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# Evaluation in vitro et in vivo de la stabilité et de la biodisponibilité de molécules liposolubles encapsulées et incluses dans une matrice modèle

Maud Gonnet

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**Evaluation *in vitro* et *in vivo* de la stabilité et de la biodisponibilité  
de molécules liposolubles encapsulées et incluses dans une matrice  
modèle**

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## Abréviations et symboles

### A

ACN	acétonitrile
AO	AntiOxidant;
AUCcum	Cumulative Area Under Curve;

### B

$\beta$ C	$\beta$ -carotène
BHT	hydroxytoluène butylé

### C

CHCl <sub>3</sub>	chloroforme
CH <sub>2</sub> Cl <sub>2</sub>	dichlorométhane
CMC	critical micellar concentration

### D

DPPC	dipalmitoyl phosphatidylcholine
------	---------------------------------

### E

E <sub>e</sub>	Elasticité d'équilibre (module élastique)
E <sub>ne</sub>	Elasticité non équilibre (module visqueux)
E/H	Eau dans huile

### G

$\gamma$	Tension interfaciale
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### H

H/E	Huile dans eau
-----	----------------

### L

LNC	lipid nanocapsule
-----	-------------------

### M

MeOH	méthanol
$\mu$	mobilité électrophorétique

### N

NCL	nanocapsule lipidique
-----	-----------------------

### O

O/W	oil-in-Water
-----	--------------

### P

PC	phosphatidylcholine
PDI	(polydispersity index) indice de polydispersité
$\pi$	pression superficielle
PEG	polyéthylène glycol

	PEO	polyethylene oxide
	POE	polyoxyethylene
	PL	phospholipide
<b><u>R</u></b>	RA	acide rétinoïque
<b><u>S</u></b>	SDS	sodium dodecyl sulphate
	SLN	solid lipid nanoparticle
	SLM	solid lipid microparticle
	S/V	surface/volume
<b><u>T</u></b>	<sup>99</sup> Tc	technétium
	TIP	température d'inversion de phase
	τ	temps de relaxation
	THF	tetrahydrofurane
<b><u>Z</u></b>	ZIP	zone d'inversion de phase

# **Introduction**

Un régime alimentaire standard apporte dans des conditions normales l'ensemble des apports énergétiques et nutritionnels requis. Cependant, dans certaines circonstances ou sous l'influence de certains facteurs physiologiques, les besoins nutritionnels ne sont pas couverts pour certains types de micronutriments. D'une part, l'étude SU.VI.MAX a rapporté que l'ensemble des besoins en vitamines et minéraux n'étaient pas atteints par l'ensemble de la population française. D'autre part, des enquêtes épidémiologiques ont associé la consommation d'aliments riches en vitamines, et en particulier les vitamines aux propriétés anti-oxydantes, à un effet positif sur la prévalence de certaines maladies (cancers, maladies cardio-vasculaires ou certaines pathologies oculaires) [1-5]. Cependant, l'augmentation de la consommation d'aliments riches en provitamine A ne conduit pas nécessairement à une augmentation de la concentration plasmatique de la vitamine A [6]. Ce phénomène peut être expliqué par une faible biodisponibilité des molécules au sein de ces matrices complexes. La biodisponibilité représente la proportion d'un aliment ingéré disponible pour les fonctions physiologiques ou le stockage. Les compléments alimentaires existant augmentent l'apport en micronutriments, mais n'améliorent pas l'efficacité d'absorption.

Les antioxydants sont des composés fragiles susceptibles d'être dégradés ou de se complexer avant d'atteindre leur cible d'action cellulaire, pouvant conduire ainsi une diminution de leur biodisponibilité. Les micronutriments (minéraux, vitamines....) sont ingérés en petites quantités et leur taux d'absorption intestinale est variable et souvent faible. Plusieurs stratégies ont été développées pour augmenter l'apport en vitamines et éléments antioxydants: enrichissement de matrice alimentaire, compléments alimentaires (gélules, capsules ...). L'encapsulation est une stratégie permettant de stabiliser les molécules considérées jusqu'à leur absorption et de contrôler leur libération durant la digestion. Dans le domaine agro-alimentaire, l'encapsulation est développée pour l'inclusion de composés d'arômes, d'antioxydants ou de micronutriments ayant des propriétés bénéfiques pour

l'organisme. L'encapsulation peut constituer à la fois un moyen de protéger ces molécules fragiles durant le stockage, et une manière d'augmenter leur biodisponibilité. L'utilisation de ce type de système peut faire l'objet de questionnements légaux et éthiques. En effet, les produits enrichis en micronutriment sont fréquemment accompagnés d'allégations nutritionnelles qui peuvent inciter à une consommation accrue. Or, pour des concentrations élevées, certains éléments tels que les antioxydants sont susceptibles de devenir pro-oxydants, et donc toxiques [2, 7, 8]. Ce type de situation est d'autant plus probable lorsque la biodisponibilité est élevée. Le référentiel connu à ce jour pour les doses journalières recommandées n'est pas adapté aux systèmes qui augmentent la biodisponibilité.

Les systèmes particuliers utilisés pour l'encapsulation de micronutriments peuvent être produits par de nombreux procédés de formulations. Les caractéristiques des particules produites dépendent du procédé employé et excipients utilisées. Les objets formulés peuvent avoir des tailles allant du millimètre au nanomètre. Quand les éléments d'intérêt sont apportés sous la forme de nano-objets, on parle alors de nano-foods. De part leur petite taille, les nano-objets ont un rapport surface/volume (ou aire surfacique) élevé ce qui augmente les interactions avec le milieu extérieur et la quantité de tensioactifs nécessaire à leur stabilisation. De nombreux groupes de travail ont cherché à évaluer les risques liés aux nano-objets et ce dans chaque domaine d'utilisation des nanotechnologies [9-12]. La FDA (food and drug administration) et l'Afssa (agence française de la santé et de la sécurité des aliments) ont établi des rapports (2007 et 2009 respectivement) concernant les risques potentiels dus à l'utilisation des nanotechnologies en alimentation humaine [12, 13]. Si les éléments constitutifs des nano-objets sont dans la liste positive des additifs et que le procédé de production est connu, l'évaluation de leur toxicité et de leur qualité nutritionnelle en revanche n'est pas encore obligatoire. Cependant, les différentes études concernant les risques engendrés par les nano-objets sur l'organisme ont mis en évidence que la taille des nano-

objets avait des conséquences sur leur toxicité car leur petite dimension augmente les voies d'entrées dans l'organisme [9-11]. Ces études ont également démontré la toxicité des nano-objets sur les voies aériennes, la peau et l'intestin. Bien que dépendante de la taille, la toxicité des nano-objets est aussi liée à leur composition, leurs propriétés de surface et à leurs caractéristiques physico-chimiques. C'est pourquoi des conclusions générales ne peuvent pas être dressées. Il ressort de ces rapports qu'avant de développer une législation commune, accroître les connaissances sur les nano-objets semble essentiel.

Les techniques telles que l'extrusion, le *spray drying*, l'émulsification ou la coacervation ont en général des coûts moins élevés que les nanotechnologies. L'encapsulation, et les nanotechnologies en particulier, sont des techniques à employer dans des conditions pour lesquelles l'innovation technologique apporte une fonctionnalité en rapport avec son coût. Ceci peut être le cas lorsque la nutrition médicale s'adresse à des personnes fragiles, généralement en soins ou en convalescence dont les besoins nutritionnels particuliers ne sont pas forcément couverts par une alimentation équilibrée classique. Les produits de nutrition médicale nécessitent une formulation complexe qui répond à des besoins physiologiques particuliers. Cette branche de l'industrie agro-alimentaire est en plein essor.

Nous nous sommes intéressés aux vitamines lipophiles et plus particulièrement à la vitamine A ainsi qu'aux caroténoïdes dont 10 % sont des provitamines A. Les caroténoïdes sont des pigments organiques connus pour avoir des propriétés anti-oxydantes [14]. Leur capacité à réagir avec des hydroperoxydes est due principalement à leur double liaison conjuguées [14, 15]. Plusieurs études ont démontrées que les caroténoïdes peuvent protéger les membranes et les lipides membranaires contre l'oxydation dans des émulsions [16], des liposomes [17-19] ou des lipoprotéines [20]. Ces propriétés anti-oxydantes les rendent sensibles à la chaleur, la lumière et l'oxygène. Le  $\beta$ -carotène ( $\beta$ C) est la source la plus importante de vitamine A de l'organisme. Toutefois, sa biodisponibilité à partir d'aliments est



très variable, l'absorption intestinale peut varier de 3 à 26 % [21-23]. C'est pourquoi, de nombreuses études visent à augmenter son apport et sa biodisponibilité à travers la supplémentation, l'enrichissement de produits alimentaires ou l'encapsulation.

Nous avons cherché à limiter la dégradation du  $\beta$ C tout en augmentant son absorption grâce à l'encapsulation. Le choix des particules s'est porté sur une technologie maîtrisée par l'Unité 646 les nanocapsules lipidiques (NCLs) [24]. Les objectifs de ce travail consistaient à: (i) Encapsuler le  $\beta$ C dans des NCLs composées d'excipients appartenant à la liste positive des additifs alimentaires. (ii) Préserver l'intégrité et les propriétés anti-oxydantes du  $\beta$ C et (iii) Evaluer l'impact de l'encapsulation du  $\beta$ C sur sa biodisponibilité.

Cette thèse débute par une revue bibliographique s'intéressant à l'encapsulation des vitamines lipophiles et des caroténoïdes et leurs applications. Après avoir présenté les méthodes employées dans la partie expérimentale, nous décrivons le travail expérimental lui-même, constitué de quatre parties. La première partie présentera la mise au point de l'encapsulation du  $\beta$ C dans les NCLs et l'impact des conditions de digestion sur la libération ou le maintien du  $\beta$ C sous sa forme encapsulée, à travers une approche *in vitro* du parcours digestif. Dans la deuxième partie, la caractérisation interfaciale des NCLs sera réalisée grâce à des méthodes permettant de modéliser les interactions entre le  $\beta$ C et des éléments constitutifs de l'interface. La troisième partie expérimentale explicitera le comportement des NCLs dans une matrice complexe modèle : la crème fraîche. Enfin la modélisation des phénomènes *in vitro* sera complétée par l'étude de l'impact de l'encapsulation d'un composé lipophile radiomarqué modèle sur sa biodisponibilité.

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**Revue bibliographique**

## **New trends in encapsulation of liposoluble vitamins**

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**Abstract:**

Liposoluble vitamins (A, D, E, K) and carotenoids have many benefits on health. They are provided mainly by foods. At pharmacological doses, they can also be used to treat skin diseases, several types of cancer or decrease oxidative stress. These molecules are sensitive to oxidation, thus encapsulation might constitute an appropriate mean to preserve their properties during storage and enhance their physiological potencies. Formulation processes have been adapted for sensitive molecule, limiting their exposure to high temperature, light or oxygen. Each administration pathway, oral, systemic, topical, transdermal and local, requires different particles sizes and release profile. Encapsulation can lead to greater efficiency allowing smaller administration doses thus diminishing potential hypervitaminosis syndrome appearance and side effects. Carrier formulation can be based on vitamin dissolution in lipid media and its stabilization by surfactant mixture, on its entrapment in a matrix or molecular system. Suitability of each type of carrier will be discussed for each pathway.

**Keywords:** Vitamin, carotenoid, carrier, antioxidant, particle

**Abbreviations:** **AMVN:** 2,2'-azobis(2,4'-dimethylvaleronitrile); **AO:** antioxidant; **βC:** β-carotene; **Ch:** cholesterol; **CD:** cyclodextrin; **DMSO:** dimethylsulfoxide; **DPPC:** dipalmitoyl phosphatidylcholine; **DPPS:** dipalmitoyl phosphatidylserine; **LDL:** low density lipoprotein; **NC:** nanocapsule; **NP:** nanoparticle; **PC:** phosphatidylcholine; **PEG:** polyethylene glycol; **PEO:** polyethylene oxide; **PL:** phospholipid; **PDI:** polydispersity index **RA:** retinoic acid; **RDI:** recommended daily intake; **SA:** stearylamine; **SNEDDS:** self-nanoemulsified drug delivery systems; **SR-B1:** scavenger receptor class B type 1; **THF :** tetrahydrofuran; **SLM:** solid lipid microparticle **SLN:** solid lipid nanoparticle.

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## Introduction

Vitamins A, D, E, K and carotenoids are liposoluble compounds which are naturally present in food or used as excipients in different industrial fields such as pharmaceuticals, cosmetics or foods. Whereas carotenoids do not belong to usual vitamins classification, they are often studied with liposoluble vitamins as 50 carotenoids among the over 600 carotenoids identified to this day are pro-vitamin A elements. Vitamins are sensitive molecules, they must be preserved from pro-oxidant elements which could affect their chemical integrity and decrease their physiological benefits.

Encapsulation constitutes a promising approach to preserve their native properties over time. The classical systems developed in nano or microencapsulation are based on reservoir or matricial particles [1]. These systems may constitute a physico-chemical barrier against pro-oxidant elements such as free-radicals, oxygen or UV. Encapsulation also represents a mean to improve biological efficiencies such as shelf life, control active components delivery and could prevent side effect apparition [2-4]. All encapsulation processes should lead to particles with a high encapsulation rate, good polydispersity index (PDI) and high shelf life. To optimize bioefficiency, which is the proportion of active administrated molecules, particles characteristics have to be adapted to their administration pathway which could be oral, topical, parenteral or local injection. Liposoluble vitamins encapsulation may also imply different requirements depending on their use. Concerning food applications, inclusion of vitamins loaded particles in food matrix will create the so called functional food or nutraceuticals. This type of functionality will imply the use of food-grade or GRAS (Generally Recognized As Safe) components for particles formulation. More excipients and techniques are approved by cosmetics industry, implying a wider particles choice. Inclusion of vitamins loaded particles in pharmaceutical preparation will imply the use of excipients responding to Pharmacopeia regulations and their adaptation to physiological conditions in terms of pH, osmolarity and particles size. If particles are incorporated in a matrix, the choice needs to be

considered closely as vitamins and carotenoids, even encapsulated, might be altered by some industrial processes (high pressure, temperature, shear stress,...).

Considering the numerous possible particles composition, it is important to note that particles type has an influence on bioefficiency. It has been shown that  $\beta$ -carotene ( $\beta$ C) plasma response was greater for gel capsules than for commercial beadlets [5]. This study highlights the need for further investigations and optimization in the field of vitamins encapsulation. Physicochemical and physiological properties of these molecules will first be overviewed. Afterwards we will discuss the link between vitamins and carotenoids behavior in phospholipids mono or bilayers and their anti-oxidant (AO) potencies as well as their behavior when encapsulated. Finally different encapsulation processes will be compared for each type of applications to determine which vehicle is the most adapted to each pathway.

## **I. Background on liposoluble vitamins**

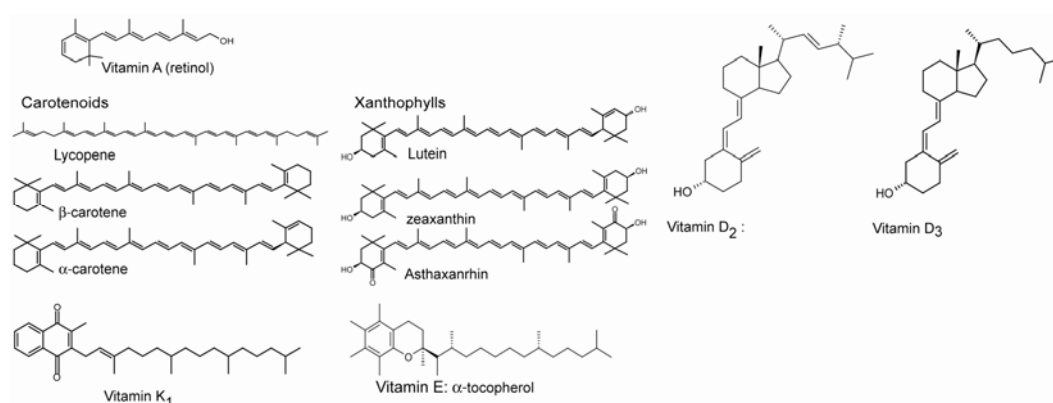
### **I.1. Vitamin A**

Vitamin A has different chemical forms in the organism depending on its location and function: retinol, retinyl esters, retinal, and retinoic acid (RA). Vitamin A is mainly ingested as retinol (Fig 1), retinyl esters or pro-vitamin A molecules such as  $\beta$ C. Retinyl esters are hydrolyzed in retinol in intestinal lumen, included in mixed micelle [6] and then absorbed in enterocyte via passive diffusion or through a specific transporter, such as scavenger receptor class B type 1 (SR-B1) [7]. Retinol is esterified in enterocytes before its inclusion in chylomicrons. Retinyl esters secretion is regulated by chylomicrons assembly [8]. They are vitamin A circulating form up to vitamin A storage sites which are mainly liver [9-12], kidneys, adipose tissue and pulmonary cells [13]. When retinol is released from storage sites, it complexed to the Retinol Binding Protein (RBP) [14]. Vitamin A-RBP complex is vitamin A circulating form. Retinol can be converted in retinal, which is involved in night vision [15]. Its oxidation is catalyzed by retinal reductase and produces RA [16-18]. RA regulates cell



proliferation, differentiation and morphogenesis (bone calcification) [19]. RA is a therapeutic agent used for leukemia, skin cancer and diseases treatment [20, 21]. RA might be efficient on other cancers such as breast cancer [22]. However a relapse and RA resistance can be observed in several patients, which might be due to RA plasma concentration decrease during treatment *per os*, as well as vitamin A hypervitaminosis syndrome.

Recommended Daily Intake (RDI) for Vitamin A is 700 and 900  $\mu\text{g}/\text{day}$  for women and men respectively. It can derive from Vitamin A or provitamin A molecules. Vitamin A deficiency occurs mainly in developing countries or in cases of unbalanced diet and malabsorption diseases. Vitamin A deficiency causes xerophthalmia, which can be cured via supplementation [23]. Vitamin A might demonstrate pro-oxidant properties when high doses ( $>10$  RDI) are ingested for a long period of time. These patients were showing hepatic fibrosis and congestion [24]. Therefore, vitamin A supplementation should be taken with care, all the more when bioefficiency is enhanced. RA is efficient as skin diseases treatment, however its poor solubility in water and its high chemical instability reduce its possible applications. Besides, as RA can have teratogen effect, its targeting could be a mean to decrease these serious side effects and enhance its therapeutic efficiency.



**Figure 1:** Lipophilic vitamins and carotenoids chemical structures

## I.2. Carotenoids

Carotenoids are organic pigments integrated in plants chromoplasts and some other photosynthetic organisms. Over 600 carotenoids are known; they are separated into two classes: carotenes which represent oxygen-free molecules and xanthophylls which are hydroxycarotenoids and consequently less hydrophobic than carotenes (Fig.1). Main carotenes are  $\alpha$ -carotene,  $\beta$ C and lycopene. Main xanthophylls are lutein, zeaxanthin, asthaxanthin and  $\beta$ -cryptoxanthin. Lutein and zeaxanthin are the two major components of macular pigment. Their main property is to filter blue light which can be highly damageable for cells [25, 26]. Carotenoids are known to have high free radical scavenging properties [27], this is due to their multiple conjugated double bonds. Consequently they are sensitive to O<sub>2</sub>, light and heat. Indeed, carotenoids can protect plasmatic lipids and membrane from oxidation [28].

Once released from food matrix, carotenoids are included in chyme lipidic fraction, emulsified by bile salts in mixed micelles [6]. Carotenoids absorption may occur via a facilitated process, through SR-B1 and passive diffusion [7, 29]. SR-B1 is involved in cholesterol (Ch), vitamins A and E absorption; therefore when ingested together, competition could decrease their absorption [29]. Carotenoids absorption rate seemed to depend on the considered molecule [30]. Lycopene had the lowest absorption rate in Caco 2 cells, lutein uptake was higher and  $\beta$ C had the greatest. Besides, molecules conformation seemed to have a significant impact on their absorption rate: *trans*  $\beta$ C had a higher intake rate than *cis*  $\beta$ C. Whereas all-*cis* lycopene seemed to be preferentially absorbed [31]. As  $\beta$ C is the greatest source of vitamin A in human diet, it is the most encapsulated and studied carotenoid. *In vivo* studies on rat showed that after its absorption in enterocytes,  $\beta$ C was converted to retinal then to retinol [17, 18], esterified and finally included in chylomicrons [16, 32]. This absorption pathway was a saturable process: therefore when  $\beta$ C was ingested in high concentration,

excessive  $\beta$ C was not converted but directly included in chylomicrons [33]. In humans, saturations occurs for lower concentrations, therefore more  $\beta$ C is absorbed intact [34]. Other carotenoids follow the same absorption pathway. Conversion ratios are carotenoid dependant: production of 1 $\mu$ g of retinol requires 12  $\mu$ g of  $\beta$ C and 24  $\mu$ g of other carotenoids [35]. The main storage site is liver parenchymal cells, as for vitamin A [36, 37]. A case study demonstrates that carotenes intake was significantly greater for healthy persons than for people suffering from bladder cancer [38]. This study strengthened the theory according to which carotenoids might have beneficial effect on health especially on cancer and cardiovascular diseases prevalence. However,  $\beta$ C, like vitamin A, becomes pro-oxidant under high oxidative stress, which induces molecule eccentric cleavage resulting in apo-carotenoids formation. When concentration of apo-carotenoids is low, they can be converted in RA [39, 40]. At higher concentration, the conversion pathway might be saturated and apo-carotenoids might accumulate in the cytosol. This situation could create a pool of highly reactive components. Although the mechanism is still unclear, it has been demonstrated that  $\beta$ C breakdown products induced by cigarette smoke, for example, are not directly related to a pro-oxidant effect in bronchial epithelial cells [41]. Neutrophils apoptosis induction by  $\beta$ C breakdown products might constitute a part of cancer risk enhancement [42]. This could explain that cancer risk increases when smokers are supplemented in  $\beta$ C [43].

### I.3. Vitamin D

Vitamin D is a seco-steroid hormone and has two main chemical forms (Fig 1). The first is vitamin D<sub>3</sub> (cholecalciferol) which is synthesized in the skin after light exposure [44] and displays several chemical structures [45]. Calcidiol is the unhydroxylated and inactive form. Calcidiol is the monohydroxylated and blood circulating form (25-hydroxyvitamin D<sub>3</sub>) [46, 47]. Calcitriol is the dihydroxylated and active form (1,25-dihydroxyvitamin D<sub>3</sub>) [48]. Calcitriol displays different actions. First, it controls calcium and phosphorus homeostasis,

intestinal transport, bone metabolism and renal calcium reabsorption, as well as blood pressure and insulin secretion [49-53]. In enterocytes, it combines to its cellular receptor and regulates calcium and phosphorous absorption [54, 55]. Second, it regulates immune system and controls cell differentiation [56, 57]. The second chemical form is vitamin D<sub>2</sub> (ergocholecalciferol). It is formed after irradiation of ergosterol [58-60]. In humans, vitamin D<sub>2</sub> potency is less than the third of vitamin D<sub>3</sub> [61]. Once released from food matrix, vitamin D is included in mixed micelle, like other lipophilic components, and enters in enterocytes via passive diffusion through a unsaturable mechanism [6, 62]. Afterwards, it is included in chylomicrons and activated in liver [63]. A sufficient light exposure and adequate dietary calcium and phosphorus intakes can fulfill physiological needs: RDI is 5 µg/day. Vitamin D deficiency occurs mainly in developing countries. It causes rickets and can be cured with UV light therapy and supplementation [64].

#### I.4. Vitamin E

Vitamin E encompasses eight molecules composed by a chromanol ring and a phytol side chain displaying identical functions: four tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) and four tocotrienols ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ). Tocopherols have saturated side chain whereas tocotrienols have three conjugated double bonds.  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  prefixes indicate position of methyl groups on chromanol ring [65].  $\alpha$ -tocopherol is the most abundant in nature (Fig 1) [66]. One  $\alpha$  tocopherol molecule can trap two peroxy radicals responsible of lipid oxidation initiation [67]. Hence, this molecule protects membrane lipids against oxidation [68]. Thanks to Van der Waals interactions, it stabilizes membranes mechanically [69]. Vitamin E has also a positive effect on fertility [70]. Vitamin E quinone possesses anti-clotting activity through the inhibition vitamin K dependant carboxylase which regulates blood clotting [71]. Vitamin E digestion is similar to vitamin A and carotenoids digestion.  $\alpha$ -tocopherol can use SR-B1 to enter in enterocyte [72]. Vitamin E

RDI is 15 mg/day and deficiency might occur in case of fat malabsorption or for premature infants. It is usually characterized by neurological problems due to poor nerve conduction, which are reversible by supplementation [66].

### I.5. Vitamin K

Vitamin K1 (phylloquinone) and K2 (menaquinone 4) represent the K vitamins. They are composed by a hydrophilic naphthoquinone head group and a hydrophobic carbon chain tail (Fig 1). Phylloquinone carbon chain contains one double bond whereas menaquinone carbon tail length is variable and can contain several double bonds. Vitamin K displays antihemorrhagic properties [73]. It is an essential co-factor of glutamate residues carboxylase. This enzyme is responsible of carboxylation of proteins glutamate residues [74, 75]. Carboxylation of these residues in prothrombin allows their complexation with calcium [76, 77]. Carbon chain double bond enables prothrombin linkage with membrane phospholipids (PLs) and activates blood clotting cascade [78]. Vitamin K is also involved in mineralization: several studies demonstrated the link between vitamin K, carboxyglutamic acid residues carboxylation and calcium deposition in bones [79]. Once released from food matrix, vitamin K is included in mixed micelle like other lipophilic components [6], enters in enterocytes and is included in chylomicrons [80]. Vitamin K RDI is 90 and 120  $\mu\text{g}/\text{day}$  for women and men respectively; its deficiency occurs mainly in developing countries. It is characterized by uncontrolled bleeding, cartilage calcification accompanied by severe bone malformation or insoluble calcium salts deposition in the walls of arteries and cartilages, which increases cardiovascular risk [81].

Each vitamin considered presents AO properties which constitute a great physiological advantage. However, this asset makes them fragile and their activity can be greatly reduced by oxidation due to oxygen, light and high temperatures exposure. As they are liposoluble, they can interact with membranes PLs.

## II. Vitamins and carotenoids as modulator of membrane fluidity and phospholipids oxidation

For several years, relations between vitamins structure and membrane characteristics have been studied in monolayers, bilayers modeled by liposomes (vesicles constituted by one or more PLs bilayers) and biological membranes. Due to their physiochemical properties, lipophilic vitamins interact with membrane PLs and therefore influence chemical and physical stability of biological membranes. First, they might exhibit AO properties and prevent membrane lipids from oxidation. PLs and vitamins interactions might also have consequences on membrane characteristics such as fluidity and permeability. These datas might give possible explanations for vitamins protective effect and be a useful tool to predict their behavior when encapsulated in particles including a PLs interface. First studies have investigated interactions between retinol,  $\alpha$ -tocopherol and membrane lipids. They seemed to have an impact on membrane structure and stability [82]. Afterwards, vitamins potencies to protect membrane lipids against oxidation have been largely investigated.

Vitamin E free radical scavenging properties have been underlined in phosphatidylcholine (PC) liposomes, human erythrocyte ghost membranes and low density lipoprotein (LDL) [68]. Vitamin E limited drastically PC oxidation in monolayers (LDL), bilayers and biological membranes. AO effect might be explained by  $\alpha$ -tocopherol parallel orientation to PLs in dipalmitoyl PC (DPPC) membrane [83]. In PLs monolayers,  $\alpha$ -tocopherol displayed maximum interactions with polyunsaturated PLs, especially when hydrophobic chains had approximately similar length [84]. In saturated and unsaturated PLs mixed monolayers,  $\alpha$ -tocopherol interacted preferentially with unsaturated PLs. This interfacial organization gave more protection to unsaturated PLs which are the most sensitive to oxidation.  $\alpha$ -tocopherol seemed to interact with several PLs at the same time and dynamically, which was possible thanks to membrane fluidity. These interactions might

explain  $\alpha$ -tocopherol great free radical scavenging properties.  $\alpha$ -tocopherol was able to form aggregates in the lipid bilayer without affecting its physicochemical properties, thus creating a reservoir [85].  $\alpha$ -tocopherol was located in bilayer hydrophobic core. This position has been confirmed with synthetic single chain PLs [86]. These PLs exhibit no symmetry and tend to form spherical micellar structures rather than bilayers [87], in combination with  $\alpha$ -tocopherol at equimolar ratio they formed bilayers. These molecules seemed to have a complementary shape. Moreover, this behavior seemed to be linked to the presence of a free hydroxyl group in  $\alpha$ -tocopherol since esterified vitamin E (by an acetate group) did not form mixed bilayers.  $\alpha$ -tocopherol inclusion in DPPC bilayer might decrease membrane fluidity [69]. The molecule partitioned membrane bilayer decreasing PLs motion, which led to gel to liquid-crystal transition broadening and membrane fluidity decrease.  $\alpha$ -tocopherol might be bound to PLs via hydrogen bonds and also via hydrophobic interactions (Fig. 2). Vitamin E acetate displayed interactions with PLs similar to those observed between PLs. However,  $\alpha$ -tocopherol and vitamin E acetate both increased membrane permeability, which is correlated with gel to liquid-crystal transition broadening.  $\alpha$ -tocopherol is efficient for PLs protection against oxidation in liposomes even at gastric pH [88]. This vitamin is a valuable component to encapsulate for supplementation but also in combination to protect fragile components from oxidation. It has also been shown that  $\alpha$ -tocopherol was mainly located at the oil/water interface in an emulsion [89].

Many studies dealing with lipophilic vitamins impact on PLs oxidation and membrane structure have been performed with vitamin A and carotenoids. A small difference in vitamin A chemical structure can have major consequences on its inclusion in membrane and thus on membrane fluidity [90]. Retinal and retinol seemed to modify less DPPC liposomes membrane structure than RA as shown by electron spin resonance and membrane permeability measurements. They remained in bilayer hydrophobic core having only a small

influence on membrane upper portion. Their inclusion in bilayer seemed to increase slightly its order and its permeability. Carboxylic acid group is more polar than aldehyde or alcohol groups; therefore RA was closer to PLs polar heads creating disorder in bilayer upper portion (Fig. 2). This might explain the great permeability enhancement (11fold) observed.

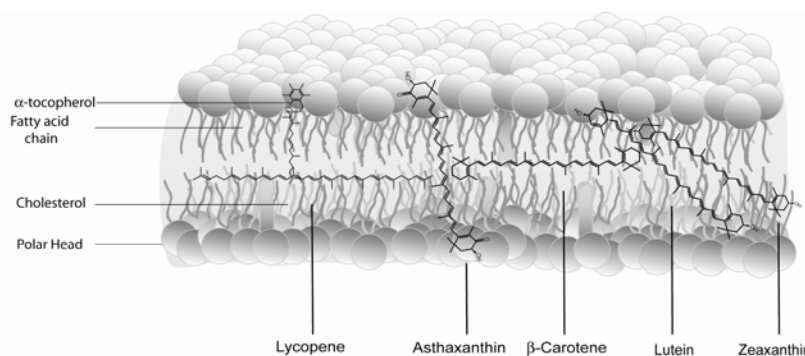
Carotenoids chemical structures seemed to be a key factor in their localization in an oil-in-water model emulsion stabilized by a PLs monolayer [91]. Due to their variety, chemical structure and polarity effect could be easily evaluated.  $\beta$  and  $\alpha$ -carotene are highly hydrophobic, thus they were distributed essentially in emulsion droplets core, whereas zeaxanthin and lutein were preferentially located at oil/water interface [91].  $\beta$ C orientation in bilayer vesicles might depend on PLs composition.  $\beta$ C was in the inner part PLs bilayer, but the molecule was oriented parallel to the bilayer plan when it was composed by dioleoyl PC and perpendicular to bilayer plan when it was composed by soybean lecithin. This position increased membrane PC protection against free radicals [92].  $\beta$ C vertical conformation might be possible if its hydrophobic core is thick enough. Thus,  $\beta$ C would display less fluidizing properties and give more protection to PLs [93]. However, it is generally accepted that  $\beta$ C position in a bilayer is closer to horizontal plane than vertical plane. Yet, this conformation gives protection to membrane PLs [94]. Another study compared carotenoids influence on PLs oxidation when included in PC liposomes [95]. Inhibitory oxidative effect was in the following order, ranged from the greatest to the lowest AO effect: Zeaxanthin= $\alpha$ -tocopherol (reference) > cryptoxanthin >  $\beta$ C > cathaxanthin and asthaxanthin > lycopene. Except for lycopene, carotenoids degradation was correlated to PLs protection. Whereas  $\beta$ C and zeaxanthin displayed very similar AO properties in solution, they acted differently when included in liposomes. This could be attributed to their different inclusion patterns in lipid bilayers.  $\beta$ C and lycopene might be less accessible for free radicals as they are located in lipid bilayer hydrophobic core, whereas zeaxanthin is spanned across the lipid bilayer, increasing



its free radical scavenging properties [96].  $\alpha$ -tocopherol and various carotenoids ability to protect membrane PLs has also been compared in PC multilamellar liposomes [97]. Inhibitory oxidative effect was in the following order, ranged from the greatest to the lowest AO effect: lycopene >  $\alpha$ -tocopherol >  $\alpha$ -carotene >  $\beta$ -crytoxanthin =  $\beta$ -carotene > lutein. Besides, carotenoids displayed a synergetic AO effect which might be due to their different location in membranes. Differences in carotenoids AO effect have been noticed between the two previous studies. Especially for lycopene which displayed the lowest AO properties in experiments performed by Woodall *et al.*, whereas it was the strongest AO for Stahl *et al.* [95, 97]. These differences might be partly attributed to the differences of concentrations in carotenoids and different type of liposomes namely uni or multilamellar liposomes used. Carotenoid AO property seemed to be environment and concentration dependant. Position and interaction with PLs might have less influence on PLs protection in multilamellar liposomes. Even though these discrepancies remain intriguing, we might think that AO molecules encapsulation might be of use to protect sensitive components included in particles. Investigations have been made to know whether carotenoids protective properties on membrane were only due to their free radical scavenging properties or to membrane permeability alteration. Experiments demonstrated that asthaxanthin inclusion in multilamellar liposomes PC membrane might increase its rigidity, thus limiting pro-oxidant compounds penetrations and enhancing protection against free radical [92]. On the contrary, lycopene did not show a significant membrane protection. This result tends to confirm Woodall *et al.* results [95]. It has been demonstrated that lutein and zeaxanthin could adopt lateral and vertical position in a mixed DPPC monolayer depending on surface pressure and their respective concentration [98]. They both increased hydrophobicity and decreased water permeability. At high concentration, they could be segregated in a vertical position. Lutein was more adaptable to external conditions than zeaxanthin, because lutein  $\epsilon$ -ring has the

ability to rotate [99]. NMR studies have shown that lutein and zeaxanthin were oriented respectively with angle of  $57^\circ$  and  $33^\circ$  with respect to the normal axis to the plan formed by the PC membrane (Fig. 2) [100]. These differences in orientation might have an impact on their AO effect: when the AO is in a normal position to membrane bilayer surface, the protective effect should be more pronounced. Molecule conformation and location in membrane may also have an impact on their encapsulation efficiency. Socaciu *et al.* investigated different processes to encapsulate carotenoids into mixed PC liposomes and their effect on membrane characteristics [101].  $\beta$ C remained in membrane hydrophobic core and displayed strong fluidizing properties. This can be explained by an increase of PLs motional freedom, head and tail, even though  $\beta$ C remained in hydrophobic core [102]. Thus the order was decreased in the PLs membrane, which became less compact [103]. On the contrary, zeaxanthin and lutein were inserted across the bilayer thus rigidifying the membrane. These discrepancies between carotenes and xanthophylls behavior demonstrate polarity influence on membrane fluidity. Membrane inner part might become more hydrophobic and less permeable to water [104]. It can also be postulated that carotenoids incorporation might be governed by their effect on membrane rigidity, as  $\beta$ C could integrate membranes seven times better than xanthophylls. These results have been strengthened by a study concerning xanthophylls and xanthophylls esters incorporation in PC liposomes [105]. Their encapsulation rate was related to their ability to interact with membrane bilayer. As lutein was able to change its position in the lipid bilayer, it gave more freedom in the structured system. Upon mechanical stress like extrusion during liposomes formulation, this feature allowed lutein maintenance in the bilayer. Zeaxanthin and its esters remained preferentially in membrane core, and could be squeezed out during extrusion. This could explain the lower encapsulation rates obtained with this last carotenoid and emphasizes carotenoid structure impact on encapsulation efficiency.

Studies performed with vitamin K<sub>1</sub> and K<sub>2</sub> monolayers spread at air/water interface underlined the impact of a *trans* double bond in the hydrocarbon chain [106]. Indeed, the double bond in vitamin K<sub>2</sub> hydrocarbon chain gives more rigidity to the molecule. This feature leads vitamin K<sub>2</sub> to adopt a more vertical alignment in the monolayer. And the packing is less dense due to the restricted motion of the hydrocarbon chains. Vitamin K<sub>1</sub> behavior has been also studied in DPPC monolayer and membrane [107]. Interactions between vitamin K<sub>1</sub> and DPPC monolayer led to monolayer condensation. It appeared that membrane fluidity was increased by vitamin K<sub>1</sub> inclusion thanks to a decrease of the gel to liquid-crystal phase transition temperature.



**Figure 2:** Vitamins schematic disposition in a lipid bilayer

To conclude, vitamins and carotenoids chemical characteristics have consequences not only on encapsulation efficiency but also on membrane lipids oxidation, membrane polarity and fluidity. These parameters could affect encapsulated active molecule release. Molecule hydrophobicity will determine its location in particles, which can be modulated by PLs composition. If AO effect is the aim of encapsulation, molecule vertical inclusion in lipid bilayer should be preferred. A stronger molecule protection will be displayed by its encapsulation in the core.

### III. Encapsulation strategies to formulate liposoluble vitamins carriers

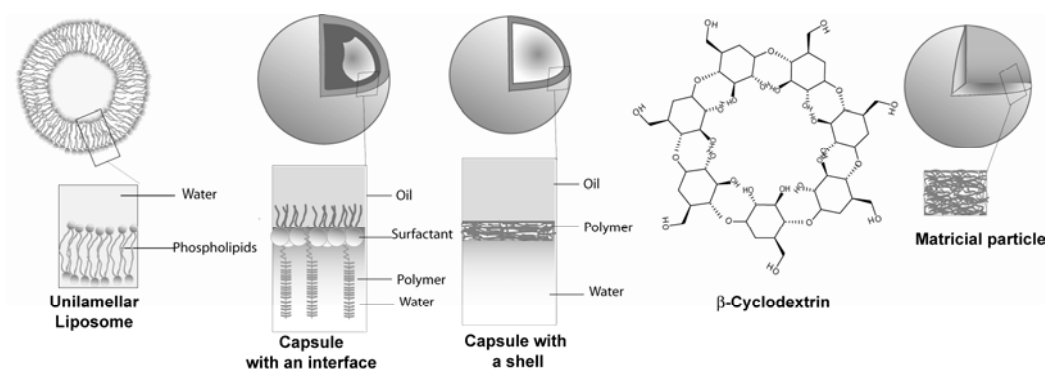
Process choice, excipients physicochemical properties and excipients/encapsulated vitamin interactions will determine particles characteristics (morphology, surface charges, permeability and encapsulation efficiency). Two major types of particles emerged: (i) lipid based formulations: in these particles vitamins are solubilised. (ii) vitamins matricial entrapment by polymer. These particles can be formulated by physico-chemical or mechanical processes. According to encapsulation processes various types of liposoluble vitamins carriers can be obtained as illustrated in figure 3.

#### III.1. Physico-chemical processes

Vitamin solubilization in a dispersed lipid phase is the first step of many encapsulation processes. One of the most used process to encapsulated vitamins in industry is vitamin dissolution in lipid and its inclusion in gelatine capsules or beadlets for example [5]. This method requires no high cost technology and can be easily up scaled. Objects produced are mainly for oral supplementation. Their stability depends on storage conditions, lipid nature and beadlets characteristics.

Emulsion can be produced by several methods: high pressure homogenization, mechanical agitation or phase inversion. They can all be adapted to industrial scale, however high pressure homogenisation is more energy consuming [1]. They produce droplets if surfactant interface is fluid or capsules if surfactant interface is rigid. High pressure homogenisation and phase inversion method produce the smallest (in the nanometer range) and the most stable droplets/ capsules. Carrier stability is dependent of surfactant characteristics, most of time a fluid interface lead to destabilisation. Carrier stability can be increased by the use of high melting point lipid which is at the solid state in the carrier. In this case, emulsification is performed at a temperature above lipid melting temperature [3, 87, 108-111]. Emulsification

can also occur under mild agitation and produce small oil droplets. This method is used for very specific formulation such as self-nanoemulsified drug delivery systems (SNEDDS) used for oral administration. A solid shell can be produced by simple or complex coacervation. Coacervation is a well known encapsulation process based on phase separation due to macromolecules desolvation [112]. Coacervation can be initiated by environmental changes able to affect polymer solubility in the solvent [1], such as heat [113], addition of salt [114] or of an opposite charged polymer [1]. This process can be easily adapted to industrial scale, and usually produces particles ranging from 5 to several hundreds  $\mu\text{m}$  [1].



**Figure 3:** Type of carrier encountered in lipophilic vitamin encapsulation

Liposomes are vesicles composed by one or more PLs bilayers. They can encapsulate hydrophilic components in their core and hydrophobic molecules in lipid layers. To encapsulate lipophilic vitamins, they are usually obtained by vitamins and PLs dissolution in organic solvent which is removed under vacuum [88, 115-117]. PLs film is redispersed in water or buffer using ultrasounds. To obtain unilamellar vesicles, centrifugation or extrusion can be used. They have high encapsulation efficiency and particles produced are in the nanometre range. Their stability might be greatly influenced by interfacial properties of encapsulated molecules and their interaction with surfactants. Their surface properties can be modified by polymer grafting. Similar structures can be produced with Ch or other

polyglycerol ether following the same procedure. These vesicles are called niosomes [118-121]. They are considered to be more stable than liposomes.

Liposoluble vitamins can also be encapsulated in polymers based objects through hydrophobic interaction in cage molecules or matricial particles. These systems are able to produce a sustained release. The most studied cage molecules are cyclodextrins. They are cyclic oligosaccharides presenting an hydrophobic space.  $\alpha$ ,  $\beta$  and  $\gamma$  cyclodextrins have respectively 6, 7 or 8 glucopyranose units. Small hydrophobic molecules can be complexed in this cavity. Complex formation and dissociation depends on molecule hydrophobicity, shape and chemical function [122, 123]. These complexes improve molecule apparent solubility. Matricial particles entrap physically and chemically the active components. Vitamin can be dissolved in an organic solvent and dropped in a polymer suspension. The polymer is composed by an hydrophobic and an hydrophilic part. Thanks hydrophobic interactions they can form complexes which can assemble in supramolecular structures like micelles [124-128]. This method leads to particles in the nanometer range and is easily adaptable to a large scale. Solvent evaporation technique has also been used to encapsulate lipophilic vitamins in microspheres [3, 129, 130]. Briefly, polymer and vitamin are homogenized in a solvent (usually organic) which is emulsified with water. The solvent is removed by evaporation or several washing. Microsphere formation is due to polymer precipitation. Solvent evaporation techniques require no heavy equipment and are easily adaptable to industrial scale. Another way to encapsulate lipophilic vitamins in a matricial particle is to freeze dry a polymer/water emulsion [131]. In this case vitamin is dispersed in emulsified polymer. Droplets are solidified during freeze drying. This technique is adaptable to industrial conditions and compatible with sensitive molecule encapsulation. However, freeze drying remains expensive and delicate to achieve. Particles size depends on emulsification parameters and particles stability on polymer state.

Some of these methods require organic solvent (liposomes niosomes, solvent evaporation method, inclusion in cyclodextrins). Apart from biocompatibility issues this aspect can restrain industrial scale as it implies solvent recycling.

Systems	Particles size nm	Particles charge	Vitamins state	Oral administration	Parenteral administration	Topical and transdermal administration	Local administration
<b>Emulsions</b>							
emulsion	30 to 60 nm	not informed (-)	dissolved	[132, 133]	-	[134, 135]	possible
SNEDDS	nm range	-	dissolved	[136, 137]	no	no	no
<b>Capsules</b>							
lipid core particles	20 µm or 170 µm	surfactant dependant	dissolved	use of ethyl acetate	no considering size	possible	[3]
	50 to 600 nm	-	dissolved	[109]	no considering size and PDI	possible	No considering PDI
SLNs	30 nm to 500 nm	-	dissolved	possible	[111, 138]	[87, 108, 110, 139]	possible
polymeric NCs	230 nm	-	dissolved	use of acetone	possible	[135]	possible
coacervate	25µm [140]	-	dissolved	[140] use of formaldehyde and gelatine [114]	No considering size	[113] use of hydroxyl butyl vinyl ether and vinyloxy ethyl methacrylate	[141] use of glutaraldehyde
	900 or 300 nm [[113]						
500 nm [141]							
<b>Liposomes</b>							
Unilamellar	100 to 250 nm	PLs and surfactant dependent	dissolved	use of organic solvent	peg coating [111]	[116]	possible
Multilamellar	200 to 900 nm			[88]	[142-148]	[117] (+ charge)	possible
Niosomes	100 to 500 nm			use of organic solvent	No considering size	[119-121]	possible
<b>Matricial particles</b>							
silicate particles	1,2 to 5,8 µm	-	dissolved	use of decyl-alcohol	no considering size	[149, 150]	possible
nano dispersion	nm range	-	crystalline	[151] use of hexane	no	-	-
	µm to mm	-	crystalline	[152]	no	no	no
starch	1,2 to 0,8 mm or 6 to 15µm	-	dissolved or crystalline	[131, 153]	no (size pb)	is carbohydrate matrix adapted ?	-
mannitol							
microsphere	5 to 50 µm	-	-	use of organic solvent	no considering size	Possible but release slow	[129, 130]
<b>Micelles</b>							
PEO-PLLA	50 to 250 nm	-	complexed		possible		[154]
polyvinyl alcohol	400 nm<	-	complexed	possible	No considering size	-	[2]
chitosan	70 to 250 nm	+ if chitosan alone	complexed via electrostatic interactions	possible	grafting [124, 155]	[126]	possible
casein	30 to 530 nm	close to 0 mV	hydrophobic interaction	[127]	[128] (dextran shell)	Immune system activation?	-
<b>Cyclodextrins</b>							
	1.5 to 2 nm	-	complexed via hydrophobic interactions	[156]	[157-159]	[160]	[159, 161]

**Table 1: Presented particles main characteristics and their possible applications**

### III.2. Mechanical processes

Mechanical processes (spray drying, spray chilling, extrusion...) require specific appliances which can be easily up scaled [1]. Their main advantages are the large quantity produced and particles high shelf life. However, the use of high shear or high temperature has

to be taken into account to limit vitamins loss. Particles produced are matricial (figure 3). When the encapsulating material is composed by polysaccharides or polymers, they are usually at the amorphous state. Particles have usually high shelf life if stored under polymer glass transition temperature. Otherwise, polymer matrix becomes viscous and encapsulated component might be released or degraded. Particles size can be modulated by modifying solution viscosity, inlet and outlet temperature. These techniques are more adapted to oral administration as they lead to large particle size ( $\mu\text{m}$  to  $\text{mm}$ ).

Spray-drying consist in atomizing a solution or a dispersion containing the encapsulating agent [1, 152]. Droplets are solidified by contact with a hot cold air flow. During spray congealing (also called spray chilling or cooling), low melting point excipient (wax, natural glyceride lipids, synthetic and semi-synthetic lipids, hydrophilic/lipophilic surfactants and co-solvents (solid PEGs))is extruded through a nozzle in a cooling chamber [1, 162]. Droplets are solidified by cold air flow, this process overcome the residual solvent issues encountered with spray drying. With these processes, particles are mostly spherical but their surface topography is polymers dependant [162]. Molecule retention and release profile can be optimized with modification of polymer length [152] or type [162]. Liposoluble molecule can be encapsulated in the crystalline form [152] or dissolved in excipients [162].

Melt extrusion consist in pushing a melted material through a die, granulates are formed after extrusion by section [1, 153]. This method is adapted to industrial conditions. Process can be adapted to low moisture conditions [153]. However temperature used are comprised between 70 to 150°C which might not preserve vitamin integrity. Polymer composition and characteristic (carbohydrate excipients)have an impact on process temperature and thus on vitamin preservation. Granulates formed by this method are in the millimetre range.



## IV. Encapsulation applied to oral administration

Liposoluble vitamins intestinal uptake depends on meal lipid content [163-165]. For oral administration applications, encapsulation should prevent vitamins from complexation in gastrointestinal tract or control its release. Their bioaccessibility and bioavailability might also be enhanced by encapsulation. If considered carriers are not sensitive to digestion process, they could be absorbed in enterocytes or via paracellular pathways such as gap junctions. Otherwise, lipidic fraction deriving from degraded carriers might be included in mixed micelles and follow their regular absorption pathway [29].

### IV.1. Lipid based carriers

A way to enhance vitamin plasma levels is to increase vitamin intake. Supplementation can be done by mixing lipophilic vitamins with dry food products [166]. This method is efficient to improve vitamin plasma level, however vitamins might not have a good bioavailability. In this preparation vitamin is adsorbed onto the surface of dry food particles which might not be the most appropriate for its protection. Matricial particles can include oil droplets. Melt extrusion has been adapted to low moisture conditions for vitamin K (dissolved in oil) encapsulation [153]. Encapsulation ranged from 66 to 99 % which is high considering the temperatures employed. Formulated granules can be added to dry food product as well.

Liposomes retain attention in several studies. They seemed to enhance vitamin bioaccessibility [88]. Vitamin E was more absorbed when included in liposomes than when it was dissolved in sardine oil. As liposomes structure was affected by incubation in buffer solution at gastric pH, absorption enhancement was probably mostly due to vitamin E greater

bioaccessibility. However, liposomes formulation requires excipients dissolution in organic solvents which might constitute a drawback for nutraceutical or food use.

Emulsions have the advantage of being applicable to most of industrial fields. They can be administered directly, incorporated as a food ingredient or nutraceutical preparation to enhance vitamin bioavailability. This was shown with small emulsion droplets size and PLs used as stabilizer. For instance, nanosolve® is a fine emulsion stabilized by lecithin/ polyol or lecithin/carbohydrate matrix. Droplets size is ranging from 30 to 60 nm [132, 133]. Vitamin E was 10 fold more bioavailable from NanoSolve® than the same oil contained in commercial gelatin capsules [167-169]. Aqua-biosorb® is another vitamin dissolution media included in gelatin capsules. It was formulated with 80 % of polysorbate, 10 % of ethanol, and 10 % of propylene glycol. Vitamin A and E bioavailability were increased respectively by 3 and 5 to 8-times with Aqua-biosorb® in comparison to capsules filled with oil, tablets or water dispersible capsules. Emulsification can also occur directly in the gastrointestinal tract with SNEDDS, which are composed by a mixture of oil and surfactant. SNEDDS can form stable emulsion under mild agitation and after their dilution in water or physiological media such as gastric juice. They have been used to increase retinol acetate bioavailability [136, 137]. Their feasibility zone was determined by varying concentrations of soybean oil, glyceryl monocaprylate and polyethoxylated castor oil. Retinol acetate concentration was fixed at 25 %. Surfactant (polyethoxylated castor oil) to co-surfactant (glyceryl monocaprylate) ratio from 1:1 to 2:1 led to SNEDDS. Retinol acetate bioavailability was assessed in rats with SNEDDS in cellulose capsules or formulated as tablet. First formulation increased retinol acetate bioavailability by 2 and the second by 1.4 when compared with retinol acetate dissolved in oil and included in capsules. SNEDDS might have enhanced bioavailability thanks to the nanoemulsification which increased bioaccessibility. Further studies concerning

RA physico-chemical interactions with SNEDDS constituents and its stability during storage could complete efficiently these results.

Nanodispersions have been developed for food applications and applied to  $\beta$ C [151]. The process was based on an emulsification-evaporation technique.  $\beta$ C was dissolved in hexane, emulsified in water containing tween 20 as emulsifier by mechanical stirring followed by high pressure homogenization. After hexane evaporation,  $\beta$ C crystals in the nanometer range were obtained and were physically stable for 12 weeks.  $\beta$ C retention was ranging from 25 to 56 %. Such formulation process lead to a high payload, however, as mentioned for liposomes, nanodispersion process might lead to solvent traces in particles which can rise questioning for further applications. Besides,  $\beta$ C crystalline form might be poorly bioefficient.

Solid lipid microparticles (SLMs) can be a good alternative to nanodispersions. They are produced by spray congealing, thus they are constituted by solid lipid excipients. Encapsulated molecule is included in a lipid matrix but as SLMs is solid their stability might be good. Vitamin E loaded (SLMs) had size greater than  $75\mu\text{m}$  [162]. Vitamin E is stabilised in SLMs, however particles size might not provide an efficient bioavailability. Lipid nanoparticles (NPs) might combine emulsions, SLMs and nanodispersions assets. One type of lipid NPs is solid lipid nanoparticles (SLNs), their core is constituted by high melting point lipid which is at the solid state in the carrier. For example,  $\beta$ C SLNs are characterized by (i)  $\beta$ C dispersion in lipids which is an important step for  $\beta$ C inclusion in intestinal mixed micelle and (ii) a stable structure thanks to the surfactant shell and lipid solid state [109]. However, SLNs formulation requires high pressure, and high temperature for melt homogenization.  $\beta$ C chemical structure can be altered by these drastic conditions. Encapsulated with  $\alpha$ -tocopherol,  $\beta$ C was stable for 20 days at room temperature. Formulations were characterized by two particles populations (85 nm and 360 nm), which is not a major issue for oral administration.

#### IV.2. Entrapment of liposoluble vitamins in molecular assemblies

Liposoluble vitamins can be encapsulated in cage molecules like cyclodextrins (CDs) or supramolecular assemblies made of micelles-like systems. CDs vitamin complexes improve molecule apparent solubility, however complex stability is dependant of pH and dissolution media composition [122, 123]. Since intestinal absorption of liposoluble vitamin is dependant on lipid meal content, it can be highly variable. Thus, increase vitamins apparent solubility in lumen might constitute a mean to improve their bioavailability.

Studies carried out with RA showed that constituents of dissolution media could interact with CDs and compete with encapsulated RA [170]. Montassier *et al.* have investigated interactions between RA and  $\beta$ -CDs, dimethyl- $\beta$ -CD and 2-hydroxypropyl- $\beta$ -CD [160]. RA/CD complexes formation has been assessed by scanning electron microscopy, differential scanning calorimetry, infra red spectra, X-rays diffraction and NMR study. Complexation increased greatly RA apparent solubility in water. However, particulate systems were not stable. Another study circumvent these stability issues and showed that CDs could enhance RA bioavailability [157]. RA loaded 2-hydroxypropyl- $\beta$ -CDs were stable during 3 months at room temperature and apparent RA solubility was increased almost 35 000 times at pH 7.4 as RA solubility in water is close to  $1.10^{-9}$  mol/l. This discrepancy might be due to different formulation method. In the studies performed by Montassier *et al.* [160], RA was dissolved in ethanol whereas RA was added directly in aqueous CD solution in the second study [157]. As ethanol increased temporarily RA solubility in ethanol/ water mixture, CDs could interact with more than one RA molecule leading to less stable complexes. Bioavailability might be enhanced (3fold) thanks to RA fine dispersion in intestinal media

[158]. The main drawback is the use of excessive RA to load 2-hydroxypropyl- $\beta$ -CDs molecules, leading to low (25%) encapsulation yields [157].

In food-industries, carotenoids are largely used as dyes, and encapsulation might be a tool to stabilize food-color. FDA has allowed  $\beta$ -CDs incorporation in foods up to 2 %.  $\beta$ C stabilization has been evaluated when encapsulated by  $\alpha$ ,  $\beta$  and  $\gamma$ -CD [156].  $\beta$ C-CDs complexes were formed via a co-crystallization mechanism and were largely more stable than  $\beta$ C dissolved in oil.  $\alpha$ -CD possessed the greatest stabilizing properties for  $\beta$ C over time when exposed to mild temperature (60°C). The complex between CDs and  $\beta$ C might protect the molecule from light degradation. Besides, this type of encapsulation increased significantly  $\beta$ C apparent solubility in water. When filtered sterile, a suspension of  $\beta$ C/methyl- $\beta$ -CD remained stable for 6 months at room temperature and exposed to normal daylight. Even though this encapsulation technique fulfills many requirements for  $\beta$ C stabilization over time and against mild temperature, precise data recording encapsulation efficiency might be useful before going further.

Vitamin A palmitate has been encapsulated by gelatine/acacia gum complex coacervation [113]. Coacervates were spherical capsules characterized by diameters of 24  $\mu$ m or more. Vitamin A was dissolved in corn oil, its encapsulation efficiency was over 63%. To increase particles stability overtime, they could be dried by hot air or freeze drying. However particles hardening required cross-linking agent like formaldehyde which is toxic.

Caseins represent almost 80 % of milk proteins and are largely used in the food industry. Vitamin D<sub>2</sub> loaded casein micelles have been developed to enrich dairy product without affecting their sensory properties [127]. An ethanolic vitamin D<sub>2</sub> solution was dropped in a sodium caseinate solution, homogenized by magnetic stirring. It led to vitamin D<sub>2</sub> non-covalent binding to sodium caseinate then micelles assemblies. Vitamin D<sub>2</sub> loaded micelles exhibited encapsulation efficiency of 27 %, with size distribution ranged from

30 to 530 nm. Vitamin D<sub>2</sub> seemed to be protected from light due to vitamin-casein interaction and micelle structure synergistic effect. This process needs to be optimized before going further as particles distribution is broad and encapsulation efficiency is low.

Mannitol gelatine matrix has been used to encapsulate  $\beta$ C: droplets were stabilized by freeze drying [131]. A buffered aqueous phase was necessary to limit or avoid mannitol crystallization during freeze-drying and increase particles stability. Divalent ions, especially zinc, could interact with mannitol thus prevent its crystallisation and limit particles and  $\beta$ C degradation during storage. Further investigations could be useful to characterize release profile of particles produced and evaluate encapsulation effect on bioavailability *in vivo*.

Melt extrusion has been adapted to low moisture conditions for vitamin D (crystalline form) encapsulation [153]. Granulates were formed right after the diehead by section of extruded material at high temperatures around 100°C. Carbohydrate composition had an impact on process temperature and thus on vitamin preservation. It ranged from 66 to 99 % which is high considering the temperatures employed.

## **V. Encapsulation applied to topical and transdermal application**

This type of administration can be adapted to deliver active molecules to skin or in blood circulation. Encapsulation for skin deposition requires vitamins protection against different stresses (light, oxygen,...) and particles penetration through skin. Cutaneous absorption can be divided in several steps: (i) drug deposition on skin, (ii) drug diffusion through the stratum corneum to reach dermis or epidermis, (iii) molecule metabolism by dermal cell or local blood circulation.

Considering vitamins and carotenoids properties, particles cited afterwards have been mostly designed for local action.

### V.1. Lipid based carriers

Since stratum corneum is composed by lipids bilayers, liposomes may pass through this barrier and could constitute an appropriate drug carrier for dermal route. Padamwar *et al.* demonstrated that composition of liposomes bilayers could influence tocopherol acetate release [116]. Low concentrations of PLs and Ch might involve lower interactions between tocopherol acetate and PLs, inducing a more efficient vitamin release from liposomes. Vitamin E acetate encapsulation efficiency was about 100% and did not depend on excipient concentration. However, tocopheryl acetate skin penetration depends on the composition and textural characteristics of the matrix in which liposomes were added. Liposomes had sizes ranging from 100 to 220 nm with a low PDI,  $\zeta$  potentials were largely negative (-29 to -55 mV). Skin penetration observed was lower when liposomes were included in polyacrylic acid polymer gels. Decrease of drug permeation could be attributed to lower liposomes diffusion in the matrix, which was also inversely correlated with gel viscosity. Storage at 30°C and 65 % of relative humidity up to three months modified nor liposome size neither their drug delivery properties.

Liposomes surface charge might impact their drug deposition potencies. This hypothesis has been tested with RA loaded vesicles [117, 121]. Multilamellar vesicles (200 to 900 nm) were formulated with DPPC and dipalmitoyl phosphatidylserine (DPPS) as negatively charged PL or SA as positively charged PL. Ch was used as membrane fluidity modifier. Surface charge of liposomes did not influence RA *in vitro* release. However, RA permeation through skin was higher in liposomes formulated with SA (positively charged) than in liposomes formulated with DPPC and DPPS (negatively charged). As membrane fluidity and permeability did not affect RA diffusion, we can also hypothesize that RA release limiting factor should be liposomes diffusion through stratum corneum. SA liposomes might diffuse deeper thanks to their positive charges, inducing a closer release of RA to

dermis. These results have been strengthened with RA liposomes and niosomes [121]. However, independently from their surface charge or their membranes fluidity, these preparations were less efficient than RA ethanolic/water solution. It is generally accepted that ethanol might affect stratum corneum structure thus enhancing drug permeation. It can be hypothesized that oil and surfactant can interact physically with stratum corneum bilayers and disorganize this structured environment. Thus, surfactant characteristics have an impact on drug permeation.

RA protection against UV radiation by encapsulation has been studied with liposomes, niosomes [120], emulsion and lipid particles [87, 108, 135]. RA was less degraded when encapsulated in each of these formulations than in methanolic solution. However, its protection was dependant of carrier characteristics: sonicated unilamellar vesicles, niosomes made with Brij®30 and Triton® CG110 and liposomes made with hydrogenated PLs were the most efficient [120]. Ourique *et al.* compared RA stability in nanocapsules (NCs) and nanoemulsions [135]. RA NCs were formulated by RA, oil, sorbitan monoleate and poly- $\epsilon$ -caprolactone dissolution in acetone and their emulsification in water. Acetone was then removed by evaporation. NCs size was about 230 nm with PDI ranging from 0.14 to 0.24 and negative  $\zeta$  potential. NCs improved significantly RA photostability when compared to nanoemulsion formulated according to the same process without polymer. These promising results need to be confirmed by studies on drug penetration and inclusion in matricial systems.

Even though, retinol solubilization in stable emulsion positively influenced its skin absorption [134]. A reservoir form might maintain biological efficiencies while decreasing side effects. For instance, RA topical administration often causes burning, erythema or peeling. RA was found to be less irritating for the skin when deposited in SLNs gels than with commercial cream RetinA® [87, 108]. Various emulsification methods can be used to



produce SLNs leading to various mean diameters from 30 nm to 500 nm with a PDI from 0.17 to 0.8 [87, 108, 110, 139]. Their drug deposition properties were evaluated first with retinol and retinol palmitate [110]. No significant increase of their penetration in the skin was found. Drugs did not reach local circulation; this might limit side effects and can be an advantage for RA local action. These results were confirmed with 13-*cis*-RA and RA loaded SLNs [87, 108, 139]. It has been hypothesized that SLNs could diffuse in stratum corneum which could explain the decrease of skin irritation.

## V.2. Entrapment of liposoluble vitamins in molecular assemblies

Chitosan is a polycationic polysaccharides derived from shellfish chitin soluble in water only at acidic pH. Its positive charge confers to chitosan particles mucoadhesive properties[171] which might enhance drug permeation. These properties have been largely studied for nasal and oral administration [172], considering previous results its charge might be of interest for dermal deposition [117]. Retinol has been encapsulated in chitosan NPs for cosmetic or pharmaceutical applications [125]. Infrared spectroscopy and NMR studies suggested that retinol and chitosan formed a complex thanks electrostatic interactions between retinol hydroxyl group and chitosan amine group. An ethanolic solution of retinol was dropped in an aqueous chitosan solution using ultrasounds to disperse the system. Encapsulation rate has been estimated between 63 and 76 % [125]. NPs were characterized by diameters ranging from 70 to 250 nm and  $\zeta$  potential from 52 to 75 mV depending on chitosan/retinol ratio. Retinol was included in micelles core which can explain NPs diameter increase with drug load.  $\zeta$  potential seemed to increase with retinol content. Chitosan NPs could be lyophilized and redispersed in an aqueous solution without affecting their diameters or their  $\zeta$  potentials. This method increased tremendously retinol apparent solubility in water

(1600 times). To estimate chitosan efficiency as dermal carrier, further investigations concerning drug deposition are needed. Particles positive charge and small size might be an advantage for retinol penetration.

Thermoresponsive coacervates were formulated with vitamin E, they can form a fine hydrogel after UV (254 nm) or heat stimulation [140] and thus release their content. Vitamin E was mixed with ethanol before its inclusion in coacervates which had a mean diameter of 900 nm or 300 nm depending on formulation temperature. This process is a quite innovative way to use coacervation; however the use of modified hydroxyl butyl vinyl ether and vinyloxy ethyl methacrylate is not suitable for health product design.

## **VI. Encapsulation applied to intravenous administration**

### **VI.1. Lipid based carriers**

Liposomes have been widely used for parenteral drug administration. RA anti-proliferative effect has been shown when encapsulated in liposomes on several cell types: aggressive B-cell lymphomas [142], murine histiocytoma M5076 tumor cells [144] and myeloid erythroid leukemia K562 cells [143]. The study performed by Sundersan *et al.* [142] revealed that encapsulated RA was more efficient at lower doses than the free molecule to inhibit cell proliferation and led also to an increased apoptosis. Investigations made on hepatic cancer *in vivo* have been performed with DPPC Ch sterylglucoside liposomes. These liposomes were more stable during storage and in rat serum than DPPC Ch liposomes [144]. Life span of mice suffering from hepatic metastasis cancer was increased by 20 % ; besides the dose injected was 4 to 16-fold time lower to ineffective doses of free RA [173]. In this case, encapsulation allows RA anti tumor potencies to act on mice hepatic tumor at lower doses. RA and retinol unilamellar liposomes ability to induce myeloid erythroid leukemia K562 cells differentiation and to affect their growth has also been studied [143]. RA and retinol liposomes had diameters around 100 nm. Liposomes were stable in buffer and serum

during 24 hours. It appeared that antiproliferative effect was 65 and 51 fold greater for RA and retinol liposomes than for free components. RA encapsulation in liposomes seemed also to prevent RA from being metabolized over time and maintain its plasmatic concentration [145-147]. Liposomes might be a mean to preserve RA therapeutic properties for the IV route. RA has been included in DPPC and SA liposomes [145, 146]. It appeared that microsomal RA metabolism was significantly decreased by encapsulation even after repeated injection. Its metabolism was also decreased by encapsulation when deposited on F9 teratocarcinoma cells. These results have been confirmed through clinical trials [147, 148]. RA liposomes were more efficient to maintain RA plasma level over time than RA beadlets administrated *per os* (such as Vesanoid<sup>®</sup> or Accutane<sup>®</sup>). Its therapeutic efficiency has been studied with patients suffering from promyelocytic leukemia. Treatment with RA liposome was combined with chemotherapy. It appeared that RA liposomes could significantly lead to remission when leukemia was diagnosed early. These results constitute a support for more complete studies as in this case only 12 patients were included in the trial. Liposomes are efficient carriers for RA delivery thanks to their high encapsulation efficiency and their sizes adapted to systemic pathway. Besides, by modifying their surface properties via PEG or dextran grafting, their stealth properties can be modulated and their efficiency upgraded [174-176].

RA has been encapsulated in SLNs coated with PEG to improve their stealth properties [111]. These particles were suitable for parenteral administration as they have size between 150 and 300 nm and largely negative surface charge ( $\zeta$  potential -38 mV). Besides, to improve their shelf life, formulations can be freeze dried. Retinoids encapsulation efficiency in SLNs can be improved by the addition of liquid lipid [138]. Indeed, during lipid recrystallization after formulation retinoids especially RA or retinol can be expelled out of the particles or be distributed to the surface of solid lipid and thus less protected. The addition of

liquid lipid limit the degree of lipid crystallinity and therefore drug expulsion. This type of formulation could constitute a good alternative to liposomes.

## VI.2. Entrapment of liposoluble vitamins in molecular assemblies

$\beta$ -CDs might constitute a good compromise to avoid organic solvent use, whereas liposomes could be preferred as their behaviour *in vivo* is largely studied.  $\beta$ -CDs and unilamellar liposomes were compared as delivery carrier for  $\beta$ C, lutein and canthaxanthin inclusion in biological membranes [159]. Carotenoids loaded  $\beta$ -CDs were prepared by mixing  $\beta$ -CDs with crystalline carotenoids. Solid samples were diluted in tris-buffer, sonicated and centrifuged to extract carotenoids/ $\beta$ -CD complexes.  $\beta$ C was equally incorporated into membranes whether it was included in  $\beta$ -CDs or liposomes, while lutein incorporation was more efficient when encapsulated in  $\beta$ -CDs. It appeared that fluids membranes (mitochondrial) facilitated apolar carotenoids incorporation such as  $\beta$ C or lycopene whereas more polar carotenoids like lutein, zeaxanthin or asthaxanthin were preferentially incorporated in rigid membrane (plasma membrane). Affinity between carotenoids and CDs might have an influence on molecule release and therefore on their inclusion in membrane. RA complexation in CDs did not affect its pharmacokinetic profile after IV administration [158]. This might be explained by the rapid dissociation of RA-CDs complexes when diluted or in presence of plasma proteins.

Carrier characteristics can be adapted to respond to their environment such as pH. pH is known to be lower around tumor cells, which is due to their high metabolism. Thus, a carrier able to release its loading only in acid media would preserve healthy tissues. RA has been complexed with PEO-b-poly(L-lysine) polymer by covalent bonding [154]. Lysine ammonium group was complexed with retinoate  $\text{COO}^-$  group. PEO-b-poly(L-lysine) retinoate complex formed nanometric micelles. For pH below 6, lysine  $\alpha$ -helixes disappeared, this

increased dramatically micelles size from 50 nm to 250 nm. For pH lower than RA pKa (between 8 and 6), the complex might be mostly dissociated and RA could be released. However, further investigations would be needed to evaluate RA release properties. These micelles could have good stealth properties considering their size and PEO coating and be appropriate for systemic administration and also for local tumor delivery.

Dextran is known to decrease liposomes opsonisation [175]. Grafting this polymer onto other carriers might have the same effect.  $\beta$ C encapsulation in casein-*graft*-dextran NPs has been achieved [128]. Briefly  $\beta$ C was dissolved in ethanol and mixed with casein-*graft*-dextran co-polymer solution. Solvent was then removed under vacuum. NPs diameter was ranging between 175 and 300 nm depending on  $\beta$ C/casein ratio and pH. Particles  $\zeta$  potential was approaching 0 mV. NPs had a casein and  $\beta$ C core and a dextran shell. They were stable up to 91 days when stored at pH 7. NPs were also stable against pH variation, dilution, FeCl<sub>3</sub> oxidation.  $\beta$ C could be released by enzymatic hydrolysis. However,  $\beta$ C stability during particles storage has not been determined. This encapsulation process is innovative as NPs formation and encapsulation induced by hydrophobic interactions between  $\beta$ C and hydrophobic segments of casein were performed at the same time. However, encapsulation efficiency was low, ranging from 4.7 to 54%. This might be explained by the fact that  $\beta$ C is encapsulated thanks hydrophobic interaction. As NPs have appropriate size for systemic pathway [177] and dextran shell, *in vivo* study would be valuable in order to determine their circulating half life and possible stealth properties.

## VII. Encapsulation applied to local injections

Intramuscular or subcutaneous administration pathways broaden acceptable carrier size range when compared with other type of administration. Particles can enter in cells as such or release encapsulated molecule in interstitial fluid. This type of encapsulation with dissolved molecule might display greater absorption efficiency.

### VII.1. Lipid based carriers

Reservoir carriers are preferred for this type of applications. Retinyl acetate has been encapsulated in lipid microspheres using different methods [3]. First, in melt dispersion technique, retinyl acetate was incorporated in the melted lipid fraction (cetyl alcohol or palmityl alcohol and Ch) and emulsified with surfactant/water mix by mechanical stirring. The emulsion was heated to reach melting temperature and rapidly cooled down at 20°C. The second method employed solvent evaporation technique: retinyl acetate/lipid mix was dissolved in ethyl acetate at 50°C and emulsified with surfactant/water mixture until solvent was evaporated. These two methods produced biodegradable particles able to encapsulate retinyl acetate. Lipid microspheres mean diameters were 170 µm with the melt technique and 20 µm with evaporation process. Adding methacrylic polymer led to better particle size distribution. Encapsulation efficiencies were greater than 70 % for both methods and retinyl acetate displayed a controlled release (27 % in 8 h). The melt method did not use organic solvent, although it might not be adapted for highly temperature sensitive components. These particles could be administered as such or included in an organogel which would slowly release carriers in interstitial fluid [178]. These formulations could produce a sustained release for several days thus improving patient convenience.

## VII.2. Entrapment of liposoluble vitamins in molecular assemblies

Polymeric structure might provide a sustained release efficiently. Indeed, matrix could be designed to degrade slowly after administration. PEO-poly(L-lactide) and poly (L-lactide) microspheres were used to encapsulate RA [129]. They were formulated via emulsion solvent extraction method which led to high loading efficiency (90 %). Microspheres were smooth and spherical with mean diameter of 5  $\mu\text{m}$ . The main advantage of this carrier is its slow RA release during 5 weeks at a constant rate. RA loaded poly(DL-lactide-co-glycolide) microspheres were also formulated according to solvent evaporation method [130]. Particles had size ranging from 10 to 100  $\mu\text{m}$  depending on preparation parameters. 2% of poly(vinyl alcohol) was necessary to obtain spherical particles with a smooth surface. RA was released over 30 days and could inhibit U87MG cell proliferation as efficiently as free RA. Thus biodegradable microsphere can be used for RA long term delivery. Smaller polymeric micelles made with PEG grafted chitosan provided a constant RA release as well [124, 155]. Chitosan grafting allowed its solubilization at non acidic pH and maintained its ability to form complexes with RA via its amine groups. Mixed micelles were constituted by a RA chitosan hydrophobic core and a PEG outer shell. NPs were spherical with diameters ranging from 50 to 200 nm and positive surface charge. This technique led to high encapsulation efficiency (> 80 %). RA was released during 35 days *in vitro*, which might be due to electrostatic interaction strength. RA properties were preserved when encapsulated and it was more efficient to inhibit cancer cell migration than free RA [124]. RA polymeric micelles constitute a good mean to bypass several problems encountered with RA treatments. Considering *in vitro* results, RA slow release might allow a sufficient RA concentration when administered locally. RA chitosan-PEG micelles seemed adaptable to industrial scale, however chitosan quality which can vary from batch-to batch might be an issue.

RA has also been encapsulated in gliadin NPs by coacervation [141]. Gliadin was purified from wheat gluten prior encapsulation process. RA gliadin NPs had a mean diameter of 500 nm and were slightly negative. Encapsulation yield was around 90 %. RA NPs were stable 4 days at 37°C in PBS. They could be cross-linked with glutaraldehyde which enhanced their stability against trypsin. However, this chemical stabilisation raises questioning considering glutaraldehyde toxicity. RA release was characterized by an initial burst effect followed by a slow release. Simple coacervation was used with gliadin particles to encapsulate vitamin E [114]. Particles were stabilized by the addition of PEG-propylene glycol block copolymer. Particles had an average diameter of 900 nm and exhibited a matricial structure. NPs could provide a sustained release of vitamin E. Further investigation on complement activation by gliadin NPs should be of interest to determine if they could be used as such for *in situ* administration. If NPs were injected, it is very likely that they would lead to the activation of immune response especially with local or parenteral injection.

Spray drying conditions can be adapted to produce smaller particles in less invasive conditions which extend fields of application. RA has been encapsulated in polymeric micelles formulated by spray drying [2]. Polymer was constituted by polyvinyl alcohol partially substituted by oleyl-amine. Spray drying was performed at low temperature (70°C as inlet temperature and 40°C as outlet temperature). Encapsulation occurred by RA inclusion in hydrophobic zone created in polymers aggregates, whose sizes did not exceed 400 nm. As revealed by DSC studies, RA was in an amorphous state when encapsulated. The highest RA inclusion was obtained with a 1:5 drug polymer ratio. With higher polymer concentration, polymer/polymer interactions were favoured and with lower polymer concentration led to lower encapsulation efficiency. RA could slowly dissociate from the polymer complex (2 % of RA after 24 hours and 5 % after 3 days). RA anti-tumor activity has been evaluated on human neuroblastoma cell line LAN-5. Cytotoxic effect was greater for linked RA than for



free. This result might be due to the reservoir effect. These particles could be used for local tumor treatment as tumor endothelium pores have sizes ranging from 100 nm to 1  $\mu\text{m}$ .

## VIII. Conclusion: matching carriers and administration pathways

The table 1 summarizes each type of particles described in this review and gives information on their potencies for each application type. Oral, topical and transdermal pathways seemed the most appropriate to patient convenience. Key point to increase the efficiency of molecule orally absorbed is enhancing molecule apparent solubility in intestinal lumen. Emulsions and CDs seemed efficient, and their formulation could be performed without organic solvent. However, emulsion based formulations are produced by methods compatible with industrial scale which is not the case for CDs. An appropriate carrier for dermal application should penetrate easily into the *stratum corneum* and remains in superficial skin layers to produce a local action and maintain biological efficiencies while decreasing side effects. Lipid based carriers seemed the most appropriate to bypass *stratum corneum*. A reservoir form might maintain biological efficiencies and decrease side effects. Positively charged liposomes, which have higher skin permeation, could have greater potencies for transdermal delivery. Besides, their core water content might hydrate stratum corneum and therefore increase liposomes penetration. However, their formulation requires organic solvent. Besides, they are often considered as unstable carriers. SLNs might be a good alternative as their surface properties can be modulated to needs and their formulation process is adaptable to industrial scale. Liposomes are also good candidates for *IV* administration as their surface can be modified to increase their stealth properties, however stability issues should be taken into account. NPs have the same grafting possibility in addition they display a reservoir of active molecule. Their physical and chemical stability

might be more easily controlled. Polymeric particles can be obtained with several types of polymers: polysaccharides, proteins and synthetic polymer which can be chemically modified and environment responsive. Polymeric structures are the most adapted to *in situ* administration. Polymer variety allows the production of particles adapted to environmental conditions. Indeed, this pathway requires mostly release profiles in accordance to pharmacological needs.

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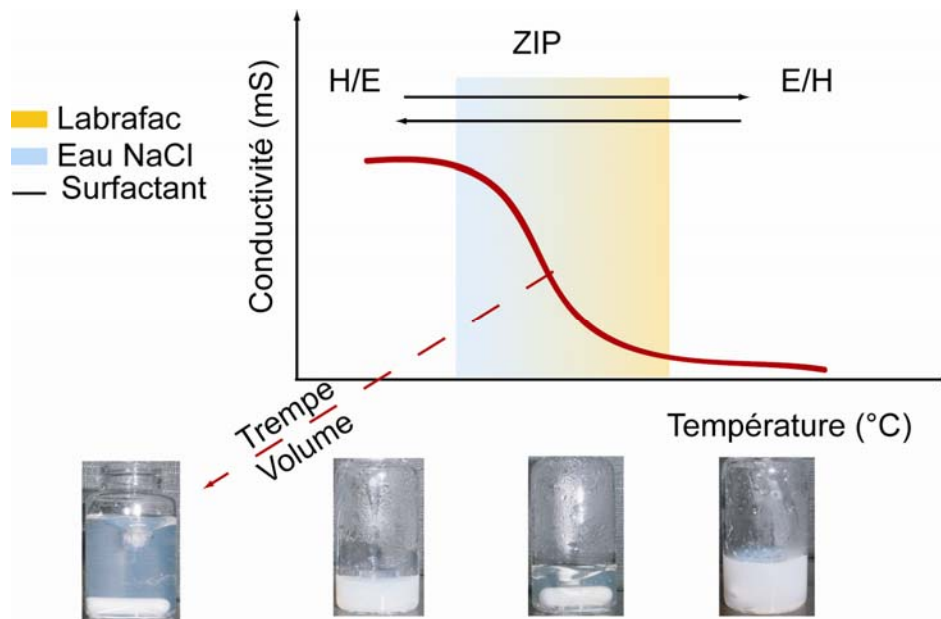
**Approche théorique des méthodes employées**

## 1. Les nanocapsules lipidiques (NCLs)

### a. Procédé de formulation

Les NCLs sont des nanocapsules semi-solides, constituées d'un cœur huileux et d'une couche dense de molécules tensioactives. La formulation des NCLs est basée sur un procédé d'émulsification par inversion de phase qui a fait l'objet d'un brevet [1]. La phase lipophile des NCLs est constituée par du Labrafac<sup>®</sup> (Gattefossé, S<sup>t</sup>-Priest, France). Le Labrafac<sup>®</sup> est un mélange de triglycérides à chaînes moyennes des acides caprique et caprylique (C8-C10). Les surfactants forment la coque. Le surfactant lipophile est un mélange commercial de phospholipides, le Lipoïd<sup>®</sup> (Lipoid<sup>®</sup>, Cham, Switzerland) issu de la lécithine de soja. Le Lipoïd<sup>®</sup> est de la lécithine hydrogénée composée à 85 % de phospholipides dont 86 % par de la dipalmitoyl phosphatidyl choline (DPPC). Le surfactant hydrophile est un tensioactif non ionique (Solutol<sup>®</sup> HS 15, BASF, Ludwigshafen, Germany). Il est composé de 70% de 12-hydroxystearate de PEG 660 et de 30 % de PEG 660 libre (Eupean Pharmacopea, IV<sup>th</sup>, 2002). La phase aqueuse est composée d'eau ultra pure et de NaCl.

La formulation est basée sur la capacité d'une émulsion à s'inverser en fonction de la température [4]. Les excipients sont homogénéisés par agitation magnétique, et chauffés jusqu'à une température  $T_2$  (**figure 1**) au-dessus de la température d'inversion de phase du système (TIP) du système. Le système est ensuite refroidi jusqu'à une température  $T_1$ . En tout, trois cycles de températures sont réalisés entre les températures  $T_1$  et  $T_2$  (autour de la TIP) sont réalisés. Lors du dernier refroidissement un choc thermique est réalisé avec l'ajout d'un grand volume d'eau froide (2.5 fois le volume initial de la formulation), ce choc permet la structuration des NCLs [5]. La suspension est ensuite homogénéisée pendant 5 min.



**Figure 1 : Formulation des NCLs ; H/E : émulsion huile dans eau ; E/H : émulsion eau dans huile**

b. Principes de la méthode d'inversion de phase :

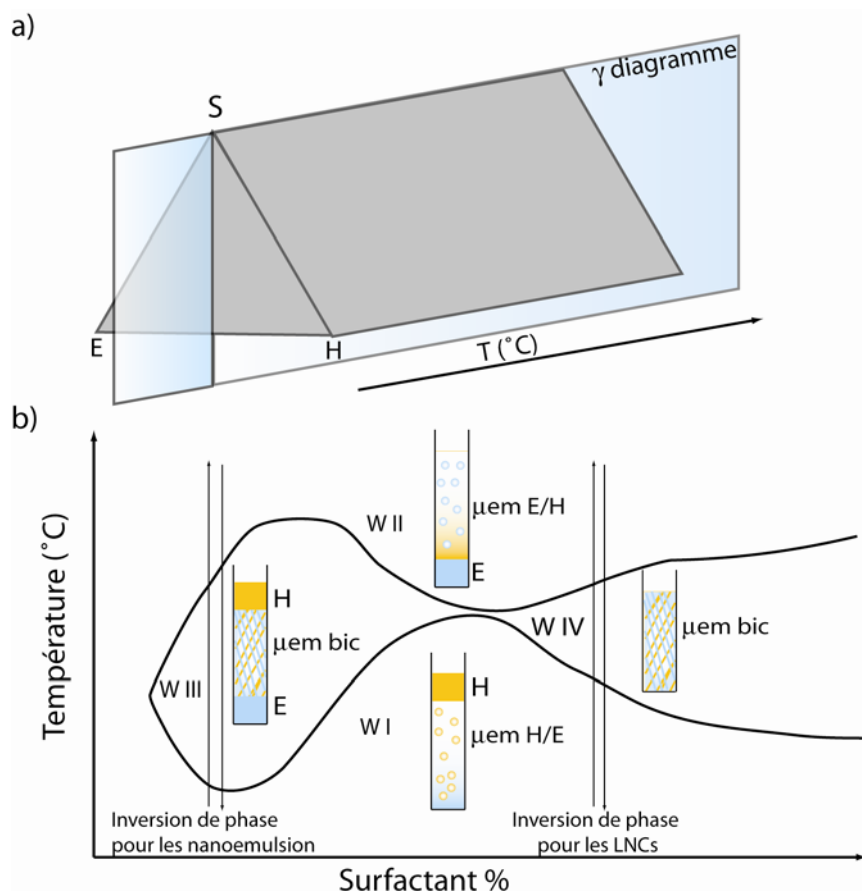
Le phénomène d'inversion de phase peut facilement être mis en évidence par conductivité (**figure 1**). La conductivité est élevée lorsque la phase continue est aqueuse (température  $\ll$  TIP), chute lors de l'inversion de phase pour atteindre un minimum lorsque la phase continue est huileuse donc non conductrice (température  $\gg$  TIP). La zone correspondant à la chute de conductivité est appelée zone d'inversion de phases (ZIP). L'inversion de phase est due à un changement de HLB (Hydrophilic Lipophilic Balance) du stéarate de PEG 660. L'augmentation de la température provoque une déshydratation des têtes polaires du stéarate de PEG, qui devient plus lipophile : on passe alors d'une émulsion huile dans eau (H/E) à une émulsion eau dans huile (E/H). La déshydratation des unités éthylène oxyde est une conséquence de la rupture des liaisons hydrogène avec les molécules d'eau. Le système suit la règle empirique de Bancroft [6], selon laquelle le sens d'une émulsion est régi par la solubilité du surfactant. La phase continue d'une émulsion est la phase dans laquelle le

surfactant est le plus soluble. La TIP est donc définie comme étant la température à laquelle le changement de polarité du surfactant s'opère ; dans cette zone, des structures de type microémulsions se créent [7]. Ce phénomène est réversible. Il correspond au changement de courbure des monocouches de tensioactif qui forment des micelles gonflées d'huile ou une émulsion H/E à basse température, et des micelles inverses gonflées d'eau ou une émulsion E/H pour des températures élevées. Pour les températures intermédiaires (températures de la ZIP) le rayon de courbure des monocouches de tensioactif est proche de zéro, provoquant ainsi la formation d'un système bicontinu [8]. La déshydratation des têtes polaires est influencée par la présence de NaCl dans la phase aqueuse, par conséquent la TIP également.

### c. TIP et approche de Winsor

Les NCLs, comme certaines nano-émulsions, sont formulées grâce à la méthode de la TIP [9, 10]. Les deux modes de formulation peuvent être différenciés grâce à l'étude du diagramme ternaire des systèmes à l'équilibre formés par le mélange E/H/Surfactant en fonction de la température, représenté par un prisme (**figure 2 a**) [11]. En faisant une coupe transversale on obtient un diagramme ternaire (E/H/Surfactant) à température constante. Après une coupe horizontale du prisme, on obtient un graphique bidimensionnel de la température en fonction de la proportion des phases aqueuse et organique, le pourcentage de surfactant étant constant. Pour différencier la formulation des NCLs de celle des nanoémulsions, on utilise préférentiellement la coupe verticale qui correspond à un rapport eau/huile constant. On obtient alors le diagramme  $\gamma$  ou diagramme de poisson (**figure 2 b**).





**Figure 2** : a) Prisme S/H/E/T représentatif de l'évolution d'un diagramme de phase ternaire E/H/S en fonction de la température b) Coupe verticale ( $\gamma$ ) du prisme S/H/E/T pour un rapport eau /huile constant ou « diagramme poisson » ; W : Winsor, H : huile, E, Eau ;  $\mu\text{em}$  : microémulsion ; bic : bicontinue.

Dans ce diagramme on observe quatre systèmes différents. Pour des températures faibles le système est de type Winsor I, il est constitué de deux phases. Dans ce système, le sens favorisé de l'émulsion est H/E. La phase supérieure est constituée par la phase organique (H), une microémulsion H/E constituant la phase inférieure. Pour les températures les plus élevées, le système est de type Winsor II ; il est également constitué par deux phases. Dans ces conditions, le sens favorisé de l'émulsion est E/H. La phase inférieure est constituée par la phase aqueuse (H), une microémulsion E/H constituant la phase supérieure. Pour les températures intermédiaires et de faibles proportions de surfactant (<12% pour le diagramme

E/H/Solutol), le système est de type Winsor III [12]. Il est composé de trois phases : une phase aqueuse inférieure, une phase organique supérieure et une phase intermédiaire. La phase intermédiaire est formée par une microémulsion bicontinue, riche en surfactant constitué par des micelles et des micelles inverses gonflées. Le refroidissement d'un système Winsor III permet la formation de nano émulsions H/E [13]. Pour des proportions plus importantes de surfactant, le système est monophasique de type Winsor IV. Il est constitué par une microémulsion bicontinue. Des cycles de température effectués autour de cette zone, suivis d'une dilution rapide par de l'eau froide, permettent la structuration des NCLs [12].

## 2. Diffusion de la lumière

### a. Mesure de la taille des particules

La suspension de NCLs une fois formulée est caractérisée par diffusion de la lumière. On détermine le diamètre moyen des NCLs et la polydispersité de la suspension ou indice de polydispersité (PDI) en utilisant Malvern Zetasizer Nano ZS (Malvern Instruments SA, Worcestershire, UK). La diffusion de la lumière (ou spectroscopie de corrélation de photons ; diffusion de la lumière quasi-élastique) est une technique utilisée pour mesurer le mouvement Brownien (diffusion) et la distribution de taille d'un ensemble de particules en solution. Le diamètre hydrodynamique d'une particule ( $R_H$ ) est calculé grâce au coefficient de diffusion et à l'équation de Stokes-Einstein.

$$R_H = \frac{KT}{6\pi\eta D}$$

D : Coefficient de diffusion

K : Constante de Boltzman

T : Température

$\eta$  : Viscosité du solvant

La taille hydrodynamique calculée à partir de cette méthode est pondérée avec l'intensité moyenne, appelée « Z average » ou taille moyenne. La distribution peut être

transformée en fonction du volume ou du nombre de particules, en utilisant la théorie de Mie. La distribution de taille en volume permet d'avoir une indication sur les proportions massiques de chaque population de particules. Le diamètre moyen de chaque suspension de NCLs sera toujours indiqué en volume. Le PDI permet de caractériser la distribution de taille des particules, un PDI > 0.3 indique une distribution hétérogène tandis qu'un PDI << 0.3 indique une distribution unimodale des particules.

#### b. Evaluation de la turbidité

L'appareil utilisé pour évaluer la turbidité des suspensions de NCLs est un Turbsican (Formulation, L'Union, France). Il permet de déterminer la proportion de lumière transmise ou rétrodiffusée à travers une suspension colloïdale. La transmission correspond au taux de lumière traversant la suspension sans être diffusée.

Le flux de lumière transmis (T) est calculé grâce à la loi de Lambert-Beer :

$$T(l, ri) = T_0 e^{-\frac{2ri}{l}}$$

Ri : Rayon interne de la cellule

l : Longueur de transport du photon, définie comme suit :

$$l(d, \Phi) = \frac{2d}{3\Phi Q_s}$$

D : Diamètre moyen des particules

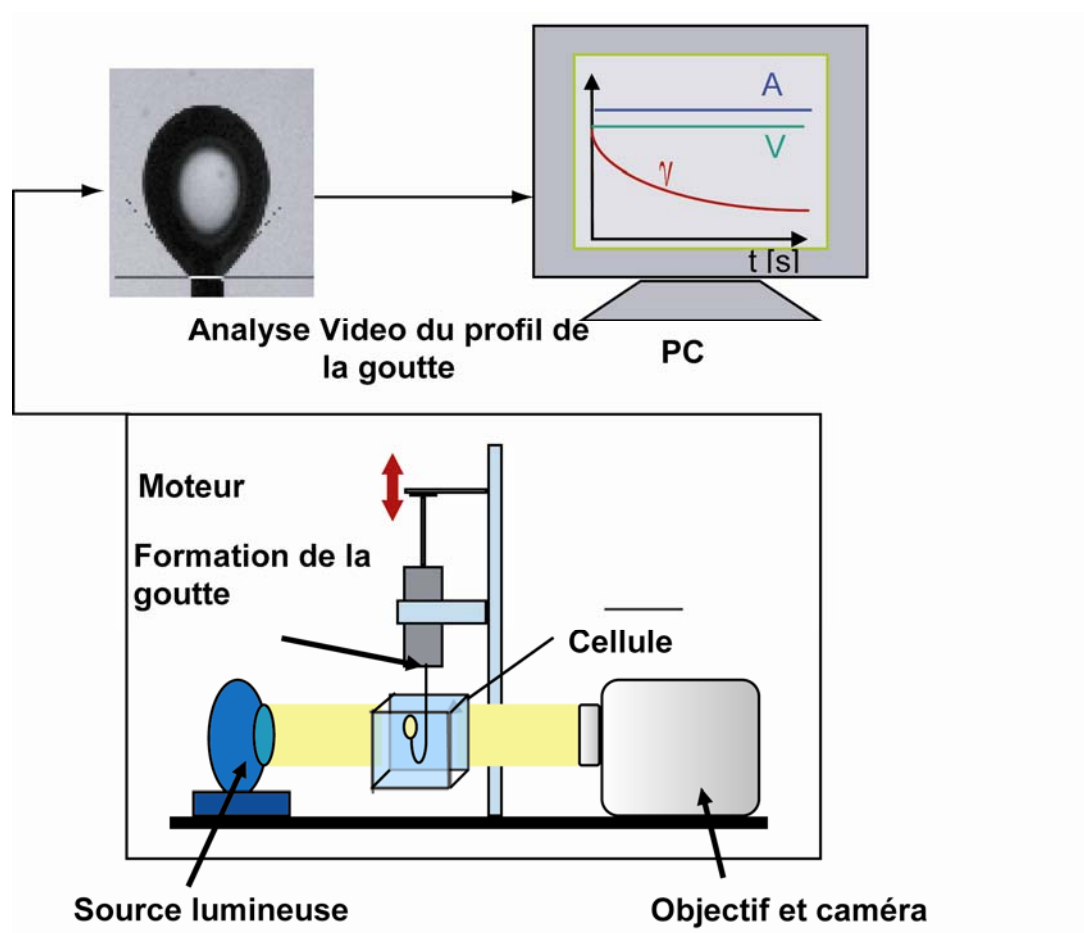
$\Phi$  : Fraction volumique

La transmission est donc dépendante du diamètre moyen des particules et de leur fraction volumique. La suspension analysée est contenue dans une cellule de verre cylindrique. La source lumineuse (diode électroluminescente dans le proche infra rouge,  $\lambda_{\text{air}}=850\text{nm}$ ) balaye la cellule de verre cylindrique de haut en bas et de bas en haut. Le détecteur de transmission, situé à  $180^\circ$  par rapport au rayon de lumière incident acquiert des données tous les  $40 \mu\text{m}$ . On

obtient alors les valeurs de diffusion en fonction de la hauteur du tube. Si pour un point du tube, la transmission augmente, alors on peut déduire que la turbidité baisse. A partir de ces données, plusieurs caractéristiques de la suspension peuvent être déterminées (mise en évidence de phénomènes de déstabilisation : crémage, sédimentation).

### 3. Caractérisation de l'interface des NCLs :

#### a. Tensiomètre à goutte



**Figure 3** : Représentation schématique du tensiomètre à goutte

La tension interfaciale huile/eau a été mesurée par la méthode de la goutte montante (**figure 3**). La goutte est formée à l'extrémité d'une aiguille recourbée à 180° reliée à une seringue, dont le piston est commandé par un moteur. La tension superficielle ( $\gamma$ ) à l'interface huile/eau ainsi que le volume et la surface de la goutte sont calculés par le logiciel en temps réel par analyse du profil de la goutte, et grâce à l'équation de Laplace-Young. Les détails de

calculs ont été présentés par Tewes *et al.* [14]. Ce modèle prend en compte la pression capillaire ou force de Laplace et la gravité.

Après formation de la goutte, les molécules tensioactives dissoutes dans la phase aqueuse et/ou huileuse s'adsorbent à l'interface. Lorsque la tension de surface est stable, le système a atteint un équilibre dynamique. Les caractéristiques d'un tensioactif, ainsi que les propriétés rhéologiques de l'interface peuvent être identifiées par dilatation ou compression de la surface de la goutte. Une modification de l'aire interfaciale engendre une variation de la concentration des tensioactifs adsorbés à l'interface. Les caractéristiques viscoélastiques des interfaces figurent parmi les paramètres qui sont corrélés à la stabilité des colloïdes. Leur évolution en fonction du temps permet de prévoir l'évolution de leur stabilité et ainsi de mettre en évidence ou d'expliquer les phénomènes de maturation des colloïdes tels que le mûrissement d'Ostwald ou la coalescence [15].

Parmi les approches expérimentales employées en rhéologie 2D, les modes harmonique ou de type rampe sont les plus utilisés. Nous avons choisi le mode rampe (**figure 4a**) en effectuant une compression rapide pendant un temps  $t_f$ , et lente pendant un temps  $t_s$  (en pointillé large). La compression rapide permet de déduire, dans le cas des comportements viscoélastiques, le temps caractéristique de la relaxation. La compression lente permet d'accéder expérimentalement aux modules viscoélastiques de l'interface en utilisant un modèle de type Maxwell généralisé (**Figure 4 c**) [14, 16-21].

La variation de pression interfaciale durant l'expérience peut s'écrire :

$$\Delta\pi = \pi(t) - \pi_i = \gamma_i - \gamma_0 \quad (1)$$

avec

$$\Delta\pi = \Delta\pi_e + \Delta\pi_{ne} \quad (2)$$

Une partie de cette variation ( $\Delta\pi_e$ ) est reliée à l'élasticité (ou module de charge) dilatationnelle  $E_e$  et caractérise les interactions latérales exercées par les molécules adsorbées. Le module élastique est représenté par un ressort sur le modèle mécanique (**figure 4c**, branche du haut). La partie  $\Delta\pi_{ne}$  est associée à la dissipation de l'énergie accumulée lors de la compression et est relié au module de perte, que l'on nommera partie non équilibre de l'élasticité ( $E_{ne}$ ). Elle s'accompagne d'une relaxation de pression liée à l'expulsion du surfactant ou fragment du surfactant hors de l'interface. Le temps de relaxation  $\tau$  représente le temps nécessaire à la monocouche pour retrouver un état d'équilibre énergétique après une perturbation. Sur le modèle mécanique, le module viscoélastique correspondant à un ressort couplé à un amortisseur (**figure 4c**, branche du bas). Les comportements rhéologiques peuvent s'interpréter par une modification de la composition de la couche interfaciale. Pendant la compression, une partie des molécules adsorbées à l'interface peut avoir changé de conformation ou avoir été expulsée dans la goutte ou dans la phase externe modifiant par conséquent les caractéristiques de la couche interfaciale.

L'approche théorique liée aux calculs à suivre a été exposée précédemment [17-19, 22, 23].  $\tau$  peut être calculé à partir des données issues de la compression rapide pour  $t > t_f$  en réalisant une régression exponentielle.

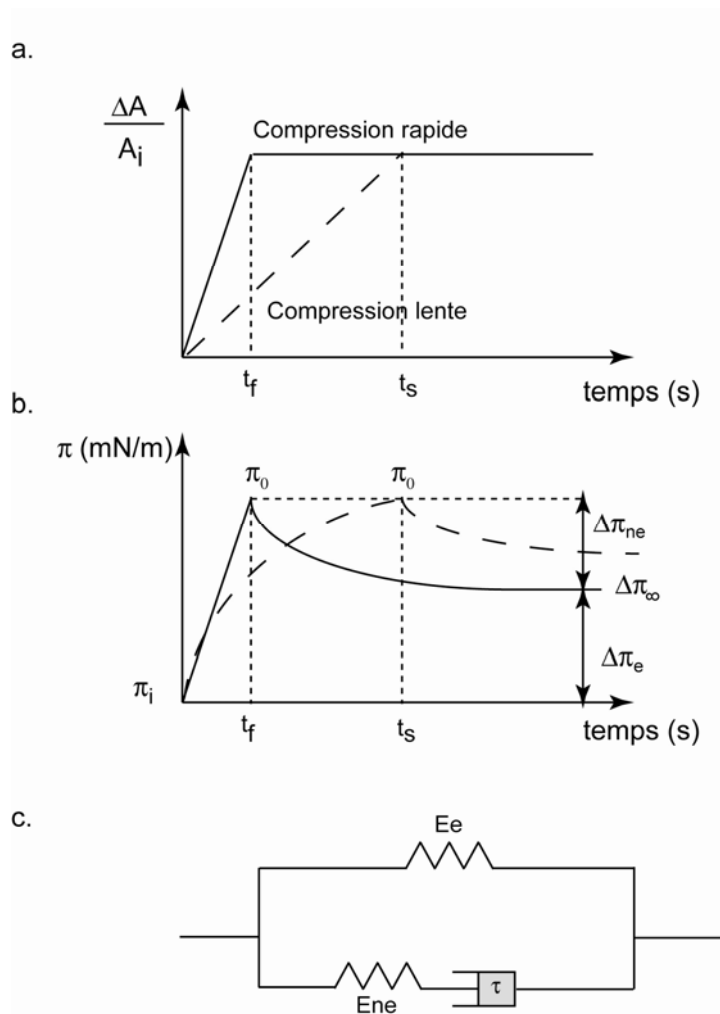
$$\pi(t) = (\pi_0 - \pi_\infty)e^{-t/\tau} + \pi_\infty \quad (3)$$

La compression lente est utilisée dans un deuxième temps pour déterminer les composantes  $E_e$  et  $E_{ne}$  grâce à la valeur de  $\tau$  calculée précédemment et à la relation suivante :

$$\frac{\Delta\pi}{Ut} A_t = E_e + E_{ne} \frac{\tau}{t} (1 - e^{-t/\tau}) \quad (4)$$

Avec  $U$  la vitesse de compression et  $A_i$  l'aire de la goutte avant compression.  $E_e$  et  $E_e$  sont déterminées grâce à une régression linéaire.

La compression rapide est réalisée à une vitesse supérieure à  $-0.5 \mu\text{l.s}^{-1}$  pour une perturbation  $\Delta\pi_{\text{max}} \leq 2 \text{ mN.m}^{-1}$ . La compression lente est réalisée à une vitesse de  $-0.03 \mu\text{l.s}^{-1}$  pour une perturbation  $\Delta\pi_{\text{max}} \leq 2 \text{ mN.m}^{-1}$ .



**Figure 4 :** a) représentation schématique des variations d'aire pour les modes de compression rapide— ou lente----, l'aire est maintenue constante après compression dans les deux cas ;b) Représentation des variations de pression après une compression rapide — ou lente ---- ; c) Modèle mécanique illustrant le comportement viscoélastique d'une monocouche d'après le modèle de Maxwell.

## b. Caractéristiques électrophorétiques des particules molles

La mobilité électrophorétique ( $\mu$ ) correspond au mouvement d'une particule colloïdale chargée dans une solution d'électrolyte par l'intermédiaire d'un champ électrique. Cette grandeur physique apporte des informations fondamentales sur les propriétés électriques de surface de ces objets. Pour une particule molle composée d'un cœur de triglycérides et d'une couronne de polymères, l'hypothèse de départ est que les molécules d'eau ainsi que les électrolytes peuvent pénétrer dans la couche de polymères [24]. La mobilité électrophorétique dépend donc de la densité de charges dans la couche perméable aux ions ( $N$ ), des forces de friction exercées par les chaînes de polymères sur le liquide dans la couche perméable aux ions, de la taille des particules et de la fréquence du champ électrique sinusoïdal appliqué [25] :

$$\mu = \frac{\varepsilon_0 \varepsilon_r}{\eta} \frac{\Psi_0 / Km + \Psi_{DON} / \lambda}{1 / Km + 1 / \lambda} + \frac{eZN}{\eta \lambda^2} \quad (1)$$

$\mu$  : Mobilité électrophorétique ( $\mu\text{mcm/Vs}$ )

$\varepsilon_r$  : Permittivité relative (80)

$\varepsilon_0$  : Permittivité du vide ( $8.85 \cdot 10^{-12} \text{ F.m}^{-1}$ )

$\eta$  : Viscosité de la solution d'électrolytes ( $\text{Pa.s}^{-1}$ )

$ZN$  : Densité de charge dans la couche perméable aux ions

$1/\lambda$  : Epaisseur de la couche perméable aux électrolytes

$Km$  est le paramètre de Debye-Hückel de la coque, il est calculé grâce au paramètre de Debye-Hückel  $\kappa$ .

$$\kappa = \left( \frac{2z^2 e^2 n}{\varepsilon_0 \varepsilon_r kT} \right)^{1/2} \quad (2)$$

$$Km = \kappa [1 + (A)^2]^{1/4} \text{ avec } A = ZN/zn \quad (3)$$

$z$  : Valence des ions de la solution d'électrolytes

$e$  : Charge électrique élémentaire ( $1.6 \cdot 10^{-19} \text{ C}$ ).

$T$  : Température (K)

$K$  : Constante de Boltzmann

$n$  : Concentration de la solution d'électrolytes ( $\text{Osm.m}^{-3}$ )

$\Psi_{DON}$  : Potentiel de Donnan correspond à une distribution inégale ions entre deux solutions ioniques



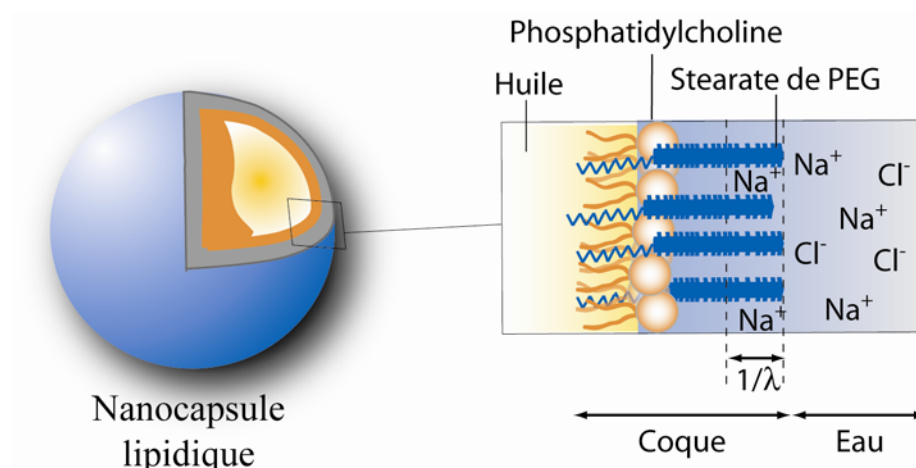
$$\psi_{DON} = \frac{kT}{ze} \ln(A + (A^2 + 1)^{1/2}) \quad (4)$$

$\psi_0$ : Potentiel de surface, potentiel à la limite entre la surface de la couche et la solution

$$\psi_0 = \frac{kT}{ze} \left[ \ln(A + (A^2 + 1)^{1/2}) + \frac{1}{A} (1 - (A^2 + 1)^{1/2}) \right] \quad (5)$$

Le potentiel  $\zeta$  est défini comme le potentiel électrique de la particule dans le plan de cisaillement pour lequel la vitesse relative de la particule est zéro. Si le plan de cisaillement correspond à la surface de la particule alors le potentiel  $\zeta$  correspond au potentiel de surface de la particule  $\Psi_0$ . Les caractéristiques de la couche perméable aux ions (ZN et  $1/\lambda$ , **figure 5**) peuvent être déterminées expérimentalement pour les NCLs [2, 26]. La mobilité électrophorétique est mesurée pour des suspensions de NCLs ayant une concentration en NaCl de 0.1, 0.3, 0.5, 1, 1.5, 2, 10, 25, 50 mM avec un zétamètre (zeta sizer nano ZS, Malvern instruments S.A.). ZN et  $1/\lambda$  sont ensuite déterminés en fittant la courbe expérimentale de  $\mu(\text{NaCl})$  des NCLs avec l'équation (1).

Les chaînes de stéarate de PEG peuvent être schématisées par une succession de dipôles [2] qui rendent la particule chargée alors que le stéarate de PEG est considéré comme un surfactant non ionique. Les NCLs de 20, 50 et 100 nm ainsi que des NCLs formulées avec du stéarate de PEG 1500 ont déjà été étudiées grâce à cette approche [2, 26].



**Figure 5:** Représentation schématique de ZN et de  $1/\lambda$  pour une NCLs dans une solution de NaCl

#### 4. Biodisponibilité et bioaccessibilité d'un composé lipophile encapsulé dans des NCLs

Plusieurs étapes sont susceptibles de limiter l'absorption intestinale des micronutriments : une faible libération de la matrice alimentaire, leur complexation avec d'autres éléments du chyme dans le tractus gastro-intestinal ou encore leur dégradation. L'acronyme ADME pour Absorption, Distribution, Métabolisme et Elimination permet de rassembler les paramètres qui influencent la bioaccessibilité et la biodisponibilité d'une molécule (**figure 6**). Dans un premier temps la molécule considérée doit être libérée de la matrice et solubilisée dans le milieu intestinal. Si la libération se produit trop tôt, dans l'estomac par exemple, cela augmente le risque de complexation de la molécule avec d'autres éléments du chyme. Ce phénomène diminue alors la proportion de molécules accessibles à l'absorption, ou bioaccessibilité de la molécule considérée. L'absorption est régie par des mécanismes variés dépendant de la molécule, de sa concentration, de la composition du chyme ainsi que de l'abondance des transporteurs membranaires et donc du patrimoine génétique [27]. Certaines molécules peuvent utiliser les mêmes voies d'entrée dans la cellule et ainsi rentrer en compétition. Ce phénomène peut diminuer

l'absorption intestinale et donc la biodisponibilité de la molécule considérée. Les composés liposolubles sont transportés dans le réseau lymphatique via les chylomicrons: c'est l'étape de distribution. La proportion de cette molécule stockée ou métabolisée sera considérée comme biodisponible, cependant, elle peut être influencée par le métabolisme hépatique ou l'excrétion urinaire.

Un autre acronyme a été développé pour lister les facteurs influençant la biodisponibilité des caroténoïdes : SLAMENGI [28] :

**S : Species** ou espèce de caroténoïdes : selon son hydrophobicité et l'isomère considéré (*cis* ou *trans*) l'absorption intestinale est plus ou moins favorisée [29]. Par exemple, l'alpha-carotène semble être moins bien absorbé que le  $\beta$ C [30]. L'isomère *trans* du  $\beta$ C est mieux absorbé que l'isomère *cis* [29] tandis que le *cis* lycopenène est mieux absorbé que le *trans* [31].

**L : Linkage**, correspondant à liaison moléculaire : le  $\beta$ C issu des légumes verts (épinards...) est associé avec les protéines des chloroplastes et le  $\beta$ C issu des racines (carottes) ou des tomates est sous forme semi-cristalline [32]. Dans les deux cas, ces interactions moléculaires sont des facteurs limitants de l'absorption intestinale. L'existence de certains caroténoïdes estérifiés peut également influencer la biodisponibilité.

**A : Amount** : quantité consommée

**M : Matrix** : matrice dans laquelle sont inclus les caroténoïdes. L'augmentation du fractionnement de la matrice, ainsi que les traitements thermiques qui déstabilisent les parois cellulaires des végétaux, conduisent à une meilleure libération du  $\beta$ C et à une meilleure biodisponibilité [33]

**E : Effectors of absorption and bioconversion**, qui correspond aux éléments du chyme qui pouvant augmenter l'inclusion des caroténoïdes dans les micelles et donc l'absorption dans l'entérocyte (la proportion de lipide, la taille des gouttelettes lipidiques) ou la limiter (la

consommation de fibres qui peut diminuer jusqu'à 42% l'absorption de  $\beta$ -carotène). Les facteurs de variation inter individus sont aussi inclus dans ces paramètres [34].

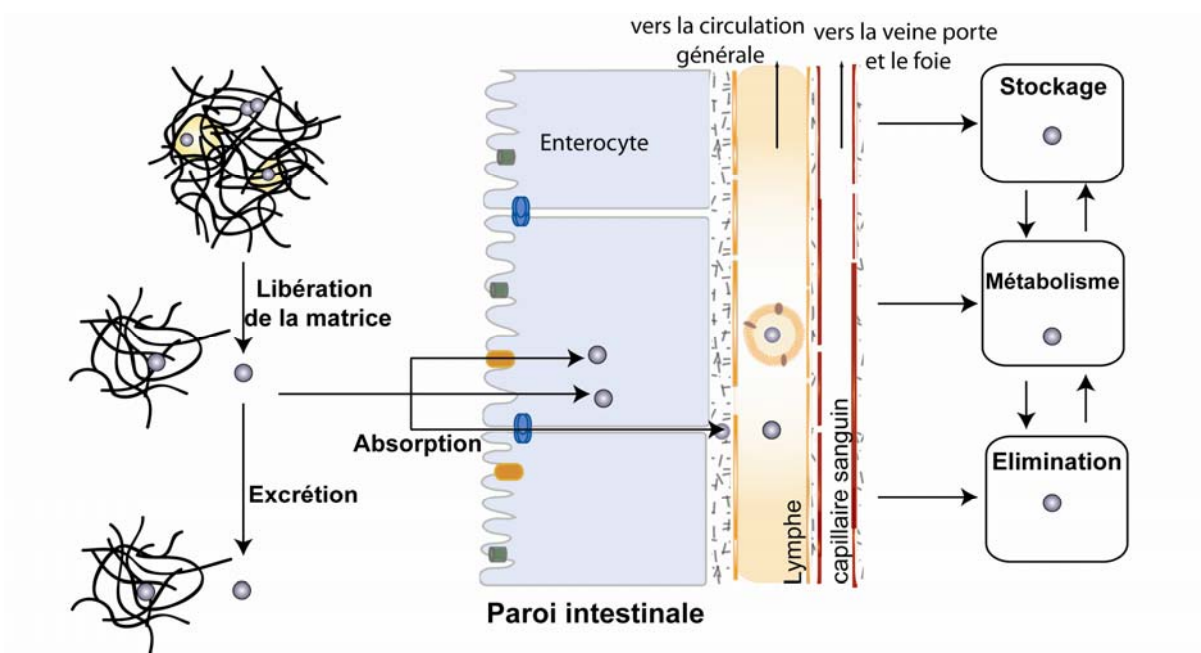
**N : Nutrient status** : statut nutritionnel de l'individu.

**G : Genetic factors** : L'absorption des caroténoïdes dans l'entérocyte peut s'effectuer par diffusion simple ou facilitée à travers des transporteurs membranaires. L'abondance ou l'efficacité de ces transporteurs peuvent être régies par des facteurs génétiques [27] . La variabilité d'absorption entre les sujets des études citées peut être en partie expliquée par ce facteur. L'efficacité de la conversion enzymatique du  $\beta$ C en rétinol peut aussi être affectée par ce paramètre.

**H : Host related factors** (facteurs dépendants de l'hôte). Déficiences dans l'absorption des lipides, mais aussi age, sexe ...

**M : Mathematical Interactions** (interactions mathématiques): correspond aux interactions entre ces facteurs.

Cette approche est plus spécifique, mais elle met en évidence les mêmes facteurs et interactions que le concept ADME.



**Figure 6** : Concept ADME (Absorption, Distribution, Métabolisation, Elimination)

a. Evaluation de la stabilité des NCLs dans des milieux de gastriques et intestinaux modèles

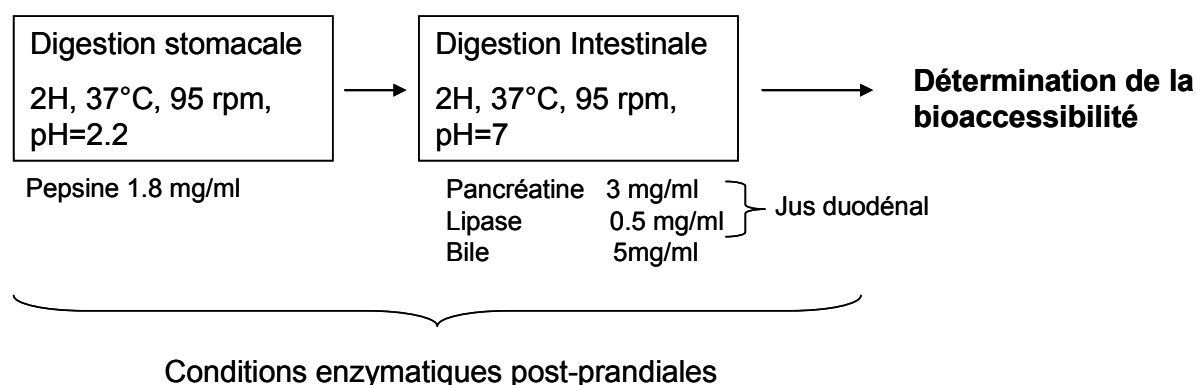
L'évaluation que nous avons menée *in vitro* permet d'évaluer la forme sous laquelle le  $\beta$ C encapsulé arrive au niveau de son site d'absorption intestinal : l'entérocyte. Différents milieux ont été développés pour mimer les conditions de digestion *in vivo* [3, 35-39]. Plusieurs paramètres influent sur la digestion : la présence d'enzymes et de sels biliaires, leur proportion, les changements de pH et d'osmolarité mais aussi des paramètres mécaniques (péristaltisme) qui assurent l'homogénéisation du bol alimentaire et son cheminement dans le tractus gastro-intestinal. Les milieux simulés diffèrent majoritairement par leur concentration en enzymes, en sels biliaires et par leur pH qui correspond aux périodes inter-prandiale ou post prandiale. Les milieux mimant les conditions inter-prandiales sont les plus pauvres en enzymes et le pH mimant les conditions gastriques est plus bas (1.6). Du fait de la mobilité gastro-intestinale et de la mise en route des sécrétions digestives, la composition enzymatique, le pH évoluent dans le temps après ingestion. Pour prendre en compte ces modifications, certains modèles de digestion *in vitro* sont composés de plusieurs milieux postprandiaux dont la composition s'enrichit en sécrétions digestives.

Nous avons choisi d'évaluer la stabilité des NCLs dans des conditions postprandiales avec des concentrations en enzymes et sels biliaires élevées. Les milieux gastriques et intestinaux ont été préparés selon le protocole mis au point par Versantvoort *et al.* [3] (**tableau 1**). Ce modèle de digestion inclut également des molécules non enzymatiques qui permettent d'approcher le plus possible des conditions réelles. Ainsi, l'ajout de mucine permet de diminuer la tension interfaciale. Les modèles de digestion *in vitro* développés spécifiquement pour déterminer la bioaccessibilité des caroténoïdes [40] ou par la pharmacopée [41] ne contiennent pas de lipase gastrique, car cet enzyme n'est pas active aux pH du milieu gastrique simulé [42].

	Jus gastrique	Jus duodenal	Bile
Solution inorganique	<ul style="list-style-type: none"> <li>- [NaCl]= 5.5 g/l</li> <li>- [NaH<sub>2</sub>PO<sub>4</sub>]= 0.5 g/l</li> <li>- [KCl]= 1.6 g/l</li> <li>- [CaCl<sub>2</sub>]= 0.8 g/l</li> <li>- [NH<sub>4</sub>Cl]= 0.6 g/l</li> <li>- pH adjusted to 1.2±0.05</li> </ul>	<ul style="list-style-type: none"> <li>- [NaCl]= 14 g/l</li> <li>- [NaHCO<sub>3</sub>]= 6.8 g/l</li> <li>- [KH<sub>2</sub>PO<sub>4</sub>]= 0.16 g/l</li> <li>- [KCl]= 1.13 g/l</li> <li>- [MgCl<sub>2</sub>]= 0.1 g/l</li> </ul>	<ul style="list-style-type: none"> <li>- [NaCl]= 10.5 g/l</li> <li>- [NaHCO<sub>3</sub>]= 11.6 g/l</li> <li>- [KCl]= 0.76 g/l</li> </ul>
Solution organique	<ul style="list-style-type: none"> <li>- [glucose]= 1.3 g/l</li> <li>- 1.2 ml glucuronic acid]= 0.04 g/l</li> <li>- [urée]= 0.17 g/l</li> <li>- [glucosamine hydrochloride]= 0.66 g/l</li> </ul>	<ul style="list-style-type: none"> <li>- [urée]= 0.2 g/l</li> </ul>	<ul style="list-style-type: none"> <li>- [urée]= 0.5 g/l</li> </ul>
Ajout après mélange des solutions organique et non organique (50/50; v/v)	<ul style="list-style-type: none"> <li>- [BSA]= 1 g/l</li> <li>- [Mucine]= 3 g/l</li> <li>- [Pepsine]= 2.5 g/l</li> </ul> <p><b>pH ajusté à 2.00±0.05</b></p>	<ul style="list-style-type: none"> <li>- [BSA]= 1g/l</li> <li>- [Pancreatin]= 9 g/l</li> <li>- [Lipase]= 1.5 g/l</li> <li>- [CaCl<sub>2</sub>]= 0.1998 g/l</li> </ul> <p><b>pH ajusté à 8.10±0.05</b></p>	<ul style="list-style-type: none"> <li>- [BSA]= 1.8 g/l</li> <li>- [Bile]= 30 g/l</li> <li>- [CaCl<sub>2</sub>]= 0.222 g/l</li> </ul> <p><b>pH ajusté à 8.20 ±0.05</b></p>

**Tableau 1** : Constituants et concentrations des différents jus digestifs du modèle de digestion *in vitro* [3].

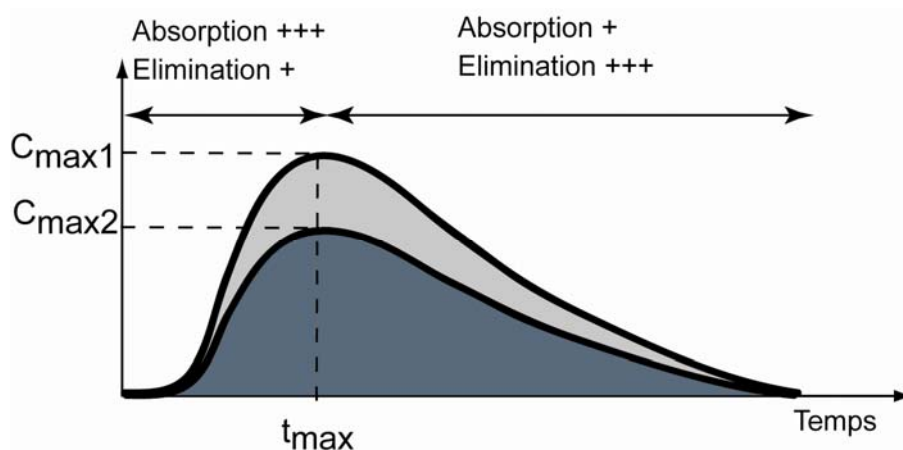
La digestion est simulée par une phase de digestion stomacale de 2H, suivie d'une digestion intestinale de 2H (**figure 7**). L'étape de digestion par la salive n'a pas été simulée pour l'étude des suspensions de NCLs. En effet, la structure nanométrique des particules excluait une altération de leur structure par effet mécanique lors de la mastication. De plus, l' $\alpha$ -amylase n'a pas été identifiée comme une enzyme destructurant pour les NCLs. Les conditions de manipulation choisies sont compatibles avec l'activité enzymatique (pH).



**Figure 7** : Protocole de digestion simulée en conditions postprandiales.

## 5. Evaluation de biodisponibilité

D'un point de vue pharmacocinétique, la biodisponibilité s'évalue en comparant l'aire sous la courbe de la cinétique sanguine de la molécule après absorption intestinale avec l'aire sous la courbe de la cinétique sanguine de la molécule après injection par voie *I.V.* de la même dose (**figure 8**). En mettant en relation les informations apportées par l'étude du concept ADME et cette définition, on peut établir que la partie ascendante de la courbe correspond à une phase d'absorption forte et de faible élimination, tandis que la phase descendante correspond à une phase de faible absorption et de forte élimination. On parle de biodisponibilité relative lorsque plusieurs formes sont comparées, la cinétique sanguine de référence n'est pas celle de la voie *I.V.*.



**Figure 8 : Evaluation de la biodisponibilité**

Pour l'évaluation de la biodisponibilité du  $\beta$ C ou d'autres caroténoïdes, l'approche pharmacocinétique présentée ci-dessus est adaptée. En effet, il est délicat de formuler une suspension particulière ayant des propriétés correspondant à celles des lipoprotéines qui transportent les caroténoïdes dans le sang. Une étude a simulé un profil de concentration plasmatique après injection par voie *I.V.* grâce à un logiciel en prenant en compte le temps moyen de demi-vie des chylomicrons dans le sang [43]. Mais de manière générale, les études qui cherchent à évaluer de la biodisponibilité des caroténoïdes n'utilisent pas cette méthode

qui n'inclue pas la bioconversion. Différents types d'études ont été développés afin de déterminer la biodisponibilité des caroténoïdes.

- Des études évaluent l'absorption grâce à la différence entre la quantité absorbée et la quantité excrétée dans les fèces [43, 44]. Cette méthode ne détermine pas l'influence de la flore intestinale, ce qui peut conduire à une surévaluation de la quantité absorbée, de plus le dosage des caroténoïdes dans les fèces est complexe.
- Des études mesurent la réponse plasmatique : on évalue la différence entre la concentration basale de  $\beta$ C, de rétinol et d'esters de rétinol après ingestion du  $\beta$ C. Dans ces études, les dosages sont réalisés sur le plasma ou sur la fraction riche en triglycérides. Cette fraction représente l'ensemble des lipoprotéines. Lorsque la concentration plasmatique est suivie après une dose unique de concentration physiologique, l'augmentation plasmatique peut être faible par rapport à la concentration basale et donc déterminée avec moins de précision. Ainsi, dans de nombreuses études, la dose de  $\beta$ C administrée a été augmentée jusqu'à des doses pharmacologiques. Ces doses ne reflètent pas la réalité des quantités absorbées à partir d'aliments non enrichis, mais cette méthode permet de limiter les erreurs dues aux concentrations plasmatiques trop faibles [33, 45, 46]. L'utilisation de molécules marquées avec des isotopes stables ( $^{13}\text{C}$ ,  $^2\text{H}$ ) comme référence interne [33, 47] ou représentant la totalité de la dose ingérée [48] augmente la précision du dosage et permet de différencier les molécules endogènes des molécules nouvellement absorbées. L'utilisation d'isotopes permet également de déterminer plus efficacement la conversion des provitamines en molécules actives [34, 49-51]. Après une administration



chronique on observe l'élévation de la concentration plasmatique moyenne[52, 53]

- Un dernier type d'études permettant l'évaluation de la biodisponibilité consiste en l'extraction et le dosage dans la fraction chylomicrons [32, 46]. Cette méthode permet d'isoler la fraction de molécules nouvellement absorbées des molécules endogènes incluses dans les LDL principalement. Cette méthode requiert un volume d'échantillons élevé (5ml). De plus, le temps de demi-vie des chylomicrons (2 à 11 min chez l'homme) doit être pris en compte [32].

Les méthodes basées sur l'AUC peuvent également sous-estimer la biodisponibilité. Les méthodes basées sur le dosage de la fraction riche en triglycérides du plasma avec étalon interne de type isotope stable semblent être les plus précises. Cependant, ces études sont fastidieuses et ont un coût élevé.

Il est difficile de donner des valeurs précises et globales de la biodisponibilité du  $\beta$ C. En effet, chaque étude a une dose administrée, une matrice et une méthode d'évaluation de la biodisponibilité spécifiques. Cependant, les mêmes facteurs principaux se dégagent : (i) une proportion de lipides de 5 à 10% permet une meilleure solubilisation du  $\beta$ C, ce qui accroît la proportion incluse dans les micelles mixtes et l'absorption dans l'entérocyte [47]. La solubilisation est meilleure dans une phase lipidique riche en acides gras à chaînes moyenne [31], mais la formation des chylomicrons et donc la libération du  $\beta$ C absorbé dans la lymphe nécessite des acides gras à chaînes longues [46]. Plus l'aliment est facile à digérer, plus la proportion absorbée est importante : 5% du  $\beta$ C sont absorbés à partir de purée de carottes commerciale contre 3% à partir de carottes écrasées [33]. Les facteurs inter-individuels (transporteurs membranaires, temps de circulation des chylomicrons, flore intestinale) ne peuvent pas être contrôlés bien qu'ils aient un impact important sur l'évaluation de la biodisponibilité.

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# **TRAVAIL EXPERIMENTAL**

## **Partie expérimentale**

**Chapitre 1** (p 83): Encapsulation du  $\beta$ -carotène dans les NCLs et évaluation de leur stabilité *in vitro*.

**Chapitre 2** (p 110): Caractérisation des propriétés interfaciales des NCLs chargées en  $\beta$ -carotène

**Chapitre 3** (p 139): Comportement des NCLs dans une matrice complexe modèle

**Chapitre 4** (p 166): Evaluation de la biodisponibilité d'un complexe lipophile radiomarqué encapsulé dans les NCLs

## **CHAPITRE I :**

**ENCAPSULATION DU  $\beta$ -CAROTENE DANS LES NCLs ET  
EVALUATION DE LEURS STABILITE *IN VITRO*.**



## **Encapsulation du $\beta$ -Carotène dans les NCLs et évaluation de leur stabilité *in vitro*.**

Notre objectif principal consistait à formuler des particules capables de protéger une molécule fragile pendant le stockage tout en préservant ses fonctionnalités. Nous avons choisi de travailler sur une molécule qui a à la fois un impact sur les qualités organoleptiques d'un produit alimentaire (contrôle de l'oxydation, couleur) et des effets bénéfiques sur l'organisme (pro-vitamine A et propriétés anti-oxydantes): le  $\beta$ -carotène. Ce composé est sensible à la lumière, ainsi qu'aux températures élevées. C'est pourquoi, il constitue un bon modèle pour élaborer une formulation adaptable à d'autres micronutriments lipophiles fragiles.

Comme cela a été développé dans l'approche bibliographique, l'absorption intestinale des caroténoïdes est sensiblement améliorée par leur dissolution dans une phase huileuse et l'émulsification fine. Les particules choisies, les nanocapsules lipidiques (NCLs) ont fait l'objet de nombreuses recherches au laboratoire. Elles ont un diamètre moyen modulable pouvant aller de 20 à 100 nm et un cœur huileux liquide [1]. Ce type de structure semble donc avoir un bon potentiel pour améliorer l'absorption intestinale du  $\beta$ C. De plus, leur enveloppe pégylées peut avoir un impact sur la stabilité du  $\beta$ C pendant le stockage ou après inclusion dans une matrice complexe.

L'objectif de cette première partie du travail expérimental était donc de formuler des NCLs de grades alimentaires ayant la capacité d'encapsuler un composé lipophile tout en maintenant son intégrité chimique et donc ses propriétés anti-oxydantes. Nous avons ainsi cherché à caractériser d'un point de vue physicochimique les particules afin de comprendre les mécanismes mis en jeu lors du procédé d'encapsulation du  $\beta$ C. De plus, nous avons du adapter le procédé de formulation avec des excipients de grade alimentaire. Dans un premier

temps la fragilité du  $\beta$ C a été évaluée afin de déterminer quels paramètres ( $O_2$ , lumière, température, temps) devaient être prioritairement contrôlés lors de l'encapsulation. Ensuite, l'hydroxystéarate de polyéthylène glycol 15 a été remplacé par un stéarate de polyoxyéthylène (POE) de grade alimentaire : le stéarate de POE 40. La stabilité de la suspension a ensuite été évaluée *in vitro* durant le stockage et dans des milieux simulant les milieux gastro-intestinaux. Cette étape permettra d'évaluer sous quelle forme le  $\beta$ C encapsulé arrive au niveau de son site d'absorption intestinale

Ce travail est en soumission dans le *journal of controlled release*.

**Référence :**

[1] B. Heurtault, P. Saulnier, B. Pech, J.E. Proust, J.P. Benoit, A novel phase inversion-based process for the preparation of lipid nanocarriers. *Pharm. Res.* 19(6) (2002) 875-880.

# **Encapsulation of $\beta$ -carotene in lipid nanocapsules might enhance its bioaccessibility**

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**Abstract :**

Lipid Nanocapsules (LNCs) were formulated according to phase inversion method and loaded with  $\beta$ -carotene ( $\beta$ C). This method was then adapted with food grade additives and optimized to minimize  $\beta$ C loss due to oxidation during formulation. LNCs had an average diameter of  $22\pm 1$  nm and a slightly negative surface charge:  $\zeta$  potential of  $-7.6\pm 4.1$  mV.  $\beta$ C encapsulation efficiency was 85 %,  $\beta$ C concentration in suspension was  $8.5\pm 0.8$  ng/ml of suspension, LNCs suspension was concentrated by 2 by osmosis. The use of a food grade POE 40 stearate led to an increase of LNCs diameter during storage at  $4^{\circ}\text{C}$  or at room temperature after 60 days, without any significant loss of  $\beta$ C. This maturation effect could be explained by Ostwald ripening phenomenon. *In vitro* LNCs stability was studied in simulated gastro-intestinal digestion fluid mimicking post-prandial conditions. LNCs integrity was assessed by light scattering and turbidimetry. It appeared that LNCs were stable in gastric media. During intestinal digestion, LNCs distinctive size peak disappearance was combined with the detection of other structures. This did not occur with bile juice, pancreatic and duodenal enzymes separately. No influence of particles size, formulation process or loading was detected, whereas long chain polymer seemed to delay LNCs digestion. We might hypothesize that LNCs are broken down by bile salts dependant lipase included in pancreatic secretion. The appearance of large ( $> 500$ nm) and small ( $< 10$  nm) structures during intestinal incubation might correspond to aggregated or vesicular structures and mixed micelles respectively. These systems are formed during lipid digestions. LNCs lipidic core might be included in mixed micelles allowing  $\beta$ C absorption by enterocytes.

**Abbreviations :**

**ACN:** Acetonitrile; **Ag:** argon;  **$\beta$ C:**  $\beta$ -carotene; **BHT:** Butylated hydroxytoluene; **DJ:** duodenal juice; **GJ:** Simulated gastric juice; **GRAS:** Generally recognized as safe; **IF:** Simulated intestinal fluid; **LNC:** Lipid Nanocapsule; **MeOH:** Methanol; **PDI:** polydispersity index; **PEG:** Polyethylene glycol; **PIZ:** phase inversion zone; **POE:** Polyoxyethylene; **PIT:** Phase Inversion Temperature; **THF:** Tetrahydrofuran.

**Keywords:**  $\beta$ -Carotene; nanoencapsulation; *in vitro* digestion.

## Introduction

Carotenoids are organic pigments integrated in plants chromoplasts and some other photosynthetic organisms. They are known to have high free radical scavenging properties due to the abundance of conjugated double bonds [1, 2]. Carotenoids can protect membrane lipids from oxidation *in vitro* in foods emulsions [3], liposomes [4-6] in lipoproteins [7] or *in vivo* [8]. General assumption is that carotenoids have a positive impact on health [9, 10]. Many epidemiological studies demonstrated an inverse correlation between vitamin intake and cancer risk [11]. These health benefits can be due to cell differentiation, cell to cell communication or antioxidant properties. Yet, under certain circumstances, such as high pO<sub>2</sub>, cigarettes smoke or asbestos exposure, carotenoids can lose their ability to scavenge free radicals, become pro-oxidant and increase cancer risk [10, 12, 13]. βC constitutes the greatest source of vitamin A in human diet, but its βC bioavailability from food is variable and dependant of meal lipid content [14-16]. Thus, many attempts have been made to increase βC intake and bioavailability via supplementation, food enrichment. Bioavailability is the proportion of active component available for metabolism or storage. Bioaccessibility is the proportion of a micronutrient released from intestinal chyme and available for intestinal absorption. To be absorbed in enterocytes, βC should be dissolved in lipid fraction and included in mixed micelles [17]. Afterwards, it enters enterocytes via passive diffusion following triglycerides pathway or via specific hydrophobic transporters such as SR-B1 [18, 19].

Encapsulation may constitute a mean to preserve βC native properties against oxidation processes. Capsule wall may constitute a mechanical barrier against pro-oxidant elements penetration such as free-radicals, oxygen, metal ions [20]. Encapsulation might also represent a mean to improve biological efficiencies such as shelf life, bioavailability, bioaccessibility, prevent vitamins from complexation in gastrointestinal tract or a tool to control vitamins

release [21-24]. If the nanocarriers are not sensitive to digestion process, they could be absorbed in enterocytes or via paracellular pathways such as gap junctions. Otherwise, lipid fraction deriving from degraded carriers might follow regular absorption pathway [25].

Several techniques have been adapted for carotenoids encapsulation. Spray drying, largely used for food supplementation and nutraceuticals, leads to large particle size [24, 26-28]. Other encapsulation processes have been developed to produce smaller particles size, such as liposomes or nano-crystals, but require, in most cases the use of organic or inflammable solvent. The main advantage of these processes is their high payload [5, 23, 29]. However, the question of residual solvent must be raised for food applications in particular. Besides, as  $\beta$ C is included in the crystalline form, bioaccessibility might be lower.  $\beta$ C can also be included in hydrophobic cages such as cyclodextrins [30]. Despite their lower payload these carriers might remain of interest if they lead to a greater bioaccessibility or bioavailability. In several studies, lipophilic vitamins bioavailability was increased by their encapsulation in an emulsified oil phase [31-33]. Several formulation processes are based on  $\beta$ C dissolution in lipid fraction and emulsification. Droplets can be stabilized and form a stable emulsion or be surrounded by a rigid core to form particles. In most cases, when  $\beta$ C is incorporated dissolved in oil, formulated carriers have larger mean diameters [24, 28]. Simple or complex coacervation can be used to produce a solid shell around oil droplets. Coacervation is based on phase separation due to macromolecules desolvation [34-37]. Emulsification can occur directly in the gastrointestinal tract with self-nanoemulsified drug delivery systems [38]. As oil phase is already finely emulsified in intestinal tract,  $\beta$ C is more easily absorbed. Lipid nanoparticles might combine emulsions and nanodispersions assets. Lipid nanoparticles produced by Hentschel *et al.* are stable thanks to lipid solid state at room temperature [39]. However their formulation requires high pressure homogenization and high temperature which can constitute a drawback for  $\beta$ C stability. Emulsion based formulation improve

vitamin bioavailability mainly thanks to a finer molecule dispersion which improve its inclusion in bile salts micelles, thus increasing bioaccessibility [25]. Vitamin dissolution in carriers might also be an important point to consider for oral administration. Therefore, encapsulation of  $\beta$ C dissolved in an oil fraction, finely emulsified and stabilized might constitute the best compromise between processes presented above.

We chose to encapsulate  $\beta$ C dissolved in lipid nanocapsules (LNCs) which are constituted by a liquid lipid core surrounded by an interface constituted by soy lecithin and polyethylene glycol (PEG) stearate. Formulation process is based on the Phase-Inversion Temperature (PIT) method [40]. The technique allows the formulation of monodisperse particles, in a nanometre size range (20-100 nm) without use of organic solvent and with low mechanical energy. Peltier *et al* [41] previously showed that bioavailability of an anticancer drug (paclitaxel) was improved by its encapsulation in LNCs. In this paper, we adapted the PIT process to formulate food grade  $\beta$ C loaded LNCs able to encapsulate  $\beta$ C and maintain its chemical integrity. *In vitro* gastro-intestinal post-prandial conditions were simulated to evaluate  $\beta$ C bioaccessibility.

## **I. Materials and methods**

### *1. Materials*

HPLC grade solvents, methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher Scientific (Loughborough, UK); Chloroform ( $\text{CHCl}_3$ ) and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). All *trans*- $\beta$ C ( $\geq 97\%$  (UV), BioChemika), POE 40 stearate were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Butylated hydroxytoluene (BHT) and Dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), NaCl,  $\text{NaH}_2\text{PO}_4$ , KCl,  $\text{NaHCO}_3$ ,  $\text{MgCl}_2$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{CaCl}_2$  and HCl (37%) were obtained from VWR Prolabo (Fontenay-sous-Bois, France). PEG 15 Stearate (Solutol HS-15<sup>®</sup>) was purchased from BASF. Labrafac<sup>®</sup>, composed by caprylic and capric acids, was obtained from Gattefossé (Gattefossé,

S<sup>t</sup>-Priest, France), and Lipoid<sup>®</sup> 75 S-3 (soy lecithin) from Lipoid<sup>®</sup>, (Cham, Switzerland). De-ionized water produced by a Milli-Q plus R system (Millipore, Paris, France). Argon (Ag) was purchased from Air Liquid (Air Liquid). Pepsin from porcine gastric mucosa (3 200-4 500 U/mg), porcine bile extract, porcine pancreatin (8xUSP), lipase type II as well as mucin and glucuronic acid were purchased from Sigma-Aldrich (St Quentin Fallavier, France).

## *2. Limitation of $\beta$ C auto-oxidation*

$\beta$ C handling validation was assessed by comparison of <sup>1</sup>H and <sup>13</sup>C MNR  $\beta$ C spectra after 3 days exposure to normal daylight and temperature. As there were no significant differences between spectra, rapid manipulations such as weighing were done under subdued light and in presence of O<sub>2</sub>. Influence of O<sub>2</sub>, light and temperature on  $\beta$ C auto-oxidation was assessed with  $\beta$ C dissolved in Labrafac<sup>®</sup> at 0.18 ± 0.02 mg/ml. Briefly, 10 ml aliquots of this solution were incubated in an oil water-bath set at 60, 70 or 80°C, in presence or absence of O<sub>2</sub> and light. Darkness was achieved by wrapping cautiously glass vials with aluminum foil and O<sub>2</sub> depleted atmosphere was performed by saturation with Ag. Every 30 min, for 2h30 min, 200  $\mu$ l were taken out in triplicate for analysis. The dosage was performed spectrophotometrically (UV Spectrophotometer UV, Uvikon 922, Kontron Instruments, Montigny Le Bretonneux, France) at 460 nm, 200 $\mu$ l of oil were dissolved with 1.2 ml of CHCl<sub>3</sub>.

## *3. Formulation of pharmaceutical grade LNCs*

LNCs containing Solutol<sup>®</sup> HS 15, were formulated according to the protocol achieved by Heurtault *et al.* [40]. For  $\beta$ C loaded LNCs, oil phase was replaced by a solution of  $\beta$ C in Labrafac<sup>®</sup> at a concentration of 0.7 mg/ml. In order to produce LNCs having an average size of 20, 50 or 100 nm, excipients were weighed as outlined by table 1. Excipients were homogenized under magnetic stirring and PIT method was applied through several fast heating and cooling steps between temperatures T<sub>1</sub> and T<sub>2</sub> which are dependant of LNCs size



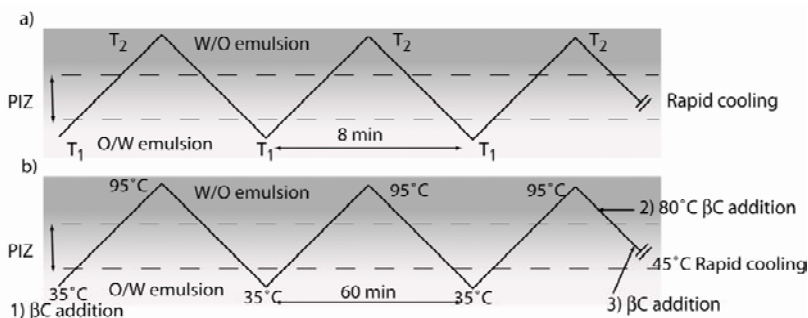
(8°C/min; table 1; figure 1.a)). LNCs were structured by the addition of 12.5ml of ultra-pure cold water (4°C) during the last cooling step. This step is called rapid cooling. Rapid cooling temperature is also dependant of LNCs size (table 1). Thus formulations lasted 25 min. This procedure was performed in the dark in order to prevent as much as possible  $\beta$ C degradation.

		20 nm	50 nm	100 nm
Excipients amounts	PLs	75 mg		
	NaCl	89 mg		
	oil	600 mg	1025 mg	1180 mg
	PEG 15 stearate	2097 mg	846 mg	486 mg
	Water	2110 mg	2975 mg	3159 mg
T1/T2/ rapid cooling temperature		60°C/90°C/75°C	60°C/95°C/80°C	60°C/98°C/84°C

**Table 1:** Excipients amounts and temperature parameters for each size of LNCs

4. Formulation process optimization for food grade carrier formulation

The process based on the PIT method described in section I.3) has been adapted by replacing PEG 15 stearate by a food grade POE 40 stearate (E 431 in the positive list of food ingredient). This required slow heating and cooling steps (2°C/min) between 35°C and 98°C to permit POE 40 stearate hydrophilic part complete dehydration and emulsion inversion. To decrease PIT and allow LNCs formation at 45 °C, salt concentration was increased in the aqueous phase from 5 % to 34 %. Therefore rapid cooling was performed at 45°C. The final proportions of water, POE 40 stearate and oil were 60/32/8 % (w/w) while phospholipids amount was unchanged in comparison to amounts shown in table 1. Several  $\beta$ C incorporation methods were studied. First, we incorporated  $\beta$ C dissolved in oil fraction [ $\beta$ C] = 0.7 mg/ml at the beginning of the cycles (Figure 1 b1)), second during the last cycle before the phase inversion 2) and third just before the rapid cooling 3). For methods 2 and 3, half of oil phase was incorporated without  $\beta$ C at the beginning of formulation and the other half was used to dissolve  $\beta$ C and was added to formulation as explained previously. All experiments were carried out under Ag enriched atmosphere and subdued light.



**Figure 1:** a) PEG 15 stearate LNCs formulation process; b) POE 40 stearate LNCs formulation

In order to decrease surfactant excess from suspension, filtration, centrifugation, dialysis, and sterical exclusion chromatography were compared. Filtration was efficient to remove most surfactant aggregates but led to a saturation of the pores which implied the use of several filter and a loss of 40% of suspension volume. Centrifugation removed most of surfactant aggregates without changing suspension volume; however it was not sufficient to purify the suspension as light scattering measurements revealed the presence of aggregates. The best results (volume reduction less than 10 % and absence of surfactant aggregates in final suspension) were obtained with the combination of two centrifugations (30 min, 50 000g, Floor standing high speed refrigerated centrifuge, KR22i, Jouan, Saint-Herblain, France) followed by filtration on 0.45  $\mu\text{m}$  and 0.2  $\mu\text{m}$  sterile filters successively (Millipore, St Quentin Fallavier France).  $\beta\text{C}$  loaded LNCs suspensions were then dialyzed for 3 H against ultra-pure water (cellulose ester, MWCO: 15 000, Eindhoven, The Netherlands). Afterwards, dialysis tubing was placed on dry PEG 35 000 (Sigma-Aldrich, St Quentin Fallavier, France) and concentrated by osmosis for 3H.

### 5. Particle characterization

LNCs average hydrodynamic diameter and PDI were determined by dynamic light scattering using a Malvern Autosizer 4700 (Malvern Instruments S.A., Worcestershire, UK) fitted with a 488 nm laser beam at a fixed angle of 90°. Measurements were taken at 25°C, with  $0.89 \times 10^{-2} \text{Pa.s}$  viscosity and a refractive index of 1.33. LNCs suspensions were diluted by

60 in ultra pure water to ensure convenient scatter intensity on detectors.  $\zeta$  potential measurements were carried out using a Zeta Sizer 2000 (Malvern Instruments, France) equipped with an AZ-4 cell. Size and  $\zeta$  potential measurements were performed in triplicate.

To measure  $\beta$ C loading in stock LNCs suspension, we used spectrophotometry at 460 nm in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$ . Briefly, LNCs were diluted by 8 in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  which allows LNCs disruption and dissolution of LNCs excipients. Standards were realized with unloaded LNCs and pure  $\beta$ C. An HPLC method was used to quantify precisely the concentration of intact  $\beta$ C during storage. Samples were taken out and stored under Ag at  $-20^\circ\text{C}$  until HPLC analysis (Waters Alliance® 2690 modular system composed by a Waters 600 controller pumps, a Waters 717 plus Autosampler injector and a W 2487; Waters S.A., Saint-Quentin en Yvelines, France). HPLC column was a Sunfire C18 (150 mm, 5  $\mu\text{m}$ , 4.6 mm, Waters S.A., Saint-Quentin en Yvelines, France) with a guard column (20 mm, 5  $\mu\text{m}$ , 4.6 mm, Waters). Isocratic elution of analytes was performed with a mobile phase composed by  $\text{MeOH}/\text{THF}/\text{ACN}$  (72/8/20, %vol) at a flow rate of 2 ml/min,  $\beta$ C retention time was 12min. Samples were prepared by the addition of 3.4 ml of  $\text{MeOH}/\text{THF}/\text{ACN}$  (50/10/40 with 0.1% of BHT, v/v) to 600  $\mu\text{l}$  LNCs samples under subdued light, filtered on 0.2  $\mu\text{m}$  filters and placed in amber glass vials.  $\beta$ C stock solution (1 mg/ml) was prepared weekly in THF and stocked under Ag at  $-80^\circ\text{C}$ . Calibrating standard solutions were prepared freshly before each analysis with unloaded LNCs and pure  $\beta$ C.

6. Evaluation of  $\beta$ C bioaccessibility *in vitro*

	Gastric Juice (GJ)	Duodenal Juice (DJ)	Bile Juice (BJ)
Inorganic solution	<ul style="list-style-type: none"> <li>- [NaCl]= 5.5 g/l</li> <li>- [NaH<sub>2</sub>PO<sub>4</sub>]= 0.5 g/l</li> <li>- [KCl]= 1.6 g/l</li> <li>- [CaCl<sub>2</sub>]= 0.8 g/l</li> <li>- [NH<sub>4</sub>Cl]= 0.6 g/l</li> <li>- pH adjusted to 1.2±0.05</li> </ul>	<ul style="list-style-type: none"> <li>- [NaCl]= 14 g/l</li> <li>- [NaHCO<sub>3</sub>]= 6.8 g/l</li> <li>- [KH<sub>2</sub>PO<sub>4</sub>]= 0.16 g/l</li> <li>- [KCl]= 1.13 g/l</li> <li>- [MgCl<sub>2</sub>]= 0.1 g/l</li> </ul>	<ul style="list-style-type: none"> <li>- [NaCl]= 10.5 g/l</li> <li>- [NaHCO<sub>3</sub>]= 11.6 g/l</li> <li>- [KCl]= 0.76 g/l</li> </ul>
Organic solution	<ul style="list-style-type: none"> <li>- [glucose]= 1.3 g/l</li> <li>- 1.2 ml glucuronic acid]= 0.04 g/l</li> <li>- [urea]= 0.17 g/l</li> <li>- [glucosamin hydrochlorid]= 0.66 g/l</li> </ul>	<ul style="list-style-type: none"> <li>- [urea]= 0.2 g/l</li> </ul>	<ul style="list-style-type: none"> <li>- [urea]= 0.5 g/l</li> </ul>
Added when inorganic and organic solution where mixed (50/50; v/v)	<ul style="list-style-type: none"> <li>- [BSA]= 1 g/l</li> <li>- [Mucin]= 3 g/l</li> <li>- [Pepsin]= 2.5 g/l</li> <li>pH adjusted to 2.00±0.05</li> </ul>	<ul style="list-style-type: none"> <li>- [BSA]= 1g/l</li> <li>- [Pancreatin]= 9 g/l</li> <li>- [Lipase]= 1.5 g/l</li> <li>- [CaCl<sub>2</sub>]= 0.2 g/l</li> <li>pH adjusted to 8.10±0.05</li> </ul>	<ul style="list-style-type: none"> <li>- [BSA]= 1.8 g/l</li> <li>- [Bile]= 30 g/l</li> <li>- [CaCl<sub>2</sub>]= 0.2 g/l</li> <li>pH adjusted to 8.20 ±0.05</li> </ul>

**Table 2:** Gastro-intestinal media composition used for *in vitro* digestion representing fed conditions as described by Versantvoort *et al.* [42]

LNCs stability in conditions simulating post-prandial gastro-intestinal media was evaluated by an *in vitro* digestion model. PEG 15 stearate LNCs of 20, 50 and 100 nm were compared with POE 40 stearate LNCs. Impact of particles size,  $\beta$ C loading and polymer size could be evaluated. Gastro-intestinal fluids were prepared according to Versantvoort *et al.* [42]. Digestive fluids composition is described in table 2. 700  $\mu$ l of LNCs were incubated with 1.68 ml of simulated gastric juice (GJ) during 2 H at 37°C in a shaking water-bath set at 95 rpm. Afterwards, intestinal digestion was simulated by the addition of 280  $\mu$ l of NaHCO<sub>3</sub> (84.7 g/l), 1.68 ml of duodenal juice (DJ) and 0.84 ml of bile juice (BJ) and a 2H incubation under the same conditions. Each medium free of LNCs, was controlled by light scattering in similar conditions. LNCs were also incubated with gastrointestinal juice depleted of enzymes. LNCs stability has also been followed over time in digestion media. In order to avoid influence of volume variation due to sampling, the volume of digestion media was increased to 150 mL. 1ml of digesta was taken out every 30 min during 2H. LNCs integrity was evaluated by dynamic light scattering (size measurement). Briefly, digesta was centrifuged (3000 g, 15min, 4°C) and filtered on 0.2 $\mu$ m filters and dissolved by 15 with ultra pure water (Milli-Q systems, Millipore, St Quentin Fallavier France) before size measurement. LNCs

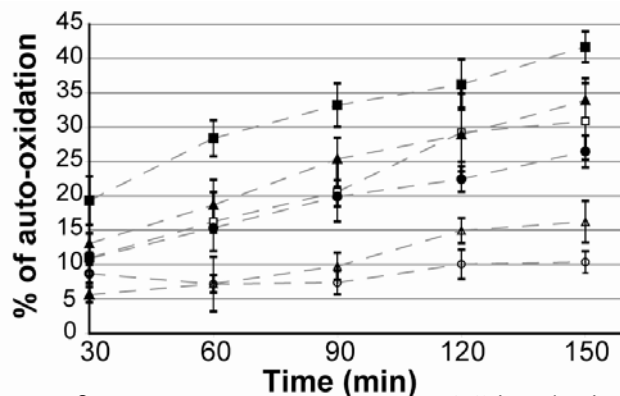
digestion was followed over time by turbidimetry. This experiment was done with 100 nm PEG 15 stearate LNCs as this LNCs suspension displayed the greatest turbidity. Light transmission measurements were performed with Turbiscan MA 2000 (Formulation, L'Union, France) to investigate LNCs suspension stability. The Turbiscan MA 2000 reading head acquires transmission and backscattering data every 40  $\mu\text{m}$  while moving up and down along the cell. Briefly LNCs and digestion media were incubated at 37°C in the Turbiscan MA 2000, mean light transmission through the digestion media was calculated over time.

### 7. Statistic analysis

Significant differences were plotted by an ANOVA followed if necessary by a Student-Newman-Keuls T test ( $p < 0.05$ ). These calculations were performed using the software PC primer.

## II. Results and discussion

### 1. Determination of appropriate conditions for $\beta\text{C}$ formulation



**Figure 2:**  $\beta\text{C}$  degradation over time;  $\beta\text{C}$  incubation at 80°C in presence of  $\text{O}_2$  and light,  $\blacksquare$ ;  $\square$  at 80°C in absence of  $\text{O}_2$  and light,  $\blacktriangle$  at 70°C in presence of  $\text{O}_2$  and light;  $\triangle$  at 70°C in absence of  $\text{O}_2$  and light;  $\bullet$  at 60°C in absence of  $\text{O}_2$  and light and  $\circ$  at 60°C in absence of  $\text{O}_2$  and light

To evaluate the potential degradation of  $\beta\text{C}$  during formulation,  $\beta\text{C}$  auto-oxidation was followed over time. We evaluated the effect of most influent factors (temperature, atmosphere and light) on  $\beta\text{C}$  degradation during a period corresponding approximately to the duration of a POE 40 stearate LNCs formulation. For all incubation conditions,  $\beta\text{C}$  auto-

oxidation increased significantly over time (figure 2).  $\beta$ C degradation was temperature dependant since at the end of the experiment, in presence of oxygen and light,  $\beta$ C auto-oxidation was 42%, 32% and 27% for 80°C, 70°C and 60°C. As O<sub>2</sub> and light could induce free radical species formation,  $\beta$ C degradation under these experimental conditions did not reach a plateau. At a given temperature, Ag and darkness reduced significantly  $\beta$ C degradation. It was clearly proved at 60 and 70°C ( $p < 0.05$ ). Therefore, manipulating in the dark and under argon should protect  $\beta$ C against auto-oxidation. Indeed, under these conditions, auto-oxidation process significantly slowed down and almost reached a plateau. For higher temperatures, 80°C, Ag and darkness decrease  $\beta$ C degradation but not significantly; temperature might constitute the major factor influencing  $\beta$ C auto-oxidation even for short incubation times. Time and temperature influenced the most  $\beta$ C stability. Besides, during the first 30 min of incubation no significant differences have been detected even at 80°C with light and oxygen. Therefore, we chose to control the couple time/temperature exposure first during POE 40 stearate  $\beta$ C loaded LNCs. As one temperature cycle last 60 min we chose to incorporate  $\beta$ C during the last cycle according to method 2) and 3) (figure 1.b)). Influence of light and O<sub>2</sub> was minimized by manipulation under Ag and subdued light.

## *2. $\beta$ C PEG 15 stearate LNCs*

$\beta$ C loaded PEG 15 stearate LNCs had mean diameters of 18.4±1.3 nm (peak width 5.4±0.5 nm; table 3), 48.9±1.8 nm (peak width 9.9±0.3nm) and 91 ± 10 nm (peak width 27±2 nm). All suspensions were monodisperse as PDI were 0.08±0.03, 0.038±0.015 and 0.06 ± 0.02 respectively. Particles were all slightly negative as shown by  $\zeta$  potential values (table 3). These results are in accordance with previously published data on unloaded LNCs PEG 15 LNCs [20, 43]. To determine an influence of particle size on  $\zeta$  potential, they should be

measured after dialysis to avoid any influence of free PEG 15 stearate [20].  $\beta$ C concentration in LNCs suspensions was  $0.024 \pm 0.001$  mg  $\beta$ C /ml of suspension,  $0.037 \pm 0.002$  and  $0.035 \pm 0.003$  mg/ml for 20, 50 and 100 nm LNCs in that order (table 3). These concentrations are in accordance with initial amount of  $\beta$ C incorporated in each formulation and with their temperature/ time exposure (table 1). Encapsulation efficiency as calculated includes  $\beta$ C loss during formulation due to its non encapsulation, its degradation during formulation or during filtration. Encapsulation efficiency was evaluated at  $97 \pm 2\%$ ,  $87 \pm 6\%$  and  $95 \pm 6\%$  for 20, 50 and 100 nm LNCs respectively.  $\beta$ C degradation during formulation is very limited and in accordance with  $\beta$ C degradation kinetics shown in figure 1.

	size (nm)	PDI	$\zeta$ potential (mV)	[ $\beta$ C] $\mu$ g/ml suspension	of encapsulation efficiency
LNCs 20 nm	18,4 $\pm$ 1,3	0,08 $\pm$ 0,3	-5,7 $\pm$ 1,3	0,024 $\pm$ 0,001	97 $\pm$ 2 %
LNCs 50 nm	48,9 $\pm$ 1,8	0,038 $\pm$ 0,015	-5 $\pm$ 1,5	0,037 $\pm$ 0,002	87 $\pm$ 6%
LNCs 100 nm	91 $\pm$ 10	0,06 $\pm$ 0,02	-8,2 $\pm$ 1,8	0,035 $\pm$ 0,003	95 $\pm$ 6%

**Table 3: 20 nm, 50 nm and 100 nm PEG 15 stearate LNCs characteristics**

Before encapsulation,  $\beta$ C was dissolved in the oil phase and no precipitation was observed during storage, thus  $\beta$ C might be located in LNCs lipid core. This hypothesis can be strengthened by previous study with paclitaxel which was incorporated in oil phase before formulation and located in LNCs core [41]. Besides, if release takes place a precipitation can be observed. According to previous studies, we confirmed that PEG 15 stearate LNCs were stable over time [40].

### 3. Adaptation to food grade standards:

$\beta$ C incorporation method 2) and 3) were in accordance with  $\beta$ C degradation kinetics. Under these conditions,  $\beta$ C is exposed to temperatures below  $80^\circ\text{C}$  for less than 30 min. Following results concern only these formulation processes. Method 3) did not lead to LNCs formation whereas method 2) lead to the formation of monodisperse LNCs formulation.  $\beta$ C loaded LNCs, formulated with food grade POE 40 stearate, had an average diameter of 22

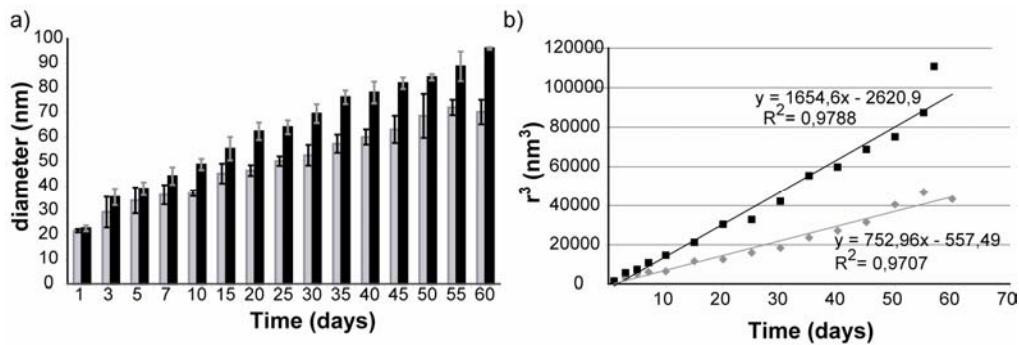
nm  $\pm$  1 nm (n=14) after dialysis. Particles suspension are monodisperse as shown by the low PDI  $0.15 \pm 0.005$  (n=14). Particles had slightly negatives  $\zeta$  potential  $-7.6 \pm 4.1$  mV (n=14).  $\beta$ C concentration in a suspension after concentration process was  $13.2 \pm 3.3$  ng/ml and loading efficiency was 85 % (n=14). Because of oil fraction reduction and the addition of oil fraction in two steps, POE 40 stearate LNCs final suspension concentration was between 2 to 3 times lower than for PEG 15 stearate LNCs. However, this concentration is sufficient for in vivo studies planned. Before encapsulation,  $\beta$ C was dissolved in the oil phase and no precipitation was observed during storage, thus  $\beta$ C might be located in LNCs lipid core. This hypothesis can be corroborated by previous study in which Nile red was incorporated in oil phase during formulation and located in LNCs core [44]. This technique led to the formation of monodisperse particles suspension characterized by  $\beta$ C dissolution in the lipid core of polymer shell particles.

#### *4. $\beta$ c loaded LNCs stability over time*

Contrary to PEG 15 stearate LNCs, POE 40 stearate particles mean diameter increased over time when stored at 4°C or at room temperature (figure 3 a). After 60 days, particles mean diameter increased by a factor 3 or 4 when stored at 4°C or room temperature respectively. As for typical dispersed systems (emulsion, nanoemulsion) particles diameters increase can be due to two types of phenomenon Ostwald ripening or coalescence. Even though, Ostwald ripening is usually considered as that the major destabilization process for nanoemulsion [45]. It has been shown that coalescence could also be responsible for emulsion droplets size increase [46]. According to Lifshitz-Slyozov-Wagner theory developed for emulsion droplets, Ostwald ripening can be quantitatively assessed by plotting cubic particles radius against time [47, 48]. A good linear correlation can be found between cubic particles radius plotted versus time at 4°C and room temperature (figure 3.b)). Thus, Ostwald ripening



might be the main destabilization process occurring during storage. Linear slope equation corresponds to the rate of ripening  $\omega$  ( $\omega = \frac{dr^3}{dt} = \frac{8}{9} \left[ \frac{C(\infty)\gamma V_m D}{\rho RT} \right]$ ) [45].  $\omega$  is dependant of diffusion coefficient of the dispersed phase in water (D); dispersed phase density  $\rho$ , interfacial tension  $\gamma$ , molar volume of the dispersed phase  $V_m$ , temperature in Kelvin T and oil bulk phase solubility  $C(\infty)$ . These parameters are temperature dependant. Indeed density is usually lower for higher temperature as well as interfacial tension or solubility. On the contrary molar volume increases with temperature. In our case, the increase of temperature results in an increase of ripening rate (figure 3.b)). This phenomenon can be facilitated by the remnant POE 40 stearate micelles which can serve as lipid carrier from one LNC to the other and facilitate maturation process [49, 50]. To limit Oswald ripening one option is to mix oil used with less water soluble oil. Another possibility is to decrease interfacial tension at O/W interface or dilute particles suspension to eliminate micelles. This last option is not possible if encapsulated component is required with a high concentration. Besides,  $\beta$ C is known to have fluidizing properties [51] and decreases order in phospholipids membranes [52].  $\beta$ C loading and POE 40 stearate might affect interfacial properties. Hydrophilic chains length of POE 40 stearate might render LNCs shell less rigid and thus more permeable and thus favor Oswald ripening that is not the case for  $\beta$ C PEG 15 stearate LNCs.



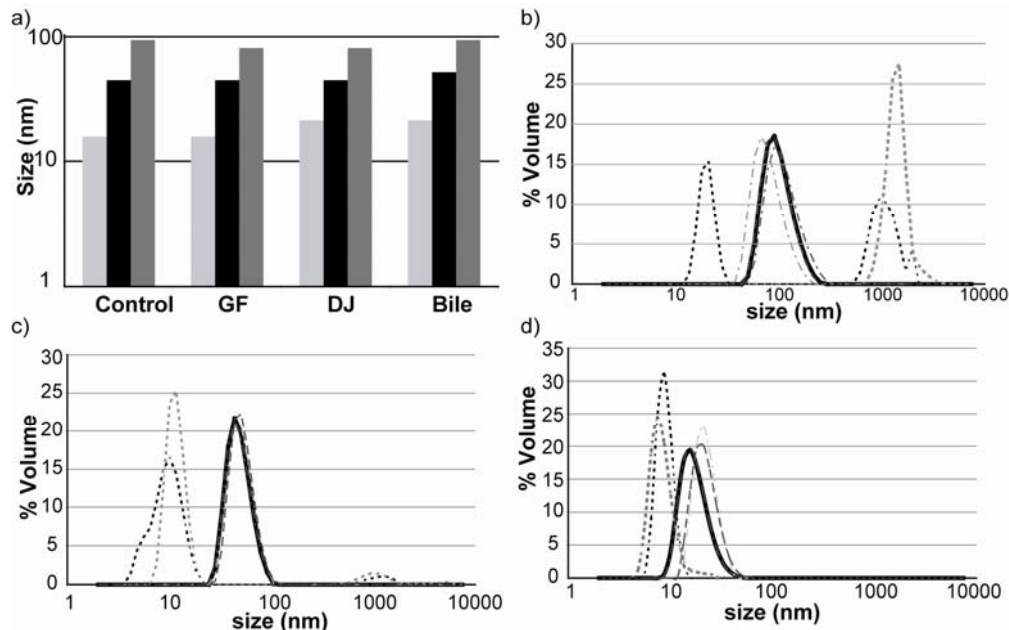
**Figure 3:**  $\beta$ C loaded LNCs size stability over time: a)  $\blacksquare$  at 4°C and  $\blacksquare$  at room temperature; b) cubic radius plotted against time:  $\blacklozenge$  4°C and  $\blacksquare$  room temperature.

$\beta$ C concentration in suspension was followed during POE 40 stearate LNCs storage. After two months, no significant degradation was detected at 4°C or room temperature. Light or oxygen did not have an influence on  $\beta$ C stability in LNCs. No precipitation of  $\beta$ C was observed. Thus POE 40 stearate LNCs are able to protect  $\beta$ C against oxidation even if their structures evolve over time.

#### *5. LNCs stability in simulated gastric and intestinal fluids*

As shown by typical size distributions, LNCs distinctive size peak was present after incubation (2 hours) in control media, GF, BJ and DJ for all types of LNCs (figure 4.b, c, d); 5.b, c)). This is also clear on histograms (figure 4.a) and 5.a)) that LNCs mean diameter is unchanged under this incubation conditions. We can then hypothesize that LNCs are stable against dilution, pH variation at 37°C and in GF followed by a digestion with BJ or DJ alone. These results are in accordance with previously published datas [53]. LNCs stability in stomachal juice is a critical point for nutritional of pharmaceutical purposes: indeed, the latest the component is released from the carrier the smallest chance it has to be degraded or complexed by chime components.

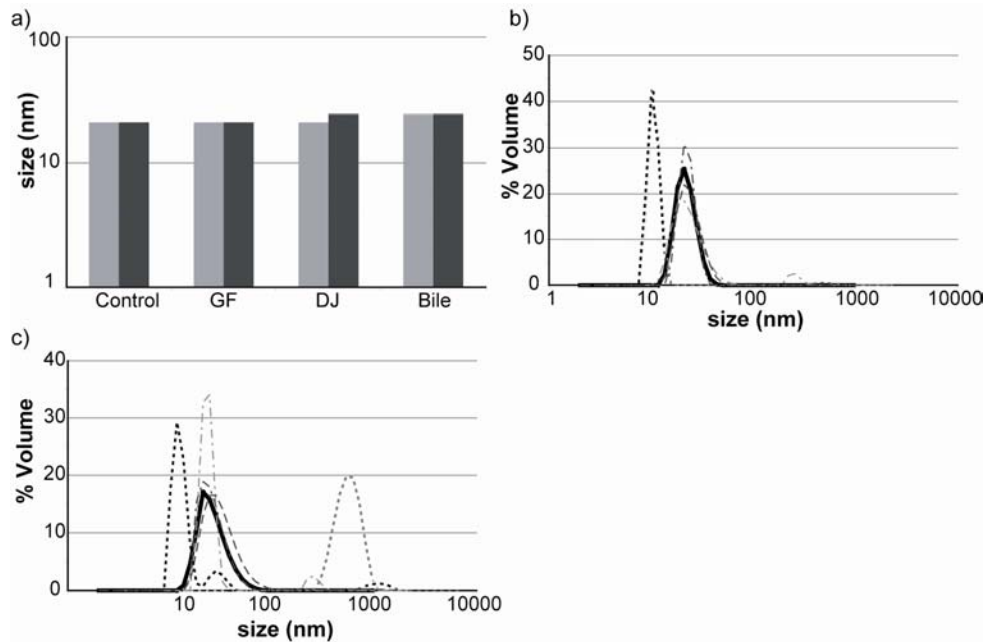
After incubation in complete intestinal fluid (IF), LNCs distinctive size peak was not distinguishable and smaller peaks appeared for PEG 15 stearate LNCs and unloaded POE 40 stearate LNCs (figure 4.a, b, c) and 5.b, c)). These peaks are similar to ones measured with IF without LNCs (data not shown), they might correspond to uni or multilamellar structures of bile components such as bile acids or lecithin [25]. Besides, for all LNCs, large diameter particles can be identified with different percentages in volume. The disappearance of LNCs distinctive size peak combined with the detection of small particles (as shown by size peak around 10 nm) or large particles (as shown by size peak above 500 nm) is the sign of LNCs destabilization in IF regardless of their size, polymer or loading. LNCs seemed destabilized by the combination of pancreatic enzymes and bile acids.



**Figure 4: Size distribution of PEG 15 Stearate LNCs suspensions after digestion, a)** mean size measured by light scattering for 20 nm LNCs (light gray), 50 nm LNCs (black) and 100 nm LNCs (dark gray); typical granulogram of b) 100 nm 15 PEG stearate LNCs c) 50 nm 15 PEG LNCs; — Control; - - GF; ··· DJ; - · Bile; ····· Complete digestion.

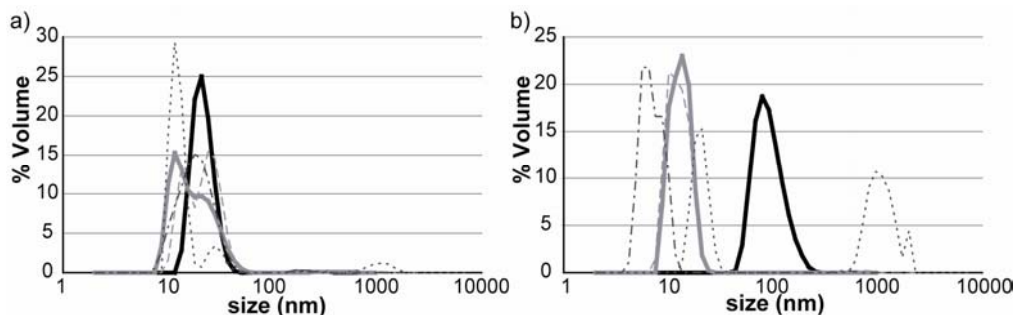
To compare destabilization process of the different LNCs, 100 nm PEG 15 stearate LNCs and  $\beta$ C loaded POE 40 stearate LNCs digestion was followed kinetically (figure 6). During POE 40 LNCs digestion, small diameter structures appeared after 30 min of incubation (figure 6.a). Particles with diameters corresponding to LNCs were also distinguished but size distribution was affected. After 1H or 1H30 of incubation, small particles and LNCs sized particles were spotted. After 2 H small particles and a small proportion of large particles were distinguished whereas LNCs distinctive size peak was no more present. For 100 nm PEG 15 stearate LNCs (figure 6. b), size peak corresponding to LNCs was no more visible after 30 min and particles of small diameters could be identified. This suggests that LNCs might already be destabilized by the combination of intestinal enzyme and biliary acids. After 1H of incubation, small particles (<10 nm) could be identified with a small proportion of large diameter particles. At  $t=1H30$ , only large particles could be identified. And after 2H, small and large particles were identified. It seems that after 30min of incubation, PEG 15 stearate LNCs were almost totally

digested whereas POE 40 stearate LNCs could be identified during 1H30. This might be the sign of a greater stability of POE 40 stearate LNCs.



**Figure 5:** a) mean size measured by light scattering for unloaded POE 40 stearate LNCs in , and  $\beta$ C POE 40 stearate LNCs in ; **Size distribution of LNCs suspensions after digestion**, b) POE 40 stearate LNCs c)  $\beta$ C POE 40 stearate LNCs; **—** Control; **— —** GJ; **— ·** DJ; **— ·** BJ; **····** IF, **····** complete digestion;

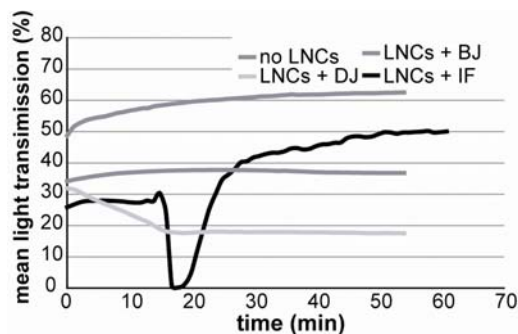
A turbidity loss of the media has been noticed during LNCs incubation in IF. We hypothesized that this clearance was correlated with LNCs destabilization. In order to precise this kinetic phenomenon, 100 nm PEG 15 stearate LNCs incubation in IF has been followed with time by light transmission. We chose these particles because their native suspension displayed the greatest turbidity; therefore changes in light transmission could be more easily identified. As shown by (figure 7) under destabilization conditions, light transmission in the media was very different from controls. Indeed, after 15 min of incubation, we observed a rapid increase of turbidity immediately followed by a great increase of light transmission. The trend observed is specific to LNCs + IF media and occurs during the first 30 min which can be correlated to the digestion process.



**Figure 6:** Kinetic destabilisation of a)  $\beta$ C POE 40 stearate LNCs and b) 100 nm PEG 15 stearate; — 0 min; — 30 min, — 1H ; — ■ 1H30 and — ■■ 2H .

Kinetics measurements (**figure 6**) indicate that POE 40 stearate LNCs seemed to be more resistant than 100 nm PEG 15 stearate LNCs, as they can be distinguished for incubation longer times. This difference might be attributed to higher polymer length which can decrease phospholipids and lipids accessibility to bile salt and lipase and therefore postpone POE 40 stearate LNCs digestion. We could compare this behavior to complement protein activation decrease occurring with particles grafted with longer PEG stearate [54]. The protein accessibility can be related to possible conformation of long chain polymer (brush and mushroom). Considering the particle size that appear in IF media during incubation and experimental media composition [55], we can hypothesize that, *in vitro*, digestion process lead to the recombination of particles and media constituents which could lead to the formation of supra-molecular assemblies made of bile salts, lipolysis product and lecithin, similar to some extent to mixed micelles formed *in vivo* during digestion. This result may have some consequences *in vivo*. For instance, mixed micelles allow triglycerides dissolution in intestinal media and their dissociation is followed by absorption in enterocytes. We can hypothesize if LNCs are disrupted, that their lipid core might be included in mixed micelles as all dietary lipids. Larger particles might be assemblies of smaller structures as media have all been centrifuged and filtered prior size measurements. Similarly, it as been shown that during intestinal digestion of SNEDDS, large vesicles ( $>100\mu\text{m}$ ) were formed during the first steps of digestion process [25]. These vesicles were not stable over time, and their sizes decreased

during digestion process to finally form mixed micelles. In our study, we can hypothesize that large particles detected correspond to aggregated structures formed during digestion. The precise characterization of the objects remains difficult since their formation implies complex dynamic processes



**Figure 7: Intestinal 100 nm PEG 15 stearate LNCs destabilisation followed by light transmission.**

### III. Concluding remarks

In conclusion, LNCs were stable in GF whatever their size, the polymer type, the formulation process or loading. LNCs stability in stomachal juice is a critical point: indeed the latest the component is released from the carrier, the smallest chance it has to be degraded or complexed by chime components. All types of LNCs studied seemed degraded by complete intestinal digestion. The combination of pancreatic enzyme and lipase with bile acids was necessary to disrupt their structure. It can be hypothesized that pancreatic lipase required surface active bile salts to reach its hydrolysis site. Bile salt dependant lipase might also play a role in LNCs digestion [56]. Indeed, this enzyme, secreted by pancreas and therefore included in pancreatin, is activated by bile salt. LNCs type affected digestion kinetics.  $\beta$ C resulting from LNCs digestion might follow an absorption pathway similar to dietary  $\beta$ C and be included in mixed micelles. Inclusion in mixed micelles could be improved thanks  $\beta$ C/oil fine dispersion in LNCs suspension which is a critical point for absorption [31, 38]. Encapsulation might protect  $\beta$ C until its inclusion in mixed micelles by avoiding its entrapment in insoluble fibers if ingested after a meal or degradation and enhance  $\beta$ C

bioaccessibility. Overall increasing  $\beta$ C bioaccessibility might increase as well its bioavailability. This mechanism could also explain the rise of paclitaxel bioavailability by encapsulation in LNCs in a recent study [41].

This study allowed the design of food-grade LNCs with POE 40 stearate encapsulating stable  $\beta$ C. These particles are characterized by a monodisperse suspension. Ostwald ripening can explain mean diameter increase during storage. It points out the influence of the shell composition on the *in vitro* stability of LNCs. Further investigations are needed to determine whether  $\beta$ C loading, POE stearate or formulation process is responsible of this behavior.

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## **CHAPITRE II :**

### **CARACTERISATION DES PROPRIETES INTERFACIALES DES NCLs CHARGEES EN $\beta$ -CAROTENE.**

## **Caractérisation des propriétés interfaciales des NCLs chargées en $\beta$ -Carotène**

Les NCLs de stéarate de POE 40 ont une structure qui permet de stabiliser le  $\beta$ C en milieu aqueux. Cependant cette structure évolue selon le modèle du murissement d'Ostwald, alors que les NCLs de PEG 15 stéarate ont un diamètre stable dans le temps. L'altération de la structure des NCLs n'a pas d'impact sur la stabilisation chimique du  $\beta$ C. Dans ce chapitre nous avons cherché à déterminer quel(s) paramètre(s) pouvait(aient) expliquer la différence de stabilité entre ces deux types de particules.

Les propriétés interfaciales des particules ont un impact sur leur stabilité au cours du temps. La tension de surface, la nature et l'intensité des interactions entre les molécules constitutives de l'interface, ainsi que la rigidité de l'interface influent sur le mûrissement d'Ostwald. De plus, la charge de surface des particules peut influencer de manière notable la stabilité d'une suspension colloïdale. Dans cette partie, l'objectif consiste à caractériser l'interface des NCLs de POE 40 et à comprendre le rôle de chaque excipient dans la stabilisation des NCLs. Dans un premier temps, l'interface des NCLs sera évaluée d'un point de vue rhéologique par la méthode de la goutte montante. Les interactions entre les molécules constitutives de la coque des NCLs seront étudiées à l'équilibre et après compression du système. Dans un deuxième temps, les propriétés électrophorétiques des NCLs de stéarate de POE 40 seront comparées à celles des NCLs d'hydroxystéarate de PEG 15 au cours du temps.

**Interfacial organization of beta-carotene loaded lipid nanocapsules by 2D  
rheological measurements and electrophoretic mobility.**

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**Abstract:**

Lipid nanocapsules (LNCs) were developed according to phase inversion temperature (TIP) process with polyoxyethylene 40 stearate (POE 40 stearate) in order to encapsulate lipophilic pro-vitamin ( $\beta$ -carotene ( $\beta$ C)). The purpose of this study is to understand the role of each components in interfacial stabilisation of LNCs and the localization of encapsulated molecule in LNCs. Adsorption kinetics of POE 40 stearate, phospholipids and  $\beta$ C at oil/water interface and the rheological properties of resulting interfacial film were studied using a drop tensiometer. Observed interfacial behaviour was interpreted using a model corresponding to a two-dimensional viscoelastic solid. Interfacial layers were characterised first by equilibrium tension and then, we calculated the rheological parameters (storage and loss dilational moduli) for each systems. It results that the encapsulated molecule ( $\beta$ C) strongly adsorbed at oil/water interface and exerted lateral interactions with both lipid (a mixture of phospholipids) and the macromolecular surfactant (POE 40 stearate). The stabilisation of the LNC by phospholipids and POE 40 stearate was not affected by the presence of  $\beta$ C in the interfacial layer. Since electrophoretic mobility of LNCs did not evolve during storage it was concluded that the shell of capsules and its electrical characteristics remained intact with time.

**Keywords:** lipidic nanocapsules, interfacial rheology, soft particles electrophoresis.

**Abbreviations:**  $\beta$ C: beta carotene; CMC: critical micellar concentration; DPPC: dipalmitoyl phosphatidylcholine; PDI: polydispersity index PEG 15 stearate: polyethylene glycol 660 12-hydroxystearate; POE: polyoxyethylene.

## I. Introduction

Carotenoids are organic pigments known to have high free radical scavenging properties [1]. This feature is due to their multiple conjugated double bonds. It has been largely demonstrated that carotenoids could protect fragile molecule from oxidation *in vitro* in food emulsions [2], in liposomes [3-5], or lipoproteins [6]. General assumption is that carotenoids and vitamins have a positive impact on health [7-9].  $\beta$ -Carotene ( $\beta$ C) is the most abundant carotenoid in human diet [10]. It can also be efficient to prevent iron-induced oxidative stress *in vivo* [11]. Thus many studies have been made to increase carotenoids and vitamins plasmatic concentration. However, an increase of their dietary intake is not always correlated with an increase of plasmatic concentration [12] due to a low intestinal absorption [13-15]. Encapsulation is a mean to increase their dietary intake and intestinal absorption [16-19]. In this study, we focused on  $\beta$ C encapsulation.

We chose to encapsulate  $\beta$ C in lipid nanocapsules (LNCs) composed of a liquid lipid core surrounded by a thick layer of soy lecithin and polyethylene glycol (PEG) stearate used as surfactants. Encapsulation process is based on the Phase-Inversion Temperature (PIT) method [20]. The technique originality lies on the absence of organic solvent and the low energy needs. Two types of particles produced by this method can encapsulate  $\beta$ C. The first type of particles uses Solutol<sup>®</sup> HS 15 (PEG 15 stearate) as hydrophilic surfactant. These particles are known to be stable during storage [21, 22]. The second type of LNCs uses polyoxyethylene glycol 40 stearate (POE 40 stearate) as hydrophilic surfactant [22]. During storage, mean diameter of these particles increases in accordance with Lifshitz-Slyozov-Wagner theory [23, 24].

Ostwald ripening is tightly related to emulsion droplets interfacial properties. Indeed, Ostwald ripening rate is dependant, among other parameters, of interfacial tension [23-25].

Interfacial tension and consequently Ostwald ripening is dependant of surfactant interfacial properties and concentration. Besides, lately it has been shown that interfacial elasticity of emulsions might have an impact on emulsions stability [26]. A low interfacial elasticity seemed to lead to an instable system whereas high interfacial elasticity seemed to stabilise the systems [27, 28].

Few papers described the behaviour of  $\beta$ C at oil/water interface [29], even if the behaviour of this antioxidant molecules was well described at air/water interface [30-32]. In liposomes,  $\beta$ C is located in membrane hydrophobic core, but it can be oriented parallel to the bilayer plan when it was composed by dioleoyl PC and perpendicular to bilayer plan when it was composed by soybean lecithin [33]. Even though  $\beta$ C remains in membrane hydrophobic core, it increases bilayer fluidity [34, 35]. In emulsion,  $\beta$ C is distributed essentially in droplets core because it is highly hydrophobic [36].

Therefore, the difference of stability of the two types of particles described above can be linked to interfacial POE 40 stearate properties. POE 40 stearate might affect interfacial properties of  $\beta$ C and phospholipids and their interactions at oil/water interface. Thus, our aim is to characterize more closely interaction between  $\beta$ C, POE 40 stearate and phospholipids by 2D interfacial rheology [37, 38] and by electrophoretic mobility evolution during storage.

## II. Materials and Methods

### II.1. Materials

All *trans*- $\beta$ C ( $\geq 97\%$  (UV), BioChemika), POE 40 stearate were purchased from Sigma-Aldrich (St Quentin Fallavier, France). PEG 15 Stearate (polyethylene glycol 660 12-hydroxystearate or Solutol HS-15<sup>®</sup>) was purchased from BASF. Labrafac<sup>®</sup>, composed by caprylic and capric acids, was obtained from Gattefossé (Gattefossé, S<sup>t</sup>-Priest, France), and



Lipoid<sup>®</sup> 75 S-3 (soy lecithin) from Lipoid<sup>®</sup>, (Cham, Switzerland). De-ionized water produced by a Milli-Q plus R system (Millipore, Paris, France). NaCl was from VWR Prolabo (Fontenay-sous-Bois, France). HPLC grade solvents, methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher Scientific (Loughborough, UK).

## II.2. Interfacial properties

### *a. Preparation of solutions*

Lipoid<sup>®</sup> was dispersed in labrafac<sup>®</sup> at 80°C at a concentration of  $1.10^{-3}$  M and slowly cooled down to room temperature. To avoid the occurrence of aggregates due to recrystallization, the solution was centrifuged at 3000g for 30 min.  $\beta$ C was dissolved in pure labrafac<sup>®</sup> or lipoid<sup>®</sup> /labrafac<sup>®</sup> solution at a concentration of 0.5mM, which corresponds to mean  $\beta$ C concentration in POE 40 stearate LNCs oil phase. A stock solution of POE 40 stearate (Sigma Aldrich, St Quentin Fallavier, France) was made at  $1.10^{-2}$ M.

### *b. Drop tensiometer*

The adsorption and rheological measurements were obtained at oil/water interface using a drop tensiometer (Tracker, ITConcept, Longesaingne, France). The oil phase was labrafac<sup>®</sup> (density ;  $d=0.945$ ) aqueous phase was ultra pure water ( $d=0.999$ ). A rising drop of 7 $\mu$ l of labrafac<sup>®</sup> was formed using an Exmire microsyringe (Prolabo, Paris, France) into an optical glass bowl filled with aqueous phase. The axial symmetric shape (Laplacian profile) of the drop was analyzed using a video camera connected to a microcomputer. From the analysis of numerical image with Laplace equation integration of the drop profile, the interfacial tension, surface area and volume could be simultaneously calculated and recorded in real time [39].

c. *POE CMC determination*

After drop formation, molecules dissolved in lipid or aqueous phase adsorb at the interface. When surface tension stabilizes, the system has reached a dynamic equilibrium. The equilibrium surface tension of a labrafac<sup>®</sup> drop in PEO 40 stearate solution was determined for concentration of PEO 40 stearate from  $10^{-6}$  to  $10^{-2}$ M. The pseudo-critical micellar concentration (CMC) was defined as the lowest concentration leading to the lowest value of surface tension as previously described by Heutault *et al.* [40]. For these experiments surface area of the drop was kept constant by using a stepping motor connected to the microcomputer.

d. *Adsorption studies*

The equilibrium surface tension was determined for  $\beta$ C,  $\beta$ C + POE stearate 40,  $\beta$ C + lipoid<sup>®</sup>,  $\beta$ C + lipoid<sup>®</sup> + POE stearate 40, POE stearate 40, lipoid<sup>®</sup> + POE stearate 40. POE stearate 40 concentration for this study was  $5.10^{-3}$  M for other compounds concentration used are given in sectionII.2.i. For these experiments surface area of the drop was also kept constant.

e. *Rheological study*

The rheological behavior was studied by applying a ramp compression on each surface layer. We performed two types of compression on the drops (figure 1.a)): first a rapid compression during a period of time  $t_f$  and second a slow compression during  $t_s$ . For viscoelastic behaviour, rapid compression allows the determination of the relaxation time of each film ( $\tau$ ). Slow compression allow experimental determination of interfacial viscoelastic moduli using a theoretical model (generalized Maxwell) (figure 1.c)) [39-45]. Interfacial pressure variation during experiment can be defined as follows (figure 1.b)):

$$\Delta\pi = \pi(t) - \pi_i = \gamma_i - \gamma_0 \quad (1)$$

with

$$\Delta\pi = \Delta\pi_e + \Delta\pi_{ne} \quad (2)$$

$\Delta\pi_e$  is related to dilatational elasticity ( $E_e$ ), which is comparable to storage modulus, and characterize lateral interactions between adsorbed molecules at the interface. Elastic modulus is represented by a spring in the mechanical model (figure 1.c)).  $\Delta\pi_{ne}$  is associated to energy dissipation accumulated during compression, it is related to loss modulus This part of elasticity will be referred as non equilibrium elasticity ( $E_{ne}$ ).  $E_{ne}$  is characterized by a pressure relaxation due to molecule chain or surfactant expulsion from the interface to bulk or oil phase as well as reorganization of the interfacial film. Relaxation time  $\tau$  is the time necessary for the film to reach equilibrium after a perturbation. On mechanical model, viscoelastic modulus is represented by a spring combined to a shock absorber (figure 1.c)).

Theoretical approach related to following calculation have been described elsewhere [41-43, 46, 47].  $\tau$  was calculated after drop fast compression (for  $t > t_f$ ), using exponential fit (Origin 8.1, Originlab).

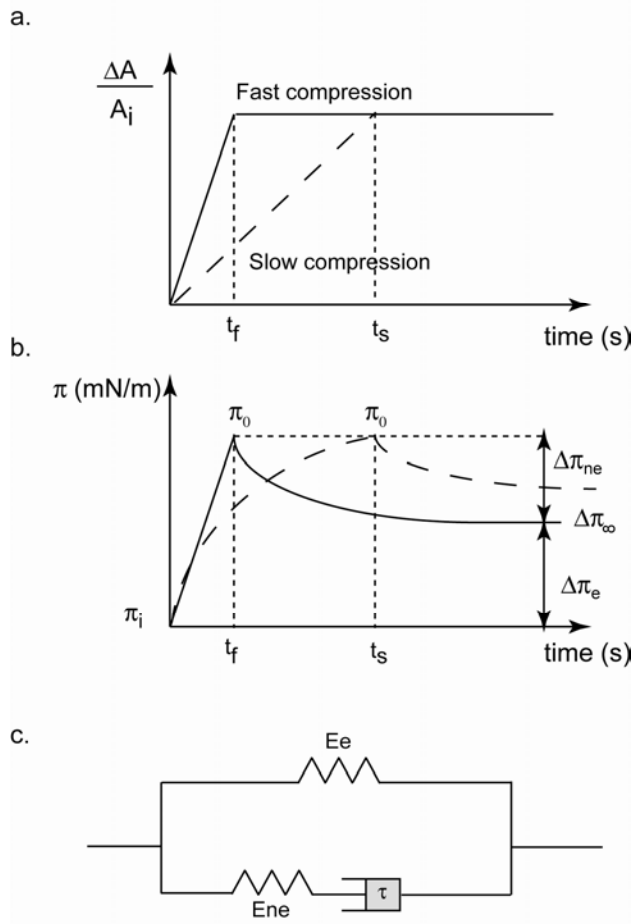
$$\pi(t) = (\pi_0 - \pi_\infty)e^{-t/\tau} + \pi_\infty \quad (3)$$

Slow compression is required for  $E_e$  and  $E_{ne}$  calculation using  $t$  value calculated previously, using following formulae:

$$\frac{\Delta\pi}{Ut} A_i = E_e + E_{ne} \frac{\tau}{t} (1 - e^{-t/\tau}) \quad (4)$$

$U$  represents compression velocity and  $A_i$  drop area before compression.  $E_e$  and  $E_{ne}$  are determined respectively from origin and slope of the linear representation of equation 4 . Fast compression was realized at a velocity higher than  $-0.5 \mu\text{l.s}^{-1}$  and slow compression was

performed at a velocity slower than  $0.03 \mu\text{l}\cdot\text{s}^{-1}$ , for each compression  $\Delta\pi_{\text{max}} \leq 2 \text{ mN}\cdot\text{m}^{-1}$  in order to keep the conditions of the linear model.



**Figure 1:** a) Schematic representation of surface area variation after fast — or slow ---- compression of the drop; b) Schematic pressure change related to fast — or slow ---- drop compression; c) Mechanical model of the film according to Maxwell Model.

### II.3. LNCs Preparation

LNCs containing PEG 15 stearate were formulated according to the protocol achieved by Heurtault *et al.* [20]. For  $\beta\text{C}$  loaded LNCs, oil phase was replaced by a solution of  $\beta\text{C}$  in Labrafac<sup>®</sup> at a concentration of 0.7 mg/ml. In order to produce LNCs having an average size of 20 nm, initial mix was composed by 75 mg of Lipoid<sup>®</sup>, 600 mg of appropriate oil phase, 89 mg of NaCl 2.097g of PEG 15 stearate and 2.11 g of ultra pure water. Excipients were

homogenized under magnetic stirring and PIT method was applied through 3 cycles of fast heating and cooling steps (8°C/min) between 60 and 90°C. LNCs were structured by the addition of 12.5ml of ultra-pure cold water (4°C) when the mix reaches 75°C during the last cooling step. This procedure was performed in the dark in order to prevent as much as possible  $\beta$ C and RA degradation. LNCs suspensions were then filtered on 0.45  $\mu$ m and 0.2  $\mu$ m sterile filters successively (Millipore, St Quentin Fallavier France).

LNCs containing POE 40 stearate were also formulated according to the PIT method described previously but several changes have been made to formulation process. Temperature cycles were performed between 35°C and 98°C to permit PEO 40 stearate hydrophilic part complete dehydration and emulsion inversion at a rate of 2°C/min. To decrease PIT and allow LNCs formation at 45 °C, salt concentration was increased in the aqueous phase from 5 % to 34 %. Therefore rapid cooling was performed at 45°C. The final proportions of water, POE 40 stearate and oil were 60/32/8 % (w/w) while lipid<sup>®</sup> amount was unchanged. Oil phase was added in two steps the first half contained no  $\beta$ C and was added at the beginning of temperature cycles and the second half containing  $\beta$ C was added at 80°C during the last cooling step. Experiments were carried out under argon enriched atmosphere and subdued light. Suspensions were then centrifuged two times during 30 min at 50 000g (Floor standing high speed refrigerated centrifuge, KR22i, Jouan, Saint-Herblain, France) and filtered on 0.45  $\mu$ m and 0.2  $\mu$ m sterile filters successively (Millipore, St Quentin Fallavier France).

All LNCs suspensions were dialyzed during 24H against ultrapure water at room temperature under magnetic stirring, dialysis water was changed after 12H (cellulose ester, MWCO: 15 000, Eindhoven, The Netherlands). 17.5 ml of LNCs suspensions were dialysed against 5L of ultra pure water.

#### II.4. LNCs characterization

LNCs average hydrodynamic diameter and polydispersity index (PdI) were determined by dynamic light scattering using a Malvern Autosizer 4700 (Malvern Instruments S.A., Worcestershire, UK) fitted with a 488 nm laser beam at a fixed angle of 90°. Measurements were taken at 25°C, with  $0.89 \times 10^{-2}$  Pa.s viscosity and a refractive index of 1.33 for water and 1.59 as refractive index for LNCs. LNCs suspensions were diluted by 60 in ultra pure water to ensure convenient scatter intensity on detectors.

To measure  $\beta$ C loading in stock LNCs suspension, we used spectrophotometry at 460 nm in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$ . Briefly, LNCs were diluted by 8 in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  which allows LNCs disruption and dissolution of LNCs excipients. Standards were realized with unloaded LNCs and pure  $\beta$ C.

Suspensions characteristics are shown in table 1.

LNCs type	average diameter of LNCs suspension (nm)	PdI	$\beta$ C concentration $\text{mg.ml}^{-1}$
Unloaded PEG 15 stearate	$18.0 \pm 0.1$	$0.11 \pm 0.01$	-
$\beta$ C loaded PEG 15 stearate	$19.1 \pm 0.3$	$0.08 \pm 0.03$	$0.024 \pm 0.001$
Unloaded POE 40 stearate	$18.9 \pm 0.2$	$0.11 \pm 0.01$	-
$\beta$ C loaded POE 40 stearate	$18.4 \pm 0.4$	$0.09 \pm 0.01$	$0.009 \pm 0.001$

**Table 1:** LNCs suspensions characteristics

#### II.5. Electrophoretic mobility

Electrophoretic mobility measurements were carried out using the zeta sizer nano ZS (Malvern instruments S.A.) based on the laser dopler effect. Measurements were made in water at 25°C, with dielectric constant of 79, a refractive index of 1.33, a viscosity of 0.89 Cp for

water and a refractive index of 1.59 for LNCs, cell voltage a current were automatically set by the zeta sizer. LNCs were diluted at 1:90 with NaCl solutions and ultrapure water. Ultra pure water produced had a pH of 6.4. Final NaCl concentrations in samples were 0.3, 1, 1.5, 2, 10, 25 and 50 mM. NaCl did not have any effect on pH at concentration used. Electrophoretic mobility measurements were performed as a function of NaCl concentration.

We applied the soft particles analysis theory developed by Ohshima [48], in order to determine the characteristics of polyelectrolyte accessible region of LNCs shell: its spatial charge density (ZN) and its depth ( $1/\lambda$ ) (figure 1). Electrophoresis of soft particles depends on the volume charge density distributed in the polyelectrolytes layer, on the frictional forces exerted by the polymer segments on the liquid flow in the polyelectrolytes layer, on the particle size and on the frequency of applied oscillating electric field [49]. The analysis was applied by using the following formula:

$$\mu = \frac{\varepsilon_0 \varepsilon_r}{\eta} \frac{\Psi_0 / Km + \Psi_{DON} / \lambda}{1 / Km + 1 / \lambda} + \frac{eZN}{\eta \lambda^2} \quad (1)$$

Where  $\mu$  represents electrophoretic mobility ( $\mu\text{m.cm.Vs}^{-1}$ ),  $\varepsilon_r$  and  $\varepsilon_0$  are the relative permittivity of electrolytes solution and vacuum (respectively 80 and  $8.85 \cdot 10^{-12} \text{ F.m}^{-1}$ ).  $\eta$  is the viscosity of electrolyte solution (Pa.s).  $1/\lambda$  represents the depth of the layer accessible to counter ions (ie  $\text{Na}^+$ ).  $Km$  is the Debye-Hückel parameter of the shell; it can be calculated via Debye-Hückel parameter  $\kappa$ .

$$\kappa = \left( \frac{2z^2 e^2 n}{\varepsilon_0 \varepsilon_r kT} \right)^{1/2} \quad (2)$$

$$Km = \kappa [1 + (A)^2]^{1/4} \text{ avec } A = ZN / zn \quad (3)$$

$z$  is the valence of electrolyte solution used to modify ionic strength.  $e$  is the electric elementary charge ( $1.6 \cdot 10^{-19} \text{ C}$ ),  $T$  is the temperature in Kelvin.  $k$  is the Boltzmann constant.  $n$  is bulk concentration of the electrolytes solution ( $\text{Osm.m}^{-3}$ ).  $\Psi_{DON}$  is the Donnan potential, it

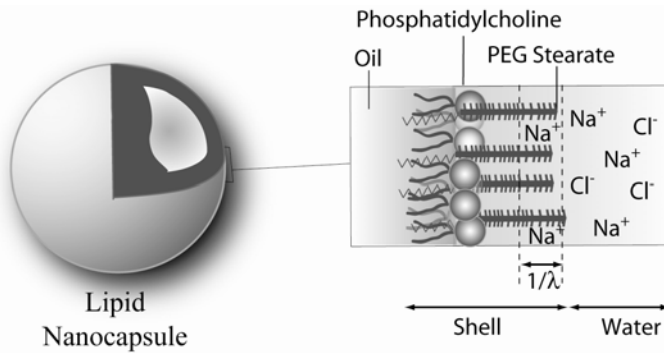
is characterized by an unequal ions distribution between to ionic solutions. It can be defined as follows:

$$\psi_{DON} = \frac{kT}{ze} \ln(A + (A^2 + 1)^{1/2}) \quad (4)$$

$\psi_0$  is the potential at the limit between the shell surface and the solution.

$$\psi_0 = \frac{kT}{ze} \left[ \ln(A + (A^2 + 1)^{1/2}) + \frac{1}{A} (1 - (A^2 + 1)^{1/2}) \right] \quad (5)$$

To determine ZN and  $1/\lambda$ , experimental  $\mu(\text{NaCl})$  was fitted with theoretical values using a Fortran program for parameter fitting with Force 2.0 software compiler. This program was based on traditional optimization method called simulated annealing with a 0.95 tolerance [50].

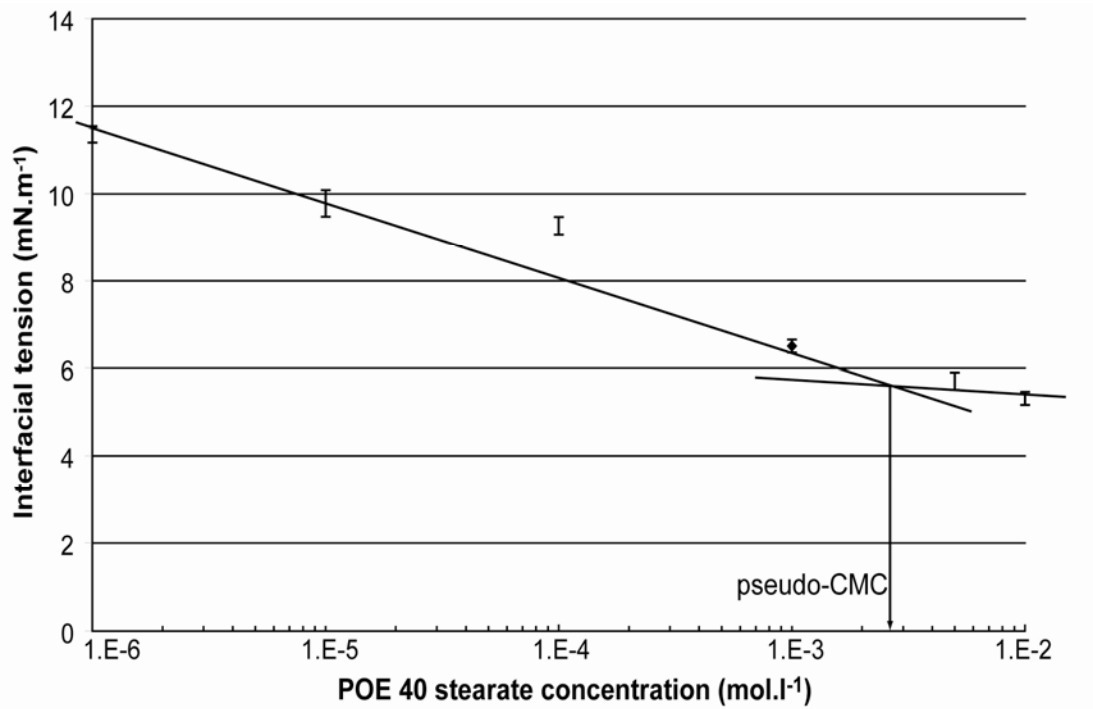


**Figure 2:** Schematic representation of ZN and  $1/\lambda$  for LNCs in a NaCl solution



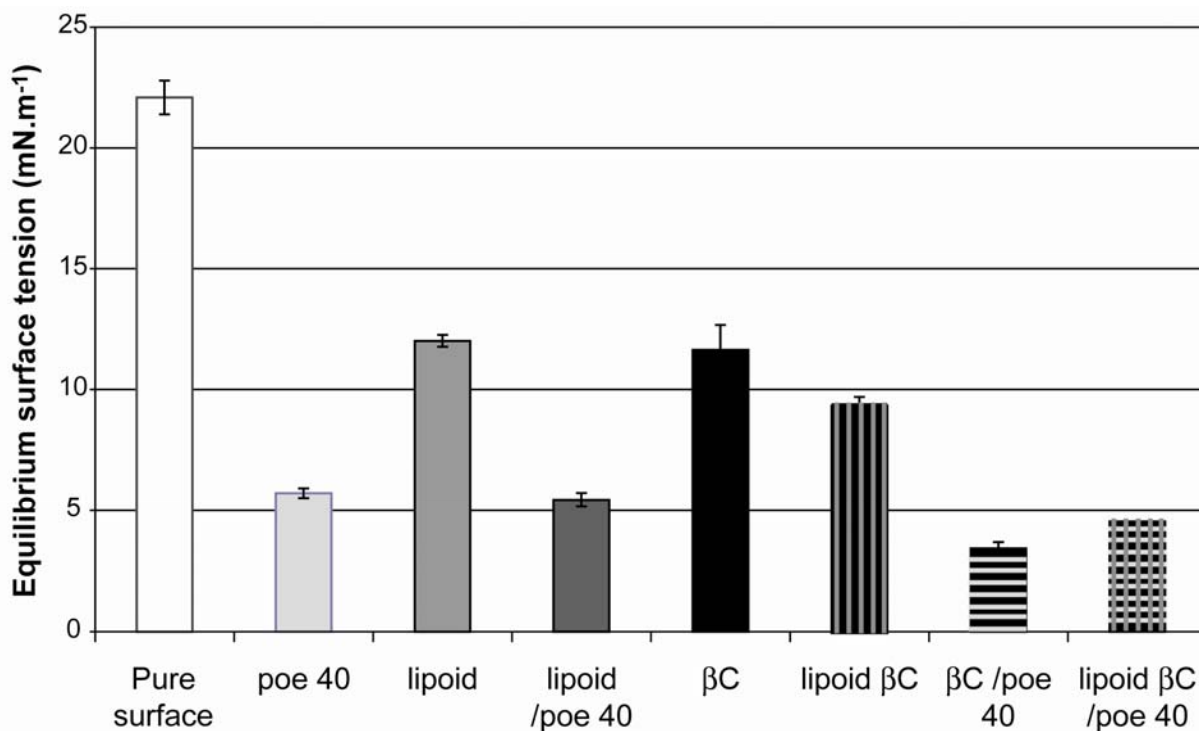
### III. Results and discussion

#### III.1. POE 40 stearate pseudo CMC determination and equilibrium surface tension



**Figure 3: Determination of the pseudo-CMC of POE 40 stearate at oil/water interface**

Figure 3 shows the equilibrium surface tension of POE 40 stearate from adsorption at different concentrations. It enables the determination of POE 40 stearate pseudo-CMC in water at water/ oil interface which is approximately  $1.10^{-3} \text{ mol.l}^{-1}$ . This value is comparable with pseudo CMC of PEG 15 stearate [40]. In order to be close to formulation condition, we chose to perform next experiments at POE 40 stearate concentration of  $5. 10^{-3} \text{ mol.l}^{-1}$ .



**Figure 4:** Equilibrium surface tension at oil/water interface

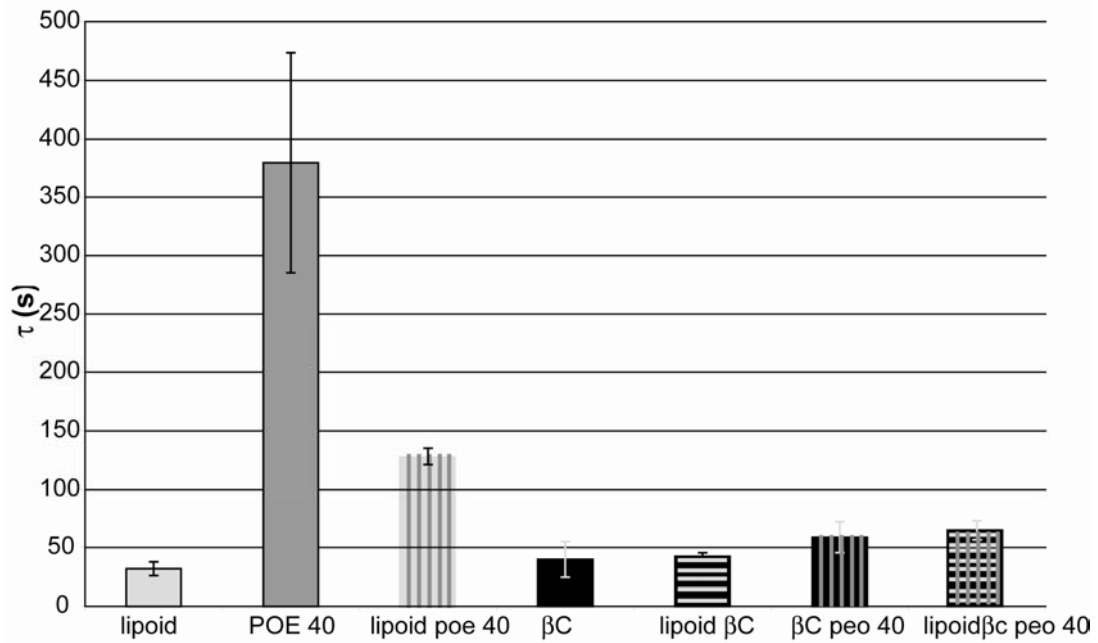
As shown by figure 4, all molecules studied exerted surface active properties at oil/water interface. Due to its well defined amphiphilic properties, and its high concentration in solution, PEO 40 stearate decreased dramatically surface tension from  $22 \text{ mN.m}^{-1}$  to  $5.7 \text{ mN.m}^{-1}$ . It corresponds to interfacial tension for PEG 15 stearate at the same concentration [40]. At a lower extent, Lipoid<sup>®</sup> adsorbed films displayed an equilibrium surface tension of  $12 \text{ mN.m}^{-1}$ . More surprisingly,  $\beta\text{C}$ , known to be highly hydrophobic, decreased dramatically interfacial tension to  $11.6 \text{ mN.m}^{-1}$ . Amphiphilic nature of  $\beta\text{C}$  was also pointed out by Joos *et al.* who measured adsorption of  $\beta\text{C}$  at hexane/water interface [29]. The long time dependent adsorption was not well described by a simple diffusion model because of probable surface reorientation phenomenon. It was estimated that hydrophobic interactions could exist and that hydrocarbon chains could be anchored in aqueous solutions and surrounded with structured water. They estimated by calculation molecular area of the packed monolayer at around  $15 \text{ \AA}^2/\text{molecules}$  which is in accordance with the collapse value of  $\beta\text{C}$  at air/water interface [30]. Measurements made by our team with  $\beta\text{C}$  at air/water interface on a Langmuir scale (data not

shown) agreed with those estimations. This indicates that  $\beta$ C molecules are mainly orientated vertically in the adsorbed layer.

When examining the interfacial mixed films, lipoid<sup>®</sup> did not significantly reduce the interfacial tension obtained with PEO 40 stearate. PEO 40 stearate might govern interaction at the interface for this combination. This was also observed by Béduneau *et al.* with PEG 15 stearate and lipoid<sup>®</sup> adsorbed layer at labrafac<sup>®</sup>/water interface [38].

On the contrary, mixed  $\beta$ C/POE 40 stearate adsorbed layer has an equilibrium surface tension of  $3.4 \text{ mN.m}^{-1}$ . This value is lower than the values obtained with single components. This indicates the influence of both constituents at the interface and the presence of cumulative effects on interfacial tension. Stearate chain and  $\beta$ C isoprene segment might interact by hydrophobic interaction. Mixed lipoid<sup>®</sup>- $\beta$ C adsorption layer has an equilibrium surface tension of  $9.4 \text{ mN.m}^{-1}$ . This value is only slightly lower than single components adsorbed film equilibrium surface tension values. These components might not have a synergetic influence on surface tension. The combination of  $\beta$ C- lipoid<sup>®</sup>/POE 40 stearate led to an equilibrium surface tension of  $4.5 \text{ mN.m}^{-1}$ . This value is slightly higher than for  $\beta$ C/POE 40 stearate combination. Molecules might compete at interface thus lowering POE 40 stearate concentration in adsorbed layer. As POE 40 stearate displayed the strongest ability to decrease equilibrium surface tension, a drop of its concentration at the interface might explain this difference.

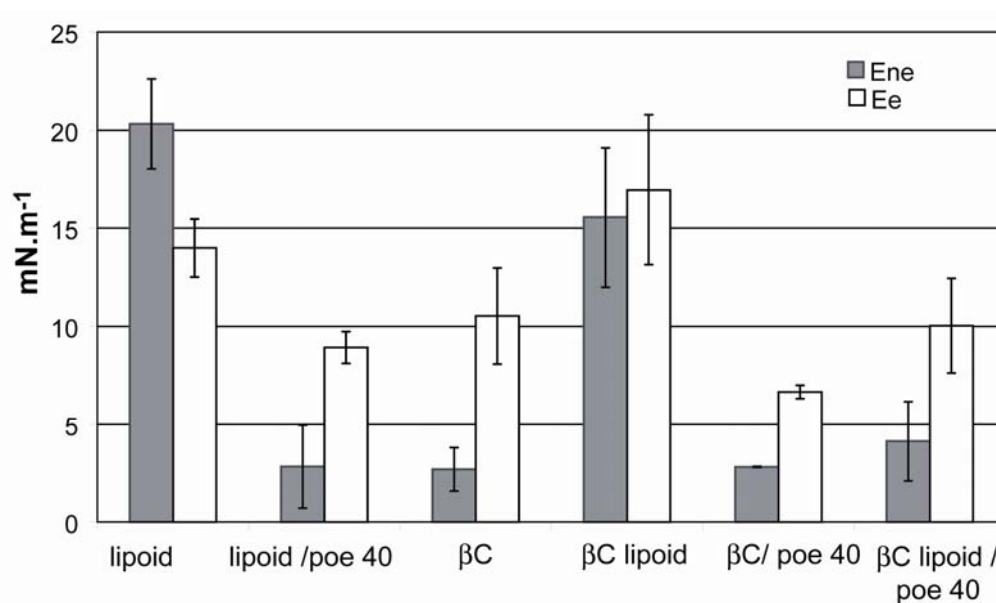
## III.2. Rheological behavior of adsorbed films



**Figure 5:** Experimental values of relaxation time  $\tau$  determined after fast compression of drops at equilibrium.

Relaxation time ( $\tau$ ) represents the time necessary for the film to reach an equilibrium energetic state after a fast compression.  $\tau$  values were determined after fast compression of adsorbed films at equilibrium (figure 5). POE stearate film was purely viscous. It corresponds to a liquid-like film. Liquid-like behaviour ( $E_e < E_{ne}$ ) is encountered in the case of flexible polymer layers (i.e. linear polymer) [51]. It indicates the reorganization of the interfacial films after the end of the compression, with probably expulsion of entire molecules towards the aqueous phase. Expulsion of POE 40 stearate from the interface during compression is consistent with previous data on PEG based polymers. Using 2D rheology, PEG 15 stearate film at oil/water interface demonstrated a liquid like interface with  $E_{ne} > E_e$  [40, 51]. Thus, molecule expulsion and rearrangements at the interface were favoured. Expulsion of PEG 15 stearate from oil/water interface has been also been identified at air/water interface [52]. This reorganization confirmed the instability of PEG 15 stearate film due to its high affinity for water. Besides, it has been previously shown that PEG 2000 chain could be expelled upon

compression from PEG2000 polymer and PEG2000-poly(d,l-lactide) block copolymers films adsorbed at the organic solvent/water interface towards the phase they have the greatest affinity for [53]. For POE 40 stearate monolayer,  $\tau$  was the highest as it reached  $380 \pm 90$  s. This high value can be explained by the fact that expelled molecules take time to re-adsorb at the interface after compression. Due to its high concentration and its strong contribution at oil/water interface, POE 40 stearate might hinder re-adsorption of expelled molecules [40]. This phenomenon might explain increases of  $\tau$  observed when POE 40 stearate is included in monolayers.



**Figure 6: Experimental values of  $E_e$  and  $E_{ne}$  for adsorbed film calculated after slow compression.**

For the other adsorbed films, viscoelastic behaviour was measured.  $E_e$  and  $E_{ne}$  were calculated from  $\gamma(t)$  responses of each film after a slow compression (figure 6). In the case of  $\beta C$ ,  $E_e$  was higher than  $E_{ne}$ , indicating a cohesive, condensed solid-like interfacial layer [51]. The predominance of lateral interactions might be explained by hydrophobic interactions between  $\beta C$  molecules. The film relaxation might be explained by a reorientation of  $\beta C$  molecules. As mentioned by Joos *et al.*,  $\beta C$  can exhibit interfacial conformational changes

during adsorption at the hexane/water interface [29].  $\beta$ C molecules can be compacted until  $15 \text{ \AA}^2/\text{molecules}$  [29, 30], afterwards  $\beta$ C terminal ring might dehydrate and rearrange in compact lattice or  $\beta$ C molecules might be expelled from interface in oil phase. As the relaxation time ( $40 \pm 15\text{s}$ ) is low, they are able to reorganize quite rapidly at oil/water interface. The molecular reorientation is also related to the hydrophobic effect.

Lipoid<sup>®</sup> film displayed high elasticity values ( $E_e = 14 \pm 2 \text{ mN.m}^{-1}$  and  $E_{ne} = 20 \pm 2 \text{ mN.m}^{-1}$ ). Contrarily to  $\beta$ C, non equilibrium part of the elasticity overcomes the equilibrium part, thus lipoid<sup>®</sup> film displayed a liquid like behaviour. Molecules interact with each other and during compression, they are easily rearranged or expelled from the interface as shown by the high  $E_{ne}$ . Lipoid<sup>®</sup> is mainly composed by phospholipids and dipalmitoyl phosphatidylcholine (DPPC) especially. DPPC is constituted by a phosphocholine head group and unsaturated fatty acid chains. It has been shown that phospholipids lateral interactions at water/dichloromethane interface were favoured by a phosphocholine head group and unsaturated fatty acid chains [37]. Thus lateral interactions displayed by lipoid<sup>®</sup> film can be explained by the properties of DPPC. The relaxation time ( $32 \pm 6\text{s}$ ) is low, thus phospholipids are able to reorganize rather rapidly after a compression.

For lipoid<sup>®</sup>/POE 40 stearate adsorbed layer,  $E_e$  ( $8.9 \pm 0.8 \text{ mN.m}^{-1}$ ) higher than  $E_{ne}$  ( $2.8 \pm 2.5 \text{ mN.m}^{-1}$ ). This is a sign of a more solid like behaviour of the film. This behaviour is different from ones observed for pure components. The addition of lipoid<sup>®</sup> in POE 40 stearate monolayer creates lateral interactions between molecules. Hydrophobic interaction might occur between phospholipids aliphatic chains and stearate. This cohesion between molecules might limit rearrangements or expulsion during compression. POE 40 stearate inclusion in lipoid<sup>®</sup> monolayer increase dramatically the relaxation time to  $128 \pm 7\text{s}$ , thus the film formed take a long time to reach another equilibrium state after a perturbation.

In  $\beta$ C/POE 40 stearate mixed film,  $\beta$ C remarkably dominates the rheological behaviour changing the system from viscous to viscoelastic. The film rigidity due to  $\beta$ C ( $E_e=10$  mN/m) is slightly decreased in mixed POE 40 stearate ( $E_e=7$ mN/m) and reflects the presence of both constituents. As for lipoid<sup>®</sup>/POE 40 stearate monolayer the addition of  $\beta$ C creates lateral interaction between molecules at oil/water interface. The results confirm assumptions made with equilibrium surface tension in previous sections. It can be hypothesized that hydrophobic interactions are created between stearic acid chain and  $\beta$ C. The existence of lateral interactions might hinder molecules vertical displacement. Lateral interactions between fatty acid chain and  $\beta$ C have been identified at air/water interface [54].  $\beta$ C increased lateral hydrophobic interactions has shown by a strong condensing effect. Its rigid structure might mechanically hinder fatty acids chains expansion and impede their rotational isomerisation. This observation has also been made at air/water interface with palmitic acid on DPPC monolayer [55] and in  $\beta$ C /dihydrophytylcellulose mixed monolayers [32]. Besides,  $\beta$ C seemed more efficient to decrease relaxation time than lipoid<sup>®</sup> as  $\tau$  value is  $59 \pm 13$ s.

Here, 2D rheology clearly reveals the viscous influence of POE 40 stearate in adsorbed film with lipoid<sup>®</sup> or  $\beta$ C by the increase of relaxation times. This great influence can be explained by its high concentration and its strong contribution at oil/water interface, POE 40 stearate might hinder the interfacial reorganization of adsorbed molecules [40].

For  $\beta$ C-lipoid<sup>®</sup> adsorbed film, non equilibrium and equilibrium part of the elasticity are equivalent.  $\beta$ C seemed to influence significantly interfacial behaviour in mixed films. The liquid-like behaviour ( $E_e < E_{ne}$ ) of lipoid<sup>®</sup> film is modified by  $\beta$ C inclusion in a more rigid monolayer ( $E_e$  goes from 13 mN/m to 17 mN/m while a drop of 5 mN/m is recorded for  $E_{ne}$ ). Monolayer molecules interact with each other at the interface, probably through hydrophobic

interaction has it has been shown between aliphatic chains and  $\beta$ C isoprene chains. However, they can also be rearranged or expelled from the interface to oil phase during compression. The influence of the two constituents is also demonstrated by the intermediate values of elasticity. As the relaxation time ( $42 \pm 3$ s) is low they are able to reorganize quite rapidly at oil/water interface.

Finally, the 3-components system shows a behaviour which is probably the consequence of multiple interactions between  $\beta$ C, lipid<sup>®</sup> and POE 40 stearate.  $\beta$ C-lipid<sup>®</sup>/POE 40 stearate film is characterized by an equilibrium elasticity ( $10 \pm 2$  mN.m<sup>-1</sup>) superior to non-equilibrium elasticity ( $4 \pm 2$  mN.m<sup>-1</sup>). Thus, this film has a more solid like behaviour. The presence of lipid<sup>®</sup> seems to slightly rigidify the film (increase of  $E_e$  compare to  $\beta$ C/POE 40 stearate film). Cohesion between molecules might be due to combined hydrophobic interactions between  $\beta$ C isoprene chain, phospholipids aliphatic chains and stearate group. The presence of PEO40 stearate leads also to an increase of  $\tau$  (65s) in respect with the binary  $\beta$ C-lipid<sup>®</sup> system (42s).

When cohesive interactions ( $E_e$ ) are more important than dissipation behaviour ( $E_{ne}$ ) it can be hypothesized that the film is less fragile and more stable. Thus POE 40 stearate, lipid<sup>®</sup> and  $\beta$ C-lipid<sup>®</sup> adsorbed films would be less stable than  $\beta$ C, lipid<sup>®</sup>/ POE 40 stearate  $\beta$ C/ POE 40 stearate and  $\beta$ C-lipid<sup>®</sup>/ POE 40 stearate monolayers.

Film associated viscosity can be evaluated by the relation  $\tau E_{ne}$  [37] and give information on the dissipative contribution in the 2D film rheology. Mean values are recorded in table 2. These calculations confirm that lipid<sup>®</sup> increases film viscosity. It has been shown that DPPC displayed a high associated viscosity because of the low hydration of phosphocholine head groups at water/dichloromethane interface [37].  $\beta$ C and  $\beta$ C/POE 40



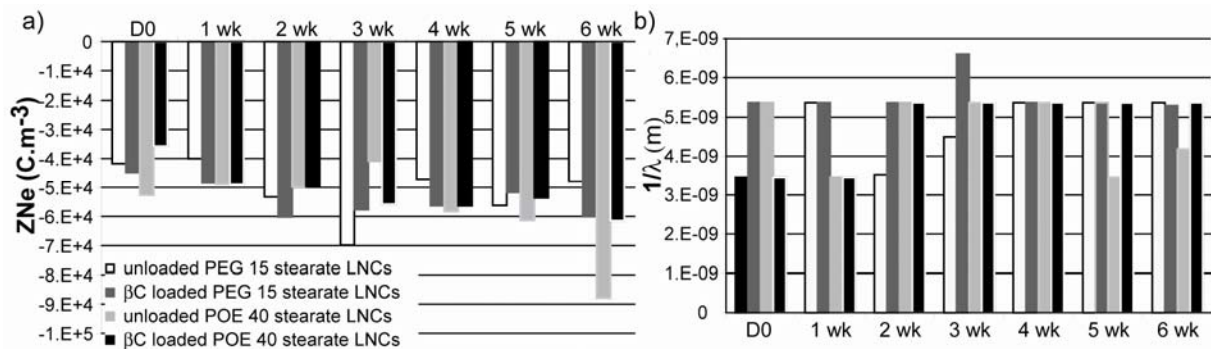
stearate displayed the lowest associated viscosity.  $\beta$ C-lipoid<sup>®</sup>/POE 40 stearate associated viscosity was higher than for  $\beta$ C and  $\beta$ C/POE 40 stearate but was more than half lower than for lipoid<sup>®</sup> films. Thus, it seems that lipoid<sup>®</sup> mobility in the mixed film is affected by the presence of POE 40 stearate. In  $\beta$ C-lipoid<sup>®</sup>/POE 40 stearate systems, lateral interactions of phospholipids fatty acids chain with stearate and isoprene chains of POE 40 stearate and  $\beta$ C respectively may stabilize interfacial layer. The presence of POE moieties mainly orientated towards the aqueous phase, could increase this stability.

	associated viscosity $\text{mN}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$
lipoid <sup>®</sup>	648±120
lipoid <sup>®</sup> /POE 40 stearate	364±20
$\beta$ C	107±41
$\beta$ C-lipoid <sup>®</sup>	661±46
$\beta$ C POE 40 stearate	166±37
$\beta$ C-lipoid <sup>®</sup> /POE 40 stearate	268±34

**Table 2: Films associated viscosity**

$\beta$ C is known to have fluidizing properties [56] and decreases order in phospholipids membranes [35]. Thus, one might have expected that  $\beta$ C-lipoid<sup>®</sup> film displayed lower rigidity. This result confirms previous conclusion that  $\beta$ C increases lipoid<sup>®</sup> film rigidity between the two types of molecule at oil/water interface.

### III.3. Electrophoretic mobility



**Figure 7: ZN and  $1/\lambda$  parameters for unloaded and  $\beta$ C loaded LNCs made with PEG 15 stearate or POE 40 stearate.**

ZNe was  $\sim 3.10^{+4}$  C.m<sup>-3</sup> (figure 7.a)) and  $1/\lambda$  was  $\sim 5$  nm (figure 7.b)) for every LNCs type. Changes in these values during storage are neither significant nor consistent. Therefore we can conclude that the two types of LNCs have the same electrostatic properties during storage. LNCs loading or POE 40 stearate do not affect particles interfacial charge properties. Thus electrostatic interaction might not play a significant role in POE 40 stearate LNCs maturation process. These results show that  $\beta$ C and POE 40 stearate have no influence on LNCs electrophoretic mobility.

#### **IV. Concluding remarks-perspectives**

POE 40 stearate has the strongest ability to decrease interfacial tension, from this point of view POE 40 stearate might decrease Ostwald ripening rate and enhance LNCs stability. However POE 40 stearate film is purely viscous, it has a liquid like behavior. Upon compression film organization is largely modified. When POE 40 stearate was associated to Lipoid<sup>®</sup> or  $\beta$ C the film was more stable as lateral interaction was favored and molecule vertical movement limited.

$\beta$ C is known to have fluidizing properties [56] and decreases order in phospholipids membranes [35]. This is related to the lateral interaction it exerts with phospholipids alkyl chains and in some extent to hydrophobic effects. In our system,  $\beta$ C insertion in the interfacial layers does not disturb strongly the organization of phospholipids and POE 40 stearate. This is also confirmed by electrophoretic mobility measurements. Lipoid<sup>®</sup>/POE 40 stearate organization is the driving force in the stabilization of LNCs shell [57]. Ostwald ripening observed with POE 40 stearate LNCs might be related to hydrophilic chain length of POE 40 stearate rather than a modification of electrophoretic properties of LNCs shell. Interaction between hydrophilic chains of POE 40 stearate might be favored in comparison to interfacial

hydrophobic interactions. This feature might render lipoid<sup>®</sup>/POE 40 stearate and  $\beta$ C-lipoid<sup>®</sup>/POE 40 stearate interfaces less stable than lipoid<sup>®</sup>/PEG 15 stearate or  $\beta$ C-lipoid<sup>®</sup>/PEG 15 stearate films. An increase of lipoid<sup>®</sup> concentration in LNCs shell might increase their stability.

One possible consequence of the presence of  $\beta$ C in LNCs shell might involve its release under certain conditions. Some observation (data not shown) indicates that  $\beta$ C was not released from LNCs when incubated in pure water. On the contrary, a significant release was measured when  $\beta$ C loaded LNC were incubated with SDS. This surfactant could make possible an extraction of the  $\beta$ C from interfacial layer, because surfactant micelles are able to solubilise hydrophobic compound. The interfacial localisation of  $\beta$ C in these colloidal carriers is a factor that might influence its accessibility and that must be taken into account for the design of new delivery systems.

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## **CHAPITRE III :**

### **COMPORTEMENT DES NCLs DANS UNE MATRICE COMPLEXE MODELE**



## **Comportement des NCLs dans une matrice complexe modèle**

Les NCLs sont capables de protéger le  $\beta$ C chimiquement pendant le stockage en suspension aqueuse pendant 60 jours. En milieu purement aqueux, le  $\beta$ C reste dans le cœur lipophile des NCLs. Un des objectifs est d'enrichir en  $\beta$ C un aliment complexe grâce aux NCLs. C'est pourquoi il est important de savoir si le  $\beta$ C reste encapsulé et intact dans un milieu pouvant contenir une phase grasse dispersée et/ou des éléments pro-oxydants susceptibles de le dégrader.

Dans ce troisième chapitre, les NCLs ont été incluses dans une matrice alimentaire modèle complexe : la crème fraîche. Les objectifs de cette étude sont (i) de déterminer l'impact de l'encapsulation sur la stabilité du  $\beta$ C dans une matrice complexe (ii) d'évaluer la libération du  $\beta$ C du cœur des NCLs vers la phase grasse (iii) d'estimer la capacité du  $\beta$ C encapsulé à protéger des éléments fragiles de la matrice contre l'oxydation comme les acides gras de la phase lipidique.

Pour cette étude, les NCLs de stéarate de PEG 15 ont été utilisées car elles permettaient d'obtenir la concentration la plus élevée en  $\beta$ C. De plus, la structure des NCLs de PEG stéarate est stable dans le temps.

**Nanoencapsulation: a new way to stabilize  $\beta$ -Carotene which involved a better protection of dairy emulsion against oxidation**

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## **Abstract**

Lipid oxidation is one of the major reactions altering food quality during storage. The use of antioxidants such as  $\beta$ -carotene ( $\beta$ C) limits lipid oxidation.  $\beta$ C multiple conjugated double bonds give to the molecule free radicals scavenging properties, but make  $\beta$ C sensitive to oxygen, heat and light. The aim of this work was to investigate nanoencapsulation impact on  $\beta$ C stability and evaluate  $\beta$ C loaded LipidNanoCapsules (LNCs) potencies to protect lipids from oxidation in food emulsions.  $\beta$ C loaded LNCs were produced by phase-inversion method. LNCs size distribution was disturbed by mechanical shear stress. During  $\beta$ C loaded LNCs storage in dairy emulsions,  $\beta$ C did not diffuse from LNCs core to lipid phase. Besides,  $\beta$ C encapsulation increased its chemical stability. Lipid oxidation, initiated by iron and ascorbic acid, was delayed in milk fat cream when  $\beta$ C was encapsulated in LNCs. LNCs are a new way to preserve  $\beta$ C integrity and prevent lipid oxidation in dairy emulsions.

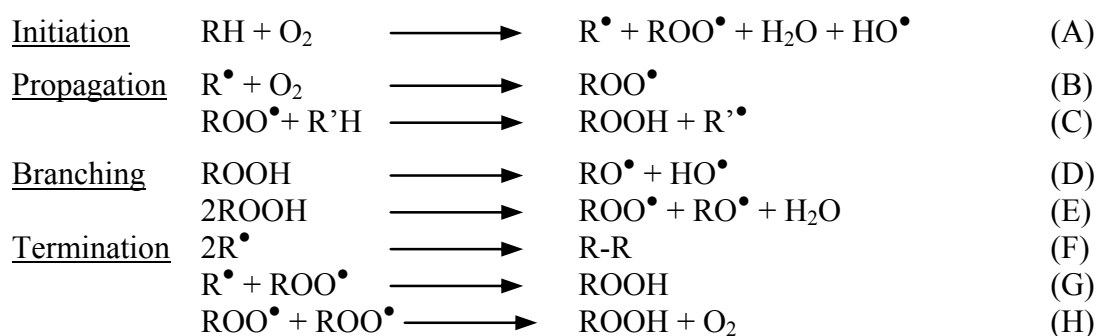
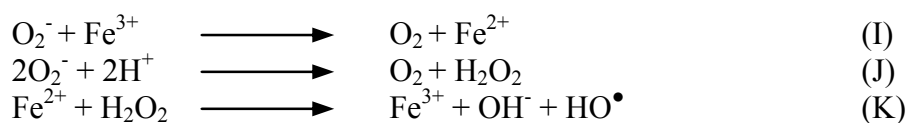
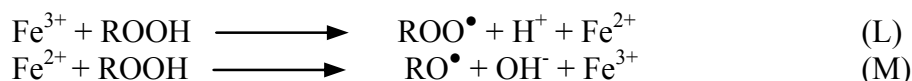
**Key words:**  $\beta$ -Carotene, encapsulation, dairy emulsion, oxidation, stability

**Abbreviations:** **AUCcum:** Cumulative Area Under Curve; **AO:** AntiOxidant;  **$\beta$ C:** beta carotene; **LNC:** Lipid NanoCapsule; **o/w:** Oil-in-Water; **PC:** PhosphatidylCholine; **PEG:** Polyethylene Glycol; **PL:** PhosphoLipid; **SDS:** Sodium Dodecyl Sulphate; **SLN:** Solid Lipid Nanocapsule

## **Introduction**

Lipid oxidation is a major issue as it causes off flavour in food products, alters nutritional and physicochemical lipid phase properties, and produces toxic breakdown products (1). Lipid oxidation mechanistic pathways are largely described in literature (2;3). Lipid autoxidation can be induced by light, oxygen, heat or metals ions (4). Production of a free radical  $R^\bullet$  is the result of the first initiation step of the reaction (Fig. 1A). Free radicals can then react with oxygen to produce peroxy radicals  $ROO^\bullet$  (Fig. 1B), which can react with another lipid molecule to form more free radical  $R^\bullet$  and hydroperoxide  $ROOH$  (Fig. 1C). Hydroperoxide can be decomposed thermally to produce two free radicals able to initiate more oxidation process (Fig. 1D, 1E). Lipid oxidation reaction is then autocatalytic. Radical reaction terminates when free radicals react with each other to produce  $R-R$  or hydroperoxide (Fig. 1F, 1G, 1H) (4).

To evaluate potencies of antioxidants (AO) to limit lipid oxidation, initiation of reaction by iron and ascorbic acid is commonly use to perform ageing accelerated tests [4]. Iron is one of transition metals which has the greatest occurrence in food products. Several studies demonstrated that lipid oxidation by iron requires the presence of both ferrous and ferric ions (5;6). Hydroxyl radicals formation catalysed by iron can be form through Haber-Weiss reaction (Fig. 1I, 1J) and the last step of this reaction is known as Fenton's reaction (Fig. 1K) (7).  $Fe^{3+}$  and  $Fe^{2+}$  are also able to react with hydroperoxide to produce free radicals (Fig. 1L, 1M) (2). Hydroperoxides formation by iron can be increased by iron complexation with EDTA or ascorbic acid (5;7-10).

**Lipid autoxidation reactions :****Haber-Weiss reactions :****Other iron induced free radicals formation:**

**Figure 1: Chemical reactions involved in lipid oxidation:** autoxidation reactions (A-H), Haber-Weiss reactions (I-K) or other iron induced free radicals formation (L-M)

*RH* : fatty acid ; *R<sup>•</sup>* : free radical; *ROO<sup>•</sup>* : peroxy radical, *OH<sup>•</sup>* : hydroxyl radical; *ROOH*: hydroperoxide.

Lipid oxidation is a complex reaction which occurs in bulk systems as well as in emulsions and varies according to physical and chemical composition of food. Matrix physical parameters such as exchange surface need to be considered to control or avoid lipid oxidation reaction. Lipids dispersed with a high surface volume ratio are more easily oxidized than bulk lipids or lipids emulsified with a low surface volume ratio (11;12).

As matrix physical parameters, chemical composition of the food matrix can mainly contribute to prevent lipid oxidation. Antioxidants (AO) are the major chemical compounds able to delay lipid oxidation reaction. However, their efficiency mainly depends on their location at interfaces (oil/air for bulk systems or oil/water for emulsions) (3;13). “Antioxidant polar paradox” theory must be considered to optimize AO efficiencies. Polar AO are more effective in bulk lipids or low surface volume oil in water (o/w) emulsion and apolar AO are

more efficient in high surface volume ratio o/w emulsions (11). pH can have also an impact on AO chemical structure hence on their surfaces properties (13)

Carotenoids are organic pigments known to have high free radical scavenging properties (14). They belong to primary or chain breaking AO. Their AO properties are mainly due to their ability to react with hydroperoxides formed by Haber-Weiss reaction for example in presence of iron and ascorbate (14;15). Several studies demonstrated that carotenoids can protect biomolecules and lipid membrane from oxidation in food emulsions (16), as in liposomes (17-19), or as lipoproteins (20). Matos *et al.* (2006) have shown that  $\beta$ -Carotene ( $\beta$ C) can also be efficient to prevent iron-induced oxidative stress *in vivo* (21). Carotenoids chemical structure greatly influences their localization in emulsion droplets or membranes (22-25). It might greatly influence their ability to interact with pro-oxidant species and therefore their AO capacities. For instance, in an o/w model emulsion stabilized by a phospholipids (PLs) monolayer, highly hydrophobic  $\beta$ C and  $\alpha$ -carotene were essentially distributed in droplets core, whereas zeaxanthin and lutein were preferentially located at their interface (22).

As demonstrated by many studies, carotenoids are able to protect food from lipid oxidation. General assumption is that AO and carotenoids have a positive impact on health (26;27). These beneficial effects on health can be due to multiple mechanisms such as cell differentiation, cell to cell communication or *in vivo* AO properties. Many epidemiological studies demonstrated that there is an inverse correlation between vitamin intake and cancer risk (28). Considering these datas, many attempts have been made to increase  $\beta$ C intake and bioavailability via supplementation or food enrichment. Yet, under certain conditions, such as high  $p_{O_2}$ , cigarettes smoke or asbestos exposure, carotenoids can loose their ability to scavenge free radicals and become pro-oxidant which increase lipid oxidation in food and cancer risk *in vivo* (27;29;30)

In this study, we focused on the most abundant carotenoid in human diet:  $\beta$ C (31). Encapsulation could be a mean to preserve  $\beta$ C from oxidation and could also constitute a way to increase its dietary intake and bioavailability (32-35). We chose to encapsulate  $\beta$ C in lipid nanocapsules (LNCs) composed of a liquid lipid core surrounded by a thick layer of soy lecithin and polyethylene glycol (PEG) stearate used as surfactants. Encapsulation process is based on the Phase-Inversion Temperature (PIT) method (36). The technique originality lies on the absence of organic solvent and the low energy needs. Stability of nanoencapsulated  $\beta$ C as well as its potencies to prevent lipid oxidation in milk fat cream were evaluated and compared to untrapped  $\beta$ C to determine its possible applications in milk products.

## **I. Material and Methods**

### **I.1. LNCs Formulation**

LNCs were formulated according to the protocol achieved by Heurtault *et al.* (36). Briefly, oil phase was composed by labrafac<sup>®</sup> (Gattefossé SA, S<sup>t</sup> Priest, France) and represented 23.65 % (w/w) of the formulation. Solutol HS 15<sup>®</sup> (BASF, Ludwigshafen, Germany) and Lipoid 75 S-3, a mix of phospholipids (Lipoid, Cham, Switzerland) were used as surfactants. They represented respectively 9.75 % and 1.5% of formulation weight. The aqueous phase was composed by NaCl (1.78 % (w/w); Sigma-Aldrich, St Quentin Fallavier, France) and ultra pure water 63.32 % (w/w) produced by Milli Q water system (Millipore, Paris, France). To formulate unloaded LNCs, oil phase was composed by pure Labrafac<sup>®</sup> while  $\beta$ C (Sigma-Aldrich, St Quentin Fallavier, France) was previously dissolved in Labrafac<sup>®</sup> at a concentration of 0.45 g.L<sup>-1</sup> to formulate  $\beta$ C loaded LNCs. Their formulation was performed in the dark to prevent  $\beta$ C degradation. LNCs were formulated according to the PIT method: all excipients were added at once and homogenized under magnetic stirring. The mix was heated from 60 to 95°C at a rate of 8°C.min<sup>-1</sup> and was then cooled from 95°C to

60°C with the same temperature gradient. Heating and cooling cycles were repeated three times. During the last cooling step, ultra-pure cold water (4°C) was added at 82°C to form LNCs. Cold water volume added is equivalent to 2.5 times of the initial formulation volume. LNCs suspension was then placed in dialysis tubing (MWCO: 15 000, cellulose ester, Eindhoven, The Netherlands). and remained 3 hours against ultra-pure water to eliminate NaCl. Afterwards, LNCs filled tubing were placed on dry PEG 35 000 (Fulka, Sigma-Aldrich, St Quentin Fallavier, France) for 3 hours in order to concentrate LNCs suspension by osmose.

## **I.2 - Physico-chemical characterization of LNCs**

Size characterization of LNCs : LNCs average hydrodynamic diameter and polydispersity index (PDI) were determined by dynamic light scattering using a Malvern Autosizer 4700 (Malvern Instruments S.A., Worcestershire, UK) fitted with a 488nm laser beam at a fixed angle of 90°. Measurements were taken at 25°C, with  $0.89 \times 10^{-2}$  Pa.s viscosity and a refractive index of 1.33. LNCs were diluted by 60 in ultra pure water in order to assure convenient scatter intensity on detectors. Measurements were performed in triplicate and mean values with standard deviations were calculated. Size distributions in volume were used.

Determination of  $\zeta$  potential of LNCs :  $\zeta$  potential measurements were carried out using a Zeta Sizer 2000 (Malvern Instruments, France) equipped with an AZ-4 cell and were based on the laser-doppler effect. Measurements were taken at 25°C, with  $0.89 \times 10^{-2}$  Pa.s viscosity and a refractive index of 1.33. LNCs were diluted by 60 in ultra pure water. Three measurements were carried out for each sample and mean values with standard deviations were calculated.

Determination of  $\beta$ C concentration in LNCs :  $\beta$ C loading of LNCs was assessed by spectrophotometry at 460 nm (Uvikon 9x3W, Bioserv, Thiais, France). One volume of LNCs suspension was diluted with 7 volumes of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (obtained from Fisher Scientific (Loughborough, UK) and VWR Prolabo(Fontenay-sous-Bois, France) respectively) to allow



LNCs disruption and dilution of LNCs excipients. Calibrations curves were realized with unloaded LNCs and pure  $\beta$ C.

Evaluation of LNCs physical resistance to shear stress : LNCs were submitted to different shear stress using a rotor stator homogenizer (silent crusher S, Heidolph, type 5F, Carl Roth GmbH, Karlsruhe, Germany). Stator and rotor diameter were 3.3 and 2.0 mm respectively. 3 mL of LNCs suspension were introduced in the 5F tool-type and were stirred at 15000, 45000 or 75000 rpm during 3 min. Suspension size distribution was then determined using a dynamic light scattering device (Zetasizer Nano, Malvern Instruments SA, Worcestershire, UK). LNCs suspension without shear stress was used as control.

### **1.3. Evaluation of $\beta$ C loaded LNCs stability during ageing in dairy emulsions**

#### 1.3.1 - Measurements of $\beta$ C chemical stability

Samples preparation : Commercial milk fat cream containing 2.2 % of proteins, 30 % of lipids and 3.4% of glucids was provided by a local supermarket.  $\beta$ C loaded LNCs suspension or pure  $\beta$ C (control sample) was added to milk fat cream to obtain a final equivalent concentration of 88  $\mu$ g of  $\beta$ C per mL. The same volume of unloaded LNCs is added in control sample instead of  $\beta$ C loaded LNCs in order to provide the same viscosity and water content. Samples were introduced in vials by respecting the following ratio: 1 volume of sample for 7 volumes of ambient air. Vials were then incubated during 3 days under magnetic agitation in the dark at two different temperatures of storage : 20°C or 50°C. Each day, an aliquot was taken off to analysis  $\beta$ C contents. All experiments were performed in triplicate.

Extraction of  $\beta$ C content and  $\beta$ C quantification : One volume of milk fat cream (containing  $\beta$ C loaded LNCs or pure  $\beta$ C and unloaded LNCs) was diluted with 7 volumes of  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  to allow LNCs disruption and dilution of LNCs excipients. Solvents were then evaporated under nitrogen à 35°C.  $\beta$ C extraction was then performed according to protocol

developed by HULSHOF *et al* (2006) (37). As  $\beta$ C is a lipophilic molecule, lipid fraction of samples was extracted by addition of a mix composed of ethanol, diethyl ether stabilized by 0.0025% of BHT, and petroleum ether (1:1:1, v/v/v). After centrifugation at 3000 g for 5 min, upper layer was taken out and evaporated to dryness at 35°C under nitrogen. 1,5 mL of KOH (5% w/v in ethanol) was then added to dry residue. Samples were then flushed with nitrogen, closed hermetically and shaken in the dark for 3hours at 200 rpm to realise saponification reaction. 1.5 mL of ultra pure water was then added and the mixture was extracted twice with 3ml of hexane. For each sample, extraction was performed in triplicate and  $\beta$ C concentration was measured by spectrophotometry at 450 nm. Calibrations curves were realised with standards solution containing different concentrations of  $\beta$ C in hexane. Extraction efficiency of  $\beta$ C was then evaluated at 80.5 % for milk fat cream containing pure  $\beta$ C and unloaded LNCs and at 74 % for milk fat cream with  $\beta$ C loaded LNCs.

### 1.3.2. - Measurements of $\beta$ C release from LNCs

*Preparation of model milk fat cream* : Aqueous phase of model milk fat cream was prepared by dissolution of 20 g of sodium caseinate (Lactalis Industrie, France) in 1l of ultrapure water (Milli Q water system, Millipore, Paris, France) containing 80 mM of NaCl. This solution was kept under slow stirring agitation at 8°C for one night before use to have a good protein solubilisation. Afterwards, pH was adjust to 6,7 with NaOH 1 M. 7 ml of aqueous phase were added to 3 ml of dried dairy fat previously heated at 50°C (melting point at 30-34°C, Lactalis Industrie, France). Aqueous and oil phases were then mixed at  $4250s^{-1}$  during 3 min with a rotor stator homogenizer (Silent crusher S, Heidolph type 12 F/M, Carl Roth GmbH, Karlsruhe, Germany). 5 identical preparations were performed and pooled together before use for  $\beta$ C release experiments. Homogenization conditions were selected to obtain larger droplet size than in commercial milk fat cream to have an efficient separation between oil and

aqueous phase for  $\beta$ C release evaluation. After emulsion preparation, oil droplets size distribution of model milk fat cream was determined by laser light scattering using Saturn Digisizer 5200 apparatus (Micromeritics, Norcross, USA). Emulsion was dispersed at 1:10 w/w in SDS solution to separate flocculated oil droplet. As expected, oil droplet size was ranged from 1 to 10  $\mu\text{m}$  for model milk fat cream whereas it was 1  $\mu\text{m}$  for commercial milk fat cream.

Storage conditions :  $\beta$ C loaded LNCs were dispersed in model milk fat cream to obtain a final concentration fixed at 88  $\mu\text{g } \beta\text{C} \cdot \text{ml}^{-1}$  of emulsion. Release study was performed in the dark at 4°C to prevent  $\beta$ C from oxidation. 2 mL samples were taken out at 0, 1, 2 and 3 days.

$\beta$ C extraction of aqueous and lipid phases : The method used to separate  $\beta$ C released from LNCs in fat globules of model milk fat cream and  $\beta$ C remained in LNCs was adapted from a method described previously by Patton *et al.* (38). 1 ml of 50 % w/w saccharose solution was added to 1 ml of the model milk fat cream in order to increase aqueous phase density. Mix was then slowly homogenized before its deposition with a needle at the bottom of centrifuge tube which contained 10 ml of NaCl 80 mM. Afterwards, samples were centrifuged at 3000 g during 10 min at 4°C without mixing these two phases.  $\beta$ C extracted from the aqueous phase (bottom layer) was identified as  $\beta$ C encapsulated in LNCs and  $\beta$ C extracted from lipid phase (upper layer) was identified as  $\beta$ C released from LNCs in fat globules of model milk fat cream. Centrifuge tubes were then frozen vertically at -20°C and cut to separate bottom phase from upper phase. Each phase is then defrosted separately at room temperature.

$\beta$ C quantification in aqueous and lipid phases : Determination of  $\beta$ C concentration remained in LNCs (aqueous phase) was determined by the addition of MeOH/CH<sub>2</sub>Cl<sub>2</sub> as described in section 1.2.3. Determination of  $\beta$ C released from LNCs to fat globules of emulsion (lipid phase) was assessed according to protocol developed by HULSHOF *et al* (2006) (37) as described in section 2.1.2.

#### 1.4. - Determination of $\beta$ C loaded LNCs effect on kinetics of lipid oxidation in dairy emulsions.

##### 1.4.1. - Samples preparation

$\beta$ C loaded LNCs were added in commercial milk fat cream to obtain a final concentration of  $88 \mu\text{g } \beta\text{C.ml}^{-1}$  of milk fat cream. Two controls were also performed and stored in the same conditions. The first one was composed of milk fat cream in which the same volume of unloaded LNCs is added instead of  $\beta$ C loaded LNCs in order to provide the same viscosity and water content. This control served as reference of lipid oxidation kinetic in milk fat cream without  $\beta$ C. Second control contained unloaded LNCs as well but free  $\beta$ C was added to milk fat cream to obtain a final concentration of  $88 \mu\text{g}$  of  $\beta\text{C.ml}^{-1}$ . The second control permitted to determine if lipid oxidation kinetic changes with  $\beta$ C incorporation mode (loaded in LNCs or free adding).

Accelerated lipid oxidation test was performed in the dark at  $50^{\circ}\text{C}$  during 33 hours. Lipid oxidation reaction was initiated by adding  $\text{FeCl}_3$  and sodium ascorbate used as redox cycling system in the media. Final concentrations of  $\text{FeCl}_3$  and sodium ascorbate were  $500 \mu\text{mol.l}^{-1}$  of milk fat cream. Every hour,  $360 \mu\text{l}$  samples were taken out to evaluate the quantity of hydroperoxides formed.

##### 1.4.2 - Quantification of primary products of lipid oxidation

Preparation of Xylenol Orange reagent : Lipid oxidation was followed by kinetic hydroperoxides formation using Xylenol Orange test. Colorimetric dosage reagent was prepared in methanol and was composed of  $100 \mu\text{M}$  Xylenol Orange,  $250 \mu\text{M}$  ferrous

ammonium sulphate, 4 mM BHT, 250 mM H<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich, St Quentin Fallavier, France). This reagent was stored at 4°C in the dark up to 7 days after preparation.

Quantification of hydroperoxides in milk fat cream: 360µL of samples were completed with methanol to obtain a concentration of 10 mg of dairy fat/ mL. 100 µl were then added to 900 µl of Xylenol Orange reagent. After 30 min of incubation at room temperature in the dark, samples were filtered to remove protein aggregates and hydroperoxides concentration was assessed at 560 nm.

Data expression: Calibration curve was realized with cumen hydroperoxide (CuOOH, Sigma-Aldrich, St Quentin Fallavier, France) in methanol. Hydroperoxide concentration in samples is express in µmol equivalent CuOOH.kg<sup>-1</sup> of dairy fat. Cumulative areas under curves (AUCcums) have been calculated using Kinetica 4.4 software after 9 and 33 hours of incubation..

### **I.5 - Statistic analysis**

Every experiments has been performed at least in triplicates, results are expressed in mean ± standard deviation. Significant differences were plotted by an ANOVA followed if necessary by a Student-Newman-Keuls T test (p<0.05). These calculations were performed using the software PC primer.

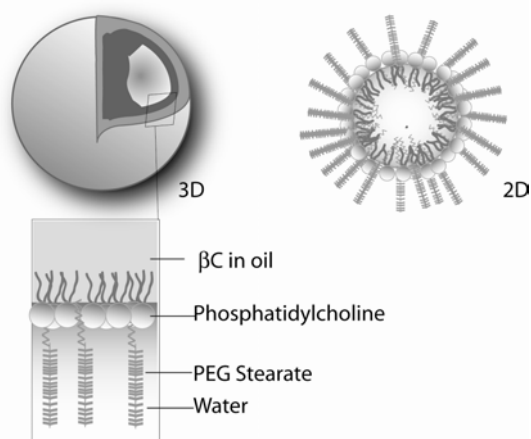
## **II. Results and discussion**

### **II.1 – βC loaded LNCs characterization and physical stability**

βC loaded LNCs have a mean diameter of 91 ± 3 nm with a polydispersity index (PDI) of 0.06 ± 0.02 which characterize a monodisperse formulation. ζ potential was slightly negative -7 ± 1 mV with a peak width of 8 ± 3 mV. These datas are in accordance with literature dealing with unloaded LNCs (39). It can also be hypothesized that βC loaded LNCs

have the same structure and physical properties than unloaded LNCs (39;40). It is generally accepted that LNCs have a core shell structure, composed by an oily core and an interface formed by lecithin phospholipids and PEG stearate (36).

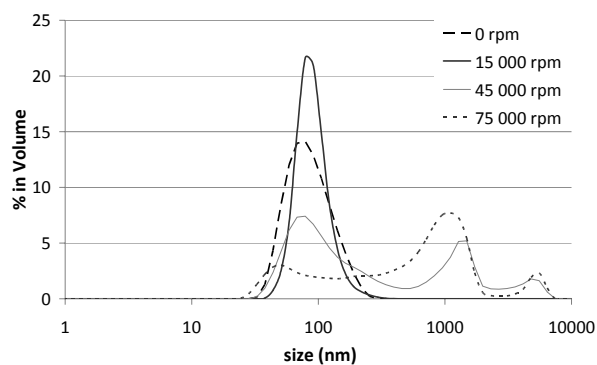
To determine location of  $\beta$ C in LNC, previous study was performed using a fluorescent probe (Nile Red) incorporated in oil phase before LNC formulation (41). Results revealed that Nile Red was only located in LNCs core. As  $\beta$ C is hydrophobic like Nile Red and was also dissolved in lipid fraction, we can hypothesize that  $\beta$ C is located in LNCs lipid core.  $\beta$ C concentrations in LNCs suspensions were  $0.035 \pm 0.003 \text{ mg.ml}^{-1}$  and  $0.096 \pm 0.07 \text{ mg.ml}^{-1}$  before and after concentration respectively. Encapsulation efficiency was evaluated at  $95 \pm 6\%$  (data not shown).  $\beta$ C loaded LNCs structure can be schematized as shown on figure 2.



**Figure 2:** Schematic representation of  $\beta$ C loaded LNCs structure.

Concerning LNCs stability, Peltier *et al.* demonstrated that a precipitation can be observed when an hydrophobic component is released from LNCs (34). This phenomenon occurs also with solid lipid nanoparticles (SLNs) (42). No precipitation of  $\beta$ C was observed during  $\beta$ C loaded LNCs long time storage (6 months), revealing that particles were stable over time without mechanical disturbances. This result was in accordance with previous works (36). LNCs stability was evaluated under mechanical disturbances as observed in

homogenization process in industry (Fig. 3). Size distribution was only slightly altered for 15 000 rpm stress as peak is wider but the distribution remained centred on the same size as control. Other peaks centred at 1300 nm and 5000 nm appeared for 45 000 and 75 000 rpm, while characteristic LNCs size peak decreased. We can also hypothesize that LNCs might be broken down as size distribution graphs were largely modified. Destabilisation seemed greater at 75 000 rpm, as initial LNCs size peak is no more distinguishable. Large diameters peaks might correspond to LNCs breaking down products which might be aggregated and form new microstructures. These results suggest that LNCs can support low shear stress created by mild homogenization, but they are sensitive to physical treatment involving high shear stress.

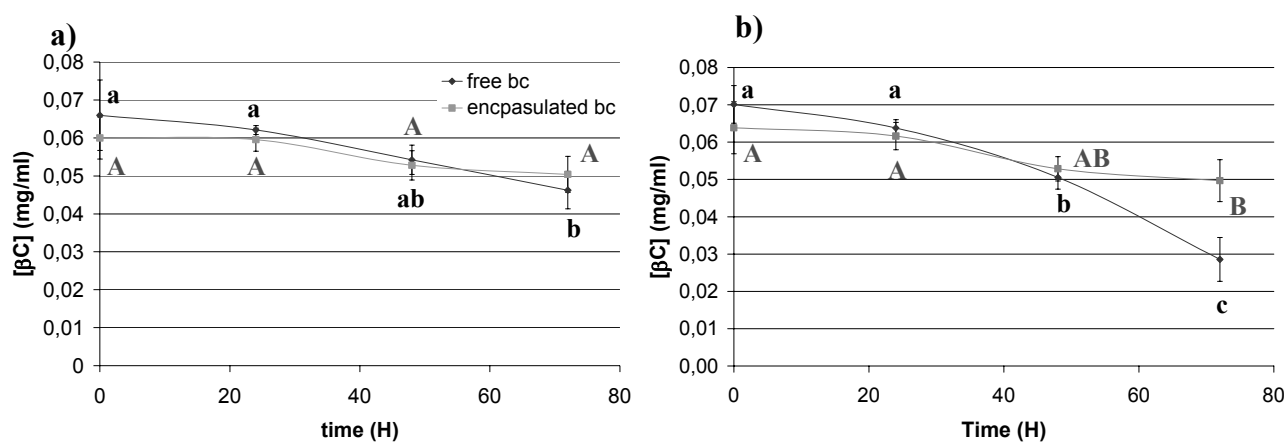


**Figure 3:** LNCs suspension volume size distribution according to shear stress applied.

## II.2 - Evaluation of $\beta$ C loaded LNCs stability during ageing in dairy emulsions

Encapsulation influence on  $\beta$ C chemical stability was evaluated in a polyphasic matrix containing dispersed fat droplets: milk fat cream.  $\beta$ C loaded LNCs or free  $\beta$ C were incorporated in milk fat cream and incubated at 20 or 50°C during 3 days. At 20°C,  $\beta$ C concentration decreased slowly during storage when  $\beta$ C was free or when it was included in LNCs (Fig. 4a). Free  $\beta$ C concentration was significantly lower in dairy emulsions after 72 hours than in dairy emulsion without ageing ( $p < 0.05$ ). After 72 hours of incubation at 20°C, loss of  $\beta$ C was 30 % when  $\beta$ C was free whereas it was only 16 % with encapsulated  $\beta$ C. With

only 3 days of ageing, we can also already observe a slight protective effect of LNCs able to increase  $\beta$ C chemical stability at 20°C.

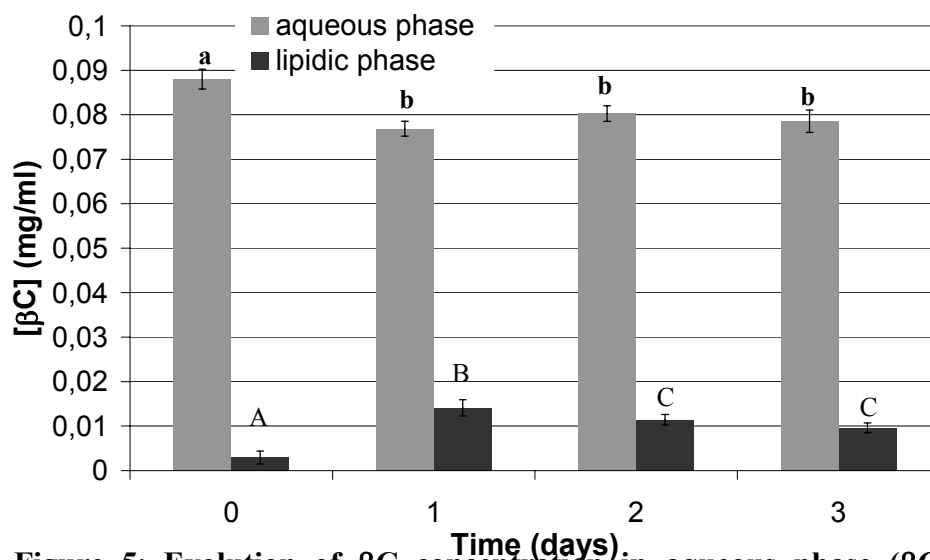


**Figure 4: Evolution of  $\beta$ C concentration in during ageing in dairy emulsions at 20°C (a) and 50°C (b).** Dots with different letters are significantly different ( $p < 0.05$ ,  $n=3$ ). Bold letters concern free  $\beta$ C and capital letters concern encapsulated  $\beta$ C.

At 50°C, free  $\beta$ C degradation was greater than at 20°C (fig.4b). Loss of free  $\beta$ C concentration was 28% after 48h of incubation and reached 59% after 72 h. Free  $\beta$ C was significantly more degraded than encapsulated  $\beta$ C especially after 48h of incubation. Indeed, loss of  $\beta$ C, when it was encapsulated in LNCs, was only 17 % after 48h and 22 % after 72h of ageing. LNCs size and interface composition might limit electrolyte penetration in LNCs shell and therefore oxidative species accessibility to  $\beta$ C (39). LNCs interface might also constitute a chemical protection thanks to phosphatidylcholine (PC) which is an effective singlet oxygen quencher in O/W emulsions (43). PC would thus act as a surface active antioxidant. However, LNCs protective effect is limited as it seems to delay and not stop oxidation processes. A thicker interfacial layer might be beneficial to decrease interfacial layer penetration and  $\beta$ C protection. The use of longer surfactant (brij 76 or POE(10) stearyl ether against brij 700 or POE(100) stearyl ether) seemed efficient to strengthen lipid protection against oxidation in o/w emulsion (44). It has also been shown that a multilayer interface could enhance lipid protection against oxidation (45) and that a more dense particle interface reduced  $\beta$ C



degradation during light exposure (46). Thus, LNCs are efficient to protect  $\beta$ C from oxidation in dairy emulsions conserved at 20 or 50°C, thanks to the presence of PLs in LNCs shell and the thickness of PEG stearate shell which might limit electrolytes penetration.



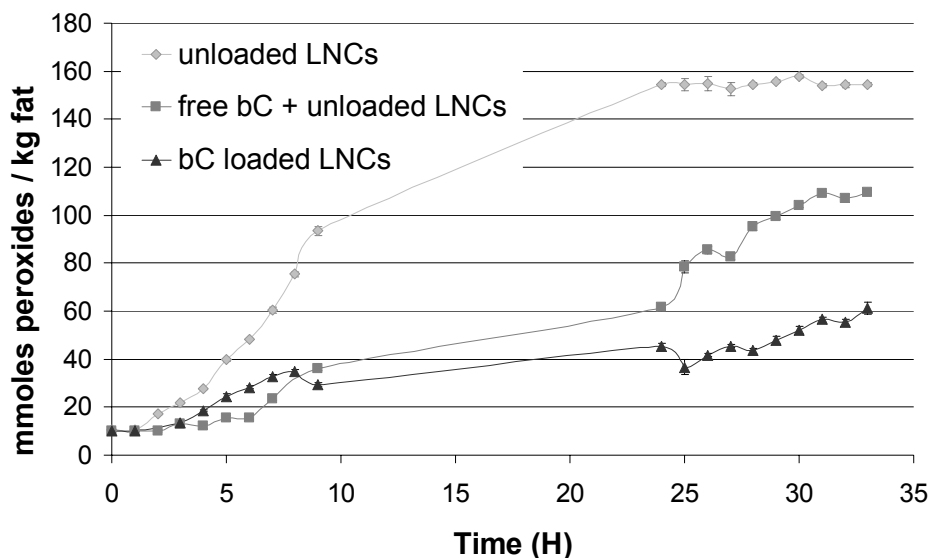
**Figure 5: Evolution of  $\beta$ C concentration in aqueous phase ( $\beta$ C loaded LNCs) and in lipid phase ( $\beta$ C release from LNCs + endogenous  $\beta$ C of milk fat) during 3 days of storage at 4°C. Bars with different letters are significantly different ( $p < 0.05$ ,  $n = 3$ ). Bold letters concern  $\beta$ C in aqueous phase and capital letters concern  $\beta$ C in lipid phase of dairy emulsion.**

As performed for chemical stability evaluation, efficiency of LNCs to limit  $\beta$ C diffusion in polyphasic matrix containing dispersed fat droplets was evaluated during 72h of storage. Temperature was fixed at 4°C to reduce  $\beta$ C degradation and experiments were performed in a model dairy emulsion to facilitate separation of oil and aqueous phases. Before storage (day 0),  $\beta$ C concentration measured in aqueous phase of model dairy emulsion corresponded to  $\beta$ C amount initially incorporated with LNCs (Fig. 5). Analysis of emulsion lipid phase revealed that dairy fat contained also a small amount of  $\beta$ C ( $0.02 \text{ mg} \cdot \text{g}^{-1}$  of emulsion). This concentration is in accordance with literature (37) and corresponds to the amount of  $\beta$ C naturally present in milk fat. After one day of incubation, a significant decrease of  $\beta$ C concentration in emulsion aqueous phase has been noticed passing from  $0.088 \text{ mg} \cdot \text{g}^{-1}$  to

0.078 mg.g<sup>-1</sup> emulsion. It was correlated with a significant increase of  $\beta$ C concentration in lipid fraction of emulsion (from 0.02 to 0.012 mg.g<sup>-1</sup> emulsion). For longer ageing time,  $\beta$ C concentration in emulsion aqueous phase was unchanged whereas a significant decrease of  $\beta$ C concentration was observed in emulsion lipid phase. Thus, we might conclude that a limited diffusion of  $\beta$ C from LNCs core to oil droplets has taken place and that equilibrium has been reached after one day. As observed for pure PLs membranes, which seemed to be fluidized after  $\beta$ C incorporation (23-25), this transfer might be influenced by  $\beta$ C loading effect on LNCs shell properties. Indeed, LNCs interface is composed of PEG stearate and PLs. A more fluid shell could also facilitate  $\beta$ C transfer from LNCs core to oil droplets. However, as  $\beta$ C transfer from LNCs to lipids droplets takes place only during 1 day and remains small after 3 days,  $\beta$ C might not increase LNCs shell fluidity in a way that could have a significant impact on its release. We can also notify that lipid fluidity is low under these experimental conditions (4°C). The lower lipid fluidity might limit  $\beta$ C diffusion from LNCs to emulsion oil droplet. The decrease of  $\beta$ C concentration in emulsion lipid phase after 1 day of ageing might be related to chemical degradation of  $\beta$ C as observed in fig.4 for free  $\beta$ C stored at 20°C in milk fat cream.

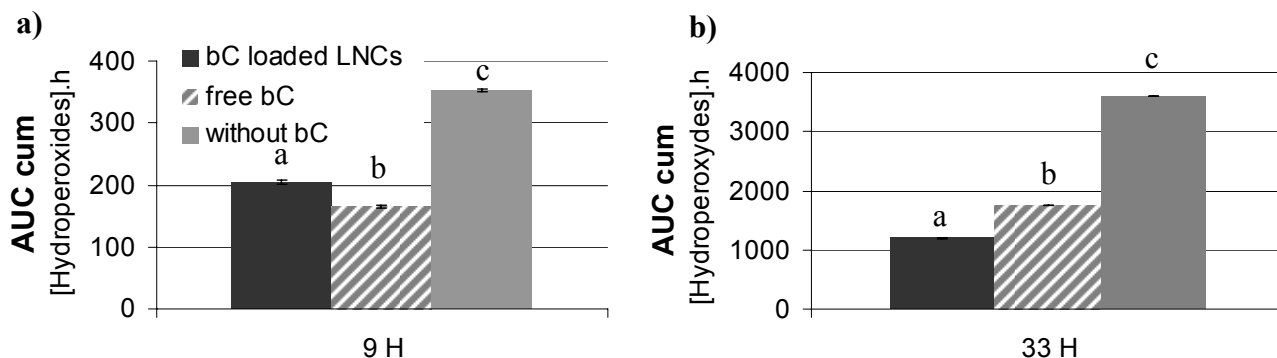
We can also conclude that encapsulation in LNCs is efficient to increase  $\beta$ C content as it remained in LNCs core even if its environment includes lipid droplets as in dairy emulsions. LNCs are also efficient to limit  $\beta$ C chemical degradation and protect  $\beta$ C from oxidation at 20 or 50°C thanks to LNCs shell composition and thickness.

### II-3 : Influence of $\beta$ C encapsulation on lipid oxidation of milk fat cream



**Figure 6:** Evolution of hydroperoxides concentration during ageing at 50°C of milk fat cream [n=3].

Lipid protection against oxidation by free or encapsulated  $\beta$ C was studied in milk fat cream under pro-oxidant conditions during 33 hours at 50°C (Fig.6). Whatever experimental conditions, hydroperoxides concentration increased with ageing time, indicating that lipids were oxidized. As expected, lipid oxidation was always more important without  $\beta$ C (unloaded LNCs) than when “free  $\beta$ C + unloaded LNCs” or “ $\beta$ C loaded LNCs” were added. When  $\beta$ C was included in LNCs, final lipid oxidation was lower than final lipid oxidation observed for emulsion with unloaded  $\beta$ C LNCs. AUCs analysis underlined that samples containing “free  $\beta$ C + unloaded LNCs” had greater protective effect than samples including  $\beta$ C loaded LNCs after 9H of incubation (fig. 7a)). After 33H of incubation  $\beta$ C loaded LNCs was more protective than “free  $\beta$ C + unloaded LNCs” (fig. 7.b)). These results demonstrated that  $\beta$ C loaded LNCs were the most efficient system to limit lipid oxidation in dairy emulsions submitted to pro-oxidant conditions.



**Figure 7: Cumulated Area Under hydroperoxides kinetics Curves (AUC cum) during 9 hours (a) or 33 hours (b) of ageing at 50°C in milk fat cream. [n=3].** AUC cum were calculated with Kinetica 4.4 [n=3]. Bars with different letters are significantly different ( $p < 0.05$ ).

Since no significant  $\beta$ C degradation was noticed between  $\beta$ C loaded LNCs and free  $\beta$ C during 33 hours at 50°C (fig. 4-b)), temperature effects on  $\beta$ C chemical stability can not explain the differences noticed in hydroperoxides formation. After 33H hours of incubation, lipid oxidation in emulsion was lower with  $\beta$ C loaded LNCs than with "free  $\beta$ C+ unloaded LNCs". One might be surprised by  $\beta$ C loaded LNCs greater protective effect, as they were dispersed in aqueous phase, while free  $\beta$ C was dissolved in lipids requiring protection. It has been shown previously that surfactant micelles containing an hydrophobic AO could delay lipid oxidation in emulsified lipids (47). Explanations for these results could be based on emulsion oxidation parameters or LNCs and fat globules characteristics ie: interfacial area with aqueous phase, diffusion of LNCs and fat globules in aqueous phase and  $\beta$ C diffusion in LNCs core or in milk fat globules.

Emulsion viscosities can have a major impact on lipid oxidation. Low viscosity increases collision rates and aqueous phase circulation around lipid droplets and therefore promotes oxidation (48). As Control and free  $\beta$ C samples were diluted with the same amount of unloaded LNCs as  $\beta$ C loaded LNCs group, viscosity should not play a significant role to explain the differences observed.

Most of pro-oxidant molecules such as iron and oxygen are solubilised in aqueous phase in emulsions. Thus, interfacial area plays an important role in oxidation processes. LNCs and milk fat globules might be considered as small and large lipid emulsion droplets respectively as LNCs are at least ten times smaller than milk fat globules. Therefore, LNCs have a greater surface/volume ratios ( $\frac{S}{V} = \frac{3}{4}R$ ) than fat globules. It has been shown that when lipid droplets size decrease (i.e. when interfacial area increase) and when oxidative reactant are in excess, oxidation rate increases (2). Pro-oxidant elements would have more chance to be scavenged by  $\beta$ C when it is included in LNCs.  $\beta$ C might be also more accessible in LNCs than in fat globules. Indeed, a small molecule such as hydroperoxide or peroxy radical can diffuse more rapidly and be more easily scavenged by  $\beta$ C in a sphere with a small diameter like LNCs than in larger lipid droplets (2). Besides, as fatty acids in LNCs have smaller chain length than most of dairy fatty acids, LNCs core might be more fluid than fat globules core. Therefore,  $\beta$ C could diffuse more easily in LNCs core.

Considering previous results, it was hypothesized that  $\beta$ C remained in lipid core and might not have other interfacial role than fluidizing properties. This parameter might affect free radical penetration in fat globule and therefore lipid oxidation over time. Milk fat globules are surrounded by a membrane constituted by PLs and proteins. This membrane might constitute a physical and chemical barrier against oxidation, by decreasing free radicals interaction with insaturated lipids as some PLs are known to have AO properties (43). PLs and  $\beta$ C combined properties might explain the low hydroperoxides formation during the first hours when  $\beta$ C was dissolved in fat globules.

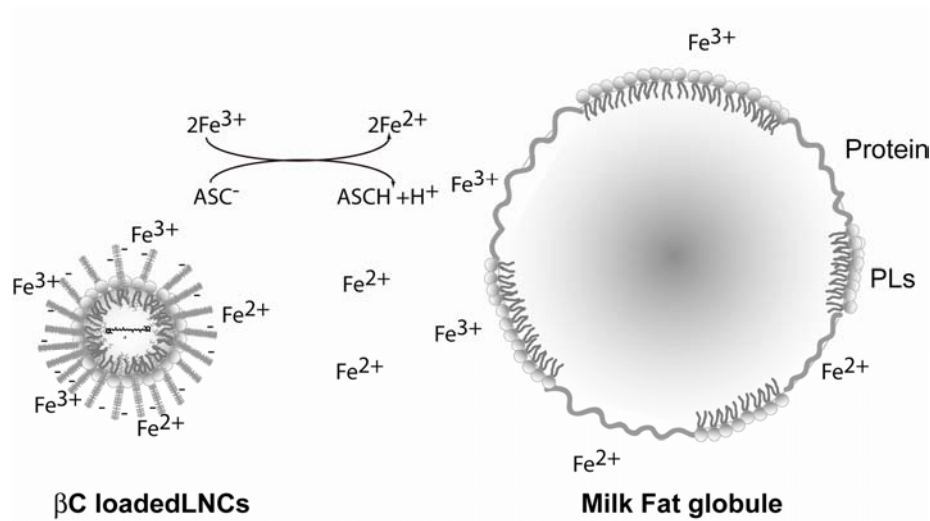
LNCs surface properties might also contribute to explain present results. Since LNCs  $\zeta$  potential is slightly negative, they might have a tendency to attract  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ . This phenomenon has previously been described with negatively charged sodium dodecyl sulphate (SDS) stabilized emulsion droplets (49;50). It has been demonstrated that  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  are able to interact with lipids included in droplets (51). Thus, divalent ion presence at o/w droplet interface is a

strong pro-oxidant factor (45). Pro-oxidant activity located in the aqueous phase might be more concentrated at LNCs surface. This would reduce oxidative stress around fat globules. Hence, free radicals might be more easily scavenged by  $\beta$ C loaded LNCs and oxidation process would be stopped. Unloaded LNCs, used for controls samples, might have the same potencies to attract iron but might have only a small ability to decrease oxidative stress thanks PLs free radicals scavenging properties.

We can also hypothesise that LNCs might be more easily surrounded by ionic Fe than fat globule because of their size and their surface charge (Fig. 8). This could explain the greater oxidation chain breaking observed with encapsulated  $\beta$ C and its ability to protect fragile emulsified lipids from oxidation.

### **III. Conclusion**

LNCs are able to protect  $\beta$ C from oxidation during storage.  $\beta$ C release of  $\beta$ C loaded LNCs during storage is limited in polyphasic matrix such as dairy emulsions. This might be due to a barrier effect created by PEG stearate shell and its composition.  $\beta$ C loaded LNCs are also able to protect sensitive elements dispersed in aqueous phase such as lipids globules from oxidation initiated by iron and ascorbate. This protective effect might be due to their high surface volume ratio compared to lipids droplets, and to their slightly negative charge.  $\beta$ C loaded LNCs ability to decrease lipid oxidation in emulsion could make them interesting food additives for food products, cosmetics or pharmaceuticals.



**Figure 8:** Hypothetic scheme of  $\beta\text{C}$  loaded LNCs role in protection of milk fat globules against oxidation induced by iron and ascorbate.

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## **CHAPITRE IV :**

### **EVALUATION DE LA BIODISPONIBILITE D'UN COMPLEXE LIPOPHILE RADIOMARQUE ENCAPSULE DANS LES NCLs**

## **Evaluation de la biodisponibilité d'un complexe lipophile radiomarqué encapsulé dans les NCLs**

Des enquêtes épidémiologiques ont mis en évidence une corrélation négative entre la consommation d'aliments riches en vitamines ou en antioxydants et la prévalence de certaines maladies comme le cancer, les maladies cardio-vasculaires ou certaines pathologies oculaires [1-5]. Cependant l'augmentation de la consommation d'aliments riches en l'un ou plusieurs de ces éléments ne conduit pas nécessairement à une augmentation de leur concentration plasmatique [6]. Cela peut s'expliquer par une absorption intestinale insuffisante, ce phénomène est d'autant plus grand avec les composés hydrophobes. Ainsi le  $\beta$ C, caroténoïde précurseur de la vitamine A, est une molécule lipophile aux propriétés anti-oxydantes dont l'absorption intestinale à partir de végétaux est variable et souvent faible avec, par exemple, une absorption de 3 à 5% à partir de purées de carottes [7], de 7% à partir de poêlée de légumes [8] et de  $23 \pm 12\%$  à partir d'épinards [8, 9]. Toutefois ces résultats sont étroitement liés à la diversité des méthodes employées pour déterminer l'absorption, le type de doses administrées (pharmacologique ou physiologique) ou encore la variabilité inter-individu. De plus, divers facteurs interviennent dans l'amélioration de l'absorption intestinale de molécules lipophiles comme la dispersion fine et la solubilisation des vitamines lipophiles et du  $\beta$ C avant ingestion [10]. Ces deux étapes agissent directement sur l'absorption intestinale des composés lipophiles et donc sur leur biodisponibilité. Ainsi, pour les caroténoïdes, plusieurs critères ont été listés et inclus dans un acronyme : SLAMENGHI, correspondant à (species, linkage, amount, matrix, effectors of absorption and bioconversion, nutrient status, genetic factors, host related factors, mathematical interactions (ou interaction entre les facteurs cités précédemment)[8]. La solubilisation permet de contourner le problème posé par les liaisons moléculaires ou « linkage ». Dans les végétaux, le  $\beta$ C est soit lié à une protéine (dans les chloroplastes) ou sous forme semi-cristalline (dans les tomates ou les carottes par exemple)

[11]. Dans les deux cas, ces interactions moléculaires sont des facteurs limitant de l'absorption intestinale. L'augmentation du fractionnement de la matrice ou l'altération des parois cellulaires par traitements thermiques, qui conduisent à une meilleure dispersion et une solubilisation accrue, permettent une libération du  $\beta$ C et une amélioration de la biodisponibilité [7].

Plusieurs stratégies ont été développées pour augmenter la biodisponibilité des vitamines lipophiles dont l'utilisation de systèmes particuliers. Les facteurs cités précédemment sont également applicables à ces particules. Certains systèmes augmentent surtout les quantités ingérées, ces systèmes agissent peu sur la biodisponibilité mais ils restent néanmoins efficaces pour augmenter les taux plasmatiques des molécules considérées [12]. D'autres particules ont un diamètre moyen important mais contiennent une phase lipidique capable de solubiliser une molécule lipophile : les particules produites par melt extrusion [13], les microcapsules lipidiques solides [14] ou encore les coacervats [15]. Au contraire, les dispersions fines fractionnent le  $\beta$ C sous forme cristalline [16, 17]. Des systèmes combinent ces deux aspects : les liposomes [18], les nanoémulsion [19-25], les nanoparticules lipidiques solides [26] ou encore les cyclodextrines [27]. Ainsi, les nanocapsules lipidiques (NCLs) possèdent ces deux caractéristiques puisque le  $\beta$ C est encapsulé par dissolution dans le cœur huileux et les particules produites sont de très petites tailles. Nous avons donc envisagé l'utilisation de ces systèmes particuliers pour améliorer l'absorption intestinale.

Idéalement l'étude de l'impact de l'encapsulation sur l'absorption intestinale de composés antioxydants lipophiles aurait dû être réalisée avec le  $\beta$ C. La méthode HPLC mise au point avait un seuil de sensibilité trop haut et donc non compatible avec un dosage précis des taux plasmatiques. D'autre part, la mise au point d'un dosage en LC-MS-MS du  $\beta$ C et de ses métabolites ou d'un marquage radioactif du  $\beta$ C (iode ou technétium) s'est révélée infructueuse. Nous avons donc procédé à l'évaluation des NCLs en utilisant un complexe

lipophile radiomarqué ( $^{99m}\text{Tc}$ -SSS) encapsulé dans la phase lipidique mimant ainsi le devenir d'une molécule lipophile quant à son absorption intestinale. L'effet particulière sur l'absorption intestinale pourra être identifié et comparé aux données disponibles *in vitro* sur le modèle cellulaire Caco-2 [28] et *in vivo* avec un composé anticancéreux lipophile [29]. D'autre part, la progression des NCLs dans le système gastro-intestinal sera évaluée à travers une étude de biodistribution.

## I. Matériels et méthodes

### I.1. Préparation du complexe [ $^{99m}\text{Tc}(\text{PHCS}_3)_2(\text{PHCS}_2)$ ] ( $^{99m}\text{Tc}$ -SSS)

La synthèse du complexe ( $^{99m}\text{Tc}$ -SSS) est une synthèse en deux étapes, avec passage par un intermédiaire de degré V chélaté par un ligand labile. L'ion pertechnétate [ $^{99m}\text{TcO}_4^-$ ] est produit par un générateur  $^{99}\text{Mo}/^{99m}\text{Tc}$  (Institut des Radio-Eléments, Fleurus Belgium) et élué dans du sérum physiologique. L'ion pertechnétate est réduit par du chlorure stanneux en présence de gluconate de sodium, cette réaction produit le complexe intermédiaire de degré V. La formation de ce complexe intermédiaire permet d'augmenter la cinétique de formation du complexe final. La synthèse du complexe final a été réalisée selon le protocole établi par Mevélec *et al.* [30]. La solution saline de pertechnétate (700 MBq dans 1 ml) est ajoutée à un kit gluconate lyophilisé composé par 7.5 mg de gluconate de calcium, 2.5 mg de chlorure de sodium et 0.075 mg de chlorure d'étain. Après 15 min d'agitation à température ambiante, 20 mg de dithiobenzoate de sodium (ligand, ENSC Rennes, France) sont ajoutés au milieu réactionnel. La solution est ensuite chauffée à 100°C pendant 30 min. Après refroidissement le complexe est extrait avec 1 ml de  $\text{CH}_2\text{Cl}_2$  (Sigma Aldrich, Ludwigshafen, Germany). La phase organique est prélevée puis lavée trois fois avec 1 ml d'eau ultra pure (système Milli-Q plus R, Millipore, Paris, France).

### I.2. Préparation des échantillons

Deux types de particules ont été formulées les NCLs de stéarate de PEG 15 de 100 nm et les NCLs de stéarate de POE 40, leur cœur lipidique incluant le complexe lipophile de  $^{99m}\text{Tc}$ -SSS. D'autre part le complexe de  $^{99m}\text{Tc}$ -SSS a été utilisé seul dans la phase huileuse des NCLs (labrafac<sup>®</sup>, Gattefossé, St Priest, France).

Les NCLs de PEG 15 et la solution témoin de  $^{99m}\text{Tc}$ -SSS dans le labrafac<sup>®</sup> (complexe 1) ont été réalisés avec la première solution de  $^{99m}\text{Tc}$ -SSS dans le  $\text{CH}_2\text{Cl}_2$ . Cette solution a été séparée en deux. La première moitié de la solution de  $^{99m}\text{Tc}$ -SSS dans le  $\text{CH}_2\text{Cl}_2$  est ajoutée à 9 ml de labrafac<sup>®</sup> puis le  $\text{CH}_2\text{Cl}_2$  est évaporé à 80°C sous agitation (complexe 1). L'autre moitié de  $^{99m}\text{Tc}$ -SSS dans le  $\text{CH}_2\text{Cl}_2$  est utilisée pour la formulation des NCLs. Les NCLs de PEG 15 stéarate (Solutol HS15, BASF, Ludwischafen, Germany) de 100 nm sont préparées selon la méthode d'inversion de phase décrite précédemment [31] : 486 mg de PEG 15 stéarate sont homogénéisés avec 75mg de lipoid<sup>®</sup> (Lipoid, Cham, Switzerland), 89 mg de NaCl (VWR Prolabo, Fontenay-sous-Bois, France), 1.18 g de labrafac<sup>®</sup> et 3.16 g d'eau ultra pure. Les proportions massiques en eau, stéarate de PEG 15 et en huile sont : 65/10/25. La moitié de la solution de  $^{99m}\text{Tc}$ -SSS dans le  $\text{CH}_2\text{Cl}_2$  est ajoutée au mélange et quatre cycles de température sont réalisés entre 60°C et 95°C à une vitesse de chauffe et de refroidissement de 8°C.min<sup>-1</sup> pour formuler les NCLs et évaporer la totalité du  $\text{CH}_2\text{Cl}_2$ . Lors de la quatrième descente de température, à 84°C, 10 ml d'eau ultra pure à 4°C ont été ajoutés à la formulation.

Les NCLs de stéarate de POE 40 et la solution témoin de  $^{99m}\text{Tc}$ -SSS dans le labrafac<sup>®</sup> (complexe 2) dans le labrafac<sup>®</sup> ont été réalisés avec la deuxième solution de  $^{99m}\text{Tc}$ -SSS dans le  $\text{CH}_2\text{Cl}_2$ . Cette solution a été ajoutée en totalité à 600µl de labrafac<sup>®</sup> puis chauffée à 80°C sous agitation jusqu'à évaporation complète du  $\text{CH}_2\text{Cl}_2$ . Les NCLs de POE 40 stéarate (Sigma Aldrich, St Quentin Fallavier, France) sont également formulées selon la méthode d'inversion de phase. Les cycles de température sont réalisés entre 35 et 98°C à une vitesse de chauffe et de refroidissement de 2°C.min<sup>-1</sup>. La trempe est réalisée à 45°C lors du troisième cycle avec

12.5ml d'eau à 4°C. Les proportions finales en eau, POE 40 stéarate et huile excipients sont 60/32/8 % (w/w), la concentration en sel de la phase aqueuse est augmentée de 5% pour les NCLs de PEG 15 stéarate à 34%. La proportion en lipid<sup>®</sup> reste inchangée. La phase huileuse, constituée par le complexe dilué dans le labrafac<sup>®</sup>, est ajoutée en deux temps selon la méthode utilisées pour l'encapsulation du  $\beta$ C (chapitre 1) [32]. La fraction restante de <sup>99m</sup>Tc-SSS/labrafac<sup>®</sup> est diluée dans 9 ml de labrafac<sup>®</sup> (complexe 2).

Les suspensions de NCLs sont ensuite filtrées sur filtres stériles (taille de pores 0.2  $\mu$ m, Millipore, St Quentin Fallavier France). Le diamètre moyen des particules ainsi que la mono dispersité de la suspension sont évalués par diffusion de la lumière à l'aide d'un Autosizer 4700 (Malvern Instruments S.A., Worcestershire, UK). Les échantillons sont dilués au 60<sup>ème</sup> dans de l'eau ultra pure. Les mesures sont réalisées à 25°C avec un indice de réfraction de 1.33 et une viscosité de  $0.89 \times 10^{-2}$  Pa.s.

### I.3. Expérimentation animale

Des rats Sprague-Dawley femelles de 8 semaines ( $310 \pm 23$  g) à jeun depuis 24 h ont été gavés avec 1 ml de suspension de NCLs de stéarate de POE 40 ou de solution de <sup>99m</sup>Tc-SSS/labrafac<sup>®</sup> et 1.6 ml de suspension de NCLs de stéarate de PEG 15. La dose gavée par rat était de 5 kBq. Les prélèvements sanguins ont été effectués à 15 min, 30 min, 1 h, 2 h, 3 h et 6 h. Trois prélèvements ont été réalisés par rat après anesthésie par voie gazeuse avec de l'isoflurane. Pour chaque type de formulation, 2 groupes de quatre rats ont été faits. Le premier groupe de rats de chaque série a été prélevé à 15min, 1h et 3h le deuxième à 30 min, 2 h et 6 h. Les deux premiers prélèvements ont été réalisés dans le sinus rétro-orbital et le dernier par ponction intracardiaque. Entre chaque prélèvement les rats ont été placés dans des cages à métabolisme individuelles où ils ont eu accès à l'eau *ad libitum*. Après le dernier



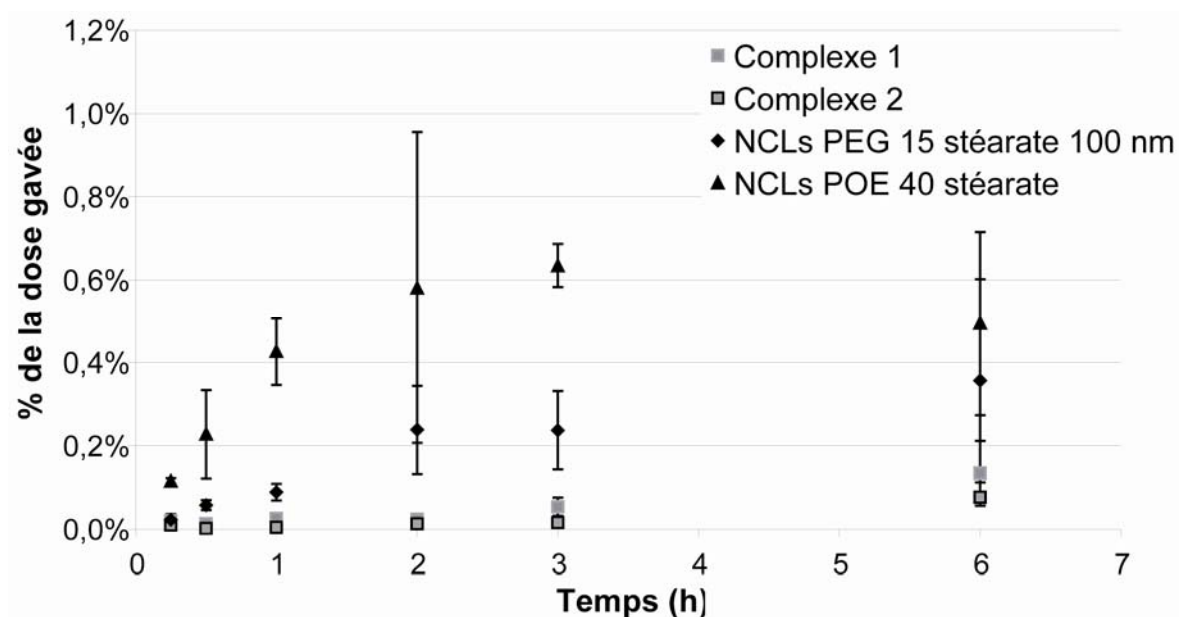
prélèvement sanguin (à 3 ou 6 h), les animaux ont été sacrifiés et différents organes (œsophage, estomac, intestin grêle, cæcum, gros intestin, reins, vessie, foie, rate et une fraction de carcasse) ont été prélevés. Chaque prélèvement a été pesé puis l'activité a été mesurée par un compteur gamma (Packard Auto-gamma 5000 series). Pour l'interprétation des données brutes, nous avons posé l'hypothèse que le sang représentait 7% du poids des animaux [33].

## II. Résultats et discussion

### II.1. Propriétés des NCLs

Les NCLs de stéarate de POE 40 présentent un diamètre moyen de 19 nm et un PDI de 0.269. La polydispersité des particules est plus élevée que pour les particules chargées en  $\beta$ C ou blanches. Les NCL de stéarate de PEG 15 ont un diamètre moyen de 95 nm et un PDI de 0.051. On peut considérer que les deux suspensions sont monodisperses.

### II.2. Cinétique sanguine



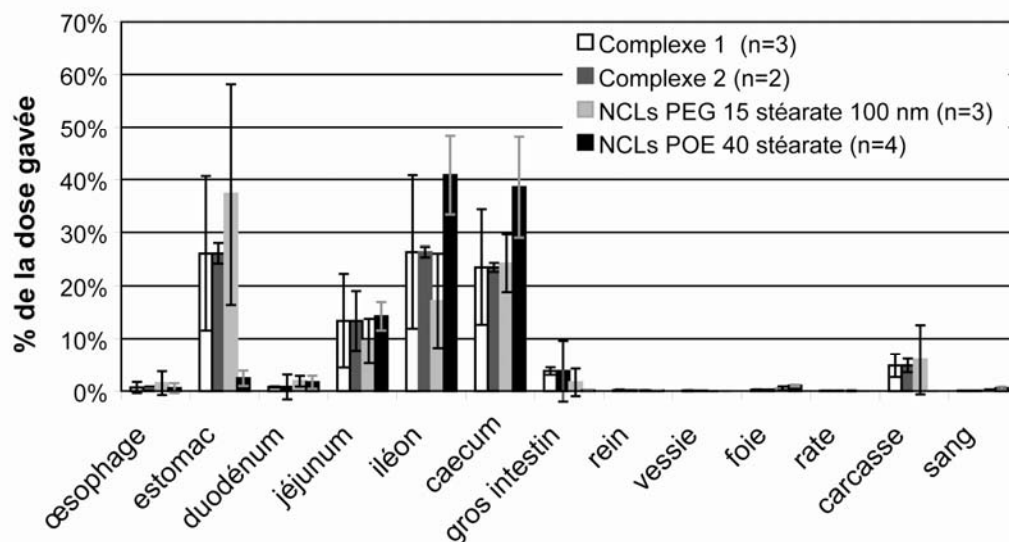
**Figure 1** : Cinétique sanguine du  $^{99m}\text{Tc}$ -SSS après gavage. (moyenne  $\pm$  écart-type)

Les résultats présentés ont été calculés pour l'ensemble du volume sanguin en pourcentage de la dose totale mesurée par animal. Pour les NCLs de POE 40 stéarate, le maximum est atteint après 3 h et correspond à  $0.6 \pm 0.05$  % de la dose gavée alors que pour les NCLs de PEG 15 stéarate de 100 nm, le maximum est atteint au temps 6 h avec  $0.36 \pm 0.24$  % de la dose gavée. Le complexe seul est lui présent dans le sang jusqu'à 6h avec  $0.13 \pm 0.08$  % ou  $0.077 \pm 0.013$  % pour les complexe 1 et 2 respectivement.

L'absorption du  $^{99m}\text{Tc}$ -SSS est donc plus importante lorsqu'il est encapsulé dans les NCLs que lorsqu'il est solubilisé dans l'huile. Cette différence d'absorption entre les NCLs et les groupes témoins mais aussi entre les deux types de NCLs (19 contre 95 nm de diamètre moyen) peut s'expliquer par la meilleure dispersion présentée par les NCLs pour laquelle la taille joue un rôle prépondérant. L'augmentation de l'absorption peut également être due à l'inhibition des pompes d'efflux telle que celle de la P-glycoprotein par les stéarates de PEG et de POE [34]. Quel que soit le système, l'absorption globale du  $^{99m}\text{Tc}$ -SSS reste faible pour tous les groupes. Il est possible que les NCLs bien qu'intactes ne soient pas absorbées ou que les NCLs soient digérées et que le  $^{99m}\text{Tc}$ , libéré sous forme complexée ou non, ne soit pas absorbé. Des travaux antérieurs concernant le paclitaxel ont mis en évidence une augmentation de l'absorption de l'anticancéreux à partir d'une suspension de NCLs de stéarate de PEG 15 50 nm [29]. Cette augmentation d'absorption à partir des NCLs en comparaison de préparations commerciales permettait une concentration plasmatique supérieure au seuil thérapeutique. Cependant, si on rapporte la concentration plasmatique à la dose gavée, la concentration plasmatique la plus élevée correspond à une absorption de 0.1% de la dose gavée à 30 min de l'anticancéreux. Tandis qu'avec les NCLs de POE 40, on observe un taux plasmatique équivalent à 0.2 % de la dose gavée à 30 min et de 0.6% à 3h. Ces résultats confirment que le taux d'absorption d'un xéniobiotique à partir des NCLs est faible. Les différences entre les taux d'absorption peuvent être dues aux caractéristiques

propres des molécules suivies (paclitaxel et  $^{99m}\text{Tc}$ -SSS) et aux caractéristiques des particules (taille ou nature du polymère). Les deux systèmes particuliers étudiés permettent cependant d'augmenter l'absorption du complexe par rapport aux témoins.

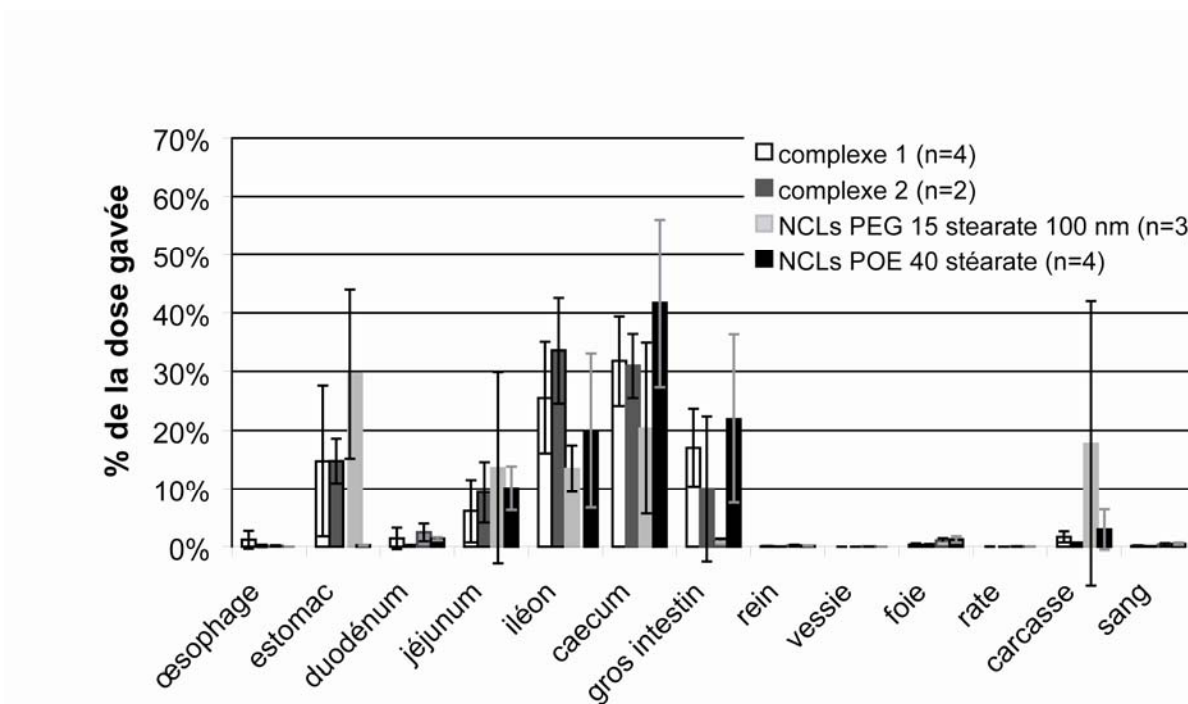
### II.3. Etude de biodistribution



**Figure 2** : Biodistribution 3h après le gavage ; (moyenne  $\pm$ écart-type)

Après 3h, la quasi-totalité de la dose est retrouvée dans le système digestif. Près d'un tiers de la dose est retrouvée dans l'estomac pour les groupes témoins (complexe 1 et 2) et pour les NCLs PEG 15 stéarate de 100 nm (figure 2). Le reste de la dose est retrouvée en ordre croissant dans le jéjunum, l'iléon, et le caecum. Pour les NCLs de POE 40 stéarate la dose est quasiment absente au niveau de l'estomac et est majoritairement détectée dans l'iléon et le caecum de manière équivalente et plus faiblement dans le jéjunum. Ces données mettent en évidence une différence importante de vitesse de vidange gastrique et de mobilité intestinale entre les différents systèmes. Dans les groupes témoins, le complexe est dilué dans une phase huileuse, ce paramètre peut expliquer une vidange gastrique lente [35]. Les NCLs de 100 nm restent dans l'estomac de manière équivalente aux témoins ce qui démontre

qu'avec une mobilité équivalente le système dispersé est plus efficace que la phase huileuse seule. Plusieurs paramètres peuvent influencer sur la mobilité gastro-intestinale, le volume de gavage, leur différence d'osmolarité, de taille [36]. Cependant après 3h, les effets du volume et de l'osmolarité doivent être mineurs [37].



**Figure 3 :** Biodistribution 6h après le gavage ; (moyenne  $\pm$ écart-type)

6h après le gavage, la majorité de la dose est dans le système digestif (figure 3), ces résultats confirment la faible concentration sanguine de  $^{99m}\text{Tc}$ -SSS pour tous les groupes. Pour les NCLs de POE 40 stéarate, la dose est retrouvée majoritairement dans le caecum, l'iléon et le gros intestin. Ces résultats corroborent la diminution de l'absorption observée à 6 h en cinétique sanguine (figure 1). Pour les NCLs de PEG 15 stéarate de 100 nm, la distribution de la dose est quasi identique à celle obtenue 3h après le gavage (30%). La mobilité des NCLs de PEG 15 stéarate de 100 nm dans le tractus gastro-intestinal semble très lente: la différence entre la dose moyenne dans l'estomac entre 3 et 6h est seulement de 7%. Pour les groupes témoins, la dose se retrouve principalement dans l'intestin grêle, et 10 à 17% de la dose totale est retrouvée dans le colon, la proportion contenue dans l'estomac est moins

importante (15%). Ces modifications de répartitions de la dose mettent en évidence la motricité digestive. 6h après le gavage les différences de mobilité des préparations dans le système digestif persistent.

L'absorption du  $^{99m}\text{Tc}$ -SSS est plus importante pour les NCLs que pour le complexe solubilisé dans l'huile. Les NCLs de stéarate de POE 40 associent une mobilité intestinale élevée et une absorption plus importante du  $^{99m}\text{Tc}$ -SSS. Les NCLs de stéarate de PEG 15 ont la mobilité intestinale la plus lente, l'absorption du  $^{99m}\text{Tc}$ -SSS est également augmentée par rapport au témoin. La dispersion de la phase lipidique a donc un effet positif sur l'absorption. Les NCLs de stéarate de PEG 15 présentant une progression plus lente dans le système digestif, n'ont pour autant pas d'absorption intestinale plus élevée comme le révèle la différence observée entre les deux types de NCLs après 6H. D'autre part, il est assez étonnant d'observer que la suspension de NCLs de stéarate de PEG 15 reste plus longtemps dans l'estomac que la solution huileuse.

Cette étude préliminaire met en évidence l'avantage lié à l'utilisation de systèmes nanodispersés pour lesquels la taille apparaît être un critère majeur. Un marquage plus proche de la molécule étudiée doit cependant être recherché pour pouvoir apprécier au mieux les différents paramètres intervenant dans le processus d'absorption intestinal.

### **III. Conclusion**

L'utilisation des NCLs étudiées permet l'augmentation de l'absorption de  $^{99m}\text{Tc}$ -SSS en comparaison du complexe libre. Cependant le taux d'absorption est très faible et la majorité de la dose gavée est toujours dans le système digestif après 6h. Pour confirmer ces résultats il serait judicieux d'augmenter le nombre de rat. Les différences entre les deux types de NCLs peuvent être dues à leur différence de taille ou de type de polymère. Il serait donc intéressant de réaliser ces expériences avec des NCLs de PEG 15 de 20 nm. Une étude sur

intestin *ex vivo* pourrait mettre en évidence la différence d'absorption à partir des différents types de NCLs. Il est également important de réaliser le même type d'étude avec le  $\beta$ C. D'autre part, les capacités des NCLs à augmenter l'absorption intestinale de nutriments ou de principes actifs lipophiles pourraient être optimisées en diminuant leur mobilité intestinale grâce à l'inclusion des NCLs dans une matrice [38, 39]. Cette matrice devra pouvoir diminuer la vitesse du transit et permettre la diffusion des NCLs.

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# **DISCUSSION GENERALE**

Les vitamines et les antioxydants ont un effet positif sur la prévalence de certaines maladies (cancers, maladies cardio-vasculaires ou certaines pathologies oculaires) [1-5]. Toutefois, un enrichissement de l'alimentation n'est pas toujours corrélé à une augmentation du taux plasmatique de ces composés [6]. En effet, certains micronutriments notamment les vitamines lipophiles ont une faible biodisponibilité. Dans le cas de composés liposolubles, plusieurs étapes peuvent freiner l'absorption intestinale : leur libération de la matrice, leur solubilisation dans la phase grasse, leur inclusion dans les micelles mixtes. De plus, les molécules ayant des propriétés antioxydantes sont sensibles à la lumière, aux températures élevées et à l'oxygène. L'encapsulation peut donc constituer à la fois un moyen de préserver leurs propriétés pendant le stockage, et de limiter l'influence de certains paramètres impliqués lors de l'absorption intestinale. L'analyse de la littérature montre que les systèmes particuliers les plus efficaces sont ceux qui combinent la solubilisation de la molécule d'intérêt dans une phase huileuse et une taille inférieure au micromètre.

Dans ce contexte, nous avons utilisé les nanocapsules lipidiques (NCLs), systèmes permettant l'encapsulation d'un composé lipophile fragile, le  $\beta$ -carotène ( $\beta$ C), dans des conditions compatibles avec une utilisation alimentaire. Ces particules présentent un diamètre moyen inférieur à 100 nm et un cœur lipidique liquide, capable de solubiliser des molécules d'intérêt. Ces deux spécificités font de ces particules des candidates idéales pour l'administration par voie orale. Les objectifs étaient de préserver le  $\beta$ C pendant le stockage et de le rendre accessible au niveau de la lumière intestinale pour améliorer son absorption. De plus, nous souhaitons incorporer ces nano-objets dans une matrice complexe, afin d'évaluer la stabilité du  $\beta$ C encapsulé et son aptitude à préserver les éléments fragiles de la matrice.

## **I. Conception de NCLs de grade alimentaire adaptées à l'encapsulation de composés fragiles**

### I.1. procédé de formulation

Le procédé de formulation classique, basé sur la méthode d'inversion de phase [7], a été modifié pour une application alimentaire et pour permettre l'encapsulation de composés lipophiles fragiles. Dans un premier temps, le polymère originel (PEG 15 stéarate de grade pharmaceutique) a été remplacé par un polymère de grade alimentaire, le stéarate de POE 40. Dans un second temps, la dégradation du  $\beta$ C dans différentes conditions de formulation a été évaluée. Il est apparu qu'une atmosphère appauvrie en  $O_2$  et en absence de lumière parvenait à préserver l'intégrité du  $\beta$ C pendant 30 min à 80°C.

Le stéarate de POE 40 a une température diversion de phase (TIP) plus élevée que le stéarate de PEG 15. En effet, la TIP augmente avec le nombre de groupement oxyéthylène [8]. Cette différence a impliqué des modifications du barème temps/température des cycles de chauffage et de refroidissement du procédé. L'addition de sel permet l'abaissement de la TIP, ce qui est plus compatible avec l'encapsulation de molécules fragiles. Cependant, la température élevée des cycles de températures a due être maintenue pour assurer le changement de conformation du stéarate de POE 40, permettre l'inversion de phase et un faible indice de polydispersité de la nanosuspension finale. La vitesse de chauffage et de refroidissement lente est également un paramètre critique pour la formation des NCLs. Ceci peut s'expliquer par la viscosité élevée du système. Par conséquent, les propriétés physicochimiques du stéarate de POE 40 ont, en premier lieu, déterminé les conditions de formulation des NCLs de grade alimentaire. L'addition de la phase huileuse en deux temps pour l'encapsulation du  $\beta$ C est une conséquence du barème temps/température employé pour la formulation de NCLs et des caractéristiques du  $\beta$ C.

L'optimisation du procédé de formulation des NCLs avec le stéarate de PEO 40 a donc permis d'encapsuler le  $\beta$ C avec un taux d'encapsulation compatible avec les études ultérieures. De plus, l'encapsulation permet la stabilisation chimique et physique du  $\beta$ C pendant deux mois à 4°C et à température ambiante.

## I.2. Stabilité des suspensions dans des milieux gastro-intestinaux simulés.

Dans cette partie, nous avons cherché à déterminer l'influence de milieux biologiques susceptibles d'influencer la stabilité des NCLs et le devenir du  $\beta$ C encapsulé au niveau de son site d'absorption. Cette étude a permis de mieux appréhender les mécanismes mis en jeu *in vivo*. Les NCLs formulées à l'aide des stéarates de POE 40 et de PEG 15 ont été incubées dans des milieux gastro-intestinaux simulant les conditions postprandiales [9]. L'évolution dans le temps des suspensions de NCLs a été évaluée par diffusion et transmission de lumière. Il est apparu que les NCLs étaient stables dans en milieu gastrique, duodéal et biliaire pris séparément. L'association des sels biliaires au milieu duodéal conduisait à la digestion des particules. Ceci a été mis en évidence par la disparition du pic de taille caractéristique des NCLs et la clarification du milieu. La présence de structures micellaires et vésiculaires dans les milieux de digestion nous permet de penser que le  $\beta$ C est inclus dans les micelles mixtes. La formation des micelles mixtes pourrait être rendue possible par la digestion des triglycérides composants le cœur des NCLs [10].

La couronne de polymères entourant les NCLs est connue pour limiter l'adsorption de protéines [11]. Ainsi, elle pourrait constituer une barrière stérique limitant l'adsorption des enzymes. Ce phénomène peut expliquer la stabilité des NCLs en présence du milieu duodéal seul. En revanche, du fait que les acides biliaires sont des surfactants puissants, il est probable qu'ils puissent seuls modifier la composition de l'interface sans modifier la distribution de

taille des NCLs. Cette activité interfaciale des acides biliaires semble nécessaire pour modifier l'arrangement des surfactants à l'interface, et rendre la surface des NCLs plus accessible aux enzymes tels que les lipases. Cette hypothèse est en accord avec des travaux antérieurs portant sur la stabilité d'émulsions. Ceux-ci ont en effet mis en évidence l'inhibition de l'activité enzymatique des lipases par différents surfactants (tween 20 ou 80 (polyoxyéthylènesorbitan monolaurate ou monooleate), ou phospholipides) [12-14]. L'activité interfaciale des acides biliaires sur la stabilité des NCLs pourrait être clarifiée par des mesures de tensiométrie à l'interface huile/eau.

## **II. Caractérisation de l'interface des NCLs**

Contrairement aux NCLs de stéarate de PEG 15, le diamètre moyen des NCLs de POE 40 évolue pendant le stockage (chapitre 1). Nous avons cherché à comprendre si la composition interfaciale des NCLs de POE 40 pouvait expliquer le mûrissement d'Ostwald observé. Les propriétés interfaciales de chaque constituant des NCLs de POE 40 ont donc été étudiées par des mesures rhéologiques bidimensionnelle, à l'interface eau/huile, par la méthode de la goutte montante.

L'étude rhéologique a mis en évidence que le stéarate de POE 40 est le constituant des NCLs qui a le plus d'influence sur la tension interfaciale. La monocouche de stéarate de POE 40 est purement visqueuse et présente un comportement de type liquide [15]. Lors de la compression de l'interface, de nombreux réarrangements des chaînes de POE ont lieu à l'interface et conduisent à l'expulsion des segments polymères vers la phase aqueuse. Le lipoid<sup>®</sup> conduit à la rigidification de l'interface ce qui s'explique par la présence de phospholipides ayant un groupement polaire phosphocholine et des chaînes grasses insaturées [16]. Les interactions hydrophobes entre le stéarate de POE 40 et le lipoid<sup>®</sup> à l'interface permettent une meilleure cohésion de la couche et une diminution des réarrangements lors de

la compression. Le film mixte est donc plus stable que les monocouches simples. Ces résultats concordent avec des études précédentes montrant le rôle important du lipoid<sup>®</sup> dans la stabilité des NCLs stabilisées par du stéarate de PEG 15 [17].

Le  $\beta$ C s'adsorbe à l'interface huile/eau pure et présente donc un caractère tensio-actif marqué. Il modifie également les propriétés des films de lipoid<sup>®</sup> et de stéarate de POE 40, probablement du fait de son orientation interfaciale. Cependant, sa présence dans des films mixte lipoid<sup>®</sup>/stéarate de POE 40 modifie peu les qualités rhéologiques de l'interface. Ce résultat est en accord avec la stabilité dans le temps des NCLs de stéarate de PEG 15 chargées en  $\beta$ C.

Le stéarate de POE est aussi efficace pour abaisser la tension interfaciale que le stéarate de PEG 15 [18]. Bien que le mûrissement d'Ostwald soit fortement influencé par ce paramètre [19, 20], la faible valeur de tension interfaciale du film lipoid<sup>®</sup>/stéarate de POE 40 ne doit pas être à l'origine du phénomène de déstabilisation. L'effet observé pourrait être plutôt relié à la taille de la partie hydrophile du stéarate de POE 40, qui est beaucoup plus grande que celle du stéarate de PEG 15. Ainsi, les interactions entre les chaînes hydrophiles du stéarate de POE 40 pourraient être favorisées au détriment de la cohésion moléculaire à l'interface, ce qui pourrait entraîner le transfert de l'huile dispersée. L'augmentation de la proportion de lipoid<sup>®</sup> dans les NCLs de stéarate de POE 40 pourrait augmenter la cohésion entre les molécules à l'interface et limiter le mûrissement d'Ostwald.

### **III. Comportement des NCLs dans une matrice alimentaire modèle**

Dans cette partie, les NCLs ont été caractérisées dans un milieu complexe : la crème fraîche liquide. Il est essentiel de savoir si les propriétés mises en évidence sur des suspensions des NCLs dans l'eau sont conservées lorsque le milieu extérieur contient des

éléments potentiellement oxydants et une phase lipidique dispersée. Le système dispersé complexe retenu, du fait de la présence d'acides gras insaturés fut un bon modèle pour évaluer la stabilité chimique du  $\beta$ C encapsulé et l'influence des NCLs sur la stabilité du produit.

Cette étude a mis en évidence que l'encapsulation dans les NCLs augmentait la stabilité du  $\beta$ C en milieu complexe à 20 ou à 50°C. Les NCLs ont donc une structure qui permet la protection d'éléments fragiles tels que le  $\beta$ C bien que leur petite taille augmente le rapport surface/volume. L'interface des NCLs peut constituer une barrière physique et limiter les interactions entre le  $\beta$ C inclus dans le cœur et les éléments pro-oxydants de la phase aqueuse. De plus, l'interface des NCLs contient des phospholipides qui présentent une capacité à piéger les radicaux libres [21]. Les phospholipides de l'interface peuvent donc former une barrière chimique. Il serait intéressant de réaliser cette étude avec les NCLs de POE 40, car la longueur de chaîne du polymère augmente la protection contre l'oxydation [22]. L'effet barrière de la couronne de stéarate de POE 40 pourrait améliorer la protection du  $\beta$ C déjà observée avec le stéarate de PEG 15.

La libération du  $\beta$ C d'une structure dans laquelle il est dissout dépend de la composition du milieu extérieur. Si la phase externe contient des structures de types micellaires ou des éléments pouvant s'associer pour solubiliser le  $\beta$ C tels que des micelles de SDS (données internes) ou d'acides biliaires et d'acides gras [10], le  $\beta$ C est libéré de sa phase dispersée. Avec les NCLs, seul 10% du  $\beta$ C encapsulé est libéré en 24 heures à 4°C et est solubilisé dans les globules gras de la matière grasse laitière. Ceci peut s'expliquer par la couronne pégylée entourant les NCLs qui crée une barrière stérique, limitant les interactions avec les globules gras et par la température faible qui limite la fluidité des phases lipidiques. Les NCLs permettent donc de compartimenter le  $\beta$ C dans une phase donnée d'un produit complexe. L'absence de système de type micellaire solubilisant le  $\beta$ C et pouvant faciliter le transfert d'une structure à l'autre peut expliquer que la libération demeure faible.

Nous avons cherché à savoir si le  $\beta$ C, une fois encapsulé, continuait à avoir un effet protecteur sur les éléments fragiles de la matrice : les lipides des globules gras de la crème. En effet, ses propriétés antioxydantes sont importantes *in vivo* : elles permettent de protéger des éléments fragiles de l'oxydation [3, 23]. Il a été également montré que le  $\beta$ C était également efficace pour protéger, *in vitro*, certains constituants de particules [24-26].

Nos résultats montrent que le  $\beta$ C seul protège les lipides contre l'oxydation, mais que cet effet est plus efficace après nanoencapsulation. Cela peut s'expliquer par les caractéristiques des NCLs. De par leur petite taille, le rapport surface/volume des NCLs est plus grand que pour les globules gras de la crème, ce qui augmente les interactions avec les initiateurs d'oxydation contenu dans la phase continue ( $\text{Fe}^{2+}$ ). De plus, les NCLs ont un potentiel  $\zeta$  négatif et la couronne de polymères est totalement pénétrable aux ions comme le démontre les études de mobilité électrophétique (chapitre 2). Ces deux particularités pourraient augmenter la concentration en éléments pro-oxydants autour des NCLs et donc diminuer le stress oxydant autour des globules gras.

La nanoencapsulation permet donc d'enrichir une matrice en micronutriments tout en protégeant ses éléments fragiles contre l'oxydation. Ces propriétés peuvent être mises à profit pour des applications en agro-alimentaire et en cosmétique, en utilisant par exemple des NCLs chargées en vitamine E ou en  $\beta$ C comme éléments protecteurs d'éléments actifs d'une crème.



#### IV. Approche *in vivo*

Dans cette partie, nous avons cherché à évaluer la capacité des NCLs à augmenter l'absorption par voie orale d'un composé lipophile. Les NCLs de stéarate de POE 40 et de PEG 15 de 100 nm ont été marquées par un complexe lipophile de  $^{99}\text{Tc}$ , le  $^{99}\text{Tc}$ -SSS. Ces NCLs ont ensuite été administrées chez le rat par gavage. Une étude de biodisponibilité et de biodistribution a été réalisée.

Il apparaît que le  $^{99}\text{Tc}$ -SSS est le plus absorbé à partir des NCLs de stéarate de POE 40 et le moins absorbé quand il est dissous dans le labrafac<sup>®</sup>. La dispersion plus fine de la phase huileuse due aux particules pourrait expliquer l'absorption plus élevée observée pour les deux types de NCLs. De plus, la présence de polymères pégylés peut inhiber une partie des pompes d'efflux et renforcer cet effet [27]. Le taux d'absorption pour les NCLs de stéarate de POE 40 est faible (0.6%) et la majorité de la dose administrée est toujours présente dans le système digestif après 6h (95%). Ce taux d'absorption faible s'avère néanmoins plus important que celui obtenu dans une étude précédente avec des NCLs de PEG 15 de 50 nm chargées en paclitaxel (0.1%) [28]. Toutefois, le  $^{99}\text{Tc}$ -SSS et le paclitaxel ont une perméabilité intestinale faible. On peut donc espérer que l'absorption intestinale du  $\beta\text{C}$  à partir des NCLs soit plus importante que pour ces molécules.

Le suivi du marqueur radioactif semble indiquer que les NCLs de stéarate de POE 40 ont la mobilité gastro-intestinale la plus rapide. Afin de ralentir la vitesse de circulation dans l'intestin grêle, ce qui pourrait favoriser l'absorption intestinale [29, 30], il serait intéressant d'étudier l'effet matrice. Cela pourrait être réalisé à partir de la matrice modèle déjà étudiée ou à l'aide de gels protéo-saccharidiques, qui sont des constituants majeurs de matrices complexes alimentaires.

Ce travail constitue une étude préliminaire qui gagnera en précision par une augmentation du nombre de rats par groupe. L'extrapolation de ces résultats pour le  $\beta\text{C}$  étant

limitée, une étude des NCLs chargées en  $\beta$ C est nécessaire à l'optimisation de ces systèmes. Le marquage du cœur de la particule et d'un élément de la coque, combiné au suivi du  $\beta$ C et de ses métabolites dans le sang, pourrait mettre en évidence l'impact de l'encapsulation sur la biodisponibilité du  $\beta$ C et déterminer si les NCLs sont intactes au niveau de l'intestin.

En outre, l'inclusion des NCLs dans une matrice pourrait diminuer la vitesse de mobilité gastro-intestinale et ainsi optimiser leur capacité à améliorer l'absorption intestinale de composés lipophiles. Une matrice contenant des acides gras à longue chaînes comme la matière grasse laitière augmenterait également la concentration plasmatique en  $\beta$ C et en esters de rétinol en stimulant la sécrétion de chylomicrons [31-36].

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# **CONCLUSION ET PERSPECTIVES**

Ce travail de thèse a mis en évidence l'intérêt de l'encapsulation d'une molécule fragile modèle ayant des propriétés anti-oxydantes comme le  $\beta$ -carotène ( $\beta$ C) pour des applications alimentaires. Le procédé d'encapsulation dans les nanocapsules lipidiques (NCLs) permet la préservation de l'intégrité chimique du  $\beta$ C. Les particules produites ont la capacité de protéger le  $\beta$ C contre l'oxydation pendant le stockage en suspension aqueuse ou après incorporation dans une matrice laitière. La libération du  $\beta$ C des NCLs est nulle dans une suspension aqueuse et limitée dans la matrice laitière. Cette libération peut être modulée, si nécessaire, par les propriétés du milieu extérieur. De plus, le  $\beta$ C encapsulé apparaît plus efficace pour protéger des éléments fragiles de la matrice contre l'oxydation que le  $\beta$ C libre. Le diamètre moyen des NCLs de grade alimentaire augmente pendant le stockage, ce qui peut s'expliquer par un murissement d'Ostwald. Les études interfaciales suggèrent que le stéarate de POE 40, le constituant majeur de la coque des NCLs, est impliqué dans ce phénomène de maturation. Dans les milieux de digestion simulés, les NCLs sont apparues stables aux variations de pH. La lyse enzymatique semble nécessiter la présence d'acides biliaires permettant la déstabilisation de l'interface et la diminution de l'encombrement stérique. Chez le rat, les NCLs semblent permettre une augmentation de l'absorption intestinale d'un xénobiotique lipophile, et ce malgré une forte rétention du composé dans le système digestif. L'inclusion des NCLs dans une matrice permettant la diminution de la vitesse de transit pourrait optimiser leur potentialité à augmenter l'absorption intestinale. Cette étude doit évidemment être complétée par des données concernant les NCLs chargées en  $\beta$ C.

Les applications possibles de ces particules peuvent concerner en premier lieu la nutrition médicale. Les produits de nutrition médicale doivent répondre à des besoins physiologiques particuliers. Cette branche de l'industrie agro-alimentaire et de la pharmacie pourrait bénéficier de cette technologie innovante pouvant s'appliquer à d'autres composés

lipophiles fragiles. Par ailleurs, la capacité du système NCLs/ $\beta$ C à protéger des éléments fragiles de la matrice extérieure pourrait être appréciée dans le domaine des cosmétiques.

Ce procédé de formulation peut être appliqué à d'autres molécules lipophiles fragiles. Ainsi, nous avons validé l'encapsulation de l'acide rétinoïque grâce aux méthodes développées pour le  $\beta$ C. Ce système peut avoir des applications différentes étant données les propriétés thérapeutiques de l'acide rétinoïque dans le domaine des maladies de peau ou dans le traitement de certains cancers.





## **Résumé**

Ce travail de thèse porte sur l'encapsulation du  $\beta$ -carotène ( $\beta$ C) dans des nanocapsules lipidiques (NCLs) formulées avec des constituants de grade alimentaire, afin d'augmenter sa biodisponibilité. Dans un premier temps, nous avons mis en évidence, *in vitro*, que les NCLs étaient dégradées par l'association de sels biliaires et d'enzymes pancréatiques. La déstabilisation des NCLs pourrait rendre le  $\beta$ C accessible à l'absorption grâce à son inclusion dans des structures de type micellaire. Dans un deuxième temps, la modélisation des propriétés rhéologiques et électriques de l'interface des NCLs a été réalisée par tensiométrie et zétamétrie. Ces études ont montré que l'encapsulation du  $\beta$ C n'avait pas d'impact sur les propriétés rhéologiques et électrophorétiques de l'interface. Une étude sur matrice modèle (émulsion alimentaire complexe) a montré que l'inclusion du  $\beta$ C dans les NCLs protégeait le  $\beta$ C et augmentait ses propriétés antioxydantes. Enfin, des études *in vivo* chez le rat ont souligné que l'encapsulation d'un composé radiomarqué lipophile augmentait son absorption intestinale. Ce système devrait par conséquent augmenter la biodisponibilité du  $\beta$ C grâce à sa solubilisation dans le cœur lipidique et la dispersion fine de la phase huileuse dans un milieu digestif.

**Mots-clés** : Biodisponibilité, encapsulation,  $\beta$ -carotène, antioxydant, nanocapsules lipidiques.

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## **Abstract**

This thesis concerns  $\beta$ -carotene ( $\beta$ C) encapsulation in lipid nanocapsules (LNCs) in food grade conditions to improve its bioavailability. First, *in vitro* studies demonstrated that the combination of bile acids and pancreatic enzyme destabilised LNCs structure. NCLs disruption might make  $\beta$ C accessible to intestinal absorption thanks to the presence of micellar type structures. Then, LNCs interface rheological and electrochemical modelling, performed by tensiometry and zetametry, revealed that  $\beta$ C inclusion in LNCs did not influence LNCs interfacial properties. Afterwards, a study on a model matrix (complex food emulsion) showed that encapsulation protect  $\beta$ C from oxidation and enhanced its antioxidant potencies. Finally, *in vivo* studies on rats revealed that encapsulation of a radiolabelled component enhanced its intestinal absorption. These particles should improve  $\beta$ C bioavailability thanks to its solubilisation in NCLs lipid core and the fine dispersion of lipid fraction.

**Keywords**: Bioavailability, encapsulation,  $\beta$ -carotene, antioxidant, lipid nanocapsules

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