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Glycosylated liposomes against *Helicobacter pylori*: Behavior in acidic conditions

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This paper is dedicated to the memory of Michel Ollivon.

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ABSTRACT

Helicobacter pylori was isolated in 1982 and confirmed as a gastric pathogenic agent at the end of the 1980s. The present work deals with liposomes formulations in which are incorporated cholesteryl tetraethylene glycol oside as model ligands for *H. pylori* adhesins. This study is devoted to the behavior of liposomes in gastric conditions. The glycosylated vesicles are stable and the pH of the internal aqueous compartment remains close to 4 even through more acidic conditions are imposed to the external phase (pH 1.2–2). Such a pH gradient depends essentially on the nature of phospholipids used and is not extensively affected by the incorporation of the targeting agent. These aspects are particularly important to the development of liposome formulations against *H. pylori*, bacteria sensitive to antibiotics which are unstable in very acidic conditions.

Introduction

Discovered by Warren and Marshall in 1982 (2005 Nobel prize), *Helicobacter pylori* was confirmed as a pathogenic agent at the end of the 1980s. In 1994, the WHO classified it as type I carcinogen because of the gastric cancers and mucosa-associated lymphoid tissue lymphomas which can occur after a chronic infection. The prevalence of this bacterium in the world is still high [1–3] and the eradication rate does not reach the 90% expected in the WHO's purpose [4]. The classical way to cure *H. pylori* infection is to use a 7 days tri-therapy based on two antibiotics (amoxicillin, clarithromycin) and one proton pump inhibitor (omeprazole, lansoprazole, pantoprazole). However, because of the high level of antibiotic resistance to *H. pylori,* and the poor patient compliance in a lesser measure [5], new medicines with better effectiveness and simpler regimen are required [6]. In a previous study [7], we described the formulation of antibiotic-loaded glycosylated liposomes which interact with the bacteria. Indeed, virulent 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 [3,8–10]. Due to an urease activity, the close environment of the bacterium is neutralized by the production of ammonia and carbon dioxide [8,11] then the agglutination experiments have been conducted at neutral pH. However, it is important to control the behavior of our formulations, containing phospholipid (DPPC or Epikuron), cholesterol and cholesteryl tetraethylene glycol oside in an acidic media in order to check their stability and the intra-liposomal pH variations. This is important all the more as a slight fluidizing effect has been already described after the embedment of cholesteryl osides in phospholipid membrane [12].

The poor stability of liposomes in the gastrointestinal tract [13] is mostly due to bile salts and pancreatic lipases [14–18] and in stomach medium, liposomes are quite stable [17,19], thus allowing a gastric targeting. More important, the pH inside the liposome should be controlled. Indeed, *H. pylori* is sensitive to many antibiotics but most of them can not be used in acidic medium because of chemical degradation [20]. The protection of the active substance against the stomachal acidity and its release in the neutral bacterium environment could overcome the problem of acidity and increase the anti-*H. pylori* treatment panel.

Materials and methods

Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, monobasic sodium phosphate were purchased from Sigma–Aldrich.

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The neoglycolipid (Fig. 1), cholesteryl tetraethyleneglycol *N*-acetylglucosamine (GlcNAcE$_4$Chol), was synthesized already reported [21]. Fluorescent probe, Oregon Green® 514 carboxylic acid, was provided by Molecular Probes™, Invitrogen. Epikuron 170 mixture (phosphatidylcholine >72%, phosphatidylethanolamine >10%, phosphatidylinositol <3%, lyso-phosphatidylcholine <4% and free fatty acids 10%) was provided by Degussa. All solvents and reagents were analytical grade.

Methods

Vesicle preparation. Liposomes were prepared by extrusion method. 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 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 homogenized by extrusion under nitrogen above the phospholipid transition temperature, 3 times through 0.4 μm and 8 times through 0.2 μm GTTP filter (Millipore, isopore™). The vesicles were characterized by quasi-elastic light scattering using a Coulter nanosizer apparatus (Model N4 MD, Coultronics, France). A mean hydrodynamic diameter of 170 nm was obtained.

Intra-liposomal pH determination. Because several drugs efficient *in vitro* against *H. pylori* are unstable in acidic conditions, we have measured the pH inside different compositions of liposomes, after incubation in various media. The pH of the aqueous internal compartments of the vesicles was determined by the use of 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. 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). Liposomes were prepared as described above and the fluorescent probe was incorporated at the concentration of 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/internal pH of the suspension was measured during 2 h as a function of time, with a pH-meter and the fluorescent probe, respectively.

Agglutination assay. PC-based liposomes containing 10% of GlcNAcE4Chol were incubated with a USP gastric medium (USP XXIV, plus pepsin) for 1.5 h. Then liposomes were separated by ultracentrifugation at 40,000 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 $CaCl_2$. 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.

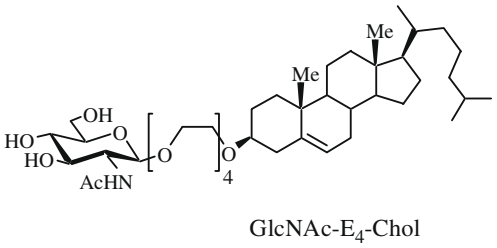

Fig. 1. Neoglycolipid used in this work.

Results

Intra-liposomal 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 Fig. 2A. Absorption ratios between 504 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 fluorescent probe concentration into the liposome aqueous cavities. The intensity ratio versus the pH of the fluorescent probe environment is shown on Fig. 2B. As expected, the Oregon green 514 carboxylic acid fluorescent probe can be used to check pH changes between pH 5 and pH 3, while the absorption ratio (504 nm/467 nm) is not sensitive outside this range. The behavior 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 ~18 mM). No significant changes were observed in the intensity ratio versus pH.

The intensity ratios obtained during a 2 h incubation of various 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 Fig. 3A–D, respectively. Some obvious differences can be seen between the various formulations. Concerning DPPC bilayers, in the absence of glycolipid (Fig. 3A) 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 Fig. 3B, the incorporation of GlcNAcE4Chol 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. 3C and D). 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 equilibrium 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 < Epik–Chol < Epik–Chol–glycolipid, in the range of pH 2–3.

When Epikuron–Chol–GlcNAcE4Chol 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.

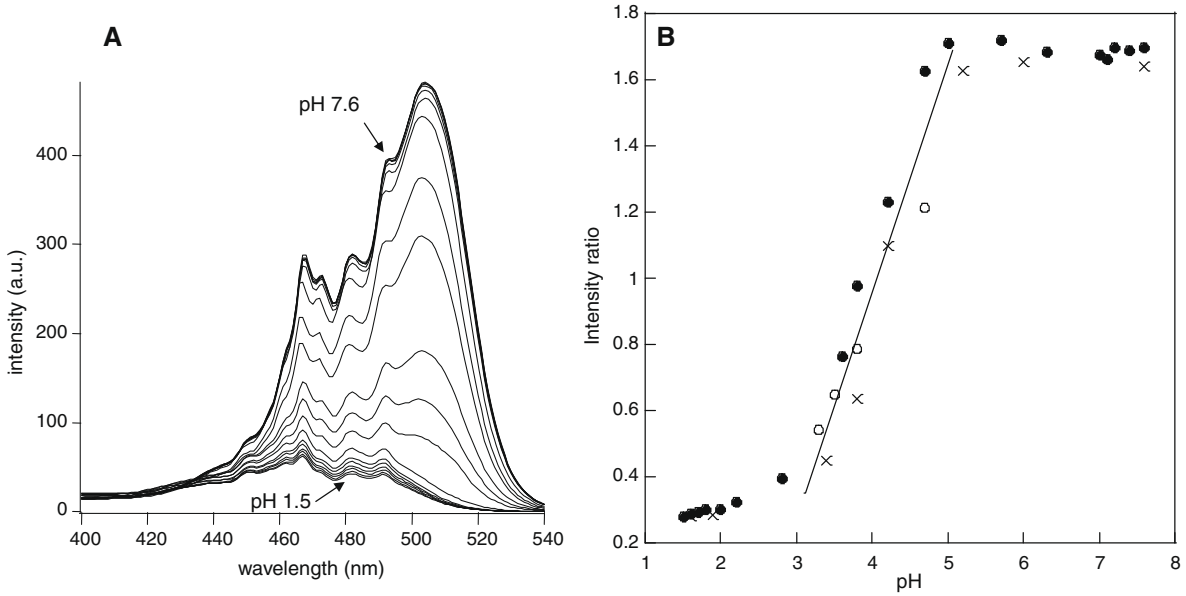

Fig. 2. (A) Raw Oregon green 514 carboxylic acid emission spectra at 570 nm recorded in Hepes buffer (10 mM, NaCl 150 mM) between pH 7.6 to 1.5. (B) Intensity ratios (I_{504nm}/I_{467nm}) versus pH; three experimental conditions were used: pure water (x), Hepes buffer (●) and Hepes buffer containing unloaded liposomes (○). The increases in intensity ratio in the pH range 3–5 were considered as linear and Kaleidagraph tools have been used to fit the values in order to obtain the intensity ratio versus pH correlation.

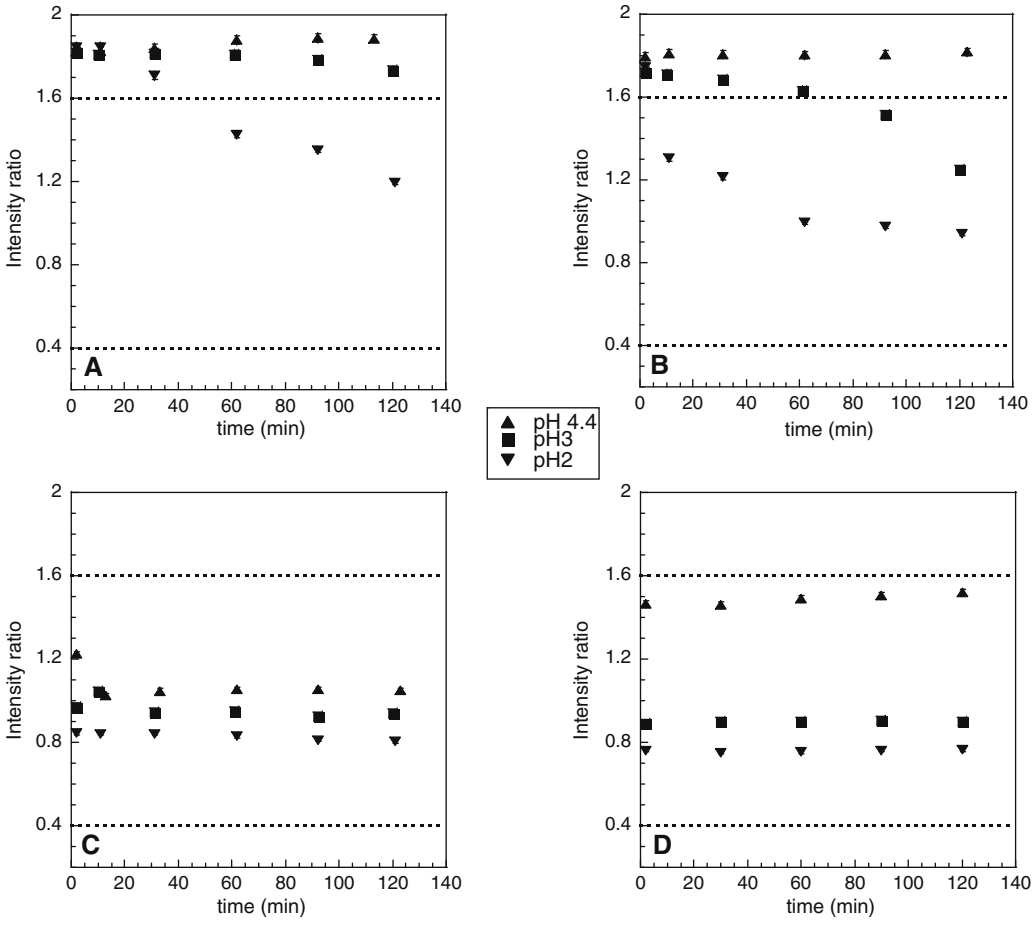

Fig. 3. Fluorescent probe intensity ratio versus time recorded at pH 4.4, 3 and 2, for liposomes composed of (A) DPPC–cholesterol, (B) DPPC–cholesterol–glycolipid, (C) Epikuron–cholesterol and (D) Epikuron–cholesterol–glycolipid. The dashed lines correspond to intensity ratio limits in which pH changes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

Table 1
Internal liposome pH after a 2-h incubation in a 10 mM Hepes buffer adjusted at pH 4.4, 3 and 2 with hydrochloric acid.

df	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

Agglutination assays

The physical stability of the liposomes and the chemical stability of GlcNAcE4Chol, following a 1.5-h incubation in simulated gastric fluid (with pepsin) was controlled by agglutination assays. The results are represented in Fig. 4. The agglutination of the glycolipid containing liposomes, resulting in an increase of optical density, is statistically similar 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.

Discussion

Proton permeation through liposomal membrane

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.

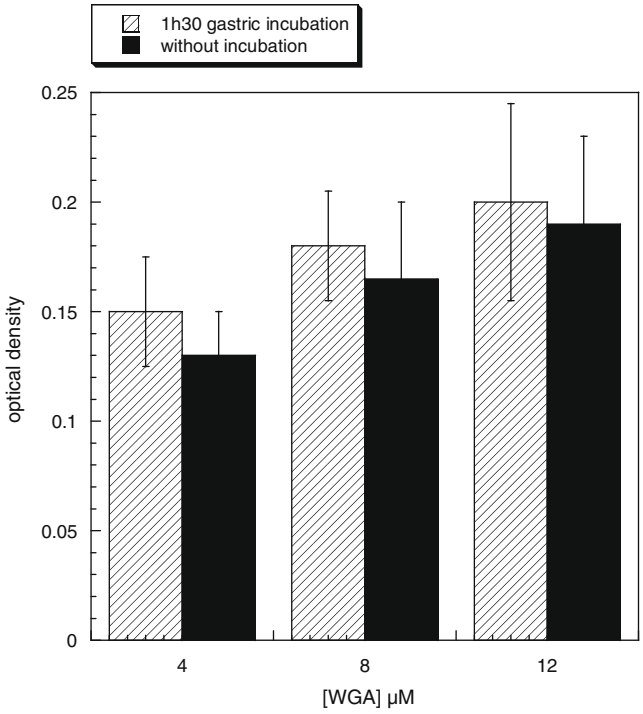

Fig. 4. Comparison of the agglutination results, after addition of WGA to $GlcNAcE_4Chol$-containing liposome (expressed as the increase of optical density at 450 nm). Dark histograms correspond to liposomes which were not incubated, and clear histograms correspond to liposomes incubated in gastric fluid and resuspended in the same buffer as untreated ones (Hepes buffer, pH 7.4).

It is generally admitted that the phospholipid bilayer of biological membranes is a rather tight diffusion barrier for hydrophilic solutes, including ions. However, proton permeability coefficients covering a range between 10^{-3} and 10^{-9} cm s^{-1} [22–25], compared with permeabilities of 10^{-10}–10^{-14} cm s^{-1} for other monovalent cations [26–28], have been described. Here, we used the DPPC/cholesterol mixtures at pH 2 to estimate the permeation coefficient. 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:

$$J = \frac{dQ}{dt} \times \frac{K_s \times D}{h} \times \Delta C \quad (1)$$

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:

$$P = \frac{J}{C_0} \quad (2)$$

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

The slope (dQ/dt) was calculated to 9.67×10^{-7} mol l^{-1} min^{-1}, from the graph giving proton concentration in the liposomes versus time (figure not shown). After size exclusion chromatography, the liposome concentration was ~2 mM (based on ammonium ferrothiocyanate assays); 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 Å^2 [29], the total bilayer surface area can be estimated at 7471 cm^2 (around 2460 cm^2 mg^{-1} of lipid, which is similar to the value of 2200 cm^2 mg^{-1} by Deamer and Nichols [30,31]). Then it was possible to calculate a flux of 1.3×10^{-7} mol cm^{-2} min^{-1} and a permeation coefficient of 2.2×10^{-5} cm s^{-1} (external pH 2).

It is interesting to mention that this permeation coefficient value is in accordance with accepted values of the literature, in the order of magnitude of 10^{-5} cm s^{-1} [32]. This calculation seems to confirm the validity of the fluorescence method we used to check the intra-liposomal pH.

pH gradient at equilibrium

Beyond the kinetics, it is interesting to mention that a pH gradient could be maintained during at least 2 h by the use of phospholipid bilayers. The pH inside the vesicles did not reach that measurable outside. Two possible artefacts should be ruled out: water movements due to salt gradients and behavior of the fluorescent probe in the presence of lipids. (i) 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 [33]; 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%) [34]. (ii) To check the behavior 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 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 3.6–4.4, depending on the vesicle composition. These values are quite interesting in terms of pharmaceutical use of such liposomes. Amongst the most prescribed antibiotics, H. pylori is susceptible in vitro to amoxicillin, clarithromycin and metronidazole, in addition with many other antibacterial agents, with MIC_{90} of 0.06 mg/l, 0.03 mg/l and 6.0 mg/l, respectively, at neutral pH [35,36]. 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 amoxicillin, penicillin G or ampicillin are susceptible to hydrolytic degradation when the pH deviates significantly from the isoelectric point. Thus the half-life of amoxicillin is 5.2 h at pH 1 but increases to 19.0 and 176.9 h at pH 2 and pH 4, respectively [37]. At 37 °C, the decomposition rate constant of penicillin G is one hundred times faster at pH 1.8 than at pH 4 [38]. 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 [39]. 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 [37,40]. From these results, it is possible to calculate that 99.3% of the drug remains intact after a 1-h incubation at pH 4, confirming also the interest of clarithromycin encapsulation in such liposomes.

Glycolipid stability and accessibility

The agglutination results confirm the stability of the cholesteryl ethyleneglycol derivative after incubation in a simulated gastric medium. Indeed, it has been reported previously that non specific interactions between PC-containing liposomes and wheat germ agglutinin (WGA) remain negligible (optical density increase at 450 nm lower than 0.005) [12]. Therefore, agglutinations reported in Fig. 4 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 demonstrates that it is possible to obtain 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 is not affected to a large extent by the incorporation of a targeting agent such as Glc-NAcE4Chol in the vesicle bilayer. These aspects are especially important to the development of colloidal formulations against *H. pylori*, bacteria sensitive to antibiotics which are unstable in gastric conditions.

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