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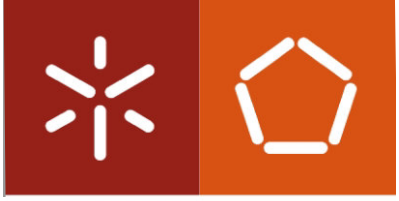

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Development of nanostructures for encapsulation of vitamins

Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação do
Professor Doutor António Augusto Martins de Oliveira Soares Vicente
e do
Doutor Miguel Ângelo Parente Ribeiro Cerqueira

Outubro de 2013

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Título

Desenvolvimento de nanoestruturas para o encapsulamento de vitaminas.

Development of nanostructures for encapsulation of vitamins.

Orientador

Professor Doutor António Augusto Martins de Oliveira Soares Vicente

Co-orientador

Doutor Miguel Ângelo Parente Ribeiro Cerqueira

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Mestrado em Bioengenharia

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ABSTRACT

Vitamins are sensitive and unstable when exposed to inappropriate temperature, oxygen, light and humidity conditions. For the food industry it is important to reduce some of these limitations and being with nanosystems arise a promising solution.

This work aims at the development of nanosystems for the encapsulation of riboflavin (water-soluble) and α-tocopherol (liposoluble) using biopolymer and their further characterization. For encapsulation of riboflavin an ionotropic polyelectrolyte pre-gelation was used as production method being chitosan and alginate used as main materials. (±)-α-tocopherol was encapsulated through the self-assembly of zein, one of the major proteins of corn with an amphiphilic character.

Initially a preliminary study was done to determine the optimal concentrations of biopolymers used and encapsulated vitamin that allow the highest efficiency and the production of nano-sized structures. For the alginate and chitosan system the optimal concentrations were 0.63 of alginate, 0.4 of chitosan and 0.065 mg/ml of riboflavin. For the zein system 2 and 1 mg/ml of zein and (±)-α-tocopherol, respectively, were used. Nanosystems were characterized in terms of average size, polydispersity index and zeta potential (through Dynamic Light Scattering) and vitamin entrapment efficiency. The average size (by number) for alginate/chitosan nanoparticles with riboflavin was 110.17 (± 47.71) nm. The nanosystems present values of polydispersity index and the zeta potential of 0.520 (± 0.041) and -29.64 (± 0.97) mV, respectively. Zein nanoparticles with (±)-α-tocopherol present an average size of 93.60 (± 1.09) nm and a polydispersity index of 0.222 (±0.020) with a zeta potential of +26.08 (± 2.51) mV. Both nanosystems have successful with encapsulation of vitamins, being the values of encapsulation efficiency (EE) and loading capacity (LC) obtained for (-)-riboflavin 55.91 (± 5.56) % and 2.18 (± 0.63) %, respectively, and for (±)-α-tocopherol was 94.95(± 4.17) % of EE and 8.53(± 1.90) % of LC. In order to complete the characterization of the nanoparticles was also done release profiles was also done for both vitamins and the diffusion coefficient (*D*) was calculated based on the fitting of model: for (-)-riboflavin at 37 °C *D* was 2.28×10^{-20} m^2/s at pH 7 and $4.95 \pm 0.74 \times 10^{-20}$ m^2/s at pH 2, and at 25 °C *D* was 1.02×10^{-20} m^2/s and 1.88×10^{-20} m^2/s for pH 7 and 2, respectively; for (±)-α-tocopherol encapsulated into zein nanoparticles at 37°C pH 7, the (±)-α-tocopherol is not released and at pH 2 *D* was 2.8×10^{-18} m^2/s. Lastly, the stability of both nanoparticles was assessed being evaluated the effect of temperature and storage time in particles sizes and PDI.

Alginate, chitosan and zein are biodegradable, biocompatible, food-grade with good physicochemical properties and can be used in the development of biopolymer-based nanosystems. This work shows that these biopolymer-based nanosystems can be used for the encapsulation of water-soluble and liposoluble vitamins with a great potential for application in food products.

RESUMO

As vitaminas são sensíveis e estáveis quando expostas a temperaturas inadequadas, oxigénio, luz e humidade. Para a indústria alimentar é importante reduzir algumas destas limitações e os nanosistemas aparecem como uma solução promissora.

Este trabalho tem como principal objectivo o desenvolvimento e caracterização de nanosistemas para a encapsulação de (-)-riboflavina (vitamina hidrosolúvel) e (±)-α-tocoferol (vitamina liposolúvel) usando biopolímeros. Para a encapsulação da (-)-riboflavina foi usado o método pré-gelificação ionotrópica, sendo o alginato e o quitosano os principais materiais. O (±)-α-tocoferol foi encapsulado através de automontagem da zeína, uma proteína do milho com carácter amfifílico.

Inicialmente foi feito um estudo preliminar com o objectivo de determinar os parâmetros e concentrações óptimas dos biopolímeros e das vitaminas. Para o sistema do alginato e quitosano as concentrações óptimas foram 0,63 de alginato, 0,4 de quitosano e 0,065 mg/ml de (-)-riboflavina. Para o sistama da zeína foram usados 2 e 1 mg/ml de zeína e (±)-α-tocoferol, respectivamente. Os nanosistemas foram caracterizados em termos de médias de tamanhos, índice de polidispersividade e potencial zeta (através de espalhamento dinâmico de luz) e eficiência de encapsulação da vitamina. A média dos tamanhos (por números) para as nanopartículas de alginato/quitosano com (-)-riboflavina foi de 110,17 (± 47,71) nm. O nanosistema apresenta valores de índice de polidispersividade e potencial zeta de 0,520 (± 0,041) e -29,64 (± 0,97) mV, respectivamente. As nanopartículas de zeína com (±)-α-tocoferol apresentam uma média de tamanhos de 93,60 (± 1,09) nm e têm um índice de polidispersividade de 0,222 (± 0,020) com um potencial zeta de +26,08 (± 2,51) mV. Ambos os sistemas foram bem sucedidos na encapsulação das respectivas vitaminas, para a (-)-riboflavina os valores de eficiência de encapsulação (EE) e de capacidade de incorporação (LC) foram 55,91 (± 5,56) % e 2,18 (± 0,63) %, respectivamente, e 94,95 (± 4,17) % de EE e 8,53 (± 1,90) % de LC para o (±)-α-tocoferol. De forma a completar a caracterização de ambas as nanopartículas, também foram avaliados os perfis de libertação de ambas as vitaminas e foram calculados os coeficientes de difusão (*D*): para a (-)-riboflavina a 37 °C o *D* foi de 2,28 $\times 10^{-20}$ m^2/s para pH 7 e $4,95 \pm 0,74 \times 10^{-20}$ m^2/s para pH 2 e a 25 °C o *D* foi de $1,02 \times 10^{-20}$ m^2/s e $1,88 \times 10^{-20}$ m^2/s para pH 7 e 2, respectivamente; para o (±)-α-tocoferol encapsulado em nanopartículas de zeína a 37 °C pH 7 não houve libertação da vitamina e a pH 2 o *D* foi de $2,8 \times 10^{-18}$ m^2/s. Por fim, a estabilidade de ambos os sistemas foi medida, sendo avaliados os efeitos da temperatura e do tempo de armazenamento nos tamanhos e PDI das nanopartículas.

O alginato, quitosano e zeína são biodegradáveis, biocompatíveis, edíveis e com boas propriedades físico-químicas e estes podem ser usados no desenvolvimento de nanosistemas biopoliméricos. Este trabalho mostra que esses sistemas biopoliméricos podem ser usados para a encapsulação de vitaminas hidro- e liposolúveis com um grande potencial para aplicação em produtos na indústria alimentar.

LIST OF PUBLICATIONS

Maria A. Azevedo, Miguel A. Cerqueira and António A. Vicente, Development of an edible, bio-based nanostructure for encapsulation of water soluble vitamins. IFT'13 - Annual Meeting & Food Expo, Chicago, 13-16 July 2013 (poster presentation).

Maria A. Azevedo, Miguel A. Cerqueira and António A. Vicente, Development of biopolymer based-nanosystems for vitamins delivery. ESBP 2013 – European Symposium on Biopolymers, Lisbon, Portugal, 7-9 October 2013 (poster presentation).

Miguel A. Cerqueira, Ana C. Pinheiro, Hélder D. Silva, Philippe E. Ramos, Maria A. Azevedo, Maria L. F. López, Melissa C. Rivera, Ana I. Bourbon, Óscar L. Ramos, António Vicente (2013). Design of bio-nanosystems for functional compounds delivery, *Food Engineering Reviews*, DOI 10.1007/s12393-013-9074-3.

Maria A. Azevedo, Ana I. Bourbon, Miguel A. Cerqueira and António A. Vicente (2013). Development and characterization of alginate/chitosan nanoparticles for controlled release of vitamin B2. To be submitted to *International Journal of Biological Macromolecules*.

Maria A. Azevedo, Ana I. Bourbon, Miguel A. Cerqueira and António A. Vicente (2013). Development α-tocopherol loaded nanoparticles through zein self-assembly. To be submitted to *Journal of Agricultural and Food Chemistry*.

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LIST OF GENERAL NOMENCLATURE

Abs – Absorbance

° C – Celsius

$CaCl_2$ – Calcium Chloride

Corp. – Corporation

cP – Centipoise

D – Translational diffusion coefficient

d (H) - Hydrodynamic diameter

Da – Dalton

DLS – Dynamic Light Scattering

DSC – Differential Scanning Calorimetry

EE – Encapsulation Efficiency

e.g. – For example

FTIR – Fourier transform infrared spectroscopy

g – Unit of measure of acceleration (m/s^2)

H – Hours

LC – Loading Capacity

K – Boltzmann's constant

M – Molar

µL – Microliters

µm – Micrometers

m^2/s – Square meter per second

mL – milliliters

min – minute

mm – millimeters

mM – millimolar

Mw – Molecular weight

nm - Nanometers

NTA – Nanoparticle Tracking Analysis

PCS – Photon Correlation Spectroscopy

PDI – Polydispersity

PBS – Phosphate Buffer Saline

QLS – Quasi-Elastic Light Scattering

RF^- – Anionic form of riboflavin

RFH – Neutral form of riboflavin

RFH_2^+ – Cationic form of riboflavin

rpm – Rotations per minute (r/min)

SD – Standard Deviation

T – Absolute temperature

TEM – Transmission Electron Microscopy

TGA – Thermogravimetric analysis

TOC – (±)-α-Tocopherol

Tris-HCl – Tris-Hydrochloride

UK – United Kingdom

USA – United States of America

η – Viscosity

UV – Ultraviolet

λ – Wavelength

ζ – Zeta Potential

CHAPTER I

MOTIVATION, OBJECTIVE AND OUTLINE

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1.1. THESIS MOTIVATION

The food industry has as main challenges: to improve the production efficiency, food safety and food characteristics. This way, the maintenance and increase of chemical, physical, microbiologic and nutritional stability of food and compounds used in food is an essential aim to achieve.

Vitamins have a special attention from food industry, because they have bioactive properties that are essential for normal maintenance, growth and development of human organism and their absence causes a specific deficiency syndrome. However, humans do not have the capacity to synthesize vitamins, except vitamin D and B_3, meaning that they must obtain vitamins through ingestion of food (Ball, 2008; Combs Jr, 2012). The enrichment of food and/or beverages with vitamins is a good idea to complete a feed with absence of vitamins, but they can also be used to mask the flavor of vitamins and mineral or to improve taste of the food products. Nonetheless, vitamins are sensitive and unstable when exposed to inadequate temperature, oxygen, light and moisture. This way, the nanoencapsulation can be a solution to keep stability of vitamins (De Britto et al., 2012; Luo et al., 2012).

Nanotechnology is an emergent and multidisciplinary field that is stimulating much interest across many areas of research and industries. This field involves the application, production and processing of materials with sizes at nano-scale and due to sizes of materials, the nanostructures when applied in food industry allow e.g. great improvements in bio-adhesive properties, appearance, texture, stability, or flavor of the product and release of bioactive compounds, higher bioavailability and can solve some problems such as compatibility and loss of activity of some functional compounds, affected by light, oxygen and temperature when dispersed in the food (Acosta, 2009; Kuan et al., 2012). In order to improve the efficiency and stability of nanostructures it is essential to find adequate materials; in the particular case of the food sector it is also important the replacement of non-food-grade materials by bio-based and biodegradable food-grade materials. Biopolymers, such as polysaccharides, proteins and lipids, open the door to new functionalities and applications due distinct advantages of biodegradability, edibility and lack of toxicity (Chassenieux et al., 2013).

In doing so, the encapsulation of vitamins can solve the problems associated with used and incorporated of vitamins in food and/or beverages and can lead to an increase of stability and to

promote their bioactivity. It is possible that encapsulation of vitamins also improves the controlled release of vitamins in human digestive system.

1.2. RESEARCH AIMS

The main objective of this thesis was the development of nanosystems for the encapsulation of (-)-riboflavin (water-soluble) and (±)-α-tocopherol (liposoluble) using biopolymer and their further characterization. The main focus areas were:

i) The development and characterization of nanosystems;

ii) Assessment of the intake of vitamins in nanosystems developed.

1.3. THESIS OUTLINE

The thesis is organized in five chapters, being this chapter the first and is described the motivation, research aims and outline of the thesis. The chapters 2 to 5 are distributed as follows:

CHAPTER II - "INTRODUCTION": Presents a review of the relevant literature about the theme of the thesis, being provide to the reader the basic information to understand the main issues of this work.

CHAPTER III – "MATERIALS AND METHODS": In this chapter is approached the materials and methodologies used for nanoparticles preparation and their characterization.

CHAPTER IV – "RESULTS AND DISCUSSION": Shows all results and their discussion. In this work was development two nanosystems with different characteristics, so this chapter has two main titles: "ALGINATE/CHITOSAN NANOPARTICLES" and "ZEIN NANOPARTICLES".

CHAPTER V – "GENERAL CONCLUSIONS": Presents the overall conclusions and recommendations and suggestions for future work.

CHAPTER II

INTRODUCTION

2.1 NANOTECHNOLOGY

On 29 December 1959 the physicist Richard Feynman (Nobel Prize for Physics 1965) gave a speech entitled "There's Plenty of Room at the Bottom" in annual meeting of the American Physical Society at the California Institute of Technology and proposed the exploration of materials at scale of atoms and molecules resulting in something that human wouldn't see (Asiyanbola and Soboyejo, 2008; Miyazaki and Islam, 2007). This way began the history of nanotechnology. However, just in 1974 the nanotechnology term was first used and defined by Norio Taniguchi (researcher of University of Sciences in Tokyo) to refer the processing, separation, consolidation and deformation of materials at scale of an atom or a molecule (Asiyanbola and Soboyejo, 2008; Kuan et al., 2012; Miyazaki and Islam, 2007). Currently, the nanotechnology is defined as a science that involves the design, synthesis, characterization and application of materials at the nano-scale, being one nanometer (nm) one-billionth of a meter (Calster, 2006; Commission, 2004; Kuan et al., 2012). Generally, all particles with a size less 1 micrometer (μm) are classified as nanoparticles (Asiyanbola and Soboyejo, 2008; Mohanraj and Chen, 2006), but different interpretations of the nanoparticle dimensions have been proposed and the more stringent classifications for nanoparticle just include particles with a size in the range of 1-100 nm (Calster, 2006; Cushen et al., 2012; Reverchon and Adami, 2006). These stringent classifications are justified by the fact that some physical properties appear when the materials reach the values 1-100 nm, but the legitimate definition classifies the sub-micron scale as nano and extends the limit to 1 μm (Buzea et al., 2007; Naahidi et al., 2013).

The materials at nano-scale change their physics, chemistry and biologic properties and these changes are due to increase of relative surface and quantum effects that begins to control the material at this scale. This way, the utilization of nanomaterials, when compared with normal utilization (micro or macro), is an added advantaged because they can have better properties such as: increased physical strength, chemical reactivity, electrical conductivity, magnetism and optical effects (Buzea et al., 2007; Chau et al., 2007; Siegrist et al., 2008). As result, nowadays, exist ever more technological sectors, e.g. medical, information technologies, energy production and storage, materials science, manufacturing, instrumentation, food, water and environmental and security, that betting on nanotechnology a way to achieve better results for various problems and to create new technologies. In doing so, the investments in nanomaterials research have

increased, making the nanotechnology an expanding technology with many applications (Commission, 2004; Miyazaki and Islam, 2007; Pitkethly, 2004).

Nanotechnology can help the food sector achieving some of its main goals, i.e. improve production efficiency, increase food safety and enhance food characteristics; to do that applications in food industry are focused on the development of nano-size delivery systems for functional ingredients and additives, and innovative packaging. Actually, food sector is one of the sectors with fewer applications at nano-scale, but it is expected that in the near future the nanomaterials in food industry increase (Cushen et al., 2012; Weiss et al., 2006). This increase can be explained by advantages related with the size reduction of materials e. g. improvement of bio-adhesive properties, that includes an increase of adhesive force and prolonged gastrointestinal transit time, possible leading to a higher bioavailability (Acosta, 2009). Based on these unique characteristics nanosytems can solve some problems occurring when using systems at macro- and microscale for delivery of functional compounds; such problems are: compatibility (e.g. aggregation and phase separation) with the food matrix (influencing e.g. appearance, texture, stability, or flavor of the product); release, that should be controlled and only activated once inside the human gut (i.e. some compounds start to be released when mixed with the food product and functional compounds lose their activity); and loss of activity of some functional compounds, affected by light, oxygen and temperature when dispersed in the food matrix. This versatility and limitless potential of nanotechnology allow its application in different systems that are part of food sector (Figure 2.1).

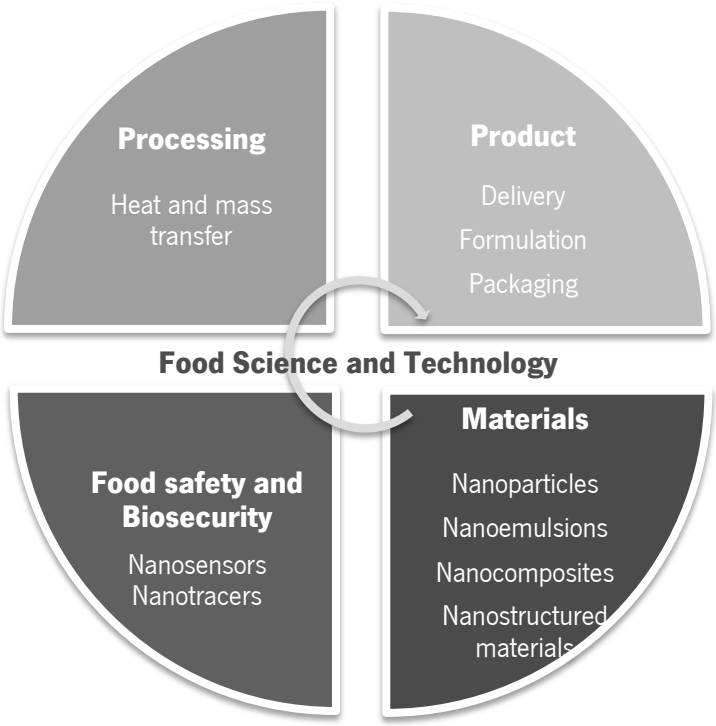


Figure 2.1 - Applications of nanotechnology in food sector. Image adapted from Blasco and Picó (2011) and Weiss et al. (2006).

2.2 EDIBLE AND BIO-BASED MATERIALS

The technological sectors that use nanotechnology have a greatest challenge: to find materials that ensure the efficiency and stability of nanosystems; and in the food sector is also important replacement of non-food-grade materials by bio-based and biodegradable food-grade materials.

In food sector, the physic-chemical properties, such as solubility, molecular weight, glass/melting transition, crytallinity, diffusivity, film forming and emulsifying properties, or costs of material are important factors in materials selection (Sagalowicz and Leser, 2010). Besides that, it is also necessary to take account the future characteristics of nanosystems, e.g. size, morphology, biodegradability, biocompatibility, cytotoxicity, drug loading, release profile and preparation method (Matalanis et al., 2011; Sundar et al., 2010). Initially, the synthetic polymers were the basis of nanosystems, because they have great properties of durability and strength. However, the use of synthetic polymers triggered a lot of environment and health problems, because they are produced from fossil fuels, are not biodegradable and the synthesis of some polymeric materials involves the use of toxic compounds or generation of toxic products. So, it is important to use renewable source-based biopolymers materials of natural origin that not involve the use of

toxic or harmful components in their development, use or degradation (U.S. Congress 1993; Wang et al., 2005).

Biopolymers are polymers produced by biological systems (e.g. microorganisms, plants and animal) or using chemical processes derived from biological starting materials such as amino acids, sugars, natural fats or oils. They are mainly composed by repeating units of saccharides, amino acids, lipids, peptides and nucleic acids, Figure 2.2, and have the characteristic of being degraded by biological activities (Chassenieux et al., 2013; Kuan et al., 2012; U.S. Congress, 1993). Polysaccharides (e.g. alginate, pectin, dextran and chitosan), proteins (e.g. zein, whey protein isolate) and lipids (e.g. medium chain triglycerides, tristearin and corn oil) are some examples of biopolymers. The utilization of biopolymers open the door to new functionalities and applications due distinct advantages of biodegradability, edibility and lack of toxicity (Chassenieux et al., 2013).

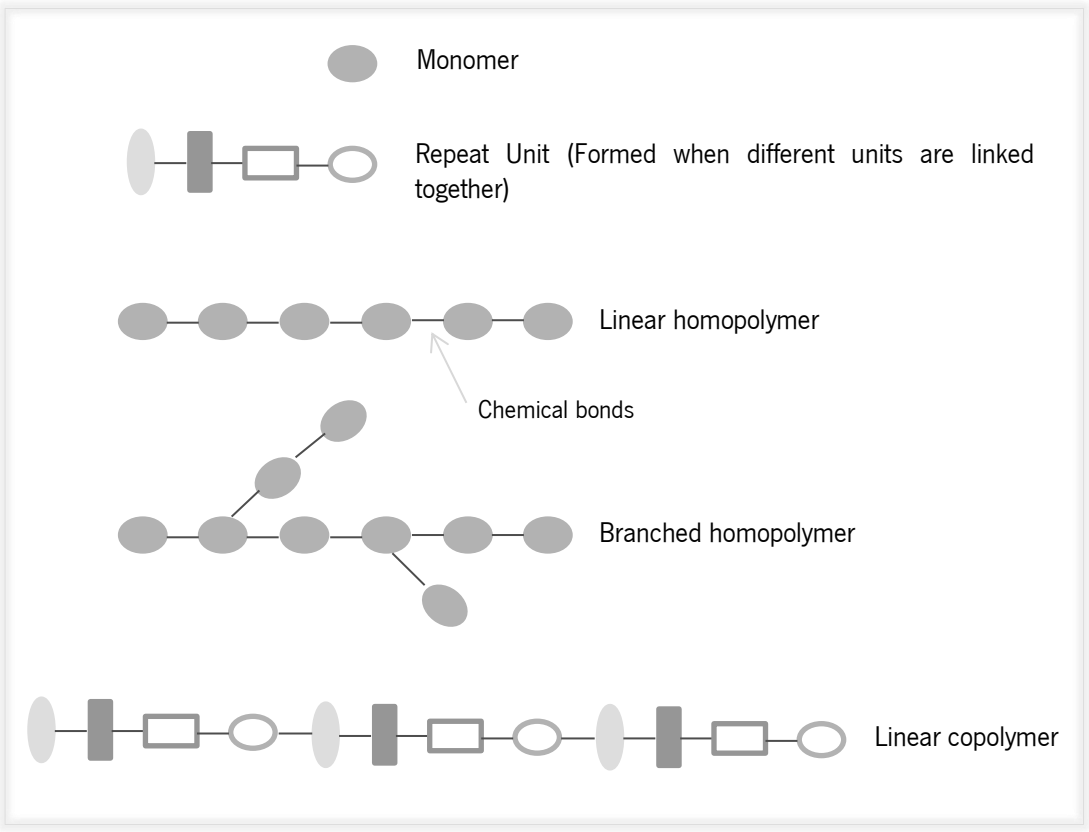


Figure 2.2 – Some basic structure of polymers. Image adapted from U. S. Congress (1993).

2.2.1 POLYSACCHARIDES

Polysaccharides are natural polymers composed by simple sugars (monosaccharide residues) that are linked by different glucosidic bonds (U.S. Congress, 1993; Yang and Zhang, 2009). The

polysaccharides are present in plants, fungi, bacteria, algae and animals, and the source/origin (environmental conditions and history) and their monomer sequence can be responsible for the molecular structure of polysaccharides (Matalanis et al., 2011; Yang and Zhang, 2009). Each polysaccharide has different chemical structures (e.g. type, number, sequence and bonding of the monosaccharide within the polymer chain), which are responsible by molecular weight, degree of branching, structure, flexibility, electrical charge and interaction between polysaccharides. Besides, depending of the ionic groups presented in the polysaccharide chain, some polysaccharides can be neutral (e.g. starch and cellulose), anionic (e.g. alginate, carrageenan, xanthan and gum Arabic), or cationic (e.g. chitosan). This differences allow to polysaccharides present different functional properties such as: solubility, thickening, gelation, water holding capacity, surface activity, emulsification and digestibility (Yang and Zhang, 2009). Based on the existing great variety of polysaccharides it is necessary when choosing one or more polysaccharides for bio-based nanosystems preparation to considerer their physicochemical properties and the desired characteristics of the bio-based nanosystems (e.g. morphology, density, refractive index, size, charge and stability) (U.S. Congress, 1993; Yang and Zhang, 2009).

For the presented work two polysaccharides (alginate and chitosan) were used as material for the development of bio-based nanosystem. Below, are presented the most important characteristics of these two polysaccharides.

2.2.1.1 Alginate

Alginate is an hydrophilic polysaccharide extracted from marine brown algae of the *Phaeophyta* family. It is a linear biopolymer composed by two uronic acids, 1,4-linked-β-D-mannuronic acid (M) and α-L-guluronic acid (G), being carboxylic groups from uronic acids responsible by their negative charge (Figure 2.3). Alginate is considered a non-toxic, biocompatible, biodegradable and mucoadhesive polysaccharide (Silva et al., 2006; Sundar et al., 2010; Vrignaud et al., 2011) and it approved for application in pharmaceutical and food industry.

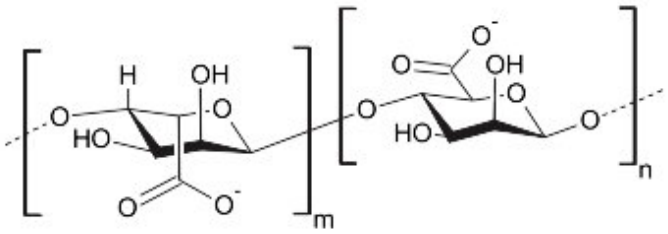


Figure 2.3 – Chemical structure of alginate. The monomeric units of alginate, 1,4-linked-β-D-mannuronic acid (M) and α-L-guluronic acid (G), can be linked in varying sequences, as blocks of alternating gulutonic and mannuronic residues, blocks of guluronic acids and of mannuronic acids (Sundar et al., 2010). Image from Vrignaud et al. (2011).

The solubility of alginate in water depends on the associated cations, for example sodium alginate is soluble in water but when a solution with multivalent cations (e.g. calcium - Ca^{2+}) is used the biopolymer can form a reversible gel (Sarmento et al., 2007; Silva et al., 2006; Sundar et al., 2010). The gelling property of alginate is promoted by the high selectivity of guluronic acid residues that in the presence Ca^{2+} organized in side-by-side blocks formatting the so called "egg box" model. The arrangement is due to an electrostatic interaction between Ca^{2+} and the negatively charged oxygen atoms (Figure 2.4). The gelling property of alginate depends of following factors: sequential order and composition of mannuronic and guluronic acid residues, molecular weight of biopolymer and concentration of counter ions in solution (Sarmento et al., 2007; Silva et al., 2006). However, the addition of a polyelectrolyte complex such as chitosan or poly-L-lysine induces a polyelectrolyte complex formation and stabilization of the alginate pre-gel nucleus into individual sponge-like particles (Sarmento et al., 2007). The hydrophilic and gelling properties, make of alginate a biopolymer with a considerable potential for encapsulation of hydrophilic components (Vrignaud et al., 2011).

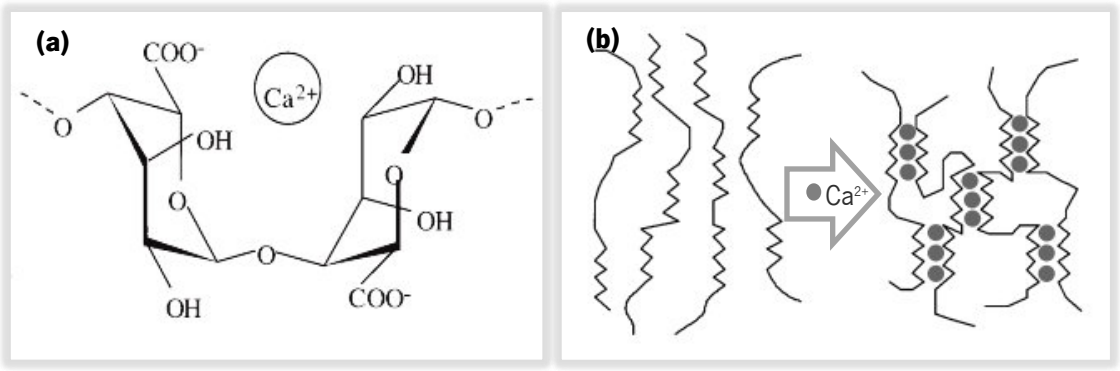


Figure 2.4 – (a) Binding of cation Ca^{2+} and guluronic acid residue of alginate; (b) "Egg box" model. Image adapted from Chavanpatil et al. (2007) and Myrvold and Onsoyen (2004).

2.2.1.2 Chitosan

Chitosan is a cationic polysaccharide derived from the *N*-deacetylation of chitin, the second most abundant natural biopolymer. The chitin is obtained from exoskeletons of marine arthropods (e.g. shells of crabs, shrimps and krill), walls of fungi (e.g. fungal mycelia) and cuticle of insects. It is a linear cationic copolymer composed by N-acetylglucosamine and glucosamine residues with β-1,4-linkage (Mourya and Inamdar, 2008; Shahidi et al., 1999; Sundar et al., 2010). Chitin has unique properties such as ability to polyoxysalt formation, chelate metal ions and optical structural characteristics. However, it is insoluble in water and in most organic solvents, being its biodegradation very slow. So, chitosan, chitin deacetylated form, appears as a great solution for these solve that problems (Kumar, 2000; Shahidi et al., 1999).

Chitosan is a linear copolymer composed by repeating units of 2-amino-2-deoxy-β-D-glucan with glycosidic linkages, where the amine groups confer of chitosan special properties (e.g. high charge density, readily available for chemical reactions and salt formation with salts) (Agnihotri et al., 2004; Sundar et al., 2010). Chitosan present three types of reactive functional groups: an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 position, respectively (Shahidi et al., 1999). The position of free amino and N-acetyl groups is responsible by solubility of chitosan; nevertheless, the chitosan solubility is improved with organic solvents as aqueous acids (e.g. formic acid and acetic acid) (Agnihotri et al., 2004; Kumar, 2000; Mourya and Inamdar, 2008). The increase of solubility is due to protonation of amino groups by acids that along the chitosan chain increase the polarity and the degree of electrostatic repulsion (Mourya and Inamdar, 2008). *N*-deacetylation of chitin during chitosan formation is not complete, and chitosan can have a degree of deacetylation between 40-98% (Mourya and

Inamdar, 2008) and molecular weight ranged between 3.8 and 2000 KDa; these two characteristics can influence chitosan properties being important factors for its use in several applications (Mourya and Inamdar, 2008; Sundar et al., 2010).

This biopolymer has great properties such as biocompatibility, biodegradability, non-toxicity, and several studies indicated chitosan with a great adsorption and muchoadhesive properties, antifungal activity, ability to promote metabolic changes, an excellent film-forming ability and micro/nanoparticles developing. Due to these properties, the chitosan have a great potential for food, environmental, pharmaceutical, medical and agriculture sectors (Agnihotri et al., 2004; Kumar, 2000; Peniche and Acosta, 2003; Sarmento et al., 2007; Sundar et al., 2010; Weber, 2000).

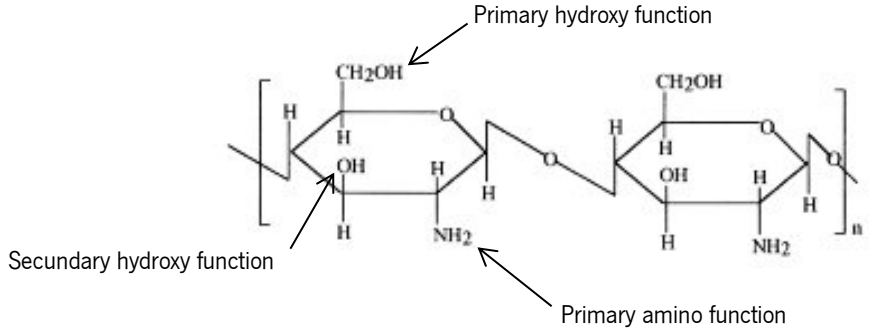


Figure 2.5 – Chemical structure of chitosan. Image adapted from Kumar (2000) and Mourya and Inamdar (2008).

2.2.2 PROTEINS

Proteins are biopolymers that are present in plants (e.g. zein, gluten and soy) or animals (e.g. casein, whey, collagen and keratin) composed by a linear sequence of amino acids linked by peptide bonds; exist 20 amino acids and many different combinations and sequences, so the proteins can have distinct structures and properties (Weber, 2000; Nelson and Cox, 2000).

Amino acids are composed by a carboxyl group (COOH) and an amino group (NH_3) linked to a carbon atom. Besides that, they have a side chain, R group, which varies in structure, size and electrical charge for each amino acid being responsible for their solubility and polarity. Taking into account properties of R group, the amino acids can be classified as aromatic, nonpolar or polar and uncharged or positively or negatively charged (Nelson and Cox, 2000). The amino

acids bind to each other by amide linkages between the amino group of one amino acid and the carboxyl group of another by removal of the elements of water, these linkages are called of peptide bonds. Polypeptide is the product of linkages between two or more amino acids and have a low molecular weight, when the linkages contain more than 50 amino acids presenting a higher molecular weight (ranged between 6/10 and 40 000 KDa), they are called of protein (McKee and Mckee, 2013; Wade, 2012).

Proteins have a great range of structural and catalytic properties, being their amino acids sequence, environmental conditions and the physico-chemical treatment (e.g. exposure to different temperatures, pressures, solvents, pH values and ionic composition) responsible for it. As a result of their diversity/complexity, they can be classified according to chemical composition, shape, structure or function as shown in Figure 2.6 (McKee and Mckee, 2013; Wade 2012; Nelson and Cox, 2000).

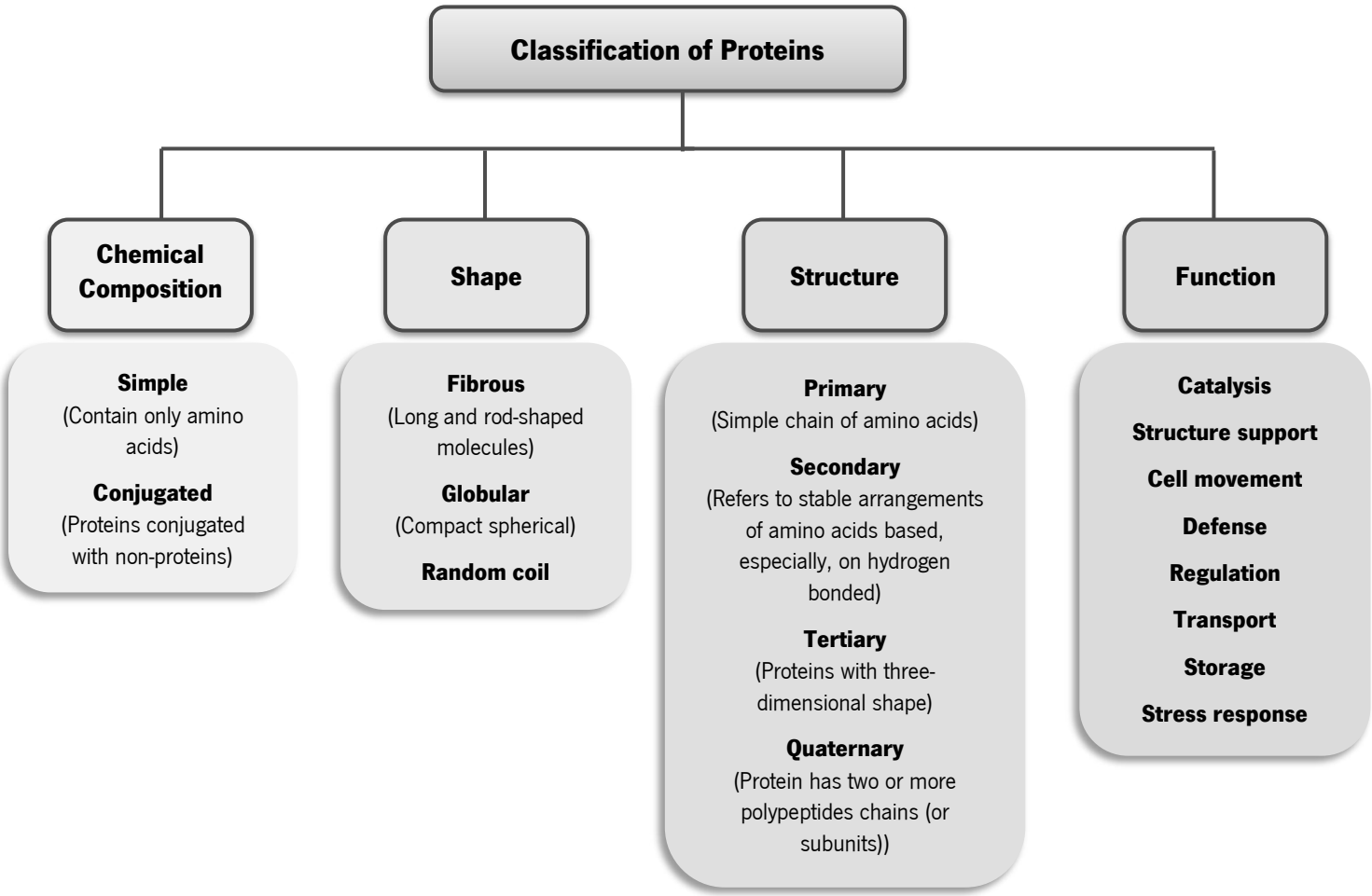


Figure 2.6 – Classification of proteins and their characteristics.

The proteins not only have a great biochemical interest, but also, due to their functional properties, such as self-assembly, emulsification, gelation, foaming, water binding capacity and gas barrier, have a big potential as technological biomaterial (Chassenieux et al., 2013; Chen et al., 2006; Weber, 2000). For example, in food sector, they can be used to produce biopolymer-based delivery systems or film forming solutions for packaging materials being these systems relative easy to prepare, biodegradable, immunogenic, nontoxic and present a greater stability *in vivo* and during storage (Sundar et al., 2010). However, different factors must be considered when proteins are chosen as biomaterial and is important to: a) establish appropriate physicochemical conditions for protein associate with other proteins or non-proteins; b) establish the electrical characteristics between the involved molecules when electrostatic interactions are used to structure formation; c) know the properties of biopolymer particles formed after protein association (e.g. morphology, physical properties, size, charge and stability) (Matalanis et al., 2011).

Due to the potential of proteins as bio-based material zein was used for nanostructure preparation. Below are presented the most important characteristics and the reason for this choice.

2.2.2.1 Zein

Corn or maize (*Zea mays*) is one of the most important cereals on food industry and their main components are starch, proteins and water. The proteins represent one of the greatest components and about 75 % are present in endosperm tissue, being zein the major storage protein of corn (Lawton, 2002; Shukla and Cheryan, 2001).

Zein was first isolated in 1821 by John Gorham and was described as a soft, ductile, tenacious and elastic protein, but only in 1939 began the commercial production due their deficiency in essential amino acids (e. g. lysine and tryptophan) and insolubility in water. However, since its isolation in 1821, zein has scientific interest as a polymeric material and their properties such as ability to form tough, glossy, hydrophobic grease-proof coatings and their resistance to microbial attack has been explored (Lawton, 2002; Shukla and Cheryan, 2001). Zein is also biodegradable and renewable and is one of the few cereal proteins that are extracted in relatively pure form. So, zein is considered as safe and food grade ingredient with a great potential for: film, coatings and plastics applications, encapsulation and controlled release of fat-soluble compounds and for that

can be used in food and pharmaceutical sector (Lawton, 2002; Luo et al., 2011; Shukla and Cheryan, 2001; Weber, 2000).

Zein is considered a prolamin and is amphiphilic due their structure containing three quarter of lipophilic and one quarter of hydrophilic amino acids residues (Luo et al., 2011). The composition of amino acids sequence is responsible for its solubility: zein is insoluble in water except in the present of alcohol such as ethanol and isopropanol, or in the presence of high concentrations of urea or alkali and anionic detergents (Lawton, 2002; Shukla and Cheryan, 2001). It has a surface charged that varies according to the pH of environment (Deo et al., 2003). During zein extraction from corn is used a suitable solvents and as a result is obtained a native form of protein in heterogeneous mixture of disulfide-linked aggregates with a molecular weight of 44 000 Da (Shukla and Cheryan, 2001). This protein is composed by a mixture of four distinct types of peptides that varying in molecular size, solubility and charge and can be separated using their different solubility. The four peptides are denominated as α-, γ-, β- and δ-zein, being α- and γ-zein the most abundant types (Lawton, 2002; Shukla and Cheryan, 2001). Exist three models for structure of zein (Shukla and Cheryan, 2001; Wang and Padua, 2012a): a) helical wheel model for zein where antiparallel helices are clustered within a distorted cylinder stabilized by hydrogen bonds (Argos et al. 1982); b) model where helices are linked by glutamine-rich bridges that are aligned in an antiparallel fashion (Matsushima et al. 1998); c) a three-dimensional structure, being a coiled-coil super helix with lutein at the core (Momany et al. 2006) (Figure 2.7).

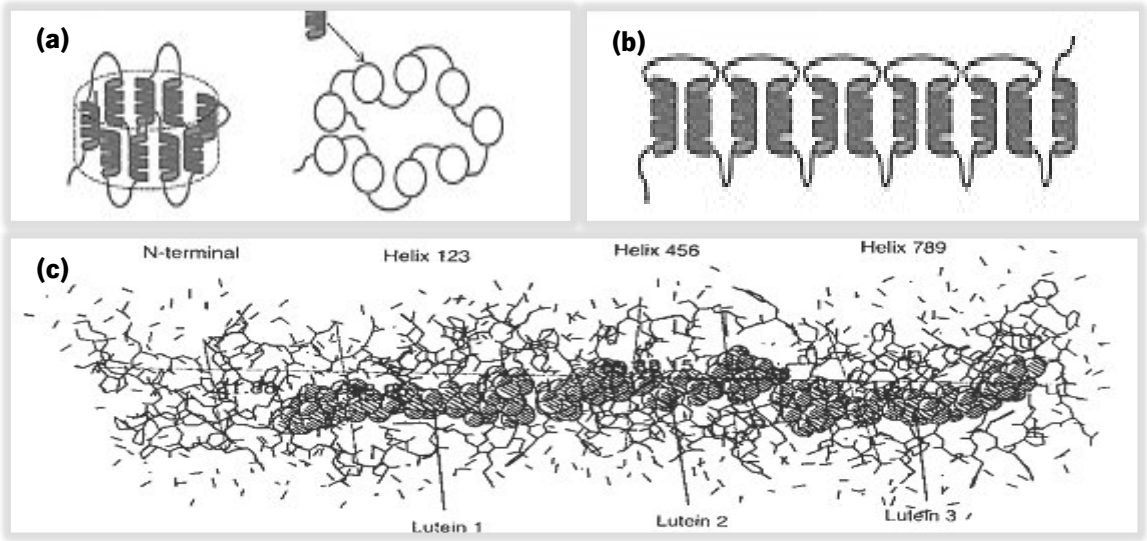


Figure 2.7 – Structure models of zein: (a) Argo's model; (b) Matsushima's model; (c) Momany model. Image (a) and (b) adapted from Y. Wang and Padua (2012) and (c) adapted from Momany et al. (2006).

2.3 NANOSYSTEMS IN FOOD SECTOR: PREPARATION AND CHARACTERIZATION

In general, the selection of materials to prepare nanosystems is very important and a great challenge, but the nanosystem and their characteristics are one of the most important factors to ensure the efficiency and stability of nanosystem selected.

Nanosystems can be classified based on (Morris 2010; Silva et al. 2011):

- the major material used in their fabrication;
- the production method (e.g. bottom-up or top-down);
- the predominant forces in the system (e.g. electrostatic, hydrogen bonding);
- the main properties of the system (e.g. mechanical and optical properties) and
- the system's overall free energy (thermodynamic or kinetic stable systems).

One of the major trends in the development of nanosystems is to combine different approaches such as: mixtures of the materials used, combination of bottom-up strategies (nanosystems are obtained by interaction of small components such as layer-by-layer and self-assembly) and top down strategies (formation of nanosystems by physical-chemical processes that involve reduction of particle size to nano-size, e.g. homogenization), and intervention of different types of forces during the production process (Choi et al., 2011; Hu et al., 2007; Lertsutthiwong et al., 2008; Yu et al., 2006) in order to achieve a desired functionality. In the last years a large number of different delivery nanosystems have been developed, often using a trial-and-error approach, which leads to a great number of developed and well characterized nanosystems however without a final and conclusive application.

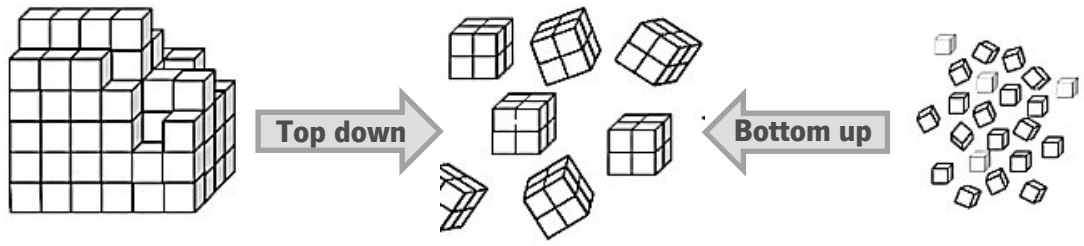


Figure 2.8 – Top down and bottom up approaches. Image adapted from Reverchon and Adami (2006).

In the food sector, the utilization of delivery systems, in particularly, at the nano scale showed to be promising as active vectors. Some of the reasons are their capacity to release bioactive compounds and their high intracellular uptake. Their capacity to improve the stability of active substance and their high encapsulation efficiency (Mora-Huertas et al., 2010).

2.3.1 NANOCAPSULES

Nanocapsules, also called nanoparticles, are constituted by an external cavity consisting of a polymeric membrane and an internal part composed by a liquid or polymeric matrix that contains the active compound (Fang and Bhandari, 2010; Mora-Huertas et al., 2010). The selection of an appropriate method for nanocapsules production is an important step in order to have nanostructures with properties allowing the desired performance and functionality. The method will depends on the physicochemical character of the polymer, the bioactive compound, the final application and the desired properties for the nanocapsules (e.g. particle size, particle size distribution, surface area, shape, solubility, encapsulation efficiency and release mechanism) (Ezhilarasi et al., 2013; Pal et al., 2011; Rao and Geckeler, 2011). The methods for preparation of nanocapsules are divided in three main techniques: polymerization (preparation of nanocapsules through polymerization of monomers using classical polymerization or polyreactions), dispersion of preformed polymers (nanocapsules are obtained directly preformed from synthetic, semi-synthetic or natural polymers) and ionotropic pre-gelation/coacervation (Pinto Reis et al., 2006; Rao and Geckeler, 2011). During and after nanocapsules formation is important their characterization and in order to evaluate main characteristics. For that is commonly used two types of methods: characterization methods (e.g. measurement of size, morphology, charge and stability) and chemical composition methods (Blasco and Picó, 2011).

2.3.1.1 Preparation Methods

Ionotropic pre-gelation/coacervation

Ionotropic pre-gelation/coacervation methods are based on the ability of polyelectrolites to cross-link in the presence of a counter-ion (Patil et al., 2010) being commonly used biodegradable hydrophilic polymers (e.g. chitosan, sodium alginate and gelatin) to form nanocapsules (Mohanraj and Chen, 2006). Ionotropic pre-gelation/coacervation involves the blend of a polymer, with positive or negative charge, and a cationic counter-ion (e.g. calcium chloride) or polyanionic (e.g. sodium tripolyphosphate). Then a second polymer is added that allows polyelectrolyte complexation and nanocapsules formation. The bioactive compound is entrapped in the core of the first polymer and stabilized after the addition of the second polymer. The polyelectrolyte solutions and bioactive compound are added in a counter-ion solution or vice-versa drop wise with a needle under magnetic stirring (Calvo et al., 1997; Sarmento et al., 2007). This

methodology is based in physical-chemical mechanisms; therefore it is affected by several parameters such as stirring, flow rate of solutions, polymers characteristics (e.g. molar mass, flexibility and charge), pH, ionic strength, concentration and polymers ratio (Ezhilarasi et al., 2013). The nanocapsules produced by this method can be used, for example, to encapsulation of riboflavin, curcumin, tea catechins and capsaicin (Das et al., 2010; Hu et al., 2007; Wang et al., 2008).

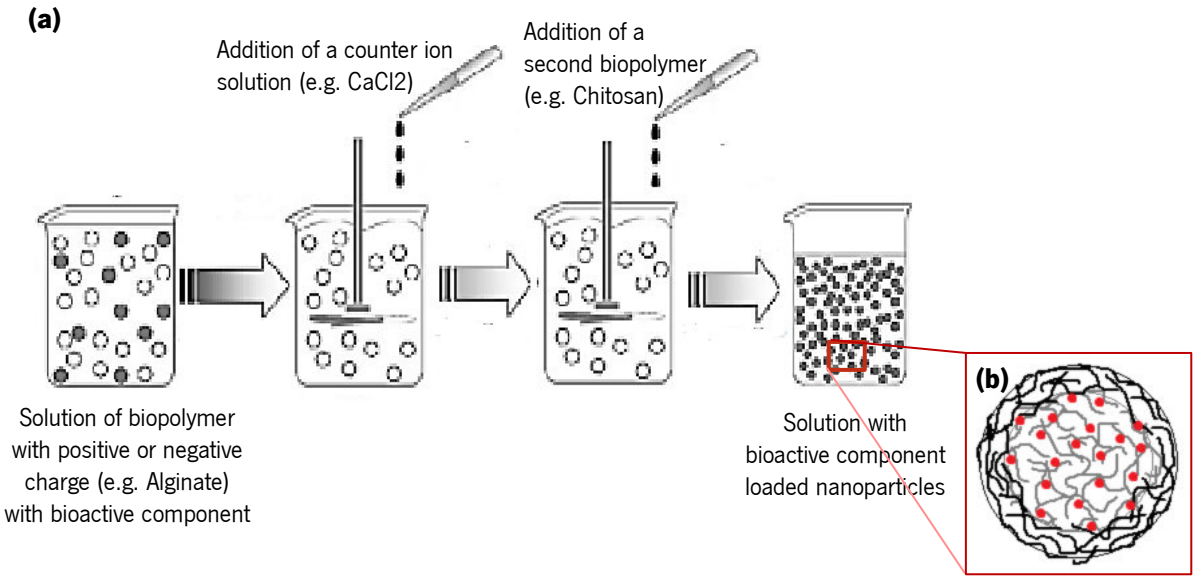


Figure 2.9 – (a) Schematic of Ionic pre-gelation/coacervation method; (b) Structure of nanoparticle with bioactive component loaded: ● Bioactive component; ∿ Chitosan; ∿ Alginate. Images adapted from (a) Nagavarma et al. (2012) and (b) Y. Zhang et al. (2011) and Mora-Huertas et al. (2010).

Self-assembly

The self-assembly process uses polymers with capacity to form spontaneously compact and stable nanocapsules. One of the main driving forces for self-assembly is amphiphilicity and some weak interactions such as van der waals, capillary, π-π and hydrogen bonds. Besides that, it is also important to exist a long-range repulsion, for example, thermodynamic incompatibility, phase separation, excluded volume and columbic repulsion, between the polymers and the medium. Thus, this method consists in a polymer structure organization without help or guidance from external agents (Reches and Gazit, 2006; Sanguansri and Augustin, 2006; Wang and Padua, 2012a). The nanocapsules formed by self-assembly are dependent on the size and shape of polymer, composition of solution and environmental stresses. Materials such as zein, casein,

chitosan and polylactic acid are examples of polymers used in the production of nanocapsules by this method. Typically, nanocapsules have an average size of 50-100 nm (Sanguansri and Augustin, 2006). Some works show that this process is able to produce nanocapsules that can be utilized in food industry e.g. in the encapsulation of vitamins (Li et al., 2011).

2.3.1.2 Characterization Methods

Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) or also known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QLS) is a not invasive, versatile and useful set of techniques to measure the particle size, polydispersity index (PDI), zeta potential and (in some cases) the shapes of particles with a size down to 1μm in solution. Normally, this technique is used in the characterization of emulsions, micelles, polymers, proteins, nanoparticles or colloids (Malvern Instruments, 2004; Pecora, 2000).

The particle size is the diameter of nanoparticles and the polydispersity index (PDI) is parameter that gave us the distribution of nanoparticles size. The PDI is dimensionless and values greater than 0.2 indicate that the sample is not monodisperse, in other words, has a very broad size distribution. The measurements of these parameters are based in a simple principle: illuminating of sample with a laser and analyzing the scattered light, where the detector position can be at either 173° or 90° depending on the model of Zetasizer Nano model. Due to different sizes of particles, the light are scattered in different directions and with different intensities, being monitored the scattered light intensity (particle size) and Brownian motion (random movement of particles carrying on the time - the large particles have a slow moving and small particles have a quick). The relationship between these parameters (movement and size of particles) allow to determine the diameter of particles using the Stokes Einstein equation, Eq. 2.1 (Dahneke, 1983; Goldburg, 1999; Malvern Instruments, 2004).

$$d(\mathrm{H}) = \frac{k\mathrm{T}}{3\,\pi\eta\,\mathrm{D}} \qquad \text{Eq. 2.1}$$

Hydrodynamic diameter, d (H);

Boltzmann's constant (k);

Absolute temperature (T);

Viscosity (η);

Translational diffusion coefficient (D).

The average size (z-average diameter) estimated by DLS is obtained through an intensity distribution, but these results can be converted in a volume distribution that can also be converted to a number distribution (Malvern Instruments, 2004).

The Zeta Potential (ζ) is other parameter that can be measurement by DLS and consist, essentially, in measurement of electrostatic/charge at the surface of the nanoparticle through a laser that passes the sample cell. The scattering is detected at an angle of 90°. This measurement can give information related with dispersion, aggregation or flocculation of dispersions, emulsions and suspensions (Kirby and Hasselbrink, 2004; Malvern Instruments, 2004).

Nanoparticle Tracking Analysis (NTA)

The NTA is a recent and innovative technique that combines a laser light scattering microscopy with a charge-coupled device camera. So, is possible the visualization and recording of particles in a solution. NTA determines particle size and measures the concentration of particles with size ranged from 30 to 1000 nm. These measurements are possible due the capacity equipment to identify and track the particles through Brownian motion than then are used in a expression derived of Stokes-Einstein equation for particle size calculation. For the measurement of particles concentration is calculated the scattering volume through dimensions of field of view and the depth of the laser beam, then scattering volume calculated is extrapolated and is determined the average of concentration (number of particles per milliliter of sample). Furthermore, the scattered light is captured by a charge-coupled device camera, being obtained an image and recorded a video of the particles of sample (Filipe et al., 2010; Gillespie et al., 2011).

Transmission Electron Microscopy (TEM)

TEM is a conventional microscopy technique based in transmission of electron and is considered the most powerful of microscopes. The aim of this technique is to provide information about morphologic, compositional and crystallographic of samples. In general, the TEM has an electron source, thermionic gun, electron beam, electromagnetic lenses, vacuum chamber, condensers, sample stage, phosphor or fluorescence and a computer (Reimer, 1984; Wang, 2000).

TEM images with present high-resolution and are two dimensional, being obtained through an electron interaction whit sample. In generally, an electron beam is emitted and will be propagate along different directions, when this electron beam interacts with sample that are present in TEM grids can happen three different interactions: unscattered electrons – transmitted beam, elastically scattered electrons – diffracted beam and inelastically scattered electrons. The contrast of image depend of the amount of sample that electron beam pass and the sample material, being a good image when a contrast of sample is greater relative to the background. Due to their advantages (e.g. high-quality and detailed images, can provide information about surface, shape, size, compounds and structure of particles), this technique is used in different fields, such as nanotechnology, medical, biological, life sciences and industrial fields (Reimer, 1984; Voutou and Stefanaki, 2008).

Release Profile

The release profile of a bioactive compound is equally important as the bioactive polymer formulation because to understand the release profile can be crucial for product development and potential applications (Kumari et al., 2010; Pinheiro et al., 2012a, 2012b). Commonly, the release profile is also determined as an alternative to characterize nanoparticles, along with others parameters (e.g. particle shape, size, zeta-potential and encapsulation efficiency) (Zambito et al., 2012).

A method used to evaluate the release profile is dialysis membrane and this method consists, essentially, in to separate the nanoparticles with bioactive compound loaded from the bulk media by a dialyzing membrane, Figure 2.10. Over the time, occurs the passage of compound loaded in nanoparticles through the membrane into the release media and a sample is withdrawn at intermittent intervals to evaluate the compound release (Souza and Deluca, 2005).

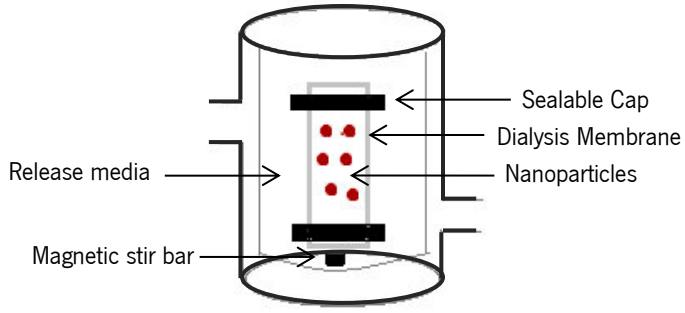


Figure 2.10 – Schematic presentation of set-up for release profile by dialysis membrane method. Image adapted from Prata et al. (2008) and Souza and Deluca (2005).

There are four mechanisms that can be responsible by release profile of bioactive compound from nanoparticles: Mechanism of Fick's diffusion, polymer matrix swelling, polymer erosion and degradation; and the different mechanisms depending on the system and environmental conditions. However, to understand the release mechanism involved is necessary to use mathematical modeling (Pinheiro et al., 2012b). The release profile depends of various parameters, such as value of diffusion coefficient, size and shape of bioactive compound and polarity of the matrix (Romero-cano and Vincent, 2002).

2.4 BIOACTIVE FOOD COMPOUNDS

The normal development and growth of human depend of different constituents, being one of them bioactive compounds. These components are substances/compounds present in animal and plants that can have different physiological functions, such as health promoting or disease preventing effect (De Vos et al., 2010). Each bioactive component has their own molecular characteristics (e.g. molecular weights, conformations, polarity and charge) that are responsible by physicochemical differences between bioactive components (e.g. solubility, partitioning, physical state, interactions, optical characteristics and chemical stability). They can be divided into bioactive molecules such as vitamins, bioactive peptides, antioxidants, fatty acids and minerals; and bioactive living cell such as probiotics (McClements et al., 2009; de Vos et al., 2010), which can be used in food industry to improve taste, aroma, stability, nutritional value and appearance of fodd products (Augustin and Hemar, 2009; Raybaudi-Massilia and Mosqueda-Melgar, 2012). Some of these bioactive compounds are sensitive to heat, moisture and/or pH and can be slowly degraded and lose their activity during utilization. This way, the food industry has a great challenge: protect these components during production, storage and consumption.

The micro- and nanoencapsulation with edible materials appears as a solution to overcome these problems, in order to protect the bioactive compound against adverse conditions and simultaneous keep the functionality of the bioactive compound. Due to their different properties, is needed to develop micro- or nanostructurs with specific physicochemical characteristics to promote compatibility and stability between bioactives compounds and the structure used. Besides that, it is also essential to select a system easily incorporated into the food and that does not interfere with the texture and taste of the food (De Vos et al., 2010).

As already mentioned the vitamins are an example of bioactive component that plants and microorganisms have the capability to produce for the normal organism functioning. The human haven't this ability, but it is recognized that vitamins are essential for maintain health and well-being of human (Spitzer and Schweigert, 2007). Below are presented the most important characteristics of them and their importance for us.

2.4.1 VITAMINS

In nineteenth century, the physiologists thought that food was a source of only four types of nutrients (protein, fat, carbohydrate and ash) and water, being vitamin only associated with the preventing of vitamin disease and their biochemical functions. Today, this opinion is different and the term *vitamin* is a common word that reveals an interrelationship between diet/food and health (Combs Jr, 2012).

Vitamins are defined as organic molecules with small dimension and low molecular weight being presented in food in small amounts. They are considered essential for normal maintenance, growth and development of human organism and their absence cause a specific deficiency syndrome. The incapacity of human to synthetize some vitamins (exception of vitamin D and B_3) lead to their necessity to get through food products (Ball, 2008; Combs Jr, 2012). In general, the pathway of vitamins biosynthesis is complex and is biologically more efficient for human to ingest the vitamins that synthetize through simples' molecules. This fact has a disadvantage that is the dependency of the other organisms to get essentials components to life (Berg et al., 2004).

In general, all vitamins have similar characteristics, but their chemical properties are very different (e.g. the vitamins A, K and C are enzymatic co-factors, E and C act as antioxidants and vitamins A and D are hormones). Besides that, vitamins can be divided into liposoluble and water

soluble vitamins according their solubility in water (Combs Jr 2012; Berg, Tymoczko and Stryer 2004). As other bioactive components, the vitamins are also sensitive and unstable when exposed to inadequate temperature, oxygen, light and moisture. So, the micro- or nanoencapsulation can help to reduce these limitations and to provide a prolonged shelf life and stability. The encapsulation of vitamins can also use as nutritional supplements, to mask the flavor of vitamins and mineral or to improve taste of the food products (De Britto et al., 2012).

2.4.1.1 Riboflavin (Vitamin B_2)

The vitamins of complex B (B_1, B_2, B_3, B_5, B_6, B_8, B_9 and B_{12}), vitamin C, H and pantothenic acid are soluble in water, being classified as water-soluble vitamins. The vitamin B_2 is a flavin and was first isolated in 1933 from egg. Initially, was called ovoflavin, but when was determined their chemical structure was called riboflavin because molecule contained a ribose-like structure (ribitol). Their molecule also contain an isoalloxazine tricyclic ring and methyl groups (Figure 2.11) and are present in all animal and plants cell being the most common sources of riboflavin wheat bran, milk and milk products, eggs, meats and vegetables (Ball, 2008; Sebrell Jr and Harris, 1954).

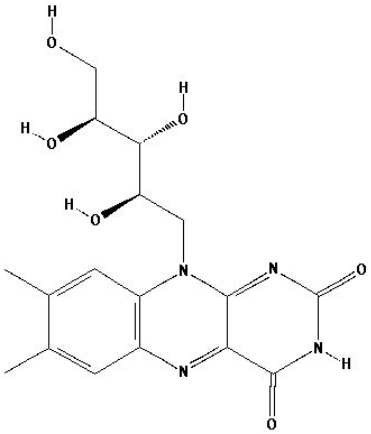


Figure 2.11 – The Chemical structure of riboflavin.
Image from http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=6759 (September 19, 2013).

The riboflavin is a precursor of coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) that act as electron carriers in oxidation-reduction reactions. These coenzymes are called flavoproteins and are essential for hundreds of enzymes FMN- and FDA-dependents, amino acids metabolism, energy production and activation of folate and pyridoxine coenzymes.

The absence of riboflavin has been linked to cancer appearance, cardiovascular disease, anemia and neurological and developmental disorders (Burgess et al., 2009; Roje, 2007).

Relatively of riboflavin stability, is known that this vitamin lose 50% of their activity when it is exposed to sunlight or any UV light. On the other hand, when riboflavin is heated, for example when some food product that contains riboflavin is cooked, the riboflavin keeps their stability. However, in milk pasteurization riboflavin loses about 20 % of it activity and in food sterilization with radiation or ethylene oxide completely destroid. For food industry is important to solve this instability and to protect riboflavin properties in food products. Actually, the riboflavin is availed in oral preparation (alone or most commonly in multivitamin preparations) or as an injectable solution and due to their properties and yellow color, it also used to enrichment and provide color and/or flour of food and beverages (Spitzer and Schweigert, 2007).

2.4.1.2 Tocopherol (Vitamin E)

The term vitamin E includes eight fat soluble components that are found in nature: four called tocopherols (α-, β-, γ- and δ-tocopherol) and four tocotrienols (α-, β-, γ- and δ-tocotrienols); being the most common and biologically active α-tocopherol. The vitamin E was first isolated from wheat germ in 1936 and the name tocopherol is derivate from Greek word *tocos* that are mean "to bring forth in childbirth" due to its essential role in the reproduction of various species. In 1937 the structure and synthesis was clarified and the ending *–ol* was added to the name for identifies the substance as an alcohol (Ball, 2008; Gonnet et al., 2010; Spitzer and Schweigert, 2007).

The vitamin E is a liposoluble vitamin as vitamin A, D and K, being necessary the presence of lipids, bile and pancreatic juice for their absorption in the human organism. This vitamin is an organic molecule composed by a chromanol ring and a phytol side chain that can be eight different conformations: tocopherols have a saturated side chain and tocotrienols have three conjugated double bonds, where α-, β-, γ- and δ- prefixes indicated methyl groups position on chromanol ring, (Figure 2.12). Although, the tocopherols and tocotrienols have different structures, they present similar functions (Gonnet et al., 2010).

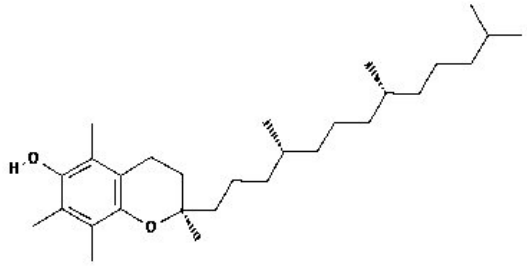


Figure 2.12 – Chemical structure of α-tocopherol.

Image from http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=14985 (September 19, 2013).

Actually, is known different positive effects of vitamin E, for example, on cardiovascular, immune, reproduction systems, and also in cancer prevention and protection of membrane lipids against oxidation. The most important of sources of vitamin E are vegetable oils, nuts, whole grains and wheat germ, having vegetables, fruits, fish and meat a relatively low content. The absence of this vitamin has been linked to neurological disorders, but is reversible whit ingestion of vitamin E supplements or through food and beverages fortified with vitamin E (Gonnet et al., 2010; Wagner and Chen, 2004). However, the food industry has a great challenge to solve: improve physical stability of vitamin E in beverages and food. Due to the insolubility of vitamin E in water it is necessary create a system that can be used in fortification of food products and improve the solubility/stability of vitamin. Another problem is the turbidity of beverages in the presence of vitamin E. For an ideal food product, it's important a good physical stability and normal appearance. This way, again, micro- nanoencapsulation appears as a good solution, being used, for example, biopolymers with starch or liposomes or clusters of molecules such as cyclodextrin (Spitzer and Schweigert, 2007; Wagner and Chen, 2004).

CHAPTER III

MATERIALS AND METHODS

3.1. DEVELOPMENT OF NANOPARTICLES

3.1.1 ALGINATE/CHITOSAN NANOPARTICLES PREPARATION

3.1.1.1 Materials

Sodium alginate with MW≈15900 Da and viscosity ≈ 200 cp from *Laminaria hyperborea* was purchased from Manutex RSX (Kelco International, Ltd., Portugal). Chitosan with 91.23% deacytelation degree was purchased from Golden-Shell Biochemical Co., Ltd (China). Calcium chloride ($CaCl_2$) was obtained from Panreac (Panreac Quimica SA, Barcelona, Spain) and the component bioactive, (-)-riboflavin, with 376.36 g/mol of molecular mass was obtained from Sigma-Aldrich Chemical Co. Ltd (St. Louis, MO, USA).

3.1.1.2 Methodology

The development of alginate/chitosan nanoparticles was based the methodology described by Sarmento et al. (2007). Ionotropic pre-gelation method followed by polycationic crosslinkg was used as production method and the biopolymers alginate and chitosan were used as main materials. Briefly, alginate was dissolved in distilled water and the chitosan was dissolved in 1 % acetic acid and the pH of alginate and chitosan solutions was initially set to 4.9 and 4.6, respectively. Through a preliminary study (ANNEX A), the optimal concentrations (selected based in the particle size and PDI) of alginate and chitosan were determined, being, respectively, 0.63 mg/mL and 0.4 mg/mL. then, 7.5 mL of 18 mM calcium chloride solution was dropped at a flow rate of 0.125 mL/min into a beaker containing 117.5 mL of alginate solution at a constant homogenization using an Ultra-Turrax (T 25, Ika-Werke, Germany) at 20 000 rpm. Then, 25 mL of chitosan solution were added dropwise into the previous solution with a stirring of 600 rpm and a flow rate of 0.278 mL/min. After the addition of chitosan solution, alginate/chitosan nanoparticles were maintained at constant stirring during 30 minutes. For the water-soluble vitamin encapsulation, (-)-riboflavin was dissolved in the alginate solution before addition of calcium chloride solution. Different concentrations of vitamin were tested (0.0475, 0.065, 0.095, 0.13 and 0.19 mg/mL) based on the maximum solubility and the optimal concentration of vitamin was determined (selected based in particle size and PDI), being 0.065 mg/mL. For nanoparticles preparation were used a syringe pump (NE-1000 Multiphaser™ Programmable Syringe Pump, New Era Pump Systems Inc.), two polypropylene syringes, one of 10 mL (Injekt

10 mL Luer) and other of 50 mL (Ominifix® 50 mL Luer), obtained from B|Braun Ltd. (Melsungen, Germany) and still needles of 0.60 x 25 mm (100 Sterican®, B|Braun Ltd.).

3.1.2 ZEIN NANOPARTICLES PREPARATION

3.1.2.1 Materials

Corn zein with 88.0 to 96.0% of protein content was obtained from Acros Organics (Geel, Belgium). (±)-α-Tocopherol (TOC) with 98% purity and molecular mass of 430.7 g/mol was purchased from Sigma-Aldrich Chemical Co. Ltd (St. Louis, MO, USA) and ethanol absolute PA (M=46.07) obtained from Panreac (Panreac Quimica SA, Barcelona, Spain).

3.1.2.2 Methodology

Nanoparticles were prepared using a self-assembly method where a zein solution (dissolved in 75% aqueous ethanol solution) was used as a main material.

Initially, were tested different parameters for zein nanoparticles preparation ANNEX A, and were determined the optimal parameters. Briefly, 10 mL of zein solution (2 mg/mL) at flow rate of 0.167 mL/min were dropwise in 50 mL of distilled water with a constant stirring of 700 rpm. After zein solution addition, zein nanoparticles were maintained in constant stirring for 15 minutes. For this process were used a syringe pump (NE-1000 Multiphaser™ Programmable Syringe Pump, New Era Pump Systems Inc.), a polypropylene syringe of 10 mL (Injekt 10 mL Luer, B|Braun Ltd) and a needle of 0.60 x 25 mm (100 Sterican®, B|Braun Ltd.).

For the encapsulation of compound bioactive, TOC was dissolved in zein solution and then was dropwise in distilled water following the procedure described before. The optimal concentration of TOC was 1 mg/mL (selected based in the maximum solubility and preliminary studies).

3.2 CHARACTERIZATION

3.2.1 NANOPARTICLE SIZE, POLYDISPERSITY INDEX AND ZETA POTENTIAL

Size, polydispersity index (PDI) and zeta potential of nanoparticles were measured through dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK) equipped with a He-Ne laser at a wavelength of 633 nm. For size and PDI determination polystyrene cuvettes were used (12 mm Square Polystyrene Cuvettes (DTS0012), Malvern Instruments, UK) and for zeta

potential disposable capillary cell (Disposable capillary cell (DTS1070), Malvern Instruments, UK).

All measurements were performed at 25 °C. Each measurement of size and PDI were measured with a detection angle of 173° and zeta potential measurements an angle of 17°, being the potential zeta values calculated by Smoluchowski's model. For all size, PDI and potential zeta measurements were performed at least three replicates.

Before the measurements, the samples of alginate/chitosan nanoparticles with vitamin encapsulated were centrifuged (MIKRO 120, Andreas Hettich GmbH & Co. KG). Through a preliminary study, the optimal velocity and time was determined (selected based in the particle size and PDI), being at 4601.7 g during 5 minutes.

NanoSight NS500 (NanoSight Ltd., UK) was also used to measure the size of nanoparticles and to analyze the nanoparticles concentration by size. For measurements the samples were diluted to a concentration suitable for the analysis (according to the equipment instructions) (NanoSight, 2010). The dilutions were made with distilled water for alginate/chitosan nanoparticles and with a 75% ethanol solution for zein nanoparticles. The diluted samples were injected into the sample chamber fitted with a 640 nm diode laser. For capturing and analyzing the data was used the software Nanoparticle Tracking Analysis (NTA) 2.0 Build 127.

3.2.2 ENCAPSULATION EFFICIENCY AND LOADING CAPACITY

Encapsulation efficiency (EE) and loading capacity (LC) were determined after the separation of nanoparticles with encapsulated vitamin and the solution with free vitamin. The separation was obtained using membrane separation method with an Amicon® Ultra-0.5 centrifugal filter device (Amicon® Ultra - 0.5 mL 3K device, Millipore Corp., Ireland). So, 0.5 mL of sample (solution containing nanoparticles with encapsulated and free vitamin) was added to the Amicon® and was centrifuged at 14 000 g during 10 minutes. From centrifugation was obtained a filtrate with free vitamin and a concentrate with nanoparticles with encapsulated vitamin that were used for EE and LC determination (please see Figure 3.1). To calculate EE, the filtrate was assayed spectrophotometrically and the amount of free vitamin was calculated by appropriate calibration curve (ANNEX C). In its turn, to calculate LC the concentrate was dried and the weight of

nanoparticles determined. EE and LC was calculated as follows (Luo et al., 2011, 2012; Sarmento et al., 2007):

$$EE\ \% = \frac{Vit_{total} - Vit_{free}}{Vit_{total}} \times 100 \qquad \text{Eq. 3. 2}$$

$$LC\ \% = \frac{Vit_{total} - Vit_{free}}{Nps_{total}} \times 100 \qquad \text{Eq. 3.3}$$

Where Vit_{total} represents total amount of vitamin; Vit_{free} the amount of free vitamin in filtrate and Nps_{total} the total weight of nanoparticles.

For the nanoparticles with (-)-riboflavin, the absorbance of filtrate was measured in a multi-detection microplate reader BioTeK Synergy™ HT (BioTek Instruments, Inc., USA) at 437 nm, which corresponds to maximum absorbance peak of (-)-riboflavin (Bajpai and Tankhiwale, 2006). For this measurement was used a plate of 96 wells. The amount of free (-)-riboflavin was calculated through the calibration curve of (-)-riboflavin in distilled water: $y = 33.77x - 0.0049$ ($R^2 = 0.999$) (please see ANNEX C).

For TOC, before to calculate free TOC in filtrate was determined the maximum absorbance peak being made a scan over the range 200 – 400 nm using UV – Vis spectrophotometer Jasco V-560 (Tokyo, Japan) (ANNEX B). Then, the absorbance of filtrate was assayed spectrophotometrically at 292 nm (maximum absorbance peak determined) and the amount of free TOC in filtrate was calculated through calibration curve $y = 7.5582x + 0.0004$ ($R^2 = 0.997$) (please see ANNEX C).

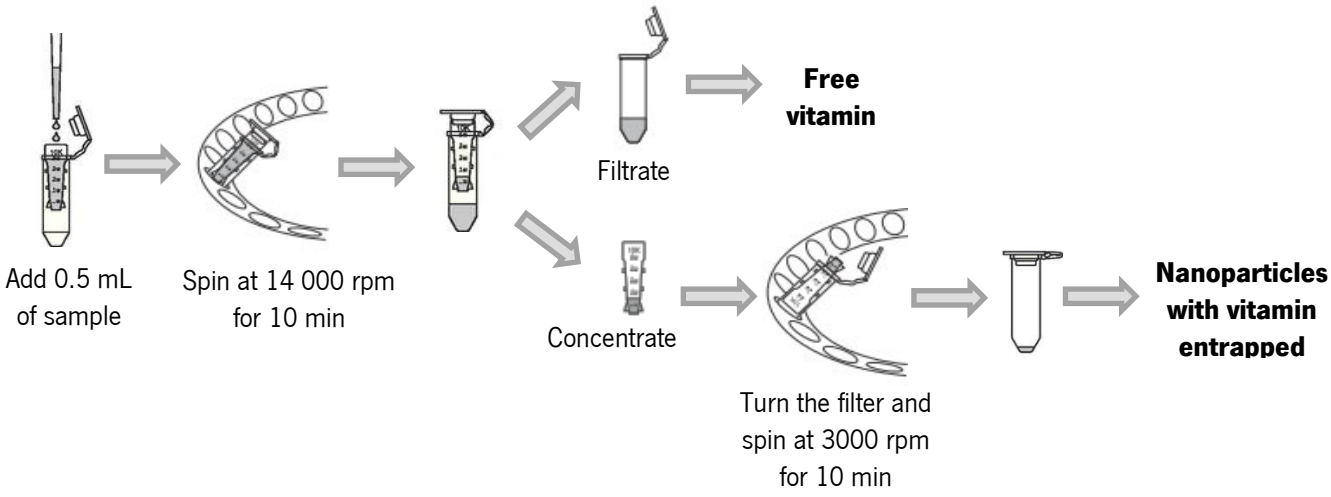


Figure 3.1 – Membrane separation method with an Amicon® ultra-0.5 centrifugal filter device. Image adapted from User Guide Amicon® ultra-0.5 centrifugal filter device.

3.2.3 MORPHOLOGY

The surface morphology of the nanoparticles with and without vitamin was evaluated through a transmission electron microscope (TEM) ZEISS 902 A (Zeiss, Germany) operating at a voltage of 80 kV. Before loading of samples into the microscope (direct deposition), the samples were drop-cast onto a carbon coated copper grid and grid was dried during 1 minute at room temperature.

3.2.4 RELEASE PROFILE

In order to evaluate the release profile of encapsulated vitamins from nanoparticles the dialysis membrane method was used (Beirão da Costa et al. 2012; Pinheiro et al. 2012).The release experiments were conducted at 37 and 25 °C and the medians utilized were phosphate buffer saline (PBS) pH 7 and Tris-Hydrochloride (Tris-HCL) pH 2.

3.2.4.1 Materials

PBS in tablets was purchased Sigma-Aldrich Chemical Co. Ltd (St. Louis, MO, USA) and was dissolved in distilled water. Tris-HCl was prepared with 0.2 M potassium chloride from Merk (Darmstadt, Germany) and 0.2 M Hydrochloric acid (36.5-38 % of purity) that was obtained from Sigma-Aldrich Chemical Co. Ltd (St. Louis, MO, USA).

3.2.4.2 Dialysis membrane method

The release profile of (-)-riboflavin from alginate/chitosan nanoparticles and of TOC from zein nanoparticles were evaluated by dialysis membrane method. Before procedure, nanoparticles with encapsulated vitamin were separated from the aqueous medium containing free vitamin using an Amicon® Ultra-0.5 centrifugal filter device and for dialysis method was used the concentrate (nanoparticles with vitamin entrapped) (Figure 3.1).

The procedure of dialysis membrane method was as follows: 5 mL of nanoparticles with encapsulated vitamin were added into a dialysis membrane (molecular weight cut-off 3500 Da; Cell-Sep H1, Membrane Filtration products, USA) and the sealed membrane was then placed into 50 mL of buffer solution (PBS or Tris-HCl) at 37 or 25 °C, with magnetic stirring. At determined time intervals, 1 mL samples were taken from the medium and replaced by fresh medium to maintain the original volume. All the experiments were performed in triplicate.

Vitamin concentration was determined at 437 nm in multi-detection microplate reader BioTeK Synergy™ HT for (-)-riboflavin and at 292 nm for TOC using a UV – Vis spectrophotometer Jasco V-560. The (-)-riboflavin and TOC concentration were calculated by calibration curves (ANNEX C): $y = 33.77x - 0.0049$ ($R^2 = 0.999$), and $y = 7.5582x + 0.0004$ ($R^2 = 0.997$), respectively.

3.2.4.3 Kinetics of Release

The release profile of encapsulated vitamin in nanoparticles was evaluated through the relationship between the size of the particles and the release fraction of vitamin at time t that can be described by equations 3.3 and 3.4:

$$\frac{M_t}{M_\infty} = 6\left(\frac{Dt}{\pi r^2}\right)^{0.5} - \frac{3Dt}{r^2} \quad \text{for} \quad \frac{M_t}{M_\infty} \leq 0.7 \qquad \text{Eq. 3.3}$$

$$\frac{M_t}{M_\infty} = 1 - 0.61\exp\left(-\frac{Dt\pi^2}{r^2}\right) \quad \text{for} \quad \frac{M_t}{M_\infty} \geq 0.7 \qquad \text{Eq. 3.4}$$

Being M_t the solute mass released at time t; M_∞ the solute mass released at infinite time when equilibrium is achieved; r is the radius of the nanoparticles and D can be calculated from Eq. 3.3 and 3.4 by non-linear parameter estimation (Beirão da Costa et al., 2012; Prata et al., 2008).

In order to determine the suitable of equations in describing the vitamin release data over the time, it was used the non-linear estimation module of Statistica®7(Statsoft, Tulsa, Ok, USA).

3.2.5 STABILITY MEASUREMENT

The freshly prepared samples were used for stability measurements. In order to evaluate the stability of nanoparticles, the samples were subjected to different parameters such as temperature and storage time and nanoparticle size and PDI measured. All measurements were performed in triplicate.

3.2.5.1 Temperature

The effect of temperature in nanoparticles was evaluated using Standard Operating Procedure (SOP) Temperature Size Trend of DLS instruments (Zetasizer Nano ZS). For these measurements

was used 1 mL of sample that was placed in a glass cell with circular aperture and stopper (12 mm Square Glass Cell for 90 sizing (PC S8501)), Malvern Instruments, UK) and was made a measurement of the nanoparticles size and PDI over the range 25 – 90 °C, with a temperature interval of 2.5 °C.

3.2.5.2 Storage Time

In order to evaluated the stability of nanoparticles throughout time, nanoparticles samples with and without vitamin were stored in a fridge at 4 °C being size and PDI of nanoparticles measured during five months.

Before the measurements samples of alginate/chitosan nanoparticles with vitamin encapsulated were centrifuged (MIKRO 120) at 4601.7 g during 5 minutes.

3.3 STATISTICAL ANALYSES

Data analyses were performed using Microsoft Windows Excel 2003 and GraphPad Prism (Version 5.00 (Trial), edition 2007, GraphPad Software, Inc., La Jolla, CA, USA). All data were reported as the mean ± standard deviation (SD) from at least three values. The analysis of variance (ANOVA) followed by Tukey's multiple-comparison (α=0.05) were employed to assess the statistical significance of results between groups. Experimental results were considered statistical significant at 95 % confidence level (p<0.05).

CHAPTER IV

RESULTS AND DISCUSSION

The main objective of this work was the development of nanosystems for encapsulation of vitamins using biopolymers and their further characterization. Therefore, two nanosystems were developed and characterized, one for water-soluble vitamins and other for liposoluble vitamins. Encapsulation of water-soluble vitamins was performed through ionotropic polyelectrolyte pre-gelation method using two charged polysaccharides, alginate and chitosan. For liposoluble vitamins a protein-based nanosystem was developed, through the self-assembly of zein. This chapter is divided in "ALGINATE/CHITOSAN NANOPARTICLES" and "ZEIN NANOPARTICLES"; where are presented the main results obtained during the development and characterization of the nanosystems and its discussion.

4.1 ALGINATE/CHITOSAN NANOPARTCILES

Alginate/chitosan nanoparticles were produced using the ionotropic pre-gelation method followed by polycationic crosslink, being used $CaCl_2$, alginate and chitosan as main materials, and (-)-riboflavin (vitamin B_2) as water-soluble vitamin model. During nanoparticles formation Ca^{2+} ions are into alginate solution, the Na^+ ions of alginate "are removed" and electrostatic interactions between $–COO^-$ groups of alginate and cross-linked Ca^{2+} ions are promoted. Then, with chitosan addition, the alginate molecules also crosslinks with chitosan through electrostatic interactions between negatively charged $–COO^-$ group of alginate and positively charged protonated amine groups of chitosan (Figure 4.1) and this way are formed alginate/chitosan nanoparticles (Bajpai and Tankhiwale, 2006).

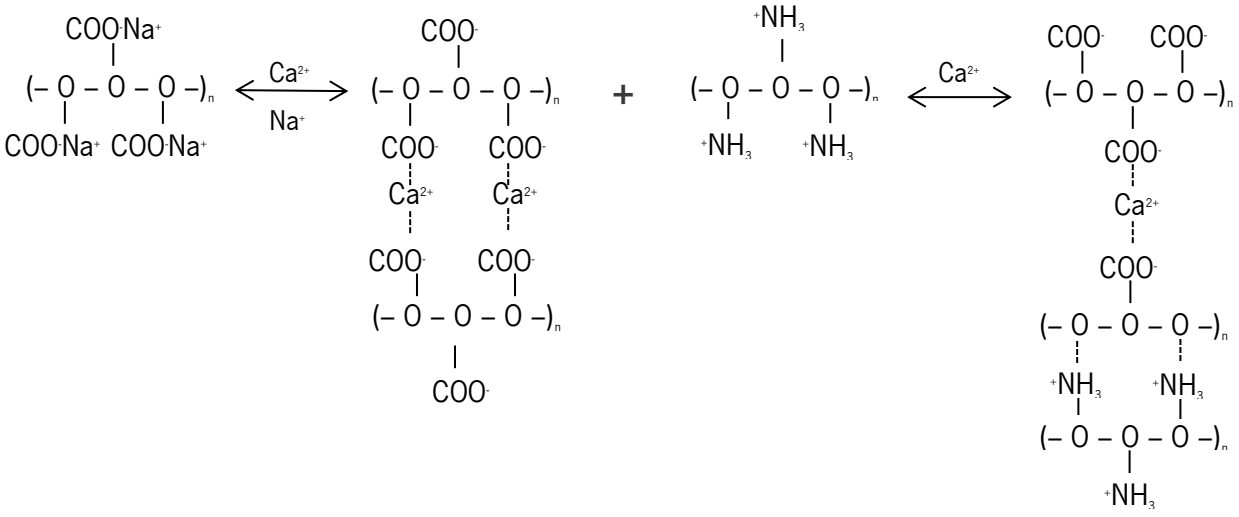


Figure 4.1 – Electrostactic interactions between Ca^{2+} ions, $-COO^-$ groups of alginate and amine groups of chitosan. Image adapted from Bajpai and Tankhiwale (2006).

Based on the methodology described by Sarmento et al. (2007), due the great complexity of the method and being used an alginate and chitosan with different properties, it was needed to test different formulations and parameters during its production. So, initially an optimization was done being the nanoparticles formulation and parameters used for their production determined.

4.1.1 OPTIMIZATION OF THE FORMULATION

The particle size and polydispersity index (PDI) are essential parameters to evaluate the stability and homogeneity of nanoparticles in solution, being considered, in this case, the most important factors during the optimization of the nanoparticles. So, the effects of different formulations and parameters in particle size and PDI were analyzed in order to optimize the formulation, being measured through DLS instrument (Zetasizer Nano ZS). The preparation conditions of alginate/chitosan nanoparticles have been optimized in terms of the polysaccharides and vitamin concentrations, flow rates of solutions, time and velocity of shake and centrifugation. In ANNEX A are summarized the effects of different formulations on particle size and PDI.

The optimized formulation was 0.63 mg/mL and 0.4 mg/mL of alginate and chitosan, respectively, with 0.065 mg/mL of (-)-riboflavin loaded.

4.1.2 CHARACTERIZATION

After previous study, the alginate/chitosan nanoparticle with and without (-)-riboflavin was characterized in terms of particle size (by z-average and number), PDI, zeta potential, morphological observation, encapsulation efficiency (EE), loading capacity (LC), release profile and stability measurements. Below, are presented the characterization of alginate/chitosan nanoparticles.

4.1.2.1 Size, Polidispersity index (PDI) and Zeta Potential

Table 4.1 shows the average of particle size, PDI and zeta potential values for alginate/chitosan nanoparticles prepared with and without (-)-riboflavin.

The alginate/chitosan nanoparticles with and without (-)-riboflavin present different sizes represented by z-average and number distribution, being in the both cases the z-average higher than the particles size by number. The z-average values are obtained by intensity of the particles in solution and the large particles scatter much more light than small particles, on the other hand, the number distribution take account the number of the particles in solution (Malvern Instruments, 2004). This way, the z-average of 512.2 (± 36.80) nm and 428.2 (± 31.09) nm for nanoparticles with and without vitamin, respectively, is due to the particles or aggregates presents in samples with a size above 500 nm that scatter more light than smaller particles. Like this, the results showed that nanoparticles of alginate/chitosan with and without (-)-riboflavin have particles or aggregates with a size above 500 nm. However, the distribution by number shows that samples have a great number of nanoparticles with a mean size of 119.5 (± 49.86) nm for samples without (-)-riboflavin and 104.0 (± 67.24) nm with (-)-riboflavin.

PDI values for nanoparticles without and with vitamin are 0.454 (± 0.066) and 0.319 (± 0.068), respectively. In general, the PDI above 0.2 indicate that the sample do not exhibit a good homogeneity (Silva et al., 2011b). For this reason, the samples are not considered monodisperses. Besides that, the standard deviations of particle size are high; also indicating those samples was not homogenous. Considering the difference between z-average and number distribution, the PDI values and the standard deviations obtained are consistent with this result.

The means of particles size by z-average and PDI for alginate/chitosan nanoparticles without and with (-)-riboflavin are significantly different (p<0.05), decreasing when the vitamin is loaded in

alginate/chitosan nanoparticles. However, the decrease of size by number distribution is not significantly different (p>0.05) showing that encapsulation of vitamin does not influence the values of size distribution by number. The decrease of size distribution can be explained by interactions between (-)-riboflavin and both biopolymers used. (-)-Riboflavin can have a cationic form (RFH_2^+) at low pH, neutral form (RFH) and anionic form (RF^-) at high pH (Choe et al., 2005), and when mixed with alginate solution at pH 4.7, the (-)-riboflavin acquire cationic form interacting strongly with negatively charged alginate. So, the smaller sizes in samples with (-)-riboflavin can be explained by the strong ionic interaction between riboflavin and alginate but may also take into account hydrogen bonding and van der walls forces that (-)-riboflavin can establish with alginate and chitosan (Sarmento et al., 2007).

The zeta potential is indicative if samples have a good colloidal stability, being particles with zeta potentials more positive than +30mV or more negative than -30mV normally considered stable (Malvern Instruments, 2004; Silva et al., 2011b). The values obtained were -30.89 ± 0.51 and -29.64 ± 0.97 mV without and with vitamin, respectively, showing that samples have a good colloidal stability. These values are also statistically significantly different (p<0.5), showing that vitamin-loaded can influence zeta potential (Table 4.1). The zeta potential for nanoparticles with (-)-riboflavin higher than the values for nanoparticles without (-)-riboflavin can also be explained by the cationic form that (-)-riboflavin acquire in solutions with pH below that favors the negative charge of the solution.

Table 4.1 – Particle size (by z-average and number), Polydispersity index (PDI) and Zeta Potential for alginate/chitosan nanoparticles with and without (-)-riboflavin, by DLS instrument (Zetasizer Nano ZS).

(-)-Riboflavin content	**Particle size**		**PDI**	**Zeta Potential (mV)**
	z-average (nm)	**number (nm)**		
Without	512.2 ± 36.80 [a]	119.5 ± 49.86 [a]	0.454 ± 0.066 [a]	-30.89 ± 0.51 [a]
With	428.2 ± 31.09 [b]	104.0 ± 67.24 [a]	0.319 ± 0.068 [b]	-29.64 ± 0.97 [b]

Values are expressed as mean ± standard deviation.

[a,b] – Different letters in same column indicate a statistically significant difference (p<0.05)

Due to differences between z-average and number distribution and in order to confirm the sizes of nanoparticles obtained by DLS, also NTA was used to measure the size of particles. The results obtained by NTA are presented in Table 4.2, being presented the mean and mode of particle size.

Table 4.2 – Particle size (mean and mode) for alginate/chitosan nanoparticles with and without (-)-riboflavin, by NTA (NanoSight).

(-)-Riboflavin content	Particle size (nm)	
	Mean	Mode
Without	168 ± 97	101
With	210 ± 109	107

Values obtained by software Nanoparticle Tracking Analysis (NTA) 2.0 Build 127 expressed as mean ± standard deviation.

Nanoparticles sizes obtained by NTA are consistent with results obtained by number distribution in DLS. Although, the standard deviation associated to the mean of particle size is high, but as the mode is a value more common in sample the size number distribution obtained by DLS was considered real size of the nanoparticles.

Particle size influences many properties of particulate materials and is a valuable indicator of quality and performance. A small particle size is important because are more efficiently absorption by the gastrointestinal track increasing their biodisponibility (Sarmento et al., 2006, 2007).

4.1.2.2 Morphological observation

The morphological observation of alginate/chitosan nanoparticles were performed by TEM. Figure 4.2 the representative photographs of the alginate/chitosan nanoparticles without (-)-riboflavin.

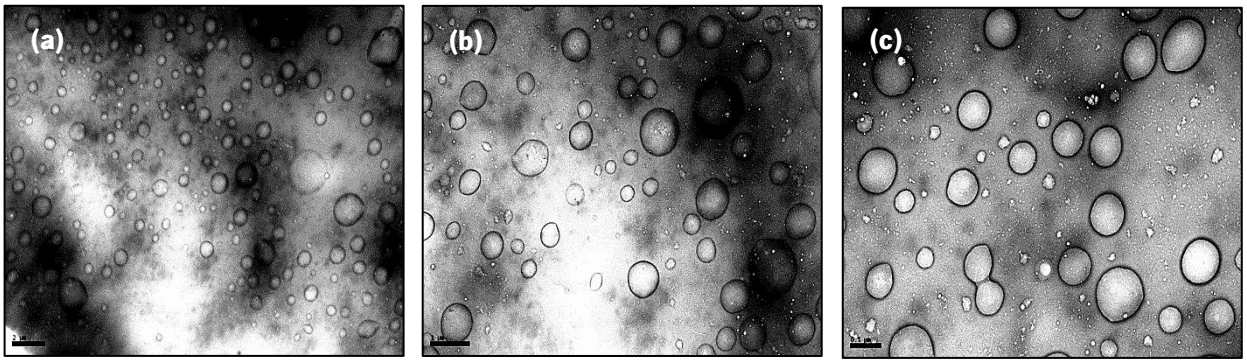


Figure 4.2 – Transmission electron microscopy (TEM) images of alginate/chitosan nanoparticles without (-)-riboflavin at a scale bar of 2 µm, 7000x (a); 1 µm, 12000x (b); 0.5 µm, 20000x (c).

TEM images show that alginate/chitosan nanoparticles have an evident spherical shape and no aggregations of nanoparticles are observed; however the size distribution of the particles was not homogeneous varying from 100 to 1000 nm. Figure 4.2a shows a broader picture of the sample and it is possible to see a great amount of nanoparticles with a smaller size (<200 nm) but also some particles with size above 500 nm. This image, not only confirms the high values of polydispersity index (>0.2) and standard deviation associated to the means of particles sizes, but also the difference between z-average and mean size by number.

This TEM pictures revealed a differences of contrast between the external polymeric membrane and internal part of nanoparticles and this difference is due to the difference between measured electrical conductivity of alginate (1.40 ± 0.2 mS/cm) and that of chitosan (1.47 ± 0.01 mS/cm), which renders alginate to be the first to become electrically charged after the incidence of the electron beam during TEM analysis (Carneiro-da-Cunha et al., 2010). So, as expected, the chitosan is the external polymeric membrane and the alginate is the internal part of the nanoparticles.

4.1.2.3 Encapsulation Efficiency and Loading Capacity

The capacity of the nanoparticles to encapsulate water-soluble vitamins was evaluated through the determination of the encapsulation efficiency (EE) and loading capacity (LC). The values of EE and LC obtained were 55.91(± 5.56) % and 2.18 (± 0.63) %, respectively. These values are lower than those reportered for other systems for (-)-riboflavin. Abd El-Ghaffar et al. (2012) obtained values of EE in the range of 80 - 100 %, however the particles sizes were more higher than the

values obtained in present work, presenting sizes of 1.4 (± 0.05) mm (El-Ghaffar et al., 2012). For others alginate/chitosan nanoparticles, e.g. with insulin-loaded, Sarmento et al. (2007) obtained EE values between 70 and 90% and LC in the range 5 – 15 %, but the mean of particles sizes were between 700 and 4000 nm (Sarmento et al., 2007) and Goycoolea et al. (2009) obtained EE values similar those obtained in this work (~50%) and the sizes of particles are between 200 and 300 nm (Goycoolea et al., 2009).

So, and besides the higher values of EE and LC obtained in others works, the results obtained show that alginate/chitosan particles at the nanoscale present good values of EE and LC.

4.1.2.4 Release profile of (-)-riboflavin from alginate/chitosan nanoparticles

The release profile allows knowing the prediction of the *in vivo* behavior of an encapsulated bioactive compound, allowing to know if the developed nanoparticles are appropriated to the proposed application (e.g. application in food industry). Release experiments were conducted at 37 and 25°C being the buffers used PBS (pH 7) and Tris-HCL (pH 2). While the temperature at 37°C tries to simulate the temperature of the human digestive system, pH 2 pretends to simulate the stomach and pH 7 the intestine. The temperature at 25°C intends to simulate the food and/or beverages at ambient temperature. So, the release profile can be defined as a method by which a bioactive compound is made available at a desired site and time and at a specific rate (Beirão da Costa et al., 2012), being, in present work, evaluated the effect of temperature and pH on vitamin release rate from alginate/chitosan.

Figure 4.3 shows the fitting of Eq. 3.3 to the experimental data of (-)-riboflavin release kinetics from alginate/chitosan nanoparticles, for pH 7 and 2 at 37 and 25°C, and it is possible to see a good agreement between experimental and model-generated values suggesting that this mathematical model describes well the physical mechanism associated to the (-)-riboflavin release. Also, it is possible to see that (-)-riboflavin release profiles showed a biphasic modulation: initially, the release profile is relatively fast ("burst effect") and then is followed by a continuous and controlled slow release phase ("lag time") (Beirão da Costa et al., 2012). One explanation for the "lag time" observed following of "burst effect", can be the vitamin distribution across the nanoparticle and this way connot diffuse easily out the membrane and the vitamin release slower (She et al., 2010). The burst effect at 37°C is for at least 50 and 5 minutes for pH 7 and 2, respectively, and at 25°C is during the first 60 minutes to pH 7 and 40 minutes for pH 2.

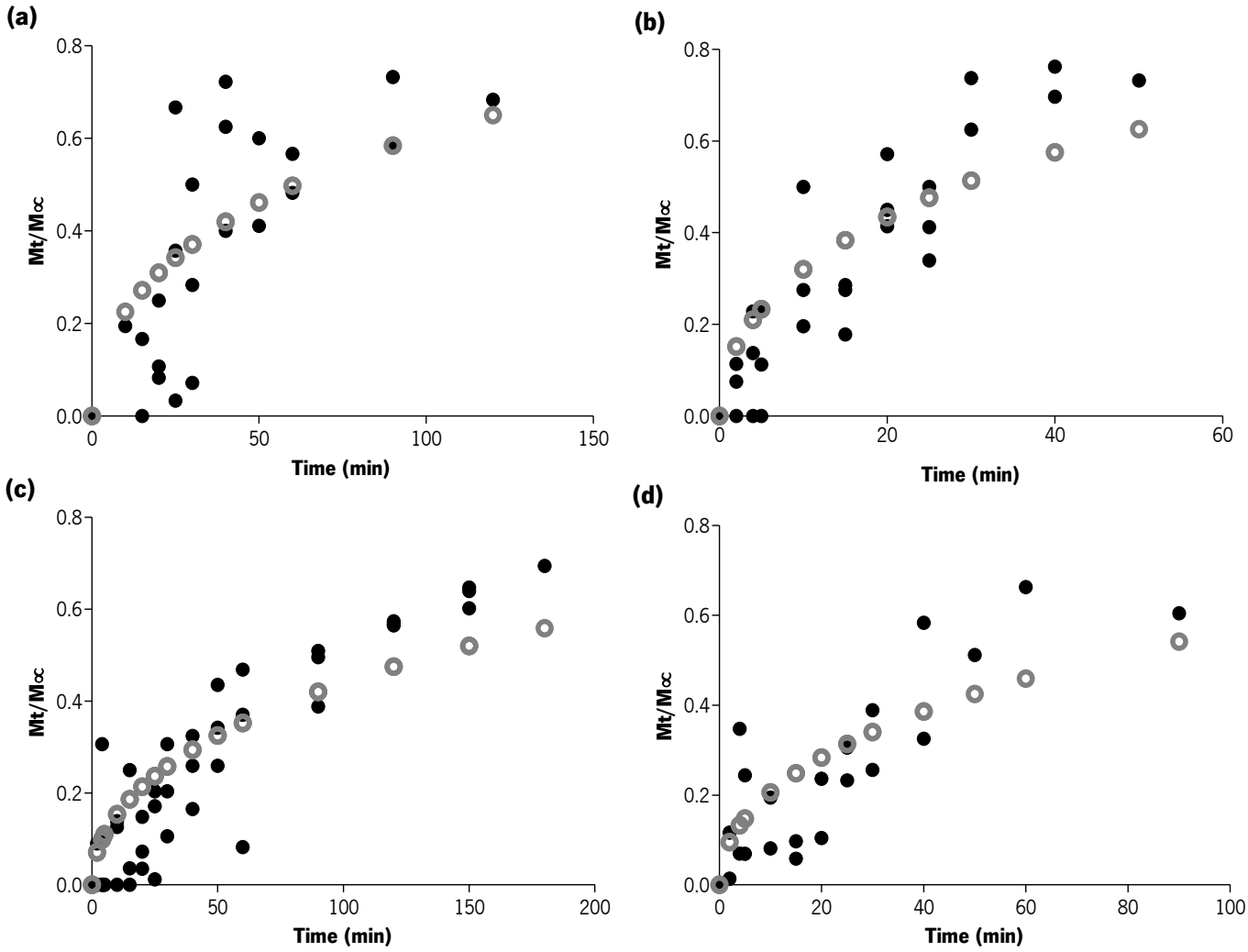


Figure 4.3 – Fitting of Eq. 3.3 to (-)-riboflavin controlled release experimental data from alginate/chitosan nanoparticles ((•) experimental results and (o) model-generated values): (a) at 37°C, pH 7; (b) at 37°C pH 2; (c) at 25°C, pH 7; (d) at 25°C, pH 2.

The initial "burst effect" can be explained based on the effect of pH in biopolymers used: alginate and chitosan. The decrease of the pH, can lead to disintegration, relaxation and swelling of the nanoparticles and, consequently, the release of the encapsulated vitamin.

Into the PBS buffer at pH 7 the NH_3^+ groups of chitosan get deprotonated to yield uncharged NH_2 groups and, this way, is reduced their electrostatic interactions with $-COO^-$ groups of alginate chains. Consequently, the nanoparticles begin to disintegrate along the release of the encapsulated vitamin. The disintegration of alginate/chitosan nanoparticles can also be due to ion exchange between Na^+ ions from the PBS and Ca^{2+} ions from the egg-box cavities of polyguluronate blocks of alginate (Bajpai and Tankhiwale, 2006). Moreover, the swelling is

increased in PBS medium, because the carboxylate groups of alginate molecules tend to ionize and give $–COO^-$ ions which facilitate the swelling of nanoparticles. The osmotic pressure should increase inside the nanoparticles due to the higher concentration of free H^+ and promote water uptake. Furthermore, electrostatic repulsion between carboxylate ions should cause macromolecular chain relaxation and increase swelling, and, thus contribute to release of vitamin (El-Ghaffar et al., 2012; Sarmento et al., 2007).

When alginate/chitosan nanoparticles are placed in Tris-HCl buffer at pH 2, the negatively charged carboxylate groups of alginate begin to protonate to form uncharged –COOH groups. This reduces the degree of crosslinking due to the decrease of ionic and electrostatic interactions among the alginate and chitosan chains within the nanoparticles, thus resulting in loosening of nanoparticle structure with subsequent faster release of encapsulated drug (Bajpai and Tankhiwale, 2006).

Based on this fitting was calculated the diffusion coefficient (*D*) for release of (-)-riboflavin encapsulated into alginate/chitosan nanoparticles, being at 37°C of 2.28×10^{-20} and $4.95 \pm 0.74 \times 10^{-20}$ m^2/s to pH 7 and 2, respectively, and at 25 °C of 1.02×10^{-20} m^2/s for pH 7 and 1.88×10^{-20} m^2/s for pH 2. At both temperatures, the *D* values in pH 2 are higher than in pH 7, which can explain the different time of "burst effect". However, comparing the *D* values, at 37 °C are higher than at 25 °C pointing out that the temperature can have influence on the diffusion of molecules through alginate/chitosan nanoparticles. The gel point (the critical point where gel first appears) of alginate and chitosan is about 32 °C and can explain the difference between *D* values at 37 °C and 25 °C because at 37 °C may have induced the gel form of alginate and chitosan leading to increase of polymers branch and, consequently, increase of nanoparticles sizes and release of bioactive compound (Liang et al., 2004).

4.1.2.5 Stability measurement

One of major applications of the vitamin-loaded alginate/chitosan nanoparticles is food industry. So, is important to guarantee that nanoparticles maintain their chemical and physical stability during processing and storage (Silva et al. 2011). The nanoparticles stability was assessed by the evaluation of the effect of some parameters such as temperature and storage time in alginate/chitosan nanoparticles without vitamin.

Temperature

The Figure 4.4 shows the effect of temperature in particle size and PDI of alginate/chitosan nanoparticles without (-)-riboflavin: until 35 °C the nanoparitcles sizes are not influenced by temperature (p>0.05), however, from 35 °C to 37.5 °C happens a significant increase of nanoparticles sizes (p<0.05). Between 37.5 °C and 90 °C the nanoparticles sizes are stable again with values ranged from 372.84 (± 138.94) to 314.65 (± 26.20) nm. Considering the values of PDI (>0.2) and the standard deviation associated of the means, the samples until 35°C are not homogenous being more homogenous with temperature increase. This way, this study shows that temperature increase is responsible not only for increase of alginate/chitosan nanoparticles sizes but also by samples more homogeneous.

As mentioned previously, the alginate and chitosan have gelling properties and some external parameters, such as pH, temperature, ionic strength, solvent composition and electrical fields, can induce these gelling properties (Carreira et al., 2010). The gelation refers to the linking of molecules that lead to progressively larger branched polymers, initially, depending on the structure and conformation of the starting polymer, yet soluble. However, the continuation of the linking process results in increasing the size of the branched polymer and the solubility decrease (Gulrez et al., 2011). In present assay, the increase of temperature may have induced the gel form of alginate and chitosan, because, as previously mentioned, the gel point of alginate and chitosan is about 32°C (Liang et al., 2004). This way, the increase of nanoparticles sizes can be explained by increase of polymers branch.

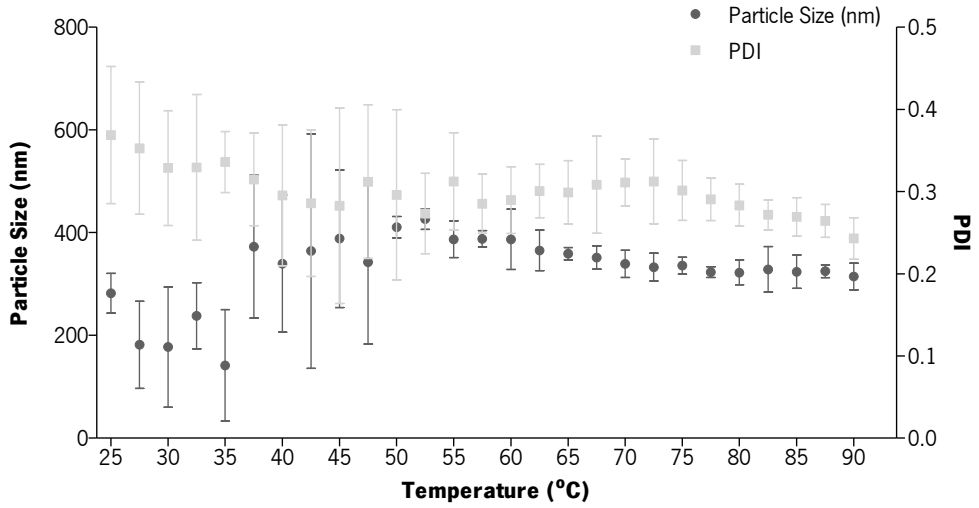
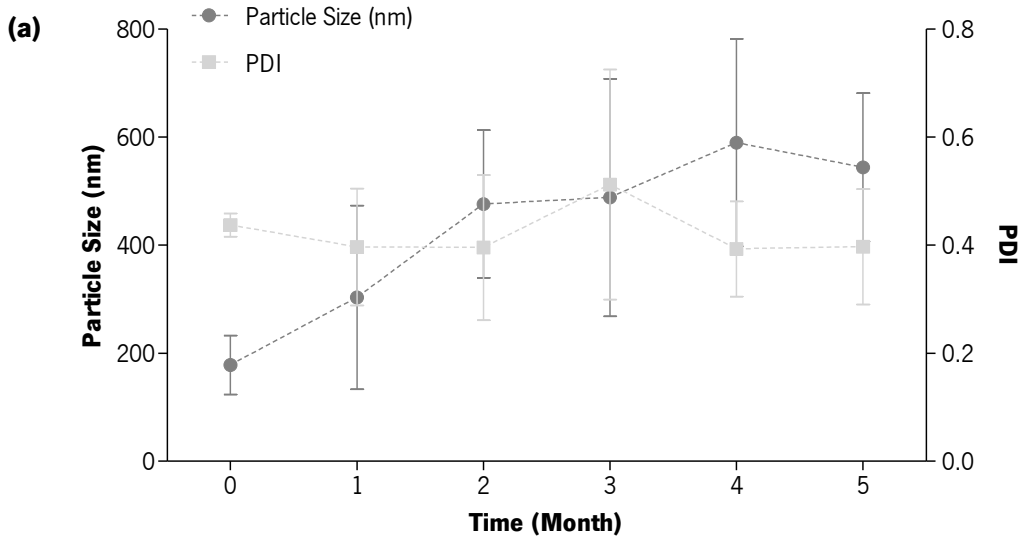


Figure 4.4 – Effect of temperature in particle size and PDI of alginate/chitosan without (-)-riboflavin.

Storage time

The stability of nanoparticles over time is important to determination of time that nanoparticles keep stable. The stability of samples was studied during five months, being used DLS for measurement of size and PDI of alginate/chitosan nanoparticles. Figure 4.5 shows that sizes of (-)-riboflavin-loaded nanoparticles are more stable than nanoparticles without (-)-riboflavin, being the PDI of alginate/chitosan nanoparticles with (-)-riboflavin smaller than (p<0.05) without vitamin. This fact can be explained by interactions between riboflavin and biopolymers used: (-)-riboflavin besides the ionic interactions that can establishes with alginate hydrogen bonding and van der Walls forces with alginate and chitosan (Sarmento et al., 2007).

Following initial nanoparticle characterization, the chemical stability of the samples was evaluated by determination of the pH after preparation of the alginate/chitosan nanoparticles without and with (-)-riboflavin, being obtained pH values of 4.6 for both systems. Over the course of five months, the pH increase to 5.4; this significant increase can be due to alteration of the charges present in the alginate and chitosan polymer chains. This way, the nanoparticles are instable and the nanoparticles sizes increase (El-Ghaffar et al., 2012; Sarmento et al., 2006). In Figure 4.5 is possible to see that the alginate/chitosan nanoparticles without (-)-riboflavin was more affected with alteration of pH than the alginate/chitosan nanoparticles with (-)-riboflavin, because the sizes increase over the course of five months and PDI are higher.



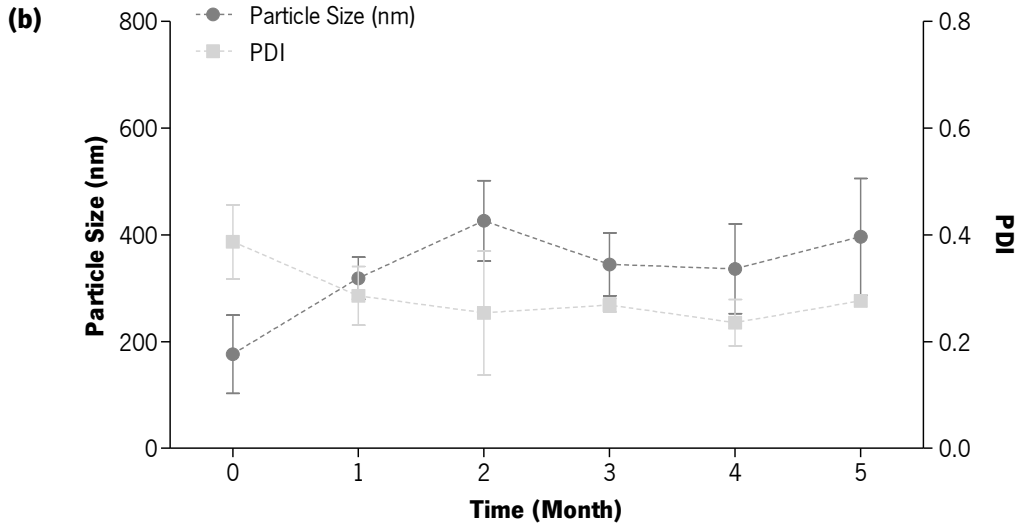


Figure 4.5 – Stability of alginate/chitosan nanoparticles: (a) without (-)-riboflavin; (b) with (-)-riboflavin.

4.2 ZEIN NANOPARTICLES

The development of nanoparticles for encapsulation of liposoluble vitamin, (±)-α-tocopherol (vitamin E), was performed using zein as main material and self-assembly as production method. As previously described, zein is insoluble in water and the main driving forces for self-assembly is amphiphilicity. But for nanoparticles formation it is also important to exist a long-range repulsion, for example, thermodynamic incompatibility, phase separation, excluded volume and columbic repulsion, between the protein and the medium. This way, in present work, the zein dissolved in 75 % aqueous ethanol solution was dropped in water and without help or guidance from external agents were formed nanoparticles of zein. When TOC is added, are established hydrophobic interactions and electrostatic interactions between TOC and zein that contribute to the encapsulation of TOC into zein (Luo et al., 2011) (Figure 4.6). Initially, was done an optimization and was determined the formulation and the optimal parameters for the production of nanoparticles (based in size of particles and PDI). This optimization is increasingly important for compound delivery and controlled release, because small particle sizes are believed to affect particle absorption at the intestinal lumen and their longevity in the blood stream (Wang and Padua, 2012b). After to determine the optimal formulation and parameters for nanoparticles preparation the vitamin was added to the system and zein nanoparticles characterized.

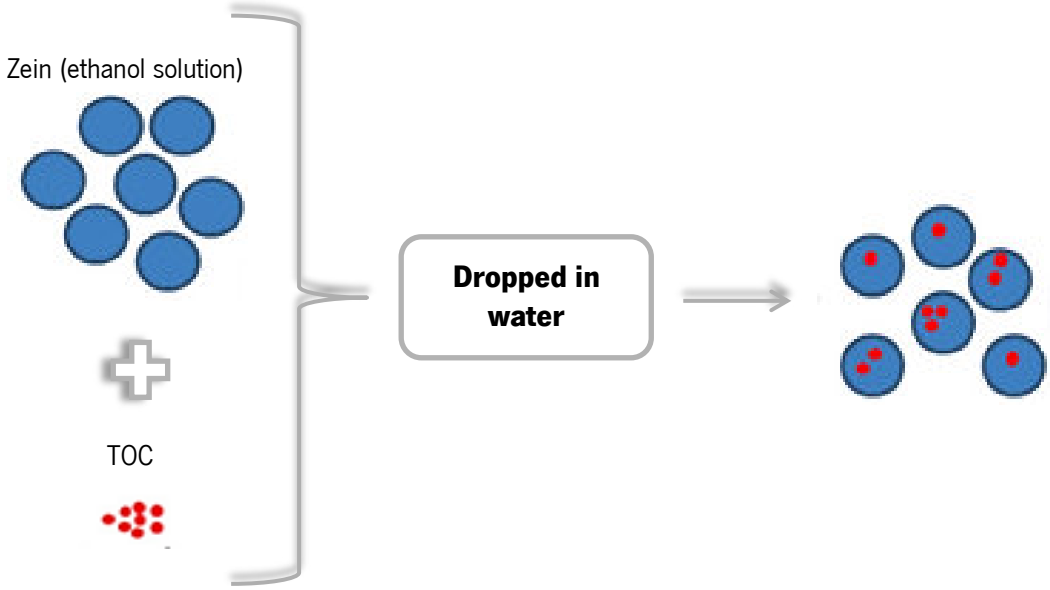


Figure 4.6 – Schematic illustration of formation of zein nanoparticles with TOC. Image adapted from Luo et al. 2011.

4.2.1 OPTIMIZATION OF THE FORMULATION

As mentioned previously, the particle size and PDI are essential parameters to evaluate the stability and homogeneity of nanoparticles in solution. So, is important to study the effect of process parameters in particle size and PDI. The preparation conditions of zein nanoparticles have been optimized in terms of the protein and vitamin ratio, flow rates of solutions, time and velocity of shake and centrifugation. In ANNEX A are summarized the effects of different formulations on particle size and PDI.

4.2.2 CHARACTERIZATION

The nanoparticles of zein with and without TOC was characterized using the same parameters used for alginate/chitosan nanoparticles characterization (particle size by z-average and number distribution, PDI, potential zeta, morphological observation, encapsulation efficiency (EE), loading capacity (LC), release profile and stability measurements). Below, are presented the results from the characterization of zein nanoparticles.

4.2.2.1 Size, Polydispersity index (PDI) and Potential Zeta

Table 4.3 shows values of particle size by z-average and by number distribution, PDI and zeta potential values for zein nanoparticles prepared with and without TOC.

Table 4.3 – Particle size (by z-average and number), polydispersity (PDI) and zeta potential for zein nanoparticles with and without (±)-α-tocopherol (TOC).

TOC content	Particle Size		PDI	Zeta Potential (mV)
	z-average (nm)	number (nm)		
Without	109.60 ± 21.78 [a]	34.96 ± 8.89 [a]	0.359 ± 0.059 [a]	25.64 ± 3.415 [a]
With	116.50 ± 28.32 [a]	50.22 ± 6.70 [b]	0.285 ± 0.093 [b]	26.08 ± 2.513 [a]

Values are expressed as mean ± standard deviation.
a,b – Different letters in same column indicate a statistically significant difference ($p < 0.05$)

The values of particle size of nanoparticles without TOC are smaller than nanoparticles with TOC, not presenting statistically significant difference (p>0.05) for particle size by z-average distribution. However, the values of particle size by number distribution are significantly different (p<0.05), increasing from 34.96 (± 8.89) to 50.22 (± 6.70) nm when TOC is added to the system. This fact can be explained by the presence of the TOC in the nanoparticle structure. However, an high TOC content can help the TOC molecules migrate to the internal parts of the nanoparticles, being establish proper interaction between the zein and TOC molecules which lead to molecules rearrangements in the molecular structure of nanoparticles to make more stable particles (Naghibzadeh et al., 2010). PDI of nanoparticles with TOC is smaller than zein nanoparticles without TOC being approximately 0.2 and 0.359 (± 0.059), respectively, indicating that the particle size of zein nanoparticles without TOC have more heterogeneous distribution.

Table 4.3 shows the potential zeta of zein nanoparticles with and without vitamin. As can be seen the sample with TOC has a zeta potential similar than sample without TOC, not being statistically significant different. Positive zeta demonstrated that the system can be used as a targeted delivery system of TOC *in vivo*, due to interactions with negatively charged biological membranes that will improve stability in the presence of biological cations (Luo et al., 2011).

In order to confirm the sizes obtained by DLS for zein nanoparticles was also used NTA, being the results presented in Table 4.4.

Table 4.4 – Particle size (mean and mode) for zein nanoparticles with and without (±)-α-tocopherol (TOC) by NTA (NanoSight).

TOC content	Particle size (nm)	
	Mean	Mode
Without	116 ± 56	88
With	149 ± 60	93

Values obtained by software Nanoparticle Tracking Analysis (NTA) 2.0 Build 127 expressed as mean ± standard deviation.

The values obtained by NTA are consistent with the values of particle size obtained by DLS, being clear the increase of sizes when TOC is encapsulated in the system. However, the results obtained by z-average distribution in DLS are more similar with NTA results. This way, the analyses done in DLS for characterization of zein nanoparticles were considered the particles sizes by z-average distribution.

4.2.2.2 Morphological observation

Figure 4.7 shows the transmission electron microscopy photographs of the zein nanoparticles without and with TOC.

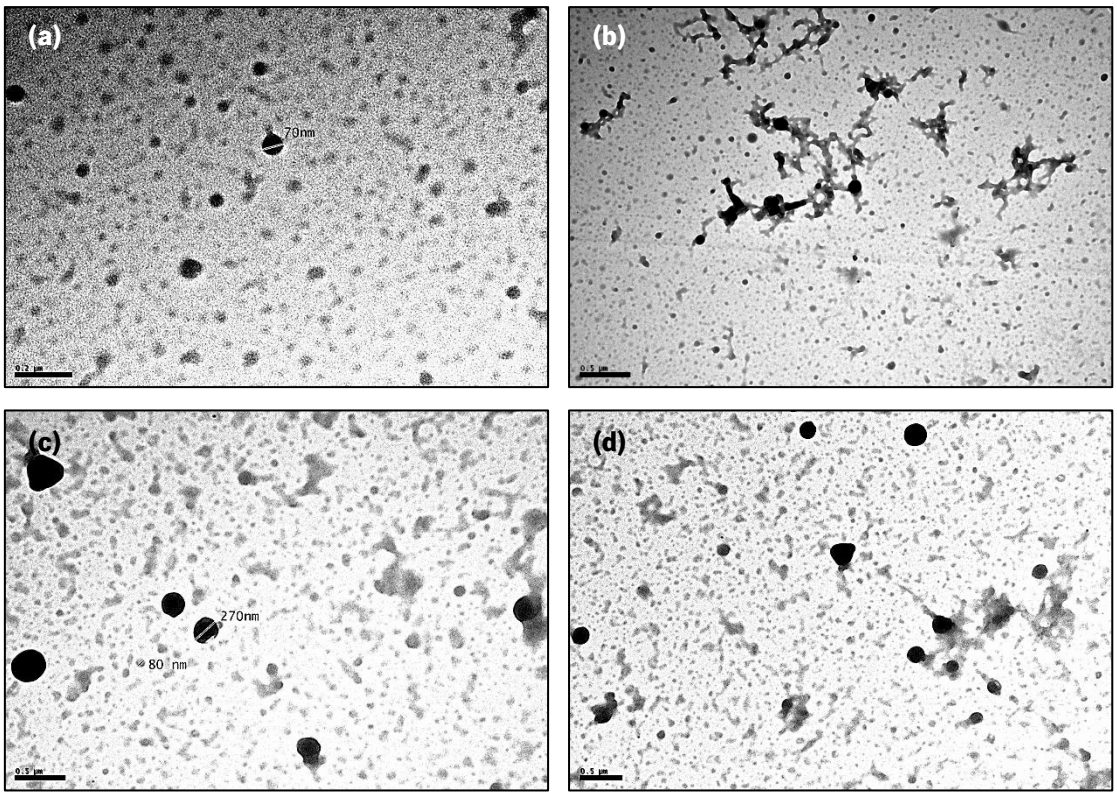


Figure 4.7 - Transmission electron microscopy (TEM) images of zein nanoparticles: (a) Scale bar: 0.2 µm, 85000x and (b) Scale bar: 0.5 µm, 30000x without TOC and (c) Scale bar: 0.5 µm, 30000x; and (d) Scale bar: 0.5 µm, 20000x with TOC.

TEM images presented in Figure 4.7 show zein nanoparticles formed with and without TOC being possible to see a structure with a continuous, compact, and uniform spherical shape. However, in Figure 4.7b and Figure 4.7d part of the nanoparticles were clumped and connected to each other and this fact can be caused by the low-energy method used in present work (Luo et al.,

2012). These agglomerate of nanoparticles can be consider responsible for the PDI values obtained to the nanosystem, 0.359 (± 0.059) for nanoparticles without TOC and 0.285 (± 0.093) with TOC. Regarding of nanoparticles sizes, as can be seen in images, they are consistent with values obtained by DLS and NTA; being possible to observe an increase in size for vitamin-loaded nanoparticles.

4.2.2.3 Encapsulation Efficiency and Loading Capacity

EE and LC were determined after separation of nanoparticles with vitamin entrapped from the aqueous medium containing free vitamin. The values of EE and LC obtained were 94.95 (± 4.17) % and 8.53 (± 1.90) %, respectively. These values are higher than those reported for other zein systems. Luo et al. (2011) with TOC obtained values of EE in the range 75 - 85 %, being the particles sizes between 200 and 800 nm. Luo et al. (2012) with vitamin D-loaded obtained EE and LC in the range 50 – 80 % and 1 – 4 %, respectively (Luo et al., 2012 and Luo et al., 2011). So, the results obtained show that zein nanoparticles obtained by self-assembly present good values of EE and LC, being that 94.95 (± 4.17) % of content TOC are encapsulated in zein nanoparticles and the high LC evidenced the electrostatic interactions between TOC and zein (Luo et al., 2011).

4.2.2.4 Release Profile of TOC from zein nanoparticles

The release properties of the TOC from the zein nanoparticles were experimentally determined by measuring the concentration of the TOC in the release media as a function of time. For this was also used a dialysis membrane method and the release experiments were conducted at 37°C and the media utilized was PBS (pH 7) and Tris-HCl (pH 2). A better understanding of the mechanism of TOC release from zein nanoparticles was obtained by analysis using the Eq.3.3 and it is possible to observe in Figure 4.8 a good agreement between experimental and model-generated values, suggesting that this mathematical model describes well the mechanism.

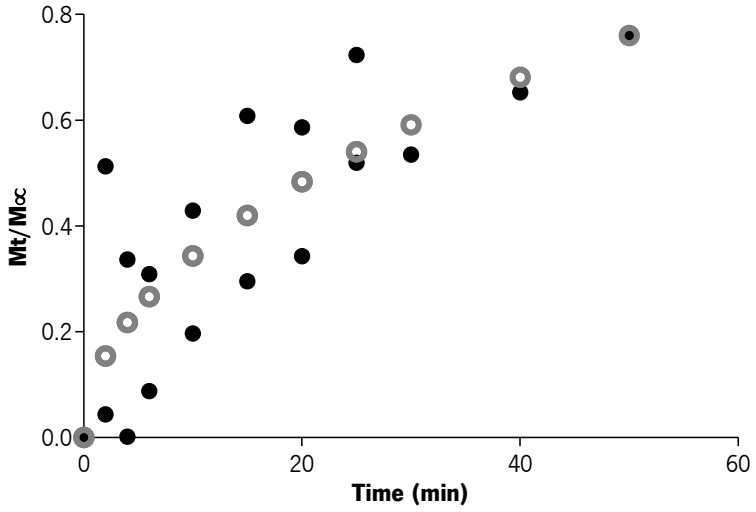


Figure 4.8 – Fitting of Eq. 3.3 to TOC controlled release experimental data from zein nanoparticles at 37°C, pH 2 ((•) experimental results and (o) model-generated values).

Into the PBS of pH 7, the TOC are not released and the hydrophobic interactions may also have contributed for this fact because zein is hydrophobic. In addition, as zein is a protein and has an isoelectric point of 6.8, zein is positively charged at pH 2-6 and negatively charged at pH 7-8. So, as TOC has a low positively charge, in pH 7 the overall electrostatic interactions between zein and TOC become attractive, which, together with stronger hydrophobic interactions, resulted in not release (Zhong et al., 2009). However, the Figure 4.8 shows that TOC was release in Tris-HCl of pH 2 and the release profile just have a "lag time" phase, release continuous and slow, being the diffusion coefficient for TOC release 2.8×10^{-18} m^2/s. This fact can also be explained by overall electrostatic interactions between zein and TOC, because as pH 2 the zein has a negative charge, the interactions are repulsive between zein and TOC and as result have a slow TOC release (Zhong et al., 2009). Moreover, when zein nanoparticles were into the buffer Tris-HCL, the particles can form aggregates that can be gradually dispersed and completely dissolved, so this fact can be other reason for TOC release in Tris-HCl medium (Parris et al., 2005; Somchue et al., 2009).

4.2.2.5 Stability

Temperature

Figure 4.9 shows the effect of temperature in zein nanoparticles size and PDI and, as can be seen, the temperature cause the increase of sizes and decrease of PDI (p<0.05). An explanation for increase of nanoparticles size is the hydrophobic interactions between zein molecules, being these interactions caused by ethanol evaporation. As ethanol is evaporated at a faster than water (ethanol boiling point at 78.37°C), the increase of temperature results in ethanol evaporation and, consequently, self-assembly was modeled from the hydrophobic and hydrophilic contributions to the interfacial free energy from zein and solvent. This way, with increase of temperature the solvent became more hydrophilic that results in changes of solvent polarity (considered the main driving force for zein self-assembly) and in increase of nanoparticles (Wang and Padua, 2012a, 2012b).

Temperature is a variable important for many proteins because the proteins denature at high temperatures and when zein is heated to 70 °C the secondary structure changes (Selling et al., 2007). As can be seen in Figure 4.9, the significant increase of sizes is from 70 °C, this way other explanation for increase of nanoparticles size is denaturation of zein.

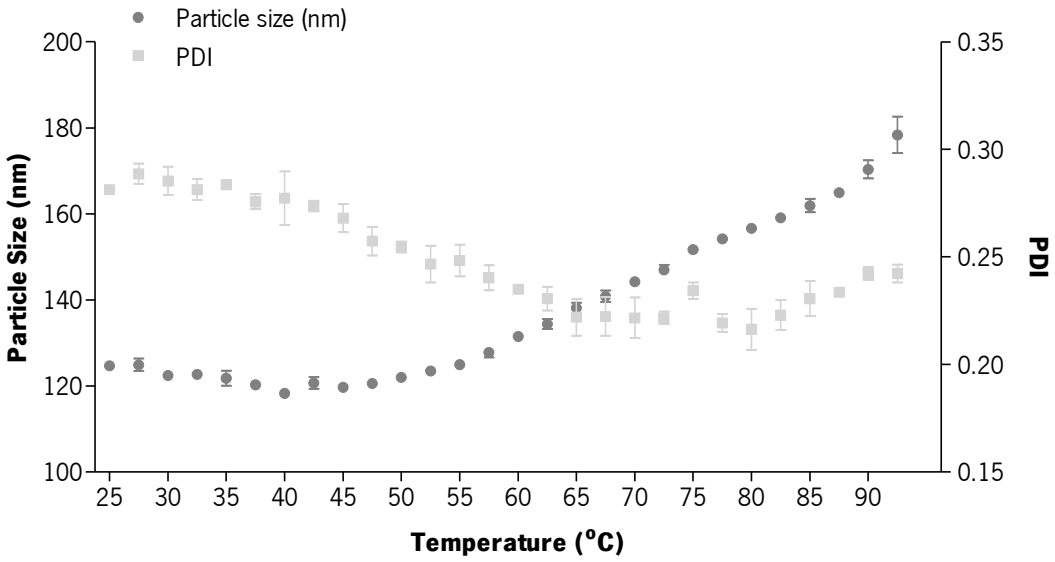


Figure 4.9 – Effect of temperature in particle size and PDI of zein nanoparticles without TOC.

Storage Time

Zein nanoparticles stability was studied during five months, being used DLS for measurement of size and PDI of nanoparticles. Figure 4.10 shows the effect of time in sizes and PDI of particles with and without vitamin. Results show that encapsulation of TOC do not interfere with stability of zein nanoparticles, presenting the samples the same stability profile. Initially, after the first month of storage the sizes of nanoparticles increase and PDI decrease, being the results obtained for first and the second month statistically different (p<0.05). Then zein nanoparticles sizes and PDI are constant from the second to the fifth month not changing particles size and PDI values (p>0.05).

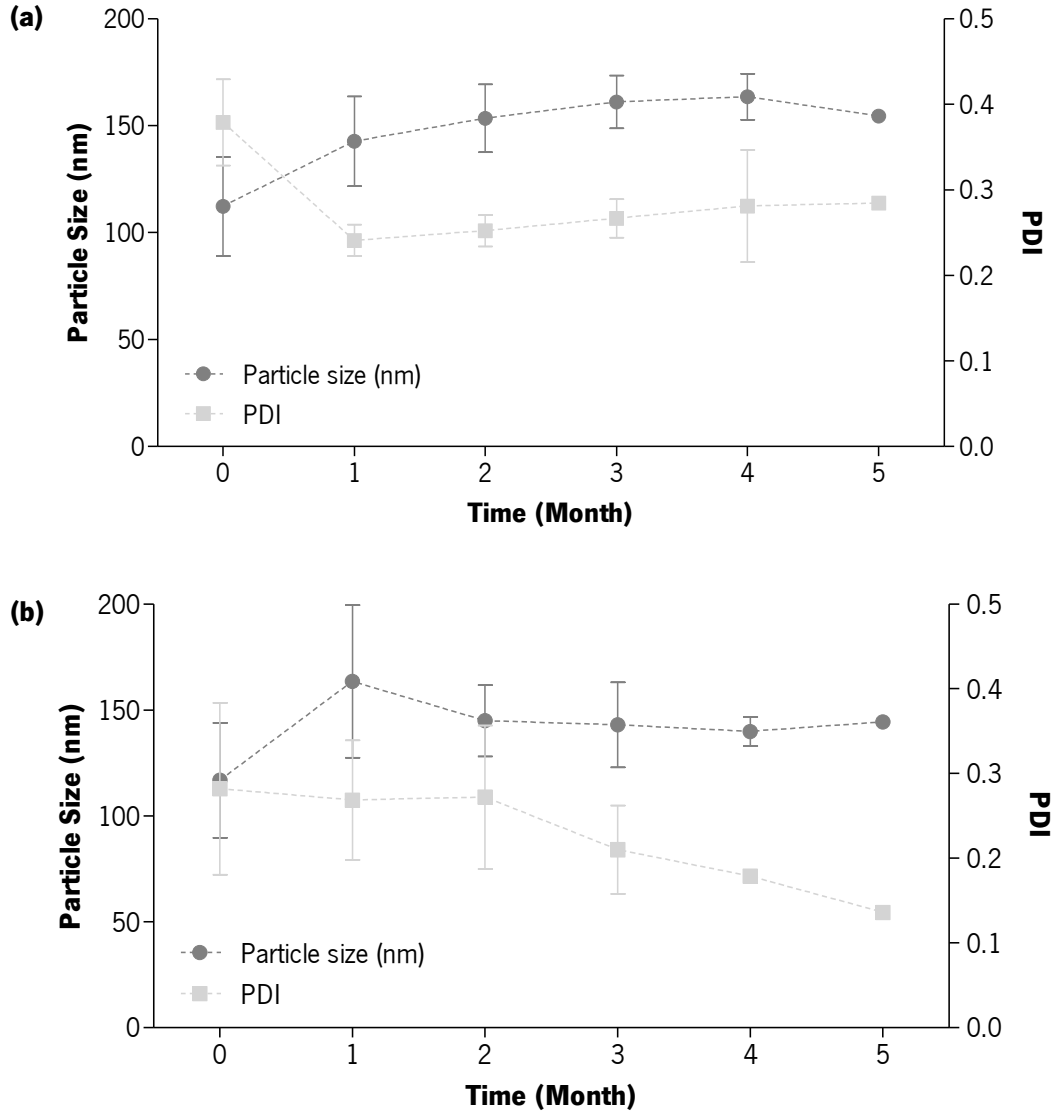


Figure 4.10 – Stability of Zein nanoparticles: (a) without TOC; (b) with TOC.

CHAPTER V

GENERAL CONCLUSIONS

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5.1 CONCLUSIONS

The main objective of this work was to develop nanostructures for the encapsulation of water-soluble and liposoluble vitamins and their further characterization. So, and in order to cover this objective two types of nanoparticles were studied: alginate/chitosan nanoparticles for encapsulation of water-soluble vitamins, and zein nanoparticles for liposoluble vitamins.

First, for alginate/chitosan nanoparticles, the interesting features of this work were the following:

- Pre-gelation ionotropic method can be used for alginate/chitosan nanoparticles production, however it has been demonstrated the importance of various parameters during the preparation of nanoparticles.

- (-)-Riboflavin can be successfully encapsulated with values of EE and LC of ~55 % and ~2 %, respectively.

- Release profiles showed that alginate/chitosan nanoparticles can be used in the retention of (-)-riboflavin.

Essentially, the results show that ionotropic pre-gelation is a promising approach to produce alginate/chitosan nanoparticles with vitamin and the nanoparticles show potential to be applied in different food and/or beverages products.

For zein nanoparticles, the main conclusions were:

- Zein nanoparticles were successfully developed as a novel delivery system for TOC, using a low-energy method self-assembly.

- Particle size and PDI of the nanostructure can be controlled with different preparation parameters.

- TOC was encapsulated without significant influence on particle size, being obtained values of EE of ~95 % and LC of ~9.

- The TOC was not release in PBS medium at 37°C but was in Tris-HCL medium.

Briefly, the zein nanoparticles are a good option for TOC encapsulation, but it is necessary an optimization to protect TOC against premature release in gastric pH and the release in intestine.

5.2 RECOMMENDATIONS

In general, the main objectives of this thesis have been achieved, however some work can be done in order to better understand the properties of the nanoparticles developed and how can they be used in food industry. So, some recommendations for improvement of the present work can be advanced:

- Nanoparticles characteristics and the relationship between vitamins and biopolymers can be achieved, for example, by FTIR (Fourier Transform Infrared Spectroscopy), DSC (Differential Scanning Calorimetry) and/or TGA (Thermogravimetric analysis).

- As both vitamins used lost their properties when exposed to the light, in order to perceive the differences between free vitamin and vitamin encapsulated, it can be interesting to do a photochemical stability measurement.

- Generally, most nutrients and vitamins are best absorbed in intestine for further utilization in the body. So, it is important to protect vitamins against premature release in gastric pH and to promote the release in intestine. This way, particularly for zein nanoparticles, I suggest a coating with, for example, chitosan or alginate.

- A release profile at 25°C in PBS and Tris-HCL medium could be useful in order to know if the temperature influences the release of TOC.

- Also it can be important to use a dynamic gastro-intestinal model that mimics the human physiological conditions (simulating of stomach, duodenum, jejunum and ileum) to evaluate the influence of physiological parameters, such as pH, temperature, peristaltic movements, secretion of digestion enzymes, bile an pancreatic juice, in both nanoparticles developed. This would allow having a clear idea clearest of what happen with nanoparticles in our organism.

- The toxicity of nanoparticles represents one of the major problems for nanotechnology, so, the *in vitro* and *in vivo* evaluation of alginate/chitosan and zein nanoparticles in different cell line and animal models can be an interesting work.

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ANNEXES

ANNEX A

OPTIMIZATION OF DEVELOPMENT OF NANOPARTICLES

I. ALGINATE/CHITOSAN NANOPARTICLES

Table A.1 – Different formulations tested for development of alginate/chitosan nanoparticles.

Formulation	Alginate (%)	Flow rate of $CaCl_2$ (mL/min)	Chitosan (%)	Flow rate of chitosan solution (mL/min)	Ultra-Turrax (rpm)	Centrifugation at the end of process
1	0.063	0.085	0.07	0.189	0	1 min at 14 000 rpm
2	0.063	0.125	0.07	0.278	0	1 min at 14 000 rpm
3	0.063	0.125	0.07	0.278	0	No
4	0.063	0.125	0.07	0.278	20 000	No
5	0.063	0.125	0.07	0.278	20 000	10 min at 14 000 rpm
6	0.063	0.125	0.04	0.278	20 000	No
7	0.063	0.125	0.04	0.278	20 000	10 min at 14 000 rpm
8	0.063	0.125	0.02	0.278	20 000	No
9	0.063	0.125	0.02	0.278	20 000	10 min at 14 000 rpm

Table A.2 – Particle size and PDI values of different formulations for development of alginate/chitosan nanoparticles.

Formulation	Particle Size (nm)	PDI
1	480.93 ± 13.84	0.542 ± 0.075
2	1115.83 ± 108.05	0.955 ± 0.078
3	412.13 ± 11.52	0.516 ± 0.015
4	863.57 ± 34.92	0.842 ± 0.019
5	389.97 ± 6.92	0.400 ± 0.052
6	539.27 ± 17.55	0.449 ± 0.031
7	296.77 ± 16.84	0.363 ± 0.031
8	699.93 ± 63.84	0.581 ± 0.053
9	292.63 ± 5.59	0.438 ± 0.014

II. ZEIN NANOPARTICLES

Table A.3 – Different formulations testes for development of zein nanoparticles.

Formulation	Zein (mg/mL)	Ratio Zein solution:distilled water (v/v)	Flow Rate (mL/min)	(±)-α-Tocopherol (mg/mL)
1	2	1:1	0.667	0
2	2	1:2	0.667	0
3	2	1:5	0.667	0
4	2	1:10	0.667	0
5	2	2:1	0.667	0
6[a]	2	1:5	0.667	0
7[b]	2	1:5	0.333	0
8[c]	2	1:5	0.333	1
9[c]	2	1:5	0.222	0
10[c]	2	1:5	0.167	0
11[c]	2	1:5	0.167	1
12[c]	1	1:5	0.167	0
13[d]	1	1:5	0.167	0.5

After zein solution addition, zein nanoparticles were maintained in the same stirring for 60 minutes and was measured the size and PDI at the time:

[a] 0, 5, 15, 25, 30, 45 and 60 minutes.

[b] 0, 5, 15, 30 and 60 minutes.

[c] 0, 5, 15, 25, 30, 45 and 60 minutes.

[d] In the end of the procedure the zein nanoparticles was maintained in same stirring for 15 minutes, being measured the size and PDI.

Table A.4 – Particle size and PDI values of different formulations for development of zein nanoparticles.

Formulation	Particle Size (nm)	PDI	Formulation	Particle Size (nm)	PDI
1	491.13 ± 17.93	0.238 ± 0.031	8.5	161.77 ± 6.98	0.408 ± 0.047
2	223.73 ± 7.87	0.201 ± 0.019	9	305.60 ± 96.49	0.358 ± 0.103
3	138.23 ± 10.88	0.361 ± 0.127	9.1	181.17 ± 19.79	0.244 ± 0.050
4	480.33 ± 258.25	0.462 ± 0.199	9.2	320.77 ± 131.79	0.340 ± 0.087
5	738.50 ± 44.98	0.252 ± 0.009	9.3	218.83 ± 44.18	0.266 ± 0.047
6.1	190.53 ± 13.64	0.459 ± 0.027	9.4	475.83 ± 247.96	0.460 ± 0.193
6.2	251.400 ± 33.81	0.512 ± 0.152	9.5	207.63 ± 35.11	0.277 ± 0.073
6.3	177.37 ± 3.07	0.447 ±0.009	10	231.77 ± 35.60	0.313 ± 0.039
6.4	224.57 ± 11.59	0.543 ± 0.099	10.1	278.30 ± 101.06	0.360 ± 0.065
6.5	208.27 ± 14.19	0.425 ± 0.072	10.2	102.50 ± 15.35	0.426 ± 0.046
6.6	203.97 ± 26.27	0.471 ± 0.078	10.3	405.77 ± 190.99	0.415 ± 0.134
7	126.83 ± 1.63	0.464 ± 0.014	10.4	140.33 ± 4.66	0.197 ± 0.025
7.1	150.30 ± 2.72	0.479 ± 0.016	10.5	231.13 ± 36.53	0.277 ± 0.035
7.2	147.03 ± 7.94	0.455 ± 0.172	11	94.71 ± 1.86	0.298 ± 0.010
7.3	219.67 ± 26.46	0.340 ± 0.152	11.1	189.10 ± 43.48	0.232 ± 0.046
7.4	266.63 ± 62.08	0.271 ± 0.037	11.2	90.61 ± 1.35	0.257 ± 0.009
8	136.83 ± 0.71	0.364 ± 0.011	11.3	102.68 ± 14.74	0.253 ± 0.055
8.1	176.40 ± 13.79	0.254 ± 0.015	11.4	172.37 ± 20.40	0.334 ± 0.169
8.2	154.37 ± 8.81	0.414 ± 0.018	12	117.13 ± 0.59	0.412 ± 0.008
8.3	145.60 ± 9.74	0.367 ± 0.066	13	116.80 ± 0.82	0.284 ± 0.012
8.4	142.03 ± 4.95	0.377 ± 0.007			

ANNEX B

MAXIMUM ABSORBANCE PEAK

I. (-)-RIBOFLAVIN

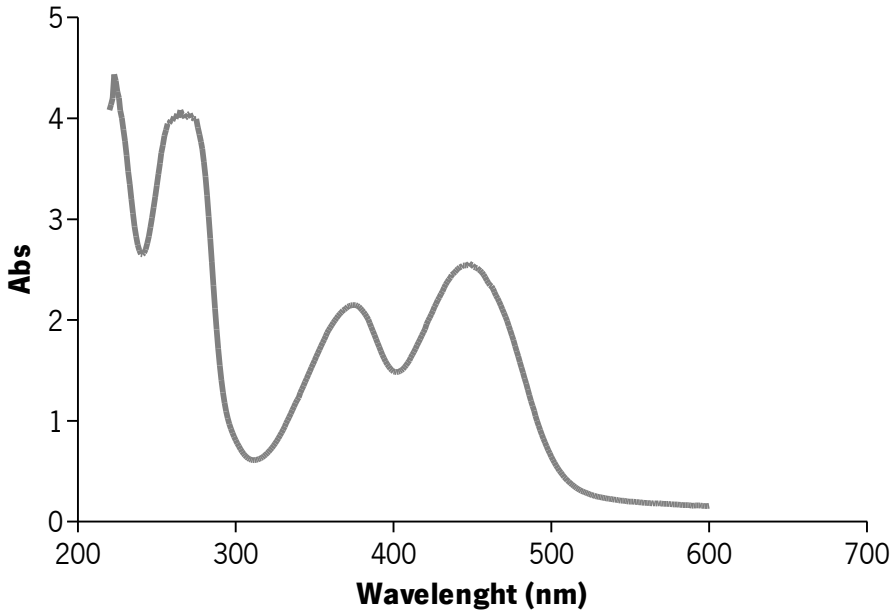

Figure A.1 – UV–Vis spectrum of (-)-riboflavin. Spectra were overlaid after scanning from 200 to 600 nm.

II. (±)-α-TOCOPHEROL

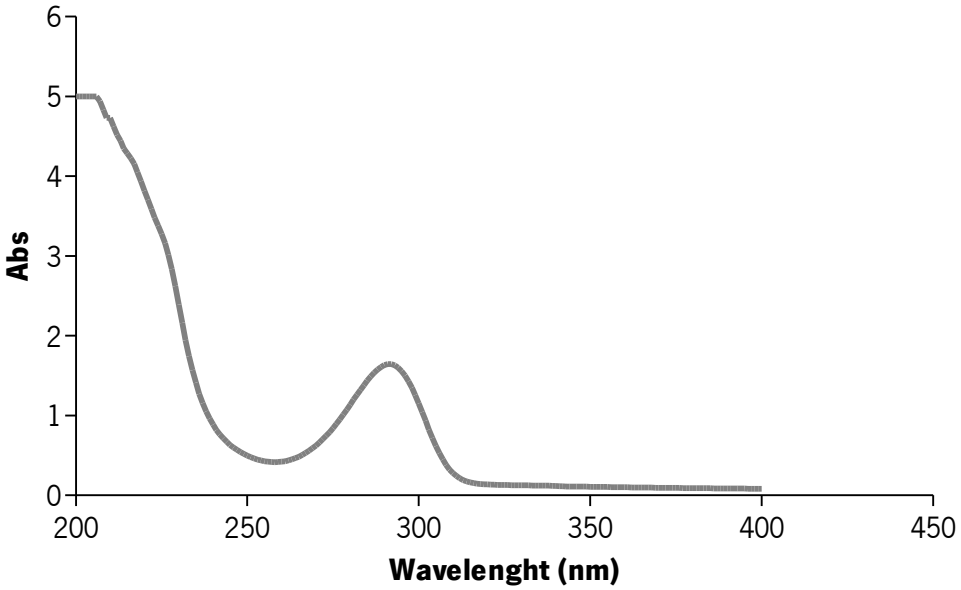

Figure A.2 – UV–Vis spectrum of TOC. Spectra were overlaid after scanning from 200 to 400 nm.

ANNEX C

CALIBRATION CURVES

I. (-)-RIBOFLAVIN

Table A.5 – Dilutions and their absorbance values for calibration curve of (-)-riboflavin.

	Dilution										
	0	1/2	1/4	1/5	1/8	1/10	1/16	1/25	1/32	1/100	1/125
[(-)-Riboflavin] (mg/ml)	0.0950	0.0475	0.0238	0.0190	0.0119	0.0095	0.0059	0.0038	0.0030	0.0010	0.0008
Absorbance (λ=437 nm)	2.465	1.488	0.738	0.642	0.343	0.311	0.186	0.130	0.088	0.033	0.025

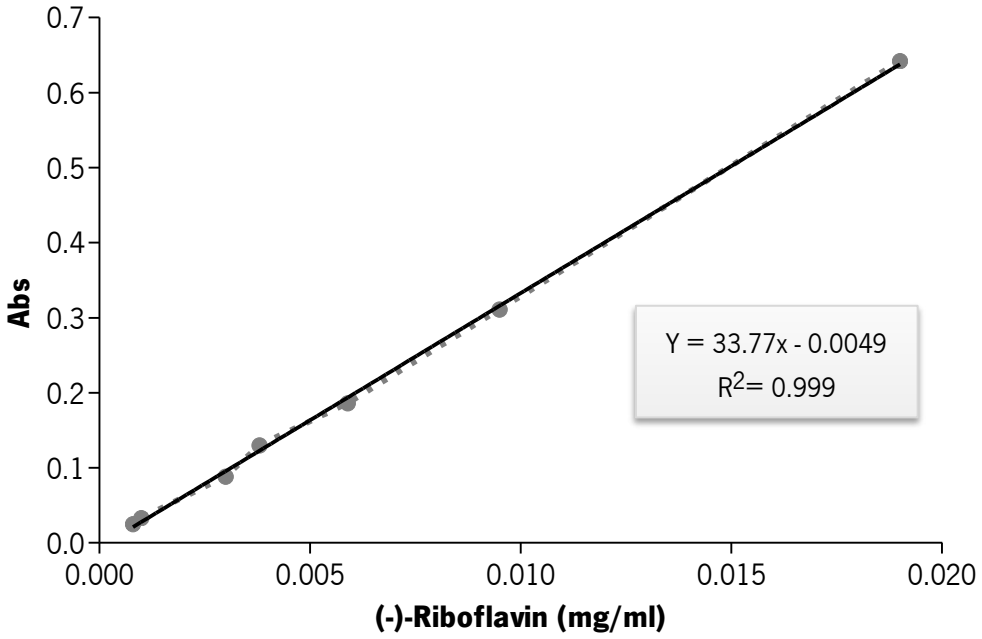

Figure A.3 – Calibration curve of (-)-riboflavin. Absorbance *versus* concentration for (-)-riboflavin.

II. (±)-α-TOCOPHEROL

Table A. 6 – Dilutions and their absorbance values for calibration curve of TOC.

	Dilution											
	0	1/2	1/4	1/5	1/10	1/16	1/25	1/32	1/100	1/125	1/150	1/1000
[TOC] (mg/mL)	0.500	0.250	0.125	0.100	0.063	0.05	0.031	0.020	0.016	0.005	0.004	0.0005
Absorbance (λ=292 nm)	2.466	1.811	1.105	0.883	0.507	0.636	0.323	0.287	0.182	0.046	0.030	0.001

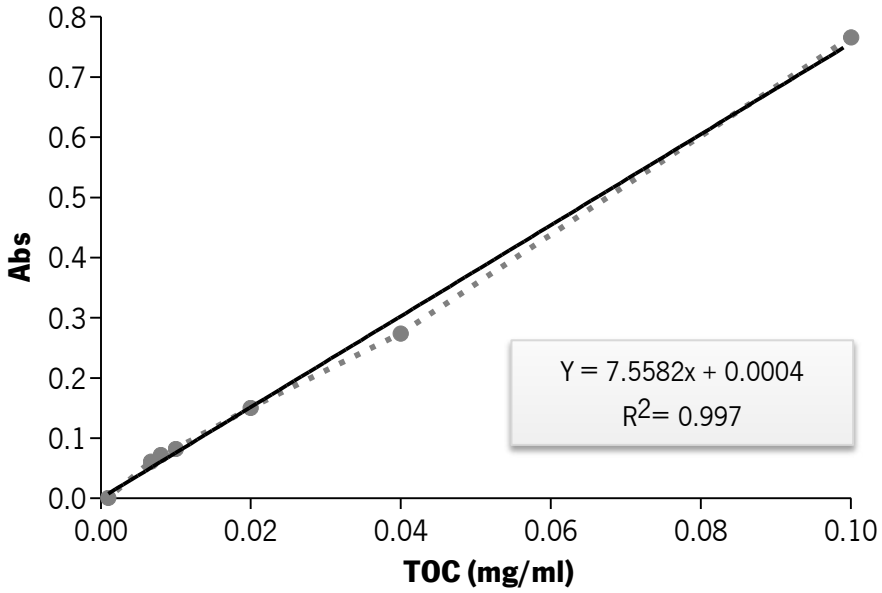

Figure A.4 – Calibration curve of (±)-α-tocopherol. Absorbance *versus* concentration for (±)-α-tocopherol.