementation of lemonade, a model food matrix, with SLNs or NLCs was also tested; the size remained stable and 70% (1.7 mg) of the added vitamin is preserved.

The biocompatibility of the formulations was subsequently assessed using fibroblasts. While non-encapsulated vitamin A evidenced toxicity at 50 μg/mL, NPs showed no toxicity up to 100 μg/mL. Preliminary permeation studies further indicate that there might be a 25% (equivalent to 38 out of 152 μg of vitamin A) permeation rate for SLNs at the intestine level.

In vitro assays simulating gastrointestinal digestion were also performed. Results showed that the NPs are not altered in the stomach, indicating that they could reach the intestine unaltered, where they can possibly be digested allowing vitamin release for its absorption.

In conclusion, we were able to produce lipid nanoparticles capable of entrapping vitamin A, stable under different conditions and non-cytotoxic. This leads us to believe that they are suitable for application in food products requiring fortification with this vitamin.

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Faithless is he that says farewell when the road darkens.

Gimli (J.R.R. Tolkien, in *Lord of The Rings – The Fellowship of the Ring*)

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First of all, I would like to express my endless gratitude to my supervisors Prof. Salette Reis and Dr. Sofia Lima for the opportunity to develop this work and for all the assistance, guidance and availability throughout this project. I also want to thank Dr. Isabel Amaral for her guidance and comprehension throughout the project.

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Table of Contents

Resumo iii

Abstract v

Acknowledgments ix

Table of Contents xi

List of Figures xv

List of tables xix

List of abbreviations xxi

1. Introduction 1

1.1. Vitamin A 1

1.1.1. Background 1

1.1.2. Biological functions 2

1.1.3. Recommended intake doses, dietary sources and vitamin A deficiency 4

1.1.4. Vitamin A absorption 5

1.2. Nanotechnology 6

2. State of the Art 7

2.1. General aspects of encapsulation 7

2.1.1. Liposomes and micelles 8

2.1.2. Solid Lipid Nanoparticles 8

2.1.3. Nanostructured Lipid Carriers 9

2.1.4. SLNs and NLCs production 10

2.2. Fortification of food with vitamin A 11

2.2.1. Cooking oil 11

2.2.2. Margarine 12

2.2.3. Fluid milk 12

2.2.4. Flours and cereals 12
2.2.5. Golden rice 12
2.2.6. Other foods 13
2.3. Nanotechnology applied to food fortification 13
2.4. Nanoencapsulation of vitamin A 14
3. Rationale of the Project 17
4. Materials and Methods 19
4.1. Materials 19
4.2. Methods 19
4.2.1. Lipid solubility tests 19
4.2.2. SLNs production 20
4.2.3. NLCs production 20
4.2.4. Physicochemical characterization of NP formulations 21
4.2.5. Stability studies 25
4.2.6. Lyophilization 25
4.2.7. Resistance to heat treatments 26
4.2.8. NPs interaction with food components 26
4.2.9. Cellular studies 26
4.2.10. *In vitro* stomach digestion 28
4.2.11. *In vitro* intestine digestion 28
4.2.12. Statistical analysis 29
5. Results and Discussion 31
5.1. RA solubility in lipids 31
5.2. NPs characterization 32
5.3. Stability studies 34
5.4. Lyophilization 37
5.5. Heat treatments 38

5.6. NPs interaction with food components 39
5.7. Cellular studies 41
5.7.1. Biosafety evaluation 41
5.7.2. Permeation across Caco-2 monolayers 42
5.8. *In vitro* stomach digestion 44
5.9. *In vitro* intestine digestion 44
Conclusions 47
Future Work 49
References 51
Annexes ..... A
Annex 1 – MTT Assay for Caco-2 cells ..... A

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List of Figures

Figure 1.1 - Structure of some important retinoids and β-carotene: a) all-trans-retinol; b) all-trans-retinoic acid; c) 3,4-Didehydroretinol; d) all-trans-retinal; e) retinyl acetate; f)11-cis-retinal; g) β-carotene. Sources: [5, 6]. 2

Figure 1.2 - Vision cycle. Adapted from [10, 11]. 3

Figure 1.3 - Vitamin A and β-carotene intestinal absorption. Adapted from [6] 6

Figure 2.1 - Some examples of NPs possible morphologies, for nanocapsules: A - simple, B - irregular, C - multiwall, and for nanospheres: D - multi-core, E - aggregate, F - matrix. Adapted from [31]. 7

Figure 2.2 - Three models for NPs: homogenous matrix (left), drug-enriched shell (middle) and drug-enriched core (right). Adapted from [36]. 8

Figure 2.3 - Schematic representation of liposomes and micelles (with transversal section). Adapted from [38] 8

Figure 2.4 - Schematic representation of SLNs structure. Adapted from [41] 9

Figure 2.5 - Benefits and drawbacks of SLNs. Sources: [37, 40, 43] 9

Figure 2.6 - Structural differences between crystalline SLNs (left) and amorphous NLCs (right). Adapted from [44]. 10

Figure 2.7 - Three types of NLCs: type I, imperfect (left); type II, amorphous (middle); and type III, multiple (right). Adapted from [45]. 10

Figure 2.8 - Rationale behind application of nanotechnology to food products. 13

Figure 4.1 – Schematic representation of lipid nanoparticles production method. 21

Figure 4.2 - Schematic representation of the electrical double layer and meaning of zeta potential. Adapted from [44]. 22

Figure 4.3 - Spectra of vitamin A solutions in ethanol 70%. The colours refer to the RA concentration in μg/mL 23

Figure 4.4 - Calibration curve of RA in 70%(v/v) ethanol/water. 23

Figure 5.1 - Appearance of the synthetized Gelucire nanoformulations: A - SLNs with RA; B - SLNs without RA; C - NLCs with RA; D - NLCs without RA. 33

Figure 5.2 - TEM images of the NPs in aqueous suspension. A, RA-loaded SLNs; B, SLNs without RA; C, RA-loaded NLCs; D, NLCs without RA. 34

Figure 5.3 - Stability of RA-loaded NPs without tocopherol. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: blue – SLN_0s, orange – NLC_0s. There are statistically significant differences for formulations after 2 weeks ($p<0.05$ vs. initial) for all zeta potentials and EE values and for size and PI of NPs stored at 4 °C. 35

Figure 5.4 - Stability of NPs without tocopherol and RA. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: green – SLN_0s, red – NLC_0s. There are statistically significant differences ($p<0.05$) for size and PI of NPs stored at 4 °C. 35

Figure 5.5 - Stability of RA-loaded tocopherol NPs over the period of 1 month. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: blue –SLNs; orange –NLCs. There are statistically significant differences ($p<0.05$) for size and PI of NPs stored at 4 °C. 36

Figure 5.6 - Stability of tocopherol NPs without RA over the period of 1 month. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: green –SLNs; red –NLCs. There are statistically significant differences ($p<0.05$) for size and PI of NPs stored at 4 °C. 36

Figure 5.7 - Examples of formulations after resuspension in water. It is possible to observe a phase separation - it is not possible to resuspend the lyophilized formulations. 37

Figure 5.8 -Evaluation of size, PI and vitamin content of particles subjected to heat treatments: on the top row, particles treated at 60 ºC; on the bottom row, particles treated at 70 ºC. The blue bars refer to the original (non-heated) particles, the orange bars to NPs heated for 5 minutes and the grey bars to NPs heated for 15 minutes. No significant changes were found for heated NPs compared to the respective original formulations (for $p<0.05$). 38

Figure 5.9 - Variations in size, PI and RA encapsulation of particles submerged in different media: in orange, NLCs; in blue, SLNs (n=3). *, significantly different ($p\leq0.05$) when compared to the original SLN; •, significantly different ($p\leq0.05$) when compared to the original NLC. 39

Figure 5.10 - Variations in size, PI and RA encapsulation of particles added to lemonade: in orange, NLCs; in blue, SLNs (n=3). *, significantly different ($p\leq0.05$) when compared to the original SLN; •, significantly different ($p\leq0.05$) when compared to the original NLC. 40

Figure 5.11 - Comparison of lemonade, A, and lemonade supplemented with NPs (NLCs), B. 41

Figure 5.12 – Results for cellular viability essays, for RA-loaded and unloaded SLNs and NLCs (n=4). Cellular viability was normalized using the viability of the positive control..... 41

Figure 5.13 - Vitamin content, size and PI of NPs in stomach-simulating media. The blue bars refer to the original particles, the orange bars to the particles at the timepoint zero and the grey bars to the particles after 2 hours in that media..... 44

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List of tables

Table 1.1 - Recommended dietary allowance and upper intake levels for vitamin A intake, as RAE.....5

Table 2.1 - Examples of nanoencapsulation of vitamin A (and pro-vitamin A) compounds. Research made in May 2018. 15

Table 4.1 - Calibration curve parameters. 24

Table 4.2 - Accuracy and precision for the calibration curve 24

Table 5.1 - RA solubility in different lipids. 31

Table 5.2 - Physicochemical characterization of the produced NPs: size, zeta potential, EE and VL. 32

Table 5.3 - Characterization of α-tocopherol NPs (n=3). 36

Table 5.4 - TEER values at the beginning of the experiment (t_0) and after 4 h (t_f), for n=2. 42

Table 5.5 – Size and PI of NPs recovered from apical and basolateral transwell compartments, at the beginning of the experiment (t_0) and after 4 h (t_f), for n=2. 43

Table 5.6 - Quantification of the non-permeated formulation ratio, based on the fluorescence detection of coumarin, at t_f (after 4h of experiment). 43

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List of abbreviations

CM	Chylomicrons
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EE	Encapsulation efficiency
FBS	Fetal bovine serum
GEL	Gelucire® 43/01
HLB	Hydrophilic-lipophilic balance
LOD	Limit of detection
LOQ	Limit of quantification
MIG	Miglyol® 812
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NLC	Nanostructured lipid carrier
NPs	Nanoparticles
OA	Oleic acid
PI	Polydispersity Index
r	Coefficient of correlation
r^2	Coefficient of determination
RA	Retinyl acetate
RAR	Retinoic acid receptors
RDA	Recommended Dietary Allowance
RE	Retinol esters
SA	Stearic acid
SLN	Solid lipid nanoparticles
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
UL	Upper intake levels
VL	Vitamin loading

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1.Introduction

Vitamins are a group of organic molecules that cannot be synthesized by the organism but are essential to life in very small amounts. This aspect differentiates them from inorganic minerals and macronutrients, which are required on a much larger scale [1]. By definition, a compound that can be synthesized by a living being in the necessary amount cannot be considered a vitamin; this means that ascorbic acid is considered vitamin C in human nutrition but should not be referred as a vitamin in case of other animals (able to properly synthesize it) [2].

Vitamins are present naturally in food and their absence or underutilization causes a specific deficiency syndrome. Their function in the organism is variable, but they do not serve structural purposes and their catabolism does not provide a significant amount of energy; instead, their applications are usually very specific and that is the reason they are needed only in small quantities [3].

Often, the term vitamin refers to a family of related compounds. In that case, each one will be referred to as a vitamer. When a substance can be metabolized to a biologically active form of a vitamin, not having that activity on its own, it is called a provitamin. Currently, thirteen substances (or group of substances) are recognized as vitamins, although a few more have been proposed as so [3]. Based on their hydrophilicity, vitamins can be divided into two major groups: fat-soluble and water-soluble vitamins.

1.1. Vitamin A

1.1.1. Background

At the end of the 19th and the beginning of the 20th centuries, several authors described a fat-soluble compound as essential to the diet (mainly in rodents). This was the first evidence of the existence of vitamin A and later on, Paul Kerrer investigated the structural chemistry of vitamin A and carotenoids [4].

Vitamin A-active compounds include both retinoids (vitamers, commonly grouped under the term “vitamin A”) and provitamin A carotenoids. The term retinoid refers to retinol,

retinaldehyde, retinoic acid and other natural and synthetic analogues, such as retinol esters, RE (Figure 1.1).

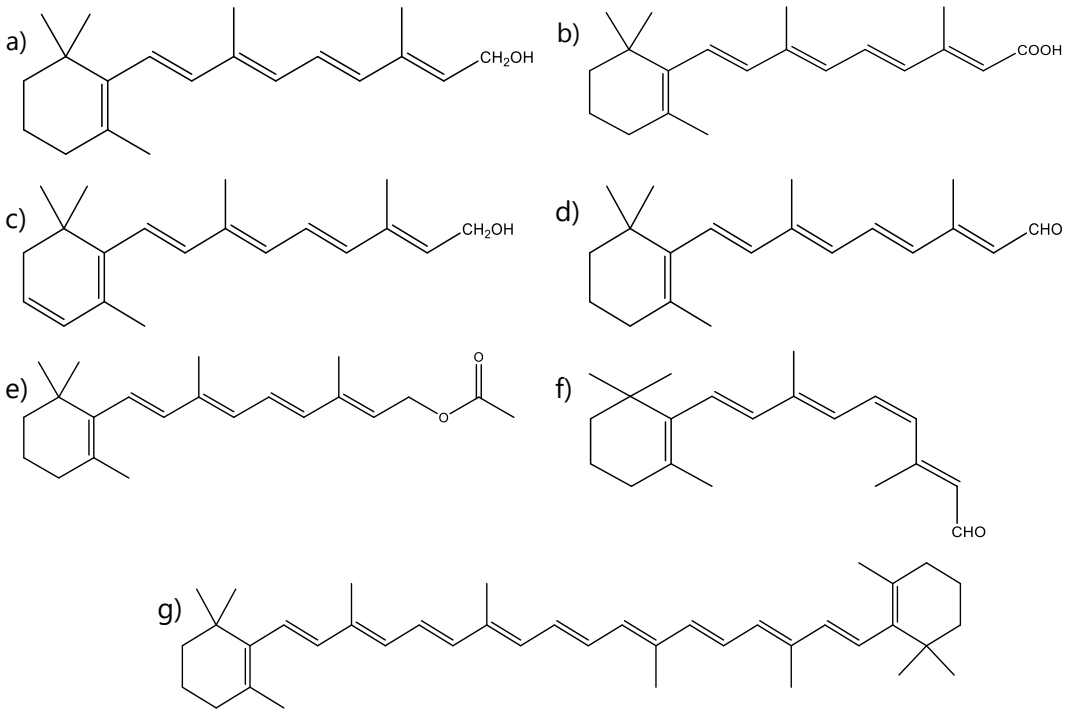

*Figure 1.1 - Structure of some important retinoids and β-carotene: a) all-*trans*-retinol; b) all-*trans*-retinoic acid; c) 3,4-Didehydroretinol; d) all-*trans*-retinal; e) retinyl acetate; f)11-*cis*-retinal; g) β-carotene. Sources: [5, 6].*

All-*trans*-retinol form has the highest vitamin activity. Nevertheless, the most common form of vitamin A in tissues is in RE (mainly palmitate, stearate and palmitate) [6]. Synthetic retinyl acetate (RA) and retinyl palmitate are often used in food processing to supplement the vitamin content of several products, such as oil and margarine [5]. Carotenoids are synthesized by some vegetables such as carrots or pumpkins [6].

Vitamin A is a fat-soluble vitamin and most of its forms have colour (retinoids are usually yellowish and β-carotene is red-brown to violet) [5]. It is quickly degraded by ultraviolet (UV) light and atmospheric oxygen [7]. However, under low oxygen stress, vitamin A and carotenoids are heat-resistant [8].

1.1.2. Biological functions

Vision

Retinoids are essential to vision processes. 11-*cis*-retinal is bound to a protein named opsin to form rhodopsin in rod cells (in the retina of the eye [9]). After absorption of light, retinal is freed from rhodopsin in its all-*trans* form. This species is then reduced to all-*trans*-retinol by membrane dehydrogenases. All-*trans*-retinol is transported to the retinal pigment

epithelium, where it is enzymatically converted to an all-*trans*-retinyl ester. The same enzyme, in complex with others, hydrolyses and isomerises the ester into 11-*cis*-retinol. This molecule is afterwards oxidized to 11-*cis*-retinal, needed in rod cells to regenerate rhodopsin [10, 11]. This cycle is known as vision cycle (Figure 1.2).

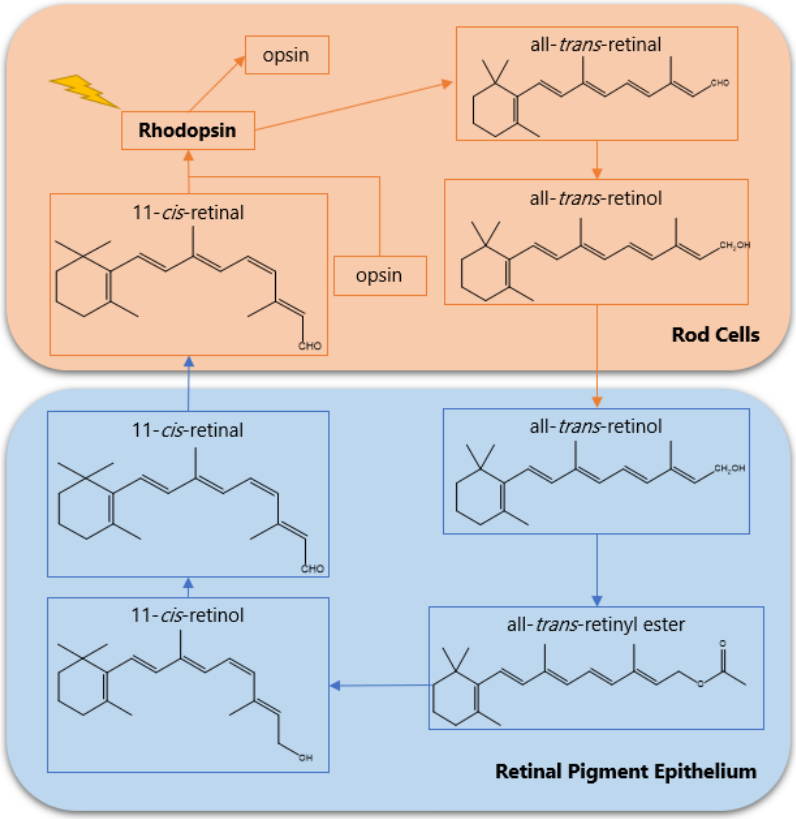

Figure 1.2 - Vision cycle. Adapted from [10, 11].

Immune response

All-*trans*-retinoic acid appears to be crucial in mucosal immune responses. Gut-associated dendritic cells transform retinol into retinoic acid and this participates in the generation of gut-tropic lymphocytes and IgA-antibody-secreting cells [12]. This form of vitamin A is linked to the development of specific receptors on lymphocytes that direct them towards the gut. Furthermore, all-*trans*-retinoic acid contributes to the differentiation of regulatory and effector T cells (a type of lymphocytes) [12].

Gene expression

Vitamin A plays a key role in gene expression. Retinoic acid receptors (RAR) and retinoid X receptors form a heterodimer that is bound to DNA (deoxyribonucleic acid). Retinoic acid can then bind to the RAR part of this dimer to cause a conformational change in the receptors,

causing the release of gene repressors. This allows the connection of gene activators that will loosen the chromatin structure and favour gene transcription. The mechanism of gene regulation is particularly important in Hox genes (genes that regulate the different types of vertebrae) [13] and genes codifying for extracellular matrix proteins [14].

Other documented functions

The role of vitamin A goes beyond the actions described above. Its importance is also documented in cell proliferation [15], reproduction [16], embryogenesis [17], neural differentiation [18] and pulmonary development [19]. Retinoids also have potential medical applications, including the treatment of acute promyelocytic leukaemia [20], thyroid carcinoma [21] and dermatological conditions [22].

1.1.3. Recommended intake doses, dietary sources and vitamin A deficiency

Different forms of vitamin A have different biological activities per mass unit. Because of this, the retinol activity equivalent (RAE) was defined in order to compare intakes of different forms of vitamin [6].

The reference values for dietary intake (RDA – recommended dietary allowance) are summarized in Table 1.1 [6]. Values for tolerable upper intake levels (UL) were also defined, since an excess of the vitamin can be harmful. These values change with age and gender but could also be influenced by other factors.

Essentially, vitamin A (retinoids) is obtained from animal-based foods, while carotenoids are supplied through vegetal foods [5]. Some common sources of retinoids include organ meats (such as liver), fish and fish oils, eggs, milk and dairy products. Margarine, milk (particularly low-fat milk) and dry milk are often fortified with RE and this fortification is crucial to prevent vitamin A deficiency [23].

An insufficient intake of vitamin A can result in its deficiency, often with considerable health deterioration. The symptom most commonly associated with this is xeropthalmia, a term that refers to the ocular damage, including night blindness, corneal ulceration and necrosis (which could result in total blindness). While the first stages of xeropthalmia can be reversed by vitamin supplementation, in severe cases the damage might be permanent. Other symptoms include keratinization (drying and hardening of soft tissues) of respiratory and urinal tract epithelium, anemia, growth retardation and an increase of both incidence and

severity of many infections. Vitamin A deficiency is, therefore, one of the causes of child mortality around the globe [24, 25].

Nevertheless, the contrary can happen as well: hypervitaminosis A, which is the excess of vitamin A in the body after an overconsumption of this compound. The symptoms of this condition include nausea, vomiting, vision blur, liver issues, loss of muscular functions and even death and they are dependent on the duration and dose of the intake. Most cases of hypervitaminosis come from the misuse of vitamin supplements [6].

Table 1.1 - Recommended dietary allowance and upper intake levels for vitamin A intake, as RAE.

	Life stage	**RDA (µg/day)**	**UL (µg/day)**
Infants	0-12 months	400	600
Children	1-3 years	300	600
	4-8 years	400	900
Adolescent/adult males	9-13 years	600	1700
	14-18 years	900	2800
	>19 years	900	3000
Adolescent/adult females	9-13 years	600	1700
	14-18 years	700	2800
	>19 years	700	3000
Pregnancy	<18 years	750	2800
	19-50 years	770	3000
Lactation	<18 years	1200	2800
	19-50 years	1300	3000

RDA – recommended dietary allowance; UL – upper intake levels; RAE – retinol activity equivalent. Adapted from [6].

1.1.4. Vitamin A absorption

Vitamin A absorption in the intestine is protein-mediated. Its uptake does not appear to be impaired by the presence of other fat-soluble vitamins; however, vitamin A may cause changes in their absorption [26].

By the action of pancreatic lipases, ingested RE in food are transformed into retinol. Due to the necessity of lipase action, low-fat diets or diseases that affect the secretion of these enzymes might decrease vitamin A uptake. Retinol then diffuses into enterocytes and here a major fraction is esterified again. After this, there is a protein-mediated transport to the lymph and blood, as the vitamin is transported in chylomicrons (CMs) [6]. The process is summarized in Figure 1.3.

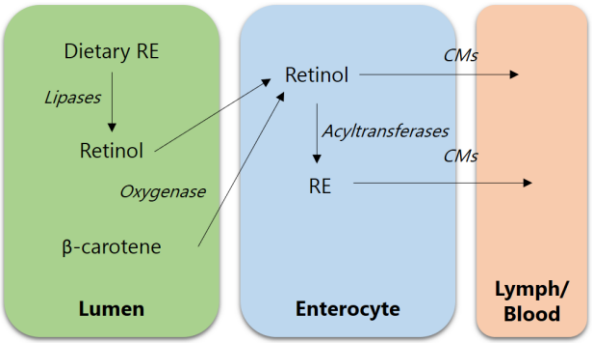

Figure 1.3 - Vitamin A and β-carotene intestinal absorption. Adapted from [6].

1.2. Nanotechnology

Nanotechnology refers to the development, characterization and application of novel materials, devices, and systems that have a functional organization in at least one dimension on the nanometer scale [27]. Applications of nanotechnology are transversal to many different areas, such as medical applications, environmental issues, cosmetics or food technology.

Nanoparticles (NPs) are examples of nanosystems that take advantage of their high surface area (caused by the diminution of radius) to display several interesting properties [28]. NPs can have different shapes and be made of a variety of materials, from metal, ceramics, to organic molecules such as polymers, proteins or lipids. Thus, NPs are highly versatile and can be tailored to the desired objectives. Considering this, nanocarriers are now seen as suitable options for encapsulating compounds of interest, in order to develop products with enhanced bioavailability, long shelf life, and targeted delivery, among other possibilities [29].

A very wide range of materials can be used in nanotechnology. However, lipids have several advantages which make them attractive as encapsulating agents for compound delivery, the main one being related to their biocompatibility. This is due to their natural occurrence in the body, which results in an easy metabolism of degradation products. Lipid nanoparticles are suitable for administration through multiple routes, including the oral [30].

2. State of the Art

2.1. General aspects of encapsulation

Encapsulation is a process by which small particles or molecules are entrapped within a wall material, resulting in capsules. The main goals of this technique are to protect bioactive compounds and to ensure controlled delivery of the encapsulated molecule. Microencapsulation refers to particles with 3-800 μm, while nanoencapsulation produces particles with diameters in the 10-1000 nm range [29].

Nanoparticles can be nanospheres or nanocapsules, depending on whether the compound of interest is uniformly dispersed in the matrix or in a cavity surrounded by a wall material, respectively [29]. NPs can be a matrix system, have simple or irregular shapes and possess one or more encapsulating agents and cores [31], as illustrated in Figure 2.1.

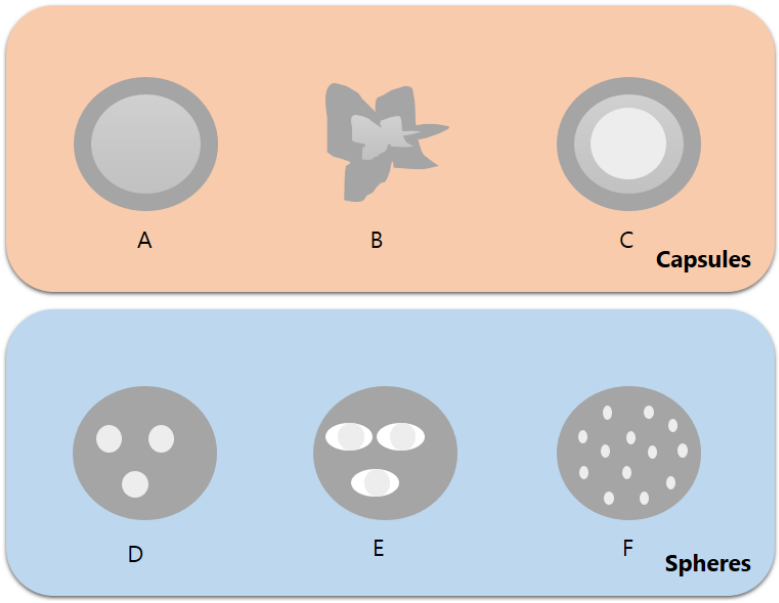

Figure 2.1 - Some examples of NPs possible morphologies, for nanocapsules: A - simple, B - irregular, C - multiwall, and for nanospheres: D - multi-core, E - aggregate, F - matrix. Adapted from [31].

Several materials can be used in the production of NPs, including ceramics [32], metals [33], polymers [34] and lipids [35]. Lipids are a very promising approach towards encapsulation of hydrophobic materials. Lipid nanoparticles can be seen as being a part of one of three possible models: homogenous distribution of the active compound in the NP, active-enriched core with active-free shell and active-free core with an active-enriched shell

(Figure 2.2) [36, 37]. The most commonly used types of lipid nanoparticles are liposomes, micelles, solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs).

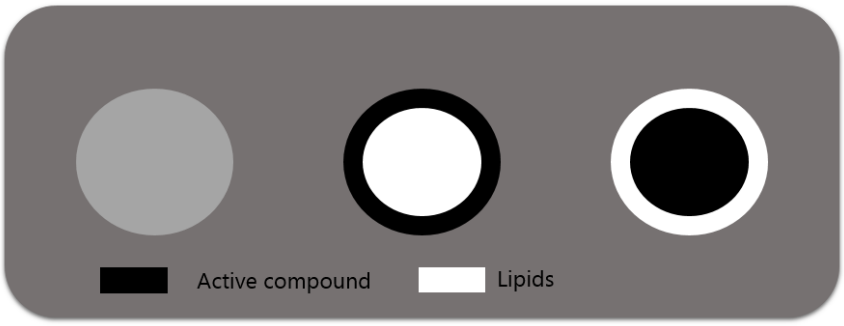

Figure 2.2 - Three models for NPs: homogenous matrix (left), drug-enriched shell (middle) and drug-enriched core (right). Adapted from [36].

2.1.1. Liposomes and micelles

Liposomes and micelles are generated by self-assembly of amphipathic lipid molecules in an aqueous environment. While micelles consist in an aggregate of surfactant molecules, liposomes are a bilayer vesicle that can be uni- or multi-lamellar (Figure 2.3). In liposomes, the polar areas of the lipids are turned outside in the bilayer, meaning they face both the outside environment and the internal aqueous core. Therefore, they present a capacity of incorporating both hydrophilic and hydrophobic compounds – in case of a fat-soluble molecule, it is entrapped in the bilayer; if the molecule is water soluble, it can be held within the aqueous core. The polar heads of lipids, in micelles, surround the core composed by the hydrophobic tails [30, 38, 39].

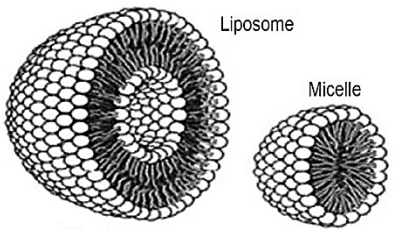

Figure 2.3 - Schematic representation of liposomes and micelles (with transversal section). Adapted from [38]

2.1.2. Solid Lipid Nanoparticles

SLNs were developed as an alternative to liposomes, emulsions and polymeric NPs [37]. Their versatility and simplicity are characteristics that made them very promising as delivery carriers [40]. They are composed of solid lipids (at room and body temperature) dispersed in an aqueous solution and stabilized by the use of surfactants [37], as illustrated in Figure 2.4.

SLNs size is small by the definition and the great challenge is often to keep a low polydispersity while developing small particles [40].

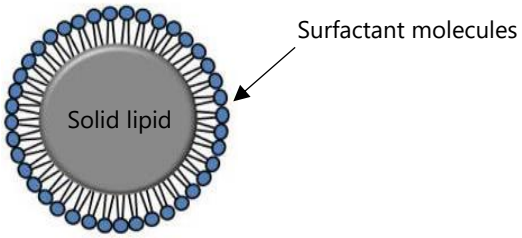

Figure 2.4 - Schematic representation of SLNs structure. Adapted from [41].

Although they are mostly used for encapsulating lipophilic compounds, they can be used with hydrophilic ones [42]. Examples of lipids used for these NPs include steroids, mono-, di- and triglycerides, fatty acids and waxes [37]. SLNs present several advantages over other delivery systems, although preparation with highly purified lipids may cause problems due to polymorphic transitions of lipids, resulting in drug expulsion during storage. Figure 2.5 summarizes the main advantages and disadvantages of SLNs.

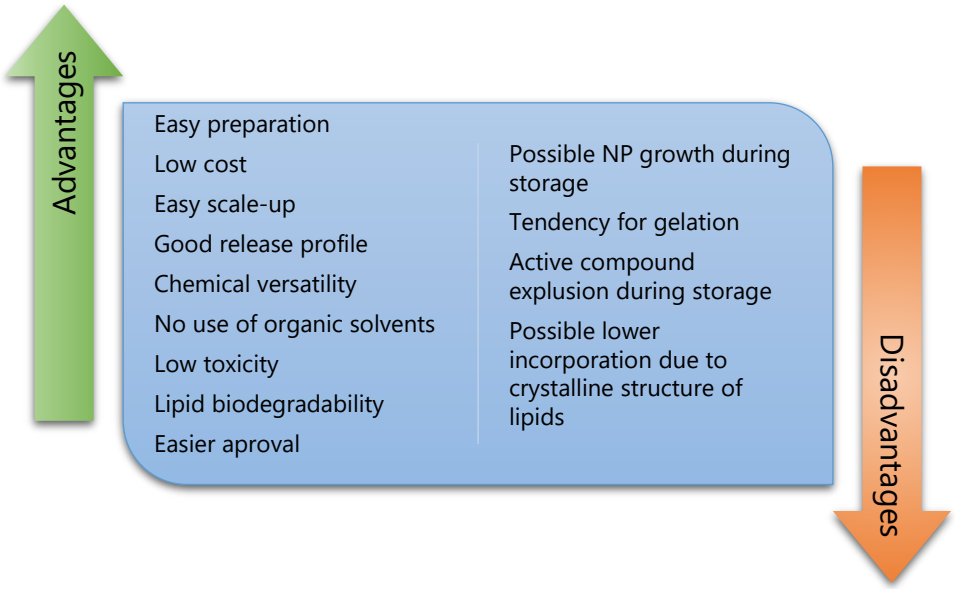

Figure 2.5 - Benefits and drawbacks of SLNs. Sources: [37, 40, 43]

2.1.3. Nanostructured Lipid Carriers

NLCs are, essentially, modified SLNs in which the solid lipid phase is composed by a mixture of solid and liquid lipids. They were developed to increase drug loading and prevent the loss of the active compound during storage [43]. Due to mixing liquid and solid lipids, NLCs do not form perfect crystals, enabling more space for drug loading and lowering the

chances of drug expulsion [40]. Figure 2.6 depicts the mains structural differences between SLNs and NLCs.

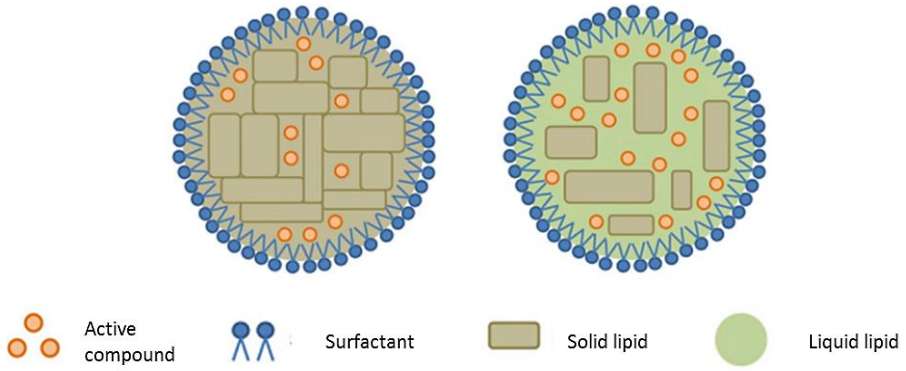

Figure 2.6 - Structural differences between crystalline SLNs (left) and amorphous NLCs (right). Adapted from [44].

There are three possible types for NLCs, depending on their structure: imperfect, amorphous and multiple (Figure 2.7). In imperfect-type NLCs (type I), the solid/liquid lipid mixing causes irregularities in matrix crystallization, responsible for drug/active compound accumulation. Type II (amorphous-type) NLCs have an amorphous (with no specific structure) matrix and this prevents recrystallization and subsequent active compound expulsion. Finally, multiple-type NLCs (type III) are essentially oil in fat in water double emulsions, used to increase drug solubility because many compounds are more soluble in liquid rather than solid lipids [45, 46].

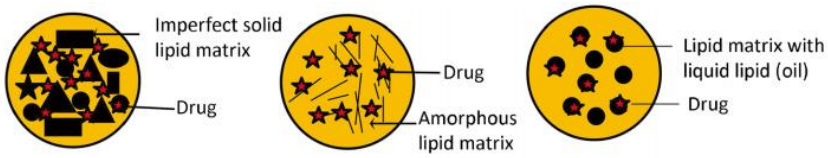

Figure 2.7 - Three types of NLCs: type I, imperfect (left); type II, amorphous (middle); and type III, multiple (right). Adapted from [45].

2.1.4. SLNs and NLCs production

There are several techniques for SLNs and NLCs production, such as high-pressure homogenization, solvent emulsification/evaporation, supercritical fluid extraction of emulsions and ultrasonication (also known as high speed homogenization) [37]. Often, these techniques are combined to achieve better results [47]. There is not a universally preferred method; the chosen technique will depend on the characteristics of the used compounds and the desired properties of the NPs [48].

High-pressure homogenization

There are two approaches towards this technique. The first is hot homogenization, in which the lipids are melted at temperatures above their melting point and the active compound is then added. The active compound loaded lipid melt is afterwards dispersed in the hot (at the same temperature of the lipid melt) aqueous surfactant solution with the aid of an Ultra-Turrax (high-pressure homogenizer) device and the emulsion with NPs is formed [37]. The alternative is cold homogenization. After lipid melting and addition of active compound, the mixture is quickly cooled by placing in dry ice or liquid nitrogen. The mixture becomes solid and is milled (50-100 μm diameter). Then, the milled powder is dispersed in the surfactant phase and this emulsion is homogenized by Ultra-Turrax [49].

Solvent emulsification/evaporation

The lipid/active compound phase is dissolved in an organic (insoluble in water) solvent and this mixture is then dispersed in an aqueous surfactant phase. The solvent is removed by evaporation under reduced pressure, which causes the NP dispersion in the aqueous phase.

Ultrasonication

The melted lipid/active compound phase is added to an aqueous phase enriched with a surfactant. This mixture is subjected by ultrasonication to produce NPs.

2.2. Fortification of food with vitamin A

Food fortification has been used in developed countries as an effective and low-cost strategy to prevent micronutrient deficiencies. It also has the advantage of being generally accepted by society [50]. Several products are commonly fortified, such as oil, margarine, cereals, milk, flours and juices [51].

2.2.1. Cooking oil

Oils are a good vehicle for vitamin A due to the hydrophobicity of this compound [50]. Nevertheless, not all oils are rich in vitamin A or this might be lost in oil processing [52, 53].

Considering its solubility in oils, vitamin A is simply dissolved in processed oils to make a homogenous product, with agitators and dosifiers already available at the factory, in either a continuous or batch process. This implies that the added cost is very low: price increment is

about 0.1-0.3% [52]. The vitamin has a high stability in oil, even if it is used for frying, and it improves vitamin bioavailability [54]. Besides, the consumption of vegetable oils is increasing globally, particularly in low incoming societies [55]. Retinyl palmitate is the chosen retinoid to apply to vegetable oils. It is added in liquid form, stabilized by α-tocopherol or a butylated hydroxyanisole/butylated hydroxytoluene mixture [52].

2.2.2. Margarine

Along with oil, margarine is a very efficient way to carry vitamin A. Margarine fortification with vitamin A is mandatory in several countries, such as Denmark, Canada, the United Kingdom or the United States [56, 57] and is an approach to imitate butter nutritional values, as many people prefer margarine over butter [54]. Adding of RE (usually retinyl palmitate) is very similar to the process used in vegetable oils [52].

2.2.3. Fluid milk

Commercial whole, low-fat and skim milk are often fortified with vitamin A to aid meeting nutritional requirements of this vitamin. Considering retinoids poor solubility in water, the pure addition of RE would be inefficient. Therefore, vitamin A fortification in the dairy industry is usually based on the addition of corn oil, sorbitan monooleate, polysorbate 80 and cholecalciferol in addition to retinyl palmitate. These oils and emulsifiers allow a better dispersion of the vitamin in the continuous phase of milk. Milk proteins might help to emulsify it as well [58]. Nowadays, methods to improve bioavailability and vitamin stability of fortified milk are being studied in order to obtain more efficient products [59-61].

2.2.4. Flours and cereals

Wholegrain cereals and flours inherent vitamin A is insignificant. However, flours are good carriers for vitamin A fortification due to the possibility of mixing in dry forms of vitamin with other additives [54]. Regardless, margarine is still preferred as a vehicle and flours are not always fortified. In the United States, wheat-soy and corn-soy are fortified, but 30-50% of the added vitamin is lost in processing and shipping [62].

2.2.5. Golden rice

Golden rice was an attempt to use genetic engineering to address the issue of vitamin A deficiency [63]. Rice was genetically modified to produce and accumulate vitamin A precursor β-carotene in the endosperm, the edible part of the rice grain [64]. This compound gives grains a golden colour, hence the name. Its bioavailability is confirmed [65] although the

genetic modification of rice is often hindered by society, resulting in an immense controversy around this product [66].

2.2.6. Other foods

Several other foods have been fortified with vitamin A around the globe. Examples include sugar [67], seasonings [68], dry milk and yogurt [69].

2.3. Nanotechnology applied to food fortification

There are several reasons to apply nanotechnology in food products, such as taste or flavour enrichment, enhanced delivery and solubility, protection against either oxidative, moisture or enzymatic degradation, antimicrobial effect (extending the products shelf life) and improvement of active compounds bioavailability [28, 70] (Figure 2.8).

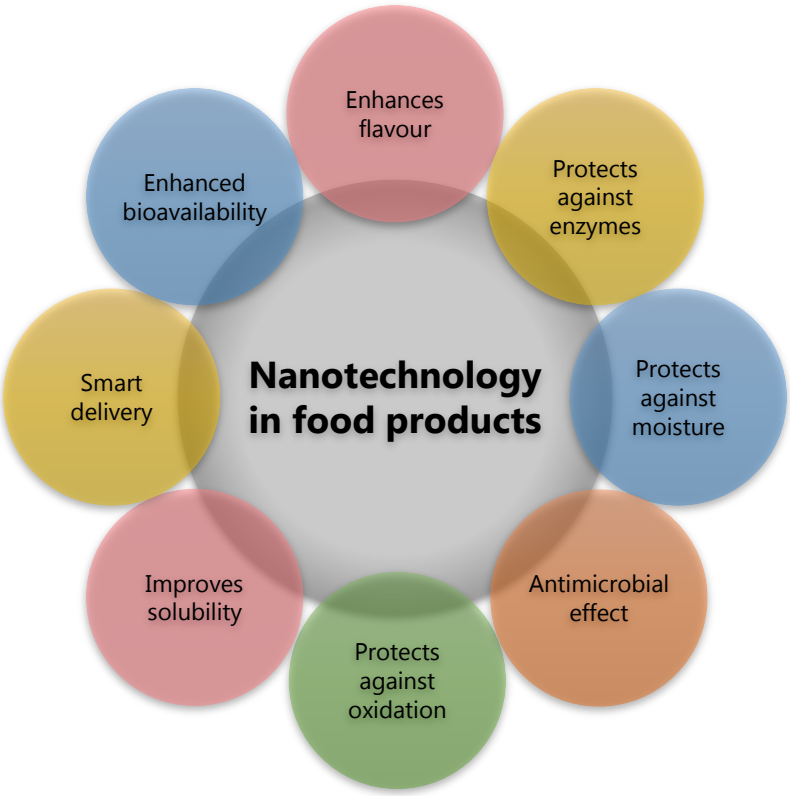

Figure 2.8 - Rationale behind application of nanotechnology to food products.

A search in databases specialized in nanomaterials-containing products in the "food and beverages" category yields 119 results, most of which refer to supplements of several kinds

[71]. However, not all claims have been certified, due to the companies' interest in keeping their production methods secret. Cerqueira *et al.* [72] have also pointed that the number of publications and issued patents including the keywords "food" and nanotechnology has risen considerably in the last two decades and might continue to do so.

Some of the major challenges of the use of nanotechnology in the food industry are related to the necessity of assuring the safety of the materials, both in terms of human health and eco-safety. The used compounds (both with a bioactive function and encapsulating agents) must be food grade and biodegradable. It is therefore essential to further investigate the behavior of the nanosystems when applied to food matrices and their fate in the environment.

Nevertheless, there are already some applications of nanotechnology in the food industry. Aquanova produces additives for beverages (such as functional drinks) based on nanoemulsions (micelles) [73]. A similar formulation for zinc and iron supplementation is produced by Taiyo International [74]. Nanotechnology is also becoming important in food packaging, mostly to prevent microbial contamination. For example, Nanobiomatters has developed materials for food packaging based on silver immobilized in clay nanocarriers [75].

2.4. Nanoencapsulation of vitamin A

Vitamin A has a great potential, both for food and medical applications. Allying this with the advantages of nanotechnology yields in wide study and research for nanocarriers capable of protecting and correctly delivering vitamin A.

Several vitamin forms can be used. β-carotene is often chosen for food applications, while for medical and cosmetic purposes active vitamin A forms are preferred. Due to recent developments in nanotechnology, a wide range of NPs types are suitable for these purposes; hence, the choice will depend on the final application. For example, some polymeric NPs are usually more suited to medical ends because of their more expensive production, when compared to lipidic formulations [76]. The rationale behind the inclusion of vitamin A in NPs is its low solubility in water and easy degradation by oxygen and light. Examples of nanoencapsulation of several forms of vitamin A (including precursor β-carotene) are shown in Table 2.1. Many authors already suggest lepidic NPs for the nanoencapsulation of vitamin A. The use of antioxidants, such as α-tocopherol, is also widely described in the literature.

Table 2.1 - Examples of nanoencapsulation of vitamin A (and pro-vitamin A) compounds. Research made in May 2018.

Particle type	Components (function)	Encapsulated compound	Application	Obs.	Ref.
Cyclodextrin complex	2-hydroxypropyl-b-cyclodextrin (inclusion agent)	All-*trans*-retinoic acid	Acute promyelocytic leukaemia therapy	Biopharmaceutical properties are unknown.	[77]
Nanofibers	Cellulose acetate (fibre component)	All-*trans* retinoic acid α-tocopherol (vitamin E)	Cosmetics	Preparation by electrospinning to explore co-encapsulation of both vitamins for skin delivery.	[78]
NLCs	Propylene glycol monostearate (solid lipid) Sunflower oil (liquid lipid) Tween 80 (surfactant) Tocopherol (anti-oxidative agent)	β-carotene	Fortification of beverages	The function of tocopherol was to protect β-carotene against oxidation.	[79]
NLCs	Palmitic Acid (solid lipid) Corn oil (liquid lipid) Ethanol (solvent) Tween 20 (surfactant)	β-carotene	Fortification of water-based foods	NPs were produced by a solvent diffusion method.	[80]
NLCs	Medium chain triglyceride (liquid lipid) Cetyl palmitate (solid lipid) Lecithin (surfactant) butylated hydroxytoluene (surfactant) Polysorbate-80 (surfactant)	All-*trans*-retinoic acid	Topical application		[81]
Polymeric NPs	Gliadin (encapsulating agent)	All-*trans*-retinoic acid	Therapeutic drug delivery		[82]
Polymeric micelles	Methoxy-poly(ethylene glycol)-poly(hexylsubstituted lactic acid) copolymer (micelle component) Quinoline Yellow (photoprotective agent)	All-*trans*-retinoic acid	Acne topical treatment		[83]

Particle type	Components (function)	Encapsulated compound	Application	Obs.	Ref.
Polymer-oil hybrid nanocarrier	poly(lactic-co-glycolic acid) (polymer) Captex 200 (liquid lipid)	All-*trans*-retinoic acid	Cancer therapy	NPs were produced by emulsion-solvent evaporation technique (solvent: dichloromethane).	[84]
SLNs	Palmitic acid (solid lipid) Epikuron 200 (surfactant) Sodium taurocholate (surfactant) Butanol (solvent)	Retinyl palmitate	Cosmetics/Dermatology (topical application)	Very effective in protecting retinyl palmitate from photo- and thermal degradation.	[85]
SLNs	Stearic acid (solid lipid) Poloxamer 188 (surfactant)	Retinoic acid	Prostate cancer treatment	Anticancer activity confirmed for concentrations of 0.2 mg/mL	[86]
Nanolipossomes	Lecithin cholesterol	Retinyl palmitate	Food applications	The authors advise choosing other types of nanoparticles due to low encapsulation efficiencies (ca. 15%)	[87]
Carbohydrate nanofibers	Cress seed mucilage (carbohydrate) Tween 80 (surfactant) Polyvinyl alcohol (surfactant)	Retinyl palmitate	Food applications		[88]
Hybrid lipid-polymer NPs	poly-ε-caprolactone polymer (polymer) Capric/caprylic triglycerides (lipid) Span 60 (surfactant) Tween 80 (surfactant)	β-carotene α- carotene Lutein	Food applications	NPs can protect carotenes from degradation from UV light for irradiation up to 50h	[89]

3. Rationale of the Project

Lately, extensive research has been applied in the areas of nanotechnology and nanoencapsulation of bioactive compounds. However, the application of this recently developed knowledge in the food industry remains low. Therefore, this project intends to apply nanotechnological developments in the food supplementation field. The nanoencapsulation of Vitamin A was selected as a relevant example for that, due to the limitations of the fortification with this compound. Nanoencapsulation of vitamin A would, in theory, overcome the issues related to the hydrophobicity and stability of this vitamin: not only it is hard to solubilize in aqueous media, it may also easily degrade or become inactive under those conditions. The instability of this substance due to exposure to light, oxidants, temperature, heat or moisture could also be improved by nanoencapsulation. Also, it would allow the controlled release of the vitamin and thus optimize the intestinal absorption and diminish its unsuccessful use [31].

Consequently, the main goal of this work was to develop and characterize lipid nanoparticles (SLNs and NLCs) encapsulating vitamin A to increase its bioavailability, by protecting it throughout the gastrointestinal tract and during storage/processing in foodstuffs. To achieve this goal, the first step was to select the adequate lipids for the incorporation of vitamin A. The following step was to produce the nanoparticles, and to characterize them in relation to their morphology, size, shape, zeta potential, polydispersity and vitamin encapsulation efficiency. The storage stability of the particles was assessed, followed by cellular studies with fibroblasts and intestine cells to determine cytotoxicity and intestinal permeation of the particles. The NPs resistance to a stomach passage was also analysed through an *in vitro* static digestion experiment. To evaluate the potential of the NPs application in a food matrix, several stability assessments were performed under mimetic food physico-chemical conditions (ionic strength, pH, sucrose and temperature). Lemonade was used as a reference food matrix for the fortification of vitamin A and characterization assays were conducted to validate the developed NPs as food supplement.

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4. Materials and Methods

4.1. Materials

Gelucire® 43/01, gelucire® 44/14, lipocire DM, cetyl palmitate, compritol® HD5 ATO, precirol® ATO 5 and compritol® 888 ATO were kindly offered by Gattefossé (Gattefossé, France). Stearic acid (SA) and oleic acid (OA) were purchased from Merck© (Merck KGaA, Darmstadt, Germany). Dynasan 116, imwitor 900 K and 491 were gently donated by Sasol (Johannesburg, South Africa). Tween 80 (polysorbate 80), retinyl acetate (RA), α-tocopherol, dimethyl sulfoxide (DMSO), sucrose, sodium chloride, sodium acetate, lipase, pepsin, porcine bile extract and pancreatin were acquired from Sigma-Aldrich (St. Luis, MO, USA). Miglyol® 812 (MIG) was obtained from Acofarma® (Terrassa, Spain). Ethanol was purchased from Fisher Chemical (UK). Acetic glacial acid was obtained from VWR International LLC (Radnor, PA, USA). Fresh lemons were acquired at a local supermarket. All water used was double-deionized water (Arium Pro, Sartorius AG, Göttingen, Germany). Weighting measurements were achieved using a Kern ABT 120-5DM digital analytical balance (Kern & Sohn; Balingen, Germany), while pH measurements were accomplished using a Crison pH meter GLP 22 with a Crison 52-02 tip (Crison; Barcelona, Spain). The cell line used in viability tests was the L929 fibroblast cell line, obtained from Cell Lines Service (CLS, Eppelheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin (10000 U/mL) mix, fetal bovine serum (FBS) and amphotericin B (Fungizone) were purchased from Gibco® (Invitrogen Corporation, UK). Caco-2 cells were acquired from the American Type Culture Collection (ATCC, Wesel, Germany) and used between passage number 40 and 55.

4.2. Methods

4.2.1. Lipid solubility tests

The lipid solubility of RA was evaluated in 11 different lipids: gelucire® 44/14, lipocire DM, imwitor 900K, gelucire® 43/01, imwitor 491, cetyl palmitate, compritol® HD5 ATO, precirol®

ATO 5, compritol® 888 ATO, dynasan 116 and stearic acid. The maximum tested solubility was 10% (w/w). 50 mg of the lipids were melted at a temperature higher (about 10 °C) than the melting point and RA was added gradually until a maximum amount of 5 mg. The lipid/RA mixture was then observed to verify the presence/absence of insoluble crystals.

4.2.2. SLNs production

Gelucire® 43/01 (GEL) and stearic acid (SA) were initially tested as solid lipids for production of SLNs. The SLNs were prepared by an organic solvent-free emulsification-sonication method. In more detail, 150 mg of solid lipid and 1 mg of α-tocopherol (except where stated, SLN_0) were melted at a temperature higher than the melting point of the lipid (60 °C for GEL and 75 °C for SA). Once the lipid was completely melted, 7 mL of 1% (v/v) Tween 80 in water, at the same temperature, was added to the lipid phase and the resulting solution was homogenized with a probe-type sonicator (model VCX-130 with a VC 18 probe, Sonics & Materials Inc., Newtown, CT, USA) for 5 min at 70% amplitude to form the nanoparticle suspension. To produce RA-loaded NPs, 10 mg (except where noted) of RA were added to the melted lipid phase and the subsequent procedure was maintained.

Since the initial test with SA have revealed a poor vitamin A encapsulation efficiency, GEL was chosen as the most suitable lipid for SLNs production.

4.2.3. NLCs production

GEL and SA were also tested as solid lipid for these NPs. The liquid lipids tested were MIG and OA, meaning that all possible combinations of one liquid lipid and one solid lipid were studied. The procedure was very similar to the preparation of SLNs: the lipid phase (150 mg of solid lipid, 45 mg of liquid lipid and 1 mg of α-tocopherol (except where stated, NLC_0) and10 mg of RA) was melted at a temperature higher than the melting point of the solid lipid and 7 mL of a 1% (v/v) Tween 80 solution at the same temperature was added. The resulting solution was subjected to sonication under the same conditions described for SLNs. Figure 4.1 shows a resumed scheme of nanoparticle production method described.

After preliminary tests, GEL/MIG NLCs were chosen for further studies, as they revealed to be the most effective in terms of vitamin A encapsulation.

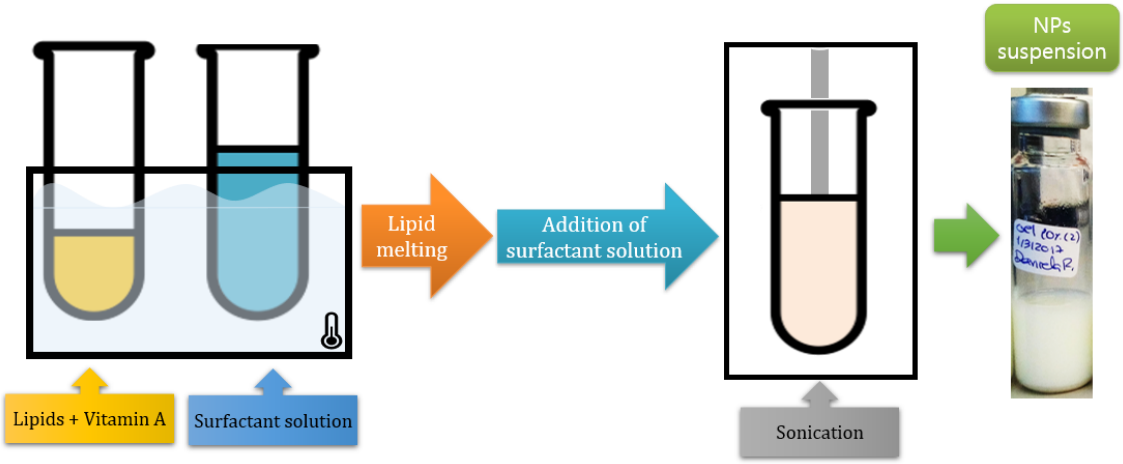

Figure 4.1 – Schematic representation of lipid nanoparticles production method.

4.2.4. Physicochemical characterization of NP formulations

Dynamic Light Scattering and Phase Analysis Light Scattering

The particle diameter, polydispersity index (PI) and zeta potential of all NP formulations was assessed. Dynamic light scattering (DLS) was the chosen method to evaluate PI and mean diameter; a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) was used to do so. With this technique, particle size was defined as its hydrodynamic diameter (since NPs were analysed in suspension) and it is calculated from the speed at which particles move in the solvent (water) due to Brownian motion.

Surface characteristics are essential to NP formulations, namely the surface charge. If a NP has a net charge on its surface, the distribution of ions surrounding the particle is changed and an electrical double layer is formed around the NP. The inner layer is known as the Stern layer and includes ions strongly bound to the particle; the outer layer (diffusion layer) is composed of ions with a weaker interaction and is limited by a theoretical boundary that defines which ions compose with the NP a stable entity. Zeta potential is the potential at this boundary (Figure 4.2) [44].

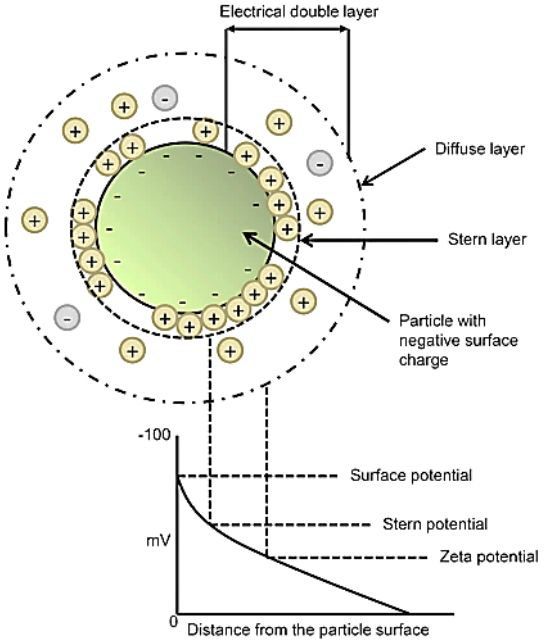

Figure 4.2 - Schematic representation of the electrical double layer and meaning of zeta potential. Adapted from [44].

A neutral zeta potential is an indicator of particles tendency for aggregation due to the decrease of repulsion forces [44]. Usually, a zeta potential of ±30 mV is required to ensure a stable system [90]. For lipid NPs, zeta potential is generally negative because of the charge given by commonly used surfactants [91]. Zeta potential was determined using a ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments Corporation), at 660 nm, at 20 °C with a detection angle of 90°, by phase analysis light scattering (PALS).

For both DLS and PALS, the formulations were diluted 100 times in double-deionised water and all measurements were performed with six runs each.

RA content

UV/Vis spectrophotometry was the selected methodology to assay the incorporation of RA into the NPs, through a direct method. This means that the NPs were separated from the suspension (which may contain non-incorporated vitamin A) and destroyed with 70% (v/v) ethanol. After a filtration step to separate the lipids, the amount of vitamin incorporated within the NPs was quantified.

All absorbance (Abs) readings were performed at 327 nm (Figure 4.3) with a V-660 UV/Vis Spectrophotometer (Jasco Inc., Easton, MD, USA). A calibration curve of UV/Vis detection of RA in an ethanol/water mixture (70% v/v of ethanol) was performed for further use in RA incorporation assays (Figure 4.4 and equation 4.1). Its accuracy (ratio between the

concentration obtained for a standard solution and its expected concentration) and precision (both repeatability, relative standard deviation of a six-fold analysis of standard solutions, and intermediate precision, RSD of duplicate analysis of standards on three consecutive days) were evaluated (Table 4.2). Considering the high accuracy and precision, and the good correlation and low detection and quantification limits (Table 4.1), the method was thought to be suitable for the quantification of vitamin A.

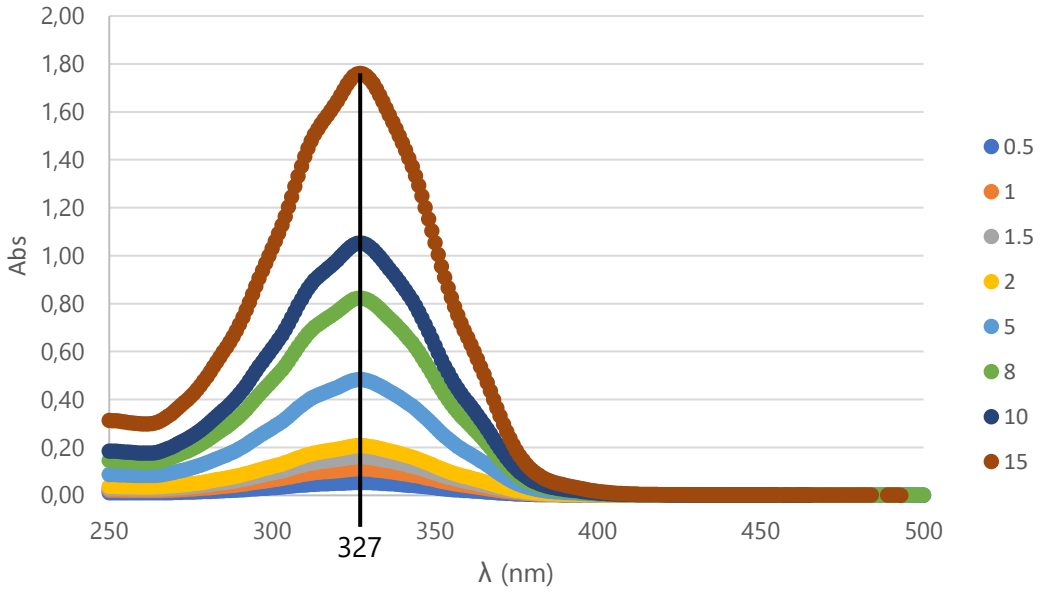

Figure 4.3 - Spectra of vitamin A solutions in ethanol 70%. The colours refer to the RA concentration in µg/mL.

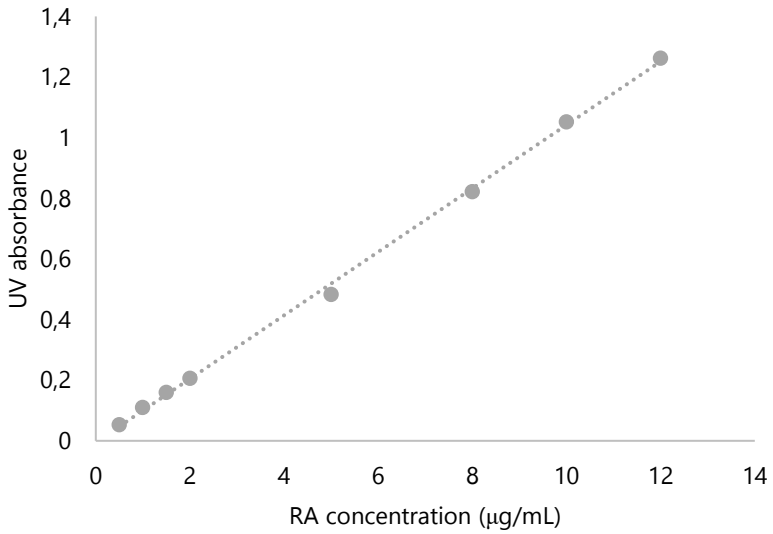

Figure 4.4 - Calibration curve of RA in 70%(v/v) ethanol/water.

$$Abs = [RA] \times (0.105 \pm 0.003) + (-0.005 \pm 0.02) \quad (4.1)$$

Table 4.1 - Calibration curve parameters.

Coefficient of determination (r^2)	0.999
Coefficient of correlation (r)	0.999
Limit of detection (LOD)	0.08 µg/mL
Limit of quantification (LOQ)	0.14 µg/mL

Table 4.2 - Accuracy and precision for the calibration curve

Standard concentration (µg/mL)	**Precision**		**Accuracy (%)**
	Repeatability (%)	**Intermediate precision (%)**	
1.0	0.12	2.4	109
5.0	0.35	3.5	93
12.0	0.15	0.6	99

The formulations were diluted 40 times in double deionized water and 2 mL of this dilution were filtrated with Amicon Ultra Centrifugal Filters Ultracell-50 kDa (EMD Millipore, Darmstadt, Germany), at 2,850 x*g* for 20 min, using a Heraeus Multifuge X1R centrifuge (Thermo Fisher Scientific, Germany). The supernatant was discarded and the pellet present in the filtrate unit was recovered through a centrifugation at 2850 *g* for 10 min. The pellet was ressuspended in 2 mL of 70% (v/v) ethanol to destabilize the particles and release the entrapped vitamin. This mixture was filtered using an 800 nm Minisart® Syringe Filter (Sartorius Stedim Biotech GmbH, Gottingen, Germany) to separate the lipids. To prevent reaching absorbance values out of the calibration range, the final solution was diluted adequately. The absorbance was analysed in the same conditions as settled for calibration curve. Each reading was performed in triplicate.

The incorporation of vitamin into the NPs was evaluated through the encapsulation efficiency (EE), defined as the quotient between the determined mass of RA in the suspension and the total amount of initially added RA in the NP preparation (eq. 4.2).

$$EE\ (\%) = \frac{RA\ mass\ in\ the\ NPs}{RA\ mass\ initially\ added} \times 100 \quad (4.2)$$

The vitamin loading (VL) of the nanoparticles was also determined and it was defined as the quotient between the determined mass of RA and the total excipients mass used for the formulation (mass of added vitamin and solid lipid, for SLNs; mass of added vitamin, liquid lipid and solid lipid for NLCs; eq. 4.3).

$$VL\ (\%) = \frac{RA\ mass\ in\ the\ NPs}{mass\ of\ added\ RA + mass\ of\ lipids} \times 100 \quad (4.3)$$

Transmission Electron Microscopy

This technique was applied to evaluate the morphology of NPs. In transmission electron microscopy (TEM), thin samples are illuminated with an electron beam (directed with a set of electromagnetic lenses) which interacts with that sample. The electrons that pass through are collected by a different set of lenses and led to a detector, such as a photographic film or a sensor (a charged-coupled device). TEM is able to analyse samples on the angstrom scale with good detail, due to the usage of an electron beam rather than light, as opposed to optical microscopes [92].

NP samples were diluted 100 times in double-deionized water and 20 µL of this dilution were placed in a copper grid for 1 minute. Then, the excess was removed by paper absorption and uracyl acetate was added as a contrast agent. After 30 seconds, the excess of uracyl was also removed. Finally, the grids were examined in a JEM- 1400 Transmission Electron Microscope (TEM Jeol JEM-1400; JEOL, Ltd., Tokyo, Japan), with an accelerating voltage of 60 kV, at an ampliation of 15000x.

4.2.5. Stability studies

The stability of NPs, both at room temperature and at 4 °C, was assessed using the parameters particle size, polydispersity and vitamin content. Firstly, NPs without tocopherol were tested ($SLNs_0$ and $NLCs_0$), but their stability in suspension was less than one week. Therefore, tocopherol was added as a possible way to overcome this issue and the evaluation was conducted up to 4 weeks.

4.2.6. Lyophilization

Lyophilization is a method which aims to increase the stability of a product, by removing its water through freezing followed by sublimation [93].

1 mg of a cryoprotectant (Aerosil®) was added to 2 mL of NP suspension and this mixture was thoroughly vortexed and afterwards frozen overnight at -80 °C. After this, NPs were lyophilized with a LyoQuest-85 plus v.407 (Telstar) freeze-drier, at -80 °C for a period of 72 h. For subsequent analysis, particles were reconstituted in the same volume (2 mL) of double-deionised water.

4.2.7. Resistance to heat treatments

Since heat treatments are common in the food industry, for either pasteurization, concentration or other purposes, the durability of the NPs under these conditions was studied. For that, 1.5 mL of formulation was added to 13.5 mL of water and placed in a water bath, at 60 or 70 °C. Samples were collected after 5 and 15 minutes and allowed to cool before assessing the size, PI and RA content of the particles.

4.2.8. NPs interaction with food components

The chemical stability of NPs in different media (simulating food matrices) was assessed by adding 1.5 mL of formulation to 13.5 mL of media. The media evaluated were solutions of NaCl (10% w/w), sucrose (10% w/w) and sodium acetate buffer at pH 5. Samples were collected at time zero and after 4, 8 and 24 h and analysed in terms of particle size, polydispersity and vitamin encapsulation the same way as described above. The studies were conducted in triplicate. This procedure was adapted from similar tests in the literature [94, 95].

To further evaluate the NPs interaction with food components, lemonade was selected as a reference matrix. Lemonade was prepared as follows: lemons were squeezed in a plastic squeezer and the juice was passed through a strainer; this filtered juice was dissolved in water in a 1:4 ratio, respectively. 1,5 mL (corresponding to 2 mg of RA for both NPs, and to 32 mg of lipid mass for SLNs and 72 mg for NLCs) of either SLNs or NLCs formulation was added to lemonade (total volume of 30 mL) and then the stability of the NPs (in terms of size, PI and vitamin content) was analysed as mentioned above, in three time-points of 24 hours. Before this essay, the absence of particles and natural vitamin A in the lemonade was confirmed by subjecting the pure lemonade to the same analysis.

4.2.9. Cellular studies

MTT assay for biosafety evaluation

L929 cells are a mouse fibroblast cell line. Fibroblasts are spread throughout the body because their function is to produce extracellular matrix. This makes them excellent for use in cytotoxicity assays, as recommended in the ISO standard regarding biomaterials safety evaluation [96]. Cells were cultivated in DMEM, supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin/Streptomycin mixture and 1% (v/v) amphotericin B, at 37ºC in a 5% CO_2 atmosphere (Unitherm CO_2 Incubator 3503 Uniequip; Planegg, Germany). The medium was replaced by

fresh, supplemented DMEM approximately every three days. When cells were confluent, they were detached from the culture flask with a scrapper (NuncTM Cell Scrappers, Thermofisher Scientific; Waltham, MA USA). After this physical detachment, cells were centrifuged at 300 x*g* for 5 minutes in a Heraeus™ Multifuge™ X1R centrifuge (Thermo Fisher Scientific; Waltham, USA) and then resuspended in fresh DMEM. Counting of viable cells was performed using a Neubauer chamber (Improved Neubauer Bright-Line, Boeco; Germany) in a Motic® AE2000 Binocular Inverted Microscope (Motic Electric Group Co., Ltd; Xiamen, Fujian, China).

The selected assay for evaluating cell viability after adding NPs was the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT can be metabolized by viable cells to a water-insoluble compound, formazan, which has a purple colour. In short, scrapped cells were cultivated in a 96-well plate (using a cell seeding density of 5×10^4 cells per well), in 100 µL of fresh culture medium. Once the cells had adhered, the medium was replaced complete DMEM containing different concentrations of SLNs, NLCs or free vitamin A. Control cells, formulation-free wells were also included in every plate. The cells were incubated for 24 hours in the same conditions as described above and afterwards the medium was replaced by 100 µL of a 0.5 mg/mL MTT solution. The plate was incubated again for 2 hours, after which the MTT solution was aspirated and 100 µL of DMSO added to solubilize the formazan crystals. Finally, absorbance was read at 570 and 630 nm using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT, USA). Cell viability was defined as described in equation 4.4.

$$Cell\ Viability\ (\%) = \frac{Abs_{570} - Abs_{630}}{Average[(Abs_{570} - Abs_{630})_{control}]} \times 100 \qquad (4.4)$$

NPs permeation on across intestinal Caco-2 monolayers

The permeation of the NPs within the intestine was assessed with Caco-2 cells monolayer model. These are human epithelial colorectal cells that differentiate when cultured in a insert filter, forming a epithelial monolayer that has been used as a model to assess absorption of orally administered formulations [97].

Firstly, an MTT assay was done with this cell line to select a non-cytotoxic NP concentration for use in the permeation studies (results in Annex 1 – MTT Assay for Caco-2 cells). These were seeded on 6-well Transwell inserts (Corning Transwell Clear, pore size 0.4 µm), at a density of 10^5 cells/cm^2. The cells were grown in supplemented DMEM which was replaced

every two days, for approximately 30 days, until the monolayer presented transepithelial electrical resistance (TEER) values above 400 Ω/cm^2 [98], which remained throughout the whole experiment. TEER measurements were performed with a a Millicell® ER-1 system voltohmmeter (Millipore Corporation, Bedford, MA), equipped with a pair of chopstick electrodes inserted into the apical medium. The permeability was assessed by adding 1.5 mL of NP suspensions labelled with coumarin diluted in supplemented DMEM (concentration of 2 mg/mL in lipids) to the apical compartment and 2.6 mL of DMEM with 3% (v/v) of ethanol to the basolateral compartment. The experiment was carried out for 4 hours and every hour a 1 mL sample was taken from the basolateral compartment and replaced with fresh DMEM/ethanol. At the end of the experiment, the content of both compartments was subjected to DLS analysis and coumarin quantified via a direct method similar to that described for vitamin A but using fluorescence detection for quantification (excitation wavelength of 340 nm; emission wavelength of 510 nm) using a using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT, USA).

4.2.10. *In vitro* stomach digestion

To evaluate if NPs could get through the stomach unaltered, a static *in vitro* digestion system was set up. 1.5 mL of the formulations were added to 30 mL of a fluid composed of a solution of salts (2.2 g/L KCl, 4.8 g NaCl, 1.5 g/L $NaHCO_3$ and 0.22 g/L $CaCl_2$) with pepsin and lipase, at pH 1.7. NPs stability (size, PI and vitamin content) was evaluated at the beginning and after two hours of the experiment.

4.2.11. *In vitro* intestine digestion

To investigate the fate of NPs in the intestine, a static *in vitro* digestion system was set up. 1.5 mL of the NP formulations were added to 30 mL of a fluid composed of a solution of salts (0.6 g/L KCl, 5.0 g NaCl and 0.25 g/L $CaCl_2$) with pancreatin (7% w/v) and porcine bile extract (4% w/v), at pH 6.5, based on previously established conditions [94]. The mixture was kept at 37 °C with constant stirring. NPs stability (size, PI and vitamin content) was evaluated at the beginning and after two hours of the experiment.

4.2.12. Statistical analysis

All statistical analyses were performed with IBM® SPSS® Statistics (SPSS 22.0, Armonk, NY, USA). Results are shown as mean value ± standard deviation from a minimum of three independent experiments. Two-tailed student's t-test and one-way analysis of variance (ANOVA) were performed to compare dependent samples and multiple groups of independent samples, respectively. When the groups presented significant statistical difference ($p \leq 0.05$), the differences between the respective groups were compared with a post-hoc test (Tukey, $p \leq 0.05$).

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5. Results and Discussion

5.1. RA solubility in lipids

The solubility of vitamin A in several lipids was studied to select possible components of the NPs. The results of the solubility studies are summarized on Table 5.1.

Table 5.1 - RA solubility in different lipids.

Lipid	RA solubility (% w/w)	Melting point (°C)
Gelucire® 44/14	10	44 [99]
Lipocire DM	10	50 [100]
Imwitor 900K	4	54-64 [101]
Gelucire® 43/01	10	43 [102]
Imwitor 491	2	66-77 [101]
Cetyl Palmitate	10	54 [103]
Compritol HD5 ATO	10	60-67 [104]
Precirol ATO 5	10	52-55 [105]
Compritol 888 ATO	10	68-74 [104]
Dynasan 116	10	61-65 [106]
Stearic Acid	10	69 [107]

10% (w/w) was the maximum tested amount. The lipids chosen for further studies are highlighted in blue.

The solubility of the compound to be incorporated in several lipids is usually the first step in developing lipid nanoparticles. Lipids in which the compound isn't soluble will have poor encapsulation and therefore must be discarded.

RA is a lipophilic molecule and this explains the high solubility in many of the tested lipids (Table 5.1).

Gelucire 43/01 and stearic acid were selected for further studies, based on the capacity to solubilize RA and their documented use for oral formulations in literature [108, 109].

5.2. NPs characterization

DLS and spectrophotometrical techniques were applied to characterize the NPs. Table 5.2 summarizes the size, zeta potential, vitamin loading and encapsulation efficiency and for all NPs produced.

Table 5.2 - Physicochemical characterization of the produced NPs: size, zeta potential, EE and VL.

	Components	Size (nm)	PI	Zeta Potential (mV)	Encapsulation Efficiency (%)	Vitamin Loading (%)
SLN_0	SA + RA	522.5 ± 7.5[(a)]	0.137 ± 0.025	-29.80 ± 0.62[(a)]	9% ± 1%[(c)]	1%
	SA	428.6 ± 6.5[(a)]	0.048 ± 0.022	-23.49 ± 0.51[(a)]		
	GEL + RA	197.1 ± 1.0	0.126 ± 0.016	-23.37 ± 1.17[(a)]	80% ± 1%	7%
	GEL	187.2 ± 0.2	0.137 ± 0.012	-26.58 ± 0.43		
NLC_0	SA + OA + RA	612.4 ± 5.2[(b)]	0.263 ± 0.008	-36.64 ± 0.61[(b)]	5% ± 1%	0%
	SA + OA	605.3 ± 7.8[(b)]	0.221 ± 0.027	-31.64 ± 0.71[(b)]		
	SA + MIG + RA	392.3 ± 2.8[(b)]	0.123 ± 0.021	-24.71 ± 0.84[(b)]	59% ± 1%[(d)]	5%
	SA + MIG	476.7 ± 1.2[(b)]	0.157 ± 0.018	-29.28 ± 0.56		
	GEL + OA + RA	228.2 ± 0.8[(b)]	0.076 ± 0.014	-28.75 ± 0.43	64% ± 1%[(d)]	6%
	GEL + OA	162.8 ± 0.8[(b)]	0.066 ± 0.010	-25.61 ± 0.47[(b)]		
	GEL + MIG + RA	268.5 ± 1.2	0.138 ± 0.007	-34.43 ± 0.67[(b)]	74% ± 1%	7%
	GEL + MIG	274.4 ± 1.4	0.178 ± 0.008	-29.96 ± 0.85[(b)]		

Values shown as mean for n = 2. SLN_0 and NLC_0 are NPs without tocopherol. (a) significantly different ($p \leq 0.05$) when compared to GEL SLN_0; (b) significantly different ($p \leq 0.05$) when compared to GEL + MIG NLC_0; (c) significantly different ($p \leq 0.05$) when compared to GEL + RA SLN_0; (d) significantly different ($p \leq 0.05$) when compared to GEL + MIG + RA NLC_0.

The size of NPs is represented as the mean of their hydrodynamic diameter. NLCs seem to have higher diameters in relation to SLNs, an increase that ranges from 11% for SA and MIG NLCs up to 46% for GEL and MIG NLCs. SA formulations also showed higher diameters when compared to GEL. A direct correlation between RA-loaded and unloaded NPs was not found – RA-loaded GEL SLNs and NLCs are statistically similar to unloaded NPs.

Size has been inversely correlated with their intestinal uptake (the smaller the NPs, the higher the uptake) for oral delivery [110]. Since SA NPs are statistically larger than GEL NPS, as shown on Table 5.2, the latter seem to be the better approach towards encapsulation of RA in food products.

The PI is an indicator of the width of NPs size distribution. Thus, particle size is usually described referring not only the hydrodynamic diameter but also its PI. Optimum values for PI are below 0.3 [91]. All NPs had PIs below 0.27, meaning the produced NPs formulations were uniform and presented a homogenous size distribution. Therefore, this was not a considered parameter in selecting NPs for further studies.

Zeta potential was also assessed. In this study, a direct connection between loading of NPs and zeta potential is not visible. There was not also a direct correlation between lipid or NP type in zeta potential. Although results might be influenced by the non-optimal formulation of NPs, this could be caused by a conjugation of all these factors. The only loaded NPs with zeta potentials below -30 mV, considered a good indicator of stability, were GEL/MIG NLCs and SA/OA NLCs. However, the latter is not the best approach due to it low RA encapsulation.

Encapsulation efficiency was generally higher for SLNs when compared to NLCs (approximately 6% increase). NLCs were developed initially to overcome some limitations of SLNs, in an attempt to achieve a higher encapsulation of active compounds, due to lower water content of the particle suspension and a minimization of loss of the encapsulated compounds during storage [36]. Although several authors have confirmed the higher EE for NLCs [111, 112], others have found no significant differences [35].

GEL NPs had good EE, with values above 70%, associated with a 7% VL, particularly for GEL SLNs and GEL/MIG NLCs. This means they could be a potentially good approach towards encapsulation of RA, because a high mass of vitamin is encapsulated in a relatively small lipid mass. They were, therefore, selected for the subsequent studies. Figure 5.1 shows the appearance of these formulations and Figure 5.2 shows their morphology as seen through TEM.

TEM microscopy confirms the idea that adding RA to the NPs does not change their major characteristics. Even though the TEM image shows a smaller particle diameter than the one measured by DLS, this happens due to the fact that DLS measures the hydrodynamic diameter while TEM measures the actual particle size, leading to a smaller value.

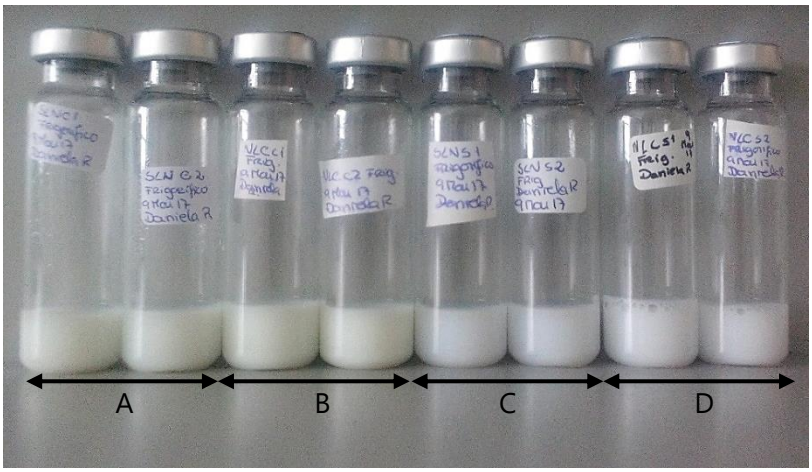

Figure 5.1 - Appearance of the synthetized Gelucire nanoformulations: A - SLNs with RA; B - SLNs without RA; C - NLCs with RA; D - NLCs without RA.

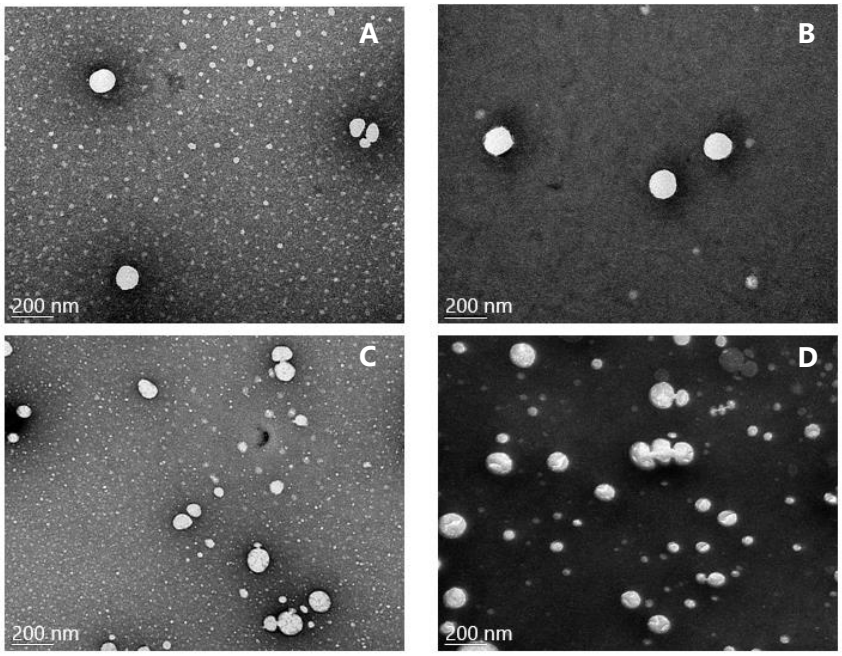

Figure 5.2 - TEM images of the NPs in aqueous suspension. A, RA-loaded SLNs; B, SLNs without RA; C, RA-loaded NLCs; D, NLCs without RA.

5.3. Stability studies

At first, the stability, both at 4ºC and at room temperature, of the GEL NPs was assessed using particles that did not contain α-tocopherol. The most relevant result is that almost all encapsulated vitamin was lost after one week (Figure 5.3 and Figure 5.4). It is also possible to see a significant increase in the zeta potential along time of NPs with RA. NPs stored at 4 °C also show a statistically relevant increase in their size and PI over time, which does not happen for NPs stored at room temperature.

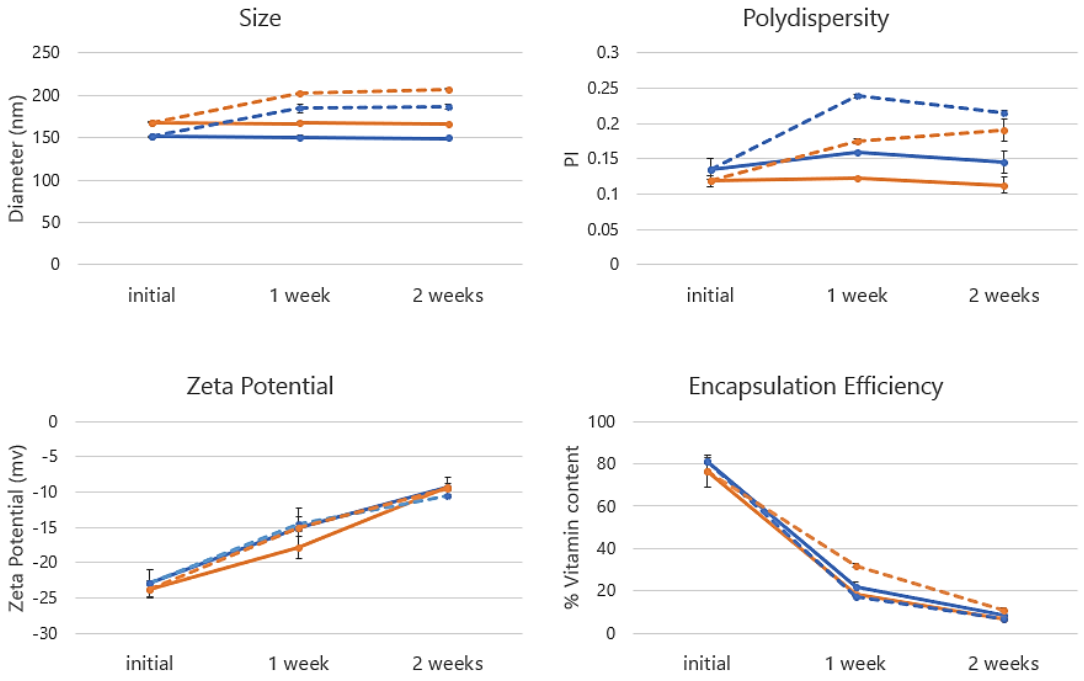

Figure 5.3 - Stability of RA-loaded NPs without tocopherol. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: blue – SLN_0s, orange – NLC_0s. There are statistically significant differences for formulations after 2 weeks ($p<0.05$ vs. initial) for all zeta potentials and EE values and for size and PI of NPs stored at 4 °C.

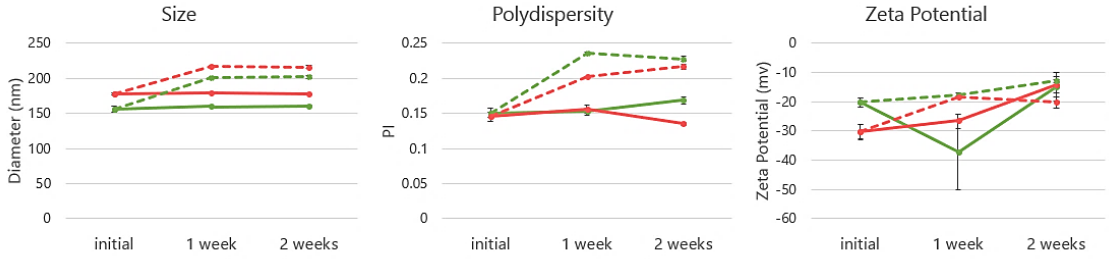

Figure 5.4 - Stability of NPs without tocopherol and RA. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: green – SLN_0s, red – NLC_0s. There are statistically significant differences ($p<0.05$) for size and PI of NPs stored at 4 °C.

To overcome this issue, several authors working with RA or β-carotene NPs had mentioned the addition of α-tocopherol to the formulations, as to prevent RA oxidation [79, 94]. Therefore, a new test was performed, adding 1 mg of α-tocopherol to the lipid phase during NP synthesis, which did not significantly alter the NPs characteristics (Table 5.3). The results are shown Figure 5.5 and Figure 5.6.

Table 5.3 - Characterization of α-tocopherol NPs (n=3).

Formulation	Size (nm)	PI	Zeta Potential (mV)	Encapsulation Efficiency (%)
SLN	187.5 ± 1.8	0.147 ± 0.009	-21.62 ± 0.93	
RA-Loaded SLN	189.6 ± 7.5	0.151± 0.004	-24.90 ± 0.80	66% ± 2%
NLC	203.7 ± 1.9	0.124 ± 0.002	-29.23 ± 0.68	
RA-Loaded NLC	203.4 ± 0.4	0.122 ± 0.002	-20.71 ± 0.49	70% ± 1%

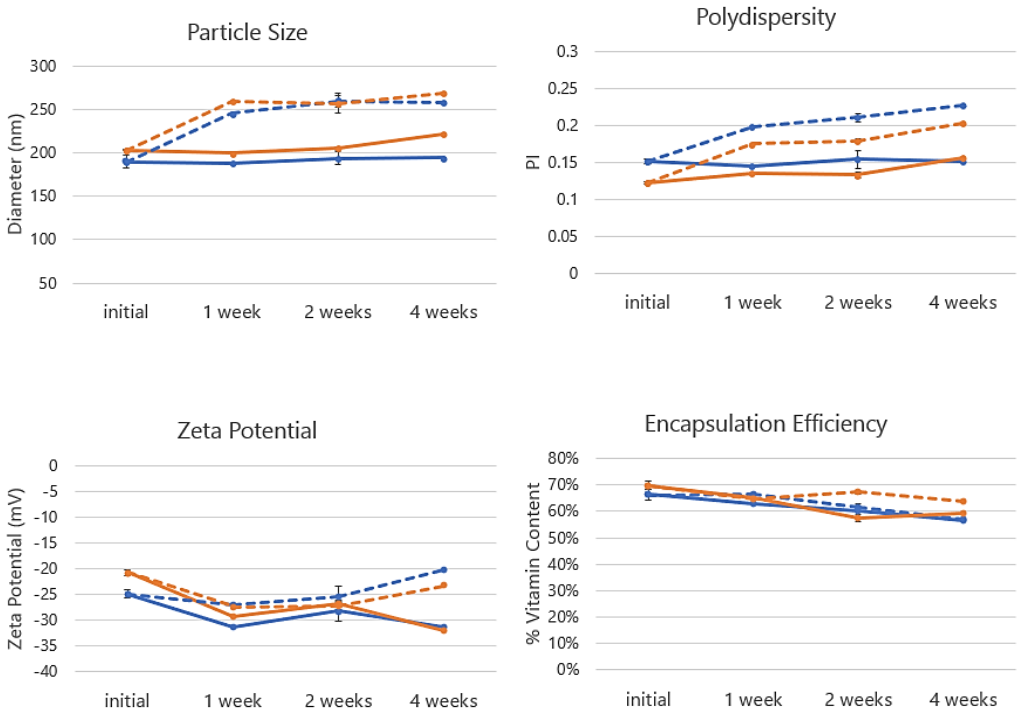

Figure 5.5 - Stability of RA-loaded tocopherol NPs over the period of 1 month. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: blue –SLNs; orange –NLCs. There are statistically significant differences (p<0.05) for size and PI of NPs stored at 4 °C.

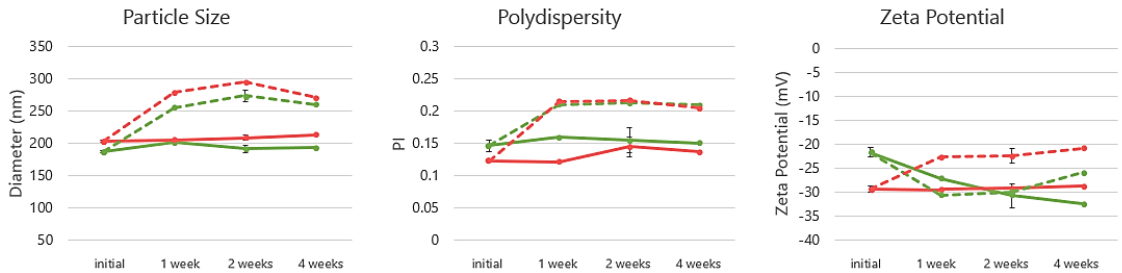

Figure 5.6 - Stability of tocopherol NPs without RA over the period of 1 month. The dashed lines represent NPs stored at 4 °C, while solid lines refer to NPs stored at room temperature. The colours represent the type of NPs: green –SLNs; red –NLCs. There are statistically significant differences (p<0.05) for size and PI of NPs stored at 4 °C.

With this, it was possible to verify that particles remained stable in an aqueous suspension for at least a month, both at 4 ºC and room temperature. No statistically significant changes were seen for the encapsulation efficiency throughout the storage. There was, nevertheless, an increase of approximately 80 nm in the size and of 0.1 in the PI of NPs that were stored in the colder temperature. Although it is a statistically relevant increase, NPs remained with a size and PI that were reasonable for the purpose of protecting and delivery of RA.

5.4. Lyophilization

Lyophilization is a broadly used technique for preservation of NPs and avoid their degradation and aggregation [113]. Theoretically, lyophilized NPs should have the same characteristics (e.g. size, PI, vitamin content) as the original formulations.

However, this process subjects the particles to extremely low temperatures for periods of up to a few days, which can damage them, even with the use of cryoprotectors. This is what happened with GEL NPs that were lyophilized. It was not possible to resuspend the NPs in water after lyophilization, although aerosil® was added as a cryoprotector (Figure 5.7). This means that the process needs to be optimized in the future, in terms of the amount and type of the cryoprotector and the duration of the process. Another approach is the co-encapsulation of the vitamin and a cryoprotector, as described in the literature [113].

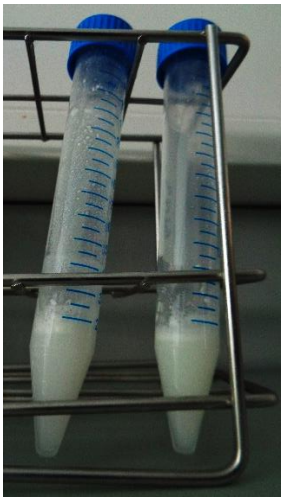

Figure 5.7 - Examples of formulations after resuspension in water. It is possible to observe a phase separation - it is not possible to resuspend the lyophilized formulations.

5.5. Heat treatments

Heat processing is very common in the food treatment, for either sterilization, concentration or other purposes. Thus, it is necessary to assess if particles would endure such temperatures, as to prevent the necessity of a post-treatment addition (that would be more expensive).

Two temperatures, frequently used in the processing of juices, were tested: 60 and 70 °C. The results for size, PI and encapsulated vitamin of the particles are shown on Figure 5.8.

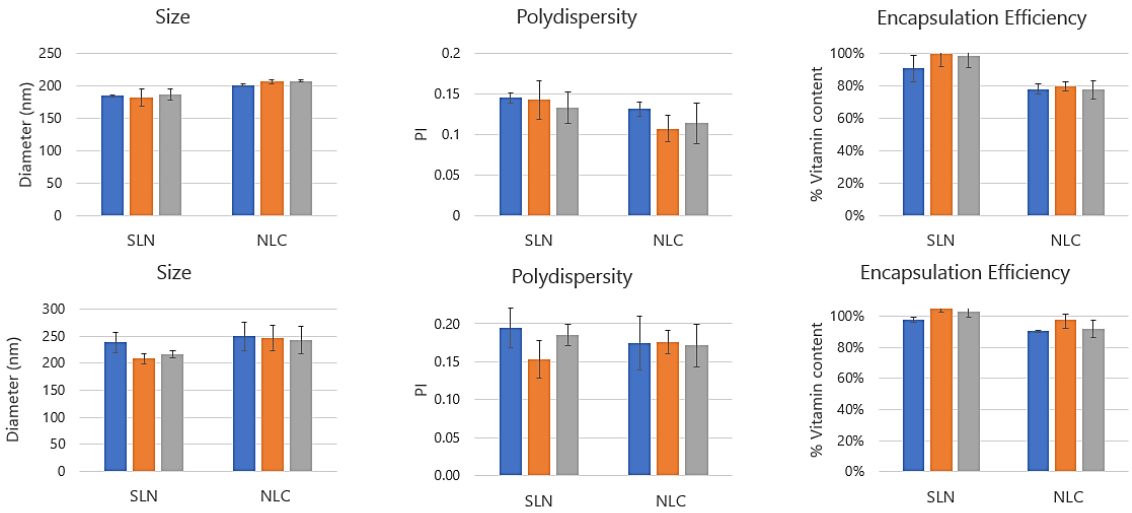

Figure 5.8 -Evaluation of size, PI and vitamin content of particles subjected to heat treatments: on the top row, particles treated at 60 ºC; on the bottom row, particles treated at 70 ºC. The blue bars refer to the original (non-heated) particles, the orange bars to NPs heated for 5 minutes and the grey bars to NPs heated for 15 minutes. No significant changes were found for heated NPs compared to the respective original formulations (for $p<0.05$).

It is interesting to see that although the tested temperatures are above the melting point of Gelucire (the solid lipid used in the NPs), 43 °C, no significant changes were found in particles submitted to these stresses. It is possible that the dispersion remains as an emulsion, even though lipid melting actually occurs, with re-solidification afterwards. Another important concept to bear in mind is that temperature affects the hydrophilic-lipophilic balance (HLB) of polyethoxylated surfactants like Tween 80. At a specific temperature (called the phase inversion temperature), the emulsion is inverted [94, 114]. In this case, that temperature (approximately 130 °C) was not achieved [114], meaning that the HLB of Tween was not affected. Therefore, it remained capable of protecting the suspension.

5.6. NPs interaction with food components

In order to understand the vitamin A-loaded NPs applicability in food matrices, it was necessary to assess their stability under food-specific environments. At first, simple solutions of compounds and conditions commonly found in food were evaluated, namely: sodium chloride (which would also simulate a high ionic strength environment), sucrose and an acetate buffer at pH 5 (a pH common in juices, milk, etc.). These simple matrices would allow an easy, but representative, prediction of the behaviour of the NPs in foodstuffs [94]. The results obtained for size, PI and encapsulation efficiency are shown in Figure 5.9.

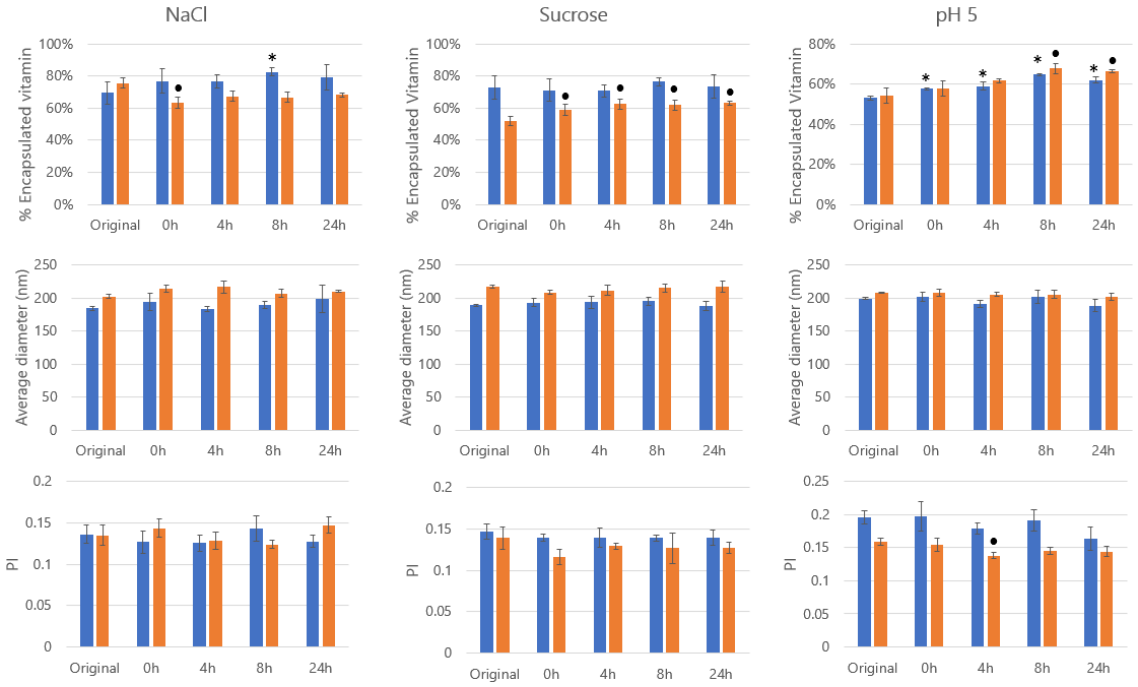

*Figure 5.9 - Variations in size, PI and RA encapsulation of particles submerged in different media: in orange, NLCs; in blue, SLNs (n=3). *, significantly different (p≤0.05) when compared to the original SLN; •, significantly different (p≤0.05) when compared to the original NLC.*

According to the results shown on Figure 5.9, the developed NPs seem to be stable in all media, with variations below 10% in the studied parameters, even though some were statistically relevant. This could mean that the particles will be stable when stored in foodstuffs under these conditions. In a previous study, nanoemulsions for β-carotene encapsulation (also stabilized with α-tocopherol) reported losses (around 30%) of the provitamin when storing the formulations in concentrated NaCl solutions (1M, which is below the concentration used in this experiment) [94]. Therefore, the proposed NPs appear to overcome this problem and could be used in food matrices.

To validate the applicability of the vitamin A-loaded NPs, a more realistic matrix was used: a lemonade, with no added sugar or preservatives. The results for the size, PI and EE of NPs are shown in Figure 5.10.

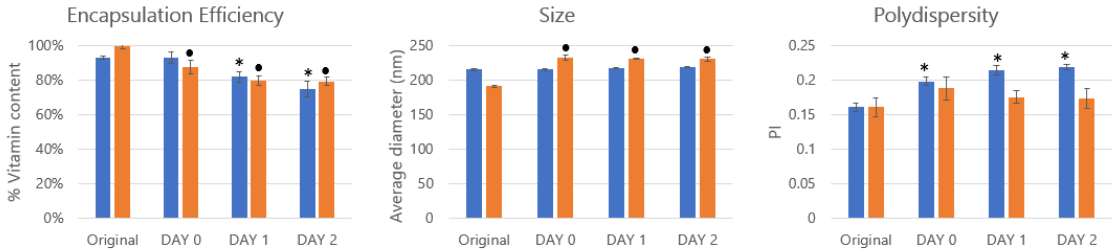

*Figure 5.10 - Variations in size, PI and RA encapsulation of particles added to lemonade: in orange, NLCs; in blue, SLNs (n=3). *, significantly different (p≤0.05) when compared to the original SLN; •, significantly different (p≤0.05) when compared to the original NLC.*

SLNs presented no changes in their average sizes throughout the experiment, even though there was an increase smaller than 0.1 in their PI (at the final time-point, the PI was approximately 0.2). NLCs show a statistically relevant increase of approximately 40 nm, probably to adsorption of some component of the lemon juice to the particles. However, this change is considered small enough for particles to remain suitable enough for their purpose. Interestingly, this increase is not followed by a change in PI.

After day 2, both SLNs and NLCs had losses of 30% of encapsulated RA. However, 70% (corresponding to 1.5 mg) of the added vitamin remained in the particles. The concentration of NPs used for this study enabled a delivery of 2.1 mg of RA, which would be higher than the recommended amounts (see Table 1.1). However, this was necessary in order to fulfil quantification limits. Figure 5.11 shows the appearance of a lemonade supplemented with an adequate amount of NPs; no visual difference is seen between supplemented and non-supplemented lemonade, which is important to develop an attractive product for costumers.

Figure 5.11 - Comparison of lemonade, A, and lemonade supplemented with NPs (NLCs), B.

Considering these results, it is possible to say that the developed vitamin A-loaded NPs appear to be suitable for addition in food matrices.

5.7. Cellular studies

5.7.1. Biosafety evaluation

It is essential to test the safety and biocompatibility of newly developed food additives. With that in mind, and considering international recommendations, a viability assay based on the quantification of mitochondrial activity using L929 fibroblasts was performed. The results are shown on Figure 5.12.

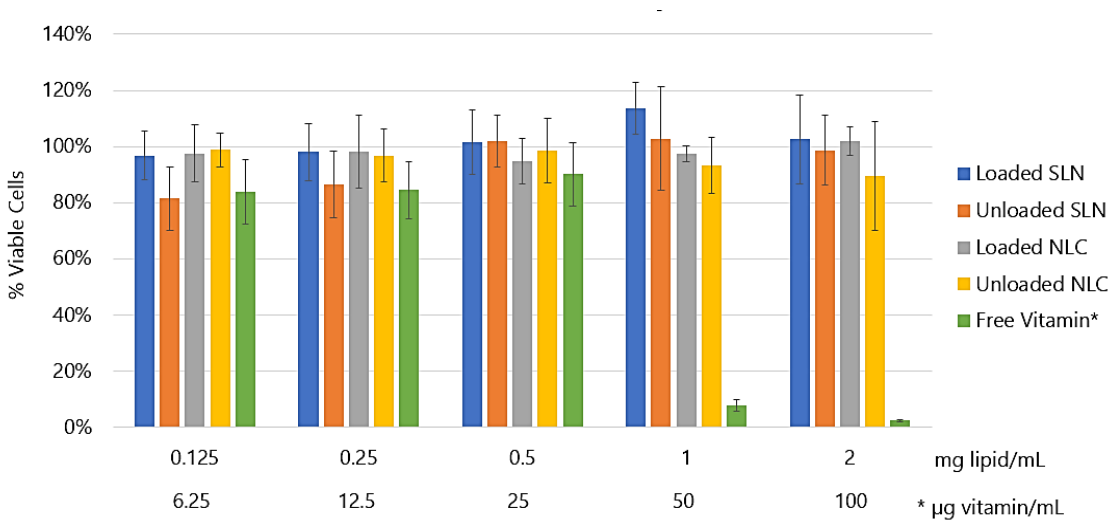

Figure 5.12 – Results for cellular viability essays, for RA-loaded and unloaded SLNs and NLCs (n=4). Cellular viability was normalized using the viability of the positive control

Analysing Figure 5.12, it is possible to observe that free vitamin A decreased cell viability to less than 10% viability for the highest concentrations (of 50 and 100 µg/mL) used. This may be due to hypervitaminosis, which is widely described in literature and which, in humans, can cause several issues, such as skin peeling, desquamation of mucous membranes, pain, among others [115]. It is, however, interesting to see that NPs seem to protect cells from the cytotoxic effect of free vitamin A (comparing formulations with equivalent amounts of RA). Both vitamin-loaded and non-loaded formulations appear to be safe for this cell line. This protection may come from one of two possibilities: either the assay was not long enough to allow the release of RA from the NPs, or the NPs enabled a sustained release of the vitamin so preventing the attainment of cytotoxic levels. It would be interesting, as a future step, to evaluate both *in vitro* cellular RA release profiles and to assess cellular uptake of the formulations, as to validate the results hereby demonstrated. Nevertheless, the fact that the lipid nanoparticles were biocompatible is a promising result that supports their safe application in food products.

5.7.2. Permeation across Caco-2 monolayers

The model of Caco-2 monolayer is a simple but relevant way of predicting the behaviour and permeation of the NPs at the crossing of the intestine barrier. Prior to the permeation studies, a MTT assay was performed to ensure the viability of the cells for the selected NP concentration (2 mg lipid/mL), as shown on Annex 1 – MTT Assay for Caco-2 cells. The preliminary results for the permeation studies are hereby presented, at Table 5.5 and

Table 5.6. These are validated by the confirmation of the integrity of the monolayer throughout the whole experiment, assured by the conservation of the TEER values above 400 Ω/cm^2 (Table 5.4).

Table 5.4 - TEER values at the beginning of the experiment (t_0) and after 4 h (t_f), for n=2.

Well	TEER at t_0 (Ω/cm^2)	TEER at t_f (Ω/cm^2)
Control without cells	150 ± 8	140 ± 7
Control with cells	432 ± 25	420 ± 17
Coumarin labeled-SLN	410 ± 16	405 ± 8
Coumarin labeled-NLC	415 ± 15	410 ± 15

Table 5.5 shows the DLS analysis of the contents of the apical and basolateral compartments at the beginning of the experiment (t_0) and after 4 h (t_f) and in Table 5.6 the amount of permeated RA is described.

Table 5.5 – Size and PI of NPs recovered from apical and basolateral transwell compartments, at the beginning of the experiment (t_0) and after 4 h (t_f), for n=2.

	Coumarin labeled-SLN	Coumarin labeled-NLC
Size at t_0 (nm)	213.0 ± 4.5	236.2 ± 1.7
Apical size at t_f (nm)	210.2 ± 6.4	235.6 ±1.1
Basolateral size at t_f (nm)	228.1 ± 12.1	230.9 ± 1.1
PI at t_0 (nm)	0.156 ± 0.006	0.167 ± 0.003
Apical PI at t_f (nm)	0.147 ± 0.001	0.157 ± 0.010
Basolateral PI at t_f (nm)	0.226 ± 0.025	0.203 ± 0.001

Table 5.6 - Quantification of the non-permeated formulation ratio, based on the fluorescence detection of coumarin, at t_f (after 4h of experiment).

Formulation	% Retained formulation at apical compartment at t_f	Equivalent permeated RA amount (µg)
Coumarin labeled-SLN	75% ± 3%	38 ± 5
Coumarin labeled-NLC	104% ± 7%	Not detected

Results indicate that at the last timepoint, it is possible to find NPs (with the same size as at t_0) in both compartments, which means that the permeation occurred but was not complete.

Table 5.6 also confirms this idea: the amount of NLCs that crossed the monolayer was undetectable. There is, nevertheless, a 25% permeation rate for SLNs, which corresponds to 38 µg of RA. This is still below the RDA values for vitamin A. It is important to notice that it was not possible to detect coumarin at the basolateral compartment, meaning that for SLNs there might be a NP internalization by the cells. The time duration of the experiment may also need adjustment to a higher period, in which it would be possible to see a higher NP permeation for both systems. Therefore, this is an ongoing experiment and in the future further studies on this matter will be developed to better understand this behaviour.

5.8. *In vitro* stomach digestion

One of the goals was to ensure that the NPs were resistant to the passage throughout the stomach. For that, particles were subjected to media with an acidic pH, salts and enzymes, present in that organ and their stability there was evaluated. The results are summarized on Figure 5.13

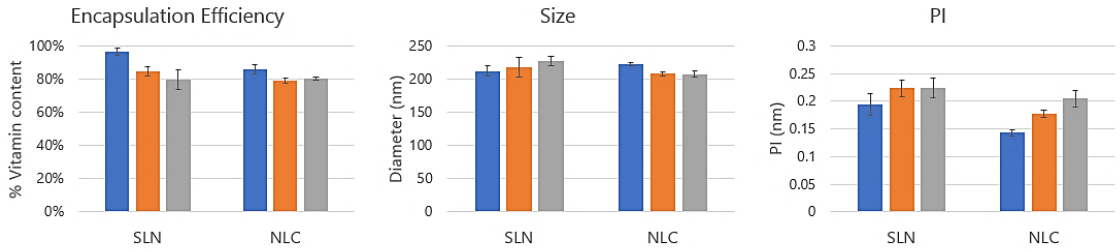

Figure 5.13 - Vitamin content, size and PI of NPs in stomach-simulating media. The blue bars refer to the original particles, the orange bars to the particles at the timepoint zero and the grey bars to the particles after 2 hours in that media.

For NLCs, there is an increase of 0.03 in the polydispersity after two hours, but their size remains roughly the same. A similar situation happens for SLNs. In terms of the incorporated vitamin, after two hours in this media, 80% of the added vitamin remained in the particles, which is a very promising result, as RA is mainly absorbed in the intestine. These results show that the particles appear to be stable in the stomach, possibly reaching the intestine.

5.9. *In vitro* intestine digestion

The small intestine is the primary place of absorption of RA [116]. To investigate the result of the interaction of NP with the intestine environment, an in vitro digestion assay was set up. Figure 1 shows the outcome of NP upon 2 hours in the defined conditions expressed as the diameter range.

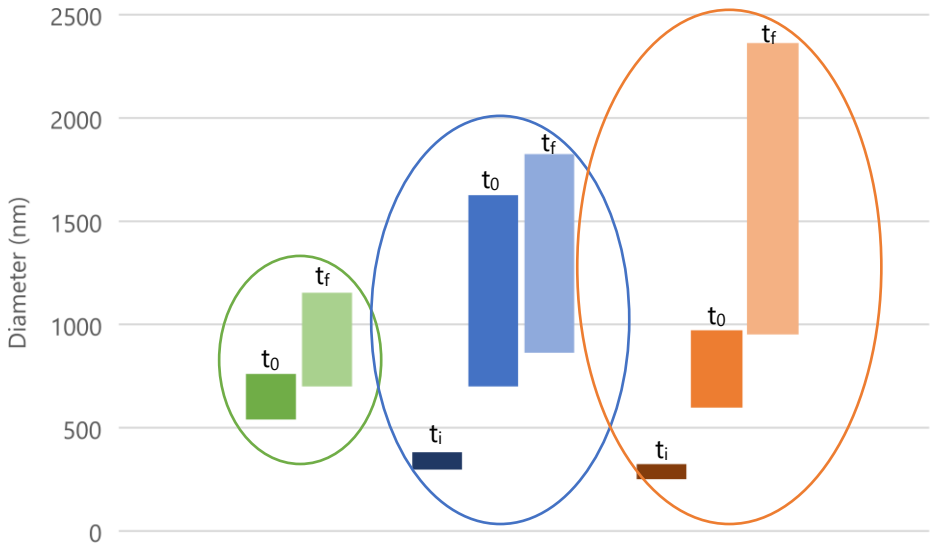

Figure 5.14 – Diameter range of NP upon 2 hours interaction with the intestine environment. Blanks (in green bars) represent the intestine mimetic medium without particles (green bars), SLNs (blue bars) and NLCs (orange bars) during an in vitro *intestine digestion. NPs were analysed before digestion (t_i), at the beginning of the experiment (t_0) and at the end (t_f).*

The bile salts present in the bile are amphiphilic and therefore create micelles in water [117]. These are detected by DLS, as shown by the green bars on Figure 5.14. The presence of bile salts and pancreatic lipases (provided by pancreatin) will destroy the NPs structure, and promote the digestion of the di- and triacylglycerols that compose the NPs [94]. This is confirmed by the substantial increase in the diameter range of NPs throughout the experiment, caused by the loss of particle structure and lipid aggregation. Gomes *et al.* achieved similar results for lipidic NPs containing β-carotene [94]. This could indicate a possible release of the encapsulated vitamin allowing its intestinal absorption. Further studies should be conducted to quantify the RA in this intestinal environment.

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Conclusions

Fortification of feedstuffs is a common practice in the food industry. This work aimed at verifying the possibility of using nanoencapsulation in lipid nanoparticles (SLNs and NLCs) for vitamin A fortification.

Regarding nanoparticles physico-chemical characterization, it was possible to produce SLNs made of Gelucire® 43/01 and NLCs made of Gelucire®43/01 and miglyol® 812, supplemented with α-tocopherol. These spherical NPs had sizes below 300 nm and PIs below 0.2, with high (approximately 80%) vitamin A encapsulation rates at a 7% loading, corresponding to 8 mg of bioactive compound.

These particles were shown to be stable both at room temperature and at 4 °C, in aqueous suspension, for a period of one month.

The developed NPs were also stable to heat treatments of up to 70 °C for periods of 15 minutes.

SLNs and NLCs were also stable in different media simulating several conditions of food products: solutions of 10% (m/v) of NaCl or sucrose and pH 5.

The developed NPs were also stable in lemonade (chosen as reference food matrix) and were capable of preserving 70% of the added vitamin after a period of two days.

Th formulations also appear to be non-cytotoxic in fibroblast cellular lines. Preliminary permeation studies have revealed that SLNs might permeate the intestine barrier, with a 25% permeation rate (equivalent to 38 out of 152 µg of vitamin A) after 4 h.

Both SLNs and NLCs were shown to remain unaltered after an *in vitro* stomach digestion assay. This indicates that perhaps they can reach the intestine unaltered. In the intestine, they are digested, possibly allowing the release of vitamin A for its absorption.

In conclusion, we were able to produce lipid nanoparticles capable of entrapping vitamin A, stable under different conditions and non-cytotoxic. This leads us to believe that they are suitable for application in food products for fortification with this vitamin.

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Future Work

The developed project shows promising results for the application of nanotechnology in food development, particularly in the supplementation of food with vitamin A. Nevertheless, more work is need at this moment, to consider the formulation of a final product.

First of all, it is necessary to confirm the increase in the vitamin bioavailability, by accessing its release from the NPs in media that would simulate the physiological medium.

More advanced biocompatibility assays are needed, such as the use of other cell lines (namely stomach cells) and the study of the impact of the NPs in animals as models of the human body behaviour and biochemistry,

Once safety is fully verified, it is also essential to confirm if the NPs do not alter the taste and texture of the products, via sensory analysis with trained panels.

Finally, to yield a new food product, it is necessary to study the scalability of the techniques hereby presented in order to create an economically interesting solution.

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Annexes

Annex 1 – MTT Assay for Caco-2 cells

An MTT assay was performed for Caco-2 cells to ensure NPs would be non-cytotoxic for this cell line and thus suitable for the performance of the intestinal permeation experiment. Figure A. 1 shows the main results obtained for it.

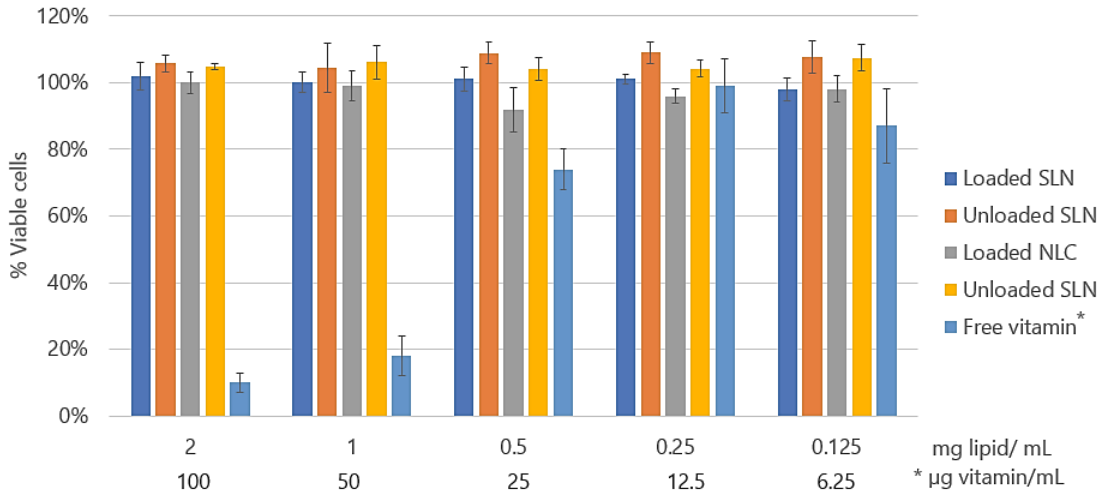

Figure A. 1 - Results for cell viability essays with Caco-2 cells, for RA-loaded and unloaded NPs (n=2).

It is possible to see that the behaviour is very similar to the one obtained for L929 MTT assays (see Figure 5.12 from chapter 5.7): none of the studied concentrations of NPs suspension was cytotoxic; nevertheless, the free vitamin showed a 80% loss of cellular viability for concentrations above 50 µg/mL.