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Research paper

Pre-formulation of liposomes against *Helicobacter pylori*: Characterization and interaction with the bacteria

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Abstract

This paper deals with the formulation of targeted liposome against *Helicobacter pylori*. We describe the characterization of liposomes loaded with antimicrobial agents (ampicillin and metronidazole) and the quantification of the interactions between such formulations and bacteria. If the encapsulation rate of ampicillin seems not strongly affected by the change of phospholipidic composition, the encapsulation of metronidazole drastically decreased in epikuron 170 liposomes compared to DPPC ones. Furthermore, as observed with X-ray diffraction measurements, the presence of metronidazole results in the disorganisation of the phospholipid bilayers. Concerning the liposome–bacteria interactions, it has been observed that the incorporation of fucosyled glycolipids in the vesicle membrane leads to liposomes that are able to interact with the bacteria either in their spiral or in their coccoid forms. Since coccoid forms are occasionally found in vivo, their recognition by the liposomes we have formulated seems promising in the fight against *Helicobacter pylori*.

Keywords: Liposome; *Helicobacter pylori*; Glycolipid; Targeting; BabA2

1. Introduction

Helicobacter pylori was discovered by Warren and Marshall in 1982 (2005 Nobel prize), and confirmed as a pathogen at the end of the 80s. In 1994, the WHO classified the microorganism as type I carcinogen because of the gastric cancers and MALT (mucosa-associated lymphoid tissue) lymphomas which can occur after a chronic infection. The worldwide prevalence of this bacterium is high [1–3] and the present eradication rate does not reach the objective defined by WHO, i.e. 90% [4]. Today, the standard treatment to cure *H. pylori* infection is a 7-days tri-therapy based on two antibiotics (amoxycillin and clarithromycin) and one proton pump inhibitor (omeprazole, lansoprazole, or 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 [5], new drugs with better effectiveness and simpler regimen are required. Indeed, *H. pylori* is sensitive to many antibiotics but a number of them cannot be used in acidic medium [6]. However, due to the urease activity, the close environment of the bacterium is neutralized by the production of ammonia and carbon dioxide [7,8]. A release of the active substance in the nearby of

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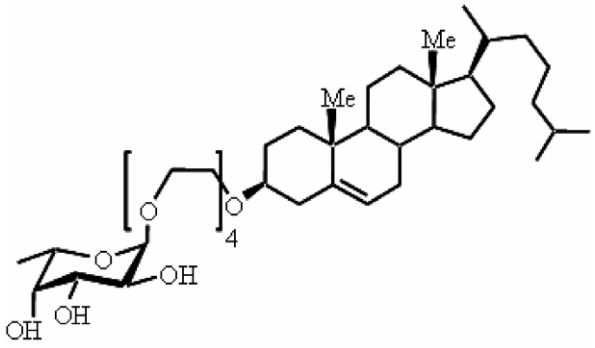
the bacterium could overcome the problem of acidity. Encapsulation of an active substance could be a good approach, by offering a protection against the stomachal acidity. In a seek for a non toxic vector able to encapsulate a wide range of drugs and easily modifiable at the surface, we have identified liposomes as good candidates for this purpose. Indeed, incorporation of specific ligands at liposome surface, 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 [9,10]. 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 four liposome formulations. All of them contain cholesterol because of its well-known bilayer stabilizing effect [11–13] in both biological and liposome membranes. Two different phospholipids were used: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) or epikuron 170. The main advantages of using epikuron are a cheap price and a composition containing at least 10% of phosphatidylethanolamine (PE), which was described to be a ligand for a *H. pylori* adhesin [14–16]. These two liposomal formulations (DPPC–cholesterol and epikuron–cholesterol) were compared with the same formulations but in which a synthetic glycolipid was incorporated. The glycolipid we used (Scheme 1) contains: a cholesterol group as anchor in the bilayer, four ethylene glycol units and fucose as ligand. Because of its flexibility, the ethylene glycol chain has the important function of a spacer separating the sugar (fucose) from the liposomal membrane. 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 [3,7,17,18]. 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 [19]. The poor stability of liposomes in the gastrointestinal tract [20] is mostly due to bile salts and pancreatic lipases [21–25]. In the stomach environment, or more generally in acidic conditions, liposomes are quite stable [24,26], thus allowing a gastric targeting.

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



Scheme 1. Schematic representation of the neoglycolipid used.

2. Materials and methods

2.1. 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-E_4-Chol) [27]. The synthesis of cholesteryl tetraethyleneglycol fucose (Fuc-E_4-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 mixture (phosphatidylcholine > 72%, phosphatidylethanolamine > 10%, phosphatidylinositol < 3%, lyso-phosphatidylcholine < 4% and free fatty acids 10%), and the DNeasy tissue kit (Ref. 69504) were provided, respectively, by Linde, Merck KGaA, Degussa, and Qiagen. All solvents and reagents were analytical grade.

2.2. Helicobacter pylori strains

Helicobacter pylori CCUG 17875 is a reference strain obtained from the Culture Collection of the University of Gothenburg. *H. pylori* 149C is a clinical strain isolated at the Istituto Cantonale di Microbiologia, Bellinzona, Switzerland.

2.3. Methods

2.3.1. Vesicle preparation

Liposomes are prepared by extrusion method [29]. Briefly, phospholipids, cholesterol and glycolipid (in a total lipid 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 μm HTTP filter (Millipore, isopore™) and 8 times through 0.2 μm GTTP filter (Millipore, isopore™)).

2.3.2. 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.

2.3.3. 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 10 mM with antibiotic free liposomes.

2.3.4. 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. One milliliter of thiocyanate reagent was added. After 1 min 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: 10–100 μg/mL). No interactions between the colorimetric method and cholesterol and/or glycolipid have been found.

2.3.5. 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 extrusion method, the rehydration of lipidic film was done with 10 mM of metronidazole or ampicillin sodium salt dissolved in pure water. The free fraction was removed by dialysis at room temperature. One milliliter of liposomes was placed into dialysis tubing cellulose membrane (cut-off 12,000–14,000 Da) 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. The amount of antibiotic in the dialysate was assessed by HPLC.

2.3.6. 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 1 mL/mn. The column was thermostated at 40 °C and the wavelength detection was set at 318 nm. The calibration curve is linear in the range 5–300 μg/ml ($r^2 = 0.9999$). The ampicillin sodium salt was assessed with a supelcosil LC18 column (Supelco®), 5 μm, 250 * 4.6 mm. The mobile phase used was NaH_2PO_4/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 calibration curve is linear in the range 5–500 μg/ml ($r^2 = 0.9996$). The HPLC analysis was performed on a Waters system (Waters 600 controller, Waters 486 tunable absorbance detector, waters 717 plus autosampler) and data acquisition was done with the Millenium 32 software.

2.3.7. 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 (4.61, 3.84, 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 available in Keller et al. [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).

2.3.8. 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 [32–34]:

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

*babA*2 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 0.5 M, pH 8, 4% v/v, water q.s 1 L) for 45 min at 110 V, 58 mA). DNA was stained with ethidium bromide and visualized by UV light.

2.3.9. 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 extrusion 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-E_4-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 (O_2 5%, CO_2 10%, N_2 85% mol), the bacteria were harvested and suspended in sterile phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4, pH 7.2) to reach a titre of approximately 10^6 CFU/mL. Ten microliters of DAPI (0.001% m/m) was added in 1 mL 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. One hundred microliters of liposomes at 10^{-4} mM was added to 100 μL of bacterial suspension, stirred and placed 1 h in dark at room temperature. Ten microliters of mixture was 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 two strains of *Escherichia coli* (CFT073 and K12) and three strains of *Staphylococcus* (*S. epidermidis* ATCC 12228, *S. aureus* ATCC 25923 and ATCC 29213).

3. Results

3.1. Size and zeta potential

The mean size of antibiotic-encapsulated liposomes lies between 147 and 163 nm with a polydispersity index of 0.10–0.11. The mean size of fluorescent liposomes (formulated with NBD-PC) lies between 136 and 172 nm with a polydispersity index of 0.07–0.12. 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 [19]. The four fluorescent formulations were negatively charged, as summarized in Table 1. 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 [19], no charge screening effect could be attributed to the glycolipid at the liposomal surface.

3.2. Phospholipids assessment

With the colorimetric method we obtained a correlation coefficient r^2 of 0.991 and 0.996 for the epikuron 170 and DPPC, respectively. We have not quantified the glycolipid in the formulations, however previous quantifications in our group [36] 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. For each formulation, the total lipid concentration was ∼29 mM after extrusion.

3.3. Encapsulation efficiency

Encapsulation efficiency was calculated using the following formula:

Table 1
Zeta potential values of the studied formulations

Formulations[a]	Name	Zeta potential (mV)
DPPC–cholesterol	F1	−4.3
DPPC–Fuc-E_4-Chol–cholesterol	F2	−2.9
Epikuron 170–cholesterol	F3	−12.2
Epikuron 170–Fuc-E_4-Chol–cholesterol	F4	−20.0

Proportions are, for F1, DPPC–cholesterol–NBD-PC (79:20:01); F2, DPPC–Fuc-E_4-Chol–cholesterol–NBD-PC (79:10:10:01); F3, Epikuron 170–cholesterol–NBD-PC (79:20:01); F4, Epikuron 170–Fuc-E_4-Chol–cholesterol–NBD-PC (79:10:10:01).

[a] Each formulation contains 1% of fluorescent phosphatidylcholine (NBD-PC).

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

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 2 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 (~1.4% vs. ~11.2%). The ampicillin encapsulation seems not strongly affected by the change of phospholipidic composition.

The Epikuron–Fuc-E_4-Chol–cholesterol (80:10:10) formulation has not been studied for several reasons. At least with metronidazole, the encapsulation efficiency with epikuron (without glycolipid) is very low and it is probable that the incorporation of glycolipid could not increase it. Indeed, we observed a slight liposomal membrane fluidizing effect due to the incorporation of glycolipid [19]. Moreover, the synthetic glycolipid amount being reduced, the bacteria–liposomes interaction studies have been privileged.

3.4. Antimicrobial agents–phospholipid interactions

The diffraction patterns and DSC signal between 20 °C and 55 °C are represented in Fig. 1a–c 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 3. 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 X-ray diffraction patterns obtained with DPPC fit well with the classical $L_{\beta'}$, $P_{\beta'}$ and L_α phases, extensively described in the literature [37–39] in which the peak around 0.1 $Å^{-1}$ corresponds to the first order of a lamellar organization. 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 (~41 °C for DPPC), the chain melt into a lamellar fluid phase (L_α). In comparison with 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 observable in Fig. 1 (comparison of Fig. 1A and B) with such a mixture and by the transition temperature decreased summarized in Table 3. 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.

Table 2
Encapsulation efficiency (mean ± SD) of the studied formulations

Formulations	Metronidazole (%)	Ampicillin (%)
DPPC–cholesterol (80:20)	11.2 ± 2.6	10.0 ± 4.0
Epikuron 170–cholesterol (80:20)	1.4 ± 1.1	13.9 ± 1.0
DPPC–Fuc-E_4-Chol–cholesterol (80:10:10)[a]	13.0	4.8

[a] In order to reduce the consumption of our synthetic glycolipid, only one measurement has been made.

3.5. Helicobacter pylori strains characterization

To identify the presence of *babA2* and *vacA* genes in *H. pylori* strains we used PCR 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 in Fig. 2, the CCUG 17875 strain contains both the fragments harbouring *babA2* (832 bp) and *vacA* (290 bp) whereas these fragments were absent in strain 149C.

3.6. Epifluorescence microscopy

As mentioned above, we used four liposomal formulations: DPPC–cholesterol–NBD-PC (F1); DPPC–Fuc-E_4-Chol–cholesterol–NBD-PC (F2); epikuron–cholesterol–NBD-PC (F3) and epikuron–Fuc-E_4-Chol–cholesterol–NBD-PC (F4). First, we compared the four formulations with fresh strains (3 days old) of *H. pylori* (17875 and 149C). We used PBS instead of liposomes as negative control. With the device used, a liposome corresponds approximately to a pixel and then individual liposomes would be difficult to distinguish. We assume that the presence of several liposomes (for example around the bacteria) is necessary to get a clear NBD fluorescence signal. Results obtained with strain 17875 and strain 149C are shown in Figs. 3 and 4, respectively. The process of bacterial aggregation is quite difficult to evaluate for several reasons. First, it depends on the type of strain used, as observed for example in Fig. 4 with the negative control of the strain 149C which presents an important "self-aggregation" or with optical density measurements we made (data not shown). Second, the size of the bacteria being at least 10 times more important than that of the liposomes, the liposome–bacteria interaction could be masked by the bacterial agglutination. Then, the epifluorescence results should not be considered as an agglutination assay but it is interesting

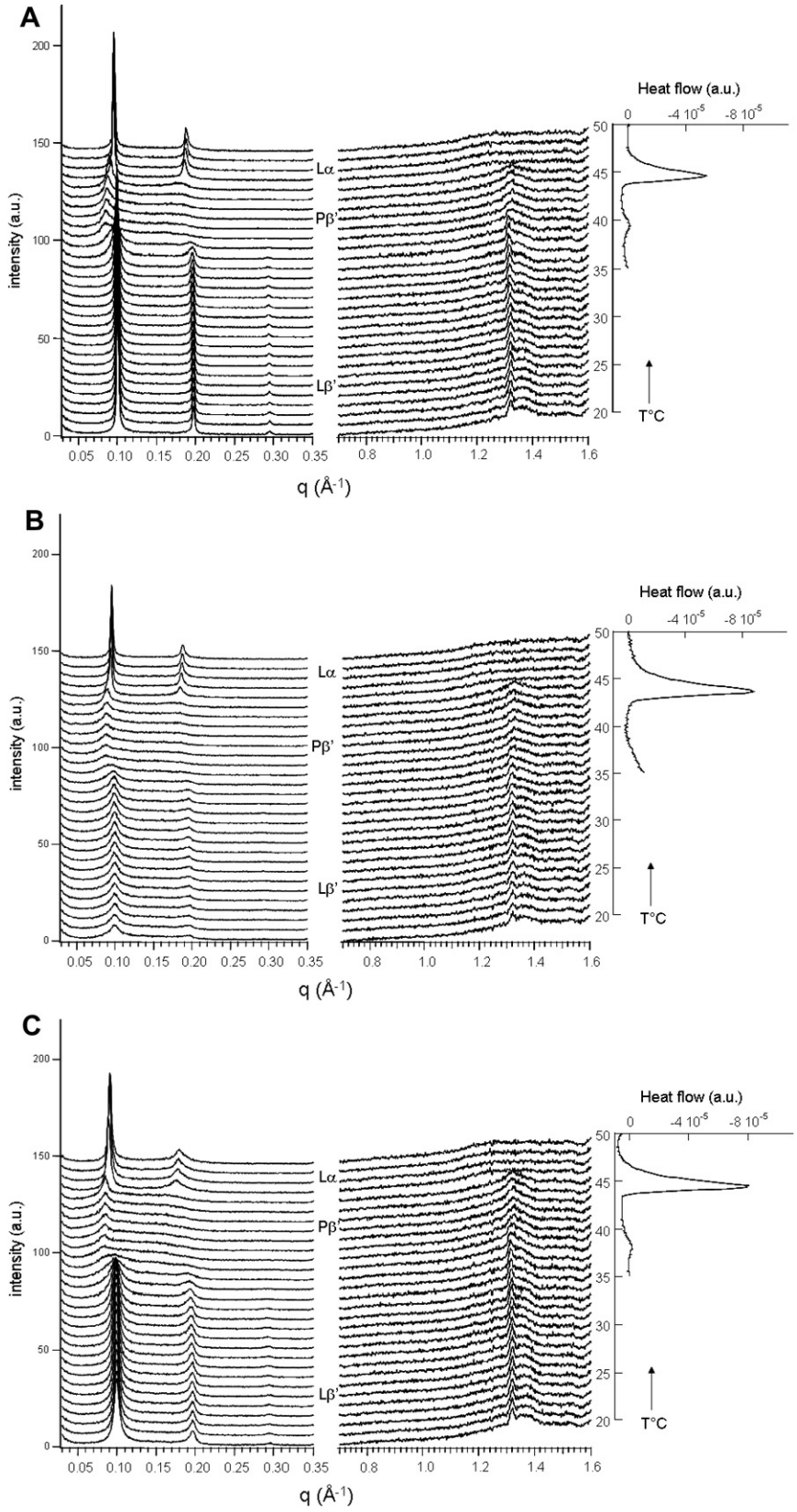


Fig. 1. X-ray diffraction coupled with the differential scanning calorimetry of DPPC hydrated with water (A), with a 5% metronidazole containing aqueous solution (B) and with a 5% ampicillin sodium salt containing aqueous solution (C). The graph at right represents the DSC signal (the temperatures are on the vertical axe) and the X-ray frames (SAXS at left and WAXS in the middle of the figure) have been obtained simultaneously during the heating.

Table 3
Summary of the most important XRD-DSC results

	d_{SAXS} (Å)			d_{WAXS} (Å)			Transition temperature[a] (°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

The correlation between the scattering vector (q) expressed in Fig. 1 and the repetitive distances (d) has been described in Section 2. In the present case, D_{saxs} correspond to the thickness of the lamellar (bilayer) organization and d_{waxs} correspond to the lateral repetitive distances.
[a] The transition temperature correspond to the T_{onset} temperature (beginning of the transition).

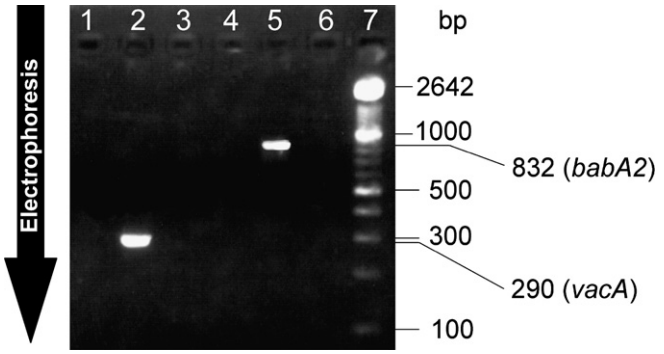


Fig. 2. PCR of *babA2* and *vacA* genes on strains 17875 and 149C. *vacA* (290 bp) for strains 17875 and 149C are in lanes 2 and 3, respectively. *babA2* (832 bp) for strains 17875 and 149C are in lanes 5 and 6, respectively. Lanes 1 and 4 are negative controls. Lane 7 is a 100 bp marker.

to compare the fluorescence superimposition due to the bacteria (DAPI) and to liposome (NBD-PC). 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 four green fluorescences is quite equivalent (Fig. 4, B1, B2, B3, and B4). For all the formulations, the superimposition of the two fluorescent dyes is very good (Figs. 3 and 4, columns C), suggesting that liposomes are "stuck" around bacteria. Because *H. pylori* can show two 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 Figs. 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 (Figs. 7 and 8, respectively).

In conclusion, with strain 17875, the best results in terms 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 result. 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.

4. Discussion

4.1. 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 trough gastric mucus dramatically decreases [40].

Liposomes made with epikuron 170 (F3 and F4) are more negatively charged than those made with DPPC (F1 and F2). This difference is probably due to the phospholipids used. Indeed epikuron is a mixture of several phospholipids (phosphatidylcholine > 72%, phosphatidylethanolamine > 10%, phosphatidylinositol < 3%, lysophosphatidylcholine < 4%, phosphatidic acid ~ 10%) and free fatty acids 10% in which the phosphate group of the phosphatic acid is non-protonated, leading to the more negative zeta potential of the epikuron vesicles [41]. 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 [19] and is probably due to the low molecular weight of our spacer (four ethylene glycol units).

4.2. 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

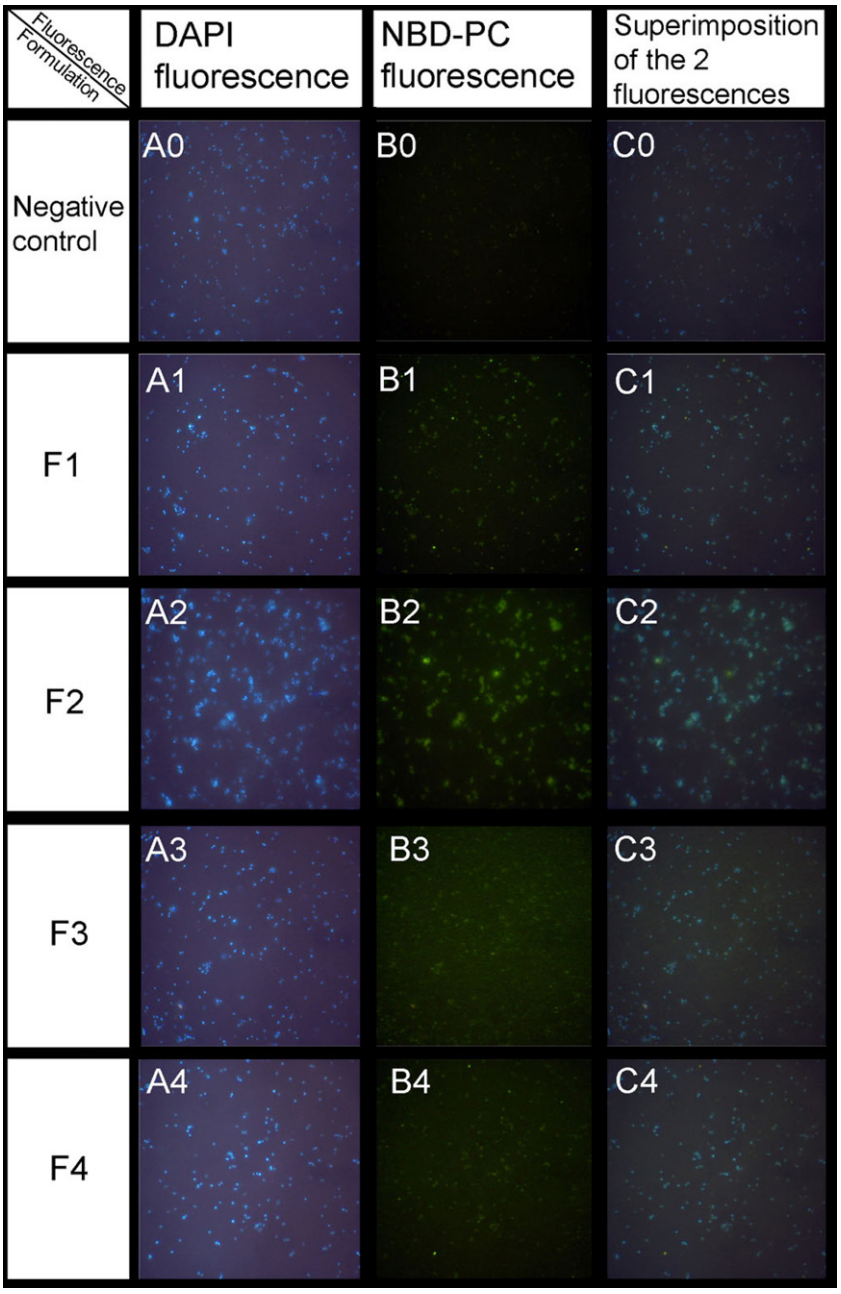


Fig. 3. Epifluorescence microscopy pictures of four liposomal formulations mixed with fresh strain 17875 of *H. pylori*. The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35] and the liposomes were labelled with NBP-PC. The formulations were DPPC (formulation F1) or epikuron 170 (formulation F3)–cholesterol–NBD-PC (79:20:01) and DPPC (formulation F2) or epikuron 170 (formulation F4)–Fuc-E_4-Chol–cholesterol–NBD-PC (79:10:10:01).

the product of the partition coefficient between hydrocarbon chains and water and the diffusion constant in the membrane [42]. The partition coefficient octanol/water (log Kow) for metronidazole was calculated to be −0.02 [43,44] 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), confirms a strong interaction between this antimicrobial agent and

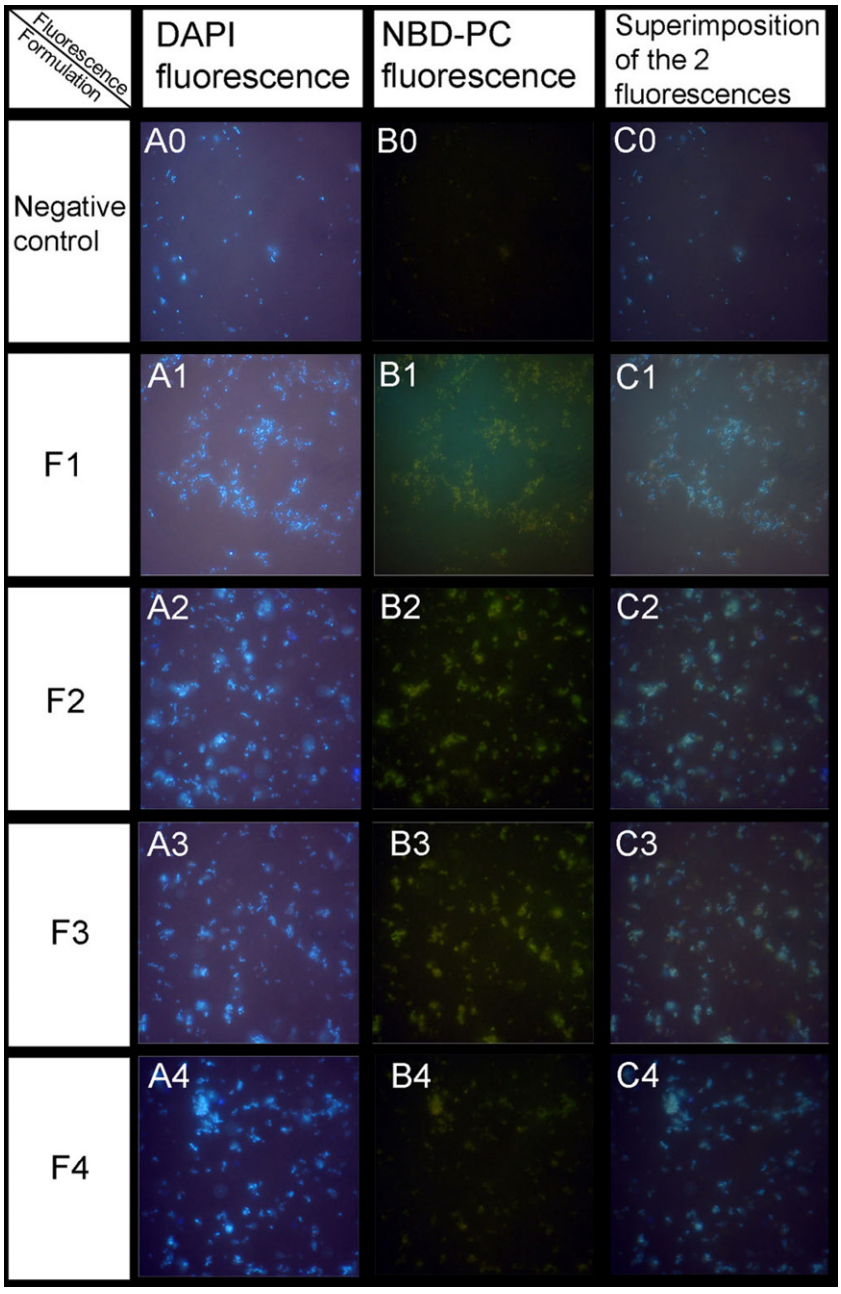


Fig. 4. Epifluorescence microscopy pictures of four liposomal formulations mixed with fresh strain 149C of *H. pylori*. The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35] and the liposomes were labelled with NBP-PC.

the phospholipids. The formation of the ripple phase is known to be very sensitive to the presence of foreign molecules [45–47]. 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 head groups [39,48]. 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), metronidazole is also more potent in terms 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. X-ray diffraction experiments on hydrated Epikuron (data not shown) have shown that it was under a fluid state at 20 °C. It is well known that the leakage of an encapsulated-drug inside a liposome dramatically increases when the vesicle is above

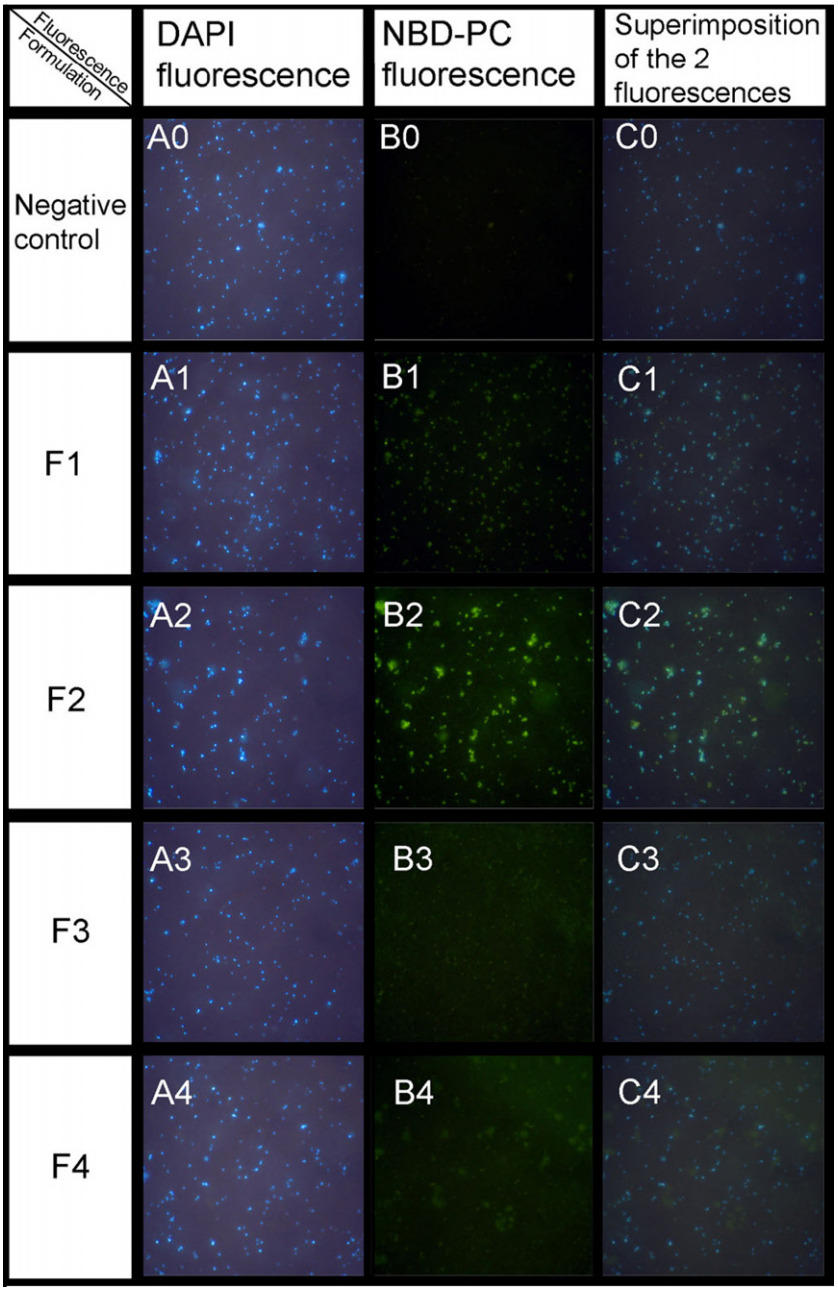


Fig. 5. Epifluorescence microscopy pictures of four liposomal formulations mixed with old strain 17875 of *H. pylori*. The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35] and the liposomes were labelled with NBP-PC.

its phase transition temperature, i.e. when the phospholipid 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 ($1.4 \pm 1.1\%$) in comparison with DPPC-based liposomes ($11.2 \pm 2.6\%$), 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 and 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

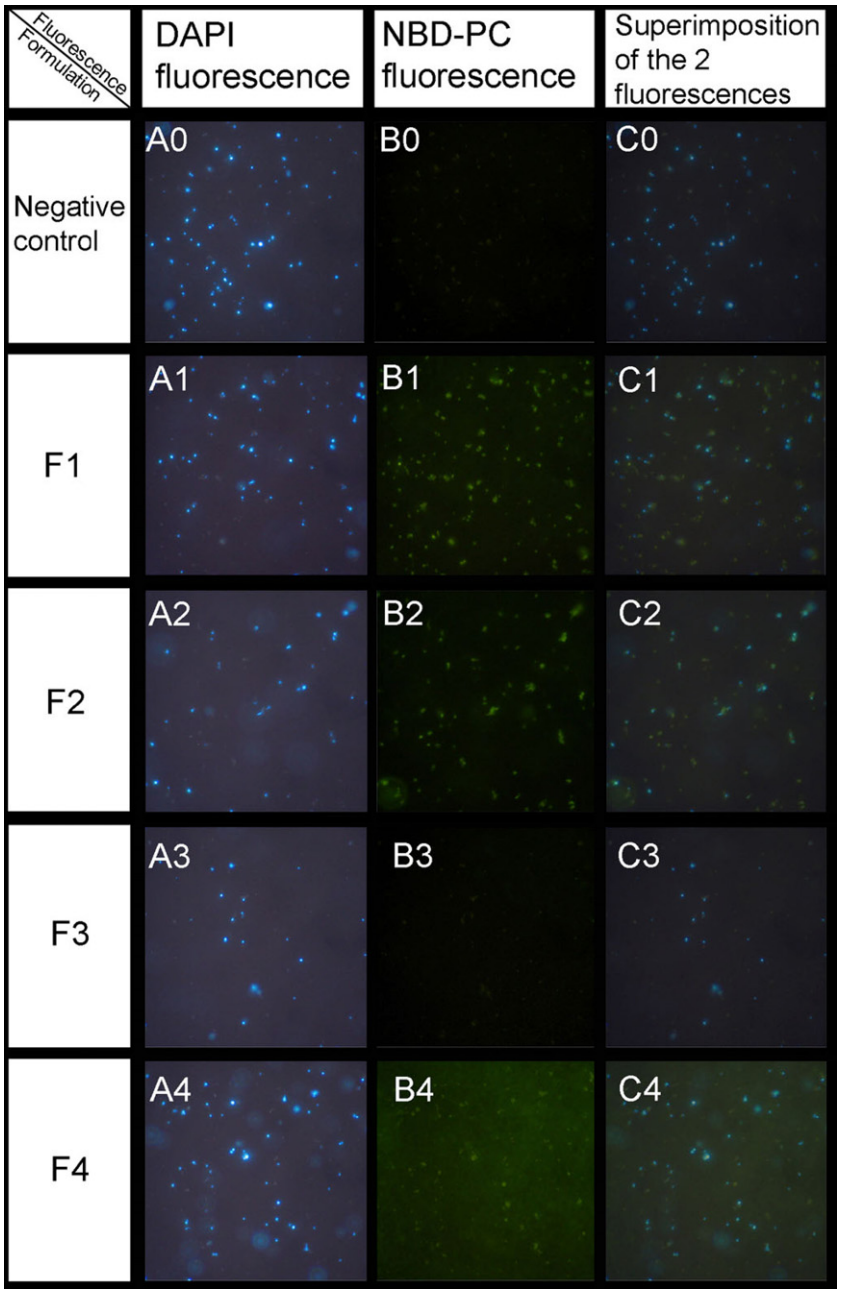


Fig. 6. Epifluorescence microscopy pictures of four liposomal formulations mixed with old strain 149C of *H. pylori*. The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35] and the liposomes were labelled with NBP-PC.

in case of allergy to β-lactams (MIC < 0.06 to 256 mg/L (extreme values)) [6]. Encapsulation efficiency for both drugs in liposomes of DPPC–Fuc-E_4-Chol–cholesterol (80:10:10) (cf. Table 2) is greatly sufficient to obtain a therapeutic effect, but the method can be improved and other drugs could probably be incorporated.

4.3. Liposome-bacteria interactions

Results obtained with epifluorescence microscopy show distinct behaviors according to the strain (17875 or 149C, fresh or old) and according to the formulation. From the results we have obtained, the interactions *H. pylori*-liposomes may be explained by four phenomena. The 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 [49]. The authors incubated cholesterol-free bacteria with cyclodextrin-mediated cholesterol and several cyclodextrin-mediated steroidal hormones (β-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 seven *H. pylori* strains tested while other steroidal hormones were

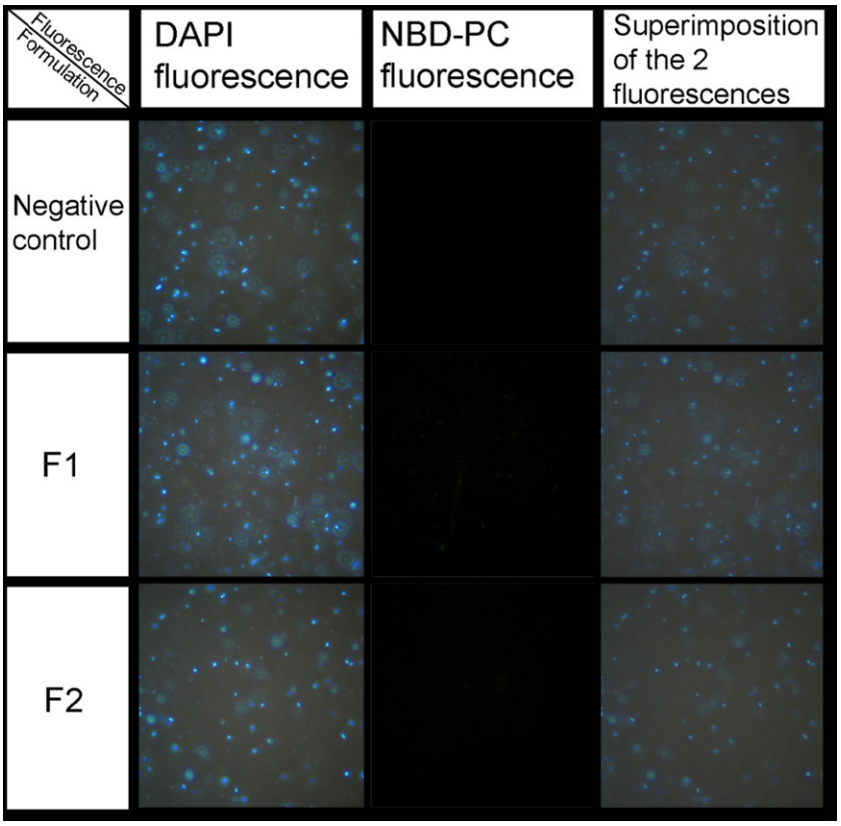


Fig. 7. Epifluorescence microscopy pictures of two liposomal formulations mixed with fresh strain CFT073 of *E. coli*. The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35] and the liposomes were labelled with NBP-PC.

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 four 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 [50,51] 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 two 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-E_4-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 [3,17]. 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 (Figs. 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 (Figs. 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. Fig. 9) [52]. The authors do not agree about the properties of the coccoid form. For some of them, it is a degeneration form of the bacterium [53] and for others, it is a resistance form [54]. But, it has been observed that the coccoid form can induce new gastric colonization in mice [55–57], it is able to survive in water [55] and probably to revert to the spiral form [58]. It should be note that antimicrobial agents could be one of the factors leading to

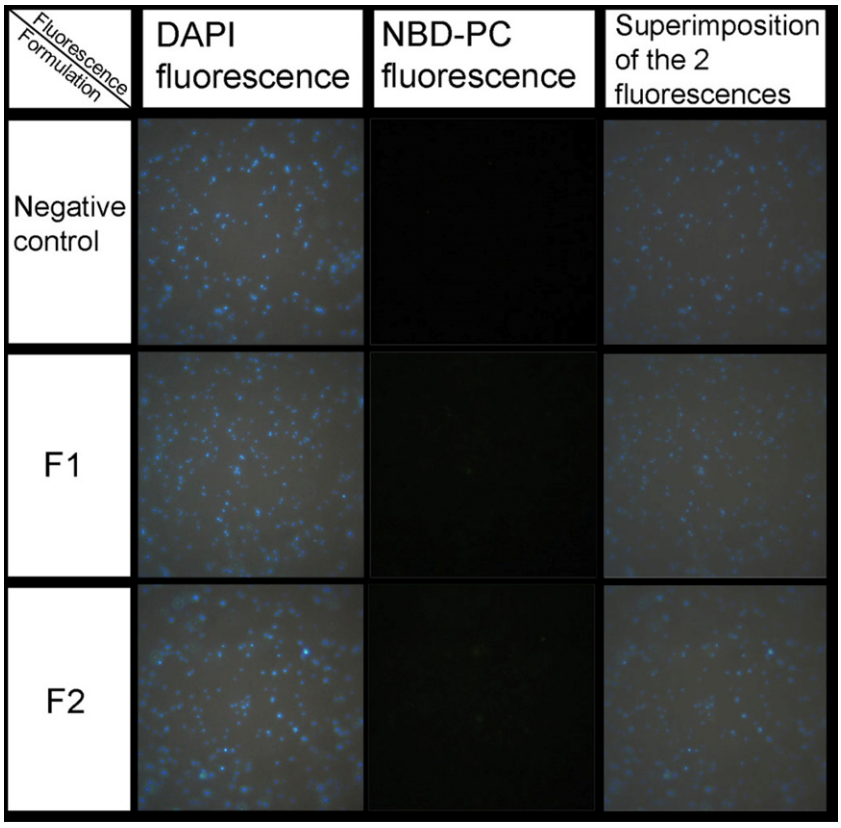


Fig. 8. Epifluorescence microscopy pictures of two liposomal formulations mixed with fresh strain ATCC 12228 of *S. epidermidis*. The bacteria were stained according to the fluorescent *in situ* hybridization technique (FISH) [35] and the liposomes were labelled with NBP-PC.

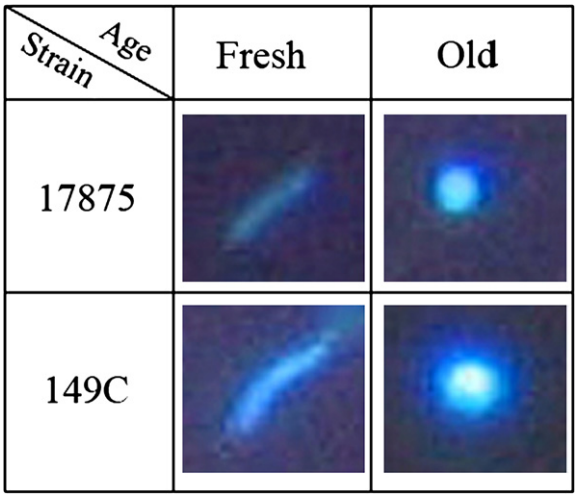


Fig. 9. Helicoid or coccoid shape of *H. pylori* in function of aging.

these morphological modifications. If the adhesion behavior of *H. pylori* is different between spiral or a coccoid forms, the last one is also able to adhere and alter gastric cells [59]. 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 (Figs. 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 [60,61]. 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*.

It is crucial to confirm that the interaction liposome–bacteria results in killing the organism. Preliminary in vitro experiments with liposomes containing ampicillin showed a clear antibacterial effect on *H. pylori* (data not published). Further experiments are planned to confirm this result, both in vitro and in vivo.

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References

[1] F. Mégraud, N. Broutet, Epidémiologie, acquisition et transmission d'*Helicobacter pylori*, La revue du praticien 50 (2000) 1414–1417.

[2] N. Broutet, Prévalence actuelle de l'infection à *Helicobacter pylori* et tendances évolutives en Europe, La lettre de l'infectiologue 15 (Suppl. 3) (2000) 28–29.
[3] S. Suerbaum, P. Michetti, *Helicobacter pylori* infection, N. Engl. J. Med. 347 (15) (2002) 1175–1186.
[4] J.C. Delchier, F. Roudot-Thoraval, A. Courillon-Mallet, H. Lamouliatte, J.F. Bretagne, J.D. De Korwin, A. Labigne, P. Vincent, F. Mégraud, J.L. Fauchère, Traitement de l'infection à *Helicobacter pylori* en pratique courante: résultats d'une enquête multicentrique nationale, La lettre de l'infectiologue 16 (Suppl. 3) (2001) 34.
[5] R. Mc Loughlin, I. Racz, M. Buckley, H.J. O'connor, C. O'morain, Therapy of *Helicobacter pylori*, Helicobacter 9 (Suppl. 1) (2004) 42–48.
[6] F. Mégraud, H. Lamouliatte, *Helicobacter pylori*, vol. 2, Clinique, Traitement., Collection Option Bio., Paris, 1997.
[7] S. Skouloubris, H. De Reuse, A. Labigne, Bactériologie et pathogénicité d'*Helicobacter pylori*, Rev. Prat. 50 (2000) 1409–1413.
[8] B. Marshall, *Helicobacter pylori*: 20 years on, Clin. Med. 2 (2) (2002) 147–152.
[9] C. Pagliaccia, X.M. Wang, F. Tardy, J.L. Telford, J.M. Ruysschaert, V. Cabiaux, Structure and interaction of VacA of *Helicobacter pylori* with a lipid membrane, Eur. J. Biochem. 267 (1) (2000) 104–109.
[10] E. Papini, M. Zoratti, T.L. Cover, In search of the *Helicobacter pylori* VacA mechanism of action, Toxicon 39 (11) (2001) 1757–1767.
[11] T.P.W. Mc Mullen, R.N. Mc Elhaney, New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry, Biochim. Biophys. Acta 1234 (1995) 90–98.
[12] H. Ohvo-Rekila, B. Ramstedt, P. Leppimaki, J. Peter Slotte, Cholesterol interactions with phospholipids in membranes, Prog. Lipid Res. 41 (1) (2002) 66–97.
[13] C. Hofsäss, E. Lindahl, O. Edholm, Molecular dynamics simulations of phospholipid bilayers with cholesterol, Biophys. J. 84 (2003) 2192–2206.
[14] B.D. Gold, M. Huesca, P.M. Sherman, C.A. Lingwood, *Helicobacter mustelae* and *Helicobacter pylori* bind to common lipid receptors in vitro, Infect. Immun. 61 (6) (1993) 2632–2638.
[15] C. Lingwood, M. Huesca, A. Kuksis, The glycerolipid receptor for *Helicobacter pylori* (and exoenzyme S) is phosphatidylethanolamine, Infect. Immun. 60 (6) (1992) 2470–2474.
[16] R.B. Umamaheshwari, P. Jain, N.K. Jain, Site specific drug delivery of acetohydroxamic acid for treatment of *H. pylori*, STP Pharma Sci. 13 (1) (2003) 41–48.
[17] D. Ilver, A. Arnqvist, J. Ogren, I.M. Frick, D. Kersulyte, E.T. Incecik, D.E. Berg, A. Covacci, L. Engstrand, T. Boren, *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging, Science 279 (5349) (1998) 373–377.
[18] R.B. Umamaheshwari, N.K. Jain, Receptor-mediated targeting of lipobeads bearing acetohydroxamic acid for eradication of *Helicobacter pylori*, J. Control. Release 99 (1) (2004) 27–40.
[19] P.L. Bardonnet, V. Faivre, F. Pirot, P. Boullanger, F. Falson, Cholesteryl oligoethyleneglycol glycosides: fluidizing effect of their embedment into phospholipid bilayers, Biochem. Biophys. Res. Commun. 329 (4) (2005) 1186–1192.
[20] J.A. Rogers, K.E. Anderson, The potential of liposomes in oral drug delivery, Crit. Rev. Ther. Drug Carrier Syst. 15 (5) (1998) 421–480.
[21] M.H. Richards, C.R. Gardner, Effects of bile salts on the structural integrity of liposomes, Biochim. Biophys. Acta 543 (4) (1978) 508–522.
[22] G.V. Betageri, S.A. Jenkins, D.L. Parsons, Liposome Drug Delivery Systems, Technomic Publishing Co. Inc, Lancaster, PA, 1993.
[23] J. Delattre, P. Couvreur, F. Puisieux, J.R. Philippot, F. Schuber (Eds.), Les Liposomes: Aspects Technologiques, Biologiques et Pharmacologiques, INSERM, Paris, 1993.
[24] R.N. Rowland, J.F. Woodley, The stability of liposomes in vitro to pH, bile salts and pancreatic lipase, Biochim. Biophys. Acta 620 (3) (1980) 400–409.
[25] A.S. Ulrich, Biophysical aspects of using liposomes as delivery vehicles, Biosci. Rep. 22 (2) (2002) 129–150.
[26] O. Freund, J. Amedee, D. Roux, R. Laversanne, In vitro and in vivo stability of new multilamellar vesicles, Life Sci. 67 (4) (2000) 411–419.
[27] M. Gelhausen, F. Besson, S. Chierici, D. Lafont, P. Boullanger, B. Roux, Lectin recognition of liposomes containing neoglycolipids. Influence of their lipidic anchor and spacer length, Colloids Surface B 10 (1998) 395–404.
[28] V. Faivre, V. Rosilio, P. Boullanger, L.M. Almeida, A. Baszkin, Fucosyled neoglycolipids: synthesis and interaction with a phospholipid, Chem. Phys. Lipids 109 (1) (2001) 91–101.
[29] F. Olson, C.A. Hunt, F.C. Szoka, W.J. Vail, D. Papahadjopoulos, Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes, Biochim. Biophys. Acta 557 (1) (1979) 9–23.
[30] J.C. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1) (1980) 10–14.
[31] G. Keller, F. Lavigne, L. Forte, K. Andrieux, M. Dahim, C. Loisel, M. Ollivon, C. Bourgaux, P. Lesieur, DSC and X-ray diffraction coupling: specifications and applications, J. Therm. Anal. 51 (1998) 783–791.
[32] M. Gerhard, N. Lehn, N. Neumayer, T. Boren, R. Rad, W. Schepp, S. Miehlke, M. Classen, C. Prinz, Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin, Proc. Natl. Acad. Sci. USA 96 (22) (1999) 12778–12783.
[33] J.C. Atherton, P. Cao, R.M. Peek, M.K.R. Tummuru, M.J. Blaser, T.L. Cover, Mosaicism in vacuolating cytotoxin alleles of *Helicobcicter pylori*. Association of specific VacA types with cytotoxin production and peptic ulceration, J. Biol. Chem. 270 (30) (1995) 17771–17777.
[34] C. Semino-Mora, S.Q. Doi, A. Marty, V. Simko, I. Carlstedt, A. Dubois, Intracellular and interstitial expression of *Helicobacter pylori* virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma, J. Infect. Dis. 187 (2003) 1165–1177.
[35] B. Bottari, D. Ercolini, M. Gatti, E. Neviani, Application of FISH technology for microbiological analysis: current state and prospects, Appl. Microbiol. Biotechnol. (2006).
[36] V. Faivre, V. Centis, P.-L. Bardonnet, F. Pirot, P. Boullanger, F. Falson, Glycosides of cholesteryl oligoethyleneglycols: synthesis and incorporation in bilayers, 30th Annual Meeting of the Controlled Release Society, Glasgow, july 19–23, 2003.
[37] G. Cevc, How membrane chain-melting phase-transition temperature is affected by the lipid chain asymmetry and degree of unsaturation: an effective chain-length model, Biochemistry 30 (29) (1991) 7186–7193.
[38] J.M. Seddon, R.H. Templer, Polymorphism of lipid-water systems, in: A.J. Hoff, R. Lipowsky, E. Sackmann (Eds.), Handbook of Biological Physics: Structure and Dynamics of Membranes, Elsevier SPC, Amsterdam, 1995, pp. 97–160.
[39] R. Koynova, M. Caffrey, Phases and phase transitions of the phosphatidylcholines, Biochim. Biophys. Acta (BBA) – Rev. Biomembr. 1376 (1) (1998) 91–145.
[40] D.A. Norris, N. Puri, P.J. Sinko, The effect of physical barriers and properties on the oral absorption of particulates, Adv. Drug Deliv. Rev. 34 (2–3) (1998) 135–154.
[41] V.C.F. Mosqueira, P. Legrand, A. Gulik, O. Bourdon, R. Gref, D. Labarre, G. Barratt, Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules, Biomaterials 22 (22) (2001) 2967–2979.
[42] A. Yamaguchi, R. Hiruma, T. Sawai, The effect of hydrophobicity of [beta]-lactam antibiotics on their phospholipid bilayer permeability, FEBS Lett. 164 (2) (1983) 389–392.
[43] G.E. Adams, I.R. Flockhart, C.E. Smithen, I.J. Stratford, P. Wardman, M.E. Watts, Electron-affinic sensitization. VII. A correlation between structures, one-electron reduction potentials, and efficiencies of nitroimidazoles as hypoxic cell radiosensitizers, Radiat. Res. 67 (1) (1976) 9–20.

[44] J.L. Davis, D. Little, A.T. Blikslager, M.G. Papich, Mucosal permeability of water-soluble drugs in the equine jejunum: a preliminary investigation, J. Vet. Pharmacol. Ther. 29 (5) (2006) 379–385.

[45] A. Tahir, C. Grabielle-Madelmont, C. Betrencourt, M. Ollivon, P. Peretti, A differential scanning calorimetry study of the interaction of Lasalocid antibiotic with phospholipid bilayers, Chem. Phys. Lipids 103 (1-2) (1999) 57–65.

[46] R.A. Videira, M.C. Antunes-Madeira, V.I.C.F. Lopes, V.M.C. Madeira, Changes induced by malathion, methylparathion and parathion on membrane lipid physicochemical properties correlate with their toxicity, Biochim. Biophys. Acta (BBA) – Biomembranes 1511 (2) (2001) 360–368.

[47] M.K. Jain, N.M. Wu, Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer: III. Phase transition in lipid bilayer, J. Membr. Biol. 34 (1) (1977) 157–201.

[48] S. Banerjee, A. Medina-Fatimi, R. Nichols, D. Tendler, M. Michetti, J. Simon, C.P. Kelly, T.P. Monath, P. Michetti, Safety and efficacy of low dose *Escherichia coli* enterotoxin adjuvant for urease based oral immunisation against *Helicobacter pylori* in healthy volunteers, Gut 51 (5) (2002) 634–640.

[49] C. Trampenau, K.D. Müller, Affinity of *Helicobacter pylori* to cholesterol and other steroids, Microbes Infect. 5 (2003) 13–17.

[50] J.I. Smith, B. Drumm, A.W. Neumann, Z. Policova, P.M. Sherman, In vitro surface properties of the newly recognized gastric pathogen *Helicobacter pylori*, Infect. Immun. 58 (9) (1990) 3056–3060.

[51] H. Pruul, C.S. Goodwin, P.J. Mcdonald, G. Lewis, D. Pankhurst, Hydrophobic characterisation of *Helicobacter (Campylobacter) pylori*, J. Med. Microbiol. 32 (2) (1990) 93–100.

[52] U. Heczko, V.C. Smith, R. Mark Meloche, A.M. Buchan, B.B. Finlay, Characteristics of *Helicobacter pylori* attachment to human primary antral epithelial cells, Microbes Infect. 2 (14) (2000) 1669–1676.

[53] J.G. Kusters, M.M. Gerrits, J.A. Van Strijp, C.M. Vandenbroucke-Grauls, Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death, Infect. Immun. 65 (9) (1997) 3672–3679.

[54] M. Benaissa, P. Babin, N. Quellard, L. Pezennec, Y. Cenatiempo, J.L. Fauchere, Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form, Infect. Immun. 64 (6) (1996) 2331–2335.

[55] F.F. She, J.Y. Lin, J.Y. Liu, C. Huang, D.H. Su, Virulence of water-induced coccoid *Helicobacter pylori* and its experimental infection in mice, World J. Gastroenterol. 9 (3) (2003) 516–520.

[56] E.M. Rabelo-Goncalves, N.F. Nishimura, J.M. Zeitune, Acute inflammatory response in the stomach of BALB/c mice challenged with coccoidal *Helicobacter pylori*, Mem. Inst. Oswaldo Cruz 97 (8) (2002) 1201–1206.

[57] P. Aleljung, H.O. Nilsson, X. Wang, P. Nyberg, T. Morner, I. Warsame, T. Wadstrom, Gastrointestinal colonisation of BALB/cA mice by *Helicobacter pylori* monitored by heparin magnetic separation, FEMS Immunol. Med. Microbiol. 13 (4) (1996) 303–309.

[58] M.I. Brenciaglia, A.M. Fornara, M.M. Scaltrito, F. Dubini, *Helicobacter pylori*: cultivability and antibiotic susceptibility of coccoid forms, Int. J. Antimicrob. Agents 13 (4) (2000) 237–241.

[59] E.D. Segal, S. Falkow, L.S. Tompkins, *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins, Proc. Natl. Acad. Sci. USA 93 (3) (1996) 1259–1264.

[60] B. Janas, E. Czkwianianc, L. Bak-Romaniszyn, H. Bartel, D. Tosik, I. Planeta-Malecka, Electron microscopic study of association between coccoid forms of *Helicobacter pylori* and gastric epithelial cells, Am. J. Gastroenterol. 90 (10) (1995) 1829–1833.

[61] W.Y. Chan, P.K. Hui, K.M. Leung, J. Chow, F. Kwok, C.S. Ng, Coccoid forms of *Helicobacter pylori* in the human stomach, Am. J. Clin. Pathol. 102 (4) (1994) 503–507.