Colloids and Surfaces B: Biointerfaces 145 (2016) 462–469

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

The influence of liposomal formulation on the incorporation and retention of PNA oligomers

Paola Ringhieri[a,b], Concetta Avitabile[c], Michele Saviano[d], Giancarlo Morelli[a,b], Alessandra Romanelli[a,b,*], Antonella Accardo[a,b,*]

[a] Department of Pharmacy, University of Naples "Federico II", via Mezzocannone 16, 80134 Napoli, Italy
[b] Interuniversity Research Centre on Bioactive Peptides (CIRPeB), via Mezzocannone 16, 80134 Napoli, Italy
[c] Institute of Biostructure and Bioimaging (IBB), CNR, via Mezzocannone 16, 80134 Napoli, Italy
[d] Institute of Crystallography (IC), CNR, Via Amendola 122, 70126 Bari, Italy

ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form 4 May 2016
Accepted 11 May 2016
Available online 12 May 2016

Keywords:
Peptide nucleic acid
Liposome
Formulation
Cholesterol
DOPG

ABSTRACT

Liposomal formulations composed of phospholipids with different unsaturation degrees, head groups and at different cholesterol content have been tested for the encapsulation of Peptide Nucleic Acid (PNA) oligomers. The best loading capability (177 μg, ER% = 87.2) was obtained for pure liposomes of phosphatidylglycerol (DOPG) with negatively charged head group. The insertion of a 10–20% of cholesterol in DOPG based liposomes provides a slight decrease (~160 μg) of the PNA loading. On the other hand, the cholesterol addition (20–30%) slows down the PNA's release (~27%) in fetal bovine serum from the liposomal formulation. Based on the encapsulation and the release properties, PEGylated DOPG liposomes with a percentage of cholesterol of 10–20% are the optimal formulation for the loading of PNA-a210.

1. Introduction

Oligonucleotide based molecules represent a potent tool to unravel and modulate the expression and the function of genes. In this context the application of oligonucleotide analogues, such as Peptide Nucleic Acids (PNAs), showing resistance to degradation *in vivo*, high affinity and specificity of binding toward complementary DNA and RNA, is widespread [1–3]. Peptide Nucleic Acids, containing a pseudo-peptide backbone in place of the sugar-phosphate backbone, have been tested as antisense, antigene molecules and recently as inhibitors of the function of miRNAs [4–7]. The limited efficiency of transfection of Peptide Nucleic Acids in cells severely hampers their application *in vivo*. In the last few years the challenge has been tackled in many different ways [8]. A recently proposed strategy entails the conjugation of PNAs to polyanionic molecules, such as polyaspartic acid peptides, followed by the packing with polycations, including polyethylenimine (PEI) and lipofectamine [5]. Extension of a PNA sequence by a 6-mer PNA polyA allows its delivery upon hybridization to a polythymidilic acid containing both N,N-dimethylaminopropyl and octyl thiophosphate triester elements [9]. Alternatively, PNAs have been hybridized to DNA and immobilized on cationic nanoparticles [10]. PNAs have been also conjugated to a variety of carriers, including peptides, PEI, cholic acid, cholesterol [7,11–13]. A very laborious approach consists in the modification of the PNA backbone by insertion of positively or negatively charged pendant groups at the alpha or gamma position [14–16]. In few cases naked PNAs have been delivered to cells: biodegradable poly (lactic-co-glycolic acid) (PLGA) nanoparticles have been used to deliver a triplex forming PNA to CD34 cells [17]. Nanoparticles based on PLGA/polyaminobeta ester derivatized on the surface with the MPG peptide were demonstrated to deliver mixtures of PNA and DNA to CF mouse model and to cause correction on the *CFTR* gene. Recently, we have reported the delivery of a PNA oligomer (PNA-a210) to K562 cells by a liposomal formulation based on egg phosphatidylcholine/cholesterol/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carbonyl-methoxy (polyethylene glycol)2000 (egg PC/CHO/DSPE-PEG2000 at 47/47/6 molar ratio) and we have demonstrated that this formulation triggers low or no toxicity to cells [18]. The PNA oligomer targets the noncoding RNA miRNA-210, which is involved in the regulation of *raptor* and *γ-globin* genes and in the erythroid differentiation of leukemic K562 cells [19]. Effective down regulation of the miR-210 was observed

* Corresponding authors at: Department of Pharmacy, University of Naples "Federico II", via Mezzocannone 16, 80134 Napoli, Italy.
E-mail addresses: alessandra.romanelli@unina.it (A. Romanelli), antonella.accardo@unina.it (A. Accardo).

http://dx.doi.org/10.1016/j.colsurfb.2016.05.034
0927-7765/

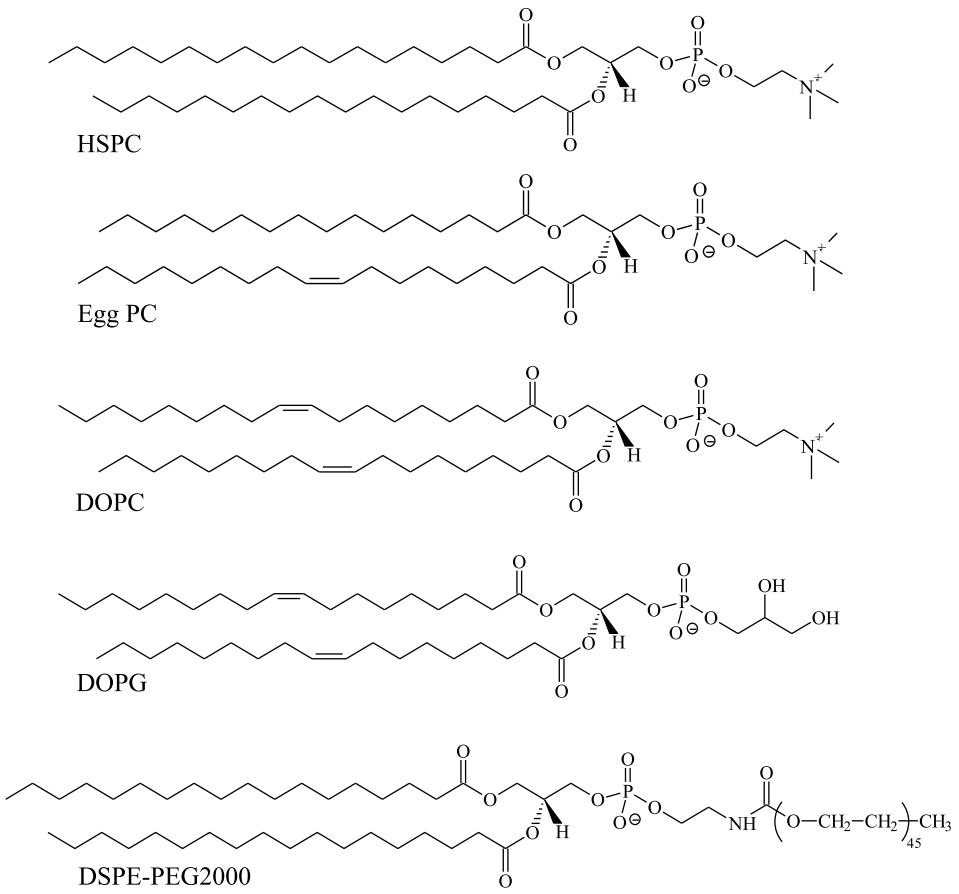

Fig. 1. Schematic representation of phospholipids used for the liposomal formulations: HSPC, egg PC, DOPC, DOPG and DSPE-PEG2000. For egg PC, which is a mixture of natural phospholipids, is here only represented the predominant fatty acid.

upon treatment of K562 cells with the liposome encapsulated PNA-a210. Similar formulations, SPC/CHO/DSPE-PEG2000 (47/47/6 molar ratio) and HSPC/CHO/DSPE-PEG2000 (56/39/5) have been reported for the encapsulation of peptides and for the liposomal drug Doxil [20]. Replacement of SPC or HSPC by egg PC, which has a higher degree of saturated chains, is expected to improve the stability of the liposomes, essential for the *in vivo* application of liposomal drugs. Goal of this work is to obtain a liposomal formulation with a higher PNA loading ability, as compared to the previously reported one [18]. The toxicity effects, likely due to lipids, will therefore be further reduced. To this aim, we tested a number of liposomal formulations, prepared using phospholipids containing two alkyl chains at eighteen carbon atoms (see Fig. 1). The effects of the unsaturation of the hydrophobic portion, of the head group and of the percentage of the cholesterol were evaluated in terms of structural features, PNA loading and release capabilities of the liposomes.

2. Materials and methods

Egg Phosphatidylcholine (Egg PC) 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPG), L-α-phosphatidylcholine hydrogenated soy (HSPC), cholesterol (CHO) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carbonyl-methoxy(polyethylene glycol)2000] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL). Dialysis tubing 8–10 kDa MWCO was purchased by Pierce Protein (USA). The PNA-a210 was prepared as described elsewhere [18]. The sequence of PNA-a210 follows: H-ccgctgtcacacgcacag-NH_2.

2.1. Liposome preparation and PNA loading

A stock solution of PNA-a210 was prepared in 10 mM phosphate buffer at pH 7.4, containing the 0.2% wt glycerol. Concentration was determined by UV–vis measurements, carried out on a Thermo Fisher Scientific Inc (Wilmington, Delaware USA) Nanodrop 2000c spectrophotometer equipped with a 1.0 cm quartz cuvette (Hellma) using a molar absorptivity (ε) of 172400 M^{-1} cm^{-1} at λ = 260 nm. All liposomal formulations, reported in Table 1, were prepared combining the thin film/sonication and the freeze/thawing methods. Briefly, the required amounts of phospholipids and cholesterol were dissolved in 3 mL of chloroform and the organic solvent was removed under a stream of N_2 to obtain a homogeneous film on the wall of the vial. Any trace solvent was further removed keeping the vial under vacuum for 15 min. Each dry lipid film was hydrated in 1.0 mL of 10 mM phosphate buffer and sonicated for 10 min. The total lipid concentration was 15 mM. Then, 500 μL of the PNA-a210 stock solution (1.0 mg/mL) were added to the preformed liposomes; the resulting mixture was frozen/thawed by dipping the glass tube for 1 min into a dry ice bath for rapid cooling and then transferred into a bath at 65 °C, allowing each sample to thaw out for 4 min, and finally vortexed for another minute. Five cycles were performed to promote the entry of PNAs into the liposomes. The liposomal suspension was extruded 10 times, using a thermobarrel extruder system (Northern Lipids Inc, Vancouver, BC, Canada) under nitrogen through a polycarbonate membrane (Nucleopore