



Formulation et caractérisation de liposomes porteurs de glycolipides synthétiques : application au ciblage d'*Helicobacter pylori*

Pierre-Louis Bardonnnet

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HAL Id: tel-00382031

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THESE
présentée
devant l'UNIVERSITE CLAUDE BERNARD - LYON 1
pour l'obtention
du DIPLOME DE DOCTORAT en CO-TUTELLE
(arrêté du 6 janvier 2005)

présentée et soutenue publiquement le 3 décembre 2007

par

Mr. BARDONNET Pierre-Louis

TITRE :

Formulation et caractérisation de liposomes porteurs de glycolipides synthétiques.
Application au ciblage *d'Helicobacter pylori*.

Directeurs de thèse :

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Dr. Vincent FAIVRE

THESE
présentée
devant l'UNIVERSITE CLAUDE BERNARD - LYON 1
et l'UNIVERSITE DE GENEVE
pour l'obtention
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d'Helicobacter pylori.

BARDONNET Pierre-Louis

A Sandrine, Eliott et à notre futur fils.

A mes parents, mon frère et ma sœur.

A toute ma famille.

A mes amis.

A Madame le professeur Françoise Falson et à Monsieur le professeur Jean-Claude Piffaretti.

Merci de m'avoir fait l'honneur de diriger cette thèse. Veuillez trouver ici le témoignage de ma reconnaissance et de mon amicale considération.

Au docteur Vincent Faivre.

Merci pour votre aide précieuse, votre soutien, votre disponibilité. Soyez assuré de ma profonde gratitude et de toute mon amitié.

Au professeur Eric Doelker.

Pour avoir spontanément accepté le rôle de répondant auprès de la section de pharmacie de l'Université de Genève. Je vous prie de bien vouloir croire à l'expression de mes sentiments les plus amicaux.

Aux professeurs Véronique Prémat, Paolo Colombo, Gilles Ponchel ainsi qu'au docteur Paul Boullanger.

Pour votre rôle de juré et/ou de rapporteur au près de l'Université Lyon I. Veuillez trouver ici le témoignage de ma sincère reconnaissance.

Table des matières

Introduction.....	5
Chapitre I : Gastroretentive dosage forms: overview and special case of <i>Helicobacter pylori</i>	11
Chapitre II : Self-organization of synthetic cholesteryl oligoethyleneglycol glycosides in water.....	66
Chapitre III : Cholesteryl oligoethyleneglycol glycosides: fluidising effect of their embedment into phospholipid bilayers.....	90
Chapitre IV : Pre-formulation of liposomes against <i>Helicobacter pylori</i> : part I – pH effects.....	108
Chapitre V : Pre-formulation of liposomes against <i>Helicobacter pylori</i> : part II – characterisation and interaction with the bacteria.....	135
Discussion générale – Conclusion.....	164
Annexe.....	175

INTRODUCTION

En 2005 le prix Nobel de médecine a été décerné aux anatomo-pathologistes J. Robin Warren et Barry Marshall « pour leurs travaux princeps sur le rôle pathogène pour la muqueuse de l'estomac et du duodénum d'une bactérie à Gram négatif, l'*Helicobacter pylori* (*H. pylori*) ». Leur découverte de 1982 remet complètement en cause le dogme selon lequel l'ulcère gastrique serait lié au stress, et incrimine une bactérie jusque là inconnue : *H. pylori*. Cette bactérie Gram négative doit son nom à sa forme en hélice et à sa niche écologique, l'estomac. Elle mesure de 2,5 à 5 µm de long et de 0,5 à 1 µm de large et peut posséder jusqu'à 7 flagelles engainées qui lui confèrent une mobilité dans le liquide stomacal ^[1,2]. Son cytoplasme dense contient le matériel nucléaire, des ribosomes, et a une structure de paroi typique des bactéries à Gram négatif avec une membrane externe et une membrane cytoplasmique séparées par un espace périplasmique, le tout d'une épaisseur de 30 nm ^[3].

H. pylori est responsable de la maladie ulcéreuse, affection fréquente, se compliquant souvent d'hémorragie, de perforation ou de sténose pylorique, et était responsable de nombreuses hospitalisations et d'une mortalité préoccupante pour une maladie bénigne. Depuis 1994, *H. pylori* est considéré par l'I.A.R.C (International Agency for Research on Cancer) et l'O.M.S (Organisation Mondiale de la Santé) comme carcinogène de type 1. Le risque de cancer gastrique augmente de façon linéaire avec la charge bactérienne en *H. pylori*. Enfin, plus ancienne est l'infection, plus grand est le risque de cancer gastrique ^[4]. Les pathologies consécutives à une infection chronique à *H. pylori* sont nombreuses. On retrouve parmi elles la dyspepsie, la gastrite, l'ulcère duodénal et l'ulcère gastrique ainsi que deux formes de cancers : le lymphome de type MALT et l'adénocarcinome. Le lymphome de type MALT (Mucosa Associated Lymphoid Tissue) est un lymphome à petites cellules, développé aux dépens des tissus lymphoïdes des annexes muqueuses, secondaire à une stimulation excessive et prolongée des lymphocytes B en réponse à l'infection à *H. pylori*. L'adénocarcinome est un néoplasme malin, se développant à partir des revêtements muqueux, des canaux excréteurs, des glandes exocrines ou des parenchymes glandulaires eux-mêmes.

Le mécanisme exact de la transmission de l'infection n'est toujours pas clairement établi. L'hypothèse la plus vraisemblable est celle d'une transmission inter-humaine, féco-orale (en particulier par l'intermédiaire des eaux de surface, dans le cas des diarrhées et des eaux non traitées) oro-orale (salive de sujets infectés) ou gastro-orale (vomissures) ^[5,6], comme le suggère la distribution de l'infection (en foyer) et sa prévalence liée à la promiscuité et à des conditions sanitaires médiocres. Bien que plusieurs animaux (porc, chat, mouton, singe) aient été considérés comme des réservoirs potentiels ^[6], on sait aujourd'hui que la muqueuse gastrique humaine est le réservoir exclusif de la bactérie ^[5]. En effet, une meilleure connaissance des bactéries des muqueuses du tractus digestif nous a montré ces dernières années que de nombreuses espèces animales possédaient leur propre espèce

d'*Helicobacter* et que chaque espèce bactérienne était quasi exclusive de son hôte ^[7]. Dans les pays en voie de développement où le système d'assainissement de l'eau n'est pas assez performant voire inexistant, une contamination féco-orale est tout à fait envisageable. De plus il existerait une synergie entre l'infection gastrique à *H.pylori* qui, par l'hypochlorhydrie qu'elle provoque dans sa phase initiale, faciliterait le passage des pathogènes entériques, et d'autre part les diarrhées, qui, par diminution du temps de transit, favoriserait l'élimination de *H.pylori* sous forme viable et donc le risque de transmission. Dans les pays développés où les logements sont plus grands, les familles plus petites, les diarrhées peu fréquentes et où « l'hygiène des selles » et le traitement de l'eau sont efficaces, la probabilité d'une telle transmission semble relativement faible ^[5]. En ce qui concerne la voie oro-orale et gastro-orale, le « turn-over » de la muqueuse gastrique est relativement rapide (2 à 3 jours). Les vomissures (voie gastro-orale) contiennent ce mucus. On retrouve par conséquent un grand nombre de bactéries dans les vomissures. En outre, il y a aérosolisation de *H.pylori* lors des vomissements, facilitant encore sa dissémination. La salive (voie oro-orale) contient elle aussi des bactéries, mais en nombre inférieur. La contamination de la salive se fait sans doute à l'occasion de vomissements ou de régurgitations, le liquide gastrique pouvant alors atteindre la bouche. La transmission d'*H. pylori* par voie oro-orale ou gastro-orale est un mode de transmission que l'on rencontre aussi bien dans les pays en développement que dans les pays développés, mais avec des fréquences différentes. En effet, la surpopulation, la promiscuité, les vomissements fréquents et le manque d'hygiène dans les pays en développement favorisent fortement la contamination. Mais dans les pays développés, une contamination oro-orale ou gastro-orale est tout à fait plausible, par exemple chez les enfants portant tout à la bouche, ou par les vomissements et/ou salive des parents à l'enfant et entre enfants. De plus, certains métiers qui impliquent un contact avec le liquide gastrique augmentent le risque d'infection à *H. pylori*.

Les risques de transmission bactérienne durant l'enfance étant étroitement liés aux conditions socio-économiques impliquant des conditions de vie différentes des populations (conditions d'hygiène, promiscuité entre les individus), il est aisé de comprendre les disparités géographiques qui existent dans la prévalence de l'infection à *H. pylori* ^[8]. Cette bactérie est présente sous toutes les latitudes et sa prévalence est élevée, puisqu'en moyenne plus de 50% de la population mondiale est porteuse de la bactérie, cependant la prévalence dans les pays en voie de développement est de l'ordre de 50 à 90%, contre seulement 5 à 20% dans les pays développés ^[2,5,6,9].

Afin de lutter contre ce pathogène et les maladies qu'il peut entraîner, la recherche du meilleur traitement a fait l'objet de très nombreuses études depuis 1986. Les monothérapies n'ont jamais été vraiment efficaces et il a fallu se tourner vers la mise au point d'associations. Les premières, relativement efficaces, étaient des associations de dérivés du bismuth à un

ou deux antibiotiques. Elles ont été remplacées progressivement par des associations d'antisécrétoires et d'antibiotiques dont les caractéristiques ont été affinées d'année en année. En 1995, la conférence de consensus française est venue officialiser les tendances qui s'étaient peu à peu détachées, suivie en 1997 d'une réunion européenne de consensus à Maastricht. Le texte français prône l'utilisation de trithérapies de sept jours associant un inhibiteur de la pompe à protons à double dose (oméprazole, lansoprazole ou pantoprazole) et deux antibiotiques (amoxicilline et clarithromycine). En cas d'allergie aux bêta-lactamines, l'amoxicilline peut être remplacée par le métronidazole ^[10].

Les critères d'un traitement optimal ont été définis comme suit : traitement efficace avec un taux d'éradication d'au moins 90 %, résultats reproductibles, schéma simple, de courte durée, avec peu d'effets secondaires, d'un coût modéré. Les trithérapies de sept jours remplissent ces critères sauf un, le plus important : le taux d'éradication. Ce taux d'éradication rapporté dans les études internationales avec ces trithérapies est supérieur à 90%, alors que plusieurs études ^[10-12] portant sur un grand nombre de patients traités conformément à la conférence de consensus d'octobre 1995 confirment que le taux d'éradication de l'infection à *H. pylori* est inférieur à 75 % en France. Parmi les facteurs les plus fréquemment associés à un échec d'éradication, on note une médiocre compliance des patients, mais surtout la résistance d'*H. pylori* aux antibiotiques ^[13]. Cette résistance concerne essentiellement deux groupes d'antibiotiques utilisés pour le traitement de cette infection, à savoir les macrolides et les nitro-imidazolés. Les fluoroquinolones et les rifamycines, encore peu utilisées, sont également concernées. D'autres antibiotiques sont bien sûr efficaces contre *H. pylori*, mais pas à pH acide, d'où la nécessité de trouver de nouvelles armes pour éradiquer cette bactérie.

Pour le moment, peu de traitements alternatifs existent. La vaccination est une voie intéressante, et au jour d'aujourd'hui de nombreux antigènes sont candidats pour créer un vaccin efficace (UreB, UreA, FlaA, FlaB, BabA, SabA, AlpAB, HpaA, Omp 18, LPS O antigènes, CagA, NAP, VacA, certaines catalases) ^[14]. Mais afin d'obtenir une vaccination efficace, il est nécessaire de trouver un "cocktail" d'antigènes au fort pouvoir protecteur, des souches recombinantes qui permettront d'exprimer ces antigènes en grandes quantités, ainsi que la détermination d'un adjuvant mucosal efficace et la mise en place d'un planning de vaccination optimal. De plus les études menées sur les animaux (souris, singe, etc.) présentent des variations quant à l'efficacité d'une telle vaccination ^[15]. Ces différences pourraient être expliquées par des facteurs bactériens, notamment la souche bactérienne utilisée pour l'épreuve dans les essais de vaccination, ainsi que des facteurs génétiques liés à l'hôte, comme des sensibilités différentes à l'infection à *H. pylori*, même au sein d'une même espèce ^[15].

La vaccination chez l'Homme contre *H. pylori* devant encore surmonter un grand nombre de difficultés, certains chercheurs ont imaginé de nouvelles approches contre cette bactérie. En effet, une des clés pour un traitement efficace contre ce pathogène est de pouvoir laisser l'antibiotique agir suffisamment longtemps dans l'estomac afin qu'il ait le temps de diffuser au plus profond de la muqueuse gastrique, ceci pour éliminer un maximum de bactéries et les potentiels foyers de réinfection. Le travail de ces chercheurs consiste à mettre au point une forme galénique capable par quelques moyens que ce soit de demeurer suffisamment longtemps dans l'estomac. Il s'agit des systèmes gastro-rétentifs (SGR).

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CHAPITRE I :

“Gastroretentive dosage forms: overview and special case of *Helicobacter pylori* “

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Article publié sous forme raccourcie dans Journal of Controlled Release 111 (1-2) (2006) 1-18.

Contents

Introduction	14
1 The stomach	15
1.1 Anatomy	15
1.2 Physiology	15
1.2.1 The mucous membrane	16
1.2.2 Gastric secretions	17
1.2.3 pH	18
1.3 The interdigestive migration myoelectric complex: IMMC	19
1.4 Gastric motility	20
1.5 Gastric emptying	21
1.5.1 Gastric emptying of liquids	22
1.5.2 Gastric emptying of solids	22
2 Gastroretentive forms	23
2.1 High density systems	23
2.2 Intragastric floating systems	24
2.2.1 HBS™: hydrodynamically balanced systems	25
2.2.2 Gas-generating systems	27
2.2.3 Raft-forming systems	29
2.2.4 Low density systems	30
2.3 Expandable systems	32
2.4 Superporous hydrogel	36
2.5 Mucoadhesive or bioadhesive systems	37
2.6 Magnetic systems	39
2.7 Conclusions	40
3 Particular case of Helicobacter pylori	41
3.1 Stomach physiopathology following H. pylori infection	41
3.2 Gastroretentive dosage forms against H. pylori	44
3.2.1 Floating drug delivery systems	44
3.2.2 Mucoadhesive drug delivery systems	45
3.2.3 Drug delivery systems with specific interaction	47
General conclusion:	49
References:	50

Figures

Fig.1: Anatomy of the stomach.	15
Fig.2: Cells of the gastric fundic mucous membrane and their secretions.	18
Fig.3: Variation of the intragastric pH during 24 h.	19
Fig.4: Schematic repartition in the stomach of nutrients in function of time.	21
Fig.5: Schematic localisation of an intragastric floating system and a high-density system in the stomach.	24
Fig.6: Hydrodynamically Balanced System (HBS).	26
Fig.7: Improvement in HBS.	27
Fig.8: Gas-generating systems.	28
Fig.9: Schematic representation of “floating pill” proposed by Ichikawa	29
Fig.10: Schematic illustration of the barrier.	29
formed by a raft-forming system.	29
Fig.11: Microballoons (a) (from Sato [73]) and foam-particles.	30
Fig.12: Schematic presentation of the structure of the low density, floating matrix tablets. ...	31
Fig.13: Different geometric forms of unfoldable systems	33
Fig.14: Different unfoldabe systems.	34
Fig.15: Swellable systems	35
Fig.16: On the left, superporous hydrogel.	37
Fig.17: Schematic representation of the strategy for the eradication of H. pylori by sustained release liquid preparation containing ampicillin.	46
Fig.18: Recognition of lipobeads by PE specific surface receptors of H. pylori.	49

Tables

Table I: The four phases of the IMMC.	20
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Introduction

Drug biodisponibility is a crucial factor to obtain a therapeutic effectiveness. One of the essential parameters which controls this biodisponibility, is the residence time of the active agent in the site of absorption. For this reason, during the last two decades, numerous gastroretentive dosage forms have been designed to exhibit a prolonged gastric residence time: high-density systems, intragastric floating systems (HBSTM systems, gas-generating systems, raft-forming systems, low density systems), expandable systems, superporous hydrogel systems, mucoadhesive systems, and magnetic systems. The advantages of such drug delivery systems are evident. They allow to use some drugs with a narrow absorption window in the upper part of the gastrointestinal tract, or drugs with a poor stability in the colon. Furthermore, the drug can act locally in the stomach, and this intimate contact with the absorbing membrane increases drug absorption. This is especially important in the case of bacteria which colonize the stomach (i.e. *Helicobacter pylori*) because the three main barriers to luminal delivery of drugs against such microorganisms are the gastric emptying, gastric acidity and epithelial mucus layer ^[1]. Indeed, *H. pylori* lives deep in the gastric mucus layer ^[2], thus increasing residence time of antibiotics should allow them to be more efficient. Moreover, topical action of antibiotics can act sometimes in synergy with a systemic effect, because some of them can be absorbed by the gastric wall, then secreted again in the stomach ^[3,4].

1 The stomach

1.1 Anatomy

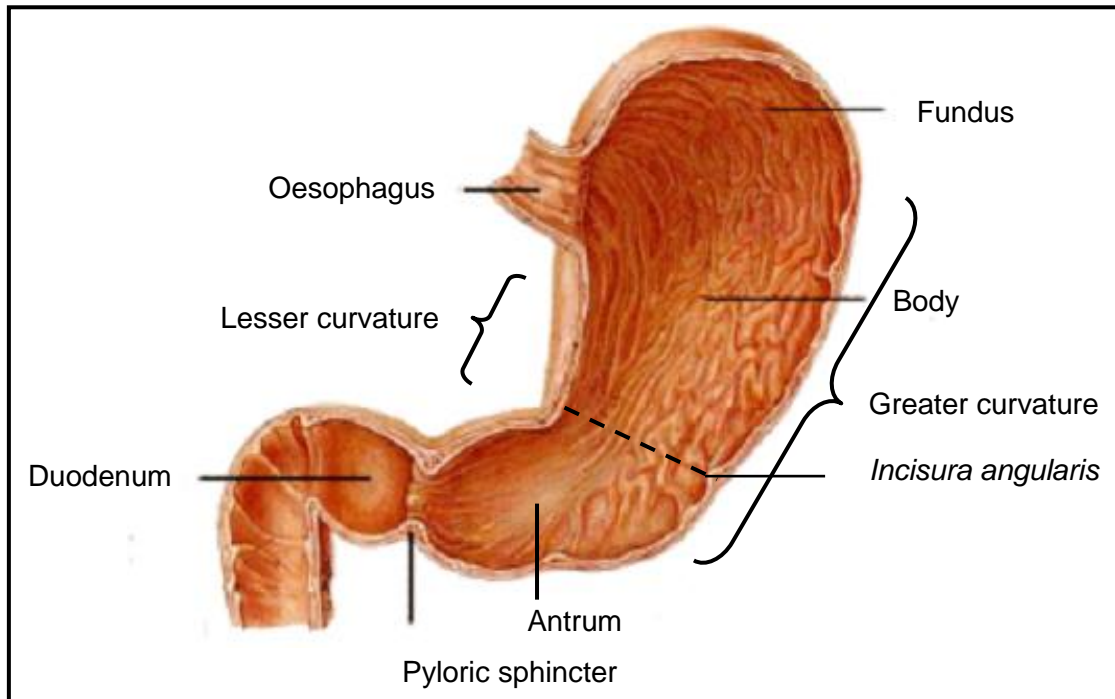


Fig.1: Anatomy of the stomach. Adapted from Internet ^[5]

The stomach is the broadest part of the digestive tract. It is located in the abdomen, between the distal oesophagus and the duodenum, under the diaphragm. The stomach is a bag in the shape of J, including two parts: the fundic reservoir and the antropyloric pump. In the post-prandial phase, the stomach (fundus and antrum) distends and can contain up to 1.5 to 2 L of food and liquid ^[6]. The greater curvature of the stomach is approximately three or four times longer than the lesser curvature. At a given point, the edge of the small curvature forms an angle which is called *incisura angularis*, where the salient mucous folds of the body of the stomach are replaced by the smoother mucous membrane of the antrum. The musculature of the stomach consists of two layers of smooth muscles: one longitudinal, external, the other circular, internal and thicker.

1.2 Physiology

The stomach has three major functions: motor, secretory and endocrine. The motor function includes the temporary retention of food and liquids, the mixture of the ingested substances with the gastric juice, and the regulation of gastric emptying. The principal substances secreted in the stomachal lumen are hydrochloric acid, pepsin, mucus, bicarbonate, intrinsic

factor and water. The stomach releases hormones in blood as gastrin and somatostatin. Motor function is mostly carried out by the antrum. The secretory function is essentially due to the fundus region and the body of the stomach ^[7].

1.2.1 The mucous membrane

The mucous membrane shows three important characteristics: surface continuity, permanent cellular renewal, and cytoprotection. Thus, the healthy gastric mucous membrane can resist to high intra-luminal concentrations of hydrochloric acid and strong peptic activity. The continuity of surface is ensured by the tight junctions, which prevent the paracellular pathway, and only let water and ions pass. This movement of ions makes it possible to create a potential gradient (primarily due to Cl^- and Na^+), which demonstrates the gastric mucous membrane integrity. This difference of potential is about -40 to -50mV in the fundus and -30 to -40mV in the antrum. The cellular renewal of the mucous membrane is a permanent phenomenon. It concerns all mucosal tissues. For the stomach, the renewal rate of the mucus cells is about 4 to 6 days ^[6]. The cytoprotection is ensured by three factors:

- the mucus, which is a genuine barrier for the diffusion.
- the capillary circulation, which brings oxygen and evacuates the compounds which crossed the mucous membrane.
- the prostaglandins, which would stimulate the blood irrigation as well as the production of mucus and bicarbonate, and would increase the synthesis of proteins, which is necessary to the maintenance and the regeneration of the cells.

In the presence of acid in the lumen, the gastro-duodenal pH approaches 2, while the immediately adjacent epithelium can reach an almost neutral pH (pH 7) ^[1,2,7,8]. This gradient of pH, which plays a role in the protection of the mucous membrane against digestion by the acid and pepsin, is certainly under the dependence of the combined bicarbonate and mucus secretion ^[8,9].

Gastric mucus consists of 95% of water and 5% of glycoproteins with molecular weight higher than 10^6 g/mol ^[6]. The charge of the mucus is essentially governed by the presence of sialic acid ($\text{pKa} \sim 2,6$) ^[10]. The mucus ensures the lubrication of the food particles, and its gelatinous consistency enables it to retain water and bicarbonate close to the epithelium. Mucus is produced together with bicarbonate in the cells of the epithelium, in the mucous neck cells of gastric glands and in the Brunner's duodenal glands. The layer of mucus varies in thickness according to the gastric region and according to the authors, between $100 \mu\text{m}$ ^[11-13] and $200 \mu\text{m}$ ^[14]. For the epithelial surface, the gastric mucus layer acts as a physical barrier against luminal pepsin, which digests the surface of the mucus gel to produce soluble degraded mucin in the gastric juice. The continuity and almost constant thickness of the

mucus gel layer observed *in vivo* is evidence that mucus secretion balances the losses by peptic digestion or mechanical erosion.

1.2.2 Gastric secretions

Gastric secretion is an isotonic solution, composed by water, hydrochloric acid, bicarbonate, NaCl and KCl salts, mucus, intrinsic factor, pepsinogen and gastric lipase. Water movements are passive, and follow the ionic movements.

The hydrochloric acid, whose role is to dissolve food particles and transform pepsinogen to pepsin, lowers the gastric pH up to 1 or 2 ^[15]. However, during the digestion process, the intragastric pH decreases slowly because of the buffer effect of nutrients (especially proteins). The stomach secretes about 1.5 L of hydrochloric acid per day. Acid secretion varies between 1-2 mL/min during rest phase, and can reach up to 6 or 10 mL/min during intense activity ^[6]. The hydrochloric acid is secreted by parietal cells, located in the fundic region, and representing 10% of the fundic cells.

The rest of fundic cells are mucus cells, which secrete mucus and bicarbonate. This mucus holds the bicarbonate at the surface of the mucous membrane, creating a physical and chemical barrier against acidity and gastric enzymes. Hence, the majority of the bicarbonate stays in the mucus layer, and quantitatively, luminal secretion of bicarbonate represents only 5% compared to the acid secretions.

Sodium is secreted by non parietal cells, with a constant flow. On the other hand, potassium is secreted by the whole of gastric cells, with intracellular or transcellular pathways.

The intrinsic factor, a glycoprotein secreted by parietal cells, is essential for the vitamin B12 absorption. Finally, chief cells, located at the bottom of the gastric glands of the fundus, secrete pepsinogen and gastric lipase, necessary for the digestion of proteins ^[7].

Endocrine cells of the stomach, also called enteroendocrine cells, secrete hormones as gastrin or somatostatin. Although very small quantities of these hormones are found in the lumen, the major part of them reach their target cells by the blood circulation. This is why secretions of the enteroendocrine cells are not considered as being part of the gastric liquid.

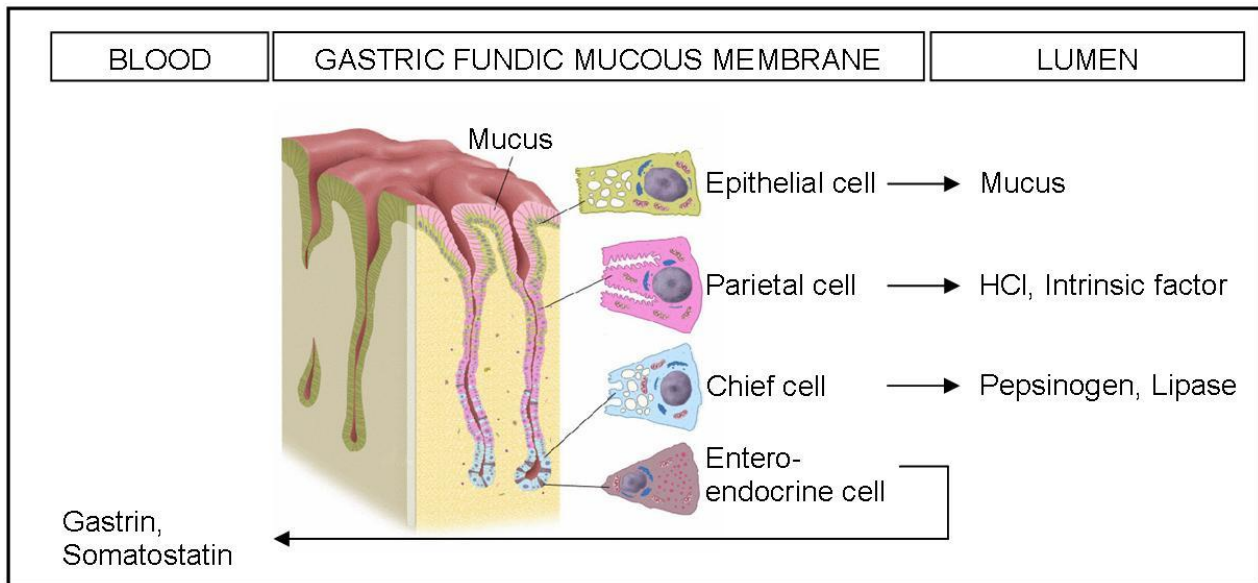


Fig.2: Cells of the gastric fundic mucous membrane and their secretions. Adapted from Internet ^[16]

1.2.3 pH

The pH of the stomach is a variable parameter. Not only because of important inter-individual differences, but also because secretion of H^+ ions is under dependence of several factors. In general, during a 24 hours period, the pH varies between 1 and 6 with an average of 3 (fig.3) ^[17]. The pH depends on the circadian rhythm, with a high evening and low morning acid secretion ^[18]. Acid secretions are also controlled by ingestion of nutrients. After the ingestion of a solid meal, three phases could be described: the cephalic phase (vision, smell, savour, mastication) and the gastric phase (distension of the stomach, increase of peptides concentration, decrease of the acidity) lead to an increase of the acid secretions; the third phase, the intestinal phase (increase of the osmolarity, the H^+ and nutrients concentrations), leads to a decrease of the acid secretions. In general, the volume of the acid secretion is proportional to the quantity of ingested proteins. Because of the buffer effect of nutrients, the decrease of the pH is relatively slow (\sim pH 5 one hour after the meal) ^[6].

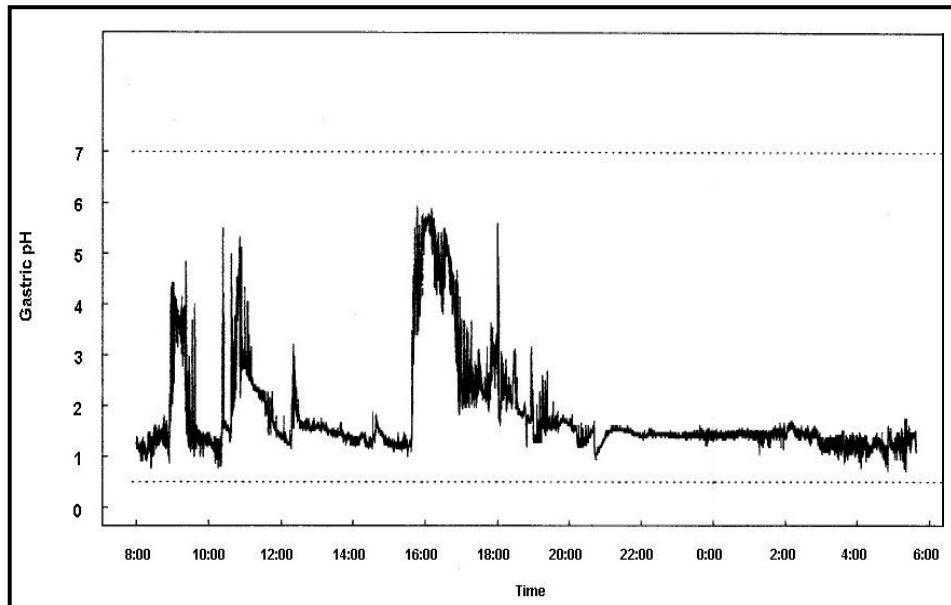


Fig.3: Variation of the intragastric pH during 24 h. From Shih ^[17].

Liquid meals have a variable secretatogue effect. For example, water or sweet drinks have almost no-effect on the secretion, unlike milk or beer which increase dramatically acid secretion. Tea and coffee have a moderate effect ^[6].

1.3 The interdigestive migration myoelectric complex: IMMC

The activity of the stomach is not the same when it is in fasted state or in post-prandial phase. But even apart from the meals, the stomach is not at rest. Indeed, there is an interdigestive muscular activity called interdigestive migration myoelectric complex (IMMC). This IMMC is a true peristaltic wave, which is born in the antrum of the stomach and propagates all along the small intestine right down the cecum. This wave is unique: there cannot be two waves at the same time. To propagate from the stomach right down to the cecum, this wave lasts 90 minutes (in average, but with important inter-individual variations), then another wave starts in the stomach. The aim of the IMMC is to evacuate indigestible solids (like the pharmaceutical solid forms) from the stomach. The four phases clearly observed during this cycle are summarized in table I ^[6,19].

The IMMC is always interrupted by a solid-liquid meal, but never after the ingestion of a drink. The IMMC is interrupted during the whole digestion, with the length of the interruption being proportional to the calorific density and the composition of the meal ^[6].

Table I: The four phases of the IMMC.

PHASE	STATE	CONTRACTIONS	LENGTH
PHASE I	Basal state	Few or no.	45-60 min
PHASE II	Preburst state	Irregular, gradually increase.	30-45 min
PHASE III	Burst state	{ Intense and powerful. Responsible for the removal of the indigestible solids.	5-15 min
PHASE IV	Short transition period	Decreasing activity.	< 5 min

1.4 Gastric motility

A gastric tonus is present in the stomach. It is characterised by two types of waves: one slow, with a three minutes cycle, one fast, with a ten or twelve seconds cycle. These contractions are not peristaltic but allow to maintain a constant pressure in the stomach. When food is ingested, in order to avoid an increase of the stomachal pressure, there is a phenomenon of gastric accommodation also called "reflex accommodation", allowing the stomach to reach a volume of 1.5 L without augmentation of pressure. Five or ten minutes after the ingestion of a meal, peristaltic movements appear in the inferior part of the body and in the antrum. Their strength and speed increase in direction of the pylorus. The gastric pacemaker, located in the body of the stomach, determines the frequency of those waves. A wave is born each 20 to 30 seconds, leading to two or three cycles per minute. Under the action of these waves, the chyme is mixed and carried towards the pylorus ^[6,7].

The evolution of a composite meal (solid and liquid) in the stomach proceeds as follows: the heterogeneous mouthfuls pile up in the fundic area. Then occurs the "gastric accommodation". Thanks to the peristaltic waves, only the periphery of the bolus is carried away towards the pylorus, and because of the retrograde flow, then goes up by the center of the stomach. Hence, the gastric homogenization takes a certain time, during which only the sides of the bolus receive hydrochloropeptic gastric secretions, whereas the center of the bolus is still impregnated by the salivary secretion. A composite meal comprises two phases: a "liquid" phase and a "solid" phase. The liquid phase is mainly made up of water, liquid foods (e.g. soup), as well as food which is dispersed in fine particles in water (e.g. mashed potatoes, compote). The solid foods consist of all nutrients swallowed in pieces of more than 1 mm side after chewing. This food represents the biggest part of the meal, but the ratio liquid/solid varies during digestion under the action of acid secretions and mechanical crushing. So, it is an homogeneous mush which leaves the pylorus. However, the pylorus "sorts" food and allows the passage of liquids more easily than solids. So, gastric emptying is a biphasic phenomenon, which is more significant during the first hour than during the third

hour. Indeed, at this time, the pylorus evacuates a greater quantity of solids. The pieces of 1 mm or less start to cross the pylorus from the beginning of gastric emptying. The crushing and the emptying of solids are simultaneous and nonsuccessive phenomena. Indeed, the gastric content evolves gradually with time from large to fine particles. During this time, the emptying of the liquids does not stop, and carries away the fine particles proportionally to their concentration in the gastric fluid. Liquid fats constitute however a special case. They float on the top of the gastric mass and are evacuated only at the end of digestion (around the 3rd hour) (fig.4).

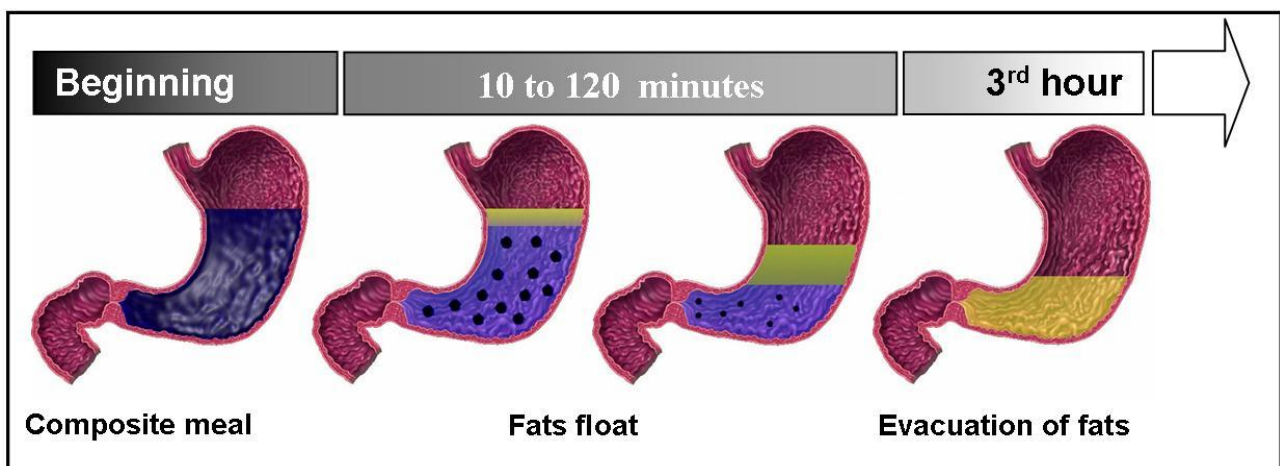


Fig.4: Schematic repartition in the stomach of nutrients in function of time.

Based on Bernier ^[6].

1.5 Gastric emptying

When the stomach is in fasted state, the pylorus is open. The first mouthful penetrates directly in the duodenum, immediately involving the closing of the pyloric sphincter. Peristaltic waves carry away the gastric chyme towards the pyloric sphincter, but in a bigger quantity compared with nutrients that will go through the pylorus. Hence, the excess of nutrients flow back to the stomach through the peristaltic contraction ring (i.e the hole created in the center of the stomach due to the muscular contractions generated by the peristaltic waves during their propagation). Thus, the transpyloric flow is a pulsating phenomenon, with a retrograde flow: it seems to be the sum of a major forward flow and minor retrograde flow ^[6]. This phenomenon was observed by Pallota and al., for a liquid and calorific meal in humans: the transpyloric flow was about 56% forward, 19% retrograde, and 25% to and fro ^[20].

1.5.1 Gastric emptying of liquids

The speed of the gastric emptying of liquids is proportional to the volume present in the stomach, and under the dependence of a gradient of pressure on both sides of the pylorus. Indeed, the volume present in the stomach decreases regularly in an exponential way. Concerning pure water, whatever the volume of liquid, half of this volume will empty each 10 minutes. Thus, for 500 mL of pure water in the stomach, 250 mL will be still there 10 minutes later. Because the gastric emptying is an exponential process, the $T_{1/2}$ value is often used: it corresponds to the time necessary to empty half of the volume present in the stomach.

Many parameters influence the gastric emptying of liquids. An isotonic solution leaves more rapidly the stomach than a hypo- or hypertonic solution. The gastric emptying of a cold drink is slower than that of a 50°C drink, the latter slower than that of a 37°C drink. The acidity of the drink also plays a role. An acid drink will slow down gastric emptying. Furthermore, sugar is also an effective retarder. Apparently, this slowing down is in relation with the calorific density of the drink. Finally, the retarder effectiveness of fats is well established, with the best effect obtained by the myristic acid (C_{14}).

Any of these parameters, individually, is more important than another. They interact together to regulate the gastric emptying. For example, with a constant volume of 250 mL, the $T_{1/2}$ of an isotonic salted serum (300 mOsm/L – 0 Cal – pH 7) is ~ 14 min ; $T_{1/2}$ of unskimmed milk (273 mOsm/L – 165 Cal – pH 6.7) is ~ 74 min ; and $T_{1/2}$ of Coca-Cola® (516 mOsm/L – 113 Cal – pH 3) is ~ 41 min ^[6].

1.5.2 Gastric emptying of solids

The gastric emptying of solids is depending on several parameters. But the size of the particles seems to be important. If a particle has a size equal or less than 1 mm, it is considered by the pylorus as a soluble substance, and is evacuated with the water right from the start of the emptying. The $T_{1/2}$ of water in a composite meal is about 60 minutes. As indicated previously, the pylorus sorts the nutrients and rejects particles of large size, which are carried away by the retrograde flow to the center of the stomach. Solids are evacuated by the pylorus slowly and regularly. Finally, indigestible nutrients, as cellulose or some pharmaceutical solid forms, are evacuated from the stomach by the IMMC ^[6].

Other parameters influence gastric emptying of solids as well as liquids. The two most important are the presence of fat substances (liquid or solid) ^[6] and the calorific density of the meal ^[21,22]. Both of them slow down the gastric emptying.

2 Gastroretentive forms

Constraints imposed by the stomach anatomy and physiology highlight a number of important parameters to be taken into account for the development of gastric retentive forms. Firstly, in the case of indigestible solids (single unit systems), **the size** is a restrictive parameter. The human pyloric diameter is $12\pm 7\text{ mm}$ ^[23]. As seen previously, during digestion, the pylorus “sorts nutrients”, and during fasted state, the IMMC is a real *housekeeper*. When the diameter is inferior to 7 mm, solids are quickly evacuated. However, in order to stay as long as possible in the stomach, it is generally accepted that the diameter must be higher than 15 mm, in particular during fasted state. The principal drawback of single unit systems is the high variability of the gastrointestinal transit time because of the “all or nothing” emptying process. Multiple unit systems, as microparticles for example, avoid this phenomenon by their statistical repartition throughout the gastrointestinal tract. When single unit systems are evacuated through the pylorus at the end of the digestion or during the phase III of the IMMC, multiple unit systems are evacuated either with a linear profile, or in bolus at the end of the digestion ^[24,25].

Secondly, as we will see, **density** is also important. The density determines the location of the system in the stomach (fig.5), and therefore, plays a role in the gastric residence time. Indeed, systems with a density inferior to that of the gastric liquid can float to the surface, and hence, stay far from the pylorus. Similarly, high-density systems sink in the bottom of the stomach and can stay longer inside because of their position in relation to the pylorus ^[24].

Finally, the **molecular weight** and the **lipophilicity** of the active agent are also important parameters. Indeed, to diffuse through the gastric mucus, molecules must have low molecular weights. Shah et al. have shown that gastric mucus was more permeable to metronidazole (171.2 g/mol) than amoxicillin (365.4 g/mol) ^[26]. Larhed et al. have demonstrated that charge of molecule had only minor effect on the diffusion coefficient in the gastrointestinal mucus, but lipophilicity was the most important physicochemical parameter. More important was the lipophilicity, more reduced was the diffusion coefficient ^[10]: this is understandable because of the mucus composition (95% of water and 5% of glycoproteins).

2.1 High density systems

Because of their bulk density superior to that of the gastric content ($\sim 1,004\text{ g.cm}^{-3}$), high density systems sink in the bottom of the stomach (fig.5). By this sedimentation process, small pellets stay in the folds of the stomach antrum (in an upright posture), and try to withstand the peristaltic waves of the stomach wall. It seems necessary to reach a threshold density close to $2.4\text{--}2.6\text{ g.cm}^{-3}$, in order to have a gastric emptying process significantly

prolonged ^[27]. To increase the pellets density, the most common used excipients are barium sulphate, zinc oxide, iron powder, titanium dioxide... Although some results are encouraging in ruminants ^[28,29], the effectiveness of this approach in human beings has not been confirmed ^[30] and no successful high-density system reached the market ^[31].

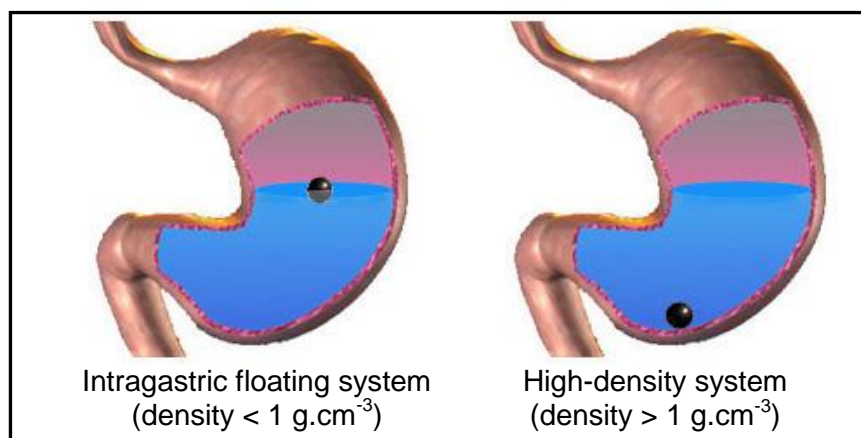


Fig.5: Schematic localisation of an intragastric floating system and a high-density system in the stomach.

2.2 Intragastric floating systems

Unlike high-density systems, intragastric floating systems have a bulk density lower than that of the gastric content. They are able to stay buoyant in the stomach without affecting the gastric emptying rate for a prolonged period of time. Hence, a controlled release of the drug in the stomach can be performed by the floating system. After the release of the drug, the residual system is emptied from the stomach. As seen previously, the fed or fasted state of the stomach influences considerably the emptying process. And although floating systems possess an inherent ability for gastric retention, they rely more on the presence of a meal, to retard their emptying ^[32-34]. Indeed, IMMC is delayed under fed conditions, and moreover in the fasted stomach, the amount of liquid is not sufficient to allow an efficient buoyancy of the drug delivery system ^[33]. So, presence of food in the stomach is important to enhance gastroretention process. Furthermore, amount of food intake, nature of food, caloric content and frequency of feeding affect profoundly gastric retention of the dosage form ^[22,35]. However, Whitehead et al. showed that meal size in 7 subjects with similar type of food but with caloric densities range between 1625 and 3777 Kcal, did not affect the gastroretentive properties of the floating beads ^[36]. Steingoetter et al. demonstrated that for three different

meal groups (i.e. hamburger meal, cheese meal, pasta meal) with respectively 36.4, 32.2 and 34.4 g of fat and 921.9, 734.6 and 934.9 Kcal, there were no differences in gastric meal emptying, tablet floating performance, or tablet residence time ^[37].

In conclusion, even if some parameters may affect gastric retention time of the dosage form in a more or less important way (according to authors and dosage forms themselves), most of the studies acknowledge that the presence of food in the stomach is the most important parameter for increasing gastric retention time.

Independently of these considerations, different intragastric floating systems using different approaches have been designed.

2.2.1 HBSTM: hydrodynamically balanced systems

Floating HBS are single-unit floating dosage forms, containing one or more gel-forming hydrophilic polymers, drug(s) and other additives. Hydroxypropylmethylcellulose (HPMC) is the most common used hydrophilic polymer; hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC), sodium carboxymethylcellulose (NaCMC), agar, carageenans or alginic acid are also used ^[38,39]. The hydrophilic polymer is mixed with the active agent and a gelatine-based capsule is often used as carrier. In the gastric fluid, the capsule is rapidly dissolved, and the swelling of polymers facilitates floating. The release of the drug is controlled by the formation of a hydrated boundary on the surface due to the hydrocolloid swelling ^[24]. The continuous erosion of the hydrated surface allows water penetration in the inner layers, thereby maintaining the hydrated surface and floating ability of the system ^[38] (fig.6). The use of excipients such as fatty excipients in the formulation, leads to low-density formulations and a slow penetration of water, avoiding the dosage form to sink. Based on this approach, Madopar LP[®] has been introduced on the market by Roche laboratories during the 80's ^[40-42]. The main drawback of floating HBS, is that this system is passive, i.e., it mainly depends on the air captured in the dry mass inside the hydrating gelatinous surface layer. Because of this passivity, the buoyancy of the system largely depends on the characteristics and the amount of hydrophilic polymer used ^[39]. The formulation of the dosage form must be a compromise between the proportion of active agent and hydrophilic polymer, and the release profile of the drug.

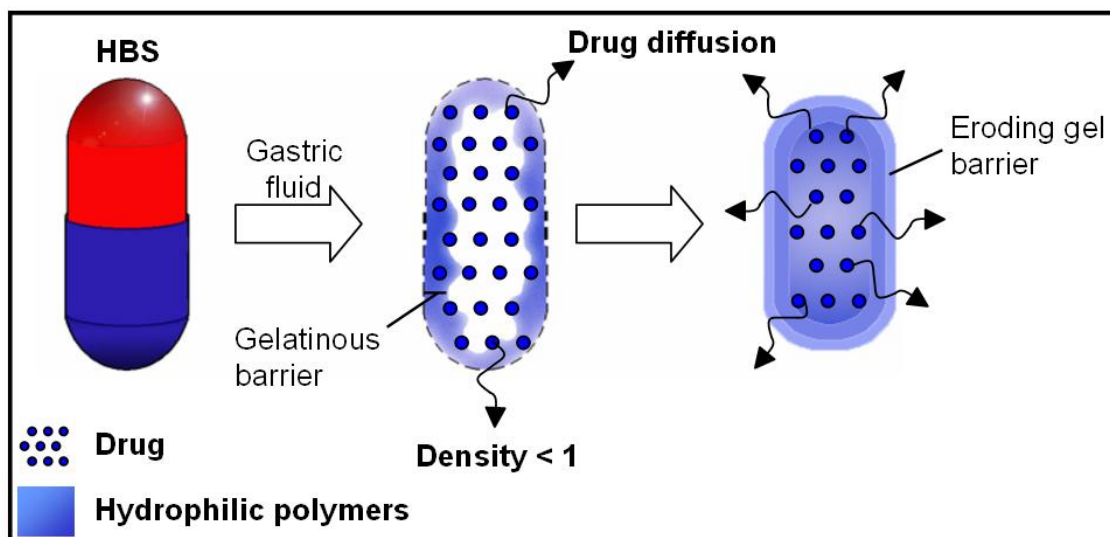


Fig.6: Hydrodynamically Balanced System (HBS).

The gelatinous polymer barrier formation results from hydrophilic polymers swelling. Drug is released by diffusion and erosion of the gel barrier. Based on Hwang^[39] and Dubernet^[24].

To overcome this problem, some investigators developed bilayer formulation. One layer supplies the floatability, and the other one the drug release. This system was used by Oth et al. to release misoprostol against gastric ulcers^[43]. Both layers contained swellable polymers and only one contained the drug (fig.7a). An adequate cohesion was observed between the two layers. Hence, it is easier to optimise the drug release profile and the buoyancy of the dosage form. With this system, Oth et al. observed a mean gastric residence time (on 10 subjects) higher than 3 hours after a single meal (breakfast), and higher than 10 hours after a succession of meals.

Other systems have been designed by investigators to improve floating HBS^[24,38]. For example, Mitra developed a multilayered floating laminate^[44]; Harrigan designed an intragastric floating drug delivery system where the drug is encapsulated in a microporous compartment in order to prevent a direct contact with the stomach wall^[45]; Gupta et al. proposed an ampicillin floating tablet, buffered by sodium citrate for maintaining a pH of about 6.0 in the direct environment of the drug^[46]; Watanabe et al. described a bilayered floating coated shell, containing a hollow polystyrene sphere^[47]; Chitnis et al. reported a bioadhesive and floaty system by coating their tablets with Carbopol® or with a synthesized bioadhesive polymer (crosslinked polymer of methacrylic acid and acrylic acid)^[48]. Finally, Krögel et al. designed a floating HBS where the buoyancy is controlled by the geometric shape of the system, consisting of an impermeable polypropylene cylinder (10/15 mm), sealed on both sides by a matrix of hydrophilic polymer (HPMC) containing the drug. The air entrapped in the core of the cylinder between the two matrices allows the system to float^[49] (fig.7b).

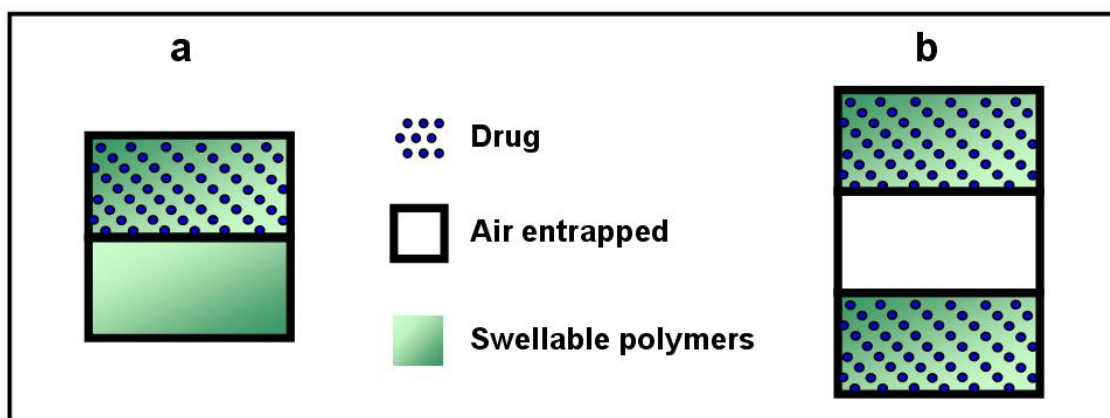


Fig.7: Improvement in HBS proposed by Oth et al. (a) ^[43]; and Krögel et al. (b) ^[49].

2.2.2 Gas-generating systems

To improve the floatability of a dosage form and to reduce its density, novel systems have been designed, consisting of a generation of gas bubbles. The easiest way to produce CO₂, is to incorporate in the formulation carbonates or bicarbonates, which react with acid (gastric acid, citric or tartaric acid). The optimal stoichiometric ratio of citric acid and sodium bicarbonate for gas generation is reported to be 0.76:1 ^[31]. An alternative is to incorporate a matrix containing portions of liquid, which gasifies at body temperature ^[50-52]. This gas generating systems have been applied for single unit systems as well as multiple unit systems.

In single unit systems, like capsules ^[53] or tablets ^[54,55], effervescent substances are incorporated in the hydrophilic polymer, and CO₂ bubbles are trapped in the swelled matrix (fig.8a). *In vivo* experiments showed an increased mean gastric residence time (up to 4 hours in the fasted dog) ^[54]. *In vitro*, the lag time before dosage form starts to float is shorter than 1 min and the buoyancy is prolonged (8 to 10 h) ^[54]. Bilayer or multilayer systems have also been designed ^[56-61]. The effervescent substances and active agents can be formulated independently and the gas-generating unit can be incorporated in any of the layers (fig.8b). Improvement of this dosage form have been made by coating the matrix with a polymer which is permeable to water, but impermeable to CO₂ ^[58] (fig.8c). The main difficulty of such formulation is to find a good compromise between elasticity, plasticity and permeability of the polymer.

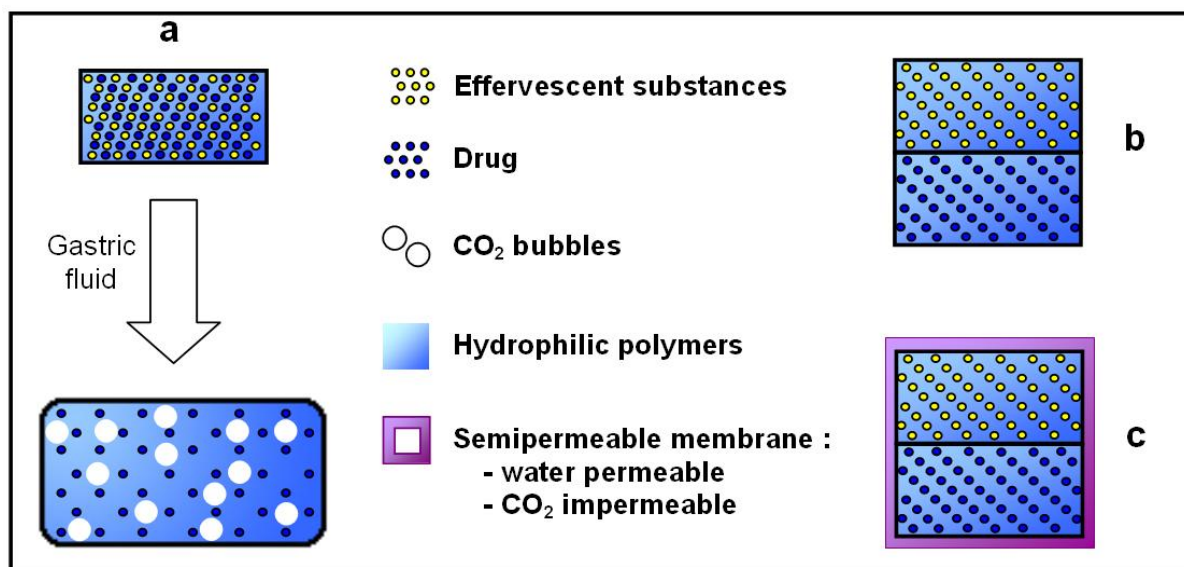


Fig.8: Gas-generating systems. Schematic monolayer drug delivery system (a) (based on Baumgartner ^[54]). Bilayer gas-generating systems, with (c) or without (b) semipermeable membrane (adapted from Dubernet ^[24]).

As written before, multiple unit systems avoid the “all or nothing” emptying process. However, it requires that the units remain dispersed and suspended individually in the gastric fluid and avoid to become an agglomerated mass floating on top ^[62]. In the beginning of the 90's, Ichikawa et al. reported a double-layered coated system in the form of granules ^[63]. It was made of an inner effervescent layer (bicarbonate and tartaric acid) and an outer swellable membrane (polyvinyl acetate and purified shellac). The system was floating completely within approximately 10 min and approximately 80% remained floating over a period of 5 h. *In vivo* studies have been carried out in beagle dogs and humans in the fed state. Most of the pills (containing barium sulfate) floated in the stomach after 10 min, and stayed floating for at least 3 h after administration as observed by periodic X-ray photographs ^[64] (fig.9). Atyabi et al. developed microparticles loaded with a model drug (theophylline) and bicarbonate ^[65]. The ion-exchange resin beads were coated with a semipermeable membrane. CO₂ is released when the bicarbonate is in contact with the hydrochloric acid of the gastric juice, which causes floatation of the device ^[66]. *In vitro*, microparticles exhibited floating times of over 24 h using a standardised procedure. Studies in human volunteers using gamma scintigraphy showed a prolonged residence time for coated beads (> 3 h in the upper stomach after a light breakfast) compared to control (uncoated beads).

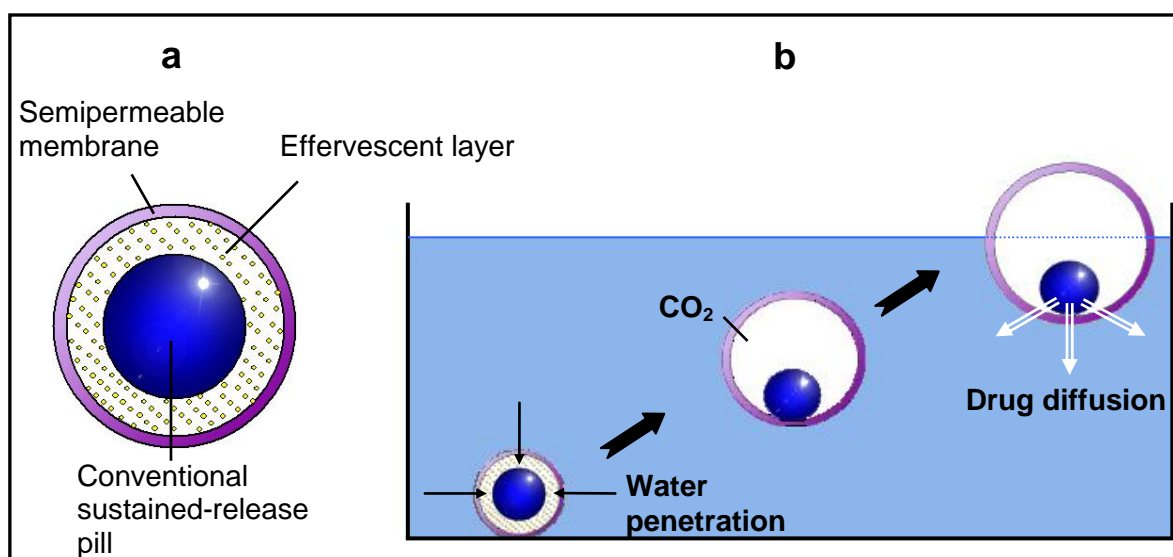


Fig.9: Schematic representation of “floating pill” proposed by Ichikawa (a). The penetration of water into effervescent layer leads to a CO₂ generation, and makes the system float (b).

Adapted from Ichikawa ^[64].

2.2.3 Raft-forming systems

A different kind of floating systems is the raft-forming system. In contact with gastric fluids, a gel-forming solution (for example a sodium alginate solution containing carbonates or bicarbonates) swells, and forms a viscous cohesive gel where CO₂ bubbles are entrapped, causing the floatation of the device (fig.10). Formulations also typically contain some antacid ingredients such as aluminium hydroxide or calcium carbonate to reduce gastric acidity. Because raft-forming systems produce a layer on the top of gastric fluids, they are often used for gastroesophageal reflux treatment ^[67-70]. A raft-forming system against this disease is available on the market (Liquid Gaviscon[®] - GlaxoSmithkline).

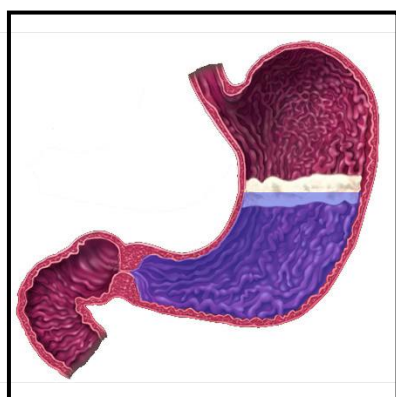


Fig.10: Schematic illustration of the barrier formed by a raft-forming system.

2.2.4 Low density systems

Gas generating systems exhibit *in vitro* and *in vivo* interesting properties, but need a lag time to reach the top of the stomach content. During this lag time, the dosage form may undergo a premature evacuation through the pyloric sphincter. Considering this point, low density systems (density < 1 g/cm³) able to be immediately buoyant have been developed. This type of system is made of low density materials, entrapping oil or air, providing an immediate floatability. Most of low density systems are multiple unit systems, and because of the low-density core, are also called “microballoons” ^[71-75] (fig.11a)

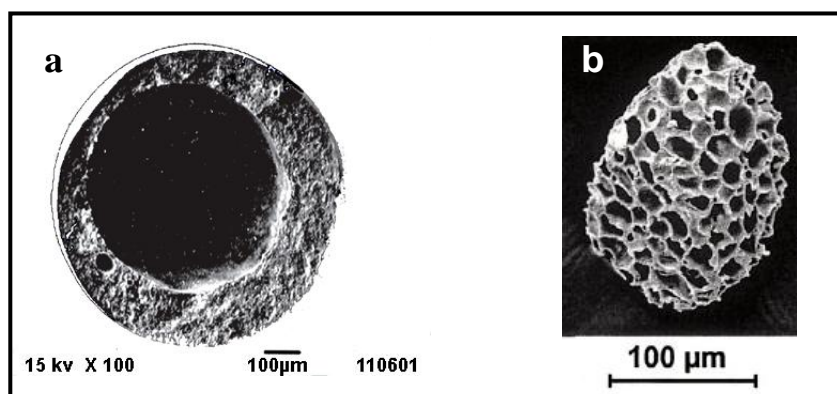


Fig.11: Microballoons (a) (from Sato [73]) and foam-particles (b) (from Streubel ^[76]).

Generally, techniques used to prepare hollow microspheres involve simple solvent evaporation or solvent diffusion/evaporation methods. Polycarbonate, Eudragit S[®], cellulose acetate, calcium alginate, agar, low methoxylated pectin are commonly used as polymers. Obviously, the quantity of polymer, the plasticizer-polymer ratio and the solvent used during preparation, influence the drug release and floating properties ^[38].

In 1992, Kawashima et al., using an emulsion-solvent diffusion method, prepared hollow microspheres loaded with drug (tranilast or ibuprofen) in their outer polymer shells. They dissolved the drug and an enteric acrylic polymer (Eudragit S[®]) in an ethanol:dichloromethane solution. This mixture was added into a stirred aqueous solution of polyvinyl alcohol (0.75% w/v) to obtain an o/w emulsion. The gas phase generated in the dispersed polymer droplet by the evaporation of dichloromethane formed an internal cavity in the microspheres. *In vitro*, the microballoons floated continuously for more than 12 h over the surface of acidic dissolution media containing surfactant ^[71]. Thanoo et al. reported the preparation of polycarbonate microspheres loaded with aspirin, griseofulvin and p-nitroaniline, by a solvent evaporation technique. They obtained spherical and hollow microsphere (confirmed by scanning electron microscopy). By this process, a high drug loading (> 50%) was achieved, and drug-loaded microspheres were found to float on

simulated gastric and intestinal fluids ^[77]. Stithit et al. used a novel preparation method by a modified emulsion-solvent evaporation to obtain floating microspheres containing theophylline. The drug-polymer (cellulose acetate butyrate and Eudragit[®] RL 100 [1:1]) dispersions are pressurized under CO₂ which dissolves within them and forms bubbles upon the release of the pressure. The microspheres have round hollow cavities due to bubbles entrapped in the dispersed drug polymer droplets. Their density is lower than 1 g/cm³ and they remain floating for more than 24 h in pH 1.2 and 7.5 buffers ^[78]. Streubel et al. developed low-density foam-based floating microparticles. These microparticles consisted of polypropylene foam powder, model drug (chlorpheniramine maleate, diltiazem HCl, theophylline or verapamil HCl) and polymer (Eudragit RS[®] or polymethyl methacrylate). They were prepared by soaking the microporous foam carrier with an organic solution of drug and polymer, and subsequent drying. The polymer and the drug were poured in an organic solvent (ethanol or methylene chloride). A suspension was obtained by dispersing the polypropylene foam powder within this organic phase. Because polypropylene foam particles acted like sponge and absorbed the organic solution, free-flowing microparticles were obtained after solvent evaporation (fig. 11b). Good *in vitro* floating behaviour was observed in most cases and a broad variety of drug release patterns could be achieved by varying the drug loading and type of polymer: more than 77% or 98% of particles kept floating for at least 8 h depending of the polymer type (Eudragit RS[®] or polymethyl methacrylate, respectively) and initial drug loading of the system (10% or 23%) ^[76]. Based on a similar approach, the same group developed also a single unit, floating drug delivery system, consisting of low density polypropylene foam powder, matrix-forming polymers (HPMC, polyacrylates, sodium alginate, corn starch, carrageenan, agar, guar gum, arabic gum), drug and filler (fig.12). All foam-powder containing tablets remained floating for at least 8 h in 0.1N HCl at 37°C. The release rate could effectively be modified by varying the “matrix-forming polymer/foam powder” ratio, the initial drug loading, the tablet geometry (radius and height), the type of matrix-forming polymer, the use of polymer blends and the addition of water-soluble or water-insoluble fillers (such as lactose or microcrystalline cellulose) ^[79].

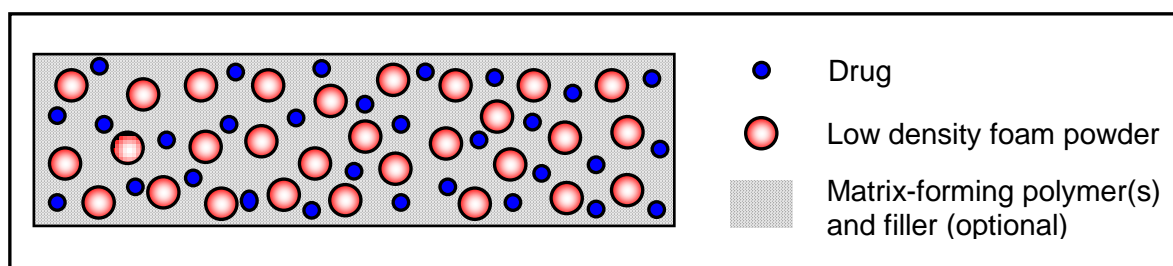


Fig.12: Schematic presentation of the structure of the low density, floating matrix tablets.

Adapted from Streubel ^[79].

Talukder and Fassihi recently developed a multiple unit floating dosage system involving cross-linked beads. They were made by using calcium and low methoxylated pectin (anionic polysaccharide), or calcium, low methoxylated pectin and sodium alginate. Two different ways of drying have been studied to evaluate the changes in bead characteristics due to process variability. One batch was dried in an air convection type oven at 40 °C for 6 h, and the other one was freeze dried. Riboflavin, tetracycline, and methotrexate were used as model drugs for encapsulation. Confocal laser microscopy revealed the presence of air-filled hollow spaces inside the freeze dried beads, which allowed them to remain buoyant over 12 hours in United State Pharmacopoeia hydrochloride buffer (pH 1.5). As expected, air-dried beads remained submerged throughout the release study. The authors also reported that calcium-pectinate-alginate beads released their contents at much faster rates than did calcium-pectinate beads (100% in 10 h vs. 50% in 10 h). Furthermore, they concluded that the nature of cross-linking, drying method, drug solubility, and production approach are all important for development of a gastroretentive drug delivery system ^[80].

Today, because hollow microspheres combine advantages of multiple unit systems and good floating properties, they are considered one of the most promising buoyant systems ^[38]. However, the effectiveness of floating hollow microspheres, similarly to the others floating systems, is under dependence of the presence of enough liquid in the stomach, and requires frequent drinking of water ^[39].

2.3 Expandable systems

Another way to keep a dosage form in the stomach, is to make it bigger than the pyloric sphincter, in order to withstand gastric transit. However, the dosage form must be small enough to be swallowed, and must not cause gastric obstruction (by the dosage form, or by an accumulation of dosage forms). Thus, three configurations are required: a small configuration for convenient oral intake, an expended form which carries out the gastroretentivity, and finally, after the release of the active agent, another small form enabling evacuation ^[81]. Unfoldable and swellable systems have been investigated.

Unfoldable systems are made of biodegradable polymers. The concept is to compress in a carrier a system which will be able to extend in the stomach. Caldwell et al. proposed different geometric forms (tetrahedron ^[82], ring ^[83] or planar membrane [4-lobed, disc, or 4-limbed cross form] ^[82]) made of bioerodible polymers. These forms are compressed in a capsule (fig.13).

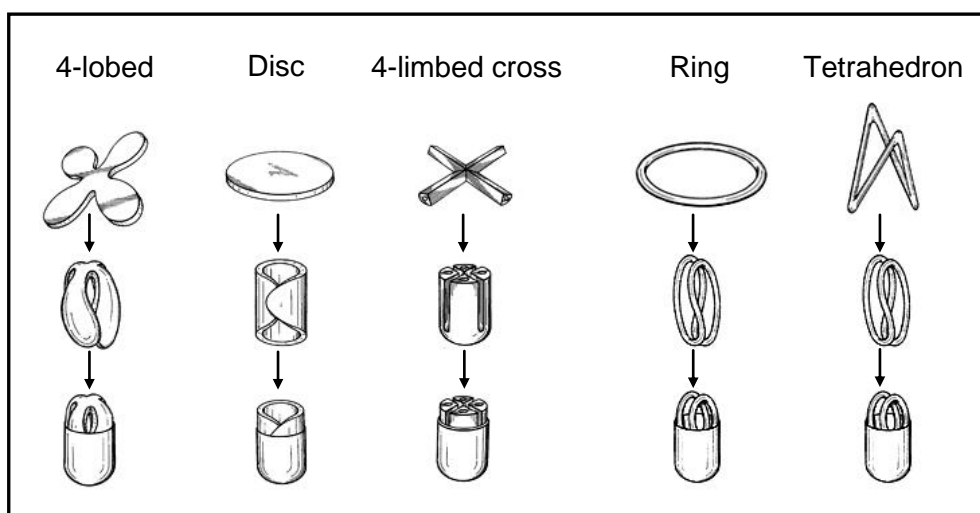


Fig.13: Different geometric forms of unfoldable systems proposed by Caldwell et al. From Caldwell ^[82,83].

Curatolo and Lo designed a kind of spring system, where the folded arms are fixed on the system by gelatine band ^[84]. In the stomach environment, the gelatine is dissolved, and the preferred extended form is restored (fig.14a). Sonobe et al. developed a “Y” form system, having the shape, size and durability to be retained in the stomach for an extended time period. The centre of the system is made of shape memory material, which assures unfolding under prolonged storage time spans. The three arms of the “Y” are made of an erodible material which serves as a drug reservoir and whose rate of degradation controls the gastric retention time. A third component provides the link between the arms and the centre of the dosage form ^[85] (fig.14b). Klausner et al. published several papers ^[86,87] on levodopa gastroretentive dosage form, based on unfolding polymeric membranes, that combines extended dimensions (~5 X 2.5 cm) with high rigidity (fig 14c). As with the previous systems, the dosage form is folded into a consistent gelatine capsule (double- or triple-zero). *In vitro* studies showed that the drug delivery system reaches its unfolded form in 15 min ^[87]. This observation was confirmed *in vivo* in beagle dogs, in which it was also observed that the dosage form maintained its large size for at least 2 h ^[86]. In humans, 67% of drug delivery systems containing levodopa were retained in the stomach 5 h post-administration, and the levodopa plasma concentration-time curve presented high similarities with the reference drug (Sinemet CR[®]), but yielded an extended absorption phase in comparison with Sinemet CR[®]. The authors also demonstrated that rigidity of the system was a crucial parameter. Indeed, a system with an extended size but with a lack of high rigidity was not retained in the stomach like the equivalent size gastroretentive dosage form ^[87].

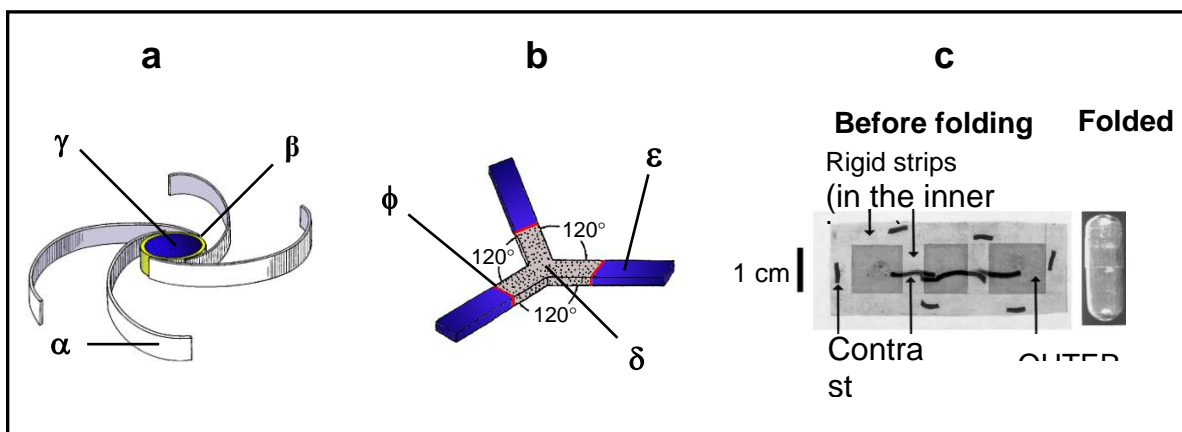


Fig.14: Different unfoldable systems. System partially unfolded proposed by Curatolo and Lo^[84] (a). α : retention arms. β : receptacle. γ : controlled release tablet. Unfolding dosage form from Sonobe et al.^[85] (b). δ : shape memory material, ϵ : erodible material, ϕ : component connecting δ and ϵ . Gastroretentive dosage form before and after folding proposed by Klausner et al.^[87] (c).

Like unfoldable systems, swellable systems use their mechanical properties to remain in the stomach. The swelling is usually due to an osmosis phenomenon. Before administration, the dosage form is small enough to be taken orally, and swells in contact with gastric liquids. The high size of the dosage form provides gastric retention, and probably because of mechanical sensation, the stomach can be maintained in the fed state, avoiding “housekeeper” waves. In the 80’s, Mamajek and Moyer designed a drug reservoir, surrounded by a swellable expanding agent, and the whole system was coated by an elastic outer polymeric membrane, controlling also the drug release (fig.15a). The outer polymer envelope was permeable to both the drug and body fluids. This external layer expanded when the swellable agent was in contact with body fluids. After the drug release, the device decreased in its volume or rigidity (due to escape of drug and expanding agent and/or bioerosion of the polymer envelope), enabling its elimination. Urquhart and Theeuwes developed a drug delivery system containing tiny pills, with a very high swelling ratio, exhibiting a 2 to 50 folds volume increase. These pills were coated by wax to control release of the drug and dispersed into the matrix (polymeric hydrogel) (fig.15b). In body fluids, the system swelled, and the tiny pills released the drug in the stomach. The reservoir left the stomach environment upon hydrolysis and bioerosion during and after the drug delivery period^[88].

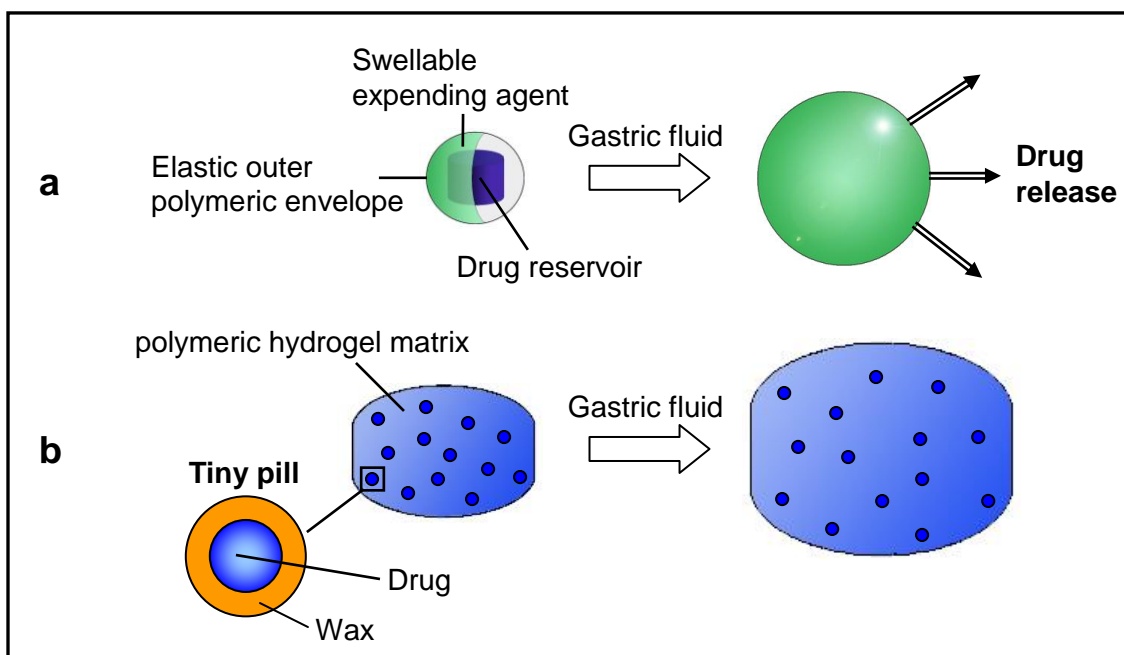


Fig.15: Swellable systems developed by Mamajek and Moyer (a) ^[89] and Urquhart and Theeuwes (b) ^[88].

More recently, Kedzierewicz and al. evaluated in humans different peroral silicone dosage forms. Three shapes were tested (slab, minimatrix and rode), and two isotopes (iodine-123, indium-111) were incorporated in swellable polymers to monitor gastric residence time by gamma-scintigraphy. These systems were designed to swell in the stomach in order to improve residence time, and to be followed by a contraction phase, necessary for the elimination of the dosage form. The slab was a device in a sheet form (20 mm width, 30 mm length, 1 mm thickness) rolled up in a zero size hard gelatine capsule. The minimatrices were small cylinders (6 mm diameter and 4 mm length). Five minimatrices were packaged into a zero size hard gelatine capsule. The rod was a cylinder quite similar to the minimatrices, but only one monolithic device (22 mm length, 6 mm diameter) was packaged into a zero size hard gelatine capsule. The study involving 12 subjects, was performed under fed conditions after ingestion of a standardized meal labelled with indium. In these conditions, a gastric residence time ranging between ~ 3 to 4.5 h was observed (3 h at least for minimatrices, 4h40 min for slabs, and 4h20 min for rods). Apart from the minimatrices, where the protocol was not optimized (due to a lack of gamma-scintigraphic images between 3 and 6 h), a real difference in the *in vivo* fate for rods and slabs was suggested by the authors. Indeed, a correlation was established between the gastric emptying of the rods and the half-gastric residence time of the meal, but this correlation was not found for the slabs. This could be explained by the fact that rods were smaller than slabs, followed the nutrients and were thus emptied in the duodenum. In contrast, the larger slabs did not move with the

meal and were refluxed for further pyloric crossing. When the stomach was empty, the slabs were removed by the action of the IMMC ^[90].

In spite of their interesting characteristics, expendable systems present drawbacks. Because of the easily hydrolysable biodegradable polymers, the storage of such device is quite difficult. Another problem which might arise with unfolding gastroretentive drug delivery system, is that the mechanical shape memory of their polymeric constitution is not long enough. Thus, the prolonged stress applied during storage reduces resiliency and impedes the ability of the dosage form to expand to the large configuration in the stomach ^[81]. Moreover, this kind of dosage form may not be cost-effective and most probably difficult to industrialize ^[39]. Finally, expendable systems should not interfere with gastric motility, must have blunt edge (to avoid mucosal damage), must be easily biodegradable and must not cause local damage because of its prolonged retention. Since a permanent retention of rigid large sized single-unit forms, may cause bowel obstruction, intestinal adhesion and gastropathy ^[31], it is important to be sure of the complete evacuation of the drug delivery system: hence, the time for “collapse” of the gastroretentive dosage form must be reproducible ^[81].

2.4 Superporous hydrogel

Superporous hydrogel is a swellable system. However, great differences exist between conventional swelling system and superporous hydrogel, explaining why they are classified separately. Indeed, due to pore size ranging between about 10 nm and 10 μ m, absorption of water by conventional hydrogel is a very slow process, and the time needed to reach an equilibrium state can take several hours ^[24,39]. During this lag time, the same risk of premature evacuation of the dosage form is encountered, as described with the gas generating systems. To overcome this problem, superporous hydrogels have been designed. To decrease the swelling time in the stomach, the pore size has been increased. With an average pore size larger than 100 μ m, superporous hydrogels swell rapidly to equilibrium size in a minute, due to water uptake by capillary wetting through numerous interconnected open pores ^[91]. Moreover, they swell to a large size (swelling ratio ~ 100 or more) (fig.16), and they are expected to develop a sufficient mechanical strength to withstand pressure by gastric contraction, by the addition in the formulation of Ac-Di-Sol[®] (croscarmellose sodium). This compound, a hydrophilic particulate material, substantially increases the mechanical strength of the system ^[92]. *In vivo* studies with dogs, showed that under the fasted condition, the superporous hydrogel composite (i.e. containing Ac-Di-Sol[®]) remained in the stomach for 2-3 h. Under fed conditions, it remained in the stomach for more than 24 h, even though the

fed condition was maintained only for the first few hours. After several hours (~30 h), fragmentation of the system occurred, and the superporous hydrogel composite emptied quickly.

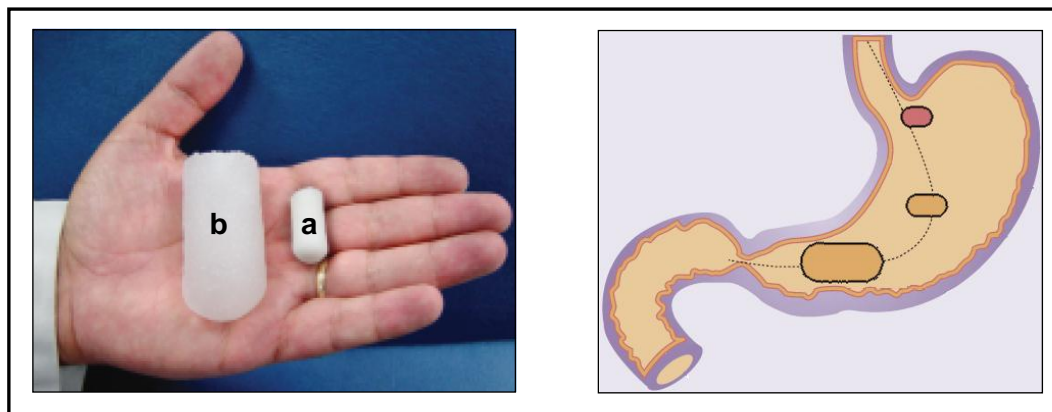


Fig.16: On the left, superporous hydrogel in its dry (a) and water-swollen (b) state. On the right, schematic illustration of the transit of superporous hydrogel. From Gutierrez-Rocca ^[93].

2.5 Mucoadhesive or bioadhesive systems

The study on mucoadhesive polymers was initiated by Park and Robinson in april 1984 ^[94]. Shortly afterwards, Smart et al. published a paper on *in vitro* tests to assess adhesiveness of various materials to mucus ^[95]. Since then, many papers have been written on polymers and bioadhesion, and many studies have been carried out to firmly stick drug delivery systems on different kind of mucosa, such as ophthalmic, vaginal, intestinal, nasal, buccal, and of course, gastric mucosa. The concept of mucoadhesion is that a dosage form can stick to the mucosal surface by different mechanisms. Different theories exist to explain these mechanisms. First is the electronic theory, involving attractive electrostatic forces between the glycoprotein mucin network and the bioadhesive material. Secondly is the adsorption theory, suggesting that bioadhesion is due to secondary forces such as Van der Waals forces and hydrogen bonding. Thirdly is the wetting theory, based on the ability of bioadhesive polymers to spread and develop intimate contact with the mucus layers. And finally, the diffusion theory, involving a physical entanglement of mucin strands and the flexible polymer chain, or an interpenetration of mucin strands into the porous structure of the polymer substrate ^[96-98]. Materials commonly used for bioadhesion are poly(acrylic acid) (Carbopol[®], polycarbophil), chitosan, Gantrez[®] (Polymethyl vinyl ether/maleic anhydride copolymers), cholestyramine, tragacanth, sodium alginate, HPMC, sephadex, sucralfate, polyethylene glycol, dextran, poly(alkyl cyanoacrylate) and polylactic acid. Even though some of these polymers are really effective, it is very difficult to achieve an efficient bioadhesion in

gastrointestinal tract because of the rapid turnover of mucus, and the relatively constant transit time. Furthermore, the stomach content is highly hydrated, and leads to a decrease in the bioadhesiveness of polymers. Indeed, Kockisch et al. compared different polymeric microspheres (poly(acrylic acid), Gantrez[®] and chitosan) in different conditions (tensile tests on porcine oesophageal mucosa and in elution experiments involving a challenge with artificial saliva). In tensile tests, poly(acrylic acid) particles exhibited a greater mucoadhesive strength and better swelling properties than those constructed from chitosan or Gantrez[®]. However, during the dynamic *in vivo* retention test involving saliva at constant flow, retention time for chitosan or Gantrez[®] particles was significantly longer (> 2 h for chitosan particles) compared to poly(acrylic acid) particles (~10 min). According to the authors, this difference may be attributed to swelling characteristics of the polymers. Because there was considerably more available water in this system (elution test) compared to tensile tests, poly(acrylic acid) polymers can hydrate more readily than chitosan or Gantrez[®] to form a non-adhesive mucilage and therefore to be 'washed' away ^[99].

In spite of these difficulties, several groups have developed bioadhesive systems with more or less success. Akiyama et al. reported in 1995 mucoadhesive microspheres using polyglycerol esters of fatty acids (PGEF) and a poly(acrylic acid) derivative. PGEF microspheres were either coated by carbopol[®] (CPC-microspheres), or poured in a carbopol[®] dispersion (CPD-microspheres). *In vitro* and *in vivo* studies with rats, showed prolonged gastrointestinal residence for CPD-microspheres compared to PGEF or CPC-microspheres ^[100]. In 1998, the same group developed bioadhesive microspheres (using carboxyvinyl polymer as bioadhesive agent) loaded with riboflavin or furosemide, and administrated them to 10 humans volunteers. By assessing furosemide in plasma and riboflavin in urinary excretions, adhesive microspheres were found to adhere to the gastric or intestinal mucosa with higher affinity in man compared to non-adhesive microspheres, resulting in prolonged gastrointestinal residence ^[101]. Several papers have been published about chitosan containing bioadhesive microparticles. Indeed, this polymer is natural, biodegradable, biocompatible, non toxic, and bioadhesive ^[102]. However, chitosan "alone" does not seem to produce interesting results. No prolonged gastric residence time was observed by Hejazi et al. for a formulation of chitosan microsphere in animals ^[103], and an erratic *in vivo* mucoadhesion of microcrystalline chitosan in human was described by Sakkinen et al.: these authors concluded that "formulations studied were not reliable gastro-retentive drug delivery systems" ^[104]. Nevertheless, by modifying chitosan (i.e. glyoxal-crosslinked chitosan ^[105], or synthesising derivatives ^[106,107]) real improvements have been obtained. For example, crosslinked chitosan microspheres are more resistant to gastric acidity than chitosan microspheres, and are slowly dissolved in the acidic stomach environment. It has been observed that the microspheres remained in the stomach of fasted gerbils even after 10 h of

administration ^[105]. To increase accuracy of mucoadhesion, some research groups have designed particles coated with lectin, characterised by their ability to bind carbohydrates with considerable specificity. For example, Ezpeleta et al. synthesized *Ulex europaeus* lectin-gliadin nanoparticles conjugates. The lectin was covalently fixed on nanoparticles (15 µg lectin/mg nanoparticle), without altering activity and specificity of *U. europaeus* lectin. Because the composition of the mucus is different according to the region of the mucous membrane ^[108], using lectin enables to target very specifically a site in the gastrointestinal tract. However, even though a good adhesiveness of the lectin is observed *in vitro* (for example, the tomato lectin for specific targeting of intestinal mucosa ^[109,110]), different studies performed *in vivo* have been less encouraging ^[111]. According to the authors, the lectin is probably binding to the intestinal mucus, but this mucus is constantly being turned over and carried out of the gut by peristalsis in a continuous flow ^[112]. Apart from few lectins ^[113], the same problem of mucus turnover is encountered with lectin conjugated with micro or nanoparticles ^[114-116]. Thus, the use of lectin targeting to the gastrointestinal tract to reduce the transit time of pharmaceutical formulations, has to date a limited success ^[112].

2.6 Magnetic systems

This system is based on a simple idea: the dosage form contains a small internal magnet, and an extracorporeal magnet is placed over the position of the stomach. The magnetic field retains the dosage form and controls its gastrointestinal transit. Ito et al. used this technique in rabbits with bioadhesives granules containing ultrafine ferrite (γ - Fe_2O_3). They guided them to the oesophagus with an external magnet (~1700 Gauss or 0.17 Tesla) for the initial 2 min, and 2 h after administration, approximately the entire amount of granules was held in the region ^[117]. Fujimori et al. formulated a magnetic tablet containing ultra ferrite (50% w/w), hydroxypropylcellulose and cinnarizine. In a study using beagle dogs, the magnetic tablet remained in the stomach for 8 h by the application of a magnetic field (1000 to 2600 G or 0.1 to 0.26 Tesla). Moreover, the absorption of cinnarizine was sustained and the area under the plasma concentration-time-curve values ($\text{AUC}_{0-24\text{h}}$) increased ^[118]. Groning et al. developed a method for determining the gastrointestinal transit of magnetic dosage forms under the influence of an extracorporeal magnet, using a pH-telemetering capsule (Heidelberg capsule). Small magnets were attached to the capsule, and administered to humans. Without external magnetic source, the dosage form transited to the intestine within 2.5 h after administration. On the other hand, with the presence of an extracorporeal magnet, in most of cases, gastric residence time of the dosage form was longer than 6h ^[119]. Two years later, the same group proposed peroral acyclovir depot tablets with internal magnets. *In vivo*

studies on five healthy male volunteers showed that in the presence of an extracorporeal magnet placed in the stomach region, the plasma concentrations of acyclovir were significantly higher after 7, 8, 10 and 12 h. Furthermore, the mean $AUC_{0-24\text{ h}}$ was ~ 2802 ng.h/mL with the external magnet, and ~ 1599 ng.h/mL without the magnet ^[120].

According to the published results, magnetic systems seem to work. However, the external magnet must be positioned with precision, and the accuracy needed for that might lead the patient to a lack of compliance ^[24,39].

2.7 Conclusions

All these drug delivery systems (high-density, floating-, unfolding-, mucoadhesive, magnetic systems etc.) are interesting and present their own advantages and drawbacks. An important feature to take into account is the stomach physiology. For example, **the moment when the drug is taken** (during or apart from the meal), is an important parameter. If a single unit system is taken during the phase III of the IMMC, because of the powerful peristaltic waves, the risks to expel the drug into the duodenum are increased. The drug evacuation is also dependent on its dissolving time. A fast dissolving drug will quickly evacuate with water through the pylorus. Otherwise, the drug will leave the stomach with the next meal or during the next phase III.

If the drug is taken during a meal, its fate is different. Hydrosoluble drugs leave the stomach with water, while liposoluble drugs leave it with lipids. And as seen previously, fats are evacuated last. In addition, the time of gastric emptying is strongly influenced by the calorific density and the bulk of the meal. Wilson et al. have shown that enteric-coated or enteric matrix tablets may be retained for a considerable time when dosed with a heavy breakfast ^[121]. For multiple unit systems, emptying process is also slower with a meal than during fasted state, because the dosage forms mix evenly with the food (density > 1), or float at the surface (density < 1). In general, drugs are evacuated more rapidly during fasted state than during prandial or post-prandial period ^[6,25,62]. Furthermore, concomitant intake of drugs such as anticholinergic agents (atropine) ^[122], opiates (codeine) ^[123] and prokinetic agents (erythromycin, metoclopramide) ^[124] highly affect the gastric retention.

Secondly, the intragastric pH varies a lot during a 24 h period. To be absorbed by the stomach, an active agent must be in its un-ionised form ^[6], which is under dependence of its **pKa** and the **stomachal pH**. Hence, in order to increase stomachal absorption, either the molecule should be un-ionised between pH 1 to 6, or the drug must be given at an optimised moment, when the stomach pH is theoretically appropriate. Furthermore, the active agent must be stable under the stomach conditions.

Finally, it is generally accepted that biological factors such as gender, age, or body mass index can affect gastric residence time of indigestible solids ^[125]. However, a recent study, based on the assessment of motility variables (gastric emptying, small intestinal and colonic transit rates, postprandial frequency of antral contractions) has shown that such biological factors do not influence so much gastric motility. Ageing seems to reduce the propulsive capacity of the colon, however, in this study, gastric and small intestinal motility was not affected, and furthermore, none of the motility variables was affected by gender or body mass index ^[126].

The stomach physiology is not the only parameter which affects the fate of drug delivery systems. Pathologies can also deeply modify the physiological gastric state, as diabetes for example ^[127]. Thus, it is necessary to develop specific formulations.

3 Particular case of *Helicobacter pylori*

Since its discovery in 1982 by Warren and Marshall, and its confirmation as a pathogen at the end of the 80's, researchers attempted in several ways to eradicate efficiently *H. pylori* from the stomach. It is well known that long time infection can lead to severe diseases, such as gastric cancers and MALT (mucosa-associated lymphoid tissue) lymphomas. In most countries, *H. pylori* infection is associated with a 4 – 6 fold increased risk of gastric cancer: this means that the majority of the gastric carcinomas in the world are related to *H. pylori* infection ^[128]. Because of the high level of antibiotic resistance to *H. pylori* and the poor patient compliance ^[129], new medicine with better effectiveness and simpler regimen are required. Given that the bacterium lives deep in the gastric mucus, a logical way to improve the efficiency of therapeutics, is to develop gastroretentive dosage forms in order to release drugs as long as possible in the ecological niche of the bacterium. For that, it is important to take into account the physiological modifications generated by a long-term colonization of the gastric mucosa by *H. pylori*.

3.1 Stomach physiopathology following *H. pylori* infection

Since 1994, the International Agency for Research on Cancer and the World Health Organisation, has been considering that *H. pylori* infection is carcinogenic to humans (group 1 carcinogen) ^[130]. Indeed, long term colonisation by *H. pylori* can cause not only several diseases such as dyspepsia, gastritis, duodenal or gastric ulcer, but also gastric cancer and MALT lymphoma. Colonisation of the gastric mucosa by *H. pylori* always causes gastritis, which may be asymptomatic or, seldom, symptomatic. Gastritis evolves in chronic gastritis, characterised by a gastric mucosal barrier dysfunction. This phenomenon was observed by

Sun et al. in gerbils, and results in an increase of the gastric mucous membrane permeability for molecules with a low molecular-weight (^{51}Cr EDTA, 342 Da) as well as high molecular weight (horseradish peroxidase, 44000 Da) ^[131]. These results support other findings in humans showing increased gastric mucosal permeability to sucrose after *H. pylori* infection ^[132,133]. Furthermore, Noach et al. observed abnormalities in the tight junction complexes in patients with *H. pylori* infection ^[134], and Posalaky et al. described an increased frequency of tight junction breaks in biopsy specimens from inflamed gastric mucosa ^[135]. *In vitro*, Byrd et al. have shown that *H. pylori* decreases or inhibits mucin synthesis ^[136,137]. Similar results have been found *in vivo*. Newton et al. found a significant 18% reduction in the proportion of polymeric gel forming mucin in the adherent mucus layer in *H. pylori* positive compared to negative subjects ^[12]. Tanaka et al. suggested that *H. pylori* infection decreases gastric mucin synthesis via inhibition of UDP-galactosyltransferase ^[138]. This effect may certainly impair the gastric mucosal barrier and contribute to the mucosal injury induced by *H. pylori* infection. Finally, *H. pylori* seems to affect the normal balance between gastric epithelial cell proliferation and epithelial cell death, interfering thus with the maintenance of gastric mucous membrane integrity. Because apoptosis regulates the cell turnover cycle in balance with proliferation, dysregulation of apoptosis associated to *H. pylori* colonization might be linked to gastric carcinogenesis ^[139]. On the one hand, some authors reported that *H. pylori* infection induces apoptosis in epithelial cells ^[140-142]. On the other hand, de Freitas et al. reported that for patients with functional dyspepsia, *H. pylori* infection induces a significant epithelial cell proliferation in the antrum area. The authors compared the cell proliferation index (PI) to the cell apoptotic index (AI), and linked them to the *cagA* (cytotoxin-associated antigen) or *vacA* (vacuolating cytotoxin) status of the strains. Indeed *cagA* and *vacA* are considered as virulence factors of the bacteria ^[128,143-145]. The *cagA* protein is secreted by *H. pylori* into the epithelial cells, wherein the tyrosine phosphorylation of the protein allows it to produce a 'growth factor' signal; this hinders the epithelial cell to maintain its normal cytoskeletal structure ^[128,144]. *VacA* is a cytotoxin secreted by the bacteria, which inserts itself into the epithelial-cell membrane to form an hexameric anionic selective channel. Its vacuolating effect induces apoptosis ^[143,146-148]. The authors found that Cag A(+) strains promotes the increase of PI, and Cag A(-) strains promotes the increase of AI, whereas the Vac A status has no influence on the PI or AI.

Furthermore, for Henriksnäs et al., the gastric mucosal barrier dysfunction induced by *H. pylori* infection, and more particularly the decrease in thickness of the firmly adherent gastric mucus gel layer, seems to reduce the ability to maintain the neutral pH at the epithelial cell surfaces in the presence of luminal acid ^[149].

Secondly, according to several authors, *H. pylori* gastritis can produce marked alterations in gastric acid secretion. Indeed, McColl et al. observed in subjects with an antral predominant gastritis, an increased release of gastrin, and consequently, an increased acid secretion. In the same paper, they noted that in other subjects, the infection produced marked body gastritis, which was associated with marked hyposecretion of acid or complete achlorhydria^[150]. This is consistent with the findings El-Omar et al., who showed that subjects with *H. pylori*-induced body-predominant gastritis, presented a profound suppression of gastric acid secretion that was partially reversible with eradication therapy^[151]. A long-term follow-up study (5 years) performed by Iijima et al., suggested that even after *H. pylori* eradication, in the majority of *H. pylori*-positive patients with marked body atrophy and profound hypochlorhydria, the pathologic process had already progressed to an irreversible stage, leaving the acid secretory levels subnormal in the majority of the patients^[152].

Thirdly, *H. pylori* infections, or at least, *H. pylori*-induced pathologies, seem to play a role on gastric motility. A study involving 58 *H. pylori* infected patients presenting active duodenal ulcer (DU), Chang et al. found that about 35% manifested either enhanced or delayed gastric emptying (of water) before treatment. After one-week triple therapy (omeprazole, amoxicillin and clarithromycin), ulcer healing and *H. pylori* eradication rates were 91.4% and 82.8%, respectively. Only 10.4% had abnormal gastric emptying, whereas normalized gastric emptying was not found in non-eradicated patients^[153]. Similarly, Konturek et al. studied the inhibitory effect of cholecystokinin (CCK) on gastric motility in DU patients. Endogenous cholecystokinin is known to inhibit the gastric emptying. In a double blind study involving 10 DU *H. pylori*-positive patients, these authors found that *H. pylori* infection was accompanied by enhanced gastric emptying and reduction in luminal release of somatostatin. This phenomena could be reversed by the eradication of *H. pylori*, indicating that both CCK and somatostatin may contribute to normalization of gastric emptying following *H. pylori* eradication in DU patients^[154]. On the other hand, Leontiadis et al. have compared the gastric emptying rate of patients with non-ulcer dyspepsia (NUD) versus asymptomatic patients (controls). The gastric emptying rate was assessed by the paracetamol absorption method. They found that NUD patients (*H. pylori*-positive and *H. pylori*-negative) had significantly delayed gastric emptying compared with controls. However, they studied the gastric emptying rate between *H. pylori*-positive and *H. pylori*-negative NUD patients, and they did not find a significantly difference. Furthermore, for patients who were initially *H. pylori*-positive, eradication of the infection did not significantly alter gastric emptying rate. Thus, in the case of NUD, gastric emptying rate is not associated with *H. pylori* status nor it is affected by eradication of the infection^[155].

3.2 Gastroretentive dosage forms against *H. pylori*

3.2.1 Floating drug delivery systems

In 1994, a patent assigned to Reckitt and Colman Products described a raft-forming formulation using triclosan. The drug was mixed with alginic acid, sodium bicarbonate, calcium carbonate and mannitol. The mixture was granulated and citric acid was added to the granules, and then packed into sachets or compressed to form tablets. In contact with the acid conditions of the stomach, the carbonate or bicarbonate salts produced effervescence, which aerated the raft structure formed by the alginates, causing it to float. However, the authors noticed that in some patients with *H. pylori* infections, the pH of the stomach contents may be elevated (possibly to as high as pH 6) reducing effervescence and, consequently, reducing the ability of the rafts to float. For this reason, they added citric acid in their formulation ^[156].

Yang et al. proposed a gas generating system, consisting of a swellable asymmetric triple layer tablet. One layer was the swellable gas-generating layer (poly(ethylene oxide), HPMC, sodium bicarbonate:calcium carbonate [1:2 w/w]). The second one was the swellable/sustainable drug containing layer (poly(ethylene oxide), tetracycline hydrochloride, metronidazole). The third one was a rapidly dissolving drug layer (bismuth salts). According to the authors, the aim of such device was to obtain a simple regimen for a standard triple therapy. Indeed, they obtained *in vitro* (in a 37°C, 0.1 M HCl solution) a duration of buoyancy and a sustained release of metronidazole and tetracycline over 6-8 h with a buoyancy lag time in the range of 17-28 min. The rapid effectiveness of the device would be carried out by the rapid dissolving layer containing the bismuth salt, which disintegrated within 10-15 min *in vitro*. However, no *in vivo* data are available concerning floatability of the drug delivery system or its efficiency against *H. pylori* ^[59].

Umamaheshwari et al. developed several drug delivery systems, especially designed to improve efficiency against *H. pylori*. In all of them, they used an antiurease drug, acetohydroxamic acid (AHA), as an active agent against the bacterium. *H. pylori* urease hydrolyses urea present in the gastric juice and extracellular fluid, to generate ammonia and bicarbonate, which effectively neutralise acidic pH in its environment ^[128,157]. Thus, urease inhibitor hinders the bacteria to protect itself against low pH, and avoid the problem of antibiotic-resistant strains ^[158,159]. This group developed polycarbonate microballoons (240 to 288 µm) by an emulsion (o/w) solvent evaporation technique. *In vitro* (in simulated gastric fluid), ~ 74 to 85% of microballoons stayed buoyant at 12h and exhibited a sustained drug release profile. *In-vitro* and *in-vivo* growth inhibition studies were performed using cultures of *H. pylori* and *H. pylori*-infected Mongolian gerbils, respectively. Microballoons showed 10

times higher anti-*H. pylori* activity compared with AHA solution ^[158]. The authors of the latter study also formulated floating-bioadhesive microspheres. The microballoons (made by a quasi-emulsion solvent diffusion method) were coated with 2% (w/v) solution of polycarbophil by the air suspension coating method. *In vitro* floating studies, detachment force measurements, and *in vivo* growth inhibition studies, demonstrated the potential of this device, which combines bioadhesive and floating properties ^[160]. Besides, cellulose acetate butyrate (CAB)-coated cholestyramine microcapsules were also proposed as gastroretentive drug delivery system by Umamaheshwari et al.. Indeed, they used CO₂ generation to provide floatability, and cholestyramine in order to have a mucoadhesive effect. Ion-exchange resin particles were loaded with bicarbonate followed by AHA, and coated with CAB by an emulsion solvent evaporation method. *In vitro* (drug release, buoyancy) and *in vivo* studies (gastric mucoadhesion in rat stomach) led the authors to conclude that this drug delivery system possessed both floating and bioadhesive properties, and may be successful in the treatment of *H. pylori* ^[161].

3.2.2 Mucoadhesive drug delivery systems

In 1998, Nagahara et al. formulated mucoadhesive microspheres containing amoxicillin. They dispersed the drug and bioadhesive polymers (carboxyvinyl polymer and curdlan [a polysaccharide]) in melted hydrogenated castor oil. Microspheres of 250 to 335 µm in diameter were obtained by a spray-chilling method followed by sieving. They compared these microspheres with an amoxicillin suspension in infected mongolian gerbils under fed conditions. The microspheres with an amoxicillin dose of 1.0 mg/kg provided the same clearance rate (20%) as the amoxicillin suspension with a dose of 10 mg/kg. This means that the amoxicillin-microspheres provided 10 times greater anti-*H. pylori* activity than the amoxicillin suspension. Moreover, adhesion of microspheres on the stomach wall was observed (~ 47% and ~ 20% remained in the stomach after 2 and 4 h, respectively). The authors concluded that these mucoadhesive microspheres containing an appropriate antimicrobial agent should be useful for the eradication of *H. pylori*. ^[162]

Recently, Liu et al. also published a study on mucoadhesive microspheres of amoxicillin. They prepared them by an emulsification/evaporation method, using ethylcellulose as matrix and carbopol 934P as mucoadhesive polymer. They demonstrated that free amoxicillin was rapidly degraded in acidic medium, however, amoxicillin entrapped in the microspheres kept stable. The *in vitro* release test showed that about 90% of amoxicillin was released in the pH 1.0 HCl solution within 4 h, while *in vivo* evaluation of mucoadhesiveness showed that during the same time, 63.6±21.9% of microspheres still remained in rat's stomach. Furthermore, they assessed a higher amoxicillin concentration in gastric tissue, after orally administration

of mucoadhesive microspheres vs. amoxicillin powder to rats at the same dose (43mg/kg). Finally, *in vivo* clearance of *H.pylori* studies revealed that for a single-dosage administration (4 mg/kg to 14.8 mg/kg), the mucoadhesive microspheres had a better effectiveness (expressed by the ratio of colony counted between amoxicillin powder and microspheres) compared to amoxicillin powder (3.2 to 9.7, respectively). In parallel, a multidosage administration regimen (3.5 mg/kg, twice a day for three consecutive days), showed a complete eradication of *H.pylori* with microspheres for 5 out the 6 rat's stomach samples involved in the study, whereas amoxicillin powder showed 4 times less efficiency. With this preliminary study on *H. pylori* clearance effect, the authors observed a tendency for an effective anti-*H. pylori* activity induced by mucoadhesive microspheres, but concluded that larger groups of animal are required to confirm these results ^[163].

Katayama et al. proposed a sustained release liquid preparation using sodium alginate. The gastroretentive property of the device was provided by the ability of sodium alginate to form a firm gel when an acid or di- or tri-valent metal ions (Ca^{2+} , Ba^{2+} , Sr^{2+}) are added. The authors expected the solution to be able to spread out, adhere to the gastric mucosa, and release continuously the antibiotic (ampicillin) (fig.17).

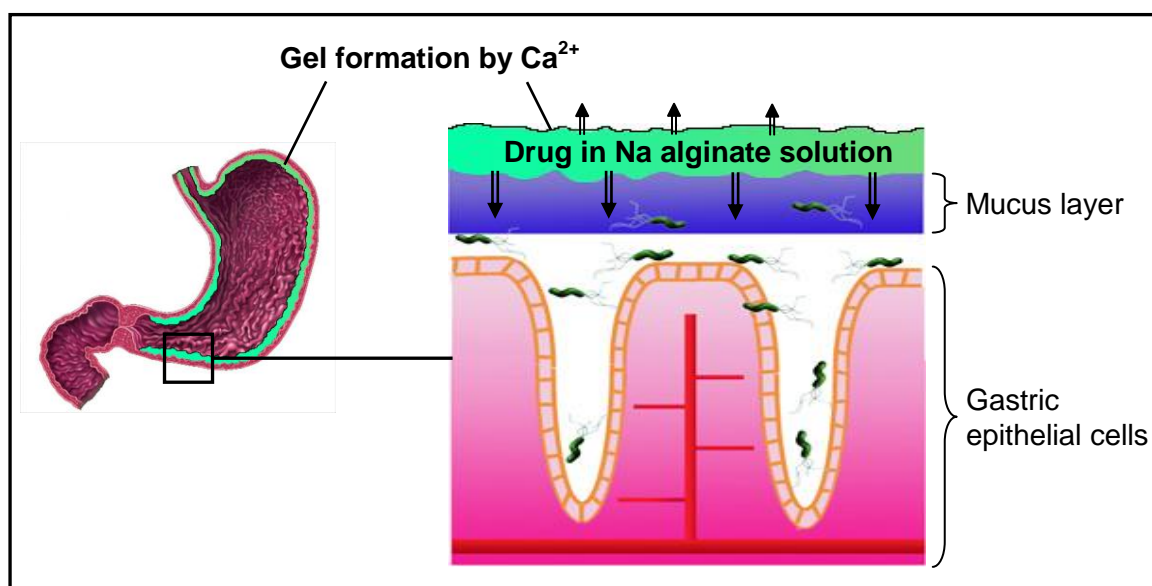


Fig.17: Schematic representation of the strategy for the eradication of *H. pylori* by sustained release liquid preparation containing ampicillin. Based on Katayama ^[164].

In vitro, the ampicillin release was retarded by calcium pre-treatment (0.10 M., 20 sec.) due to gel-formation. To evaluate the gastric residence time of the preparation, the authors compared in isolated perfused rat stomachs the remaining percent of ampicillin for an aqueous ampicillin solution vs. the sodium alginate preparation. When a calcium pre-

treatment was performed, the total remaining percent of ampicillin at 120 min was ~ 0.3% and 8% for aqueous ampicillin solution and the sodium alginate preparation, respectively. Moreover, it was observed that the sodium alginate preparation remained mainly on the gastric mucus. *In vivo* studies were also performed by the administration of aqueous ampicillin solution or sodium alginate preparation through a gastric tube to fasted rats. Because sodium alginate is insoluble in acidic conditions, the authors pre-administrated ranitidine (an H₂-blocker) to rats just before calcium solution. In these conditions, the total remaining percent of ampicillin at 60 min was near zero for aqueous ampicillin solution, and ~ 87% for sodium alginate solution ^[164].

Hejazi and Amiji recently published several papers about tetracycline-loaded crosslinked chitosan microspheres. They used different crosslinking methods. First, they prepared chitosan microspheres by ionic cross-linking and precipitation with sodium sulfate. However, it was observed that such chitosan microspheres did not provide a longer residence time in the fasted gerbil stomach. Moreover, the tetracycline concentration profile in the stomach, following administration in microsphere formulation, was similar to that of the aqueous solution ^[103]. They then tried a second crosslinking method which uses a chemical crosslinker as glyoxal. Radioiodinated (¹²⁵I) glyoxal-crosslinked chitosan microsphere suspension was administered in fasted gerbils, and animals were sacrificed at different time points to assess the radioactivity in tissues and fluids. After 2 h in the fasted stomach, ~ 17% of the crosslinked chitosan microspheres were still present (vs. ~ 10% for the non-crosslinked chitosan microspheres), and the tetracycline concentration profile in the stomach from the crosslinked microsphere formulation was higher (AUC_{0.5-10 h} ~ 870 µg.h/g of tissue) than that of the aqueous solution (~ 447 µg.h/g of tissue) and the non-crosslinked microsphere formulation (~ 358 µg.h/g of tissue). 10 h after administration, ~ 11% of crosslinked chitosan microspheres remained in the stomach ^[105].

3.2.3 Drug delivery systems with specific interaction

As already described for the bioadhesive systems, it is sometimes possible to use a specific ligand (a lectin for example) coupled with the dosage form, to target specifically a site in the gastrointestinal tract. Similarly, Umamaheshwari et al. proposed drug delivery systems with a specific targeting against *H. pylori*. To reach this goal, the authors proposed two different drug delivery systems. First, they developed nanoparticles bearing AHA coated with fucose. Indeed, it is well known that some strains of *H. pylori* express an adhesin, BabA2, which interacts with fucosylated histo-blood group antigen Lewis b (Le^b) ^[147,157,165,166]. This Le^b blood group antigen is expressed on the surface of gastric cells, and the BabA2 adhesin is essential for the bacterium adhesion ^[147]. Hence, by using fucose as ligand on the surface of

nanoparticles, Umamaheshwari et al. enabled a specific targeting against *H. pylori*. Chitosan-glutamate nanoparticles were prepared by an ionotropic gelation method, and the (L)-fucose was covalently bound to nanoparticles. The interactions between (L)-fucose conjugated chitosan-glutamate nanoparticles and *H. pylori* were characterised *in situ*, by an adherence assay with FITC-labelled strains (fluorescein-isothiocyanate) on sections of human stomach. A “plug and seal” effect between *H. pylori* and nanoparticles was observed. Furthermore, *in vitro* growth inhibition studies showed that (L)-fucose conjugated chitosan-glutamate nanoparticles exhibited ~2 fold inhibitory efficacy compared to chitosan-glutamate nanoparticles and plain drug ^[167]. By combining a bioadhesive effect (provided by chitosan and glutamate) and targeting capacity (provided by fucose), this drug delivery system presented interesting properties. However, not all the strains of *H. pylori* express the BabA2 adhesin ^[147,157,165,166]. Consequently, such system cannot be used in all *H. pylori* infections, and will never provide a 100% eradication rate. Then, Umamaheshwari’s research group proposed a second drug delivery system, based on phosphatidyl-ethanolamine (PE) containing lipid to develop a receptor-mediated drug delivery system. Indeed, PE seems to be a major receptor in promoting *H. pylori* adhesion to intact cells ^[168-170]. They used as carrier, a new hybrid vesicle, called lipobead, combining complementary advantages of liposomes and polymeric beads. This system consists of a lipid bilayer shell (PE) that is anchored on the surface of a hydrogel polymer (polyvinyl alcohol xerogel) core (fig.18). The specific binding between lipobeads and PE specific surface receptor of *H. pylori* was confirmed by an *in situ* adherence assay and radiolabelling assay with human stomach cells and KATO-III cells, respectively. Furthermore, strong agglutinations between PE-lipobeads and 2 different strains of *H. pylori* (69A and 1101) were demonstrated. The *in vitro* growth inhibition studies showed a better efficacy of PE-lipobeads than polyvinyl alcohol (PVA) bare beads and plain AHA (the 3 formulations containing the same amount of AHA). After 6 h the percent of growth inhibition (% GI) values were 100%, 25% and 35% and for lipobeads, PVA bare beads and plain AHA, respectively. After 24 h the % GI values were 100% for the three dosage forms. Contrary to PVA bare beads and plain AHA, the % GI of lipobeads was independent of drug concentration and incubation time. PVA bare beads and plain AHA take a longer time to supply the same efficiency as lipobeads. According to the authors, these results can be explained by the fact that drug targeting was predominant as a result of specificity of the lipobeads towards PE specific receptors on the bacterial surface glycocalyx. By protecting the drug from the gastric environment, and providing a good targetability, this drug delivery system could be successful in the treatment of *H. pylori*. But additional *in vivo* studies are required to evaluate the real efficiency of this system ^[159].

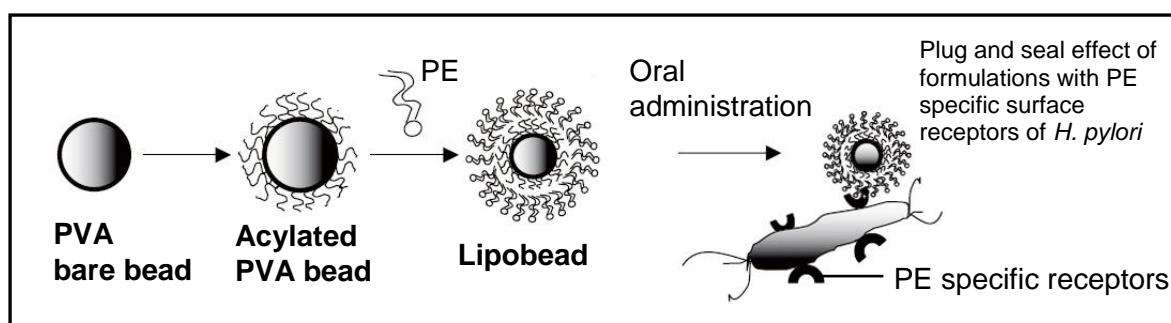


Fig.18: Recognition of lipobeads by PE specific surface receptors of *H. pylori*. Based on Umamaheshwari ^[159].

General conclusion:

To develop an efficient gastroretentive dosage form is a real challenge. Indeed, the drug delivery system must remain a sufficient time in the stomach, which is not compatible with its normal physiology. Among all the dosage forms described herein, some of them provide interesting solutions, although many of them present drawbacks. In the particular case of the *H. pylori* eradication, to be really efficient, the ideal dosage form should not only stay in the stomach, but also target the bacteria. However, knowledge about this pathogen discovered twenty years ago, is still poor. More data are necessary, for example, to identify an “ubiquitous” receptor (i.e. which exists for all *H. pylori* strains) at the surface of the bacteria which could provide a strong interaction with a ligand. Furthermore, the bacteria can exist in the stomach under a coccoid form ^[171,172], which corresponds either to a degeneration ^[173] or to a resistance form ^[174]. This transformation to a coccoid form can occur when the bacteria are subject to stress conditions, such as antimicrobial agents, aerobiosis or temperature modification, basic pH, or nutritional depletion ^[175-179]. Even though *H. pylori* expresses important pathogenic genes under coccoid form ^[180], it modifies deeply its ultrastructure ^[174] and consequently, its adhesiveness for potential ligands ^[176,181]. Thus, the development of an efficient gastroretentive dosage form against *H. pylori* is closely linked to a better understanding of its pathogenicity mechanisms.

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Les difficultés à surmonter pour la mise au point d'un médicament à temps de résidence gastrique prolongée sont donc nombreuses. L'estomac n'est pas, par nature, une partie du corps où des « objets » quels qu'ils soient, peuvent rester pendant une période prolongée. Parmi les différentes formes décrites précédemment, aucune ne semblent être parfaites. Les « single units systems » présentent un temps de rétention trop variable, du à l'effet de « tout ou rien » lors du passage du pylore. Les systèmes bioadhésifs sont peu efficaces à cause de l'hydratation très abondante de l'estomac et du turn-over rapide de la muqueuse gastrique. Les systèmes générant un gaz ont un temps de latence trop important durant lequel le médicament a de fortes probabilités d'être évacué. Les systèmes de faibles densités doivent, pour être efficaces, être avalés avec beaucoup d'eau. Tous les systèmes mécaniques présentent des risques d'obstructions de l'estomac et des problèmes de conservation sur le long terme, et enfin, les systèmes magnétiques sont peu confortables pour le patient, et il est fort à parier que l'observance du traitement avec de tels systèmes ne sera pas des meilleures. Le cahier des charges que devra remplir le médicament « idéal » pour résider suffisamment longtemps dans l'estomac et lutter efficacement contre H. pylori pourrait donc être le suivant :

- Médicament sous forme de « multiple units systems » afin d'augmenter leur répartition statistique le long du tractus digestif. Les vecteurs micro- ou nanoparticulaires sont donc clairement indiqués.
- Système suffisamment petit pour pouvoir diffuser au travers du mucus gastrique et ainsi atteindre les Helicobacters. Une taille inférieure à 200nm serait donc nécessaire ^[1]. Nous nous orientons donc vers des systèmes nanoparticulaires (liposomes, nanoparticules).
- Une parfaite innocuité. Comme n'importe quel médicament, la balance bénéfices/risques se doit d'être favorable au nouveau médicament, et engendrer le moins d'effets indésirables possibles au patient. La forme liposomale remplit pleinement cette condition. La composition phospholipidique de la bicouche membranaire est structurellement très proche des membranes cellulaires et ne présente aucunes toxicités, ce qui n'est pas forcément le cas des polymères utilisés pour les micro- ou nanoparticules.
- Système permettant un ciblage de la bactérie. Les intérêts sont multiples. Dans un premier temps on peut imaginer qu'en restant « fixé » sur la bactérie, le système demeurera dans l'estomac. Le principe actif est amené au plus près de sa cible, ce qui permet de potentialiser son effet, mais également de réduire les effets indésirables. Le vecteur final devra donc avoir une surface facilement « modifiable »

afin de pouvoir y greffer un ligand en vue d'obtenir un ciblage, et si possible permettra l'encapsulation de principes actifs de natures variées.

*Pour toutes ces raisons les liposomes nous ont semblé être de bons candidats. La technologie pour leur fabrication reste accessible et leur manipulation en laboratoire est assez aisée. Le ciblage de la bactérie se fera par l'intermédiaire du fucose, incorporé en surface du liposome. Ce sucre jouera le rôle de ligand pour l'adhésine bactérienne BabA2. BabA est une protéine OMP (Outer Membrane Protein - protéine de la membrane externe) de 75-kDa qui possède deux allèles : BabA1 et BabA2. BabA1 est « silencieux » du fait de l'absence du codon d'initiation, contrairement à BabA2 qui contient un motif répétitif de 10 paires de base correspondant au codon d'initiation ^[2]. Par conséquent seul BabA2 est responsable de l'adhésion. L'adhésine BabA2 est impliquée dans l'interaction avec l'antigène fucosylé de groupe sanguin Lewis^b (α -1,3/4-difucosylé) exprimé à la surface des cellules gastriques. (La présence de cette adhésine ne peut être généralisée à toutes les souches d'*H. pylori* mais elle est souvent présentée comme un facteur de virulence.) ^[3-6]. C'est donc en se basant sur cette interaction adhésine – sucre que nous espérons cibler la bactérie. La fonctionnalisation des liposomes se fera par incorporation de néoglycolipides de structure suivante : Ancre-Espaceur-Sucre. Le choix de l'ancre hydrophobe s'est porté sur le cholestérol : il s'insère dans les bicouches phospholipidiques de liposomes, permettant la fixation à leur surface du groupement glycosylé (cf. Fig.1).*

*En théorie, l'emploi d'une ancre hydrophobe, telle que le cholestérol, permet un ancrage « solide », et une stabilisation de la bicouche phospholipidique ^[7-9]. En effet une ancre de type alcool de Guerbet, par exemple, aurait fluidifié la bicouche ^[10]. La longueur de l'espaceur est importante. Trop court, celui-ci n'offrirait pas les meilleurs performances pour le ciblage que nous envisageons ^[11]. C'est pourquoi nous utiliserons le tétra-éthylène glycol. De part la mobilité qu'il confère à la tête sucrée, et l'éloignement suffisant de la paroi du liposome, il permet une bonne reconnaissance *in vitro* des lectines végétales ^[12]. En utilisant par exemple un espaceur mono ou di-éthylène glycol, la reconnaissance du groupement glycosidique par les lectines végétales risquerait d'être bien moins bonne. Pour le groupement de reconnaissance, les deux groupements glycosidiques utilisés seront la N-acétylglucosamine et le fucose. Une fois synthétisé, et pour avoir ensuite une idée de leur comportement au sein de la bicouche phospholipidique lors de la fabrication et l'utilisation des liposomes, nous nous intéresserons à l'organisation supramoléculaire de ces néoglycolipides en présence d'eau en quantité croissante, dans une gamme de température allant de 20 à 60°C.*

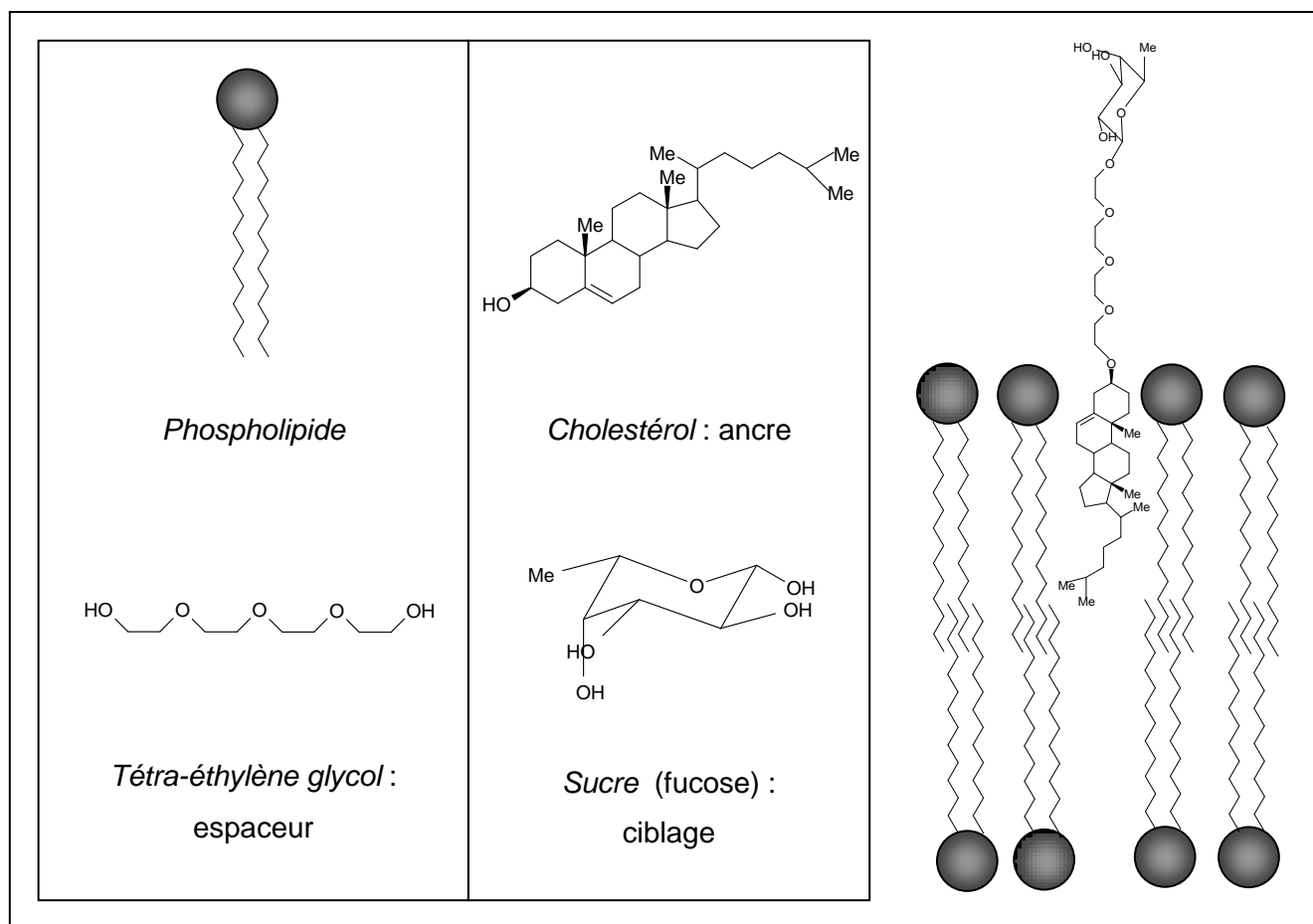


Fig.1: Représentation schématique du glycolipide et de son insertion au sein de la bicouche phospholipidique d'un liposome.

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CHAPITRE II:

Self-organization of synthetic cholesteryl oligoethyleneglycol glycosides in water.

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Contents

Introduction	69
1. Materials and methods.....	70
1.1. General synthetic methods.....	70
1.2. X-ray diffraction	72
1.3. Determination of critical micellar concentration	72
1.4. Surfactant adsorption area	73
1.5. Molecular graphism.....	73
2. Results and discussion	73
2.1. Lamellar region	73
2.2. Bicontinuous phases	76
2.3. Discontinuous phases	81
References.....	86

Figures

Fig.1: Reaction scheme	70
Fig.2: Diffractograms of anhydrous glycolipids.....	74
Fig.3: Four different arrangement of the glycolipid within the lamella	76
Fig.4: Diffractograms obtained at 20°C (a) and 60°C (b) for GlcNAc-E4-Cholesterol 1 in absence or in presence of water	77
Fig.5: Diffractograms obtained at 20°C with a GlcNAc-E4-Cholesterol/water 75:25 mixture by the use of the Elettra Synchrotron beam	77
Fig.6: R3m schematic representation	79
Fig.7: Diffractograms between 20°C and 60°C obtained with the GlcNAc-E4- Cholesterol/water 70:30 mixture	80
Fig.8: Diffractograms between 20°C and 60°C obtained with the GlcNAc-E4- Cholesterol/water 60:40 mixture	81
Fig.9: Diffractograms between 20°C and 60°C obtained with the GlcNAc-E4- Cholesterol/water 50:50 mixture	82
Fig.10: Im3m schematic representation.....	82
Fig.11: Surface tension versus concentration of GlcNAc-E4-Cholesterol.....	83

Tables

Table I: analysis of the anhydrous glycolipid SAXS patterns	74
Table IIa and IIb: main results obtained from molecular graphism tools.....	75
Table III: peak positions, tetragonal and rhombohedral indexations for glycolipid 1 / water (75:25) at 20°C	78
Table IV: a and c parameters of the rhombohedral mesh phase at 20°C for mixtures containing 70% and 60% of GlcNAc-E ₄ -Cholesterol 1.....	80
Table V: phases observed during the hydration of GlcNAc-E4-Cholesterol 1	85

Introduction

Drug targeting of therapeutic vector is an interesting approach to increase the pharmacological effect of drugs and to reduce potential side-effects. Among receptor-ligand couples investigated ^[1], the lectin-sugar recognition systems have been fully investigated in the pharmaceutical field, especially for the development of drug carriers, tailored for selective delivery ^[1-3]. In this context, we focused part of our work on glycosides of cholesteryl oligoethyleneglycols. A carbohydrate was used as the ligand for molecular recognition; a tetraethyleneglycol moiety was used as a spacer between the carbohydrate and the lipid fractions to favour recognition of the head group; cholesterol was selected as the hydrophobic anchor.

Uncharged glycolipids belong to the family of the non-ionic surfactants. From a pharmaceutical point of view, non-ionic surfactants present interesting properties 1) they are usually usable with the other surfactants, 2) they are not sensitive to high mineral content water, 3) their physico-chemical properties are not strongly affected by electrolytes, contrariwise to ionic surfactants ^[4].

In the majority of non-ionic surfactants, the polar group is either a polyol or a polyether obtained by polymerization of ethylene oxide. The starting materials submitted to polyethoxylation are fatty alcohols, acids or amines, alkyl phenols or glycerides. The behavior in presence of water of some cholesterol or sterol derivatives have been described in the literature. Among them, can be noted short-chain polyoxyethylene cholesterol ether containing 3, 10 or 15 EO units (^[5] ^[6]) or polyoxyethylene phytosterol containing 5, 10 or 20 EO units (^[7,8]). Other sterol surfactants, generally named "Chol-PEG" have been extensively reported in the literature, however they have polymeric PEO chains acting as steric stabilizer ^[9]. In the case of phytosterol surfactants, it was found that the pentaoxyethylene derivative formed a lamellar phase for important surfactant concentration in water (^[7,8]); when the concentration decreased to less than 40%, the lamellar phase separates. For 10-13 EO units, hexagonal phases are formed, followed by large lamellar and reverse micellar regions at increasing surfactant concentration. For longer hydrophilic chains, micellar, discontinuous cubic and hexagonal phases were found. In the case of trioxyethylene cholesterol ether, a lamellar phase could be observed for surfactant concentration higher than 75%; at lower concentrations, a phase separation with an excess of aqueous phase occurs ^[5]. When the number of EO units increased to 10 and 15 ^[6], the phase diagrams of the binary water/CholEO_n systems display successive lamellar, ribbon, hexagonal and cubic phases.

As mentioned before, the polar group of the non-ionic surfactants can be polyols such as carbohydrates (glucose, sucrose) or acyclic compounds (sorbitan, polyglycerol). Because of the large variety of glycolipid structures (in terms of carbohydrate head groups and lipophilic

parts) ^[10-12], it is not possible to give an overview of their phase diagrams as a function of concentration or temperature. To our knowledge, cholesteryl glucosides described in the literature (^[8,13-15]) have only been studied for their biological aspects and not from a physico-chemical point of view. However, a strong difference between the polyoxyethylene and the sugar-based surfactants should be noted. The first ones are very sensitive to the temperature and they become less water soluble at higher temperatures, whereas the second ones exhibit a classical temperature dependence, *i.e.* their solubility in water increases with temperature.

This paper deals with the synthesis and aqueous self-organization properties of two cholesteryl oligoethyleneglycol glycosides whose structures could be defined as a mix of polyethoxy and polyhydroxy surfactants described above.

Materials and methods

General synthetic methods

Compounds 1 and 2 (fig.1) were prepared by glycosylation of acceptor 5 ^[16] with donors 3 ^[17] and 4 ^[18] respectively. The synthesis of 1 has already been reported in the literature ^[16], that of compound 2 was realized following the same reaction pathway as that reported earlier for other fucosyl neoglycolipids ^[19]. Thus, donor 4 was reacted with 5 in dichloromethane at room temperature, in the presence of Bu₄NBr as the promoter, to afford compound 6 in 67% yield. It is afore to mention at this point that the reaction lead to a total α -stereoselectivity, contrariwise to results obtained previously with other acceptors ^[19]. Then, compound 6 was deprotected to 2 in 76% yield by hydrogen transfer, using cyclohexene as hydrogen donor and Pd(OH)₂ as the catalyst, without affecting the cholesterol double bound.

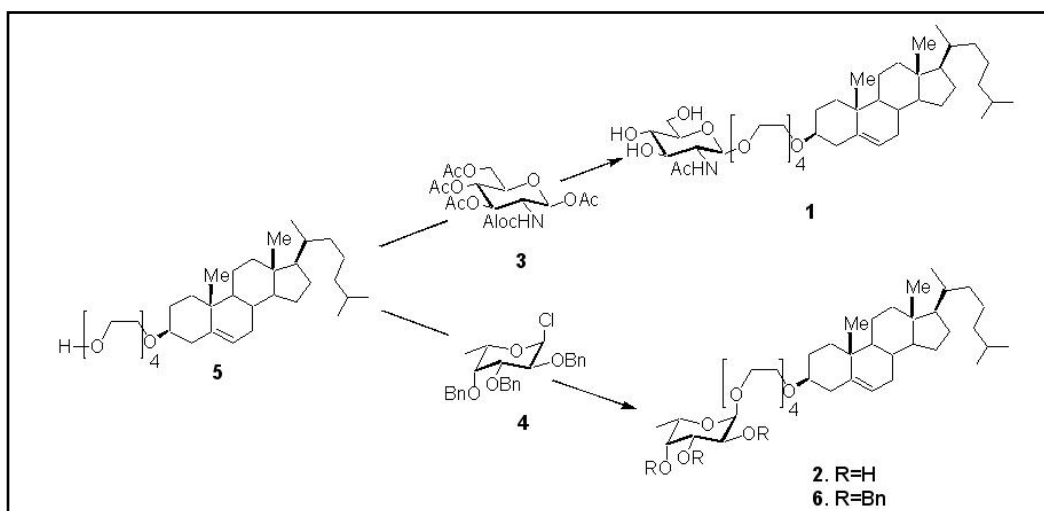


Fig.1: Reaction scheme

11-(Cholest-5-en-3 β -yloxy)-3,6,9-trioxaundecyl 2,3,4-tri-*O*-benzyl- α -L-fucopyranoide **6**. A mixture of donor **4** (1.572 g, 3.47 mmol), acceptor **5** (1.998 g, 3.55 mmol) and Bu₄NBr (1.583 g, 4.81 mmol) in dry alcohol-free CH₂Cl₂ (35 mL), was stirred at room temperature in the presence of 4Å molecular sieves (4.0 g), under argon, for 3 days. After filtration on Celite and evaporation, the residue was purified by column chromatography on silica gel, with a mixture of acetone and petroleum ether (2:5) as the eluent. Compound **6** (2.27 g, 67 % yield) was obtained as a clear oily material. $[\alpha]_D^{22}$ -34.4 (*c*=1.0, CHCl₃); ¹H NMR (200 MHz, CDCl₃) δ 7.27-7.39 (m, 15 H, 3 C₆H₅), 5.34 (d, 1H, H-6_{chol}), 4.62-5.02 (m, 7H, 3 CH₂Ph, H-1), 4.05 (dd, H-2, *J*₁₂ 3.4 Hz, *J*₂₃ 10.1 Hz), 3.90-3.98 (m, 2H, H-3, H-5), 3.55-3.76 (m, 16H, 7 OCH₂, H-3_{chol}, H-4), 3.16-3.20 (m, 2H, FucOCH₂), 0.83-2.34 (m, 40H, cholesterol), 1.11 (d, 3H, *J*₅₆ 6.4 Hz, H-6), 0.68 (s, 3H, H-18_{chol}); ¹³C NMR (50 MHz, CDCl₃) δ 138.75-139.10 and 127.49-128.45 (C₆H₅), 141.03 (C-5_{chol}), 121.59 (C-6_{chol}), 97.79 (C-1), 79.55, 79.38 (C-3_{chol}, C-3), 77.89, 76.48 (C-4, C-2), 74.88, 73.33, 73.13 (CH₂Ph), 70.38-70.95 (OCH₂), 67.35 (CH₂Ochol), 66.99 (CH₂OFuc), 66.27 (C-5), 56.84 (C-14_{chol}), 56.23 (C-17_{chol}), 50.25 (C-9_{chol}), 42.39 (C-13_{chol}), 39.14-39.86 (C-12_{chol}, C-24_{chol}, C-4_{chol}), 37.31 (C-1_{chol}), 36.93 (C-10_{chol}), 36.26 (C-22_{chol}), 35.85 (C-20_{chol}), 32.01 (C-7_{chol}), 31.96 (C-8_{chol}), 28.43, 28.30 (C-2_{chol}, C-16_{chol}), 28.08 (C-25_{chol}), 24.36 (C-15_{chol}), 23.89 (C-23_{chol}), 22.89, 22.63 (C-26_{chol}, C-27_{chol}), 21.13 (C-11_{chol}), 19.44 (C-19_{chol}), 18.79 (C-21_{chol}), 16.71 (C-6), 11.92 (C-18_{chol}). Anal calc for C₆₂H₉₀O₉ (979.40) : C 76.04, H 9.26. Found C 76.03, H 9.28.

11-(Cholest-5-en-3 β -yloxy)-3,6,9-trioxaundecyl α -L-fucopyranoide **2**. Compound **6** (1.396 g, 1.43 mmol) dissolved in ethanol (20 mL) and freshly distilled cyclohexene (13 mL) was refluxed for 7 hours in the presence of Pd(OH)₂ (292 mg). After filtration on Celite and evaporation, the residue was purified by column chromatography on silica gel, with a mixture of ethyl acetate and methanol (5:1) as the eluent. Compound **2** (0.772 g, 76 % yield) was obtained as a clear oily material. $[\alpha]_D^{22}$ -56.8 (*c*=0.8, CHCl₃); ¹H NMR (200 MHz, CDCl₃) δ 5.34 (d, 1H, H-6_{chol}), 4.92 (d, 1H, H-1, *J*₁₂ 3.4 Hz), 3.93 (m, H-5), 3.72-3.83 (m, 3H, H-2, H-3, H-4), 3.64-3.70 (m, 15H, 7 OCH₂, H-3_{chol}), 3.17-3.19 (m, 2H, FucOCH₂), 0.83-2.34 (m, 40H, cholesterol), 1.30 (d, 3H, *J*₅₆ 6.6 Hz, H-6), 0.68 (s, 3H, H-18_{chol}); ¹³C NMR (50 MHz, CDCl₃) δ 140.91 (C-5_{chol}), 121.61 (C-6_{chol}), 99.17 (C-1), 79.55 (C-3_{chol}), 71.95 (C-4), 70.13-70.84 (OCH₂), 71.05 (C-3), 69.22 (C-2), 67.23 (CH₂Ochol, CH₂OFuc), 66.15 (C-5), 56.80 (C-14_{chol}), 56.20 (C-17_{chol}), 50.20 (C-9_{chol}), 42.35 (C-13_{chol}), 39.05-39.81 (C-12_{chol}, C-24_{chol}, C-4_{chol}), 37.26 (C-1_{chol}), 36.88 (C-10_{chol}), 36.23 (C-22_{chol}), 35.81 (C-20_{chol}), 31.97 (C-7_{chol}), 31.92 (C-8_{chol}), 28.36 (C-2_{chol}, C-16_{chol}), 28.03 (C-25_{chol}), 24.32 (C-15_{chol}), 23.87 (C-23_{chol}), 22.86, 22.60 (C-26_{chol}, C-27_{chol}), 21.10 (C-11_{chol}), 19.41 (C-19_{chol}), 18.76 (C-21_{chol}), 17.24 (C-6), 11.70 (C-18_{chol}). Anal calc for C₄₁H₇₂O₉ (709.03) : C 69.46, H 10.24. Found C 69.34, H 10.44.

X-ray diffraction

Static X-ray diffraction experiments at 20°C and 60°C were performed using the following device: XRD patterns were recorded in transmission mode using quartz capillaries (1.5 mm diameter, GLASS W. Müller, Berlin, Germany), the X-ray generator was a long line-focus sealed tube (ENRAF NONIUS; Cu anode operating at 40 kV and 20 mA), two gas-filled linear detectors (1024 channels each, filled with argon-ethane mixture) were used to collect the data. With the settings used, scattering vectors q ranging from 0.04 to 0.37 Å⁻¹ and from 1.24 to 1.85 Å⁻¹ were accessible. The scattering vector is defined as $q = 4\pi \sin(\theta) / \lambda$ where 2θ is the scattering angle. From this scattering vector, it is possible to calculate the distances by the use of the following equation $q = 2\pi/d$. The calibration of the detectors was carried out on the peaks of the 2L β form of pure tristearin (4.59, 3.85 et 3.70 \pm 0.01 Å and 44.97 \pm 0.05 Å) and that of the silver behenate (58.38 \pm 0.01 Å). In order to determine the peak positions, diffractograms were fitted with Gaussian model by the use of the IGOR pro software (WaveMetrics, Inc.). The full width of the Gaussian curve at half the maximum was calculated with the following equation: $\text{width} = 2\sqrt{2 \ln 2} \sigma$ in which σ is the standard deviation.

For some glycolipid concentrations, dynamic X-ray scattering experiments were realized by modifying the temperature of the sample during the measurements. In such experiments, the XRDT measurements were coupled with a thermal analysis by Differential Scanning Calorimetry (DSC). DSC was performed using Microcalix, a microcalorimeter especially designed for installation in an X-ray beam ^[20].

Determination of critical micellar concentration

Experiments were conducted in ultrapure water, obtained by osmosis from a MilliRO6 Plus Millipore apparatus (pH 5.5, surface tension > 72 mN/m at 20°C). All glassware were cleaned with Texapon detergent and then abundantly rinsed with distilled water. The critical micellar concentration of the glycolipid was determined by surface tension measurements performed by the Wilhelmy plate method using a Krüss K10ST tensiometer (Germany). The surface tensions of the glycolipid solutions were measured after one night. In absence of glycolipid, the surface tension of water was checked during 24 hours. No change was observed during the experiment time, indicating that the evaporation of the subphase was extremely limited and confirming that the surface tension changes observed in presence of the glycolipid was only due to the absorption of the latter at the air/water interface.

Surfactant adsorption area

The glycolipid adsorption area at the air-water interface was calculated using the Gibbs adsorption equation.

$$\Gamma = -\frac{0.4343}{RT} \left[\frac{d\gamma}{d \log C} \right]$$

Since the surface tension of the glycolipid solutions have been measured after 16 hours, the curves represent equilibrium data, then the true area per adsorbed molecule could be calculated.

Molecular graphism

In order to approximate the dimensions of the glycolipids, the DS ViewerPro suite (Accelrys, Inc.) was used. The Dreiding force field was used to minimize the energy. General force constants and geometry parameters for the Dreiding force field are based on simple hybridization rules rather than specific combinations of atoms. The following three steps methodology was used: (i) building the molecular structure from the chemical bonding diagram, (ii) searching for plausible arrangements of the molecule, (iii) optimizing the generated structure by energy minimization. The zig-zag and the helical models have been taken into account as plausible arrangements of the polyethyleneglycol spacer fragment with regard to the literature ^[21,22].

Results and discussion

1.1. Lamellar region

The SAXS spectra of anhydrous glycolipids **1** and **2** are displayed on figures 2 (a,b,c), at 20°C and 60°C ; no WAXS diffraction peak could be detected. For both glycolipids, independently on the temperature, three diffraction peaks could be observed in ratios 1, 1/2, and 1/3, probably corresponding to the three first orders of a lamellar phase. The periods of the organization are reported in table I. A similar lamellar arrangement has been described previously for anhydrous polyoxyethylene cholesteryl ethers containing 10 or 15 EO units ^[6] or polyoxyethylene phytosterol containing 5 or 10 EO units ^[7]. No significant increase in size could be noted for both glycolipids with the temperature between 20°C and 60°C.

Table I: analysis of the anhydrous glycolipid SAXS patterns. “Width” is referring to the full width of the Gaussian curve at half high, calculated for the peak corresponding to the first order

	20°C	60°C
GlcNAc-E₄-Cholesterol (1)		
distance (Å)	60.3 ± 0.6	61.1 ± 0.4
width (Å ⁻¹)	0.028	0.014
Fuc-E₄-Cholesterol (2)		
distance (Å)	58.7 ± 0.2	58.1 ± 0.2
width (Å ⁻¹)	0.009	0.009

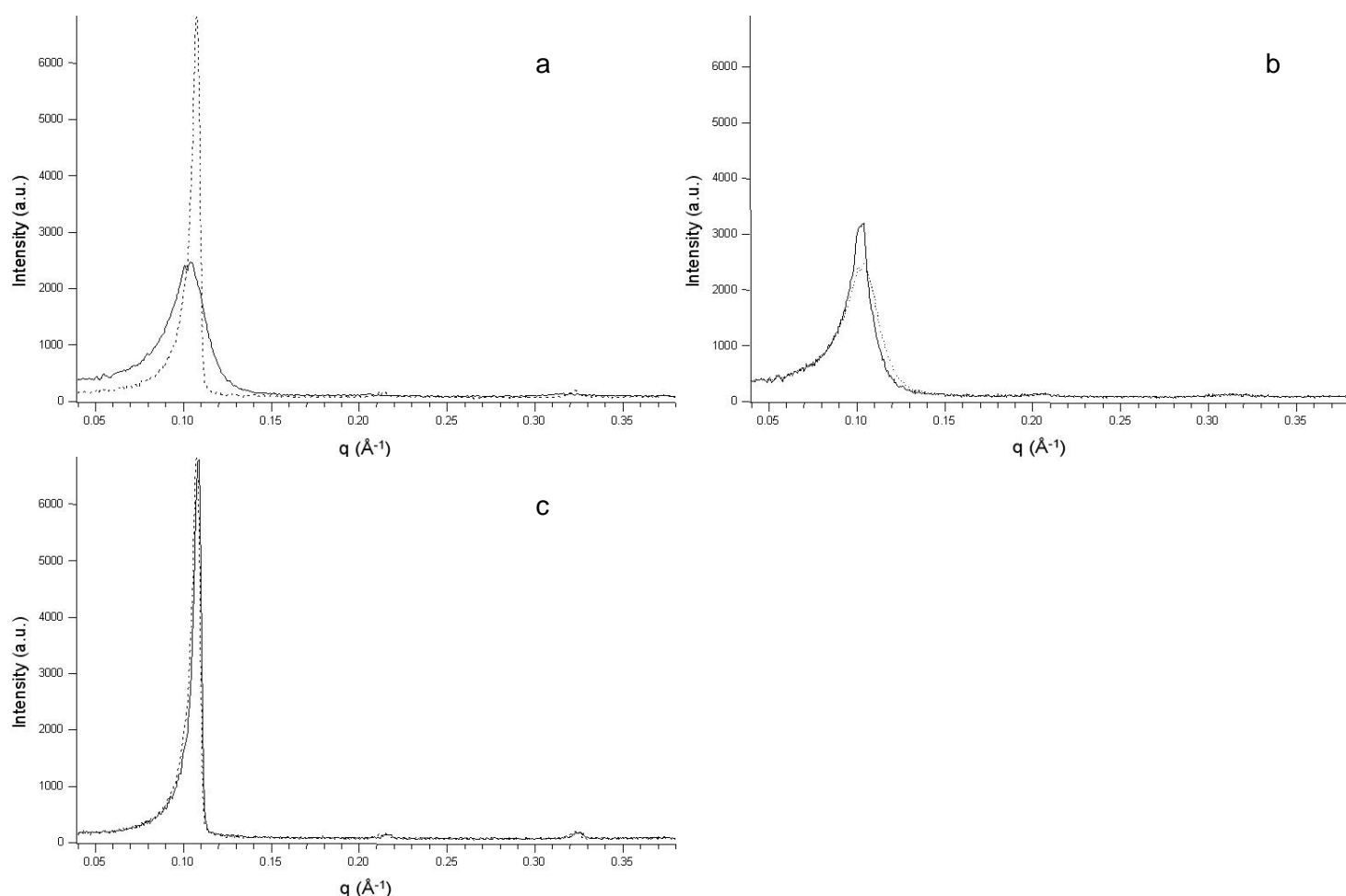


Fig.2: Diffractograms of anhydrous glycolipids; (a) at 20°C GlcNAc-E₄-Cholesterol **1** (full line), Fuc-E₄-Cholesterol **2** (dotted line); (b) GlcNAc-E₄-Cholesterol **1** at 20°C (dotted line) and 60°C (full line); (c) Fuc-E₄-Cholesterol **2** at 20°C (dotted line) and 60°C (full line).

However, two significant differences can be pointed out by comparing glycolipids **1** and **2**, *i.e.* the lamellar distance and the full width of the Gaussian curve (at half height) are higher in the case of GlcNAc derivative **1** than in that of Fuc derivative **2**. The second point suggests a more regular arrangement in the fucose derivative layer, whereas the first one could be discussed with consideration to molecular graphism.

The most important results obtained by minimization of glycolipid conformations are summarized in tables IIa and IIb.

Table IIa and IIb: main results obtained from molecular graphism tools

	PEO helical model		PEO zig-zag model	
GlcNAc-E₄-Cholesterol (1) extended distance (Å)	29.4		37.2	
Fuc-E₄-Cholesterol (2) extended distance (Å)	28.8		34.7	
	GlcNAc-E₄-Cholesterol	Fuc-E₄-Cholesterol	N-acetylglucosamine	fucose
Molecular volume (Å³)	655.9	616.4	161.7	121.9

Assuming that the dimensions of the glycolipids strongly depend on the conformation of the PEO chain which stands between zig-zag^[21,23] and distorted helical conformations^[22,24,25], it is possible to calculate lengths of the whole glycolipid molecules; the results are reported in table IIa. The calculated lengths were 29.4 Å and 28.8 Å in the helical model, and 37.2 Å and 34.7 Å in the zig-zag model, for glycolipids **1** and **2** respectively. The validity of the models has been verified after Drieding minimization with regard to the length of the PEO chain. Indeed, it has been observed in crystals that the length per EO monomer was 2.8 Å in the helical arrangement and 3.6 Å in the zig-zag arrangement. The distances measured in our calculations are 2.7-2.8 Å and 3.6 Å respectively, suggesting that the energy minimization of the whole molecule did not affect the PEO chain conformation. The slightly longer distances calculated for **1** could be due to the higher molecular volume of the *N*-acetylglucosamine moiety compared with fucose, as reported in table IIb. This correlated well with the lamellar repetitive distances, extracted from the diffraction results. Also, the arrangement of the glycolipid within the lamella could be discussed. Two distinct organizations could be taken into account: 1) interdigitated structures, in which cholesterol anchors or hydrophilic chains melt, 2) a classical bilayer with two cholesterol layers in a sandwich of ethylene glycol plus sugar layers (fig.3 a, b and c). The “interdigitated” models do not fit with the layer distance measured on the diffractograms since such layers should have thickness around 45 Å, which is lower than the 58-60 Å experimentally measured. The classical bilayer model is more

appropriate and the lengths of both glycolipids in the “PEO helical arrangement” fitted very well the experimental values. Such a “PEO zig-zag arrangement” could accommodate a tilt angle to the normal of the bilayer around 30 to 36° depending on the glycolipid (fig.3 d).

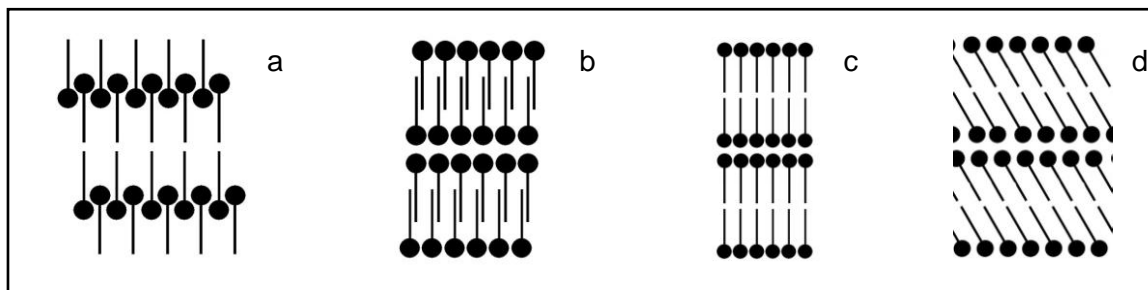


Fig.3: Four different arrangement of the glycolipid within the lamella. Interdigitated structures, in which cholesterol anchors (a) or hydrophilic chains (b) melt. Classical (c) or tilted (d) lamellar phase.

Infra-red spectroscopy data ^[26] on anhydrous GlcNAc-E₄-Cholesterol **1** allow us to partially discriminate between the previous possibilities. The “zig-zag” model would lead to a specific band at 1500 cm⁻¹ (J.B. Brubach, internal report). The lack of such absorption on the previously published data argues against the “zig-zag” model. This result could be confirmed by other infra-red measurements, since PEO helices display specific absorptions in the range 800 to 1400 cm⁻¹.

Additions of water to the GlcNAc-E₄-Cholesterol glycolipid **1** at 20°C and 60°C lead to diffractograms reported in figures 4a and 4b respectively. For a glycolipid/water 80:20 (w/w) mixture, the lamellar arrangement remains visible. However, two significant changes could be observed: 1) an increase of the bilayer thickness (from 60.3 ± 0.6 Å to 63.7 ± 0.5 Å at 20°C and from 61.1 ± 0.4 Å to 63.3 ± 0.5 Å at 60°C) that could be attributed to the hydration of sugar headgroup and 2) a decrease of the full width of the Gaussian curve at half high, that could be due to an increase of the PEO chains degree of freedom, allowing a well-ordered arrangement of the glycolipid.

1.2. Bicontinuous phases

From diffractograms of glycolipid-water mixtures recorded at 20°C (fig.4a), it is apparent that the intensities of the diffraction peaks corresponding to the lamellar “positions” (around $q = 0.1 \text{ Å}^{-1}$ for the first order) decreased drastically with the amount of water and at least two peaks appeared around 0.065 Å^{-1} and 0.086 Å^{-1} q -values.

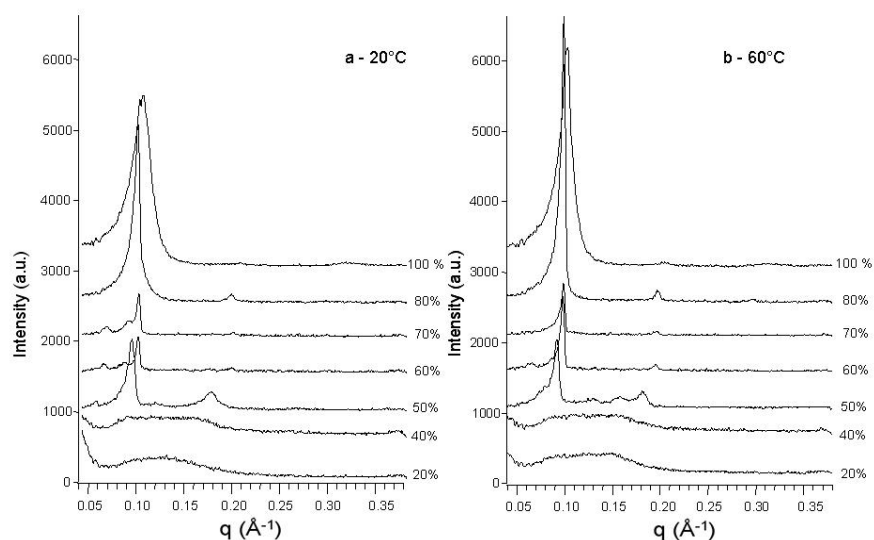


Fig.4: Diffractograms obtained at 20°C (a) and 60°C (b) for GlcNAc-E4-Cholesterol 1 in absence or in presence of water. The percent corresponds to the ratio of glycolipid in the mixture.

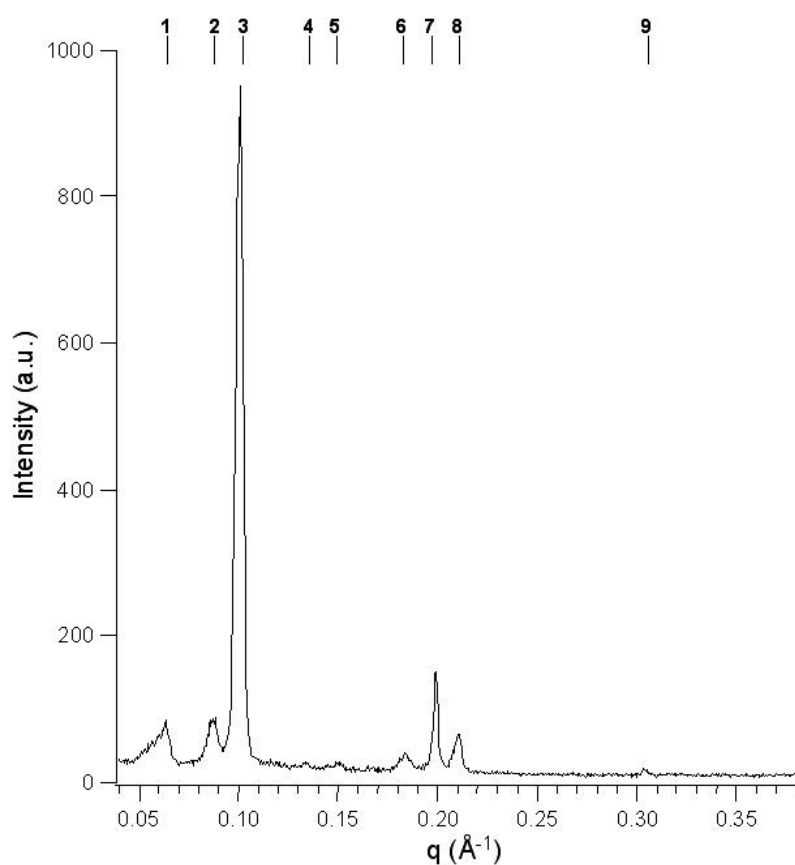


Fig.5: Diffractograms obtained at 20°C with a GlcNAc-E4-Cholesterol/water 75:25 mixture by the use of the Elettra Synchrotron beam. The numbers from 1 to 9 correspond to the peak indexation used in table III.

Table III: peak positions, tetragonal and rhombohedral indexations for glycolipid 1 / water (75:25) at 20°C. The diffractogram were obtained by the use of the Austrian-SAXS line facilities in the ELETTRA Synchrotron (Trieste, Italy)

position	d_{obs} (nm)	tetragonal			rhombohedral		
		plane	d_{cal} (nm)	% error	plane	d_{cal} (nm)	% error
1	9.99	110	10.15	1.6	101	10.37	3.7
2	7.18	200	7.18	0	110	7.18	0
3	6.25	101	6.25	0	003	6.25	0
4	4.70	211	4.71	0.2	113	4.72	0.4
5	4.18	301	3.94	6.1	030	4.15	0.7
6	3.41	330	3.39	0.6	131	3.39	0.6
7	3.16	202	3.13	0.9	006	3.13	0.9
8	2.99	411	3.11	3.9	042	2.96	1.0
9	2.07	303	2.08	0.5	060	2.07	0
mean error		$\pm 1.5\%$			$\pm 0.8\%$		
a (nm)		14.36			14.36		
c (nm)		6.94			18.76		

Because, it was difficult to get further insights into the organization of the mixture, we studied the glycolipid/water 75:25 (w/w) mixture at 20°C with synchrotron radiation (fig.5). Nine reflections could be distinguished on this diffractogram. Various intermediate phases, such as rectangular ribbons or bicontinuous cubic phase, have been tested to index the peaks observable on the diffractogram; however high order reflection cannot be fitted with these structures ^[27]. Only two lattices, the rhombohedral mesh and the tetragonal mesh, can give a convincing explanation to the SAXS pattern. The results of the indexation are reported in table III.

Concerning the rhombohedral R3m phase, the calculated Bragg peak positions were obtained according to the following equation ^[28]:

$$d_{hkl} = \frac{a}{\sqrt{\frac{4}{3}(h^2 + k^2 + hk) + l^2 \frac{a^2}{c^2}}}$$

in which h, k and l are the Miller indexes, and a (= b) ≠ c are the unit cell parameters. The condition limiting possible reflections is $-h+k+l = 3n$, with n integer.

Concerning the tetragonal mesh phase, the following equation has been used ^[28]:

$$d_{hkl} = \frac{a}{\sqrt{h^2 + k^2 + l^2 \left(\frac{a}{c}\right)^2}}$$

The condition limiting possible reflections is $h+k+l$ = pair number.

Both these organizations correspond to stack of “punctured” bilayers, together with an ordered arrangement of punctures within each bilayers ^[27]. The rhombohedral mesophase (R3m space group) contains a hexagonally close-packed array of punctures and the tetragonal mesophase (space group I422) contains a square array of punctures (fig.6). By using the two indexing schemes, the lattice parameters are $a = 14.36$ nm and $c = 18.76$ nm in the R3m space group and $a = 14.36$ nm and $c = 6.94$ nm in the I422 space group. The position mean errors are 0.8 % and 1.5 % in rhombohedral and tetragonal model respectively. Such error values are very close to that reported in the literature, confirming they are realistic models ^[29-32]. However, based on these position mean errors, the rhombohedral structure seems better than the other one. Furthermore, a correlation between the d_{003} reflection of the rhombohedral structure and the periodicity in the lamellar arrangement has been reported in the literature ^[33,34]. Such a correlation, observed in our results, confirms the probable R3m arrangement of glycolipid **1** for a glycolipid/water 75:25 (w/w) mixture at 20°C.

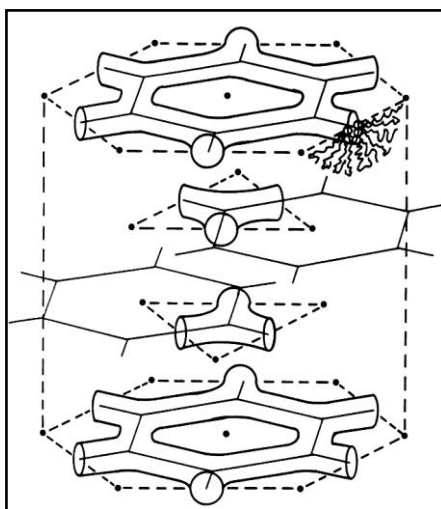


Fig.6: R3m schematic representation. From ^[35]

Because the three first reflections (d_{101} , d_{110} and d_{003}) are very similar at 70% and 60%, we think that the organization in the mixture did not change significantly for these concentrations. With this assumption, it is possible to calculate the unit cell parameters at these two concentrations (table IV).

Table IV: a and c parameters of the rhombohedral mesh phase at 20°C for mixtures containing 70% and 60% of GlcNAc-E₄-Cholesterol **1**

	70 %	60 %
a (nm)	14.14	14.66
c (nm)	18.75	18.84

Indeed, d_{110} and d_{003} are directly correlated to parameters a and c, respectively. For these two mixtures, a transition in the 20°C-60°C temperature range has been observed, as shown in figures 7 and 8. With the 70% glycolipid containing mixture, a transition to a lamellar phase could be detected around 52°C; the lamellar period is 63.9 ± 0.5 Å at 60°C. With 60% of glycolipid, the peaks corresponding to the rhombohedral mesh phase decrease with increasing temperature; however the transition is not completely ended at 60°C and the SAXS pattern probably corresponds with a mixture of R3m and lamellar phases. At this concentration, and at 60°C, the lamellar period is 64.0 ± 0.6 Å. This R3m to lamellar phase transition is consistent with other observations of a decrease in hydration number, or a decrease in water concentration near the head group, at higher temperatures reported for spectroscopy and water self-diffusion measurements ^[4] or micellar growth experiments ^[36].

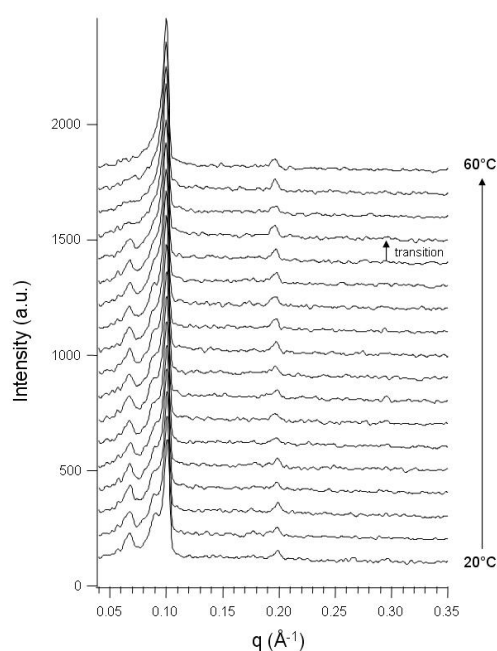


Fig.7: Diffractograms between 20°C and 60°C obtained with the GlcNAc-E₄-Cholesterol/water 70:30 mixture. Temperature rate: 1°C/min.

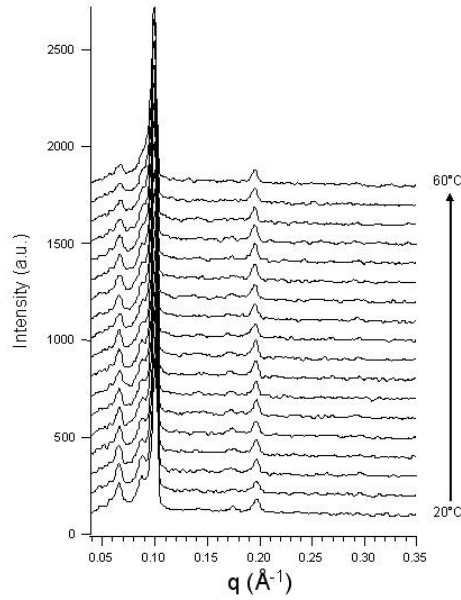


Fig.8: Diffractograms between 20°C and 60°C obtained with the GlcNAc-E4-Cholesterol/water 60:40 mixture. Temperature rate: 1°C/min.

1.3. Discontinuous phases

For the mixture containing 50% of water, at 20°C, the SAXS pattern significantly changed compared with that recorded at 60% and 70% of water. The peak corresponding to the d_{110} reflection of the R3m phase disappeared, those corresponding to the d_{101} and d_{003} reflections were displaced towards smaller q -values (0.0566 \AA^{-1} , 0.0937 \AA^{-1} respectively) and decrease in intensity; at the same time, new reflections appeared at 0.1180 \AA^{-1} and 0.1762 \AA^{-1} (fig.4a). However, it is not possible to affect unambiguously the whole pattern to a known structure. Between 20°C and 60°C, an organization change could be observed around 44°C (fig.9). The diffraction peaks at 0.0915 \AA^{-1} , 0.1291 \AA^{-1} , 0.1572 \AA^{-1} and 0.18202 \AA^{-1} fit very well with the Im3m space group (fig.10) in which the four first reflections are correlated to the lattice parameter (a) by factors $\sqrt{2}$, $\sqrt{4}$, $\sqrt{6}$, and $\sqrt{8}$. The Im3m (or Q^{229}) space group, belongs to the cubic phases, characterised by diffraction patterns in which the reciprocal spacings (s_{hkl}) of the Bragg peaks are given by the following equation:

$$s_{hkl} = \frac{q_{hkl}}{2\pi} = \frac{(h^2 + k^2 + l^2)^{1/2}}{a}$$

where h , k , and l are the Miller indices, and a is the lattice parameter. The indexation (hkl) of the first allowed Bragg reflections in the Im3m space group are 110, 200, 211, 220, 310, 222, 321, 400,....

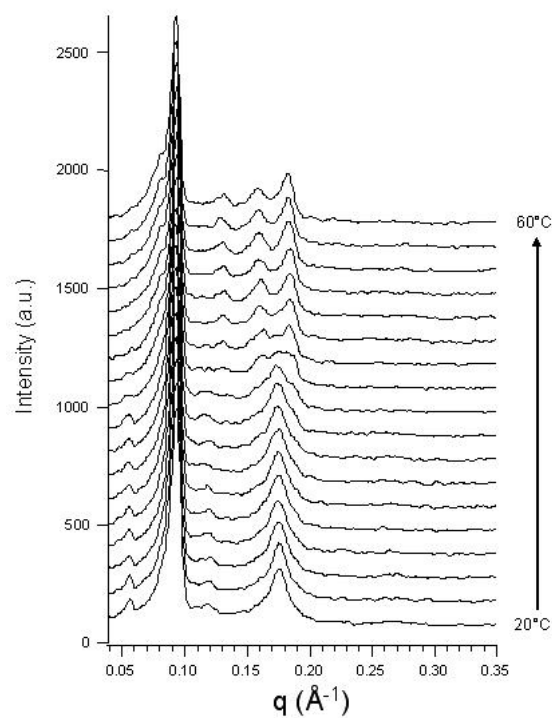


Fig.9: Diffractograms between 20°C and 60°C obtained with the GlcNAc-E4-Cholesterol/water 50:50 mixture. Temperature rate: 1°C/min.

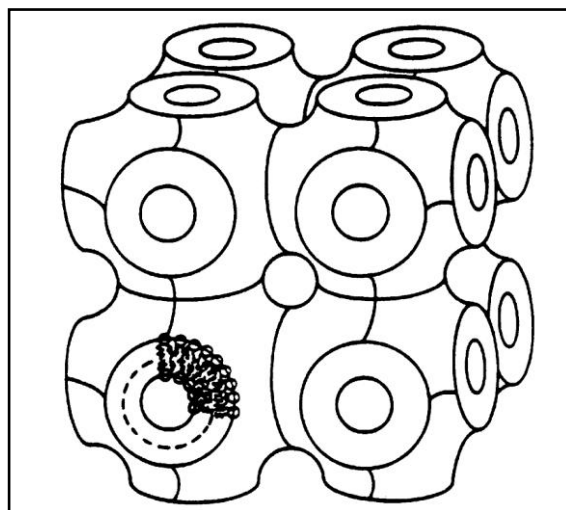


Fig.10: Im3m schematic representation. From ^[35]

In the investigated organization, the lattice parameter (a) could be calculated at $97.5 \pm 0.4 \text{ \AA}$. The Im3m cubic phase could be either a bicontinuous or a micellar structure; its topology could be either normal (type I, “oil-in-water”) or inverted (type II, “water-in-oil”). The bicontinuous structure is characterized by orthogonal networks of water channels, connected six-by-six, and separated by lipid bilayer folded as an infinite periodic minimal surface (P-minimal surface) ^[35,37]. The micellar structure is constituted by quasi-spherical micelles

closely packed in the body-centred model ^[38]. Due to the presence of PEO chains in the glycolipid and to the way by which the Im3m cubic phase is obtained (addition of water to a lamellar structure), it could be assumed that the topology of our system is normal (type I). It is also probably possible to discriminate between the bicontinuous and the micellar arrangement. Indeed, the Im3m bicontinuous phase is usually inverted, although one example of type I has been reported ^[35]. Furthermore, the lattice parameter ($a = 97.5 \text{ \AA}$) is not compatible with that of a bicontinuous structure in which this parameter should overlap at least the thickness of two bilayers. Then it can be concluded that at 60°C, for a 50/50 glycolipid/water mixture, glycolipid **1** self-organizes as quasi-spherical micelles, packed in the body-centred mode.

By increasing the amount of water (from 50% to 60%), it is possible to lose the cubic organization of the micelles. These “less organized micelles” are stable with the temperature and do not seem very sensitive to the glycolipid concentration in the range (20% to 40%), as can be seen on the corresponding SAXS patterns in figures 4a and 4b.

Surface tensions versus GlcNAc-E₄-Cholesterol concentrations in water are reported on figure 11. The time required to reach surface tension equilibrium was around 6 to 8 h (data not shown). The value of the CMC could be estimated around 0.091 mM and the calculated Gibbs area to 55.8 Å²/molecule.

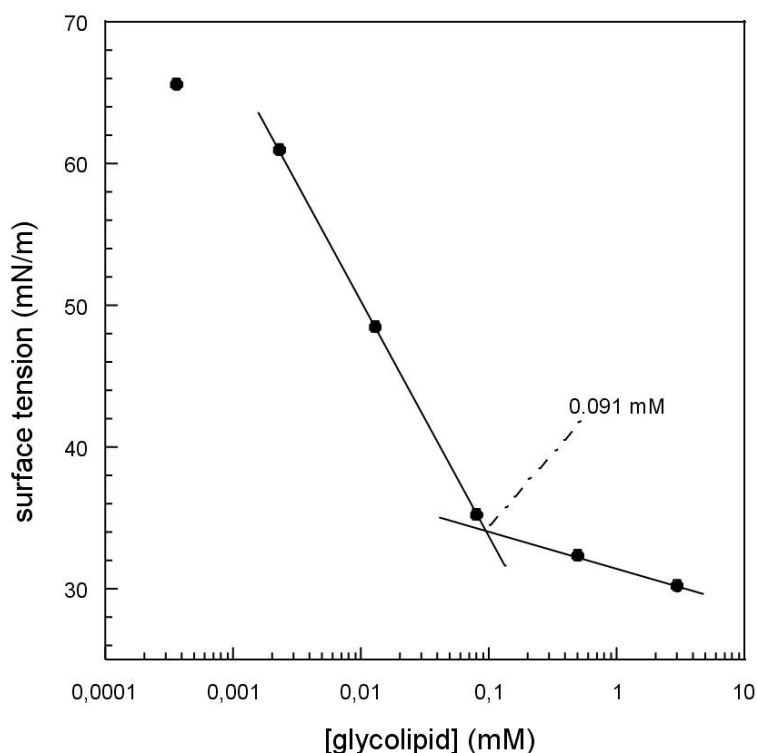


Fig.11: Surface tension versus concentration of GlcNAc-E₄-Cholesterol.

Compared with polyoxyethylene derivatives of fatty alcohol, the sterol-based surfactants have a hydrophobic tail whose characteristics are : (i) a higher number of carbon atoms (28 vs 10-14 for the aliphatic fatty alcohol), (ii) a higher steric hindrance, and (iii) a higher rigidity (the four condensed rings constitute a fairly stiff skeleton). The long time required to reach equilibrium surface tension is most likely due to the characteristics of the hydrophobic tail in sterol surfactants in which the alignment at the interface is hindered by the rigid skeleton.

The low value of surface tension at the CMC is close to that reported previously for polyoxyethylene phytosterol derivatives containing 10 oxyethylene units ^[7]. However, it should be noted that the CMC value is much higher in the present study (91 μ M versus 10 μ M). This difference could be explained by the nature of the sterol anchors (29 carbons in phytosterol and 27 in cholesterol), by the length of the polyoxyethylene moieties and by the presence of a hydrophilic sugar residue at the end of the latter in glycolipid **1**. For polyoxyethylene phytosterol, the shape parameter (*s*) was reported to be close to 1. This parameter (*s*) is defined as $v_0/(a \cdot l_0)$, where v_0 is the volume of the hydrocarbon tail, *a* is the head group area, and l_0 is the extended length of the tail. A shape parameter close to 1 is indicative of a rod-like shape of the molecule unfavorable to micellization for geometrical reasons. Therefore, it was shown that polyoxyethylene phytosterol containing five oxyethylene units were insoluble in water and does not form a single phase until a concentration of approximately 30 wt%, where a lamellar phase formed ^[7]. The hydrophilic headgroup is too small for the surfactant to pack into discrete aggregates in water; hence there is a phase separation into a water phase and a lamellar liquid crystalline region. In the case of glycolipid **1**, micellization is possible for glycolipid concentrations lower than 50% (see X-ray diffractograms in figures 4a and 4b), thus suggesting that the sugar moiety was able to induce the formation of discrete aggregates. Furthermore, the Im3m organization previously depicted requires quasi-spherical micelles. In that case, by considering a micelle with a core radius *r*, made up of *n* molecules, simple geometrical considerations allow to calculate the volume of the core $v = nv_0 = 4\pi r^3/3$, the surface area of the core $A = na = 4\pi r^2$, and hence $r = 3v_0/a$. In tightly packed micelles, the radius *r* cannot exceed the length l_0 of the tail. Introducing this limit in the expression of *r*, a range of $0 \leq s \leq 1/3$ was found for spherical micelles ^[39]. It is generally admitted that the surface area occupied by cholesterol at the air-water interface is around 19 \AA^2 . Furthermore, cholesterol adopts only a close-packed arrangement at the interface, as attested by the low-extent of the surface pressure-area isotherm and the high collapse pressure observed in Langmuir films. By using of the Gibbs equation, the calculated area per molecule of glycolipid **1** at the air-water interface is 56 \AA^2 . Molecular graphism tools allow to calculate the shape parameter for GlcNAc-E₄-Cholesterol **1** in hydrated conditions : the volume v_0 (363.7 \AA^3), and the length l_0 (17.9 \AA) of the hydrophobic tail are close to the data reported in the literature ^[40]. By using these values, a shape

parameter of 0.36 could be calculated, thus confirming the quasi-spherical shape of the micelles. Contrariwise to non-glycosylated cholesterol derivatives, the cone shape of glycolipid **1** is favorable to the formation of discrete structure in solution. For this reason, no phase separation between a water phase and a lamellar liquid crystalline region was observed. Successive transitions resulted of changes in curvature, induced by hydration of glycolipid headgroup.

In this work were studied the self-organizations of two cholesteryl tetraethyleneglycol containing glycolipids **1** and **2**, in the dry state. For one of them (**1**) the self-arrangements were studied in presence of increasing amounts of water. As summarized in table V, successive structures were observed, from lamellar phase to micelles. In presence of water, the observed phases were different to those described for cholesteryl oligoethyleneglycols. Such differences are probably due to the presence of the sugar moiety which increases hydration ability of the hydrophilic head group, inducing shape parameters in the range 1 to 0.36, responsible of curvature changes and phase transitions.

Table V: phases observed during the hydration of GlcNAc-E4-Cholesterol **1**. The characteristic parameters (d, a, c) of the different phases are expressed in Å.

	20°C	Transition T°C	60°C
100 %	L α d = 60.3 \pm 0.6	-	L α d = 61.1 \pm 0.4
80 %	L α d = 63.7 \pm 0.5	-	L α d = 63.3 \pm 0.5
70 %	R3m a = 141.4, c = 187.5	~52°C	L α d = 63.9 \pm 0.5
60 %	R3m a = 146.6, c = 188.4	~50°C	L α + R3m d = 64.0 \pm 0.6
50 %	q = 0.0566 Å ⁻¹ , 0.0937 Å ⁻¹ , 0.1180 Å ⁻¹ and 0.1762 Å ⁻¹	~44°C	Im3m a = 97.5
40 %	micellar phase	-	micellar phase
20 %	micellar phase	-	micellar phase

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Cette première partie du travail a permis la synthèse de 2 glycolipides ainsi que leur caractérisation d'un point de vue physico-chimique. Etant donné que ces glycolipides vont être utilisés en milieu aqueux, il était important de connaître leur comportement dans l'eau puisque les glycolipides, et les lipides en général, présentent un polymorphisme important en fonction du degré d'hydratation. Cela a encore été confirmé avec nos 2 glycolipides, qui présentent un degré d'hydratation élevé due à la présence de la chaîne éthylène-glycol et des résidus sucrés à son extrémité. Ainsi ils s'organisent de façon lamellaire lorsqu'ils ne sont pas hydratés, puis passent dans un système rhomboédrique, cubique, et enfin micellaire au fur et à mesure de l'hydratation du milieu. Compte tenu du déplacement de l'organisation des glycolipides vers des structures discrètes (de type micelles) quand leur hydratation augmente, l'insertion de ces glycolipides dans des membranes de vésicules semble tout à fait envisageable. L'ajout de molécules de glycolipides, à des taux « raisonnables », ne devrait pas induire de changements de courbure susceptibles de s'opposer à la formation de liposomes. Nous allons donc, dans cette deuxième partie, nous intéresser à la préparation et à la fonctionnalisation des liposomes, c'est-à-dire à l'incorporation du glycolipide dans la bicouche phospholipidique. Nous étudierons les conséquences physico-chimiques qu'entraîne l'incorporation de ces structures au sein de la bicouche et enfin, nous vérifierons l'accessibilité des sucres en surface des liposomes grâce à l'utilisation de lectines végétales.

CHAPITRE III :

**Cholesteryl oligoethyleneglycol
glycosides: fluidising effect of their
embedment into phospholipid bilayers**

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Article publié dans Biochemical and Biophysical Research Communication, 329 (4) (2005) 1186-1192.

Contents

Introduction	93
1. Material and methods	93
1.2. Materials	93
1.3. Liposome preparation	94
1.4. Differential scanning calorimetry	95
1.5. Steady-state fluorescence polarization measurements.....	95
1.6. Zeta potential measurements	95
1.7. Calculation of the fixed aqueous layer thickness (FALT)	95
1.8. Size measurements	96
1.9. Agglutination assays	96
2. Results.....	97
2.1 Steady-state fluorescence anisotropy measurements	97
2.2 Differential scanning calorimetry	97
2.3 Zeta potential	99
2.4 Size and agglutination	100
3. Discussion	100
References.....	103

Figures

Fig.1: Structures of cholesteryl glycolipids.	94
Fig.2: Thermotropic profiles of DPPC-liposomes containing the DPH probe.....	98
Fig.3: NaCl concentration dependence of the zeta potential of liposomes containing 10 mole% of cholesterol, Fuc-E4-Chol or GlcNAc-E4-Chol.....	99

Tables

Table I: Differential scanning calorimetry results.....	98
Table II: Values of the fixed aqueous layer thickness calculated from the zeta potential measurements.....	99
Table III: Increase of the optical density of liposome suspensions following the addition of lectin at a concentration of 1 μ M.....	100

Introduction

Liposomes have been proposed as drug delivery systems more than thirty years ago ^[1]. Such close vesicles can keep the encapsulated drug hidden from the environment when the bilayer structure is maintained into biological fluids. For example, the doxorubicin-encapsulated liposome, DOXIL[®], or the amphotericin B-loaded liposome, AMBISOME[®] has been approved for the treatment of AIDS-related Kaposi's sarcoma or fungal infection respectively. The way of action of these commercial formulations is passive. Thus, DOXIL remains in the circulating blood for long periods allowing an improvement of the pharmacological effects of the doxorubicin. In addition, active drug targeting of liposomes should also be an interesting approach to increase the pharmacological effect of drugs and to reduce potential side-effects. Among many receptor-ligand couples investigated ^[2,3], the lectin-sugar recognition systems have drawn attention in the pharmaceutical field especially on the development of drug carriers tailored for selective delivery ^[4,5]. In the present work, glycosides of cholesteryl oligoethyleneglycols were synthesized. The α -L-fucose was used as carbohydrate ligand for molecular recognition. This sugar has been chosen because of its role in several bacterial infections. For example, it is described in the literature that some strains of *Helicobacter pylori* express an adhesin, BabA2, which interacts with fucosylated histo-blood group antigen Lewis b (Le^b) ^[6]. This Le^b blood group antigen is expressed at the surface of gastric cells and the BabA2 adhesin is essential for the bacterium adhesion. Furthermore, this adhesin is also described as a virulence factor ^[7]. Hence, the use of fucose as ligand on the surface of drug delivery systems enables a specific targeting against *H. pylori* ^[8]. The oligoethylene moiety was used as a spacer between the carbohydrate and the lipid fraction allowing a good recognition of the headgroup by the target protein. Cholesterol was selected as hydrophobic anchor because of its stabilizing effect on bilayer ^[9]. However, the unstability of liposomes in the biological fluids could be a limitation to their applications ^[10]. Therefore it is important to check the effect of the incorporation into bilayers of whole cholesteryl glycolipids, compared with that of pure cholesterol. Steady-state fluorescence polarization, differential scanning calorimetry, zeta potential and agglutination experiments have been performed in order to investigate the inclusion of the glycolipids.

Material and methods

1.2. Materials

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), Hepes buffer, wheat germ agglutinin (WGA), and agglutinin from *Ulex europaeus* (UEA-I) were

purchased from Sigma-Aldrich. 1,6-diphenyl-1,3,5-hexatriene, lipid S75, cholesterol, and nitrogen 2-1°, 4,5 were provided respectively by Acros organics, Lucas Meyer, Laserson and Linde. Dimethylformamide and absolute ethanol were purchased from Fluka. The amount of phospholipids in all preparations was determined with the enzymatic test kit PAP 150 from bioMérieux. Compound GlcNAc-E₄-Chol (fig.1) was prepared as already reported ^[11]. The synthesis of compound Fuc-E₄-Chol, realized by methodologies already reported to prepare α -L-fucopyranosides of Guerbet alcohols ^[12], will be published in due course. It is noteworthy that the pure α -anomer was prepared for the following experiments. This point is very important with a view to targeting biological organisms such as *H.pylori* or other pathogens [6,13].

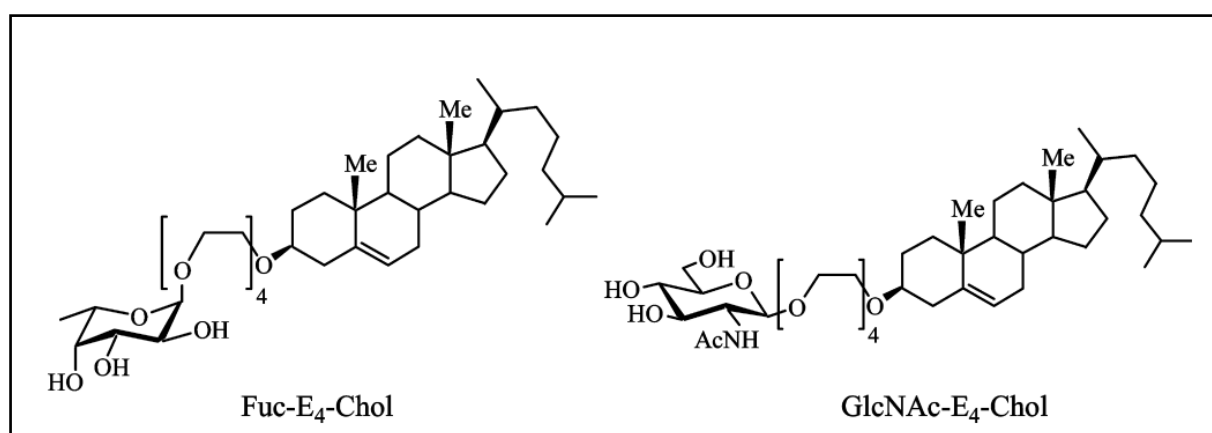


Fig.1: Structures of cholesteryl glycolipids.

1.3. Liposome preparation

After dissolving the appropriate amounts of lipids (phospholipid and glycolipid) in ethanol, 2 ml of such solution were injected in 4 ml of water. Vesicle formation was characterized by the appearance of opalescence in colloidal dispersion. The mixtures were stirred for 10 min and the organic phases were concentrated under vacuum to a volume of 1.8 ml. Then, the ethanol traces were eliminated with the addition of 6 ml water and a further concentration precisely to a volume of 2.0 ml. The liposome suspension was diluted with hepes buffer solution (20 mM) in order to obtain the desired final concentrations (3 mM of total lipids in a 10 mM buffer solution). The liposome suspensions were stored under nitrogen. Three types of phospholipids were investigated during these studies: DMPC, DPPC and a mixture containing 70 percent of phosphatidylcholine (Lipoid S75).

1.4. Differential scanning calorimetry

The calorimetric experiments were performed with a Microcal MC-2 scanning calorimeter. Before starting the experiments, the samples were degassed under vacuum and equilibrated in the calorimeter. The reference cell contained a 10 mM hepes buffer solution and the phospholipid concentration in the samples was 1 mM. Heating scans from 7°C to 65°C at a scan rate of 1°C/min were made.

The main transition temperatures (T_m) were taken at the onset of the transition at the intercept of the baseline with the tangent to the left side of the thermal peak. The enthalpy changes (ΔH) and the transition widths at half-height ($\Delta T_{1/2}$) were obtained from the area and the shape of the peak. The main transition temperatures, the transition half-heights and the enthalpy changes were determined with accuracy of $\pm 2\%$, $\pm 5\%$ and $\pm 12\%$ respectively.

1.5. Steady-state fluorescence polarization measurements

Liposomal bilayer fluidity was assessed by incorporating a fluorescent probe (DPH, 1 mM in dimethylformamide) into phospholipid vesicles containing either neoglycolipid or cholesterol. The mixture was stirred for 3 min and incubated at 4°C during one night. The total lipid concentration was equal to 350 μ M. The molar ratios of lipid/fluorescent probe were ~ 400 . The fluorescence measurements were carried out using a Spex Fluoromax spectrometer. The cell holder was thermostated with a Haake D8 apparatus and the temperature was controlled in the measurement cell with a Fluke 179 true RMS multimeter thermometer ($\pm 0.1^\circ\text{C}$). The excitation wavelength was set to 359 nm and emission to 450 nm.

The studied temperature ranges were 15-45°C for the DMPC containing liposomes and 20-45°C for the DPPC containing liposomes. Thirteen measurements were made in these ranges.

1.6. Zeta potential measurements

Zeta potentials of the liposomes were measured using a Malvern Zetamaster-S instrument. The measurements were made in Hepes buffers (pH 7.4), containing or not univalent salts (NaCl).

1.7. Calculation of the fixed aqueous layer thickness (FALT)

As described in the literature^[14-16], it is possible to estimate the fixed aqueous layer thickness of liposomes by using the zeta potential values.

The zeta potential was calculated from electrophoretic mobility applying the Smoluchowski equation ^[17]. In our study, we calculated FALT using the Gouy-Chapmann theory ^[18,19] which expressed the zeta potential $\psi(L)$ as the electrostatic potentials at the position of the slipping plane L (nm):

$$\ln \psi(L) = \ln A - \kappa L \quad \text{Eq. 1}$$

where A is regarded as a constant and κ is the Debye-Hückel parameter. For univalent salts, the Debye-Hückel parameter could be expressed as:

$$\kappa = \sqrt{C} / 0.3 \quad \text{Eq. 2}$$

where C is the molality of electrolytes.

If zeta potentials are measured with changing concentration of NaCl (from 0 to 150 mM) and plotted against κ , the slope L gives the position of the slipping plane, or thickness of the fixed aqueous layer in nanometer units.

1.8. Size measurements

Size measurements were made by quasi-elastic light scattering determinations with a Malvern Zetamaster[®]-S instrument (Orsay, France).

1.9. Agglutination assays

Agglutination assays were performed in order to observe the recognition between the sugar residues and specific lectins. Depending on the incorporated glycolipid, wheat germ agglutinin (WGA) or lectin from *Ulex europeaus* (UEA-I) was added to liposome samples enriched with 1 mM divalent cations (Ca^{2+} , Mn^{2+} , Zn^{2+}) which are essential for the interaction ^[20]. Turbidity changes at 450 nm were measured with a UV-160A recording spectrophotometer from Shimadzu at 25°C after 15 min. following the lectin addition. The lectins did not exhibit by themselves any measurable absorbance under the experimental condition.

Results

2.1 Steady-state fluorescence anisotropy measurements

The movements of the DPH probe within bilayers have been used to evaluate changes in the membrane fluidity of liposomes in the presence of neoglycolipids. Because of its structure, DPH provided information on the changes occurring in the hydrophobic core of the membrane. The phospholipid-cholesterol or phospholipid-glycolipid composite displayed typical sigmoidal thermograms (fig.2). Although the profiles of the anisotropy versus temperature curves were generally similar, the order parameters changed significantly from one mixture to the other. The presence of Fuc-E₄-Chol in DPPC-based bilayers broadened the phase transition, i.e., the temperature range in which the fluid and gel domains co-existed and also shifted the phase transition temperature mid-point (T_{mid}) towards lower temperatures. Furthermore, the shift and the T_{mid} broadening appeared to be dependent on the cholesteryl derivative concentration and on the nature of the sugar head group. Thus, the decrease of phase transition temperature was proportional to the glycolipid-phospholipid ratio and the transition temperature change was less pronounced with the *N*-acetylglucosamine than with the fucose head group. Contrary to cholesterol, it could be mentioned that the glycolipid inclusion had a fluidising effect on the bilayer in the gel phase temperature range. Similar profiles were obtained with DMPC-based bilayers (data not shown).

2.2 Differential scanning calorimetry

The DSC results are summarized in the table I. Compared to pure DPPC liposomes, the incorporation of increasing amounts of glycolipids progressively reduces the transition temperature (T_m), the enthalpy of the transition (ΔH), and the cooperativity of the gel to liquid crystalline phase transition. Indeed, there is a relationship between the shape of DSC peaks and the cooperative interaction among DPPC molecules at the phase transition temperature. Actually, a sharp peak (low $\Delta T_{1/2}$) is derived from a higher cooperative interaction among DPPC molecules, while a broad peak (important $\Delta T_{1/2}$) is derived from a lower cooperative interaction. However, it is more complex to compare the observations between cholesterol and glycolipids. With the glycolipids the lowering effect on the transition temperature is more important, whereas the effects on enthalpy and cooperativity are less pronounced than with cholesterol. At least, the two studied glycolipids give very similar results.

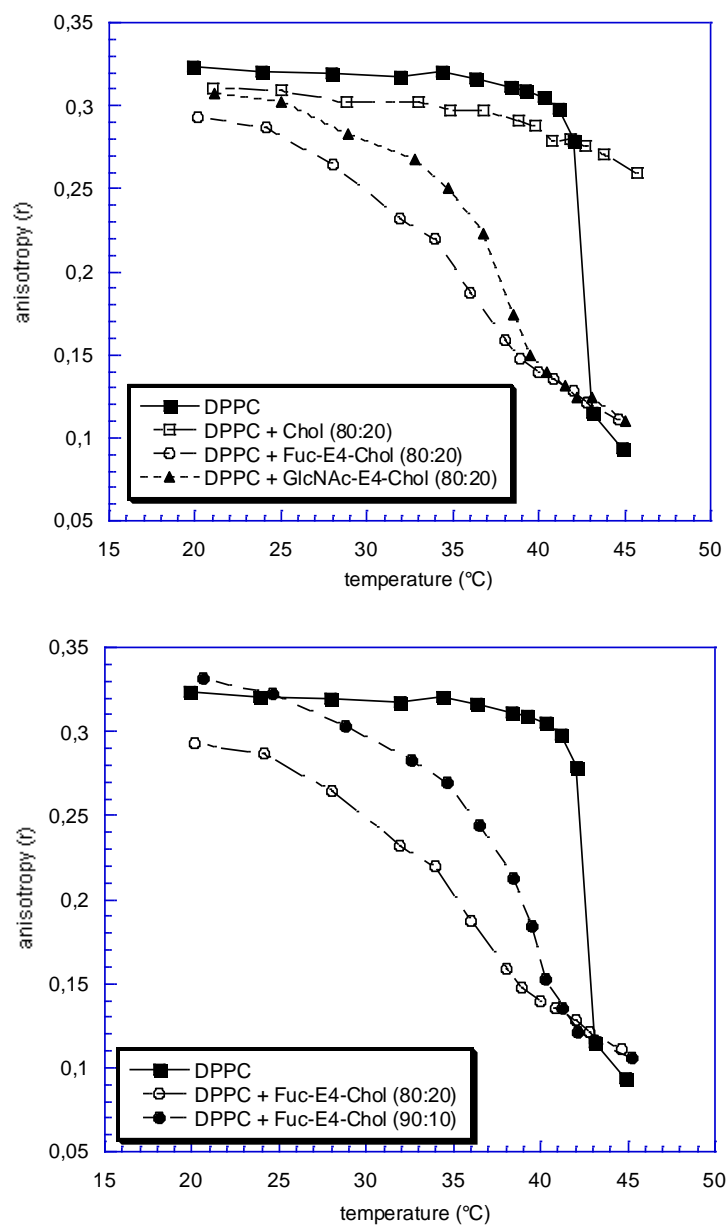


Fig.2: Thermotropic profiles of DPPC-liposomes containing the DPH probe: (top)-influence of the type of incorporated molecule, (bottom)-influence of the phospholipid-glycolipid ratio. The symbols used are explained in the inset.

Table I: Differential scanning calorimetry results.

	DPPC	DPPC/cholesterol		DPPC/Fuc-E ₄ -Chol		DPPC/GlcNAc-E ₄ -Chol	
		9 : 1	8 : 2	9 : 1	8 : 2	9 : 1	8 : 2
T_m (°C)	41.0	39.2	36.3	35.2	30.6	37.1	31.8
ΔH (kJ/mol)	32.2	15.4	6.9	25.2	21.8	24.3	21
$\Delta T_{1/2}$ (°C)	1.5	1.5	5.5	2	4.5	2	3.6

2.3 Zeta potential

The zeta potential of the different formulations is described in the figure 3. The surface charge of the phospholipid liposomes is independent of the incorporated compound (cholesterol or glycolipid). However, an important dependence on the ionic strength of the zeta potential could be observed. In solutions of low ionic strength, the zeta potential is negative and decreases in magnitude with increasing NaCl concentrations. By using equations 1 and 2, and by plotting zeta potentials versus κ , the slope gives the thickness of the fixed aqueous layer. The R^2 value of each linear fit is equal to or higher than 0.99 and the estimated FALT is around 1nm for the three studied formulations as summarized in table II.

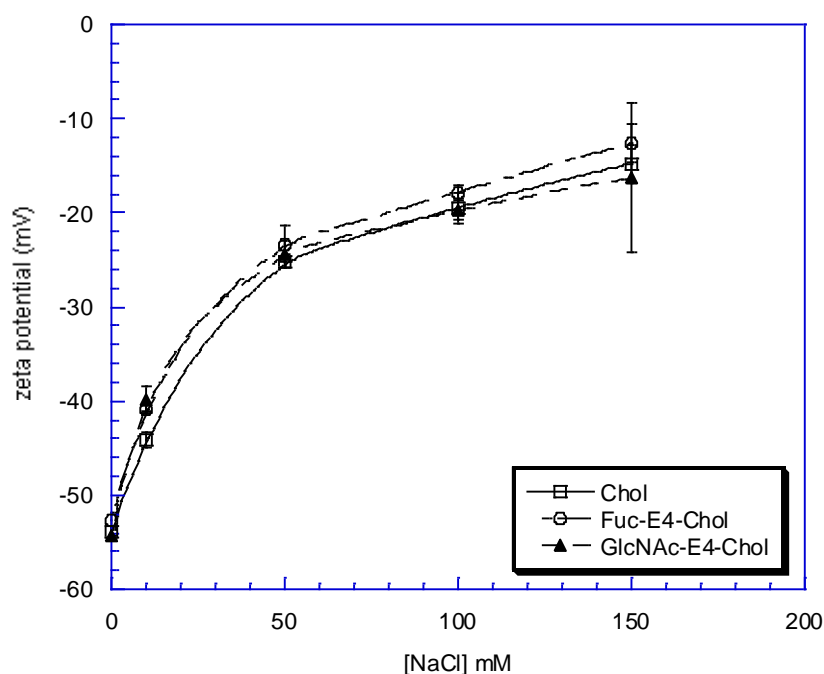


Fig.3: NaCl concentration dependence of the zeta potential of liposomes containing 10 mole% of cholesterol, Fuc-E4-Chol or GlcNAc-E4-Chol. The symbols used are explained in the inset.

Table II: Values of the fixed aqueous layer thickness calculated from the zeta potential measurements.

	Fixed aqueous layer thickness (nm)
PL + Cholesterol	1.03 ± 0.06 nm
PL + GlcNAc-E ₄ -Chol	0.95 ± 0.12 nm
PL + Fuc-E ₄ -Chol	1.12 ± 0.06 nm

The phospholipid (PL)/incorporated molecule ratio is 90/10.

2.4 Size and agglutination

The mean sizes of the liposome populations used for the agglutination experiments lie between 147 and 170 nm. The specificities of the lectin-sugar recognitions are clearly evidenced by the results of agglutinations reported in table III. Indeed, with the presence of wheat germ agglutinin, only the suspensions of GlcNAc-E₄-Chol bearing liposomes displayed an important increase in optical density, whereas cholesterol or Fuc-E₄-Chol containing liposomes did not. In the same way, the presence of lectin from *U. europaeus* led to the specific agglutination of the fucosylated liposomes only.

Table III: Increase of the optical density of liposome suspensions following the addition of lectin at a concentration of 1 μ M.

	Optical density increase at 450 nm	
	UEA-I	WGA
PL + Cholesterol	≤ 0.005	≤ 0.005
PL + GlcNAc-E ₄ -Chol	≤ 0.005	0.295 ± 0.024
PL + Fuc-E ₄ -Chol	0.100 ± 0.004	≤ 0.005

The phospholipid (PL)/incorporated molecule ratio is 90/10.

Discussion

The effect of the glycolipid incorporation on the hydrophobic core of the bilayers has been monitored with the environment-sensitive fluorescent membrane probe DPH. The average location of this probe has been shown to be around 8 Å from the center of the bilayer. Fluorescence quenching experiments have earlier shown that DPH was evenly distributed between the gel and fluid phases, allowing studies without bias toward any particular phase of the membrane ^[21]. The presence of other structures (e.g., micelles) in the liposome suspensions could have altered the interpretation of fluorescence experiments. Nevertheless, we have previously demonstrated that the phospholipid/Fuc-E₄-Chol ratio of purified liposomes, prepared by the method used in this paper ^[22], remained constant before and after size exclusion chromatography. Furthermore, the phospholipid/Fuc-E₄-Chol ratio in liposomes was shown to be identical with that of the initial weighted film, thus suggesting that no glycolipids were lost by formation of aggregates.

For both phospholipids (DPPC and DMPC), the anisotropy measurements have clearly shown a higher mobility of the probe in the gel phase when glycolipids were incorporated instead of cholesterol. This fluidizing effect has been observed already in GlcNAc-E₄-Chol/PC dry films ^[23] and also in the gel phase of another family of fucosylated glycolipids ^[12]. In the latter, very structurally different hydrophobic anchors (Guerbet alcohols vs cholesterol)

led to similar thermotropic profiles, thus suggesting that the membrane fluidity changes were essentially due to the hydrophilic moiety. Moreover, it was also reported that phospholipids with identical chains displayed rotational relaxation times for the (CH₂)_n units higher for cationic than for zwitterionic lipids. The increased translational motion and the looser membrane packing of cationic lipids were attributed to their head group repulsion ^[24]. In this work, the increase of probe mobility could be due to the PC head group spacing by the glycolipids. Another reason for the increasing mobility of the fluorescent probe could also be attributed to the difference in molecular shapes. Double-chain lipids with large head groups (such as PC) have a truncated cone shape leading to vesicle formation, whereas steryl oligoethylene glycols containing less than 20 oxyethylene units have a cylindral shape ^[25]. Contrary to the cholesterol which acts as a filler molecule, the cylindral-shaped molecules (GlcNAc-E₄-Chol or Fuc-E₄-Chol) induce strong perturbations in the packing of the lipid bilayers, resulting in an increase of the probe mobility.

In pure phosphatidylcholine bilayers, the head group is oriented approximately parallel to the plane of the bilayer and the incorporation of cholesterol does not change this conformation ^[9]. Because of steric hindrance, an increase of the surface charges in the liposomes loaded with glycolipids could be expected, either due to the reorientation of the phospholipid head groups following the theory of Makino et al. ^[26] or to the hiding of surface charges by the polyethylene glycol layer. As shown in figure 3, there are no significant changes in the zeta potential values, at given NaCl concentrations, whatever be the incorporated compounds. Therefore, there is no evidence suggesting the reorientation of the phospholipids head group. If the incorporation of the spacer in the hydrophilic network of the bilayer is not balanced by a reorientation of the phospholipid head groups, we could assume that the phospholipid heads are moving aside, leading to a looser packing of the lipid chains and then to a decrease of the fluorescent probe anisotropy.

By using the scaling laws describing the conformation of grafted flexible polymers immersed in good solvents ^[27-29], it is possible to predict the conformation of the glycolipid spacer. This model could be applied because the oligomer is highly soluble in the buffer used and considering the expected oligomer chain length the shell of the vesicles could be approximated to a planar surface. By comparing the average distance between grafting sites, d , with the Flory radius, $R_F = N^{3/5}a$ (given for a coiled chain in a good solvent with N monomer units of length a), two principal regimes have been defined ^[28]. For $d > R_F$, the polymer develops as separate coils (mushroom model), whereas for $d < R_F$, the chains adopt a stretched state (brush regime). In the second model, the thickness of the grafted polymer layer is $L = aN(a/d)^{2/3}$. Concerning glycolipids GlcNAc-E₄-Chol and Fuc-E₄-Chol, at a molar fraction of 10%, the calculated extended length of the spacer in the brush regime is 1.3 nm. In this calculation, the sugar moieties were not taken into account and therefore it is

reasonable to assume that the length of the whole head group (spacer + sugar) is higher than 1.3 nm. The experimental FALT around 1 nm, let us assume that the carbohydrate moieties are not as far as possible from the phospholipid head group layer. This point is inconsistent with the generally accepted idea considering that the increase of lectin recognition measured with increasing spacer chain length is essentially due to a better accessibility of the sugar residues. However, this result is consistent with another study using infrared spectroscopy and describing the interaction between GlcNAc-E_n-Chol (n = 1-4) and phospholipids ^[23]. In this paper, the interactions between the carbonyl group of the GlcNAc and the phosphate group of PC were shown to be independent of the oligoethoxy spacer length. Besides, several studies have explained the cryo-protectant role of sugars in liposome lyophilization by the formation of hydrogen bonds between the carbohydrate hydroxyl groups and the phosphate group of the phospholipids. Thus, depending on their size and shape, sugars affect the interactions between the phospholipid head-groups and their area which induces spacing in the bilayer ^[30,31]. However, despite this interaction, it was clear from the agglutination results (table III) that the lectins were able to interact specifically with the target sugar moieties as observed in this work but with other glycolipids also ^[32]. The optical density increase is higher with the GlcNAc-E₄-Chol / WGA couple than with Fuc-E₄-Chol / UEA-I, however it is not possible to conclude to a better recognition in the first case. The number of recognition sites is more important in the case of WGA compared to UEA-I (4 vs 2, ^[20]), then the aggregates do not have identical morphologies.

The calorimetric measurements confirm the results obtained with other techniques. The incorporation of the glycolipid in the phospholipid bilayers leads to complex effect compared to the cholesterol inclusion: the T_m decrease is more important but the ΔH decrease is less important. These results are very similar for both glycolipids GlcNAc-E₄-Chol and Fuc-E₄-Chol. The T_m decrease is classically observed after the incorporation of cholesteryl-derivatives in phospholipid bilayers ^[33,34] and could be attributed to a loss of packing. It is important to note here that transition temperature was obtained at the onset of the peak, corresponding to the beginning of the transition. The higher energy (ΔH) needed for the transition with the glycolipids compared with cholesterol could be the expression of interactions in the head group layer. A number of studies have determined the structure of the bilayer and the gel to liquid crystalline phase transition temperature as a function of hydration ^[35]. Thus, the hydration water molecules located in the head group region of the bilayer occupy about 30 Å³ per molecule and their removal decreases the area per lipid molecule and squeezes the hydrocarbon chains close together, causing increase in the calorimetric parameters. The hydration competition between the phospholipid and glycolipid head groups ^[23] could shift water molecules from the phospholipids to the glycolipids. Finally,

compared to cholesterol, the more important cooperativity observed at important glycolipid / DPPC ratios suggests an intermolecular stabilization by the sugar moieties.

In conclusion, it can be assessed that the incorporation of glycolipids GlcNAc-E₄-Chol and Fuc-E₄-Chol in phospholipid bilayers leads to a spacing aside of the hydrophobic tails in the core of the membrane. This effect, relying on the cylindrical shape of the molecules, was displayed by fluorescent measurements. This could explain the lower T_m observed with cholesteryl glycolipids compared with cholesterol. No re-orientation of the phospholipid head group has been displayed. However, the head group of the glycolipid did not adopt an extended brush model and the interactions between the carbohydrates and the phospholipid layer could probably explain the lower decrease of ΔH observed with cholesteryl glycolipids than with cholesterol.

The use of liposomes as drug carrier is strongly dependent on their stabilities in biological fluids. In oral administration, the massive liposome destabilisation observed in the gastric-intestinal tract takes place in the intestinal segment, essentially after mixing with bile salts ^[36]. Nevertheless, the stomachal environment (low pH, pepsin, ...) does not affect the structure of lipidic vesicles in many instances, allowing their gastric use. In continuation of our efforts, experiments are actually made to quantify the impact of the glycolipid fluidising effects on the drug-release properties of liposomes and to compare these results with those obtained with polymeric nanoparticles.

Acknowledgements

The authors thank A. Naik for the access to calorimeter.

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De part leur forme et l'hydratation importante de la partie polaire (chaîne d'OE + sucre) altérant l'organisation des phospholipides, l'incorporation des glycolipides dans la bicouche liposomale déstabilise quelque peu cette dernière. En revanche, même incomplètement étendue en surface des liposomes, la partie espaceur + sucre permet une reconnaissance spécifique de la formulation par les lectines végétales. Le système semble donc pourvu d'un potentiel de ciblage. Cependant, l'utilisation des liposomes comme vecteur de médicament est fortement dépendante de leur instabilité dans les fluides biologiques, contrairement aux autres systèmes (microparticules, nanoparticules...). Les résultats de différentes études menées sur la stabilité des liposomes lors de l'utilisation par voie orale, sont souvent contradictoires. En effet, la stabilité dépend des phospholipides utilisés, des caractéristiques de surface, de la taille, de la charge...induisant de grandes variabilités d'une recherche à l'autre ^[1,2]. Néanmoins, les conclusions de ces études tendent toutes vers la même idée : les liposomes, aussi stables soient-ils, sont dégradés en milieu intestinal par les sels biliaires et les lipases pancréatiques ^[3-7]. Mais il semblerait qu'en milieu stomacal en général, ou à des pH acides en particulier, les liposomes soient stables, permettant leur emploi dans un but de ciblage gastrique ^[6,8]. Toutefois, les liposomes ne sont, en théorie, pas imperméables aux protons. Nous allons donc étudier, dans cette avant-dernière partie du travail, la variation du pH intraliposomale en fonction du pH externe, et vérifier la stabilité chimique du glycolipide en milieu acide, en s'assurant de la reconnaissance spécifique par les lectines végétales. De plus nous nous intéresserons à l'organisation des phospholipides en fonction du pH et de la température.

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CHAPITRE IV:

Pre-formulation of liposomes against
***Helicobacter pylori*: part I – pH effects.**

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Contents

Introduction	111
1. Materials and methods.....	112
1.1. Materials	112
1.2. Methods.....	113
1.2.1. Vesicle preparation	113
1.2.2. X-ray diffraction experiments.....	113
1.2.3. Intra-liposomal pH determination.....	114
1.2.4. Zeta potential measurements.....	114
1.2.5. Phospholipid measurement.....	114
1.2.6. Agglutination assay	115
2. Results.....	115
2.1. X-ray experiments.....	115
2.2. Intraliposomal pH variations.....	117
2.3. Zeta potential measurements.....	120
2.4. Agglutination assays	121
3. Discussion	122
References.....	128

Figures

Scheme 1: Neoglycolipids used in this work and in previously reported studies	112
Fig.1: Diffractograms of fully hydrated DPPC (a) and Epikuron 170 mixture (b) in a pH 7.4 Hepes buffer at 20°C (bottom) and 37°C (top)	116
Fig.2: Small-angles diffractograms of fully hydrated Epikuron 170 in Hepes buffer after addition of increasing amounts of HCl (a) or equivalent amounts of NaCl (b) in the medium.....	116
Fig.3: Repetitive distances observed in fully hydrated epikuron depending on the addition of HCl (●) or NaCl (○)	117
Fig.4: (a) Raw oregon green 514 carboxylic acid emission spectra at 570 nm recorded in Hepes buffer (10 mM, NaCl 150 mM) between pH 7.6 to 1.5. (b) Intensity ratios (I504 nm/I467 nm) versus pH	117
Fig.5: Fluorescent probe intensity ratio versus time recorded at pH 4.4, 3 and 2, for liposomes composed of (a) DPPC-cholesterol, (b) DPPC-cholesterol-glycolipid, (c) Epikuron-cholesterol and (d) Epikuron-cholesterol-glycolipid	119
Fig.6: Zeta potential values of epikuron-cholesterol liposomes	120
Fig.7: Comparison of the agglutination results, after addition of WGA to GlcNAc-E4-Chol- containing liposome (expressed as the increase of optical density at 450 nm).....	121

Tables

Table I : Internal liposome pH determined from the fluorescent intensity ratios of the Oregon green probe after a two-hours incubation in a 10 mM Hepes buffer adjusted at pH 4.4, 3 and 2 with hydrochloric acid.....	120
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Introduction

Helicobacter pylori was discovered by Warren and Marshall in 1982 (2005 Nobel prize), and confirmed as a pathogenic agent at the end of the 80's. In 1994, the WHO classified it as type I carcinogen because of the gastric cancers and MALT (mucosa-associated lymphoid tissue) lymphomas which can occur after a chronic infection. In the world, the prevalence of this bacterium is still high ^[1-5] and the eradication rate does not reach the WHO's purpose, i.e. 90% ^[6]. Today, the classical way to cure *H. pylori* infection is to use a 7 days tri-therapy based on 2 antibiotics (amoxycillin, clarithromycin) and one proton pump inhibitor (omeprazole, lansoprazole, pantoprazole) or occasionally, an association of bismuth salt with one or two antibiotics. However, because of the high level of antibiotic resistance to *H. pylori* and the poor patient compliance ^[7], new medicines with better effectiveness and simpler regimen are required. Indeed, *H. pylori* is sensitive to many antibiotics but many of them can not be used in acidic medium ^[8]. However, due to the urease activity, the close environment of the bacterium is neutralized by the production of ammonia and carbon dioxide ^[9,10]. A release of the active substance close to the bacterium could overcome the problem of acidity. The active substance encapsulation could be a good approach, by offering a protection against the stomachal acidity. In a seek for a non toxic vector, able to encapsulate a wide range of drugs and easily modifiable at the surface, we have chosen liposomes. Indeed, incorporation of specific ligands at liposome surfaces, could allow a targeting to *H. pylori* and would allow an increased stomachal retention time of the drug. The other advantage of liposomes is their similarity with cell membranes. Most of *H. pylori* strains secrete a vacuolating protein, VacA, which strongly destabilizes the phospholipid membrane of epithelial cells ^[11,12]. If the liposome is very close to the bacterium, it could be expected that the release of encapsulated drug could be done by the vacuolating effect of the protein. This paper deals with 4 liposome formulations. In all of them we used cholesterol (Chol) because of its well known bilayer stabilizing effect ^[13,14] in biological and liposome membranes as well ^[15]. Two different phospholipids were used: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or epikuron 170 (Epik). The main advantages of using epikuron are a cheap price and a composition containing at least 10% of phosphatidylethanolamine (PE), which was described as a ligand for a *H. pylori* adhesin ^[16-18]. The two liposomal formulations obtained in this way (dppc-cholesterol and epikuron-cholesterol) were compared with the same formulations in which was incorporated a synthetic glycolipid having the following structure: cholesteryl tetraethylene glycol oside (Sugar moiety-E₄-Chol). Indeed, some strains of *H. pylori* express an outer membrane protein (BabA2) which is able to link the fucosylated Lewis b (Le^b) histo-blood group antigen, present on human gastric epithelial cells ^[5,9,19,20]. In a previous study, such fucosyl neoglycolipids embedded at the surface of liposomes were

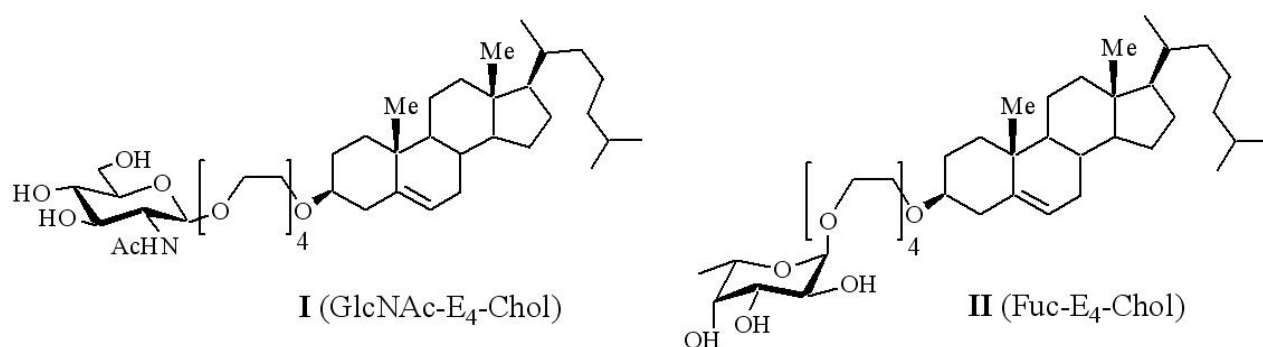
shown to display good interactions with a plant lectin, and a minimal destabilization of the liposomal membrane ^[21]. The poor stability of liposomes in the gastrointestinal tract ^[22] is mostly due to bile salts and pancreatic lipases ^[23-27]. However, in stomach medium, or more generally in acidic conditions, liposomes are quite stable ^[26,28], thus allowing a gastric targeting.

The first part of this work is devoted to the behavior of liposomes in gastric conditions. The lipid organization at different pH was explored by X-ray diffraction. The liposome internal pH variations in acidic conditions were measured by incorporation of fluorescent probes (Oregon Green[®] 514 carboxylic acid).

1. Materials and methods

1.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, monobasic sodium phosphate were purchased from Sigma-Aldrich. The neoglycolipids (scheme 1) were previously synthesized in the laboratory, as already reported for the cholesteryl tetraethyleneglycol *N*-acetylglucosamine (GlcNAc-E₄-Chol, **I**) ^[29]. The synthesis of cholesteryl tetraethyleneglycol fucose (Fuc-E₄-Chol, **II**) was realized by methods already reported to prepare α -L-fucopyranosides of Guerbet alcohols ^[30] ; they will be published in due course. Fluorescent probe, Oregon Green[®] 514 carboxylic acid, was provided by Molecular Probes [™], Invitrogen. Epikuron 170 was provided by Degussa. All solvents and reagents were analytical grade.



Scheme 1: Neoglycolipids used in this work and in previously reported studies.

1.2. Methods

1.2.1. Vesicle preparation

Liposomes were prepared by Bangham method ^[31]. Briefly, lipids and phospholipids were dissolved in chloroform, then the solvent was removed under a nitrogen stream, followed by drying under vacuum for 12 h. The lipid film thus formed on the glass was then hydrated with a 10 mM HEPES buffer (pH 7.4, final total lipid concentration : 20 mM). To achieve vesicle formation, the flask was vortexed and sonicated several times for approximately 25 min. The liposome sizes were controlled by extrusion under nitrogen above the phospholipid transition temperature (T_m), 3 times through 0.4 μm HTTP filter (Millipore, isoporeTM) and 8 times through 0.2 μm GTTP filter (Millipore, isoporeTM). The vesicles were characterized by quasi-elastic light scattering (QELS) using a Coulter nanosizer apparatus (Model N4 MD, Coultronics, France). A mean hydrodynamic diameter of 170 nm was obtained.

1.2.2. X-ray diffraction experiments

For all the studied phospholipid mixtures, static X-ray diffraction experiments at 20°C and 37°C were performed using the following device. XRD patterns were recorded by transmission using quartz capillaries (1.5 mm diameter, GLASS W. Müller, Berlin, Germany). In these experiments, the X-ray generator used was long line-focus sealed tube (ENRAF NONIUS; Cu anode operating at 40 kV and 20 mA). Two gas-filled linear detectors (HECUS; 1024 channels each, filled with argon-ethane mixture) were used to collect the data. With these settings, scattering vectors q ranging from 0.04 to 0.37 \AA^{-1} (Small-angle X-ray scattering, SAXS) and from 1.24 to 1.85 \AA^{-1} (Wide-angle X-ray scattering, WAXS) were accessible. The scattering vector is defined as $q = 4.\pi. \sin(\theta) / \lambda$ where 2θ is the scattering angle. From this scattering vector, it is possible to calculate the repetitive distances by the use of the following equation $q=2\pi/d$. The calibration of the detectors was carried out by using the well defined positions of the peaks of 2L β form of pure tristearin ($4.59, 3.85, 3.70 \pm 0.01$ \AA and $44.97 \pm 0.05\text{\AA}$) and of silver behenate ($58.38 \pm 0.01\text{\AA}$). In order to determine the peak positions, diffractograms were calibrated with Gaussian model by the use of the IGOR pro software (WaveMetrics, Inc.). The XRD apparatus used was coupled with a differential scanning microcalorimeter especially designed for installation in an X-ray beam ^[32]. The calorimeter was used as the temperature controlled sample holder.

In these experiments, lipid films were prepared as described in the “vesicle preparation” section and the rehydration was made with adequate buffer in order to reach a final lipid concentration of 15% (w/w).

1.2.3. Intra-liposomal pH determination

Because several drugs efficient *in-vitro* against *Helicobacter pylori* are unstable in acidic conditions, we have measured the pH inside different compositions of liposomes, after incubation in several media. The pH of the aqueous internal compartments of the vesicles was determined by the use of a pH-sensitive fluorescent probe (Oregon green 514, Molecular probes). Oregon green 514 carboxylic acid is a pentafluorofluorescein derivative on which protonation results in a decrease of extinction coefficients and quantum yields, as well as a blue shift of the absorption spectra. This pH-dependent absorption shift indicates that this probe is usable for excitation wavelength ratiometric measurement. The excitation wavelength pairs used here was the ratio 504 nm/467 nm and the emission intensity was monitored at 570 nm with a spectrofluorimeter (Fluorolog Spex, Jobin-Yvon). Briefly, the methodology was the following. Liposomes were prepared as described above and the fluorescent probe was incorporated at the concentration 10^{-7} M in the buffer used to rehydrate the lipid film. A gel exclusion chromatography (sephacryl S1000) was used in order to separate the encapsulated and non-encapsulated fluorescent probe. At the end of the column, scattering at 488 nm and fluorescence at 555 nm were used to isolate the fraction displaying the presence of both liposome and probe. The fraction containing the free Oregon green was removed. Just after the separation, an emission spectra at 570 nm was recorded to confirm the pH of the liposome inner phase (7.4). Then hydrochloric acid was added and the external and internal pH of the suspension were measured during two hours as a function of time, with a pH-meter and the fluorescent probe respectively.

1.2.4. Zeta potential measurements

Zeta potentials of the liposomes were measured using a Malvern Zetamaster[®] 3000 HS instrument (Orsay, France). The measurements were made in a 10 mM HEPES buffer supplemented respectively with HCl or NaCl, depending on the pH or ionic strength desired.

1.2.5. Phospholipid measurement

The amount of phospholipids was determined by a colorimetric method based on the formation of a complex between phospholipids and ammonium ferrothiocyanate^[33]. A known volume of liposome suspension was evaporated and the phospholipid residue was dissolved in chloroform (2 mL). Then, the thiocyanate reagent was added (1 mL). After vortexing for 1 min, the mixture was centrifuged for 10 min at low speed and the chloroform red layer was withdrawn with a Pasteur pipette. The absorbance was recorded at 488 nm and compared with a standard phosphatidylcholine solution (range: 10 to 100 µg/mL).

1.2.6. Agglutination assay

Agglutination assays were performed in order to check the physical stability of the liposomes and the chemical stability of the glycolipids in gastric media. PC-based liposomes containing 10% of GlcNAcE4Chol I were incubated with a USP gastric medium (USP XXIV, plus pepsin) for 1.5 h. Then liposomes were separated by ultracentrifugation at 40000 rpm for 1.5 h, at 4°C (Beckman Model L7-55 centrifuge, Beckman Instruments, Palo Alto, CA) and resuspended in a 10 mM HEPES buffer enriched with 1 mM CaCl₂. Wheat germ agglutinin (WGA) was added to liposomes and turbidity changes at 450 nm were measured at 25°C, 15 min after the addition of lectin. Agglutination measurements were compared before and after incubation.

2. Results

2.1. X-ray experiments

Figures 1a and 1b show the X-ray diffractograms (small-angle and wide-angle) obtained in a 10 mM HEPES buffer (pH 7.4, NaCl 150 mM) at 20°C and 37°C, for DPPC and epikuron respectively. DPPC patterns, at 20°C, display three peaks detectable with the SAXS, probably corresponding to the three first order of a lamellar phase, and one in the WAXS corresponding to the gel phase. The repetitive distances are 64.2 Å (SAXS) and 4.25 Å (WAXS). At 37°C, the DPPC/water mixture lost organisation (decrease on the intensity and enlargement of the SAXS peaks) and the repetitive distances become 69.4 Å and 4.26 Å.

The patterns of the epikuron/water mixture at 20°C and 37°C are quite similar to that depicted for DPPC at 37°C. For the low q, the repetitive distances are 71.0 Å at 20°C and 69.5 Å at 37°C suggesting a slight modification of these phospholipids in the temperature range. For the important q (short distances), no repetitive distance could be detectable by using the Igor Pro tools to fit the WAXS patterns.

Epikuron/water mixture was studied in the presence of increasing amounts of hydrochloric acid, in order to reach the pH 6, 4, 3 and 1.5. The corresponding SAXS patterns are displayed in figure 2a. To distinguish between charge and ionic strength effects, an amount of NaCl equivalent to that of HCl required to reach pH 1.5 was also added in another experiment. The results are presented in figure 2b; for all SAXS patterns, two peaks could be detected as at pH 7.4. Independently of the conditions (pH and ionic strength), the mean position correlation of 1.99 ± 0.02 between the two peaks suggests the presence of two orders as reported before. The repetitive distances obtained from these diffractograms are presented on figure 3. An important increase in the distance is observed in the presence of NaCl, with an enlargement of the diffraction peaks, whereas only slight variations of the

lamellar distances are observed in the presence of HCl. Furthermore, at pH 1.5, the peaks become sharper and the two orders appear more definite.

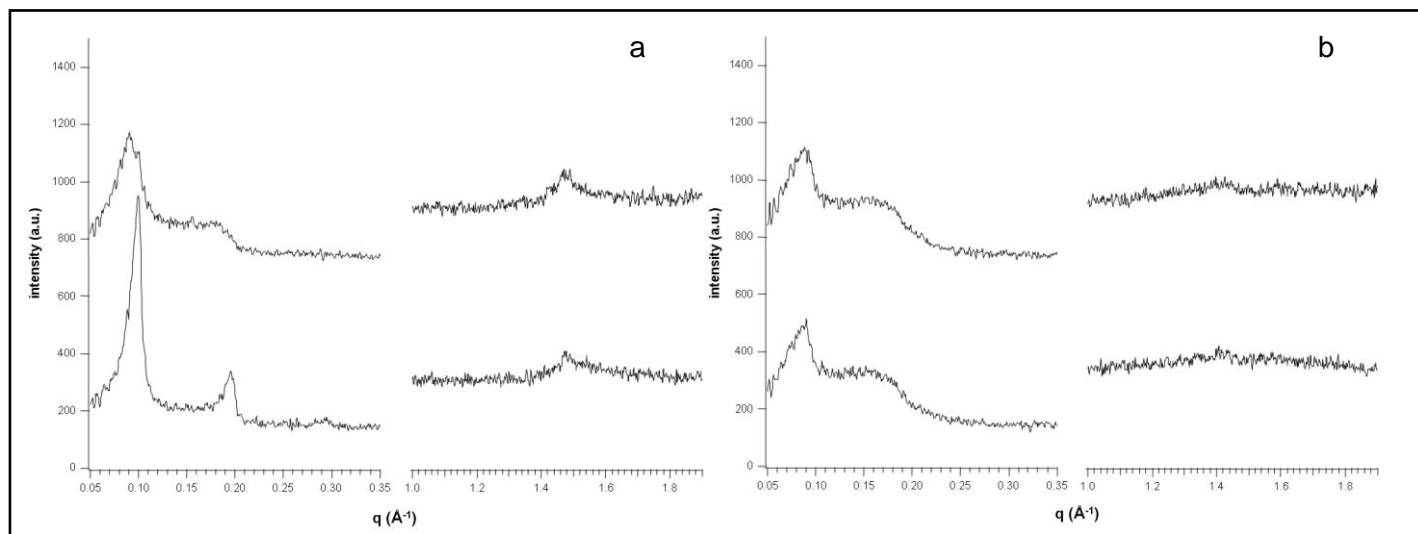


Fig.1: Diffractograms of fully hydrated DPPC (a) and Epikuron 170 mixture (b) in a pH 7.4 Hepes buffer at 20°C (bottom) and 37°C (top). The small-angles and the wide-angles X-ray scatterings are represented on the left side and on the right side of the figures respectively.

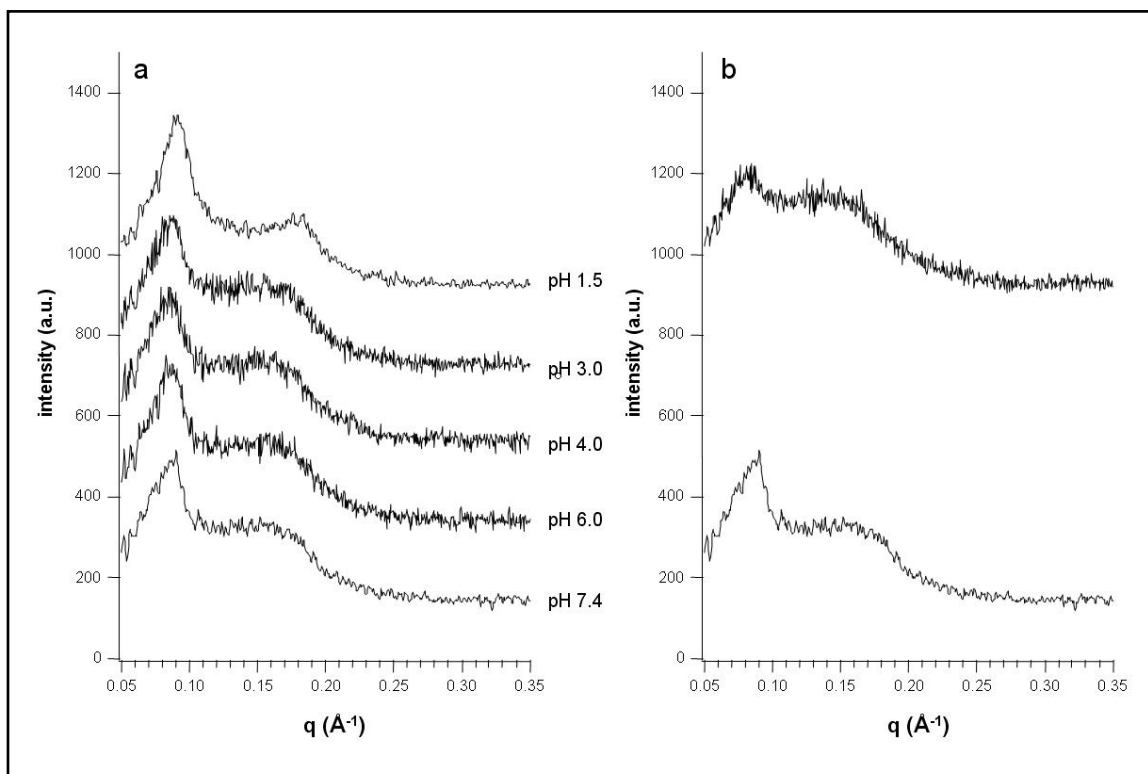


Fig.2: Small-angles diffractograms of fully hydrated Epikuron 170 in Hepes buffer after addition of increasing amounts of HCl (a) or equivalent amounts of NaCl (b) in the medium.

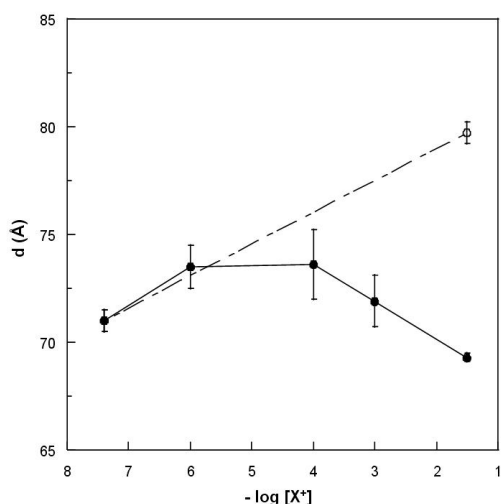


Fig.3: Repetitive distances observed in fully hydrated epikuron depending on the addition of HCl (●) or NaCl (○). In abscissa is reported $-\log [X^*]$, in which $X^+=H^+$ in the case of HCl ($-\log [X^*]=\text{pH}$) or $X^+=\text{excess of sodium ion}$ (compared to the initial buffer) in the case of NaCl.

2.2. Intraliposomal pH variations

The emission spectra at 570 nm, recorded in HEPES buffer at decreasing external pH (from 7.6 to 1.5) are reported in figure 4a. A drastic decrease of intensity could be noted. Absorption ratios between 504 nm and 467 nm were used, instead of intensity at a definite wavelength. These measurements avoid the drawbacks due to encapsulation fluctuations between different formulations and were independent on the amount of fluorescent probe into the liposome aqueous cavities. The intensity ratio versus the pH of the fluorescent probe environment is shown on figure 4b.

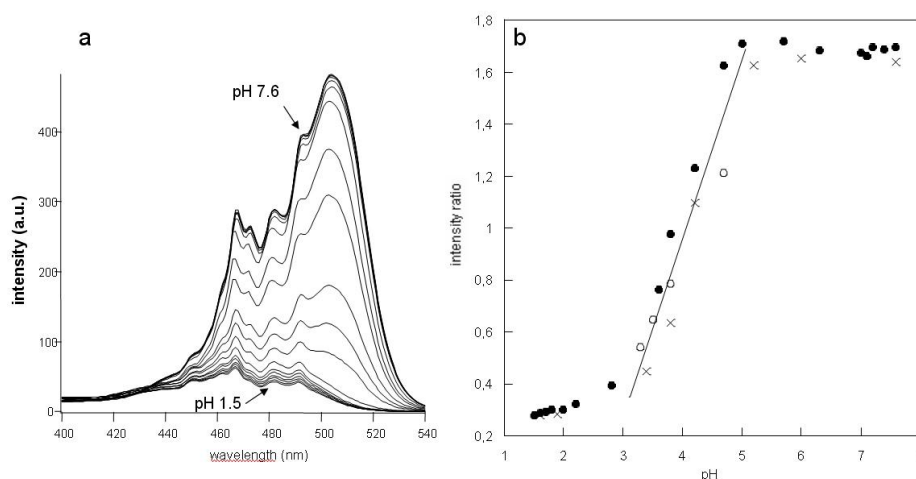


Fig.4: (a) Raw oregon green 514 carboxylic acid emission spectra at 570 nm recorded in Hepes buffer (10 mM, NaCl 150 mM) between pH 7.6 to 1.5. (b) Intensity ratios ($I_{504 \text{ nm}}/I_{467 \text{ nm}}$) versus pH; three experimental conditions were used: pure water (x), Hepes buffer (●) and Hepes buffer containing unloaded-liposomes (○). The increases in intensity ratio in the pH range 3 to 5 were considered as linear and Kaleidagraph tools have been used to fit the values in order to obtain the intensity ratio versus pH correlation.

As expected, the oregon green 514 carboxylic acid fluorescent probe can be used to check pH changes between pH 5 and pH 3, also the absorption ratio (504 nm/467 nm) is not sensitive outside this range. The behaviour of the fluorescent probe during addition of hydrochloric acid was recorded in three experimental conditions: 1) in pure water, 2) in Hepes buffer (pH 7.4), 3) in Hepes buffer (pH 7.4) containing unloaded liposomes (up to a total lipid concentration ~18mM). No significant changes were observed in the intensity ratio versus pH .

The intensity ratios obtained during a 2 h incubation of several liposome formulations (DPPC/Chol 80:20; DPPC/Chol/GlcNAcE4Chol 80:10:10; Epik/Chol 80:20 and Epik/Chol/GlcNAcE4Chol 80:10:10) at pH 2 (± 0.2), 3 (± 0.1) and 4.4 (± 0.4) are shown in the figures 5a to 5d respectively. Some obvious differences can be seen between the various formulations. Concerning DPPC bilayers, in the absence of glycolipid (fig.5a) a weak decrease is observed for the lowest pH (pH 2). However, because of the sensitivity of the probe, this does not mean that the internal pH did not changed but it remains higher than 5 when the external pH was 3 or more. As described in figure 5b, the incorporation of glycolipids I in the liposomes affords a significant change at pH 3 and a higher decrease at pH 2, which could be due to an increase of proton permeability. In the case of the epikuron containing bilayers, the improvement of permeability is not so clear; only a slight difference could be noted for the shortest time and highest external pH (fig.5c and 5d) . However, the pH equilibration time between both sides of the epikuron membrane seems to be shorter than with DPPC, suggesting that the epikuron mixture is less proton-tight than DPPC layers. Table 1 reports on permeability to protons, in term of pH instead of fluorescence intensity ratios. It is apparent that the pH inside the liposomes did not reach the outside value, after acidification, and furthermore that the pH gradient increases with the acidity of the external medium. In view of the above results, the increase in permeability as a function of membrane compositions can be reported as DPPC-Chol < DPPC-Chol-glycolipid I < Epik-Chol < Epik-Chol-glycolipid I, in the range of pH 2-3. With Epikuron, at pH 4.4, the differences between inside and outside pH are not statistically significant.

When Epikuron-chol-glycolipid I mixtures, were incubated for 2 h with simulated gastric fluid (USP XXIV – pH 1.2), an inside pH around 3.6 was measured, whatever the mixture contained pepsin or not, thus indicating that proton permeability is not affected by the presence of pepsin. As the external pH is a little bit lower than in the previous group of experiment, the internal pH is lower too.

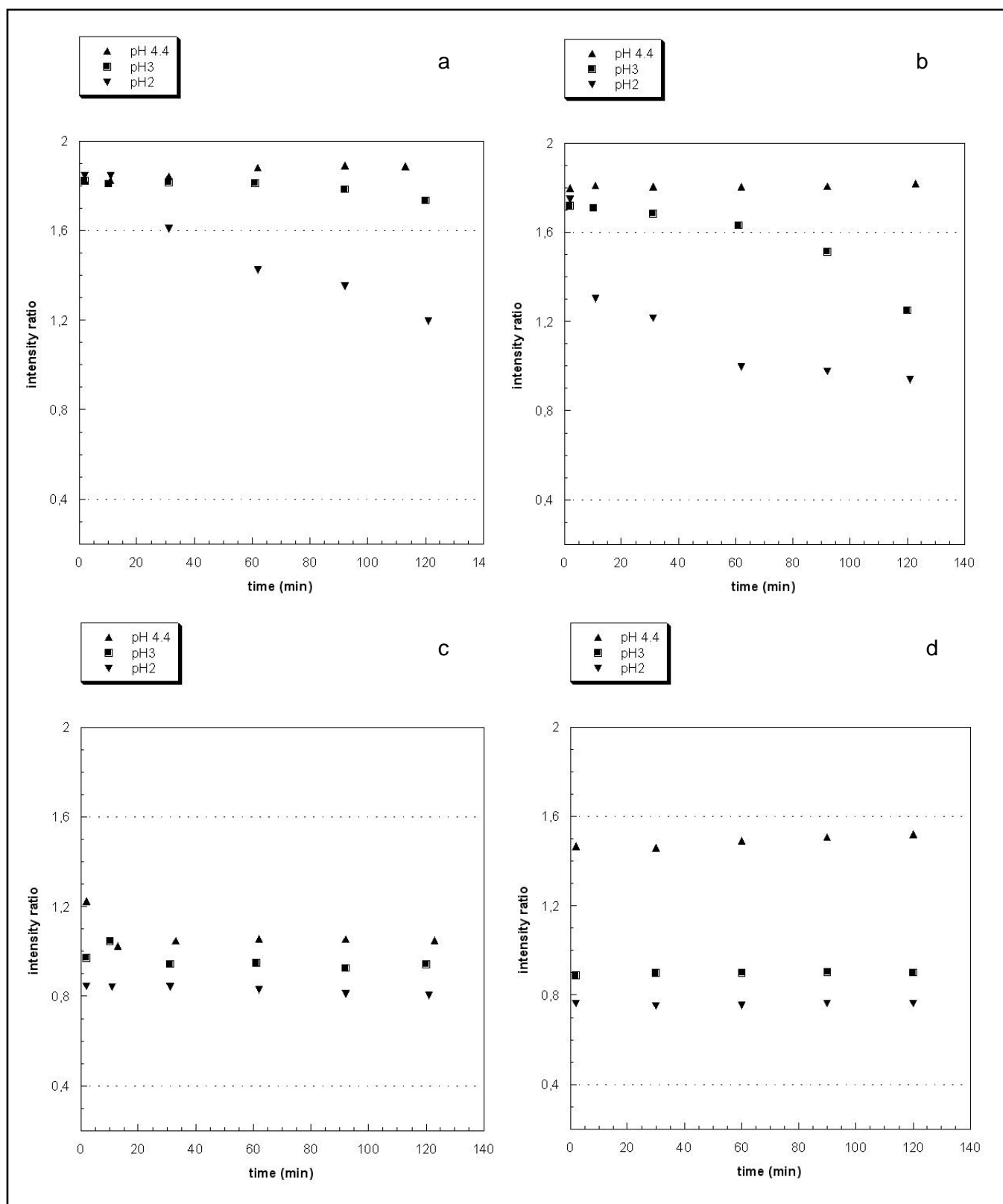


Fig.5: Fluorescent probe intensity ratio versus time recorded at pH 4.4, 3 and 2, for liposomes composed of (a) DPPC-cholesterol, (b) DPPC-cholesterol-glycolipid, (c) Epikuron-cholesterol and (d) Epikuron-cholesterol-glycolipid. The dashed lines correspond to intensity ratio limits in which pH changes could be detected (upper: pH 5; lower: pH 3)

Table I : Internal liposome pH determined from the fluorescent intensity ratios of the Oregon green probe after a two-hours incubation in a 10 mM Hepes buffer adjusted at pH 4.4, 3 and 2 with hydrochloric acid. The detailed compositions of the bilayers are described in the text.

	External pH		
	pH 2 (± 0.2)	pH 3 (± 0.1)	pH 4.4 (± 0.4)
DPPC-Chol	4.4	>5	>5
DPPC-Chol-Glycolipid	4.0	4.4	>5
Epik-Chol	3.8	4.0	4.1
Epik-Chol-Glycolipid	3.7	3.9	4.8

2.3. Zeta potential measurements

Zeta potential values obtained with the Epik-Chol liposomes at different pH and/or ionic strength are reported on figure 6. The zeta potential remains in the range [-12 mV to -7 mV] for pH in the range 7.5 to 2.8. However, a drastic increase to 4.6 ± 0.5 mV is observed when the pH is decreased to 1.8. The addition of sodium chloride, at the same concentrations than that of hydrochloric acid required to decrease the pH, leads to similar zeta potential profiles. However, there is a significant difference for the high ionic strengths: the zeta potential value is higher after the addition of HCl than that of NaCl.

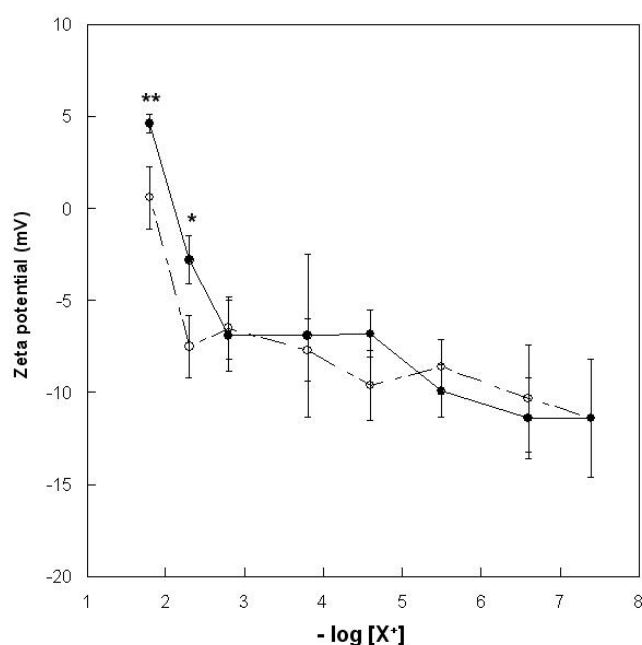


Fig.6: Zeta potential values of epikuron-cholesterol liposomes depending on the addition of HCl (●) or NaCl (○). In abscissa is reported $-\log [X^+]$, in which $X^+=H^+$ in the case of HCl ($-\log [X^+]=\text{pH}$) or $X^+=\text{excess of sodium ion}$ (compared to the initial buffer) in the case of NaCl. Variance analysis * $p<0.1$; ** $p<0.05$).

2.4. Agglutination assays

The physical stability of the liposomes and the chemical stability of glycolipids **I**, following a 1.5 h incubation in simulated gastric fluid (with pepsin) was controlled by agglutination assays. The results are represented in figure 7. The agglutination of the glycolipid **I** containing liposomes, resulting in an increase of optical density, is statistically identical after or before incubation. Independently on the liposome treatment, the agglutination slightly increases with the concentration of wheat germ agglutinin in the buffer. As WGA is a lectin specific to the sugar head-group of the glycolipid used (*N*-acetyl-glucosamine), these results strongly suggest a very good stability of the preparation in simulated gastric fluid, especially in terms of chemical stability of the synthetic glycolipid **I**.

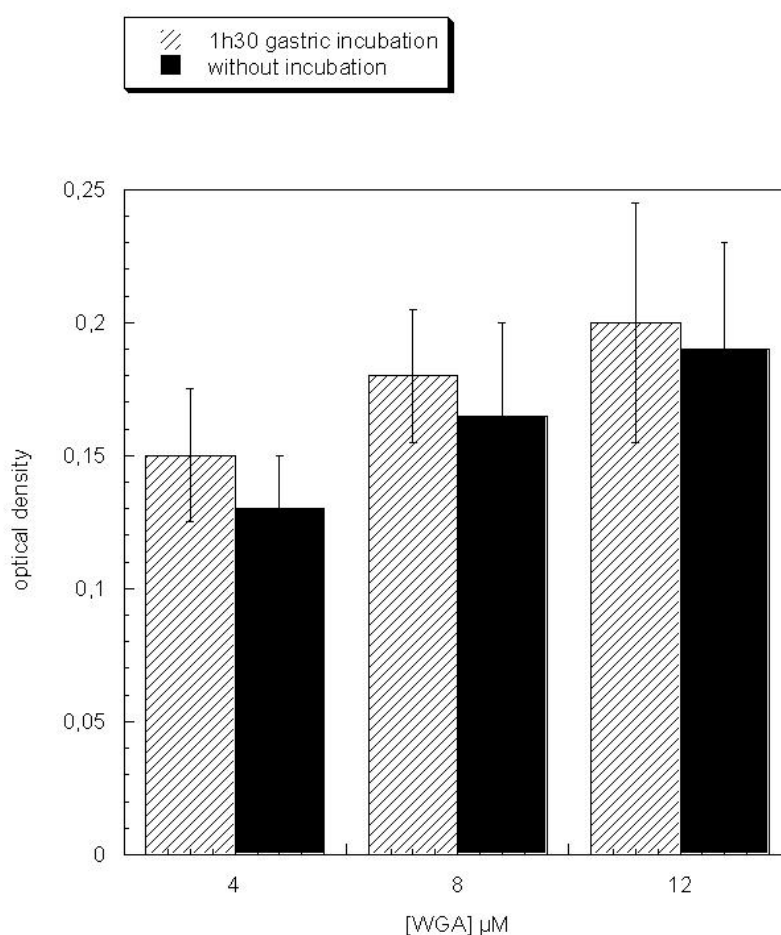


Fig.7: Comparison of the agglutination results, after addition of WGA to GlcNAc-E4-Chol-containing liposome (expressed as the increase of optical density at 450 nm). Dark histograms correspond to liposomes which were not incubated, and clear histograms correspond to liposomes incubated in gastric fluid and resuspended in the same buffer as untreated ones (Hepes buffer, pH 7.4).

3. Discussion

The X-ray diffraction patterns obtained with DPPC fit well with the classical $L_{\beta'}$ and $P_{\beta'}$ phases, extensively described in the literature ^[34-36]. The $L_{\beta'}$ corresponds to a lamellar gel phase in which the hydrocarbon chains are tilted with regard to the layer normal; tilting allows the packing mismatch to be accommodated. As the temperature is raised, a pre-transition from $L_{\beta'}$ to $P_{\beta'}$ phase occurs in hydrated lipid bilayers. Although both of them are gel phases, a flat bilayer is observed in $L_{\beta'}$ phase while periodic ripples appear in $P_{\beta'}$, a phase with corrugated surface profile. Finally, at temperature above the transition temperature ($\sim 41^\circ\text{C}$ for DPPC), the chain melt (not studied in the present work). The SAXS diffraction patterns of the Epikuron mixture are not sensitive to the temperature (between 20 and 37°C) and are quite similar to the DPPC diffractograms at 37°C . However, compared to DPPC, there is no repetitive distance in the WAXS q-range. It should be noted that this absence of WAXS peak is also true in very acidic conditions in which the SAXS peaks seem sharper. The fully hydrated epikuron mixture could to be organized as a ripple phase in which the hydrocarbon chains could be in a fluid state. Nevertheless, the formation mechanism of the ripple lipid structures is not compatible with this assumption, for the forthcoming reasons. Indeed, it has been shown that the formation of ripple surfaces was mainly dependent on the interfacial energies, governed by the lateral interactions inside phospholipid bilayers. The apparent interfacial area per molecule results from the balance between cohesive forces within the chains (hydrophobic interaction) and repulsive interactions between the headgroups ^[36,37]. The driving force for the formation of ripple phases ^[38] is the increased hydration in the headgroup region that causes an imbalance between the opposite forces previously described, thus leading to the specific physical frustration of the ripple phase. In the latter, the compromise equilibrium area per molecule does not fully satisfy the packing requirements of either the headgroups or the hydrocarbon chains. Nevertheless, this constrained arrangement implies keeping the structured organization of the hydrocarbon tails (gel phase). This is contradictory with the absence of peak we observed in WAXS patterns concerning the epikuron mixture. Furthermore, it has been shown experimentally ^[39] and by molecular dynamics simulations ^[40] that the WAXS peak in powder spectra of the ripple phase is sharper than the WAXS peak of the gel phase. This may be interpreted as lending support to a ripple phase structure with a high degree of short-range order.

As the epikuron mixture did not organize as a ripple phase, and because of the 1.99 ± 0.02 ratio observed between the two SAXS peaks, it could be reasonably assumed that the observed diffraction pattern is due to a fluid lamellar phase (L_α) ^[35]. Compared to pure phospholipid layer, the enlargement of the peak and the absence of three or more order (as

for DPPC in the present report) are due to a “mixture” effect leading to a less define repetitive distance.

With regard to our results, two aspects could be discussed concerning the pH variation inside liposomes : 1) the difference of pH decreasing rate, depending on the formulation type and 2) the pH value at equilibrium.

It is generally admitted that the phospholipid bilayer of biological membranes is a rather tight diffusion barrier for hydrophilic solutes, including ions. However, despite discordance in the published data, several studies on hydrogen ions passing through unilamellar phospholipid vesicle membranes indicate a rather high permeability of protons and/or hydroxyl ions as compared to other inorganic monovalent ions such as K^+ , Na^+ or Cl^- . Depending on the studies, the proton permeability coefficient covers a range between 10^{-3} to $10^{-9} \text{ cm.s}^{-1}$ [41-44] compared with permeabilities of 10^{-10} to $10^{-14} \text{ cm.s}^{-1}$ for other monovalent cations [45-47]. In the present work, it is possible to estimate the permeation coefficient of our liposome bilayers by using the results obtained with DPPC/cholesterol mixtures at pH 2. For the other formulations, the proton diffusion is too fast, or our technique is not sensitive enough in the range of pH. The results have been treated with the Fick's first law of diffusion:

$$J = \frac{dQ}{dt} \times \frac{1}{A} = \frac{K_s \times D}{h} \times \Delta C \quad \text{eq.1}$$

where J is the proton flux, dQ/dt is the amount of proton diffused per unit of time, A is the total liposome surface area, K_s the proton partition coefficient, D the diffusion coefficient, h the diffusional path length, and ΔC the proton concentration gradient.

Then the permeability coefficient, P , was determined by the following equation:

$$P = \frac{J}{C_0} \quad \text{eq. 2}$$

where C_0 is the proton concentration in the donor compartment, i.e. the aqueous external phase in our case.

The slope (dQ/dt) was calculated to $9.67 \times 10^{-7} \text{ mol.l}^{-1}.\text{min}^{-1}$, from the graph giving proton concentration in the liposomes versus time (figure not shown). The latter was obtained from the fluorescence intensity ratio vs. time and the ratio vs. pH graphs. After size exclusion chromatography, the liposome concentration was $\sim 2 \text{ mM}$; 2 ml of suspension used in the

spectrofluorimeter contained 2.4×10^{18} molecules of lipids. Considering that the vesicles are unilamellar and assuming each phospholipid to occupy an average interfacial area of 62 \AA^2 [48], the total bilayer surface area can be estimated at 7471 cm^2 (around $2460 \text{ cm}^2 \cdot \text{mg}^{-1}$ of lipid, which is similar to the value of $2200 \text{ cm}^2 \cdot \text{mg}^{-1}$ by Deamer and Nichols [49,50]). Then it was possible to calculate a flux of $1.3 \times 10^{-7} \text{ mol} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}$ and a permeation coefficient of $2.2 \times 10^{-5} \text{ cm} \cdot \text{s}^{-1}$ (external pH = 2).

It is interesting to mention that this permeation coefficient value is in accordance with accepted values of the literature. Indeed, as summarized by T. H. Haines [51], the permeability across the bilayers of vesicles became measurable in the early 1980s (see above) ; it is now well established and in the order of magnitude of $10^{-5} \text{ cm} \cdot \text{s}^{-1}$.

Although it is still unclear, several models have been proposed for proton leaks across lipid bilayers. According to the defect mechanism, protons could permeate bilayers via defects, or transient pores, as is widely assumed for monovalent metal cations. The comparison of the kinetics of leakage of metal cation and that of protons, in the defect model, imply that they behave through different mechanisms [52,53]. The weak acid mechanism postulates that there are molecular species present in or on one side of the bilayer which can be protonated, then translocate and diffuse to the other side and then release a proton [54-56]. The possible candidates for such a transport include lipid hydrolysis products or contaminants as free fatty acids. This hypothesis seems attractive to explain the faster pH decrease observed with the epikuron mixture compared with DPPC. Indeed, there are important traces of free fatty acid in Epikuron 170 which could act as proton carriers under their protonated form. Their translocation could be also favored by the lack of organization observed in the epikuron bilayer (see RX experiments) compared to DPPC, at the working temperature (25°C). However, the application of this theory is contradictory with several experimental features. First, the translocation would be easier in a more fluid environment, whereas the proton flux seems to be independent of it [46,57]. In this case, it is difficult to understand why a higher flux is observed with glycolipid I compared to the cholesterol (since an increase of membrane “fluidity” was observed after the incorporation of glycolipids I and II, compared with cholesterol, it was the most intuitive approach to explain the flux variations [21]). Second, the pK_a of free fatty acids embedded into phospholipid layer (mostly phosphatidylcholine) is higher than that in solution or than that of short chain acids [58-60]. It is possible that the free fatty acid remains uncharged in the whole range of pH used in this work, avoiding the flux of protons by this way. Other mechanisms, maybe complementary with the previous one could be more appropriate to understand our results. The water-wire hypothesis, modified as a proton-wire or a hydrogen bonded chain, lays down that protons are transported across the membrane via a hopping mechanism (Grotthus type conductance). It has been suggested that transient single-file chains of water can span the membrane, facilitating proton transport

^[54,61]. The positively charged proton would be partly stabilized in the fatty membrane by the hydrogen-bonded network of the water file. However, evidences against the water wire model have been put forward by Krishnawoorthy et al. who quantified the water in the bilayer using probes ^[62] and by Marrink et al ^[63] who have estimated the free energy necessary for the formation of such a water wire at 26 kcal/mol, which allows proton permeability only by assuming that they permeate almost instantaneously. Contrariwise to the membrane-spanning wire, the “water cluster” approach of Haines put forward that small clusters of water molecule, existing in both layers of the membrane, could be sufficient to stabilize a proton. Proton translocation could then take place when two clusters meet and a proton hops from one to the other ^[51]. More recently, Tepper and Voth described possible leakage with a concerted mechanism combining both water wire and cluster hypotheses ^[64]. Using a multistate empirical valence bond method to study directly water molecule structure surrounding a delocalised proton on its way through the membrane, they conclude that membrane-spanning networks rather than single-file chains, are formed around the proton. In all three previous models, dealing with water, the inclusions of molecules such as cholesterol and other sterols trends to dehydrating the lipid bilayer and act as a barrier within the hydrophobic core of the membrane. It is admitted ^[41,51,62], although not generally ^[50], that this barrier decreases the probability of cluster-contact or H-bonding (allowing the membrane-spanning network) across membranes, resulting in a decrease in proton flux. Tepper and Voth also described a strong contribution of hydrogen-bond acceptors in the lipid headgroup domain ^[64]. Furthermore, it is well known that the accessible surface area to the solvent decreases drastically from the amino group of the phospholipid head to the carboxylic ester function ^[65]. This may explain the differentiated permeabilities observed between cholesterol and glycolipid I containing bilayers. The sugar group and/or the poly(oxyethylene) chain of the glycolipid could act as a “reservoir” of hydrogen-bond acceptors, leading to proton accumulation at the outer layer of the membrane, thus increasing the amount of protons available for the diffusion through the bilayer.

Beyond the kinetics, it is interesting to mention that a pH gradient could be maintained during at least 2 hours by the use of phospholipid bilayers. The pH inside the vesicles did not reach that measurable outside. Two possible artefacts should be ruled out: 1) water movements due to salt gradients and 2) behaviour of the fluorescent probe in the presence of lipids. 1) Since hydrochloric acid was added in the medium to decrease the pH, the ionic strength inside and outside the vesicles changes during the experiments. As the ion content of the external aqueous phase was increased, water movements from the inner compartment may be possible ^[66], however this would result in a decrease of pH, not compatible with the “higher” pH measured in the liposomes. Furthermore, it has been described that even at high gradient of solute the liposome volume changes, due to osmotic mechanisms, remain small

(around 6%)^[67]. 2) To check the behaviour of the fluorescent probe in the presence of lipids, we compared the calibration curves with or without unloaded liposomes in the buffer. There is no significant difference between the two calibrations, suggesting that a probe-lipid interaction could not be the cause of the apparent pH gradient observed. The first hypothesis to explain such pH gradient could be the presence of an electrostatic barrier due to charge fluctuations at the liposomal surface. Because of pH-changes, ionisation of the liposome forming molecules should be taken into account. Indeed, several phospholipids groups are ionisable. In the Epikuron 170 mixture (phosphatidylcholine > 72%, phosphatidylethanolamine > 10%, phosphatidylinositol < 3%, lyso-phosphatidylcholine < 4% and free fatty acids 10%) could be mentioned: the phosphate groups of phospholipids, the amino function of phosphatidylcholine and phosphatidylethanolamine, or the carboxylic acid function of the free fatty acids. As reported previously, the free fatty acids and the amino groups should remain protonated in the pH range of this study. The apparent pK_a of the phosphate group is matter to some controversy since it was estimated by various authors at values ranging from <1 to <3, probably as a consequence of electrostatic interactions^[36,58,68]. It should be noted that the surface potential of our liposomes changes drastically below pH 2.2 as displayed by the zeta potential versus pH curve. Since a zeta potential increase was observed for successive additions of NaCl instead of HCl, it could not be due only to a change of ionization state. However, the zeta potential values being significantly different at this low pH (this is not true at pH < 2.2), a contribution of a protonation from PO^- to POH could not be excluded. Despite quite similar zeta potential variations, the addition of HCl or NaCl in the medium leads to different diffractograms, confirming the non equivalent state of the bilayers as a function of the cations. At the highest concentrations, the bilayer thickness increases from 71 Å to 79.7 Å with NaCl, whereas it decreases to 69.3 Å with HCl. In the case of HCl, the peaks at pH 1.5 became sharper and the second order was more visible, suggesting a more structured organization in the bilayers while the peak became broader in presence of sodium chloride. The swelling of the phospholipids in presence of monovalent salts such as NaCl has been already described. As measured in this work, an increase of the repeat spacing near 10 Å has already been reported in the literature for phospholipids with a phosphatidylcholine headgroup, dispersed in high salt concentrations^[69,70]. The enlargement and displacement of the diffractogram peaks in the presence of sodium chloride fit well with several papers describing a greater spread in the headgroup tilt distribution, and a water ordering effect due to monovalent salts^[71]. By comparison to the addition of NaCl, very low pH (around 1.5) display a significant zeta potential increase, a sharper diffraction peak and a lower repetitive distance, suggesting a better organization of the bilayer resulting probably from electrostatic considerations. At this pH, the protonation of the phosphate group could be expected which decreases the mutual repulsion of like

charged polar groups, thus allowing the organization of the bilayers. According to this hypothesis, the higher ordered structure promoted by HCl may be the cause for the difference of pH measured inside and outside the liposomes in acidic media. The protonation of the phosphate group may reduce the availability of hydrogen-bond acceptors in the lipid headgroup layer, thus decreasing the accessibility to solvent and modifying the proton permeation, as described above.

The inside pH obtained in the most acidic external conditions (pH 1.2 in simulated gastric fluid or pH 2 in Hepes buffer) was in the range 3.6 to 4.4, depending on the vesicle composition. These values are quite interesting in terms of pharmaceutical use of such liposomes. Amongst the most prescribed antibiotics, *Helicobacter pylori* is susceptible in vitro to amoxycillin, clarithromycin and metronidazole, in addition with many other antibacterial agents with MIC₉₀ of 0.06 mg/l, 0.03 mg/l and 6.0 mg/l, respectively at neutral pH [72,73]. Furthermore, the chemical reactivity of the β -lactam containing antibiotics has been extensively studied, due to the great importance of these compounds as antibacterial agents. Compounds such as amoxycillin, penicillin G or ampicillin are susceptible to hydrolytic degradation when the pH deviates significantly from the isoelectric point. Thus the half-life of amoxycillin is 5.2 h at pH 1 but increases to 19.0 h and 176.9 h at pH 2 and pH 4 respectively [74]. At 37°C, the decomposition rate constant of penicillin G is one hundred times faster at pH 1.8 than at pH 4 [75]. From these results, it is obvious that the encapsulation of β -lactam containing antibacterial agents, in liposomes able to maintain an internal pH around 4, is very promising in order to increase their chemical stability. On the other hand, clarithromycin can be inactivated by hydrolytic removal of the sugar moiety, in acidic conditions yielding the decladinose acid degradation product [76]. The clarithromycin degradation half-life has been calculated around 1.3 h at pH 2 but it increases to 15.8 and 96.7 h at pH 3 and 4 respectively [74,77]. From these results, it is possible to calculate that 99.3% of the drug remains intact after a one-hour incubation at pH 4, confirming also the interest of clarithromycin encapsulation in such liposomes.

In the aim to developing a targeting system against *Helicobacter pylori*, it was necessary to control the chemical stability of the targeting ligand. The agglutination results confirm the stability of the glycolipid I after incubation in a simulated gastric medium. Indeed, it has been reported previously that non specific interactions between glycolipid I and wheat germ agglutinin (WGA) remain negligible (optical density increase at 450 nm lower than 0.005) [21]. Therefore, agglutinations reported in figure 7 are certainly due to interactions between the *N*-acetylglucosamine moiety and its specific agglutinin, and they remain similar with or without incubation in gastric fluid.

As a conclusion, the work reported in this paper demonstrated that it is possible to obtain stable vesicles in which the pH of the internal aqueous compartment remains close to 4, even though more acidic conditions are imposed to the external phase (pH 1.2 – 2). Such a pH gradient depends essentially on the type of phospholipid used (pure DPPC or epikuron mixture) and it is not affected to a large extent by the incorporation of a targeting agent such as glycolipid I in the vesicle bilayer. These aspects are especially important to the development of liposome formulations against *Helicobacter pylori*, a bacteria sensitive to antibiotics which are unstable in gastric conditions. In the second part of this study, will be depicted the characterization of liposomes loaded with antimicrobial agents and quantification of the interaction between such formulations and the bacteria.

Acknowledgment

The authors would like to thanks A. Touhiri for its contribution to this work.

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Les expériences de diffraction des rayons X, aux petits et grands angles, ont mis en évidence la structure lamellaire de la DPPC et de l'epikuron lorsqu'ils sont complètement hydratés. La structure supramoléculaire des phospholipides en milieu acide demeure inchangée, ce qui nous permet d'envisager un ciblage gastrique. Cependant les liposomes sont reconnus comme étant perméables aux protons, ce qui peut poser problème si le principe actif encapsulé est sensible aux pH acides. Les résultats obtenus afin d'investiguer la perméabilité aux protons de quatre formulations liposomales sont fort intéressants, puisqu'un gradient de pH entre l'extérieur et l'intérieur du liposome semble pouvoir être possible, garantissant pendant au moins deux heures un pH intra-vésiculaire compatible avec l'encapsulation de principes actifs sensibles aux $\text{pH} < 4$. De plus, des calculs de perméabilité de la membrane DPPC/cholestérol à pH 2 se recoupent avec les valeurs retrouvées dans la littérature. La perméabilité aux protons apparaît comme clairement liée à la composition des liposomes, les altérations de la membrane induites par l'incorporation des glycolipides allant de pair avec une perméabilité aux protons plus élevée. Autre point intéressant, la présence de pepsine ne modifie pas la perméabilité de la bicouche phospholipidique aux protons.

Les limites de la perméabilité aux protons, et donc la présence d'un gradient à « l'équilibre », pourraient s'expliquer par des phénomènes électrostatiques à pH acide, entraînant une organisation plus stable de la bicouche phospholipidique, comme le suggère les résultats de diffraction aux rayons X, ainsi qu'une protonation des groupes phosphates, créant naturellement une barrière à la diffusion des protons.

Enfin, l'agglutination des liposomes avec des lectines végétales après incubation en milieu gastrique simulé, nous permet de conclure sur la résistance suffisante des néoglycolipides greffés en surfaces.

Au vu de ces résultats, toutes les conditions semblent réunies pour permettre à ces liposomes d'avoir une activité thérapeutique au niveau gastrique. Ainsi, dans la partie suivante de ce travail, seront notamment étudiées la capacité des vésicules précédemment décrites à encapsuler des molécules actives contre la bactérie puis les interactions entre cette dernière et différentes formulations.

Une annexe conclura ce travail afin de d'approfondir les interactions entre la phosphatidyléthanolamine et *H. pylori*, qui n'ont pu être mises en évidence avec des liposomes à base d'epikuron.

CHAPITRE V :

Pre-formulation of liposomes against

***Helicobacter pylori*: part II –**

characterisation and interaction with the

bacteria.

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Contents

Introduction	138
1. Material and method	139
1.1. Materials	139
1.2. Method.....	139
1.2.1. Vesicle preparation	139
1.2.2. Size measurements	140
1.2.3. Zeta potential measurements.....	140
1.2.4. Phospholipids assessment.....	140
1.2.5. Encapsulation efficiency assessment.....	140
1.2.6. HPLC assessments.....	141
1.2.7. X-ray diffraction.....	141
1.2.8. Polymerase chain reaction (PCR)	142
1.2.9. Epifluorescence microscopy.....	142
2. Results.....	143
2.1. Size and zeta potential.....	143
2.2. Phospholipids assessment.....	144
2.3. Encapsulation efficiency	144
2.4. Antimicrobial agents – phospholipid interaction.....	144
2.5. <i>H.pylori</i> strains characterization	146
2.6. Epifluorescence microscopy	147
3. Discussion	153
3.1 Size and zeta potential:.....	153
3.2 Encapsulation efficiency:.....	153
3.3 Liposome-bacteria interactions:.....	155
References.....	157

Figures

Fig.1: X-ray diffraction coupled with the differential scanning calorimetry of DPPC hydrated with water (A), with a 5% metronidazole containing aqueous solution (B) and with a 5% ampicillin sodium salt containing aqueous solution (C)	145
Fig.2: PCR of <i>babA2</i> and <i>vacA</i> genes on strains 17875 and 149C.	146
Fig.3: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with a fresh culture of <i>H.pylori</i> 17875	148
Fig.4: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with fresh strain 149C of <i>H.pylori</i>	149
Fig.5: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with old strain 17875 of <i>H.pylori</i>	150
Fig.7: Epifluorescence microscopy pictures of 2 liposomal formulations mixed with fresh strain CFT073 of <i>E. coli</i>	152
Fig.8: Epifluorescence microscopy pictures of 2 liposomal formulations mixed with fresh strain ATCC 12228 of <i>Staph. epidermidis</i>	152
Fig.9: Helicoid or coccoid shape of <i>H. pylori</i> in function of ageing	156

Tables

Table I: Composition and zeta potential of liposomale formulations.....	143
Table II: Encapsulation efficiency	144
Table III: Summary of the XRD-DSC results.....	146

Introduction

Because of their composition, liposomes are very similar to cellular membranes and are widely used in cosmetology and other topical forms. But their relative instability in biological fluids is their main disadvantage and limits the oral and parenteral administration. However, further commercial forms are available on the market as for example doxorubicin-encapsulated liposome (DOXIL[®]) or the amphotericin B-loaded liposome (AMBISOME[®]). DOXIL[®] is used against ovarian cancer and the pegylation around the liposome allows the system to evade detection and destruction by the immune system, which increases the time the drug is in the body. AMBISOME[®] is used against fungal infections, visceral leishmaniasis or for patients with renal impairment where classical amphotericin B deoxycholate cannot be used. For both drugs (DOXIL[®] and AMBISOME[®]) the administration is intravenous. In the particular case of *Helicobacter pylori*, which colonizes the stomach, the stability of liposomal formulations in the gastric environment is crucial. In the first part of this work, we focused on the behavior of liposomes in gastric conditions and the lipid organization at different pH and we found that our formulations were stable at gastric pH and that the internal pH was controlled, limiting the degradation of the drug. Therefore an oral administration of antibiotics-encapsulated liposomes is conceivable. Furthermore, because of their amphiphilic structure, liposomes can encapsulate a wide range of antibiotics, as for example fluoroquinolone (enrofloxacin ^[1,2], enoxacin ^[3]), aminoglycosides (amikacin, gentamicin, and tobramycin ^[4]), macrolides (erythromycin ^[5], azithromycin ^[6]), cephalosporins (cefotaxime ^[7], cefoxitine ^[8]), tetracycline (tetracycline and doxycycline ^[9]) or imidazole (clotrimazole and metronidazole ^[10], ketoconazole, miconazole and econazole ^[11]). To eradicate *H. pylori* further approaches are conceivable. Indeed the pathogenicity of *H. pylori* depends of several factors, i.e. the resistance to acidity by the production of a urease ^[12], the motility due to the flagella ^[13,14], the adherence to the epithelium gastric thanks to multiple adhesins (at least eighteens) ^[12,15-18], the capacity to evade the immune system ^[19-23] and the vacuolating effect of the VacA protein ^[24,25]. It should be possible to act against the bacterial adherence, avoiding in this way the chronic infection by *H. pylori*. To be efficient it is necessary to treat before the first symptoms and be able to inactivate as many as possible adhesins, which is very difficult to do. Alternatively, it should be possible to generate antibiotic-containing liposomes and to target them to *H. pylori* through its membrane adhesins. This is the way we have chosen by targeting BabA2, an outer *H. pylori* membrane protein, which is able to link the fucosylated Lewis b (Le^b) histo-blood group antigen present on human gastric epithelial cells ^[12,13,15,26]. In this second part of our work we generated four liposomal formulations, two without neoglycolipid (dppc-cholesterol and epikuron-cholesterol), and two with the neoglycolipid (cholesteryl tetraethylene glycol fucose) in order to target *H. pylori*. We characterized the

physico-chemical properties of liposomes (size, zeta potential, encapsulation efficiency), and observed by epifluorescence microscopy the interactions between fluorescent liposomes and fluorescent *H. pylori* strains.

1. Material and method

1.1. Materials

The 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, ref. P0763), cholesterol (ref. C8667), dialysis tubing cellulose membrane (ref. D9777), sodium phosphate monobasic (ref. S-5011) and ampicillin sodium salt (ref. A9518) were purchased from Sigma-Aldrich. The neoglycolipids were previously synthesized in the laboratory, as already reported for the cholesteryl tetraethyleneglycol N-acetylglucosamine (GlcNAc-E₄-Chol) [27]. The synthesis of cholesteryl tetraethyleneglycol fucose (Fuc-E₄-Chol) was realized by methodologies already reported to prepare α -L-fucopyranosides of Guerbet alcohols [28] and will be published in due course. Fluorescent probe 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC, ref. N3787) was provided by Molecular Probes™, Invitrogen. Brain Heart Infusion agar CM375, vitox SR090J (hydration fluid) and vitox SR090K (vitox supplement) were purchased from Oxoid. The metronidazole (ref. 68035), ammonium thiocyanate (ref. 09950) and ferric chloride hexahydrate (ref. 44944) were purchased from Fluka. The nitrogen 2-1°, 4.5; the fluorescent dye 4',6-Diamidino-2-phenylindole (DAPI, ref. 124653), Epikuron 170, and the DNeasy tissue kit (ref. 69504) were provided respectively by Linde, Merck KGaA, Degussa, and Qiagen. All solvents and reagents were analytical grade.

1.2. Method

1.2.1. Vesicle preparation

Liposomes are prepared by Bangham method [29]. Briefly, lipids and phospholipids (in a total lipidic concentration of 30 mM) are dissolved in chloroform, and the solvent is removed under vacuum (rotavapor Büchi EL). The lipidic film formed on the glass wall is hydrated with ultra pure water. To achieve vesicles formation, the flask is vortexed and sonicated several times during ~ 25 min. The liposomes size is controlled by extrusion (Lipex Biomembranes Inc.) under nitrogen above the phospholipid transition temperature (T_m) (3 times through 0.4 μ m HTTP filter (Millipore, isopore™) and 8 times through 0.2 μ m GTTP filter (Millipore, isopore™)).

1.2.2. Size measurements

Size measurements were made by quasi-elastic light scattering determinations with a Malvern Zetamaster® 3000 HS instrument (Orsay, France). The samples were diluted in deionised water before measurements.

1.2.3. Zeta potential measurements

Zeta potentials of the liposomes were measured using a Malvern Zetamaster® 3000 HS instrument (Orsay, France). The measurements were made in NaCl 10mM with antibiotic free liposomes.

1.2.4. Phospholipids assessment

The amount of phospholipids was assessed after extrusion by a colorimetric method based on the formation of a complex between phospholipids and ammonium ferrothiocyanate ^[30]. A known volume of liposomes was evaporated and the phospholipids residue was dissolved in 2 mL of chloroform. 1 mL of thiocyanate reagent was added. After 1min. of vortex, the mixture was centrifuged 10 min. at low speed and the chloroformic red lower layer was removed with a Pasteur pipette. The absorbance was read at 488 nm and compared with a standard phosphatidylcholine solution (range: 10 to 100 µg/mL).

1.2.5. Encapsulation efficiency assessment

Six liposomal formulations were done. The amount of phospholipids (dppc or epikuron 170) was 80% (mol/mol) and the rest 20% was either cholesterol, or 10% of neoglycolipid and 10% of cholesterol. During Bangham method, the rehydration of lipidic film was done with 10mM of metronidazole or ampicillin sodium salt dissolved in pure water. The free fraction was removed by dialysis at room temperature. 1mL of liposomes was placed into dialysis tubing cellulose membrane hermetically sealed in 200 mL of stirred ultra pure water, changed every hour, until a steady state was reached. Each dialysis curve was fitted to the pseudo-first order model $y = C_{\max} * (1 - \exp(-k * t))$, where C_{\max} is the maximum concentration, k is a constant and t is the time and solved with Prism software (GraphPad software, Inc.). The correlation coefficient r^2 was included between 0.996 and 0.999. For liposomes without neoglycolipids, 2 to 4 repetitions have been made, and to avoid a product consumption, only one formulation of neoglycolipid containing liposomes were investigated in this encapsulation efficiency study. The amount of antibiotic in the dialysate was assessed by HPLC.

1.2.6. HPLC assessments

For the metronidazole assessment, we used a Gemini column (Phenomenex®) 5 µm, C18, 250*4,6 mm, with a water / acetonitrile (80:20, v/v) mobile phase, at the flux of 1mL/mn. The column was thermostated at 40 °C and the wavelength detection was set at 318 nm. The ampicillin sodium salt was assessed with a supelcosil LC18 column (Supelco®), 5 µm, 250*4,6 mm. The mobile phase used was NaH₂PO₄ / acetonitrile (85:15, v/v) with a 1 mL/mn flux. The column was thermostated at 30 °C and we set the wavelength detection at 210 nm. The HPLC analysis was performed on a Waters system (Waters 600 controller, Waters 486 tunable absorbance detector, waters 717plus autosampler) and data acquisition was done with the Millenium 32 software.

1.2.7. X-ray diffraction

X-ray diffraction (XRD) has been performed by using the Austrian small-angle X-ray scattering beam line of the ELETTRA synchrotron (Trieste, Italy). Simultaneously, the DSC signal has been measured with a home-made microcalorimeter, Microcalix.

Two gas-filled linear detectors (1024 channels, filled with argon-ethane mixture) are used to collect the data. XRD patterns have been recorded by transmission using glass capillaries (0.01 mm wall thickness, 1.5 mm diameter, GLASS W. Müller, Berlin, Germany). Samples are prepared by filling capillaries with about 20 µl of sample using a special syringe. Because of the sample-to-detector distances, vector q-values ranging from 0.05 Å⁻¹ to 0.35 Å⁻¹ (Small-angles X scattering – SAXS) and from 0.60 Å⁻¹ to 1.60 Å⁻¹ (Wide-angles X scattering – WAXS) were accessible. The scattering vector is defined as $q = 4.\pi. \sin(\theta) / \lambda$ where 2θ is the scattering angle. From this scattering vector, it is possible to calculate the repetitive distances by the use of the following equation $q=2\pi/d$. The calibration of the detectors was carried out by using the well defined positions of the peaks of 2Lβ form of pure tristearin (4.59, 3.85, 3.70 ± 0.01 Å and 44.97 ± 0.05 Å) and of silver behenate (58.38 ± 0.01 Å). In order to determine the peak positions, diffractograms were calibrated with Gaussian model by the use of the IGOR pro software (WaveMetrics, Inc.).

DSC was performed using a microcalorimeter especially designed for beam-line installation. More detailed procedure and the description of calorimeter cell used are described in Keller et al, 1998 ^[31].

Both XRD and DSC data have been collected and synchronized with a National Instrument LabVIEW supported data acquisition system (H. Amenitsch, HCI, Hecus M. Braun-Graz GmbH).

1.2.8. Polymerase chain reaction (PCR)

The bacterial DNA was extracted according to the recommendations provided in the Qiagen Dneasy tissue kit (Ref 69504). For the BabA2 and VacA amplification, the following primers were used [26,32-34].

babA2 F : 5'-AAT-CCA-AAA-AGG-AGA-AAA-AGT-ATG-AAA-3'.

babA2 R : 5'-TGT-TAG-TGA-TTT-CGG-TGT-AGG-ACA-3'.

vacA F : 5'-GGT-CAA-AAT-GCG-GTC-ATG-G-3'.

vacA R : 5'-CCA-TTG-GTA-CCT-GTA-GAA-AC-3'.

Amplification was initiated by heating the mixture at 95 °C for 5 min followed by 35 cycles with the following thermal profile: 92 °C for 60 s, 52 °C for 60 s, 72 °C for 60 s.. The electrophoresis on agarose 1.5 % (m/v) was carried out in TBE (Tris 10.8% m/v, boric ac. 5.5% m/v, EDTA 0,5M-pH8 4% v/v, water q.s 1L) for 45 min. at 110 V, 58 mA). DNA was stained with ethidium bromide and visualized by UV light.

1.2.9. Epifluorescence microscopy

During liposome preparation, 1% (mol/mol) of NBD-PC was added instead of phospholipid. The total amount of lipids (30 mM) was dissolved in chloroform and liposomes were prepared by the Bangham method exactly as described above. Four formulations were done: dppc (formulation F1) or epikuron 170 (formulation F3) – cholesterol – NBD-PC (79:20:01) and dppc (formulation F2) or epikuron 170 (formulation F4) – Fuc-E₄-Chol – cholesterol – NBD-PC (79:10:10:01). Because we wanted here to observe the interaction between the liposomes and the bacteria, no antibiotic was entrapped.

The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35]. After a 3-days growth on agar media (Brain Heart Infusion Agar Oxoid, laked horse blood, supplements Vitox SR90K and SR69, Oxoid) in a microaerophilic atmosphere (O₂ 5%, CO₂ 10%, N₂ 85% mol), the bacteria were harvested and suspended in sterile phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄, pH 7.2) to reach a titre of approximately 10⁶ CFU/mL. 10 µL of DAPI (0,001% m/m) were added in 1mL of bacterial suspension, stirred, and stocked in dark at room temperature during 7 min. The mixture was centrifuged 5 min. at 3000 g and the supernatant was removed and replaced by fresh PBS, 2 times. 100 µL of liposomes at 10⁻⁴ mM were added to 100 µL of bacterial suspension, stirred and placed 1h in dark at room temperature. 10 µL of mixture were placed between a microscope glass slide and a lamella, and observed by epifluorescence microscopy at 1000× magnification, using one optic filter for DAPI (emission wavelength at 456 nm) and another one for NBD-PC (emission wavelength at 534 nm). Two strains of *H.*

pylori were used, CCUG17875 (here denoted 17875) and 149C. In order to compare the specificity of interaction with *H. pylori*, similar experiments were made with formulations F1 and F2, and 2 strains of *Escherichia coli* (CFT073 and K12) and 3 strains of *Staphylococcus* (*S. epidermidis* ATCC 12228, *S. aureus* ATCC 25923 and ATCC 29213).

2. Results

2.1. Size and zeta potential

The mean size of antibiotic-encapsulated liposomes lies between 147 to 163 nm with a polydispersity index of 0.10 to 0.11. The mean size of fluorescent liposomes (formulated with NBD-PC) lies between 136 to 172 nm with a polydispersity index of 0.07 to 0.12. It is interesting to note here that the size of the liposomes did not depend on the incorporation of drug in the vesicles or fluorescent probes in the bilayer. Furthermore, the sizes we measured in the present work are similar to that of empty glycosylated liposomes described previously [36]. The 4 fluorescent formulations were negatively charged, as summarized in Table I. Epikuron-based formulations are more negatively charged than dppc-based formulations. Indeed, zeta potentials of the DPPC formulations are comprised between 2.9 and 4.3 mV, while the epikuron formulations lead to zeta potential values from 12.2 to 20 mV. However, as also described in a previous paper, it seems not possible to affect a specific role of the glycolipid incorporation on the liposome surface charges.

Table I: Composition and zeta potential of liposomale formulations

Formulations *	Name	Zeta Potential
Dppc / cholesterol	F1	-4.3mV
Dppc / Fuc-E ₄ -Chol / cholesterol	F2	-2.9mV
Epikuron 170 / cholesterol	F3	-12.2mV
Epikuron 170 / Fuc-E ₄ -Chol / cholesterol	F4	-20.0mV

* Each formulation contains 1% of fluorescent phosphatidylcholine (NBD-PC). Proportions are, for F1: Dppc / cholesterol / NBD-PC (79:20:01); F2: Dppc / Fuc-E₄-Chol / cholesterol / NBD-PC (79:10:10:01); F3: Epikuron 170 / cholesterol / NBD-PC (79:20:01); F4: Epikuron 170 / Fuc-E₄-Chol / cholesterol / NBD-PC (79:10:10:01).

2.2. Phospholipids assessment

With the colorimetric method we obtained a correlation coefficient r^2 of 0.991 and 0.996 for the epikron 170 and dppc, respectively. For each formulation, the total phospholipid concentration was ~29 mM after extrusion. We have not quantified the glycolipid in the formulation, however previous quantification (V. Faivre, unpublished data) have shown that the phospholipid-cholesteryl tetraethyleneglycol glycoside ratio remains constant, and equal to the theoretical one, during the whole liposome preparation process. We have then considered that the lost of glycolipid was proportional to that of phospholipid in this work.

2.3. Encapsulation efficiency

Encapsulation efficiency was calculated using the following formula:

$$EE(\%) = (C_{orig} - C_{max}) / C_{orig} \times 100$$

where C_{max} is the maximum concentration reached at the end of the dialysis and C_{orig} is the antibiotic concentration in the original loading solution in which the liposomes were formed.

The encapsulation efficiency results summarized in Table II depend on the antibacterial agent and/or on the phospholipid type. The encapsulation of metronidazole drastically decreased in epikuron 170 liposomes compared to the DPPC ones (~1.5 % vs. ~14 %). On the opposite, the ampicillin encapsulation seems not strongly affected by the change of phospholipidic composition.

Table II: Encapsulation efficiency

Formulations	Metronidazole		Ampicillin	
	EE min.	EE max.	EE min.	EE max.
Dppc / cholesterol (80:20)	11.2%	13.9%	7.2%	12.9%
Epikuron 170 / cholesterol (80:20)	0.6%	2.1%	13.2%	14.6%
Dppc / Fuc-E ₄ -Chol / cholesterol (80:10:10)	13.0%		4.8%	

2.4. Antimicrobial agents – phospholipid interaction

The diffraction patterns and DSC signal between 20 °C and 55 °C are represented on the Figures 1a, 1b and 1c for DPPC films rehydrated with water, 5% ampicillin solution and 5% metronidazole solution respectively. The most important data extracted from these results are expressed in table III.

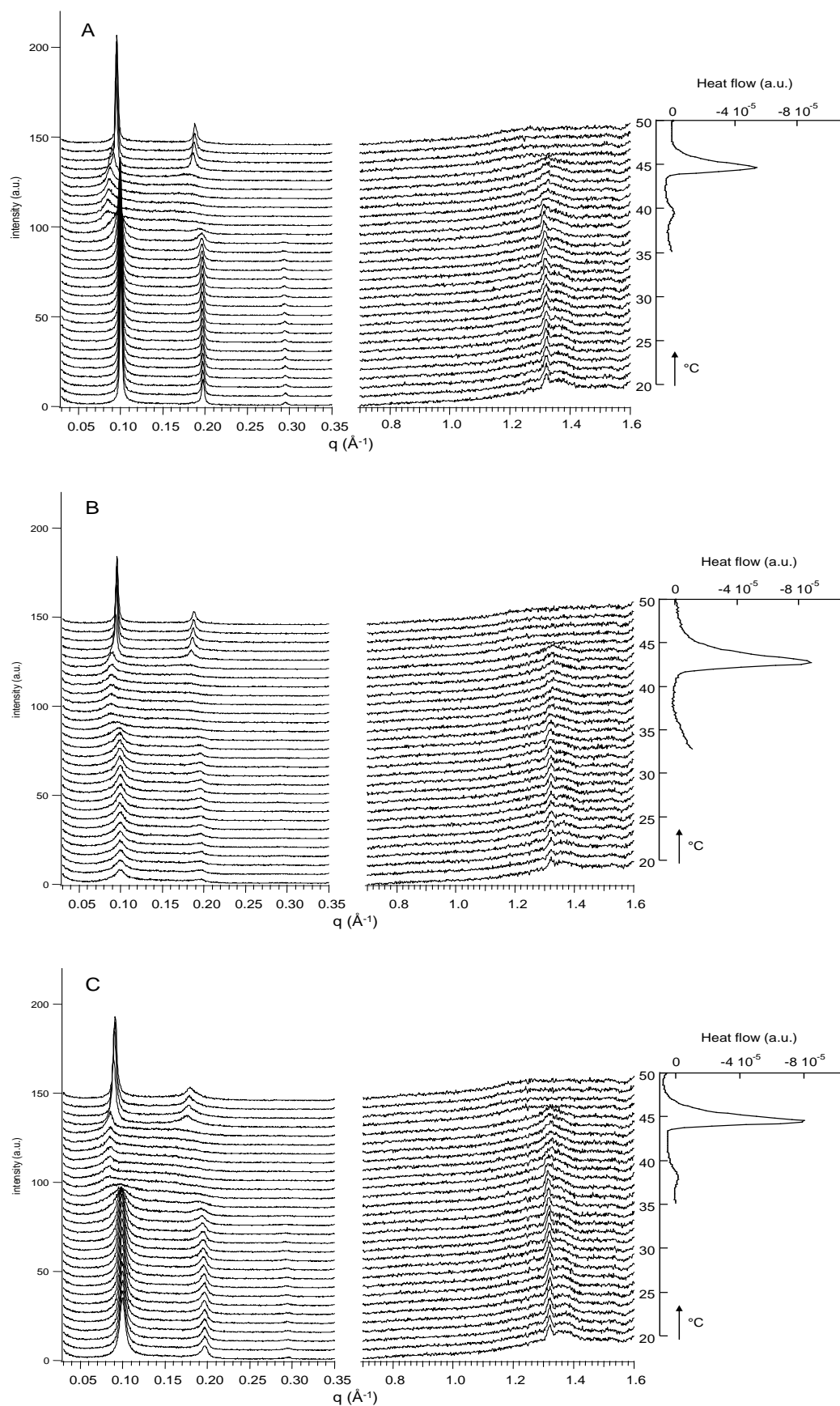


Fig.1: X-ray diffraction coupled with the differential scanning calorimetry of DPPC hydrated with water (A), with a 5% metronidazole containing aqueous solution (B) and with a 5% ampicillin sodium salt containing aqueous solution (C).

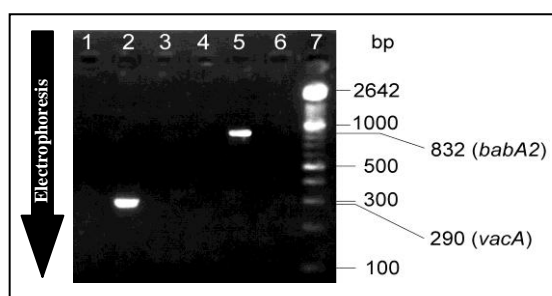
Table III: Summary of the XRD-DSC results

	d_{SAXS} (Å)			d_{WAXS} (Å)			transition temperature (°C)
	$L_{\beta'}$	$P_{\beta'}$	L_{α}	$L_{\beta'}$	$P_{\beta'}$	L_{α}	
DPPC	62.5	73.1	65.6	4.76-4.59	4.74	-	43.8
+ metronidazole	63.0	70.6	65.6	4.76-4.55	4.73	-	42.0
+ ampicillin	62.7	74.2	68.9	4.76-4.53	4.74	-	43.6

Concerning the DSC experiments, a slight shift of the DPPC transition temperature could be observed by adding the antibacterial agents in the aqueous medium. The shift is more significant with metronidazole (42.0 °C) than with ampicillin (43.6 °C). The classical L_{α} to $P_{\beta'}$ to $L_{\beta'}$ transitions are observable for DPPC with increasing temperature (see details in the part I of this work). In comparison to the pure DPPC/water mixture, the addition of metronidazole leads to a decrease of the $P_{\beta'}$ interbilayer distance (70.6 vs 73.1 Å), whereas the addition of ampicillin leads to a clear increase of the interbilayer distance in the L_{α} domain (68.9 vs 65.6 Å). The short distances (WAXS) seems not affected by the presence of the antimicrobial agent. However, the most important information given by the DRX pattern is the loss of organization of the DPPC/metronidazole/water system as expressed by the lower diffraction peak intensities measurable with such a mixture. The presence of metronidazole in the aqueous phase results in the disorganization of the DPPC layers, suggesting interactions between the phospholipids and this antibacterial agent.

2.5. *H. pylori* strains characterization

To identify the presence of *babA2* and *vacA* genes in *H. pylori* strains we used the PCR technique followed by gel electrophoresis of the amplicons. The size of the fragments obtained was compared to a molecular weight ladder (DNA molecular weight marker XIV, 100 base pair ladder, Roche Diagnostics). As shown on figure 2, the CCUG 17875 strain contains both the fragments harbouring *babA2* (832 bp) and *vacA* (290 bp) whereas the 149C strain contains neither *babA2* nor *vacA* genes.



vacA (290 bp) for strains 17875 and 149C are in lanes n°2 and 3, respectively. *babA2* (832 bp) for strains 17875 and 149C are in lanes n°5 and 6, respectively. Lanes n°1 and 4 are negative controls. Lane n°7 is a 100 base pair marker.

Fig.2: PCR of *babA2* and *vacA* genes on strains 17875 and 149C.

2.6. Epifluorescence microscopy

As mentioned above, we used 4 liposomal formulations: dppc – cholesterol – NBD-PC (F1); dppc – Fuc-E₄-Chol – cholesterol – NBD-PC (F2); epikuron – cholesterol – NBD-PC (F3) and epikuron – Fuc-E₄-Chol – cholesterol – NBD-PC (F4). First, we compared the 4 formulations with fresh cultures (3 days old) of *H. pylori* (17875 and 149C). We used PBS instead of liposomes as negative control. Results obtained with strain 17875 and strain 149C are shown in Figures 3 and 4, respectively. Apparently, *H. pylori* aggregates more with the formulation F2 (Fig. 3-A2). However the process of aggregation is quite difficult to evaluate, because it depends also of the type of strain used. For example, strain 149C “self aggregates” (negative control of Fig. 4). The intensity of the green fluorescence is higher for the F2 than for the F1 formulation (Fig. 3-B2 vs. B1) with strain 17875. With formulations F3 and F4, the intensity of the green fluorescence is very poor (Fig. 3-B3 and B4). For strain 149C, the intensity of the 4 green fluorescences is quite equivalent (Fig. 4-B1, B2, B3, and B4). For all the formulations, the superimposition of the 2 fluorescent dyes is very good (Fig. 3 and 4, columns C), suggesting that liposomes are “stuck” around bacteria. Because *H. pylori* can show 2 different forms, its “normal” helicoid shape and a coccoid shape, we performed the same experiments with older cultures, where most of the bacteria were under their coccoid form. Results obtained with strain 17875 (6 days old) and strain 149C (11 days old) are shown in Figure 5 and 6, respectively. For strain 17875, there is again a marked difference between the F2 and F3 formulations, with a higher intensity for the green fluorescence of the F2 formulation (Fig. 5, B2 vs. B1). The formulations F3 and F4 give again a very low green fluorescence (Fig. 5, B3 and B4). With 149C strain, the green fluorescence of the F1 and F2 formulations is equivalent (Fig. 6, B1 and B2), and similarly to strain 17875, the intensity of the green fluorescence with the F3 and F4 formulations is very low (Fig. 6, B3 and B4). With other bacterial species (*E. coli* or *Staphylococcus*) no green fluorescence was observed, whatever the formulation (F1 or F2) or the strain used. As examples, data are shown for *E. coli* CFT073 and *S. epidermidis* ATCC 12228 (Figures 7 and 8 respectively).

In conclusion, with strain 17875, the best results in term of interaction *H. pylori*-liposomes are observed with the formulation F2, regardless of the age of the strain. The formulation F1 gives quite good results too, but the intensity of the green fluorescence is always lower than F2 formulation. F3 and F4 formulations give in each case poor results. With strain 149C, the F1 and F2 formulation lead to equivalent results, regardless of the age of the bacteria, whereas the interaction *H. pylori*-liposomes seems to be better with fresh bacteria for the F3 and F4 formulations than with older bacteria.

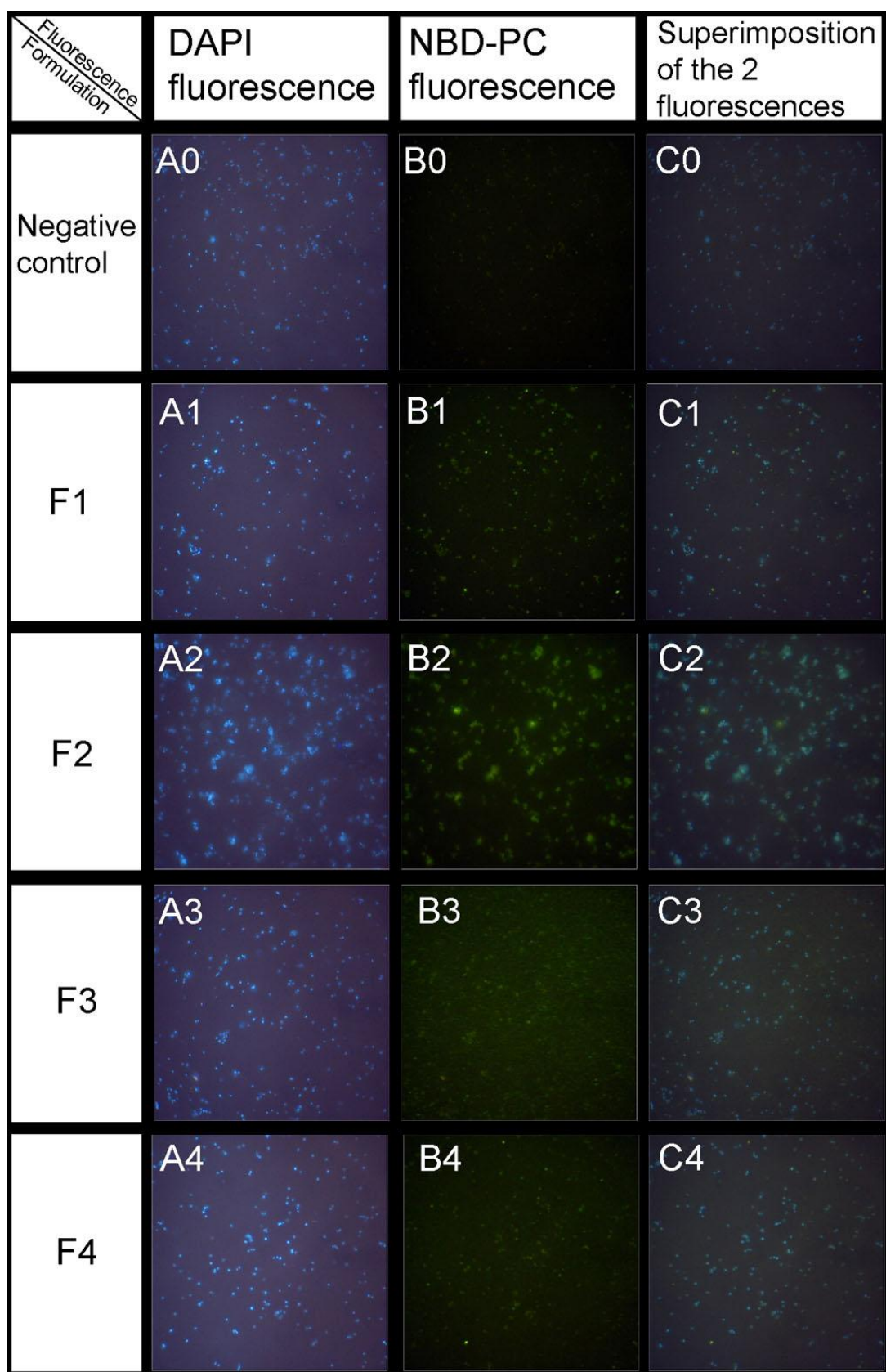


Fig.3: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with a fresh culture of *H.pylori* 17875.

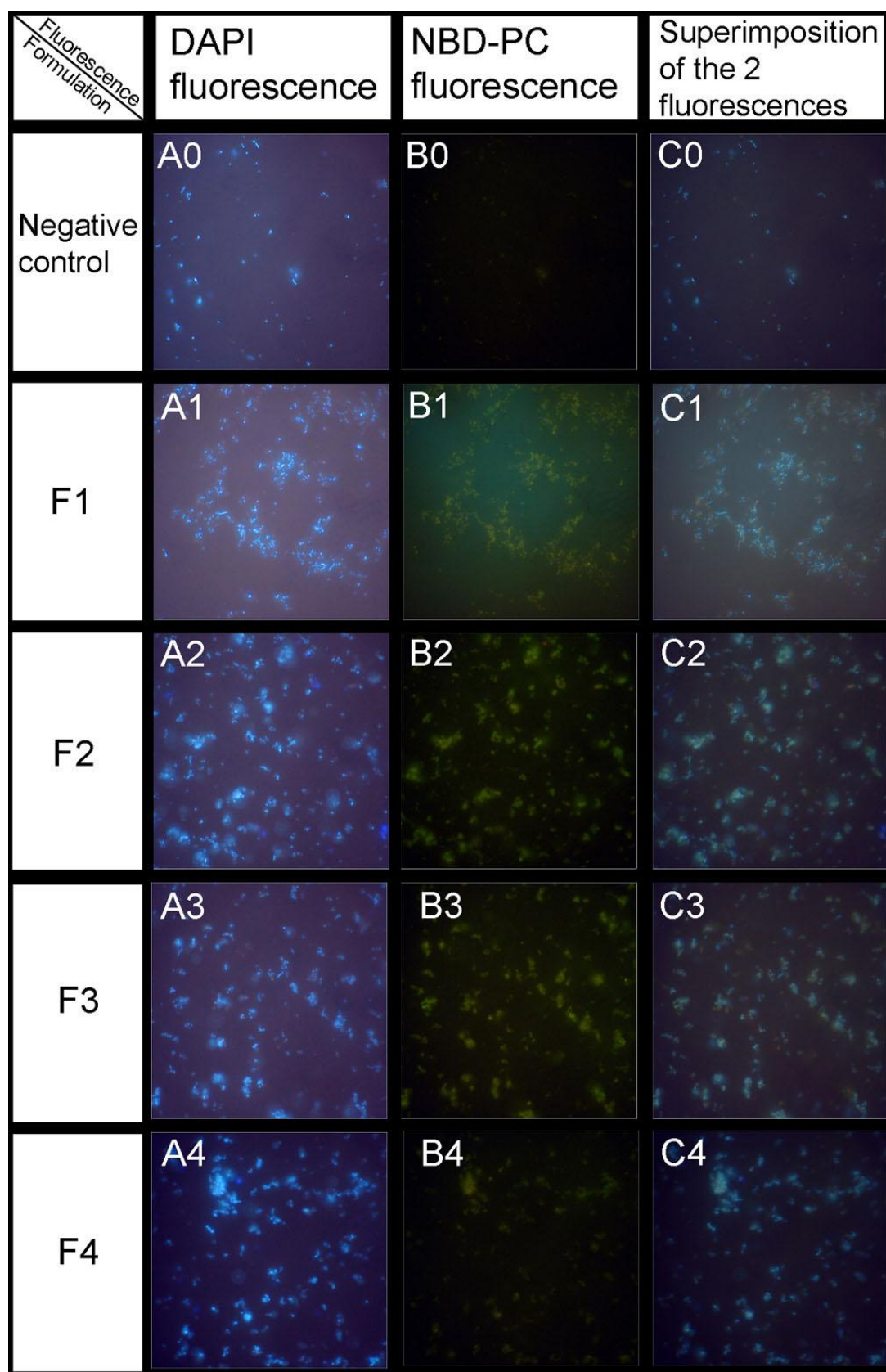


Fig.4: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with fresh strain 149C of *H.pylori*.

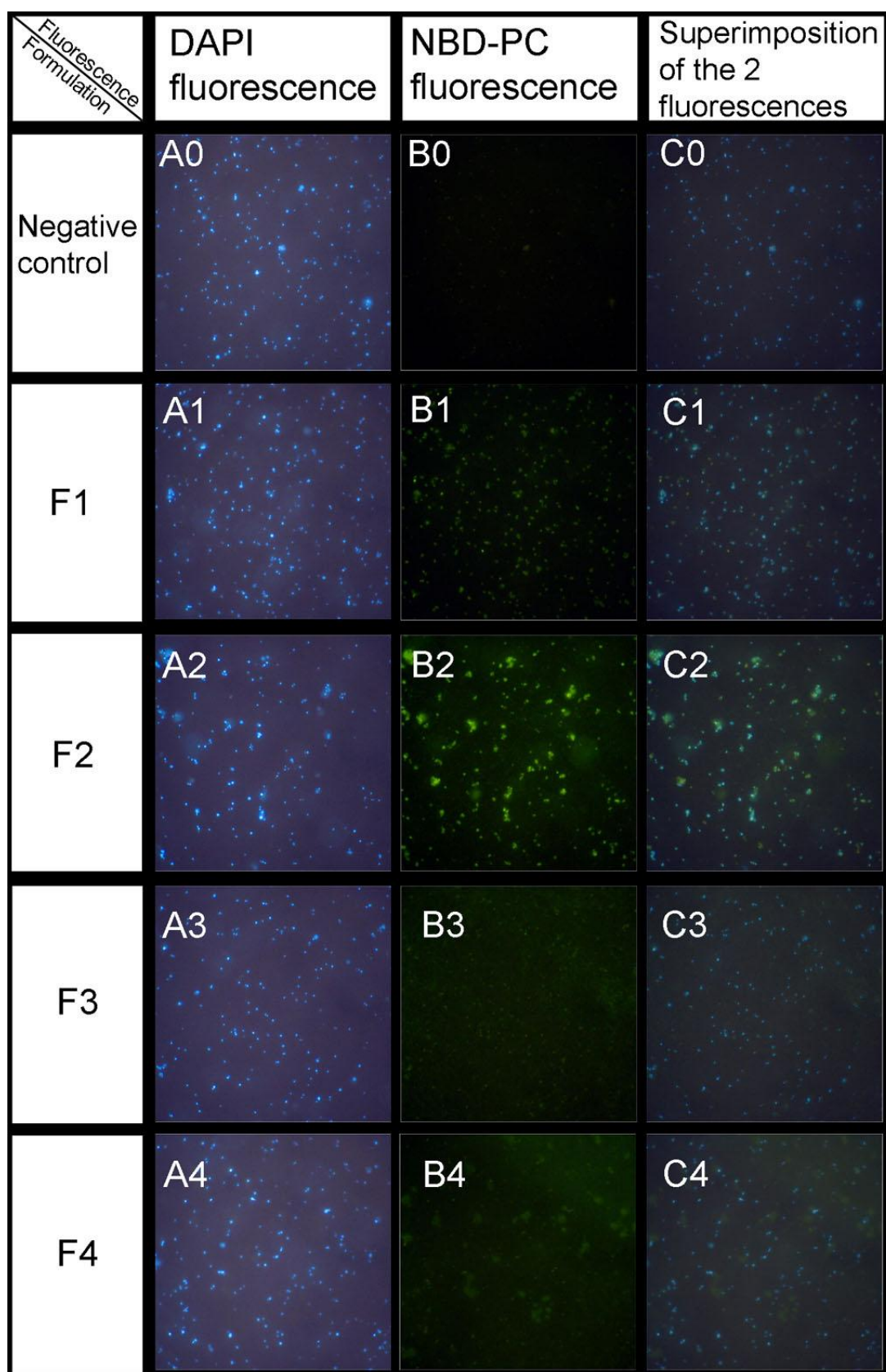


Fig.5: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with old strain 17875 of *H.pylori*.

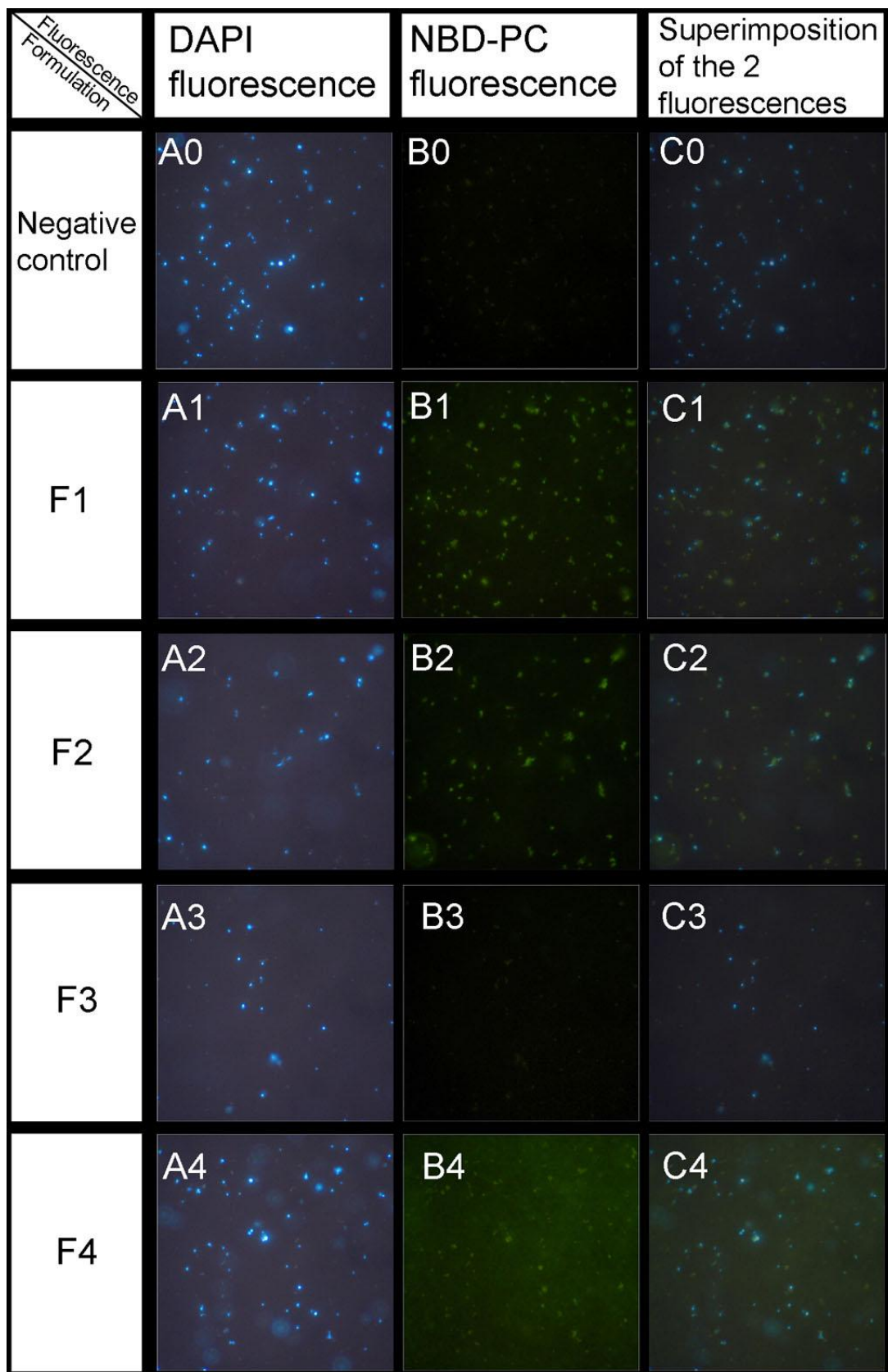


Fig.6: Epifluorescence microscopy pictures of 4 liposomal formulations mixed with old strain 149C of *H.pylori*.

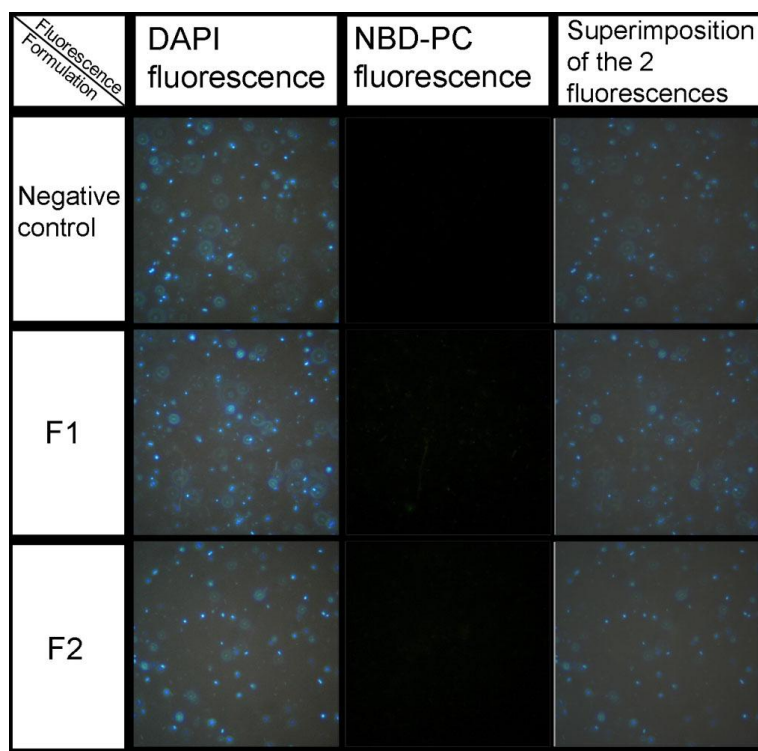


Fig.7: Epifluorescence microscopy pictures of 2 liposomal formulations mixed with fresh strain CFT073 of *E. coli*.

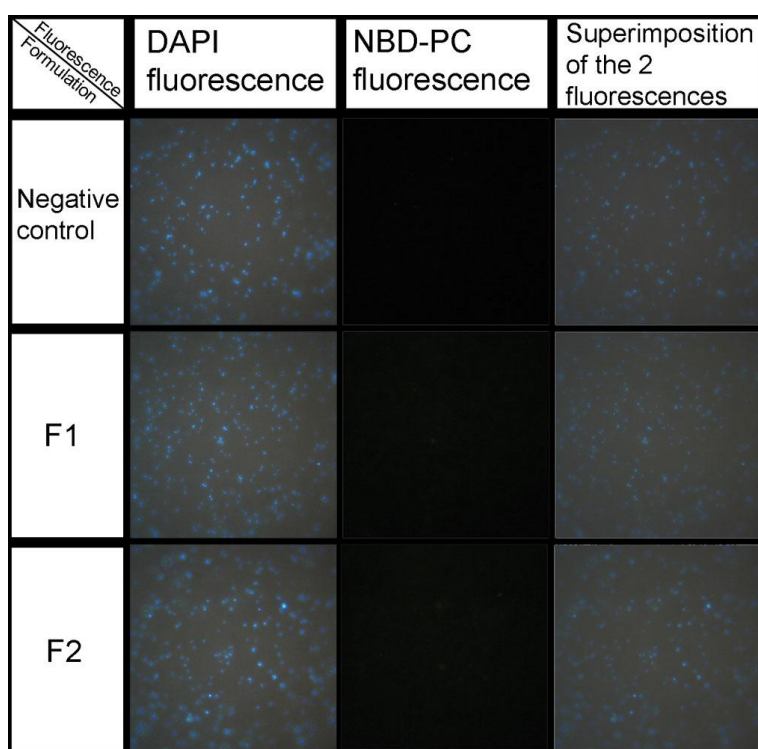


Fig.8: Epifluorescence microscopy pictures of 2 liposomal formulations mixed with fresh strain ATCC 12228 of *Staph. epidermidis*.

3. Discussion

3.1 Size and zeta potential:

Due to extrusion, the size of the liposomes we have produced is inferior to 200 nm. Size is an important point because beyond 200 nm, diffusion of particles through gastric mucus dramatically decreases^[37]. However, this is particularly true for healthy gastric mucus; but in the case of inflammation due to chronic infection by *H. pylori*, the properties of the gastric mucous membrane are deeply modified (hydrophobicity and phospholipid composition of gastric wall, gastric mucosal healing process, sucrose permeability, etc.)^[38-40]. Thus, we expect that particles with a mean diameter inferior to 200 nm have a good probability to reach bacteria under the gastric mucus.

Liposomes made with epikuron 170 (F3 and F4) are more negatively charged than those made with dppc (F1 and F2). This difference is due to the phospholipids used. Indeed epikuron is a mixture of several phospholipids (phosphatidylcholine > 72%, phosphatidylethanolamine > 10%, phosphatidylinositol < 3%, lyso-phosphatidylcholine < 4%) and free fatty acids (~10%) in which the phosphate group of the phosphoric acid is non-protonated, leading to the more negative zeta potential of the epikuron vesicles (see part I of this work, Mosqueira et al.^[41]). Furthermore it is possible that the reorientation of the phospholipid head groups following the theory of Makino et al.^[42] for epikuron is different than that for dppc. Indeed, the hydrophilic groups of phospholipids are located on the surface of liposomes and are considered as dipoles, the phosphatidyl group conferring a negative charge, and the choline group conferring a positive charge. Depending on the ionic strength and the temperature, the orientation of the dipole is different, conferring a charge to the liposome which varies with the ionic strength and the temperature of the medium. From these zeta potential results, it is not possible to observe a charge screening effect of the ethyleneglycol part of our glycolipid. This confirms a previous study^[36] and is probably due to the low molecular weight of our spacer (four ethylene glycol units).

3.2 Encapsulation efficiency:

The encapsulation efficiency of liposomes is governed by the ability of the formulation to retain drug molecules in the aqueous core or in the bilayer membrane of the vesicles. Furthermore, in drug leakage via a solubility-diffusion mechanism, the permeability coefficient is controlled by the product of the partition coefficient between hydrocarbon chains and water and the diffusion constant in the membrane^[43]. The partition coefficient octanol/water (log Kow) for metronidazole was calculated to be -0.02^[44,45] and estimated to -

0.00 and -0.01 by KowWin software (V. 1.67; © 2000 U.S Environmental Protection Agency) and ACD LogP software (ACD/Labs V.8.00), respectively. No calculated log Kow was found in the literature for ampicillin sodium salt, however KowWin and ACDLogP estimated it to -3.41 and -2.40 respectively. Thus metronidazole is probably located at the interface of the lipidic membrane and the aqueous core whereas ampicillin sodium salt is encapsulated in the aqueous core of the liposome. These assumptions could be validated by the X-ray diffraction coupled with DSC experiments. Indeed, compared to pure DPPC or ampicillin-DPPC mixture, the decrease of the diffraction peak intensities at 20 °C, the modification of the $P_{\beta'}$ phase which tends to disappear (disappearance of the pre-transition temperature and important decrease of the repetitive distance observable with the metronidazole-DPPC mixture), confirm a strong interaction between this antimicrobial agent and the phospholipids. The formation of the ripple phase is known to be very sensitive to the presence of foreign molecules ^[46-48]. The formation of ripple surfaces was mainly dependent on the interfacial energies, governed by the lateral interactions inside phospholipid bilayers. The apparent interfacial area per molecule results from the balance between cohesive forces within the chains (hydrophobic interaction) and repulsive interactions between the headgroups ^[49,50]. The interaction of foreign molecules such as metronidazole with polar head group and/or the chains of the phospholipids would lead to a modification of the previous balance and then to the disappearance of the ripple phase formation. From the partition coefficient calculation and the X-ray diffraction results, it is clear that metronidazole is a better candidate to interact with phospholipid membrane compared to the ampicillin salt. Because of its molecular weight (172 g/mol vs. 371 g/mol), is also more potent in term of diffusion ability through the bilayer. For all these reasons, we can assume that metronidazole will be more sensitive to the physico-chemical parameters of the liposome membranes than ampicillin sodium salt. In the first part of this work we found that at 20 °C, the phospholipidic membrane made with epikuron was under a fluid state. It is well known that the leakage of an encapsulated-drug inside a liposome dramatically increases when the vesicle is above its phase transition temperature, i.e. when the phospholipidic membrane is under a fluid state. On the other hand, the dppc-based formulations were under a gel state at 20 °C, a more solid and structured state which avoid a too rapid release of the encapsulated-drug. This is particularly true for amphiphilic drugs which are encapsulated at the interface of the lipidic membrane and the aqueous core. These differences concerning the drug localization and the membrane fluidity can explain the high difference of encapsulation efficiency that we observed for metronidazole between epikuron and dppc-based liposomes. Because of its amphiphilic structure and the relative fluidity of the phospholipidic membrane, the metronidazole is not long enough retained inside the epikuron based-liposome, leading to poor encapsulation efficiencies (0.6 to 2.1 %) in comparison with dppc-based liposomes (11.2 to 13.9 %), where

the membrane is under a gel state. For the ampicillin sodium salt, which is encapsulated in the aqueous core of the liposome, it seems that the membrane fluidity does not change significantly the encapsulation efficiency. Although ampicillin or metronidazole are not innovative compounds against *H. pylori*, they are interesting as model drugs for encapsulation into liposomes. Furthermore, ampicillin has a high efficiency against *H. pylori* (Minimum Inhibitory Concentration (MIC) < 0.015 to 0.06 mg/L (extreme values)) and metronidazole can be an alternative in case of allergy to β -lactams (MIC < 0.06 to 256 mg/L (extreme values)) ^[51]. Encapsulation efficiency for both drugs in liposomes of dppc - Fuc-E₄-Chol – cholesterol (80:10:10) (cf. Table 2) are greatly sufficient to obtain a therapeutic effect, but the method can be improved and other drugs could be incorporated, as for example the anti-urease acetohydroxamic acid. This 75 Da molecule can permeate intact bacterial cell and inhibits the urease activity of *H. pylori*. Without its urease, the bacterium is unable to buffer its environment, leading to its death ^[52-55]. This approach can be interesting to overcome the problem of antibiotics resistance. Ecabet sodium is also very interesting because of its anti-urease activity, coupled with an antimicrobial efficiency and its ability to regenerate the gastric mucin ^[56-58]. For these reasons, ecabet sodium is widely used in Japan against *H. pylori*.

3.3 Liposome-bacteria interactions:

Results obtained with epifluorescence microscopy show distinct behaviours according to the strain (17875 or 149C, fresh or old cultures) and according to the formulation. From the results we have obtained, the interactions *H. pylori* – liposomes may be explained by 4 phenomena. First of them is the presence of cholesterol in all the formulations. Indeed, a specific affinity of *H. pylori* to this steroid was previously described ^[59]. Authors incubated cholesterol-free bacteria with cyclodextrin-mediated cholesterol and several cyclodextrin-mediated steroidal hormones (beta-estradiol, testosterone, progesterone, hydrocortisone, dexamethasone) and then assessed steroid contents of the bacteria by gas liquid chromatography. They found high amount of cholesterol in all of the 7 *H. pylori* strains tested while other steroidal hormones were not found. Furthermore, in the same experiment, no significant amount of cholesterol was found with *S. epidermidis* and *E. coli* strains. This specific affinity for cholesterol described above is in agreement with our results. With *H. pylori* and the 4 liposomal formulations we observed the green fluorescence of liposomes superimposed on the blue fluorescence of bacteria, whereas no such results were obtained with *Staphylococcus* or *E. coli* strains. Thus, the presence of cholesterol in liposomes is probably the principal reason of the interaction *H. pylori* – liposomes. However, formulations with epikuron (F3 and F4) give poor results, especially with the coccoid forms. This can be

explained by the electrostatic repulsion, the second phenomenon which plays a part in the interaction between liposomes and bacteria. Indeed, *H. pylori* is negatively charged ^[60,61] and formulations made with epikuron (F3 and F4) are more electronegative than dppc-based formulations (F1 and F2). This is probably why the best results were obtained with less electronegative liposomes, while with too electronegative liposomes, electrostatic repulsion might have prevented strong interactions. However, when we focused on less electronegative liposomes (F1 and F2), we observed a marked difference between F1 and F2 for the strain 17875, but not for strain 149C. This is due to the presence or not of Fuc-E₄-Chol in the formulation, which is the third important point. The interaction seems to be enhanced when neoglycolipid is present in the formulation (F2) with strain 17875, while nothing changes with strain 149C. This is because strain 17875 expresses the *babA2* gene. As mentioned above, BabA2, an outer membrane protein, is able to specifically link the fucosylated Lewis b (Le^b) histo-blood group antigen presents on human gastric epithelial cells ^[12,13,15,26]. Thus, we assume a specific interaction between the fucose at the surface of the liposome and the BabA2 adhesin of *H. pylori*. This interaction could explain the better results obtained with formulation F2 and strain 17875 (Fig. 3 and 5, B2), whereas with strain 149C, which does not express the *babA2* gene, no difference was observed between the F1 and F2 formulations (Fig. 4 and 6, B1 vs. B2). The fourth phenomenon which seems to play a role is the age of the cultures. Indeed, a weak interaction was observed with F3 and F4 formulations with the fresh culture of strain 149C (Fig. 4, B3 and B4) while this interaction disappeared with older cultures (Fig. 6, B3 and B4). During aging, the morphology of *H. pylori* changes and the spiral shape of the bacterium becomes coccoid (cf. Figure 9) ^[62]. Authors do not agree about the properties of the coccoid form. For some of them, it is a degeneration form of the bacterium ^[63] and for others, it is a resistance form ^[64].

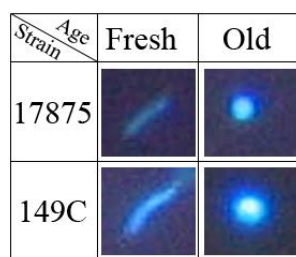


Fig.9: Helicoid or coccoid shape of *H. pylori* in function of ageing.

However, it is generally agreed that the coccoid form is not a cultivable but still viable form. Furthermore, it seems that it can induces new gastric colonization in mice ^[65-67] and it is able to survive in water ^[65], and probably to revert to the spiral form ^[68]. Besides ageing, the

factors reported in the literature to lead to this morphological modification are antimicrobial agents, environmental stress, aerobiosis modification, temperature variation, basic pH, nutritional depletion ^[68-72]. This morphological modification of the bacterium is concomitant with deep structural changes. Coccoid forms present an intact membrane and ATP stocks ^[64,72], an oxidative metabolism ^[73], they are able to adhere and alter gastric cells ^[74], and they express *ureA*, *cagA* and *vacA* genes ^[75]. But the adhesion behaviour of *H. pylori* is very different if it has a spiral or a coccoid form. In a study lead by Khin et al. ^[76], the authors showed that agglutination with lectins was dependant of the bacterial form (spiral or coccoid) and the strain. For example, for some strains, coccoid forms showed the same agglutination properties with specific lectins as the spiral forms, whereas with other lectins or other strains, the agglutination was observed only with the spiral forms, or only with the coccoid forms. The authors observed for all of the 3 strains used an agglutination with fucose specific *Tetragonolobus purpureas* (Lotus A) lectin for both the spiral and coccoid forms. Thus, it may be the BabA2 protein is still expressed, which is in accordance with our results. Indeed, whatever the age of the culture of strain 17875, we observed an interaction with fucosylated liposomes (Fig. 3 and 5, B2). The ability of glycosylated liposomes to interact with both spiral and coccoid forms is interesting because some authors found coccoid forms of *H. pylori* in vivo ^[77,78]. The role of coccoid forms in pathogenesis remains unclear and it is still not known whether coccoid forms represent a normal stage in the life cycle of *H. pylori* or not. However, our liposomal formulations seem able to target both coccoid and spiral forms, and are promising in the fight against *H. pylori*.

Acknowledgment

The authors would like to thanks H. Amenitsch for the access to the Austrian small-angle X-ray scattering beam line of the ELETTRA synchrotron (Trieste, Italy) and C. Benagli, E. Grasselli and M. Tonolla from Istituto Cantonale di Microbiologia, (Bellinzona, Switzerland) for their valuable help.

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DISCUSSION GENERALE – CONCLUSION

Table des matières

Introduction	166
1. Stratégies alternatives aux traitements classiques	166
2. De la difficulté d'étudier <i>Helicobacter pylori</i>	167
3. Choix du récepteur cible	169
4. Choix de la forme vecteur	171
Références bibliographiques	172

Figures

Fig.1: Forme bacillaire (a) et coccoïde (b) d' <i>Helicobacter pylori</i>	168
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Tableaux

Tableau I : Prévalence de BabA2 retrouvée dans la littérature.	170
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Introduction

Comme nous l'avons vu dans l'introduction de ce travail, les conséquences d'une infection chronique à *H. pylori* ne sont pas anodines. La prévalence de l'infection est élevée, et le taux d'éradication demeure insatisfaisant. De plus, comme pour bien des microbes, la résistance aux antibiotiques devient un phénomène de plus en plus prononcé au fil du temps. La nécessité de développer de nouvelles stratégies pour diversifier l'arsenal thérapeutique dans la lutte contre *H. pylori* est bel et bien une réalité.

Dans ce contexte, ce travail de thèse s'inscrit dans la thématique générale du ciblage de médicament par des systèmes vecteurs porteurs de ligands. Le type de vecteur envisagé est un liposome, le ligand est un sucre et la cible est la bactérie, *H. pylori*. Ce travail décrit de façon systématique la construction d'un tel vecteur et tente de décortiquer toutes les interactions concourantes à l'utilisation de ce dernier ; depuis la synthèse du glycolipide permettant la présentation du ligand sucre à la surface des liposomes jusqu'aux interactions, *in-vitro*, avec la cible biologique.

Les principaux résultats expérimentaux ayant été discutés tout au long des chapitres précédents, la discussion générale qui va suivre portera essentiellement sur la « philosophie » de ce travail, ses limites et ses perspectives possibles.

1. Stratégies alternatives aux traitements classiques

Parmi elles, la vaccination semble une voie séduisante. En agissant en amont de l'infection, elle prévient tous risques de pathologies tout en permettant de protéger un maximum de personnes, même celles qui n'auraient pu être vaccinées. En effet, en protégeant la masse, on élimine les risques de transmissions interhumaines, et l'on protège ainsi les minorités non vaccinées. Cependant, comme nous l'avons vu (cf. introduction), le chemin est encore long avant de voir un vaccin efficace arriver sur le marché.

Les autres alternatives ne sont pas si nombreuses. Soit de nouveaux antibiotiques sont mis à disposition, et une partie du problème (celui des résistances aux antibiotiques) est résolu, soit ce sont de nouvelles formes galéniques qui voient le jour. La deuxième solution paraît être la plus prometteuse. En effet, l'arrivée de nouveaux antibiotiques ne résout que temporairement le problème des résistances, et rien ne prouve qu'ils offriront une action bactéricide *in vivo* plus efficace que ceux actuellement utilisés. Une partie de l'échec du traitement contre *H. pylori* est certes due aux résistances, mais au minimum deux autres causes peuvent être soulignées : l'inactivation *in-vivo* par le pH gastrique de principes actifs efficaces *in-vitro* et l'existence de foyers d'infections aux plus profonds des aspérités que peuvent offrir les cellules gastriques, cachés sous le mucus, peu accessibles aux

antibiotiques si le temps de résidence gastrique n'est pas suffisant. Voilà pourquoi l'idée de formes galéniques originales dans la lutte contre *H. pylori* paraît être une bonne solution si celles-ci permettent d'atteindre les derniers foyers d'infections, par le biais, par exemple, d'un ciblage. Enfin, comme nous l'avons vu précédemment, l'utilisation de vecteurs pourraient permettre l'encapsulation d'autres antibiotiques, normalement inactifs à pH acides, et ne sont, à priori, en rien incompatibles avec les futurs antibiotiques.

2. De la difficulté d'étudier *Helicobacter pylori*

La route est encore longue avant de mettre au point « le médicament idéal ». D'autant plus que l'étude d'*H. pylori* n'est pas une chose aisée. Tout d'abord sa culture n'est pas facile. Le milieu de culture doit bien évidemment être adapté mais là encore, l'expérience montre que certains milieux donnent de meilleurs résultats que d'autres. Ainsi dans mon travail, j'ai remarqué que les géloses à sang de cheval étaient plus efficaces que celles faites à partir du sang de mouton. De plus *H. pylori* est sujet à de forts taux de mutations spontanées et/ou de recombinaisons, ainsi qu'à des variations de phase pour plusieurs de ses gènes. Cela laisse penser que les souches d'*H. pylori* continuent à changer rapidement après leur transmission d'un patient à un autre et même lors de colonisations à long terme ^[1] rendant encore un peu plus difficile leur caractérisation. La culture est relativement lente et la micro-aérobiose est un facteur limitant. En effet 45 minutes à l'air libre suffisent pour décimer une culture. S'il est nécessaire de travailler avec des bactéries vivantes, l'expérience doit être menée rapidement. Enfin durant la colonisation de l'estomac, et dans certaines conditions de cultures, on retrouve *H. pylori* sous plusieurs formes :

- une forme spiralée
- une forme en « U »
- une forme « doughnut » (beignet)
- une forme coccoïde (fig.1)

La prévalence de chaque forme semble dépendre de l'état d'infection des tissus, mais en général avec le temps, toutes les souches prennent la forme coccoïde ^[2].

Les suppositions sur le rôle de la forme coccoïde de l'*H. pylori* sont encore aujourd'hui controversées. Si tout le monde s'accorde à dire que la forme coccoïde n'est certes pas cultivable mais demeure viable, certains considèrent cette forme comme une forme de dégénérescence de la bactérie ^[3], et d'autres comme une forme de résistance ^[4].

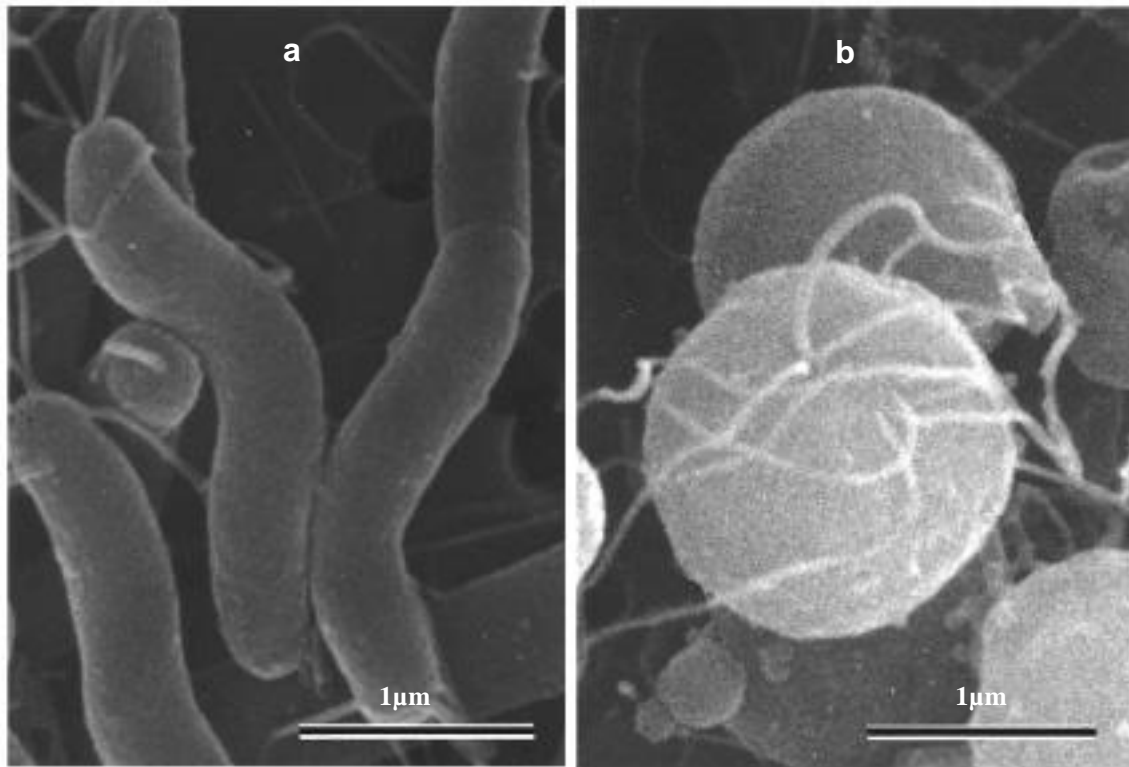


Fig.1: Forme bacillaire (a) et coccoïde (b) d'*Helicobacter pylori*. ^[5]

Les raisons pour lesquelles la bactérie passe de la forme spiralée à la forme coccoïde ne sont pas encore très claires. Cette transformation semble avoir lieu lorsque la bactérie est placée dans des conditions de stress, comme la présence d'agents antimicrobiens, une modification de l'aérobiose ou des changements de température, un pH basique, un appauvrissement nutritionnel, des temps d'incubation trop importants ^[6-10].

Lorsque la bactérie est cultivée sur boîte de Pétri (Brain Heart Infusion Agar, Sang de cheval, conditions anaérobiques) plus de 95% des bactéries se retrouvent sous forme coccoïde après 7 jours de culture ^[7]. Lorsque la bactérie est cultivée en milieux liquide (Brucella Broth Medium, sang de cheval, conditions micro-aérobiques) 85 % des bactéries se retrouvent sous forme coccoïde après seulement 3 jours de culture ^[11]. Le temps que mettent les bactéries pour passer d'une forme spiralée à une forme coccoïde, dans une culture en micro-aérobiose, est fonction des souches utilisées.

In vivo, la forme coccoïde a déjà été observé dans l'estomac humain ^[12,13]. Si la forme coccoïde n'est pas cultivable, elle demeure toutefois bien vivante. Il semble même qu'elle puisse induire de nouvelles colonisations gastriques, ce qui a déjà été observé chez la souris ^[14-16]. De plus la forme coccoïde peut survivre dans l'eau, laissant supposer qu'une infection à *H. pylori* par le biais de l'eau est possible ^[14]. D'autres recherches ont montré que même sous forme coccoïde, l'*H. pylori* exprime les gènes extrêmement importants à sa

pathogénicité : *ureA*, *cagA* et *vacA* ^[17]. Cela laisse supposer que la forme coccoïde est une forme de transmission de la bactérie, et qu'elle joue un rôle crucial dans la réinfestation après une thérapie antibiotique. Une étude ^[9] a même montré qu'*H. pylori* était capable de repasser de la forme coccoïde à la forme spiralée, confortant ainsi cette hypothèse. L'adhésion de la forme coccoïde présente quelques différences par rapport à la forme spiralée. Sous forme coccoïde, l'*H. pylori* adhère de façon moins importante aux cellules MKN45 (cellules de carcinome gastrique humain) ^[7]. En revanche, même sous forme coccoïde, *H. pylori* est capable de fixer le plasminogène ^[18]. Des travaux portant sur l'agglutination de la forme coccoïde de l'*H. pylori* par les lectines (protéines d'origine végétale capable de se fixer sur certains oligosaccharides des membranes cellulaires) ^[19] révèlent que l'agglutination varie en fonction de la forme de la bactérie (spiralée et coccoïde) et des souches étudiées.

Alors quel avenir pour le développement de nouveaux traitements ? Dans un premier temps, il est primordial de mieux connaître *H. pylori*. Le succès dans la lutte contre cette bactérie passera par une meilleure connaissance de ses faiblesses, de ses récepteurs potentiels, de sa physiologie... Une fois bien caractérisée, que se soit dans sa forme spiralée ou coccoïde, il sera plus facile de trouver un traitement efficace contre ce pathogène.

3. Choix du récepteur cible

Le choix de l'adhésine BabA2 comme cible peut être discuté. Comme évoqué précédemment, la synthèse de cette protéine ne peut être généralisée à toutes les souches d'*H. pylori*. Qu'en est-il dès lors de sa prévalence ? Cette adhésine se retrouve-t-elle assez fréquemment pour prétendre être une cible intéressante ? Les données, encore peu nombreuses, retrouvées dans la littérature, semblent indiquer que la prévalence de BabA2 varie considérablement d'une étude à l'autre, variation qui semble, entre autre, être liée à la zone géographique de l'étude. Quelques prévalences sont regroupées dans le tableau I, avec en regard de chacune, le lieu de l'étude.

Tableau I : Prévalence de BabA2 retrouvée dans la littérature.

Souches exprimant BabA2 (pour 100 souches)	Nombre total de souches	Patients souffrants de	Lieu de l'étude	Référence
100%	101	AG, UD, UG, G	Taiwan	[20]
84,9%	179	AG, UD, UG, LM, D	Japon	[21]
71,9%	114	AG, UD, LM, G	Allemagne	[22]
36,1%	76	UD, UG, D	Corée	[23]
0%	9	UD, UG, LM, G, D	Suisse ^a	Résultats du laboratoire non publiés

AG=Adénocarcinome Gastrique, UD=Ulcère Duodénal, UG=Ulcère Gastrique, LM=Lymphome de type MALT, G=Gastrite, D=Dyspepsie.

^a Provenance des patients : alentours de Lugano, canton du Tessin.

Ces résultats sont toutefois à prendre avec précautions. En effet, les données épidémiologiques concernant l'expression de BabA2 sont encore peu nombreuses et la façon dont sont menées ces différentes études ne permet pas toujours de les comparer. De plus certaines informations contenues dans ce tableau semblent en contradiction avec les conclusions d'autres auteurs. En effet, l'expression de BabA2 par une souche d'*H. pylori* est parfois considéré comme un facteur clinique pertinent et un marqueur de pathogénicité (pour les pays occidentaux, et surtout lorsqu'il est associé aux gènes *vacA* et *cagA*) [22,24,25]. BabA2 devrait donc se retrouver très fréquemment dans les maladies graves (ulcères gastriques ou duodénaux, lymphome MALT et adénocarcinomes) or ce n'est pas toujours le cas (Suisse). En outre, il existe des différences surprenantes entre la prévalence de BabA2 en Allemagne et en Suisse, pourtant pays voisins (même si l'échantillon allemand est 12 fois supérieur à l'échantillon Suisse). D'après la littérature, on sait que la prévalence de BabA2 dans les pays de l'Est est extrêmement importante, rendant d'ailleurs impossible l'utilisation de BabA2 comme marqueur de pathogénicité [20,21]. Il semble étonnant alors d'observer une si faible prévalence pour la Corée. Enfin, aucunes études pour le moment ne permettent d'affirmer que la synthèse de la protéine membranaire BabA2 est maintenue lorsque la bactérie est sous sa forme coccoïde.

Le récepteur idéal devra donc être plus ubiquitaire, dans le sens où toutes les souches d'*H. pylori* devront le posséder, aussi bien sous leur forme spiralée que coccoïde. Du fait de la découverte relativement « récente » de cette bactérie, ce récepteur n'a peut être pas été encore trouvé. Cependant, d'après l'étude bibliographique approfondie menée lors de ce travail, certaines pistes pourraient se révéler intéressantes ; notamment l'utilisation de

vecteurs recouverts de lectines végétales. Dans la littérature ^[19] ont ainsi été décrites d'intéressantes interactions entre différentes souches d'*H. pylori* et les lectines suivantes :

- avec les lectines Con A (Concanavaline A, spécifique des résidus mannose et glucose), PEA (*Pisum sativum*, spécifique des résidus mannose), VFA (*Vicia faba*, spécifique des résidus mannose et glucose), HPA (*Helix pomatia*, spécifique des résidus *N*-acetyl galactosamine), VSA (*Vicia sativa*, spécifiques des résidus mannose et glucose) les deux formes (spiralée et coccoïde) présentent une agglutination (pour les trois souches étudiées : RH54, NCTC 11637 et V₂).
- avec les lectines SBA (*Glycine max*, spécifique des résidus *N*-acetyl galactosamine), VVA (*Vicia villosa*, spécifique des résidus *N*-acetyl galactosamine) et MBA (*Vigna radiata*, spécifique des résidus galactose) seule la forme spiralée s'agglutine (pour la souche V₂).
- avec les lectines LcH (*Lens culinaris*, spécifique des résidus mannose), et NPA (*Narcissus pseudonarcissus*, spécifique des résidus mannose), seule la forme coccoïde s'agglutine (pour les souches : RH54, NCTC 11637, V₂).

4. Choix de la forme vecteur

Pour les raisons décrites dans les chapitres précédents de ce travail, la voie que nous avons suivie, en développant des liposomes glycosylés semble pertinente sur différents points (diminution limitée du pH interne des liposomes, taux d'encapsulation corrects, glycosylation aisée de la surface du vecteur, reconnaissance avec la bactérie...). Cependant, il est évident qu'elle n'est pas exempte d'inconvénients. Comme dit plus haut, les liposomes ne sont pas les systèmes les plus stables *in vivo*, notamment dans le tractus digestif, où ils sont dégradés par les phospholipases pancréatiques et solubilisés par les sels biliaires. Toutefois, cette déstabilisation devrait rester minime dans le milieu gastrique. De même, lors des périodes de stockage, la stabilité des liposomes n'est pas toujours optimale. L'oxydation des phospholipides par l'oxygène, la fusion des vésicules entre elles, la fuite de principe actif... sont autant de problèmes fréquemment rencontrés avec ces systèmes. *In vitro*, nous avons réussi à obtenir un gradient de pH pendant deux heures, mais qu'en est-il sur un temps plus long, et les résultats seraient-ils identiques lors d'expériences *in vivo* ? Enfin, d'un point de vue beaucoup plus pragmatique, la réalisation de tels liposomes sur le plan industrielle est onéreuse, et il est fort peu probable que l'amélioration qu'apporte ces liposomes par rapport à un traitement classique justifie l'investissement aux yeux des industrielles.

En ce qui concerne la formulation galénique, plusieurs améliorations pourraient donc être envisagées. Les perfectionnements pourraient avoir lieu au niveau du vecteur lui-même, en remplaçant par exemple les liposomes par des nanoparticules polymériques, reconnues pour être plus stables. Ces vecteurs de tailles nanométriques offrent les mêmes avantages que

les liposomes (répartition statistique tout le long du tractus digestif évitant ainsi le risque d'évacuation prématurée de l'estomac par le phénomène de « tout ou rien », diffusion possible à travers le mucus gastrique, protection du principe actif de l'acidité gastrique), mais de part leur conception même, ils seraient sans doute moins sensibles aux conditions agressives du milieu gastrique. Si en théorie ces vecteurs semblent être de bons candidats, leur mise au point nécessitera sans doute un travail important, ne serait-ce que par le choix du polymère qui déterminera à la fois la stabilité du système, le relargage du principe actif au niveau gastrique, et se devra d'être biodégradable et totalement inerte vis-à-vis de l'organisme.

De nombreux travaux ont été fait dans ce domaine, avec entre autres des nanoparticules à base de poly(styrène) (taille comprises entre 600 ^[26] et 800 nm ^[27]) ; de gliadine (taille ~500 nm ^[28,29]) ; de poly(ε-caprolactone) et de dextran (taille ~200 nm ^[30]). Certaines lectines végétales (Concanavaline A, *Pisum sativum*, *Vicia faba*, *Helix pomatia*, *Vicia sativa*) ont été décrites comme agglutinant à la fois les formes spiralées et coccoïdes d'*H. pylori*, et ce pour au moins trois souches différentes ^[19]. Dans la mesure où il est possible de fabriquer des nanoparticules de petites tailles recouvertes de lectines, il est tout à fait envisageable d'imaginer le médicament de demain comme une suspension de nanoparticules à base de polymère biodégradable, de taille inférieure ou égale à 200 nm, et recouvertes d'une des lectines citées plus haut.

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ANNEXE

Table des matières

Introduction	177
1. Matériel et méthode	177
1.1. Préparation et caractérisation des liposomes.....	177
1.2. Interaction liposomes – <i>H.pylori</i> :	177
1.3. Préparation des liposomes.....	178
1.4. Préparation des suspensions bactériennes.....	178
1.5. Interactions liposomes – <i>H.pylori</i> , observation de l'agrégation par mesure de densité optique ou par microscopie	179
2. Résultats – Discussion.....	179
2.1. Mesure de la densité optique	179
2.2. Observation au microscope	181
Références bibliographiques	182

Figures

Fig.1: Mesure de la DO après 1heure pour 2 souches bactériennes : 65A et 88C en présence ou non de liposomes	180
Fig.2: Mesure de la DO après 14 heures pour 2 souches bactériennes : 65A et 88C en présence ou non de liposomes	180

Tableaux

Tableau I : Agrégation des bactéries en présence des différentes formulations	181
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Introduction

Différents travaux ^[1-4] ont permis de démontrer que l' exoenzyme S-like adhesin d'*H. pylori* était capable de fixer spécifiquement certains lipides, à savoir : les lipides à tête phosphatidyléthanolamine, la gangliotriaosylcéramide et la gangliotétraosylcéramide. Cette adhésine, capable de se fixer spécifiquement et de façon dose-dépendante à la phosphatidyléthanolamine, se retrouve chez d'autres espèces bactériennes : *Escherichia coli* ^[5,6], *Helicobacter mustelae* ^[7], *Haemophilus influenzae* ^[8], *Chlamydia pneumoniae*, *Chlamydia trachomatis* ^[9], et *Campylobacter upsaliensis* ^[10]. Dans le dernier chapitre de cette thèse, l'interaction entre des liposomes constitués d'epikuron (mélange de phospholipides contenant de la phosphatidylethanolamine) et la bactérie n'ayant pu être observée, nous avons voulu mettre en évidence l'interaction phosphatidyléthanolamine – *H.pylori* en formulant des liposomes porteur de taux croissants de DMPE : 0%, 5% et 10% (mol/mol), respectivement. Comme pour toute formulation classique de liposomes, nous avons incorporé du cholestérol (20% m/m) qui en plus de stabiliser la membrane liposomiale ^[11-13], fait parti, avec la phosphatidyléthanolamine, de ces ligands plus ou moins spécifiques d'*H.pylori* décrits dans la littérature ^[1,5,14].

1. Matériel et méthode

1.1. Préparation et caractérisation des liposomes

La dipalmitoylphosphatidylcholine (DPPC) (Ref P0763), la L- α dimyristoyl phosphatidyléthanolamine (DMPE) (Ref P5693) et le cholestérol (Ref C8667) proviennent de chez Sigma-Aldrich. L'azote 4.5 classe 2-1°A vient de chez Linde.

1.2. Interaction liposomes – *H.pylori* :

Le Brain Heart Infusion (BHI) Agar CM375, le Brain Heart Infusion Broth CM225, le vitox SR090J (milieu d'hydratation) et le vitox SR090K (supplément vitox), viennent de chez Oxoid. Le DMEM:F12 medium (Dulbeco's Medium :Ham's F12 ; 1/1 mix) et la L-glutamine 200mM (Ref K0282) proviennent de chez Biochrom AG (Ref F4815). Le sérum de veau fétal (inactivé par la chaleur) (Ref 26140087) a été acheté chez Invitrogen Corporation.

1.3. Préparation des liposomes

Les liposomes sont préparés par la méthode de Bangham ^[15]. Brièvement, on prépare un film lipidique par dissolution des lipides dans du chloroforme, puis évaporation de la phase organique sous vide (rotavapor Büchi EL). Le film est ensuite réhydraté par du tampon PBS (phosphate buffer saline) (10mM ; pH 7,4) à l'aide du vortex et du bain à ultrason. On obtient ainsi une suspension liposomiale ayant une concentration en lipides totaux de 12mM, dans un volume finale de 5mL de tampon PBS. Trois formulations à base de DPPC-cholestérol-DMPE sont préparées (notées PLB7, PLB8 et PLB9): 80-20-0, 75-20-5 et 70-20-10 mol% respectivement. Les liposomes sont stockés sous azote.

1.4. Préparation des suspensions bactériennes

Les souches 65A, 70A, 83A2, 88C, 98A, 99A2, 123C, 149C, et CCUG 17875 ont été utilisées. Afin d'éviter une auto-agrégation des *H.pylori* ^[16] suite à une culture en milieu solide, nous cultivons les bactéries en milieu liquide. Pour ce faire, les *H.pylori* sont d'abord cultivés 3 à 5 jours sur gélose (Brain Heart Infusion agar-CM375, sang de mouton ou de cheval et Vitox SR90K) en micro-aérophilie (O₂ 5%-CO₂10%-N₂ 85% mol.). Les bactéries sont prélevées et resuspendues dans 1mL de BHI broth (soit pour 1L, 47g de BHI broth CM225, 50mL de sang de cheval, 20mL de vitox SR90K, eau stérile qsp 1L). Elles sont remises en suspension par pipetage. La mesure des densités optiques (DO) à 620nm (spectrophotomètre Perkin Elmer UV/Vis Lambda 2S) permet d'évaluer la concentration bactérienne du milieu ^[17]. On prépare ainsi un inoculum à 1.10⁸ CFU/mL dans un volume final de 4mL de DMEM Ham's complet (soit pour 1L, 887,5mL de DMEM Ham's:F12, 100mL de fetal calf serum et 12,5 mL de L-glutamine). Les récipients sont incubés 2 à 3 heures en micro-aérophilie à 37°C. On prélève ensuite 2mL de suspension bactérienne que l'on centrifuge 5mn à 5300 rpm (centrifuge 5417C eppendorf) puis on re-suspend le sédiment dans 2mL de PBS, ceci deux fois de suite. Trois tests sont systématiquement réalisés pour s'assurer de la qualité des souches que nous utilisons : un test à l'uréase (positif en cas de présence d'*H.pylori*), un test à l'agar sang (négatif si il n'y a pas d'autres bactéries dans le milieu testé) et un test au BHI agar (qui lorsqu'il est négatif avec le contrôle négatif, prouve qu'il n'y a pas eu de contaminations croisées).

1.5. Interactions liposomes – *H.pylori*, observation de l'agrégation par mesure de densité optique ou par microscopie

On dépose dans chaque puits des microplaques 166µL de suspension bactérienne à 1.10^8 CFU/mL avec 84µL de liposomes (5mM de lipides totaux dilués dans du PBS stérile) en évitant soigneusement la formation de bulles d'air. On remplace les suspensions liposomiales par du PBS pour les contrôles négatifs. Les microplaques sont incubées en micro-aérophilie, puis la DO est mesurée à 630nm (universal microplate reader EL800 Biotek Instrument Inc., KC Junior software) à T=1H et T=14H. Les mesures de DO sont faites en triple. Le faisceau étant perpendiculaire aux puits de la microplaque, une agglutination des *H.pylori* devrait se traduire par une augmentation de la DO.

L'observation au microscope (contraste de phase, Zeiss standard 25, grossissement x 400) se fait entre lame et lamelle en déposant une goutte de mélange des puits de la microplaque, prélevée à l'aide d'une pipette pasteur.

2. Résultats – Discussion

2.1. Mesure de la densité optique

Les résultats sont présentés dans les figures 1 et 2 pour deux souches bactériennes (65A et 88C). Par rapport au PBS seul, la DO varie peu en présence ou non des bactéries. En revanche l'ajout de liposomes dans le PBS fait logiquement augmenter la DO. On constate qu'après 1H ou 14H, la DO semble être plus importante en présence des liposomes et des bactéries, et ce de façon proportionnelle à la quantité de PE présente dans les liposomes. L'augmentation de DO observée avec la formulation PLB7 ne contenant pas de DMPE, notamment avec la souche 65A, peut provenir, soit de la simple addition des opacités des liposomes et des bactéries, soit d'une agglutination liée à la présence de cholestérol (cf chapitre V).

D'autres tests ont été faits avec d'autres souches bactériennes, et d'autres temps d'incubation, et les mêmes profils de réponses sont systématiquement obtenus. Il semblerait donc qu'il y ait une interaction bactérie – liposomes porteurs de phosphatidylethanolamine.

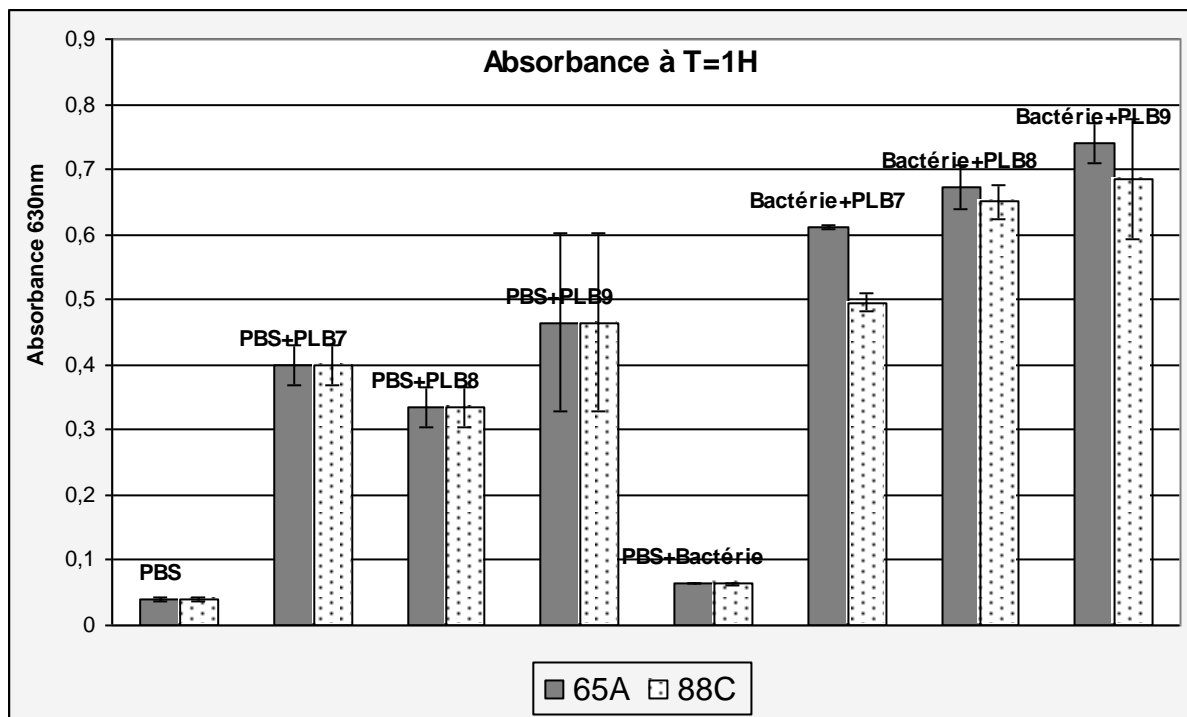


Fig.1: Mesure de la DO après 1heure pour 2 souches bactériennes : 65A et 88C en présence ou non de liposomes.

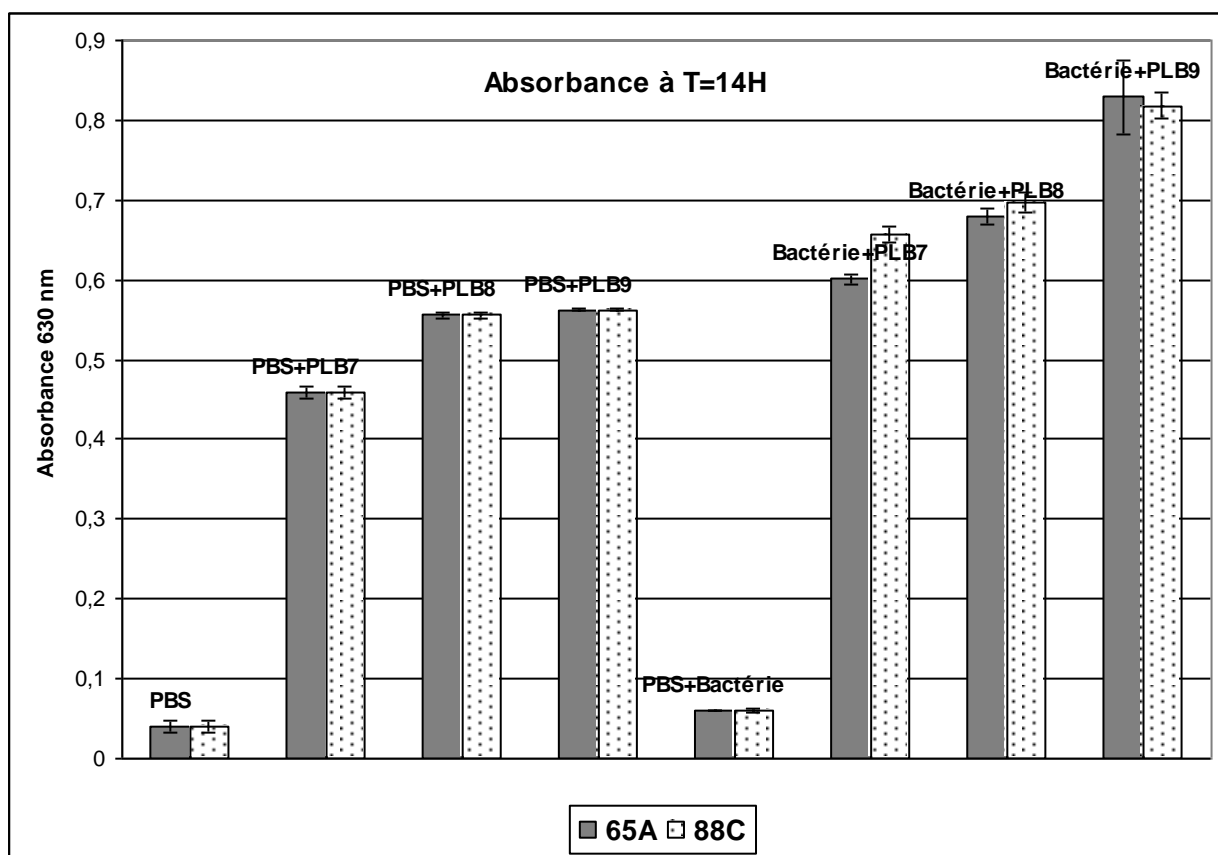


Fig.2: Mesure de la DO après 14 heures pour 2 souches bactériennes : 65A et 88C en présence ou non de liposomes.

2.2. Observation au microscope

Les résultats sont présentés dans le tableau I. Là encore une tendance semble se dégager, sans pour autant être très nette, mais surtout, ces observations ne sont pas quantifiables, donc difficiles à comparer. Les bactéries dans le tampon ont parfois tendance à se coller les unes aux autres, mais ceci est variable en fonction des souches utilisées. L'ajout de la formulation PLB7 ne semble pas apporter de grands changements quant à une éventuelle agrégation des bactéries entre elles. En revanche, il semblerait que la formulation PLB8 mais surtout PLB9 entraîne la formation d'agrégats au sein des bactéries.

Tableau I : Agrégation des bactéries en présence des différentes formulations.

	Bactérie + PBS	Bactérie + PLB7	Bactérie + PLB8	Bactérie + PLB9
Souche 88C	-	+/-	++	++
Souche 98A	+/-	+/-	+	++
Souche 99A2	+	+	+	+(+)
Souche 149C	+/-	+/-	+	+

Légende : - signifie qu'il n'y a pas d'agrégats et que les bactéries sont séparées les unes des autres. +/- signifie qu'il n'y a pas d'agrégats à proprement parler, mais que les bactéries semblent plus facilement se coller. + signifie qu'il y a des agrégats. ++ signifie qu'il y a de nombreux agrégats.

L'interprétation reste purement subjective et demeure donc difficile. De plus l'observation ne se fait que sur une goutte et ne reflète pas forcément ce qui se passe dans le puits. L'étape de pipetage peut également biaiser les résultats en cassant les éventuels agrégats qui se seraient formés dans les puits.

Toutefois, l'ensemble de ces résultats sur l'interaction entre des liposomes porteurs de groupements phosphatidyléthanolamine et *H. pylori* semble confirmer que cette dernière est possible. Ces résultats confortent donc l'hypothèse formulée dans la dernière partie de ce travail, selon laquelle la faible interaction entre la bactérie et des vésicules constituées d'epikuron serait liée à la charge de surface des liposomes ainsi formulés.

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RESUME en français

Ce travail traite de la formulation et de la caractérisation de liposomes porteurs de glycolipides synthétiques, en vue du ciblage d'une bactérie : *Helicobacter pylori*. Après avoir passé en revue les différents systèmes à temps de résidence gastrique prolongé, il décrit la synthèse et l'utilisation de néoglycolipides de type "ancree-espaceur-sucre", constitué respectivement du cholestérol, du tétraéthylène glycol et enfin du fucose (ou *N*-acétylglucosamine). Ont été étudiées dans ce travail l'organisation supramoléculaire des néoglycolipides seuls en fonction de leur état d'hydratation, les altérations de la bicouche liposomale suite à l'incorporation du néoglycolipide, l'accessibilité des sucres à la surface des liposomes, la variation du pH intraliposomal en fonction de pH externes acides, et enfin, l'interaction de quatre formulations de liposomes contenant ou non les néoglycolipides avec 2 souches d'*H. pylori*.

TITRE en anglais

Formulation and characterisation of synthetic glycolipids-loaded liposomes to target *Helicobacter pylori*

RESUME en anglais

This thesis is about the formulation and characterisation of synthetic glycolipids-loaded liposomes in order to target a bacterium: *Helicobacter pylori*. Gastroretentive systems are first reviewed. Secondly, the synthesis and use of the system "anchor-spacer-sugar", i.e. cholesterol, tetraethylene glycol and fucose (or *N*-acetylglucosamine) respectively, are described. During this work, we studied the neoglycolipids supramolecular organization in function of their hydration rate, the alteration of the liposomal bilayer following the neoglycolipid incorporation, the accessibility of the sugar moieties at the liposomes surface, the intraliposomal pH variation in function of acidic external pH, and finally, the interaction between four liposomal formulations bearing or not neoglycolipids with two strains of *H. pylori*.

DISCIPLINE

Pharmacie

MOTS-CLES

Helicobacter pylori, liposome, glycolipides, fucose, cholestérol, adhésines bactériennes.

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