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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

UNIVERSITÉ PARIS-SUD 11
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DU FONDAMENTAL A L'APPLIQUÉ

Nanoemulsões como sistemas de liberação de oligonucleotídeos anti-topoisomerase II de *Plasmodium falciparum*

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PORTO ALEGRE, 2012

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Tese apresentada por **Fernanda Bruxel** para a obtenção do grau de **doutor,** no curso de Doutorado em Ciências Farmacêuticas.

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ABBREVIATIONS

+/-	Positive (cationic lipid)/ negative (oligonucleotide) charge ratio
^{33}P-PO	5'-end labeled ^{33}P-phosphodiester antisense oligonucleotide
^{33}P-PS	5'-end labeled ^{33}P-phosphorothioate antisense oligonucleotide
DOTAP	1,2-dioleoyl-3-trimethyl ammonium-propane
E-lecithin	egg-lecithin
LCT	long-chain triglycerides
MCT	medium-chain triglycerides
NE	nanoemulsion
NE/PO	nanoemulsion/phosphodiester antisense oligonucleotide complex
NE/PS	nanoemulsion/phosphorothioate antisense oligonucleotide complex
OA	oleylamine
ON	antisense oligonucleotides
PCS	photon correlation spectroscopy
PDI	polydispersity index
PEG	polietilenoglicol
PGE1	prostaglandina E1
pLDH	lactate dehydrogenase
PO	antisense phosphodiester oligonucleotide
PS	antisense phosphorothioate oligonucleotide
S-lecithin	soy-lecithin
sPO	sense phosphodiester oligonucleotide
sPS	sense phosphorothioate oligonucleotide

SUMMARY

Nanoemulsions as delivery systems for oligonucleotides targeting *Plasmodium falciparum* topoisomerase II

The present study describes the technological, physicochemical and biological aspects of complexes formed between cationic nanoemulsions and oligonucleotides (ON) targeted toward *Plasmodium falciparum* topoisomerase II. Formulations were optimized by means of a 2^3 full factorial design. Two formulations composed of medium-chain triglycerides, egg yolk lecithin and either oleylamine or DOTAP cationic lipids were selected based on their physicochemical properties. Phosphodiester or phosphorothioate ONs were adsorbed onto cationic nanoemulsions. The adsorption isotherms indicated a progressive ON adsorption until a plateau was reached, following a Langmuir adsorption model. It was observed that a higher amount of ON was adsorbed onto the DOTAP-based nanoemulsions compared to those based on oleylamine. Further evidence of ON adsorption was obtained through the inversion of the zeta potential. Topographical and morphological analysis of the complexes, by transmission electron and atomic force microscopy, indicated ON adsorption onto the nanoemulsions. Strucutural organization in a lamellar phase was also identified by energy dispersive X-ray diffraction. ON binding and hemolysis increased along with the charge ratio of the complexes in healthy or *Plasmodium falciparum*-infected erythrocytes. Fluorescent probes co-encapsulated into the complexes (fluorescein and Nile red) were located inside the infected erytrocytes at the late stages of the parasite life cycle by confocal microscopy. Under non-hemolytic conditions, *Plasmodium falciparum* growth inhibition effect of around 80% and a delay in the parasite life cycle were observed *in vitro*. The overall results showed that the use of ON/cationic nanoemulsions complexes may represent a promising strategy for the treatment of *P. falciparum* infections.

Keywords: malaria, oligonucleotides, nanoemulsions, AFM, TEM, EDXD.

RESUMO

Nanoemulsões como sistemas de liberação de oligonucleotídeos anti-topoisomerase II de *Plasmodium falciparum*

O presente estudo descreve aspectos tecnológicos, físico-químicos e biológicos de complexos formados entre nanoemulsões catiônicas e oligonucleotídeos (ON) anti-topoisomerase II de *P. falciparum*. As formulações foram otimizadas através de um planejamento fatorial completo 2^3. Duas formulações compostas por triglicerídeos de cadeia média, lecitina de gema de ovo e pelo lipídeo catiônico oleilamina ou DOTAP foram selecionadas com base nas suas propriedades físico-químicas. ON fosfodiéster ou fosforotioato foram adsorvidos nas nanoemulsões catiônicas. As isotermas de adsorção indicam uma progressiva adsorção dos ON até que um platô seja atingido, descritos através do um modelo de adsorção de Langmuir. Foi detectada uma maior quantidade de ON adsorvida em nanoemulsões contendo DOTAP em comparação com as constituídas de oleilamina. Evidências suplementares da adsorção dos ON foram demonstradas através da inversão do potencial zeta. Análises topográficas e morfológicas, obtidas através de microscopia eletrônica de transmissão e de força atômica, sugerem a adsorção dos ON nas nanoemulsões. A organização estrutural em fase lamelar foi identificada através de difração de raios-X de energia dispersiva. A associação dos ON e hemólise aumentam com a relação de cargas dos complexos em eritrócitos sadios ou infectados por *P. falciparum*. Sondas fluorescentes (co-associadas nos complexos: fluoresceína e vermelho do Nilo) foram localizados em eritrócitos infectados nas etapas tardias do ciclo evolutivo dos parasitos por microscopia confocal. Em condições não hemolíticas, um efeito de inibição de cerca de 80% do crescimento de *P. falciparum* e um atraso no ciclo biológico do parasito foi observado *in vitro*. O conjunto dos resultados demonstra que o uso de complexos ON/nanoemulsões catiônicas podem representar uma estratégia promissora para o tratamento das infecções por *P. falciparum*.

Unitermos: malária, oligonucleotídeos, nanoemulsões, MET, MFA, EDXD.

RÉSUMÉ

Nanoémulsions comme systèmes de libération des oligonucléotides anti- topoisomérase II de *Plasmodium falciparum*

La présente étude décrit des aspects technologiques, physico-chimiques et biologiques des complexes formés entre nanoémulsions cationiques et des oligonucléotides (ON) anti-topoisomérase II du *Plasmodium falciparum*. Les formulations ont été optimisées au travers d'un plan factoriel 2^3 complet. Deux formulations composées par des triglycérides à chaîne moyenne, de la lécithine de jaune d'œuf et un lipide cationique oléylamine ou DOTAP ont été choisis en fonction de leur proprietés physico-chimiques. ON phosphodiester ou phosphorothioate ont été adsorbés sur des nanoémulsions cationiques. Les isothermes d'adsorption indiquent une adsorption progressive des ON jusqu'à ce qu'un plateau soit atteint, décrit par un modèle d'adsorption de Langmuir. Il a été détecté une quantité plus élevé d'ON adsorbés sur des formulations contenant le DOTAP par rapport à celles constituées par oléylamine. Des évidences supplémentaires de l'adsorption des ON ont été démontrées par l'inversion du potentiel zeta. L'analyse topographique et morphologique des complexes, obtenue par microscopie électronique à transmission et à force atomique, suggère l'adsorption des ON sur les nanoémulsions. L'organisation strucutrale dans une phase lamellaire a été identifiée par diffraction des rayons X d'énergie dispersive. L'association des ON et l'hémolyse augmentent avec le rapport de charge de complexes sur les érythrocytes sains ou infectés par *Plasmodium falciparum*. Des sondes fluorescentes (coassociées dans les complexes: fluorescéine et rouge du Nil) ont été localisées dans les érytrocytes infectés dans les étapes tardives du cycle d'évolution des parasites par microscopie confocale. Dans des conditions non hémolytiques, un effet d'inhibition d'environ 80% de la croissance de *P. falciparum* et un retard du cycle du parasite ont été observés *in vitro*. Les résultats globaux montrent que les complexes ON/nanoemulsions cationiques peuvent represerter une stratégie prometteuse contre les infections par *Plasmodium falciparum*.

Mots-clés: paludisme, oligonucléotides, nanoémulsions, AFM, TEM, EDXD.

INTRODUCTION

Malaria is a parasitic disease affecting 247 million people worldwide, causing more than 800,000 deaths annually, mainly of children, women and immunocompromised patients. The disease occurs in more than 100 countries, mainly those located in tropical and subtropical regions of the world. Africa and Asia are the regions most affected, but in Brazil 315,000 new cases are detected annually (WHO, 2011b). Malaria is caused by *Plasmodium* parasites, which are transmitted through the bites of an infected Anopheles mosquito. There are more than 100 species of *Plasmodium*, however, only five can cause the disease in humans: *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum* and *P. knowlesi*. The latter species originally infected monkeys, but was recently recognized as causing malaria in humans. The patient symptoms by any of these parasites may be severe, but those caused by *P. falciparum* are considered to be the most serious and can even be fatal (CDC, 2011; WHO, 2011b).

The *Plasmodium* life cycle involves the mosquito and human hosts. In humans, there is a liver and an erythrocytic phase. Merozoites are released from hepatocytes and invade erythrocytes in the bloodstream, where they multiply through ring stages, trophozoites and schizonts over a period of 48 hours for *P. falciparum* species (BRAGA and FONTES, 2005; GRIFFITH *et al.*, 2007; CDC, 2011). The chemotherapeutic strategies to treat *Plasmodium* infections remain a real challenge considering the complexity of the parasite life cycle and its biological versatility (VALE *et al.*, 2005; SANTOS-MAGALHAES and MOSQUEIRA, 2010).

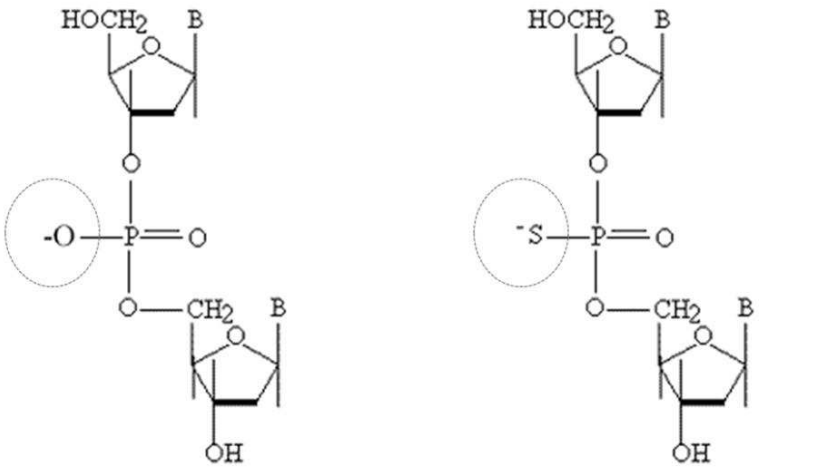
Efforts have been made to combat malaria worldwide, with international funds reaching 2 billion dollars in 2011. Internationally agreed goals and targets to eradicate the disease are reported in the Global Malaria Action Plan, created by the Roll Back Malaria Partnership, which includes over 500 institutions (CDC, 2011). However, the main challenge in controlling the disease is the drug resistance (EKLAND and FIDOCK, 2008; WHO, 2011b). Resistance has been reported to all antimalarial drugs, including artemisinin derivatives, which had been kept as a last effective alternative (NA-BANGCHANG and CONGPUONG, 2007; EKLAND and FIDOCK, 2008; DONDORP *et al.*, 2009; WITKOWSKI *et al.*, 2010).

The Global Plan for Artemisinin Resistance Containment was established in response to confirmation of artemisinin-resistance in Cambodia and Thailand, and concerns that resistance could either spread or emerge spontaneously elsewhere (WHO, 2011a). Thus, the development of new therapeutic strategies is an essential aspect of malaria control.

In this context, the potential use of nucleic acids in malaria treatment has been investigated (NOONPAKDEE *et al.*, 2003; FÖGER *et al.*, 2006). The use of single-stranded nucleic acids, named antisense oligonucleotides (ON), has been extensively described in the literature. Such molecules are able to interfere specifically in gene expression, inhibiting parasite growth (RAPAPORT *et al.*, 1992; RAMASAMY *et al.*, 1996; KANAGARATNAM *et al.*, 1998; WANIDWORANUN *et al.*, 1999; NOONPAKDEE *et al.*, 2003; BAKER and NAGUIB, 2005; FÖGER *et al.*, 2006). The specificity, the stability of the interaction between an ON sequence and a target messenger RNA and the resulting biological effects are dependent on many factors, such as the secondary structure of the target RNA, the location of the hybridization site within the RNA, the GC content in the ON and the annealing temperature. To the best of our knowledge, no specific algorithm that indicates the best sequence for a given target is available. In fact, sequences have been very often selected from the screening of various sequences, generated by computer models and expert researchers (CHAN *et al.*, 2006).

Recently, some authors have described the specific inhibition of *P. falciparum* growth by ONs targeted toward the parasite topoisomerase II enzyme (NOONPAKDEE *et al.*, 2003; FÖGER *et al.*, 2006). This enzyme is responsible for controlling the topological structure of DNA, by inserting or removing phosphodiester bridges in the DNA molecule. It plays an essential role in parasite replication and transcription processes (ZAHA *et al.*, 1996). The predicted protein product of the *P. falciparum* topoisomerase II gene differs from the sequence of the human enzyme in its possession of two asparagine-rich insertions (CHEESMAN et al., 1994).

Despite the great therapeutic potential of ONs, therapeutic applications are limited by their poor intracellular penetration and stability. ON transport into cells is limited by the high molecular weight and the polyanionic nature of these molecules, due to electrostatic repulsion with cellular membranes (JAASKELAINEN and URTTI, 2002). The stability in biological fluids is also hampered by a rapid degradation by nucleases, limiting their applications *in vivo* (OPALINSKA and GEWIRTZ, 2002). Some strategies have been proposed to overcome these drawbacks, such as the chemical modification of ONs within inter-nucleoside linkages, sugars or nitrogenated bases (COUVREUR and MALVY, 2000). The replacement of non-binding oxygens of the phosphodiester group by an atom of sulfur generates the phosphorothioate ON (Figure 1). This modification strategy is very often used due to the improved stability of the phosphorothioate ON in biological fluids (TANG and HUGHES, 1999; STAHEL and ZANGEMEISTER-WITTKE, 2003). Nevertheless, previous reports in the literature have described that these ONs are mainly related to non-specific interactions with proteins (OPALINSKA and GEWIRTZ, 2002; TOUB *et al.*, 2006).



Phosphodiester antisense oligonucleotide (PO)

Phosphorothioate antisense oligonucleotide (PS)

Figure 1. Schematic representation of phosphodiester (PO) and phosphorothioate (PS) antisense oligonucleotides.

To circumvent these drawbacks, the association of ONs to lipid carriers has been proposed (NAM *et al.*, 2009; VERISSIMO *et al.*, 2010), an example of which is cationic nanoemulsion (TEIXEIRA *et al.*, 1999). The use of intravenous nanoemulsions is well established in the literature because of their application in parenteral nutrition since the 1960s (FLOYD, 1999). The use of the parenteral route is especially advantageous when the target is located inside the red blood cells, as in malaria infections (WHITE *et al.*, 2009).

In the ON delivery field, nanoemulsions are composed of an oil core stabilized by a binary mixture of phospholipids and cationic lipids. The addition of cationic lipids is a key consideration for the association of the ON with the colloidal structure through electrostatic interactions between the negatively-charged ON and the positively-charged cationic lipid (TEIXEIRA *et al.*, 1999; TRIMAILLE *et al.*, 2003; MARTINI *et al.*, 2007). After complexation, an increase in both the ON intracellular penetration and stability has been demonstrated (TEIXEIRA *et al.*, 2003; HAGIGIT *et al.*, 2008). Recent reports have also described the possibility of modulating the association and release of nucleic acids from nanoemulsions, by selecting the type of cationic lipid and/or phospholipid, as well as the charge ratio of the final complexes (MARTINI *et al.*, 2007; FRAGA *et al.*, 2008; MARTINI *et al.*, 2008).

Considering the potential of cationic nanoemulsions as ON carriers and the proposed antisense strategy for malaria treatment (NOONPAKDEE *et al.*, 2003; FÖGER *et al.*, 2006) , we recently described an analytical spectrophotometric method to evaluate the association of anti-malarial ON with cationic nanoemulsions (BRUXEL *et al.*, 2011). In this context, to complement our results, this study describes the technological, physicochemical and biological aspects of the complexes formed between nanoemulsions and phosphodiester or phosphorothioate ONs, targeted toward *P. falciparum* topoisomerase II. This dissertation is presented in four chapters, as follows:

— **Chapter 1** presents a review article summarizing different aspects of nanoemulsions as delivery systems intended for parenteral administration;

— Studies concerning the optimization and characterization of ON/nanoemulsions complexes are described in **Chapter 2**;

— The structural organization of the complexes formed between ON and nanoemulsions is investigated in **Chapter 3**;

— Finally, **Chapter 4** reports the results of biological experiments on the interaction of complexes with erythrocytes and their inhibition of *P. falciparum* growth.

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OBJECTIVES

MAIN OBJECTIVE

This doctoral dissertation describes an investigation on cationic nanoemulsions as delivery systems for antisense oligonucleotides against the topoisomerase II enzyme of *Plasmodium falciparum*, and their potential in inhibiting parasite growth.

SPECIFIC OBJECTIVES

- To optimize the cationic nanoemulsions composition by means of a 2^3 full factorial design
- To determine the phosphodiester and phosphorothioate oligonucleotides adsorption isotherms for the optimized cationic nanoemulsions
- To characterize the physicochemical properties and morphology of the optimized cationic nanoemulsion/oligonucleotide complexes
- To evaluate the structural organization of complexes formed between oligonucleotides and the cationic nanoemulsions
- To investigate the interactions between cationic nanoemulsion/oligonucleotide complexes on healthy and *Plasmodium falciparum*-infected erythrocytes
- To evaluate the ability of the nanoemulsion/ON complexes to inhibit *Plasmodium falciparum* growth

CHAPTER 4

Cationic nanoemulsion as a delivery system for oligonucleotides targeting malarial topoisomerase II

According to previous literature, an alternative strategy based on the antisense oligonucleotides (ON) against the *Plasmodium falciparum* topoisomerase II has been considered for malaria treatment. Since phosphodiester and chemically modified phosphorothioate ON can be efficiently adsorbed on DOTAP-nanoemulsions, resulting in low hemolytic nanoemulsion/ON complexes, it was considered a promising strategy for the ON delivery. In this context, their biological effects were evaluated in *Plasmodium falciparum* cultures, what is presented in this chapter 4. Firstly, complexes were produced at different +/- charge ratios and their physicochemical properties were determined before and after dilution in culture medium, where their stability was studied over time. In parallel, their interaction with erythrocytes parasitized or not by *P. falciparum* was evaluated through hemolysis, binding experiments and confocal microscopy. The latter technique allowed the intracellular localization of ON and nanoemulsions in parasitized erythrocytes. Finally, the parasite growth inhibition and reinfection capacity were evaluated. The results showed that oligonucleotide-loaded nanoemulsions were found to be located inside the infected erythrocytes, inhibiting efficiently parasite growth (until 80%) and causing a delay in *P. falciparum* life cycle.

Cationic nanoemulsion as a delivery system for oligonucleotides targeting malarial topoisomerase II

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4.1 INTRODUCTION

Malaria is one of the most widespread parasitic diseases. It is currently endemic in more than 100 countries in tropical and subtropical areas. The mortality rate related to malaria is currently estimated at over one million people per year and has increased, most likely due to parasite drug resistance (WHO, 2008). Since the emergence of chloroquine resistance in the 1950s, antimalarial drug resistance for *Plasmodium falciparum*, is considered a major contributor to the global resurgence of malaria observed over the past 40 decades and one of the main hindrance for an effective control (HASTINGS *et al.*, 2007; NA-BANGCHANG and CONGPUONG, 2007). The emergence of artemisinin resistance is one of the greatest threats to renewed efforts to eradicate malaria (GREENWOOD *et al.*, 2008; DONDORP *et al.*, 2009). There is therefore an urgent need for the development of new drugs for malaria treatment.

Antisense oligonucleotides (ON) have been used to selectively modulate the expression of genes as well as to inhibit protein synthesis. Over the past decade, they have been considered as a potential strategy for malaria, especially since several studies have shown that antisense ON targeting of different enzymes, antigens, and other targets can inhibit *in vitro P. falciparum* growth (BARKER *et al.*, 1996; KANAGARATNAM *et al.*, 1998; WANIDWORANUN *et al.*, 1999; NOONPAKDEE *et al.*, 2003). However, antisense ON-based therapy is limited by both rapid degradation of ON in biological fluids as well as their inability to efficiently cross cell membranes due to their hydrophilic character and high molecular structure (OPALINSKA and GEWIRTZ, 2002).

To circumvent these drawbacks, the association of ON with both polymer- or lipid-based colloidal carriers has been proposed. For instance, Föger and co-workers (FÖGER *et al.*, 2006) have recently described, for the first time, the incorporation of phosphorothioate antisense ON targeting malarial topoisomerase II into biocompatible chitosan nanoparticles. The complexes showed a more pronounced sequence specific inhibition of parasite growth compared to free antisense ON. Moreover, the

association of ON with nanoparticles resulted in their protection against nuclease attack *in vitro*.

Lipid injectable emulsions have been used as sources of calories and essential fatty acids for patients for at least 40 years. Their low toxicity makes them a good alternative as an intravenous delivery system (DRISCOLL, 2006). Triglyceride-based emulsions stabilized by phospholipid and cationic lipid combinations have been studied as potential delivery systems for ON (TEIXEIRA *et al.*, 2001; TEIXEIRA *et al.*, 2003). ON molecules can be associated to oil droplets through ion-pair formation, due to the positively charged nanoemulsions and the negatively charged ON (TEIXEIRA *et al.*, 2001). More recently, the cationic lipid composition of an emulsion has been optimized to obtain the best conditions for the adsorption and release of a model ON (oligothymidilates) from nanoemulsions (MARTINI *et al.*, 2007; MARTINI *et al.*, 2008). Both electrostatic and hydrophobic interactions were found to play a role in the complexation process.

The purpose of this study was to evaluate the potential of a cationic nanoemulsion (NE) as a delivery system for antisense ON targeting *P. falciparum* topoisomerase II. The ON sequences used here were previously described to be specific by Noonpakdee et al. (NOONPAKDEE *et al.*, 2003) and Föger et al. (FÖGER *et al.*, 2006). Phosphodiester antisense ON (PO) and chemically modified phosphorothioate antisense ON (PS) were complexed to the cationic emulsion. The physicochemical properties of the whole colloidal system as well as its interaction with erythrocytes though hemolysis, binding experiments and confocal microscopy were determined. Finally, we have evaluated the potential of such a system to inhibit *in vitro* parasite growth.

4.2 MATERIAL AND METHODS

4.2.1 Materials

Medium-chain triglycerides – MCT (Lipoid AG, Germany), egg lecithin – Lipoid E-80 (Lipoid AG, Germany), dioleoyltrimethylammonium propane - DOTAP (Sigma, USA), nile red (Sigma, USA), and glycerol (Merck, Brazil) were used for the preparation of NE. Ultrapure water was obtained from a Milli-Q apparatus (Millipore, France). The ON sequences were obtained as antisense (5' ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT 3') or sense (5' AGA ATC GTA TGG TTC AAA AGA ATA TTA CAT 3') within the structural region of *P. falciparum* topoisomerase II. Phosphodiester antisense ON (PO, MW = 9146 g/mol), phosphodiester sense ON (sPO, MW = 9261 g/mol), full chemically modified phosphorothioate antisense ON (PS, MW = 9610 g/mol), full chemically modified phosphorothioate sense ON (sPS, MW = 9727 g/mol), 5'-end covalently conjugated with fluorescein isothiocyanate phosphodiester antisense ON (FITC-PO) and phosphorothioate antisense ON (FITC-PS) were purchased from Eurogentec (Angers, France). 5'-end labeled ^{33}P-phosphodiester antisense ON (^{33}P-PO) or ^{33}P-phosphorothioate antisense ON (^{33}P-PS) were synthesized using T4 polynucleotide kinase (Biolabs, U.K.) and ^{33}P ATP (Isotopchim, France).

4.2.2 Preparation and characterization of NE

NE composed of 8% (w/w) MCT, 2% (w/w) egg lecithin, 0.132% (w/w) DOTAP, 2.25% (w/w) glycerol and water to 100% (w/w), were prepared through spontaneous emulsification as previously described (MARTINI *et al.*, 2008). Briefly, a limpid ethanolic solution containing the oily phase components was slowly added to a water phase containing glycerol, under moderate magnetic stirring. The excess of solvents mixture (ethanol/water) was then removed under reduced pressure at 50°C until the desired final volume (5 ml). The final cationic lipid concentration was 2 mM, as previously optimized (MARTINI *et al.*, 2008). The mean droplet size and zeta

potential were determined through photon correlation spectroscopy and electrophoretic mobility (Zetasizer Nano ZS, Malvern Instrument, UK), respectively, at 20°C. The NE were adequately diluted in water for size and polydispersity index (PDI) determinations or in 1 mM NaCl solution for zeta potential measurements.

4.2.3 Preparation and characterization of NE/ON complexes

Antisense ON (PO and PS) adsorption on cationic NE was performed at the end of the manufacturing process, resulting in NE/ON (NE/PO and NE/PS) complexes. Increasing concentrations of NE were added to water solutions of PO and PS at 10 µM (final concentration) and incubated during 15 minutes at room temperature, as described elsewhere (TEIXEIRA *et al.*, 1999). The physicochemical properties and morphology were evaluated (as described in section 2.2) for NE/PO and NE/PS complexes. They were prepared at four different +/- charge ratios (ratios calculated between the number of positive charges from the cationic lipid present in NE, and the number of negative charges from the phosphate groups of ON) that are +0.5/-, +2/-, +4/- and +6/-.

4.2.4 Hemolysis experiments

Experiments were performed using both non-parasited and *P. falciparum*-infected erythrocytes Blood was obtained from healthy O^+ human blood and erythrocytes were collected after centrifugation. A suspension of erythrocytes (5%) in RPMI medium (Invitrogen, France), supplemented with 25 mM HEPES (Sigma, France), 25 mM $NaHCO_3$ (Sigma, France), 0.5% Albumax (Invitrogen, France) and hypoxanthine 367 µM (Sigma, France) was prepared (same conditions as for the *in vitro* experiments) and carefully distributed in 24-well plates.

Three sets of experiments were performed with NE/ON complexes: (a) NE/PO and NE/PS complexes obtained at different +/- charge ratios (+0.5/-, +2/-, +4/-, and +6/-),

in a final PO and PS concentrations of 10 µM were incubated with non-infected erythrocytes for 15 minutes; (b) An hemolysis kinetics was performed on the complex at a charge ratio of +4/-, which was incubated with non-infected erythrocytes for 5, 15, 30, 60, and 180 minutes; (c) the complex at a charge ratio of +4/- was incubated with *P. falciparum*-infected erythrocytes for 15 minutes and 44 hours hours. Besides, the hemolytic effect of unloaded NE was also evaluated over infected and non-infected erythrocytes for 44 hours. The samples were then centrifugated and supernatants were analyzed with respect to hemoglobin release by measuring the absorbance at 570 nm. NaCl 0.9% (w/v) and Triton 100 (5% w/v) were used as negative and positive controls, respectively. Results were expressed as the percentage of the amounts of hemoglobin release caused by complexes as percent of the total amount.

4.2.5 Erythrocytes binding experiments

Prior to binding experiments, ^{33}P-PO and ^{33}P-PS were obtained as previously described (AYNIE *et al.,* 1996). Briefly, 5'-radiolabeling of ON (PO and PS) were carried out as follows: 5 µl of a solution of PO or PS (10µM), 1 µl of T4 polynucleotide kinase, and 1µl of β-33P-ATP (sp act 7.4 Bq/mmol) were incubated for 30 minutes at 37°C. The reaction was stopped by heating the preparations at 80°C for 15 minutes ^{33}P-PO and ^{33}P-PS was recovered after purification by exclusion chromatography and centrifugation at 2400 rpm for 1 minute. The purity of ^{33}P-PO and ^{33}P-PS was evaluated using an automatic TLC-linear analyzer. The binding of complexes was assessed by three sets of experiments, as described in section 2.4. After incubation, cells were separated by centrifugation and treated with Triton 100 (1% v/v) at room temperature. The amount of radioactivity in the cell lysate was determined through liquid scintillation counting (LS6000 TA, Beckman- Coulter, USA), using Hionic-Fluor liquid scintillation cocktail (PerkinElmer, France). Results were expressed as the percentage of radioactivity found in cell pellets of the total amount of radioactivity with cells.

4.2.6 Stability of complexes in culture medium

Stability of complexes was carried out at 37°C in supplemented RPMI culture medium (composition described in section 2.4). The complexes tested where NE/PO and NE/PS at charge ratios of +0.5/-, +2/- and +4/-. The stability of unloaded NE (at the same proportion as present in the +0.5/-, +2/- and +4/- complexes) was also evaluated. Samples were diluted 10 times in the culture medium (as for the *in vitro* tests), whereas the mean droplet size and PDI were determined directly 15 minutes, 1, 8, 24 and 44 hours after dilution.

4.2.7 Growth inhibition of *P. falciparum in vitro*

P. falciparum 3D7 strain was maintained in O^+ human erythrocytes in albumin RPMI supplemented medium (composition described in section 2.4), under continuous culture using the candle-jar method (TRAGER and JENSEN, 1976). The parasites were synchronized to the ring stage by repeated sorbitol treatment (LAMBROS and VANDERBERG, 1979). A 5% (v/v) erythrocytes suspension with 0.5% parasitemia (number of parasites per 100 red blood cells) was incubated with the tested formulations: sense phosphodiester ON (sPO), sense phosphorothioate ON (sPS), antisense phosphodiester ON (PO) and phosphorothioate ON (PS) solutions, NE/sPO, NE/sPS, NE/PO and NE/PS complexes in the charge ratio of +4/-. All of them were prepared at a concentration of ON of 10 µM (final concentration of 1 µM in the cell suspension). Parasites were also incubated with culture medium (negative control) or with 4 µM chloroquine (positive control) in 96-well culture plates. In addition, the unloaded NE was tested at the same concentration as the one present in the +4/- complexes (6.2 mg of inner phase/mL of culture medium). After 44 h incubation at 37 ^oC, the plates were subjected to 3 freeze-thaw cycles to achieve complete hemolysis. The parasite growth was determined by the ELISA-Malaria antigen test (DiaMed, France), for the detection of *P. falciparum* lactate dehydrogenase (pLDH), as well as by microscopic examination under oil immersion of Giemsa stained thin blood smears.

Results were expressed as the percentage of reduction of parasite growth over the control receiving only the culture medium.

Parallel parasite cultures at 2% of parasitemia were used for the reinfection test. The NE/PO and NE/PS complexes were incubated with infected red blood cells containing parasites in early stage growth forms (ring) and/or mature growth forms (trophozoites) for 44 hours. Blood smears were prepared after 24 and 44 hours, by Giemsa staining. Parasitemia were determined by microscopic counting of at least 5000 erythrocytes under oil immersion.

4.2.8 Confocal fluorescence microscopy

Confocal laser scanning microscopy was used to determine the localization of FITC-PO and FITC-PS naked or complexed to NE (loaded with the lipophilic fluorescent Nile red) at +4/- charge ratio. Red blood cells infected or not with parasites at different stages of development were incubated 15 minutes with the tested formulations and then washed. The Glass slides were examined with a Zeiss LSM-510 confocal scanning laser microscope equipped with a 1mW Helium Neon laser, using a Plan Apochromat 63× objective (NA 1.40, oil immersion). Fluorescence was observed with a long-pass 488 emission filter under 543nm laser illuminations. The pinhole diameter was set at 71µm. Stacks of images were collected every 0.42µm along the z-axis.

4.2.9 Statistical analysis

Results were expressed as mean ± standard deviation of three independent experiments and were analyzed using the Student t-test.

4.3 RESULTS

4.3.1 Properties of NE and NE-ON complexes

Unloaded NE presented a droplet size of approximately 140 - 200 nm and a zeta potential positive value of about +55 mV. The association of either PO or PS to NE has been performed prior to further utilization. The amount of PO or PS complexed to NE was close to 100% since no ^{33}P-PO or ^{33}P-PS radioactivity was found in the external aqueous phase after separation by ultrafiltration/centrifugation. Their size and zeta potential values are given in Table 4.1.

The complexation of either PO or PS on NE at a +/- charge ratio higher than 4 led to positively charged complexes, while the complexes at +0.5/- charge ratio led to negatively charged ones. The zeta potential values of +2/- complexes were very close to zero, displaying important variations in their size between preparations. Indeed, these complexes exhibited the highest droplet size and PDI values, with the presence of some aggregates. In any case, differences between the physicochemical properties of NE/PO and NE/PS complexes were observed.

Table 4.1. Size (nm) and zeta potential (mV) of NE and NE/ON complexes.

		Mean droplet size (nm) ± SD*	PDI	Zeta potential (mV) ± SD*
NE		171 ± 29	< 0.2	54 ± 4.3
NE/PO	+0.5/-	206 ± 23	< 0.3	-20 ± 1
	+2/-	343 ± 239	< 0.4	9 ± 18
	+4/-	216 ± 20	< 0.3	37 ± 2
	+6/-	182 ± 33	< 0.2	41 ± 1
NE/PS	+0.5/-	178 ± 6	< 0.2	-23 ± 3
	+2/-	281 ± 172	< 0.3	12 ± 19
	+4/-	204 ± 18	< 0.3	38 ± 7
	+6/-	177 ± 7	< 0.3	43 ± 6

* Standard deviation between means.

4.3.2 Erythrocytes hemolysis and binding

Interactions between NE/PO or NE/PS complexes and erythrocytes were first evaluated through hemolysis experiments (Figure 4.1A and B). As it can be seen, between a NE/ON charge ratio of +0.5/- and +4/-, hemolysis was lower than 10% (Figure 4.1A). However, increasing the NE/PO and NE/PS complexes charge ratio to +6/- induced a higher hemolytic effect that reached a value of 25% ($p<0.05$). No differences were observed between NE/PO and NE/PS. Concerning the influence of time on hemolytic effect of complexes, as shown in Figure 4.1B, for both NE/PO and NE/PS complexes at charge ratio +4/-, hemolysis reached quickly a maximum and did not change over time. Moreover, the hemolytic effect of complexes at a charge ratio of +4/- (9% to 12%) on *P. falciparum*-infected erythrocytes remained quite similar to the hemolytic effect on healthy erythrocytes (5 to 10%) even after an incubation time of 15 minutes or 44 hours. Finally, the hemolytic effect of unloaded NE was significantly higher ($p<0.05$) than those of the +4/- NE/PO and NE/PS complexes at the same concentration, achieving 30% and 42% hemolysis on non-infected and infected erythrocytes, respectively (data not shown).

4.3.3. ON binding to erythrocytes

To evaluate cell binding of complexes, ^{33}P-labeled complexes were incubated with erythrocytes and the bound fraction was estimated after centrifugation. As the charge ratio increased, the radioactivity associated to erythrocytes increased, regardless the type of ON (Figure 4.1C). Considering all charge ratios tested, the binding to erythrocytes was higher for NE/PS complexes ($p<0.05$). Complexes obtained at a charge ratio of +4/- presented a binding over time comprised between approximately 10% and 15%. After 44 hours of incubation, the values increased, regardless of the complex composition, by up to approximately 20% (Figure 4.1D). Considering the binding of NE/PO and NE/PS complexes on *P. falciparum*-infected erythrocytes, the results were close to those observed with non infected cells, with a binding remaining

constant after 15 minutes or 44 hours of incubation (approximately 14% to 17%) for both PO and PS.

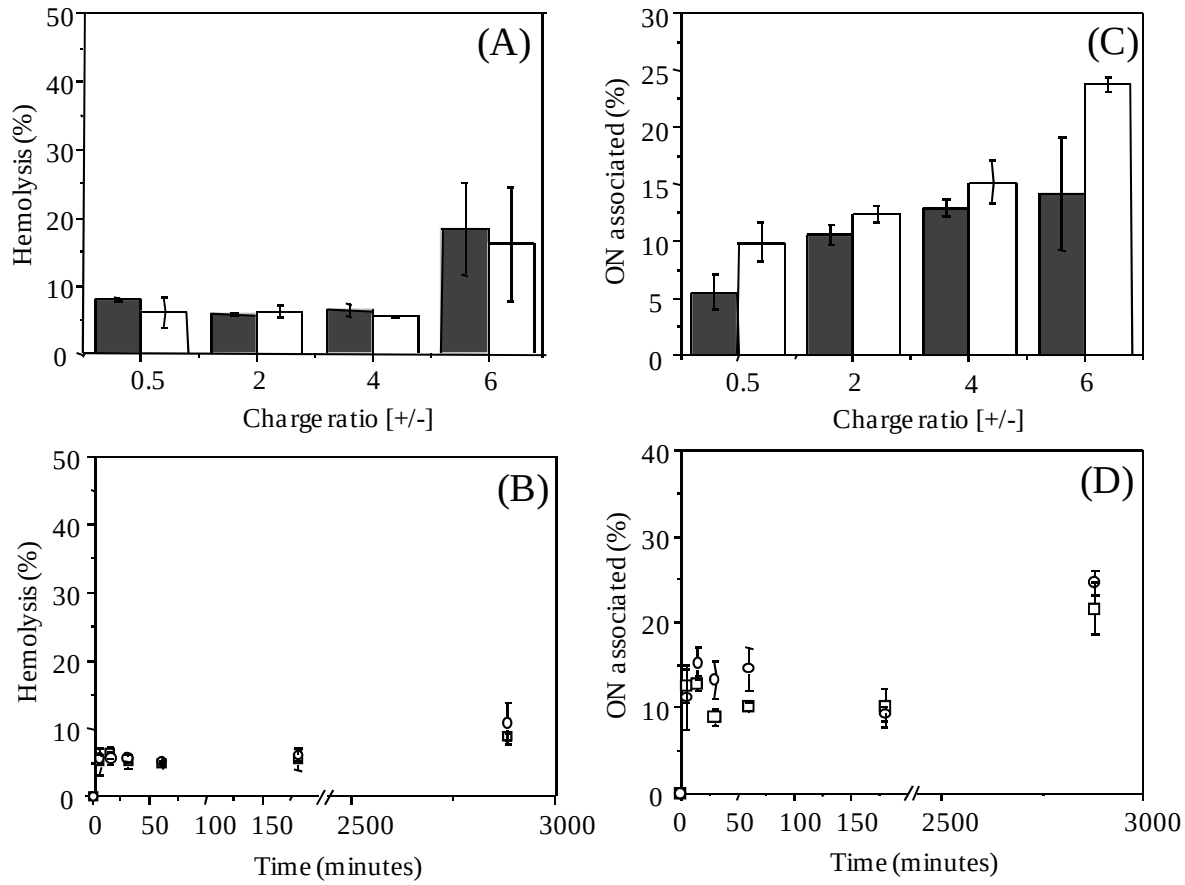

Figure 4.1. Effect of the charge ratio of complexes and incubation time on hemolysis (A and B) and ON erythrocyte binding (C and D) with non-infected erythrocytes. NE/PO (gray bars) and NE/PS (white bars) complexes were obtained at different charge ratios (+0.5/-, +2/-, +4/- and +6/-) and incubated for 15 minutes with non-infected erythrocytes (A and C). NE/PO (squares) and NE/PS (circles) complexes were obtained at a charge ratio of +4/- and incubated for 2880 minutes with non-infected erythrocytes (B and D).

4.3.4 Complexes stability in culture medium

Considering the lowest percentage of hemolysis obtained for complexes at +0.5/-, +2/- and +4/- charge ratios, they were selected for a stability study, which consisted in measuring the droplet mean size and PDI changes over time. These experiments were carried out after sample dilution in albumin supplemented culture medium (in the same

dilution factor as for the *in vitro* tests). Determination of the mean droplet size and PDI of +0.5/-, +2/- and +4/- NE/PO complexes showed that the mean droplet size of the positively charged complexes (+2/- and +4/- NE/PO) increased over the time (compared to time zero, $p<0.05$), but remained between 150 and 280 nm (Figure 4.2). At all charge ratios tested, the PDI was maintained below 0.2 until 8 hours of incubation, increasing to over 0.3 only after 24 hours. Unloaded NE, diluted in the same proportion, displayed the same stability profiles as the loaded ones.

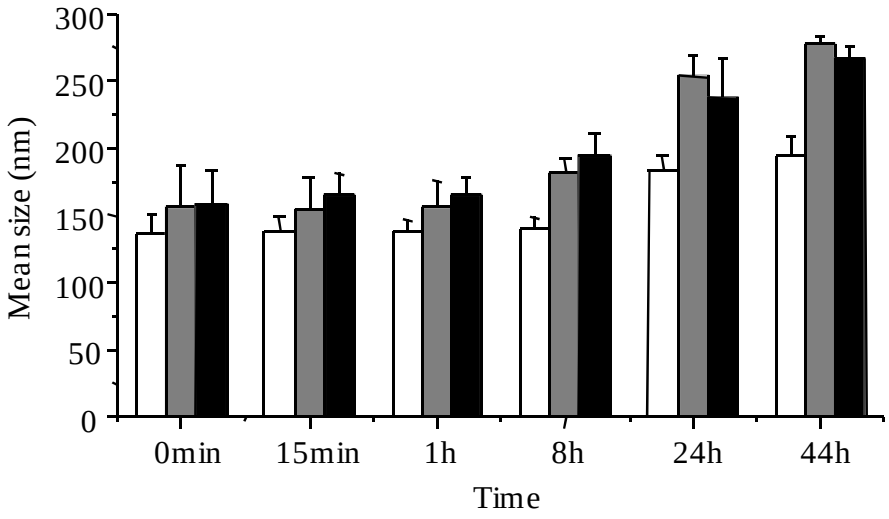

Figure 4.2. Medium droplet size and polydispersity index (PDI) characterization of +0.5/- (white bars); +2/- (gray bars) and +4/- (black bars) charge ratio NE/PO complexes during time, after dilution in RPMI supplemented culture medium.

4.3.5. Growth inhibition of *P. falciparum in vitro*

Considered as the optimal formulations, +4/- NE/PO or PS complexes were chosen for the *in vitro* evaluation of *P.falciparum* growth, measured by the detection of the pLDH protein (Figure 4.3A). Antimalarial effects of free 1 µM PO or PS (antisense) and sPO or sPS (sense control sequences), as well as +4/- NE/PO or NE/PS (antisense complexes) and NE/sPO or NE/sPS (sense control complexes), were evaluated in *P.*

falciparum synchronized ring-stage parasites for 44 hours. The final concentration in cell suspension was the same as for hemolysis, binding and stability experiments (1 µM). As can be seen, about 50% of inhibition of parasite growth was observed for the unloaded NE, used at the same amount as the one present in +4/- complex formation. Free PO, PS, sPO or sPS exhibited a low parasite growth inhibition (from 7 to 24%), regardless the type of ON tested. However, after they were adsorbed on the NE in a charge ratio of +4/-, there was a higher parasite growth inhibition (65-81%) for all complexes, compared to the free ON ($p<0.05$). In addition, NE/PS complex significantly reduced parasite growth compared to the NE/sPS complex ($p<0.05$), while NE/PO complexes did not (compared to NE/sPO complexes). All formulations tested presented antimalarial activity similar to that of chloroquine, used as positive control.

Besides the detection of parasite growth *in vitro* through the evaluation of pLDH protein, microscopic counts of all forms of parasites were performed in parallel cultures, over different stages of development: early stage (ring) forms (Figure 4.3B) and mature or late stage (trophozoites and schizonts) forms (Figure 4.3C). After 24 hours of treatment, more pronounced inhibition effects on parasite growth were observed, while after 44 hours these effects decreased. Even after treatment, the parasites persist in infecting new red blood cells, but a delay in the development cycle was observed after treatment with free PS or NE/PS complex. As shown in Figure 4.3C, after 24 hours of treatment, there was a reduction of ring forms levels and consequently, after 44 hours, a reduction of also late stage forms of parasites, compared to control, receiving only culture medium.

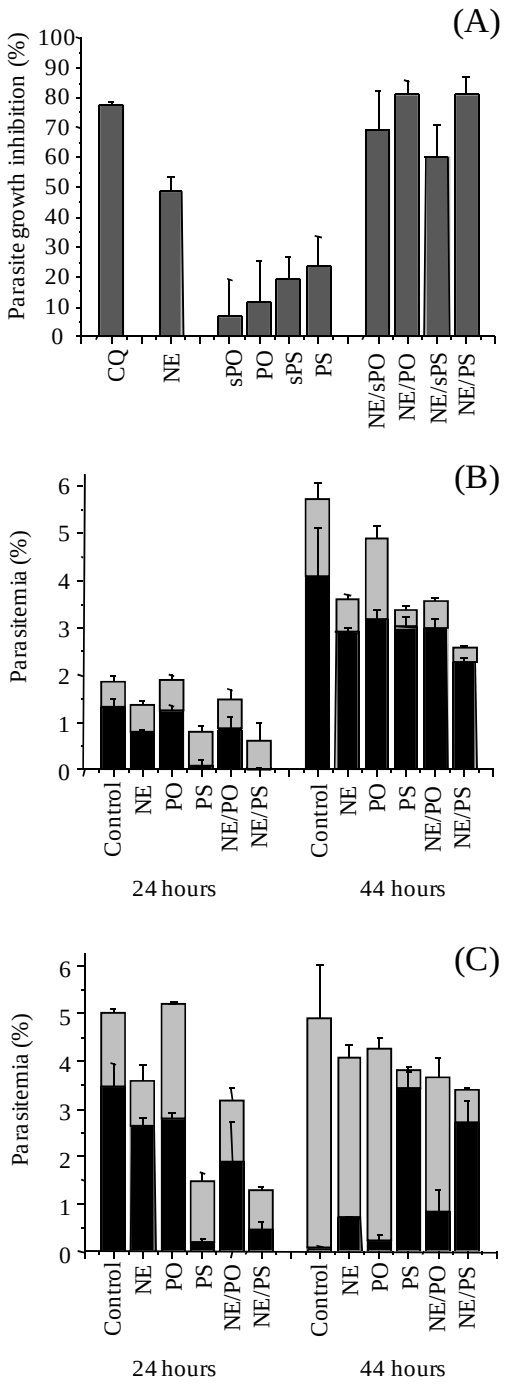

Figure 4.3. (A) *P. falciparum* growth inhibition, quantified by the pLDH ELISA test, after 44 hours of exposure to treatments (dark gray bars). (B) reinfection test over ring stage form parasites, detected by microscopy counting (Giemsa staining) 24 and 44 hours after treatment. (C) reinfection test over mature (trophozoite and schizont forms) parasites, detected by microscopy counting (Giemsa staining) 24 and 44 hours after treatment. All treatments were compared to a 100% of parasite growth control (Control). Chloroquine (CQ); nanoemulsion (NE); sense ON controls (sPO and sPS); antisense ON (PO and PS); sense NE/ON control complexes (NE/sPO and NE/sPS); antisense NE/ON complexes (NE/PO and NE/PS). Light gray bars: late stage parasites (schizonts and trophozoites); black bars: early stage parasites (ring).

4.3.6. Interactions of *P. falciparum*-infected erythrocytes by confocal microscopy

Cell and parasite uptake of NE/PO and NE/PS complexes was investigated by confocal microscopy studies. Non-infected red blood cells and erythrocytes infected with different stages parasites were incubated with the tested formulations for 15 minutes (Figure 4.4). Fluorescence was only observed into the infected erythrocytes, mainly into those containing parasites at the late stages of development. Red and green fluorescence, from the labeled NE and ON, respectively, were co-located within the parasite (Figure 4.4D). Moreover, the free ON was not observed within the erythrocytes, infected or not (data not shown).

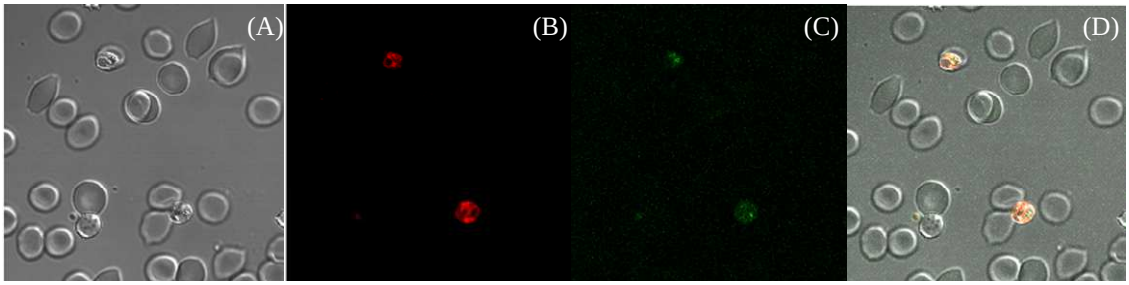

Figure 4.4. Confocal microscopy images of *P. falciparum* infected-erythrocytes after incubation with dual labeled +4/- NE/PS complexes (fluorescein and Nile red) during 15 minutes. Bright field (A and D) and fluorescence images (B and C) recorded with 488 nm excitation and 543 nm emission wavelengths.

4.4 DISCUSSION

We have recently reported the adsorption of a linear homopolythymidilate ON (16-mer) on the o/w interface of NE (MARTINI *et al.*, 2008). The main physicochemical characteristics of the NE obtained in this study are in accordance with that reported in previous studies using NE obtained through the spontaneous emulsification procedure under similar conditions (TRIMAILLE *et al.*, 2001; TRIMAILLE *et al.*, 2003; FRAGA *et al.*, 2008; MARTINI *et al.*, 2008). As studied by Martini et al., the highest possible value of zeta potential obtained for these systems could be reached with 2

mM of the cationic lipid covering the surface and was, therefore, attributed to the presence of the lipid at the oil-in-water (o/w) interface of NE (MARTINI *et al.*, 2008).

The first finding of the present study was to demonstrate the ability of such a system to bind a high length 30-mer ON bearing either PO or PS moieties since only small ON were previously complexed by NE. The results obtained suggest that complexation occurs, regardless the length of the ON, since similarly electrostatic interactions are involved in this process. For complexes with positive zeta potential values, no differences between the physicochemical properties of NE/PO or NE/PS complexes were observed ($p>0.05$). Considering that PS differs from PO only by the modification of the phosphodiester linkage, where non-bonding phosphate oxygen is replaced by sulphur, the ability for ion pairing remained unchanged when high amounts of positive charges are available.

The +/- charge ratio indicates the amount of positive charges from the cationic lipid over the amount of negative charges provided by ON molecules. We studied NE/PO and NE/PS complexes at four different charge ratios (from +0.5/- to +6/-), with growing NE concentrations added to equal concentrations of ON. The +2/- NE/PO and NE/PS complexes presented evidences of instability after 30 minutes of complexation (data not shown), what could be related to their low zeta potential values and high PDI as previously described (TEIXEIRA *et al.*, 1999; ROLAND *et al.*, 2003; MARTINI *et al.*, 2008).

The interactions of NE/PO and NE/PS complexes (obtained at different charge ratios) with erythrocytes were assessed though hemolysis, binding and confocal microscopy experiments. The binding experiments were performed so as to collect data on total cell binding (internalized and/or adsorbed at the outer cell membranes). As the charge ratio increased, binding to erythrocytes increased. Such a result could be attributed to the electrostatic interactions of the positive moieties of NE/PO and NE/PS complexes with negatively-charged membranes of erythrocytes containing sialic acid residues (VAN DAMME *et al.*, 1994). Similar features were previously reported for DNA-cationic liposomes complexes with however a higher percentage of binding

(SAKURAI *et al.*, 2001a; SAKURAI *et al.*, 2001b). A plausible explanation for these differences could be the physicochemical properties of the carriers. In fact, nanoemulsions exhibit a lower density than liposomes, thus oil droplets can be efficiently separated from cells during binding experiments by centrifugation.

We also examined the hemolytic effect of NE/PO and NE/PS complexes. Irrespective of composition, complexes obtained at charge ratios up to +4/- shows similar hemolysis, below 10%. For the highest charge ratio tested (+6/-), as well as for unloaded NE, the hemolytic effect was significantly increased ($p<0.05$), what could be related to the presence of more positive available charges for the interaction with red blood cells. Besides, it is also probably related to the nature of cationic lipid present in the NE. Previous results showed a higher hemolytic effect for oleylamine containing NE (data not shown). It has been well-documented that single-tailed cationic lipids were more toxic than their double-tailed counterparts since they can interact strongly with cell membranes and may interfere with membrane function through various mechanisms, consequently leading to cell toxicity (SENIOR *et al.*, 1991; LASCH, 1995; TANG and HUGHES, 1999).

For this reason, the NE/PO and NE/PS complexes obtained at the highest charge ratio, without a marked hemolytic effect were chosen for further studies. Thus, the hemolysis and binding of +4/- NE/PO and NE/PS complexes were evaluated over time in healthy and *P. falciparum*-infected erythrocytes. The results showed similar hemolytic effect and binding to infected or not erythrocytes, after both 15 minutes and 44 hours of incubation, suggesting no marked effect of 2% of parasites on these parameters. Contrary, accentuated hemolysis was observed for unloaded NE in the presence of parasites. The designed incubation time of 44 hours is related to *P. falciparum* life cycle, as for the following tests of *in vitro* evaluation of parasite growth.

In this context, it was necessary to verify the maintenance of physicochemical properties of NE/PO and NE/PS complexes in the presence of the culture medium, since it was supplemented with albumin, a serum protein. It is well known that

adsorption of components on the surface of cationic systems can occur, depending on their charge density (LUCK *et al.*, 1998; GESSNER *et al.*, 2002). For comparison of the results, the same NE concentration was also diluted in culture medium, since different properties are observed with salinity and dilution variations (RABINOVICH-GUILATT *et al.*, 2004). Even though there was a small increase in the mean droplet size of positively charged +2/- and +4/- NE/PO complexes (zeta potential of about +10 to +40mV), it was maintained under 300 nm. Thus, considering that the interaction with the red blood cells occurs from 15 minutes, the complexes in the three different charge ratios were considered stable.

The antisense ON sequence against topoisomerase II has been studied against *P.falciparum* K1 strain (NOONPAKDEE *et al.*, 2003; FÖGER *et al.*, 2006). Specific inhibition of parasite growth of 47% by free ON (NOONPAKDEE *et al.*, 2003) and 87% by chitosan antisense nanoparticles (FÖGER *et al.*, 2006) were obtained for this strain at an ON final concentration of 0.5 µM. In our study, at 1 µM final concentration, about 20 to 25% of inhibition was observed for free PO and PS, against about 80% for their complexes at the charge ratio of +4/- (Figure 4.3A). Similar parasite growth inhibition was obtained for NE/PO and NE/PS complexes, corroborating the red blood cell binding results, since no difference in binding was obtained at this charge ratio (+4/-).

The antimalarial activity of +0.5/− and +2/− NE/PO and NE/PS complexes were also tested (preliminary results not shown), but unlike Föger et al. (2006), which had great ereffect with negatively charged nanoparticles-ON, we found a better parasite growth inhibition by positively charged +4/− NE/ON complexes, which had a final positive zeta potential value (+30mV). Unloaded NE led to a parasite growth of 50%. Considering that they have higher positive zeta potential (about +50mV) compared to NE/PO and NE/PS complexes, their interaction with negatively charged cell membranes is enhanced.This interaction leads to a higher haemolytic effect. Therefore, unloaded NE may not be the Best control for comparing the results.

Nonspecific effects were observed for the free PO and PS, as already reported by other authors at high concentrations (RAPAPORT *et al.*, 1992; RAMASAMY *et al.*, 1996; KANAGARATNAM *et al.*, 1998; WANIDWORANUN *et al.*, 1999; STEIN, 2001). Contrary to them, no inhibition of 3D7 *P. falciparum* strain was observed at concentrations lower than 1 µM of PO or PS, even for NE/PO or NE/PS complexes (preliminary results not shown). The non-specific effects have been characterized as similar to polyanions effects, such as dextran sulfate, by blocking the recognition of sialic acids of erythrocytes membrane by merozoites, during the reinfection processes (RAPAPORT *et al.*, 1992; BARKER *et al.*, 1996; NOONPAKDEE *et al.*, 2003). However, in our studies these effects disappeared for NE/PS complexes, which were more effective that the NE/sPS complexes ($p<0.05$) but not with the NE/PO complexes for which non-specific effects were still observed. This might be due to another mechanism such as a sequence non specificity in the 3D7 strain, due to a different mRNA conformation, or a different protein expression profiling, for exemple (KONCAREVIC *et al.*, 2007). Finally, during the reinfection test, there was a higher reduction in parasitemia after treatment of erythrocytes infected by mature parasites compared to those infected by ring stage forms. Late stage forms seem to be more permeable than ring forms (ELFORD *et al.*, 1985; KIRK, 2001), as observed by confocal microscopy, by both green and red (loaded NE) fluorescence inside trophozoites parasitized erythrocytes.

4.5 CONCLUSIONS

NE provide a positively-charged interface which allows the adsorption of ON, at different charge ratios. The adsorption of PO or PS remained quite similar, suggesting that it was governed by electrostatic interactions between positively and negatively charged moieties from cationic lipids and ON, respectively. The binding with erythrocytes increased proportionally to the +/- charge ratio of the complexes, with no marked hemolysis at low charge ratio (under +4/-). In such conditions, PO and PS can bind with *P. falciparum*-infected erythrocytes in a similar extent. ON most probably

take advantage from the destabilization of the cell membrane due to the positive charges of NE, which could increase membrane permeability and allowed ON molecules to associate with cells. Our results showed that the association of ON to NE increases their activity against *P. falciparum* chloroquine-sensitive strain *in vitro,* validating the tested approach. Those systems are interesting for further studies in chloroquine-resistant strain, with the improvement of the ON sequence and backbone chemistry modification. Besides, studies including the determination of the mRNA and protein levels are in progress to better understand the results obtained. The optimized system will then be transposed to ON targeting *P. berghei* topoisomerase II for a further *in vivo* evaluation on the *P.berghei* mouse model.

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GENERAL DISCUSSION

Malaria is a widespread parasitic disease, currently endemic in more than 100 countries in tropical, and subtropical regions. Malaria is returning to areas from which it had been eradicated and is spreading to new areas, such as Central Asia, and Eastern Europe. The number of mortalities worldwide resulting from malaria is estimated at 800,000 people per year and has increased, most likely due to parasite drug resistance (CDC, 2011; WHO, 2011b). The emergence of artemisinin resistance is one of the greatest threats to the efforts to eradicate malaria (EKLAND and FIDOCK, 2008; WITKOWSKI *et al.*, 2010; WHO, 2011a). Thus, there is an urgent need for the development of new drugs for malaria treatment. Recently, some studies have shown the ability of single-strand antisense oligonucleotides (ONs) to inhibit *P. falciparum* growth (NOONPAKDEE *et al.*, 2003; FÖGER *et al.*, 2006). However, ONs-based therapy is limited by the rapid degradation of ONs in biological fluids and the inability to efficiently cross cell membranes, due to their hydrophilic character and large molecular structure (OPALINSKA and GEWIRTZ, 2002).

In recent years, there has been a considerable progress in developing nanostructured systems for the delivery of nucleic acids such as ONs (FATTAL and BARRATT, 2009). The association of ONs with cationic nanoemulsions have been described in the literature as a promising strategy to improve nucleic acids transfection to mammalian cells, protecting them against nuclease attack (NAM *et al.*, 2009; VERISSIMO *et al.*, 2010). Previous reports have noted the potential of cationic nanoemulsions composed of a triglyceride oil core stabilized by phospholipid and cationic lipid combinations as a delivery system for ONs. Reportedly, such delivery systems are able to improve the ON delivery to cells after intravitreal and intratumoral administration, protecting ON against nuclease degradation (TEIXEIRA *et al.*, 2003; HAGIGIT *et al.*, 2008).

In this context, the aim of this study was to evaluate the potential of cationic nanoemulsions as delivery systems for ON targeting *P. falciparum* topoisomerase II and their potential in inhibiting parasite growth. Experiments were firstly focused on the optimization of the cationic nanoemulsion composition by means of a factorial design. The physicochemical characterization of the ON/cationic nanoemulsion

complexes was then carried out and their effect on *P. falciparum*-infected cells determined.

In the first part of this study, we aimed to optimize the nanoemulsion composition obtained by means of the spontaneous emulsification procedure. Based on the data summarized in Chapter I, we selected triglycerides and phospholipids due to the well-documented biodegradability and biocompatibility of these compounds in parenteral applications. However, for the nucleic acid nanoemulsions, a third component is usually required to obtain a system with a positive charge, enabling the adsorption of nucleic acids through the formation of an ion pair (TEIXEIRA *et al.*, 1999; TEIXEIRA *et al.*, 2003). Thus, formulations composed of a mixed interfacial film of lecithin and either 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or oleylamine (OA) were evaluated. DOTAP was chosen since this is one of the cationic lipids most used in the development of nanocarriers for gene therapy whereas the OA selection was based on obtaining information on the effect of the acyl chains (single or double) on the physicochemical and biological properties of the complexes.

Factorial designs are used to provide the maximum amount of information from a small number of experiments (SUCKER, 1971; MONTGOMERY, 1991). Such designs allow the simultaneous quantification of the effects caused by the independent variables and the interactions between them. In this study, the influence of the three main components of the formulations (i.e. oil, phospholipid, and cationic lipid) was evaluated simultaneously through a qualitative 2^3 factorial design (Chapter II). Eight formulations were obtained from the combination of the different components: oils (TCL - soybean oil or MCT – medium-chain triglycerides), surfactants (soy or egg yolk lecithin) and cationic lipids (DOTAP or OA). From these combinations, two formulations could be selected to produce monodisperse nanoemulsions, exhibiting at the same time the smallest homogeneous droplet size and the highest positive charge. The experiment did not allow the selection of a single formulation, because the nanoemulsions composed of TCM and egg yolk lecithin presented very similar physicochemical properties, regardless of the type of cationic lipid added (OA or

DOTAP). Thus, it could be concluded that the presence of a primary (OA) or quaternary (DOTAP) amine polar head group, as well as the presence of one (OA) or two (DOTAP) unsaturated carbon chains, did not appear to significantly affect the physicochemical properties of these formulations.

The adsorption of phosphodiester and phosphorothioate ONs, targeting *P. falciparum* topoisomerase II, onto the optimized nanoemulsions was evaluated applying an ultrafiltration/centrifugation method using previously validated conditions (BRUXEL *et al.*, 2011). ON adsorption onto cationic nanoemulsions can be evaluated qualitatively and quantitatively through adsorption isotherms and the main interactions are electrostatic (TRIMAILLE *et al.*, 2003; MARTINI *et al.*, 2008). According to the results presented in Chapter II, the adsorption capacity of the ONs was different for the two selected formulations. Although an equivalent zeta potential (around +50 mV) and similar adsorption profiles were obtained, the nanoemulsions containing DOTAP showed higher ON adsorption capacity than those containing the OA cationic lipid. This may be related to additional hydrophobic interactions (between ON and the double-stranded cationic lipid DOTAP) occurring during the nanoemulsion/ON complex formation, as previously reported by other authors (TEIXEIRA *et al.*, 2001; HAGIGIT *et al.*, 2008; MARTINI *et al.*, 2008). From the linear regression analysis of the experimental data, it was found that the Langmuir model represents better the ON adsorption onto the selected cationic nanoemulsions in comparison with other models. This model is one of the most widely used for monolayer sorption onto a surface with a finite number of equivalent binding sites (SINKO, 2006).

Different approaches can be used to investigate the effect of ONs on the properties of complexes. Zeta potential measurements are very often employed to investigate the electrostatic interaction between the phosphate groups of the ON and ammonium groups of the cationic lipids (TEIXEIRA *et al.*, 2001; TRIMAILLE *et al.*, 2003; MARTINI *et al.*, 2008). Irrespective of the cationic lipid and ON nature, the zeta potential clearly dropped to negative values in a concentration-dependant manner, indicating the ON adsorption at the oil-water interface of the nanoemulsions (chapter

II). It should be noted that the adsorption studies were performed with the bulk pH in the range of 6-7, since above these values (i.e., in an alkaline environment) a progressive decrease in the positive charge was detected due to the effect of the egg-lecithin phospholipids.

Transmission electron microscopy (TEM) and atomic force microcopy (AFM) are powerful techniques for the characterization of the ONs association with colloidal carriers (BUSTAMANTE *et al.*, 1997; HASKELL, 1998). The presence of ON at the interface of the nanoemulsions was suggested by the higher contrast observed at the interface of the oil droplets by TEM, using uranyl acetate as the staining agent (Chapter II). Topographical analysis by AFM allowed the observation of the ON/nanoemulsion complexes in their natural state, through three-dimensional high-resolution images. Flattening of the oil droplets was observed, which was reduced in the nanoemulsion/ON complexes by the presence of high amounts of adsorbed ON (+0.2/- charge ratio). The ON molecules were detected on the surface of the droplets, acting as a kind of protective coating of the nanoemulsions, preventing the fusion of droplets during aggregation (Chapter III).

To better understand the structural organization of the nanoemulsion/ON complexes, energy dispersive X-ray diffraction (EDXD) analysis was performed. The EDXD technique allowed us to gain a better insight into the nature of the supramolecular of the ON-lipid interactions (ZUHORN *et al.*, 2007; LU *et al.*, 2010). A lamellar structural organization was observed for both the blank nanoemulsion and ON/ nanoemulsion complexes. Thus, an overlap of lipid layers at the interface of nanoemulsions could be proposed (Chapter III). The addition of ON to the DOTAP-emulsion led to disorganization of the lipid arrangement, which was related to the amount of ON added. In addition, the expansion of the lattice spacing indicates the probably insertion of the ON molecules in the lamellar arrangement, between the lipid layers.

Hemolysis and the binding of the ON/nanoemulsion complexes to healthy and *P. falciparum*-infected erythrocytes were evaluated (Chapter IV). The complexes

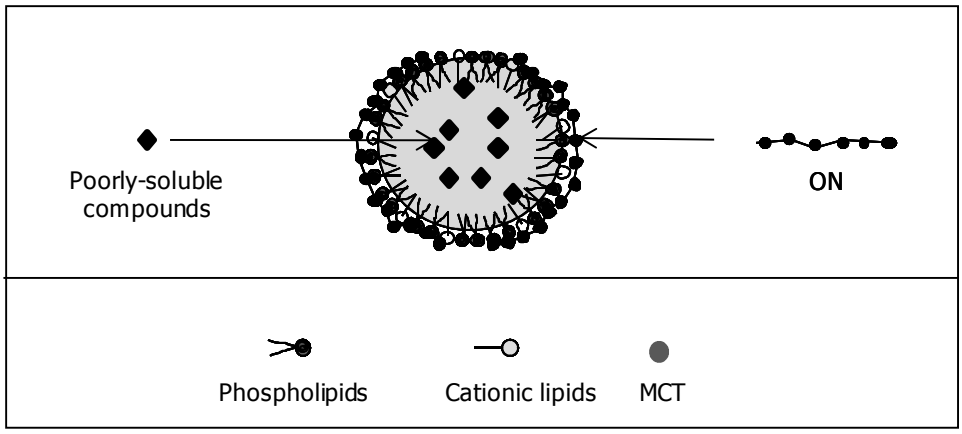
exhibited low hemolysis over time at low charge ratios, suggesting that the ON affected the toxicity. However, the OA-containing formulation presented a greater hemolytic effect than that containing DOTAP at high charge ratios. Previous reports in the literature have shown that single-tailed cationic lipids are more toxic than their double-tailed counterparts since they can interact strongly with cell membranes and may interfere with membrane function (PINNADUWAGE *et al.*, 1989; SENIOR *et al.*, 1991; LASCH, 1995).

Thus, further studies were performed with only the DOTAP-based nanoemulsions. A progressive binding of ON to erythrocytes with increasing charge ratio was detected whatever the nature of the ON. It was observed by confocal microscopy that both green (FITC-PO and FITC-PS) and red (loaded NE) fluorescence was co-located inside trophozoite-parasitized erythrocytes, suggesting that late-stage forms seem to be more permeable than ring forms.

We found that anti-topoisomerase II ON inhibited parasite growth at concentrations higher than 1 μM (Chapter IV). However, nonspecific effects were detected at the highest concentrations, which may not be related to the ON sequence, as previously described (RAPAPORT *et al.*, 1992; RAMASAMY *et al.*, 1996; KANAGARATNAM *et al.*, 1998; WANIDWORANUN *et al.*, 1999; STEIN, 2001). An effect of the formulation on the ON activity was also observed, since free ON showed an inhibitory effect of only 20%, while an inhibition of 80% was detected in the presence of nanoemulsions. Furthermore, after treating late stages (trophozoites) of *P. falciparum* with the nanoemulsion/ON complexes, there was a delay in the parasite life cycle, especially considering the phosphorothioate ON. It can be proposed that the final charge of the cationic nanoemulsion/ON complexes caused a destabilizing effect on the erythrocyte membranes, increasing their permeability to ON and validating the vectorization strategy.

Taking into account, technological, physicochemical and biological considerations, this study indicates the potential of cationic nanoemulsions as carriers for ONs, opening new prospects for other *P. faciparum* targets and *Plasmodium* species. The

co-encapsulation of a poorly-soluble anti-malarial drug into the oil core aiming at the development of a multiple drug delivery system exhibiting different action mechanisms is currently under development, as detailed in the schematic representation:



Schematic representation of a nanoemulsion and the co-enapsulation of a poorly-water soluble drug into the oil core and an antisense oligonucleotide adsorbed at the emulsions oil/water interface.

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CONCLUSIONS

— The full-factorial design allowed us to optimize the composition of the cationic nanoemulsions. Formulations composed of medium-chain triglycerides, egg-lecithin and either OA or DOTAP cationic lipids were selected based on the smallest homogenous droplet size and the highest zeta potential.

— Irrespective of the cationic lipid used, phosphodiester and phosphorothioate ON targeted toward topoisomerase II of *Plasmodium falciparum* can be efficiently adsorbed onto the cationic nanoemulsions selected by the factorial design, following a favorable Langmuir adsorption model.

— The presence of ON at the nanoemulsion interface was suggested by the zeta potential, TEM, AFM studies. From the EDXD experiments the structural organization of the nanoemulsions and complexes seem to involve lamellar arrangements.

— The replacement of a non-binding oxygen of the phosphate group (phosphodiester ON) by a sulfur (phosphorothioate ON) did not lead to major changes on the physicochemical properties of the complexes.

— The formulation containing DOTAP exhibited a less pronounced hemolytic effect when compared to that with OA, which was, in all cases, reduced in the presence of increasing amounts of ON adsorbed onto the nanoemulsions.

— The binding of ON/nanoemulsion complexes to erythrocytes was related to the +/- charge ratio. Fluorescent markers incorporated into the oil core or the 5’-end ON were found inside the late stages of *Plasmodium falciparum* in infected cells.

— The antisense ON adsorbed onto DOTAP nanoemulsions exhibited a more pronounced *in vitro* activity against *Plasmodium falciparum*, inhibiting the parasite growth by around 80% and causing a delay in its development cycle.

DISCUSSION GÉNÉRALE

Le paludisme est une maladie parasitaire répandue, actuellement endémique dans plus de 100 pays dans les régions tropicales et subtropicales. Le paludisme se retrouve dans les zones d'où il avait été éradiqué, et se répand à de nouvelles zônes géographiques, telles que l'Asie centrale et l'Europe de l'Est. La mortalité due au paludisme est estimée à plus d'un million de personnes par an. Elle a augmenté probablement en raison de la résistance des parasites aux médicaments (CDC, 2011; WHO, 2011b). L'émergence de la résistance à l'artémisinine est l'une des plus grandes menaces aux efforts pour éradiquer le paludisme (EKLAND et FIDOCK, 2008; WITKOWSKI *et al.*, 2010; WHO, 2011a). Ainsi, le développement de nouveaux médicaments pour le traitement du paludisme est urgemment nécessaire. Récemment, certaines études ont démontré la capacité de fragments d'acides nucléiques simple brin, nominés oligonucléotides antisens (ON), d'inhiber la croissance parasitaire de *P. falciparum* (NOONPAKDEE *et al.*, 2003; FÖGER *et al.*, 2006). Toutefois, la thérapie avec des ON est limitée par leur dégradation rapide dans les fluides biologiques et par leur incapacité à traverser les membranes cellulaires efficacement, en raison de leur caractère hydrophile et de leur grande structure moléculaire (OPALINSKA et GEWIRTZ, 2002).

Ces dernières années, il y a eu un progrès considérable dans le développement des systèmes de libération nanostructurés pour les acides nucléiques (FATTAL et BARRATT, 2009). L'association des ON avec des transporteurs cationiques a été décrit dans la littérature comme une stratégie prometteuse pour améliorer la transfection des acides nucléiques dans les cellules de mammifères, en les protégeant contre les attaques des nucléases (NAM *et al.*, 2009; VERISSIMO *et al.*, 2010). La littérature précédente a montré l'intérêt des nanoémulsions cationiques composé par un noyau de triglycérides stabilisés par des combinaisons de lipides cationiques et phospholipides comme un système de libération pour des ON. Ces systèmes de libération ont pu améliorer la livraison des ON aux cellules après l'administration intravitréenne et intratumorale, en protégeant les ON de la dégradation par des nucléases (TEIXEIRA *et al.*, 2003; HAGIGIT *et al.*, 2008).

Ainsi, l'objectif de cette étude était d'évaluer le potentiel des nanoémulsions cationiques comme transporteur pour des ON antipaludiques ciblant l'enzyme topoisomerase II du *P. falciparum*. Tout d'abord les expériences se sont concentrées sur l'optimisation de la composition des nanoémulsions cationiques au moyen d'un plan factoriel. Après cela, la caractérisation physico-chimique des complexes nanoémulsion cationique/ON et leur effet sur des cellules infectées par le *P. falciparum* ont été abordés.

Dans la première partie de cette étude, nous avons cherché à optimiser la composition des nanoémulsions obtenues par émulsification spontanée. A partir des données résumées dans le chapitre 1, nous avons sélectionné des triglycérides et des phospholipides en raison de la biodégradabilité et biocompatibilité bien documentée de ces composants pour des applications par voie parentérale.

Toutefois, pour des nanoémulsions contenant des acides nucléiques, un troisième élément est généralement nécessaire pour fournir une charge positive au système, permettant l'adsorption des acides nucléiques par la formation de paires d'ions (TEIXEIRA *et al.*, 1999; TEIXEIRA *et al.*, 2003). Ainsi, les formulations composées par des films interfaciaux mixtes de lécithine et soit 1,2-dioléoyl-3-triméthylammonium-propane (DOTAP) ou oléylamine (OA) ont été évalués. Le DOTAP a été choisi puisque ce lipide cationique est l'un des plus utilisés dans le développement de nanovecteurs pour la thérapie génique, tandis que le lipide OA a été sélectionné pour la comparaison. Nous avons cherché à obtenir des informations sur l'effet des chaînes acyle (simple ou double) sur les propriétés physicochimiques et biologiques des complexes.

En fait, le plan factoriel est utilisé pour fournir un maximum d'informations avec un petit nombre d'expériences (SUCKER, 1971; MONTGOMERY, 1991). Ce plan permet la quantification simultanée des effets causés par les variables indépendantes et les interactions entre eux. Voici, l'influence des trois principaux composants des formulations (c'est à dire l'huile, les phospholipides et les lipides cationiques) a été évaluée simultanément à travers un plan factoriel qualitatives 2^3 (chapitre 2). Huit

formulations ont été obtenues à partir de la combinaison des différents composants: des huiles (LCT - l'huile de soja ou MCT – des triglycérides à chaîne moyenne), des surfactants (des lécithines de soja ou de jaune d'œuf) et des lipides cationiques (DOTAP ou OA). À partir de ces combinaisons, deux formulations ont été sélectionnées, en produisant des nanoémulsions monodispersées et montrant, en même temps, la plus petite taille des gouttelettes et la charge positive la plus élevée. L'expérience n'a pas permis la sélection d'une formulation unique, car les nanoémulsions composées par les MCT et la lécithine du jaune d'œuf présentent des propriétés physico-chimiques très semblables, quel que soit le lipide cationique ajouté (OA ou DOTAP). Ensuite, il pourrait être noté que la présence d'une tête polaire amine primaire (OA) ou quaternaire (DOTAP), ainsi que la présence d'une (OA) ou deux (DOTAP) chaînes carbonées insaturées ne semblent pas affecter sensiblement les propriétés physico-chimiques de ces formulations.

L'adsorption des ON phosphodiesther et phosphorothioate ciblant la topoisomérase II de *P. falciparum* sur des nanoémulsions optimisées a été évaluée par une méthode d'ultrafiltration/centrifugation, en utilisant des conditions précédemment validées (BRUXEL *et al.*, 2011). L'adsorption des ON sur les nanoémulsions cationiques peut être évaluée qualitativement et quantitativement par des isothermes d'adsorption et les principales interactions impliquées sont de nature électrostatique (TRIMAILLE *et al.*, 2003; MARTINI *et al.*, 2008). Selon les résultats présentés dans le chapitre 2, la capacité d'adsorption de l'ON a été différente pour les deux formulations sélectionnées. Bien qu'un potentiel zeta équivalent (environ +50 mV) et des profils d'adsorption similaires aient été détectés, la capacité d'adsorption d'ON était plus élevée pour les nanoémulsions contenant DOTAP que pour les émulsions contenant le lipide cationique OA. Cela peut être liée à l'existence d'autres interactions hydrophobes (entre de l'ON et du lipide cationique DOTAP double-brin) lors de la formation des complexes nanoémulsion/ON, comme il été précédemment rapporté par d'autres équipes de recherche (TEIXEIRA *et al.*, 2001; HAGIGIT *et al.*, 2008; MARTINI *et al.*, 2008). D'après l'analyse de régression linéaire des données expérimentales, il a été constaté que le modèle de Langmuir représente mieux

l'adsorption des ON sur les nanoémulsions cationiques sélectionnées, en comparaison avec d'autres modèles. Un tel modèle est l'un des plus largement utilisé pour l'adsorption de monocouche sur une surface avec un nombre fini de sites de liaison équivalentes (SINKO, 2006).

Des approches différentes peuvent être utilisées pour étudier l'effet de l'ON sur les propriétés des complexes. Les mesures du potentiel zeta sont très souvent employées puisque l'interaction électrostatique entre les groupes phosphates de l'ON et les groupes ammoniun des lipides cationiques peuvent être suggérées (TEIXEIRA *et al.*, 2001; TRIMAILLE *et al.*, 2003; MARTINI *et al.*, 2008). Indépendemment de la nature du lipide cationique et de l'ON, le potentiel zeta est clairement réduit à des valeurs négatives, d'une manière dépendante de la concentration, en indiquant de l'adsorption de l'ON sur l'interface huile-eau des nanoémulsions (chapitre 2). Il faut mentionner que des études d'adsorption ont été effectuées aux bulk pH dans la gamme 6-7, puisque au-delà de cette valeur (dans un environnement alcalin) une diminution progressive de la charge positive a été détectée du fait de l'effet des phospholipides de la lécithine d'œuf.

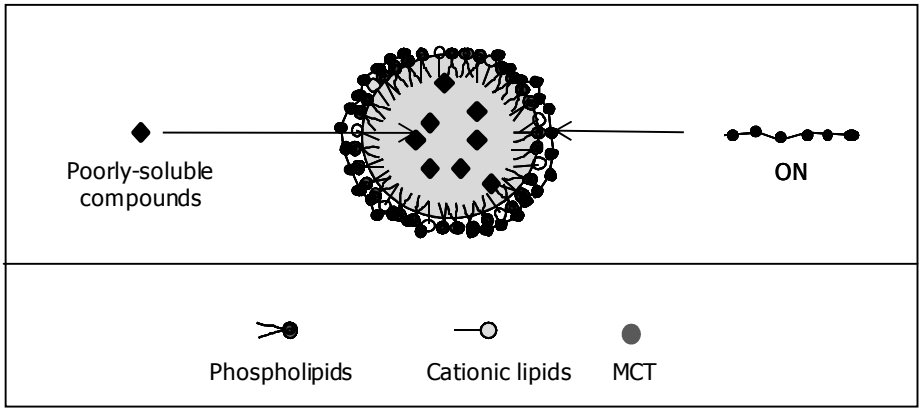
La microscopie électronique de transmission (TEM) et la microscopie à force atomique (AFM) sont des tecnhiques puissantes pour caractériser l'association des ON sur des transporteurs colloïdaux (BUSTAMANTE *et al.*, 1997; HASKELL, 1998). La présence de l'ON à l'interface des nanoémulsions a été suggérée par le plus fort contraste observé à l'interface des gouttelettes d'huile par TEM, en utilisant l'acétate d'uranyle comme agent contrastant (chapitre 2). L'analyse topographique par AFM a permis l'observation des complexes nanoémulsion/ON par des images en trois dimensions et en haute résolution. Un processus d'aplatissement des gouttelettes d'huile a été observé, qui ont été réduites en complexes nanoémulsion/ON, par la présence de quantités élevées d'ON adsorbés (rapport de charge +0,2/-). Les molécules d'ON ont été détectée à la surface des gouttelettes, en agissant comme un type de revêtement protecteur pour les nanoémulsions, empêchant la fusion des gouttelettes lors de de l'agrégation (chapitre 3).

Afin de mieux comprendre l'organisation des complexes nanoémulsion/ON, des analyses par diffraction de rayons X d'énergie dispersive (EDXD) ont été effectuées. La technique de EDXD nous a permis d'avoir un meilleur aperçu de la caractérisation supramoléculaire des interactions ON-lipide (ZUHORN *et al.*, 2007; LU *et al.*, 2010). L'organisation d'une structure lamellaire a été observée pour les deux nanoémulsions blanches et pour les complexes nanoémulsion/ON. Ainsi, un chevauchement des couches lipidiques à l'interface des nanoémulsions pourrait être proposé (chapitre 3). L'ajout d'ON à l'émulsion-DOTAP conduit à une désorganisation de l'arrangement des lipides, ce qui était lié à la quantité de l'ON ajouté. De plus, une expansion à propos du paramètre de maille indique une probable insertion des molécules d'ON dans l'arrangement lamellaire, entre les couches lipidiques.

L'hémolyse et l'association des complexes nanoémulsion/ON aux érythrocytes sains et infectés par *P. falciparum* ont été évalués (chapitre 4). Les complexes présentent une faible hémolyse au cours du temps avec des faibles rapport de charges, suggérant un effet de l'ON sur la toxicité des complexes. Cependant, la formulation contenant l'OA présente un effet hémolytique supérieur par rapport à celle contenant le DOTAP avec des rapport de charges élevés. La littérature précédente montre que des lipides à une seule queue sont plus toxiques que leurs homologues à deux queues, car ils peuvent interagir fortement avec des membranes cellulaires et peuvent interférer dans la fonction des membranes (PINNADUWAGE *et al.*, 1989; SENIOR *et al.*, 1991; LASCH, 1995). Ainsi, des études complémentaires ont été réalisées uniquement avec les nanoémulsions contenant le lipide cationique DOTAP. Une association progressive d'ON sur des érythrocytes avec l'augmentation du rapport de charge a été détectée, quelque soit la nature de l'ON. Les fluorescences vertes (FITC-PO et FITC-PS) et rouges (nanoémulsion marquée) ont été co-situées à l'intérieur des érythrocytes parasités par des trophozoïtes âgés, par microscopie confocale, ce qui suggère que les stades tardifs du parasite semblent être plus perméables que les formes en anneau (trophozoïtes jeunes).

Nous avons démontré que les ON anti-topoisomérase II ont inhibé la croissance du parasite à des concentrations supérieures à 1 µM (chapitre 4). Cependant, des effets non spécifiques ont été détectés avec les concentrations plus élevées, ceux qui ne peuvent pas être liés à la séquence de l'ON, comme décrit précédemment (RAPAPORT *et al.*, 1992; RAMASAMY *et al.*, 1996; KANAGARATNAM *et al.*, 1998; WANIDWORANUN *et al.*, 1999; STEIN, 2001). Un effet de la formulation sur l'activité d'ON a également été observé, puisque les ON libres ont montré un effet inhibiteur de seulement 20%, tandis qu'une inhibition de 80% a été détectée en présence des nanoémulsions. En outre, après le traitement des stades tardifs (trophozoïtes âgés) de *P. falciparum* avec des complexes nanoémulsion/ON, il y avait un retard dans le cycle de vie du parasite, surtout prenant en compte les ON phosphorothioates. Nous pouvons supposer que la charge finale des complexes nanoémulsion cationique/ON pourrait avoir causé un effet déstabilisant sur les membranes des érythrocytes, ce qui augmente leur perméabilité aux ON et valide la stratégie de vectorisation.

Prenant en compte les considérations technologiques, physico-chimiques et biologiques, cette étude indique le potentiel des nanoémulsions cationiques comme transporteurs pour les ON, ouvrant de nouvelles perspectives pour d'autres cibles et espèces de *P. faciparum*. La co-encapsulation d'un médicament antipaludique faiblement soluble au sein d'un coeur huileux, en visant l'élaboration d'un système multiple de libération de médicaments, avec des mécanismes d'action différents, est actuellement en développement, tels que présentés dans la représentation schématique:



Représentation schématique d'une nanoémulsion et de la co-encapsulation d'un médicament faiblement hydrosoluble dans le coeur huileux et d'un oligonucléotide antisens adsorbés à l'interface huile/eau de l'émulsion.

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