Title

Delivery of oligonucleotides into bacteria by fusogenic liposomes

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Running Head

Liposomes for the delivery of oligonucleotides into bacteria

Abstract

Oligonucleotides able to hybridize bacterial RNA via *in situ* hybridization may potentially act as new antimicrobials, replacing antibiotics, and as fast *in vivo* diagnostic probes, outperforming current clinical methodologies. Nonetheless, oligonucleotides are not able to efficiently permeate the multi-layered bacterial envelope to reach their target RNA in the cytosol. Cationic and fusogenic liposomes are here suggested as vehicles to enable the internalization of oligonucleotides in bacteria. Here we describe the formulation of cationic DOTAP-DOPE liposomes, their complexation with small negatively charged oligonucleotides and the evaluation of the intracellular delivery of the oligonucleotides into bacteria. This strategy uncovers the potential of performing FISH *in vivo* for real-time detection and treatment of infections.

Key words

Lipoplexes, liposomes, oligonucleotides, nucleic acid mimics, bacteria, FISH

1. Introduction

Synthetic oligonucleotides able to hybridize to complementary RNA represent a promising new class of agents for therapeutic or diagnostics purposes [1]. In particular, synthetic oligonucleotides have been increasingly used for the detection of bacteria in clinical microbiology via fluorescence *in situ* hybridization (FISH) [2, 3]. Oligonucleotides are not able to efficiently permeate the multi-layered bacterial envelope, thus FISH is still dependent on the permeabilization of bacteria with chemical compounds toxic to mammalian cells limiting FISH applications to *in vitro*. Nevertheless, FISH has the potential to become a broader tool and embrace *in vivo* diagnostic and even treatment of bacterial infections [4]. In order to fulfill this

potential, vehicles able to intracellularly deliver oligonucleotides through the bacterial envelope without the need for a toxic permeabilization step of bacteria are necessary.

Liposomes are among the most explored and biocompatible delivery vehicles in nanomedicine [5], but their application in bacteria is still almost exclusively limited to the transport of traditional antibiotics [6]. A representation of the intracellular deliver mechanism of liposomes with bacterial membranes is illustrated in Figure 1. Cationic liposomes can easily be complexed with negatively charged oligonucleotides, via electrostatic interactions. Generally, cationic liposomes are prepared after the formation of a lipid film followed by the hydration and spontaneous arrangement of the lipids, resulting in vesicles. The resulting liposomes are then mixed with the oligonucleotides leading to highly cooperative complexes, named lipoplexes [7, 8] (Fig.1). In particular, liposomes made from the cationic lipid dioleoyltrimethylammoniumpropane (DOTAP) and the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) are well-characterized carriers for oligonucleotides in eukaryotic cells [9, 10], but not yet in bacteria. DOTAP-DOPE liposomes are interesting vehicles for the intracellular delivery in bacteria due to the presence of the fusogenic lipid DOPE which may promote fusion of the liposomes with the bacterial envelope [8, 5]. In addition, very few studies have combined liposomes with small nucleic acid mimics (NAMs) [8, 11]. NAMs, and among them locked nucleic acids (LNA) and 2'-Omethyl RNA (2'OMe), have shown to be very useful to improve the resistance to endonucleases and increase the hybridization affinity towards target RNA, compared to unmodified DNA and RNA oligonucleotides [12].

Although cationic liposomes can easily complex oligonucleotides including NAMs, their ability to safely transport oligonucleotides through biological fluids, such as human serum and mucus, is frequently challenged by the limited colloidal stability of cationic liposomes [12-14]. The insertion of a poly(ethylene glycol) (PEG) moiety in the liposomal formulation produces a "shielding" effect which increases the particle's colloidal stability and the circulation time [15, 16]. In addition, it also "shields" the cargo, maintaining the association efficiency between the

liposomes and the oligonucleotides [17]. Although PEGylation of the liposomes may decrease their efficiency in delivering the cargo into cells, insertion of the PEG after the hydration step, the so-called post-PEGylation, has been proved to overcome this disadvantage [18].

We have already obtained successful internalization of NAMs in Gram-negative bacteria using post-PEGylated DOTAP-DOPE liposomes, in buffer and within complex biological media [8]. This enabled the *in situ* hybridization of the NAMs to their target bacterial RNA, without the need of pre-treating the bacteria with toxic permeabilization compounds [8].

2. Materials

Prepare and store all reagents at room temperature unless indicated otherwise. Diligently follow all waste disposal regulations when disposing waste and contaminated materials.

2.1 Lipoplexes

1. Round bottomed flasks.
2. Rotary evaporator.
3. Nitrogen flow.
4. Glass beads.
5. Probe sonicator.
6. Orbital incubator.
7. Dynamic Light Scattering (DLS) instrument and correspondent folded capillary Zeta cells.
8. DOTAP: Dissolve 25 mg of lyophilized (2,3-Dioleoyloxy-propyl) trimethylammonium-chloride in 1 mL of chloroform. Store at -20 °C (*see* **Note 1**).
9. DOPE: Dissolve 25 mg of lyophilized 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine in 1 mL of chloroform. Store at -20 °C (*see* **Note 1**).

10. DSPE-PEG 2 kDa: Dissolve 25 mg of lyophilized 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethyleneglycol)-2000) in 1 mL of chloroform. Store at -20 °C **(*see* Note 1)**.
11. Labeled NAMs: Add ultrapure sterile water to lyophilized NAMs to achieve a final concentration of 100 μM. Keep this stock at -20 °C. Prepare several 20 μM aliquots for routine use and store at -20°C (*see* **Note 2 and 3**).
12. HEPES buffer: 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid hemisodium salt, pH 7.4 (*see* **Note 4**).

2.2. Fluorescence *in situ* hybridization (FISH)

1. Sterile syringe: 0.2 μm pore size.
2. Slides and coverslips.
3. Immersion oil.
4. Incubator (37 ºC).
5. Epifluorescence microscope.
6. HEPES buffer: 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid hemisodium salt, pH 7.4 (*see* **Note 4**).
7. Phosphate buffered saline (3x): 0.180 M NaCl, 0.003 M KCl, 0.009 M Na_2HPO_4, 0.002 M KH_2PO_4. Adjust pH to 7.4 (*see* **Note 5**). Autoclave the solution (121 °C for 15 minutes). Store at 4°C.
8. 4% (v/v) paraformaldehyde solution: Heat 65 mL of water to 60 °C and add 4 g of paraformaldehyde with constant agitation. Keep the solution in the heat and add drops of 2 M NaOH until the solution is clear (wait 1-2 minutes to get a clear solution). Remove from the heat and add 33 mL of 3x PBS. Mix and adjust pH to 7.4 (*see* **Note 5**). Filter the solution through a syringe filter with 0.2 μm porosity. Store at 4 °C.
9. Hybridization solution: 0.90 M NaCl, 0.50 M urea, 0.05 M Tris-HCl, pH 7. Filter the solution through a syringe filter with 0.2 μm porosity. Store at 4 °C.

10. Washing solution: 0.005 M Tris base, 0.015 M NaCl, 0.1% (v/v) Triton-X, pH 10. Autoclave the solution (121°C for 15 minutes). Store at 4 °C.
11. TSB (tryptone soya broth): Prepare according to the manufacture.
12. TSA (tryptone soya agar): Prepare according to the manufacture. Adjust pH to 7.3 and autoclave the solution (121 °C for 15 minutes) (*see* **Note 6**). Distribute it throughout polystyrene Petri dishes and let it solidify.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Preparation of liposomes and lipoplexes

1. Mix the appropriate volume of DOTAP and DOPE dissolved in chloroform (1:1 M ratio) in a round bottomed flask, previously washed with chloroform (Fig. 2).
2. Dry the resulting mixture by rotary evaporation at 40 °C maintaining the pressure below 60 mbar. Let it evaporate for 15 minutes. Alternatively, flush-dry the mixture with an inert gas until all the chloroform is evaporated.
3. Add 2 glass beads to the round bottomed flask and hydrate the lipid film with previously warmed HEPES buffer to a final concentration of 5 mM of each lipid. Agitate the flask until all the lipid film is removed from the flask walls by the beads.
4. Place the turbid solution in a microtube and sonicate until the solution is clear (amplitude: 10%) (*see* **Note 7**).
5. Measure the size and zeta potential of the liposomes using a DLS instrument (25 °C, 120 min equilibration time). In order to do so, first mix 30 μL of liposomes with 970 μL of HEPES buffer and transfer it to a folded capillary zeta cell (using a 1 mL syringe) and place the cell in the instrument (*see* **Note 8 and 9**).
6. Add the 20 μM NAMs to a solution of liposomes diluted in sterile ultrapure water in order to form lipoplexes at ± charge ratio of 15. Incubate for 30 minutes (*see* **Note 10**).

3.2. PEGylation of lipoplexes

1. Evaporate DSPE-PEG 2 kDa under nitrogen flow and re-dissolve the resulting lipid film in sterile ultrapure water to a concentration of 720 μM DSPE-PEG (Fig. 2).
2. Add the diluted DSPE-PEG to the lipoplex solution to achieve a final concentration of 120 μM DSPE-PEG in the mixture. The concentration of DOTAP and DOPE should be 600 μM each, after this addition. Incubate at 37 °C for 1h in an orbital incubator (200 rpm).

3.4. Fluorescence *in situ* hybridization (FISH)

1. Harvest a colony from an agar plate into the TSB and let it grow overnight. Dilute the resulting inoculum to nearly 1.6 x 10^6 cells/mL and let it grow until the mid-exponential phase of the bacteria is achieved (*see* **Note 11**).
2. Dilute the inoculum to 1.6 x 10^6 cells/mL and distribute 1 mL of the bacterial suspension on microtubes. Centrifuge as 16800 x g, for 15 min and remove the supernatant.
3. Prepare the FISH positive control by doing a standard permeabilization. Add 400 μL of 4% paraformaldehyde and incubate for 1h, followed by a centrifugation at 8600 x g for 5 min. Remove the supernatant. Add 500 μL of 50% ethanol and incubate for 30 min. Repeat the previous centrifugation step and remove the supernatant (*see* **Note 12**).
4. Prepare the controls for NAMs internalization: Add 50 μL of 0.4 μM NAMs diluted in hybridization solution to a microtube containing permeabilized bacteria (positive control, permeabilized as described in the previous step) and the same to another microtube containing non-permeabilized bacteria (control without liposomes). Let it incubate for 1h at 37 °C.
5. Prepare the controls for bacteria autofluorescence: Add hybridization solution to a microtube containing the bacteria.
6. Prepare the testing samples: Dilute 10 x the previously prepared lipoplexes (with and without PEG) in hybridization solution. The final concentration of the oligonucleotide

should be the same as in the positive control. Add 50 μL to each test microtube. Let it incubate for 1h at 37 °C.

7. Centrifuge all microtubes at 8600 x g for 5 min and discard the supernatant. Add previously warmed (at 37 °C) washing solution and incubate at 37 °C for 15 min (*see* **Note 13**).
8. Repeat the centrifugation step and resuspend the pellet in 1 mL of sterile distilled water.
9. Take 20 μL from each microtube and place it in each well of the diagnostic microscope slides. Allow it to dry at 37 °C.
10. Mount the slides with immersion oil and a coverslip and visualize the fluorescent bacteria on the slides under the epifluorescence or confocal microscope using the appropriate filters and/or lasers. (*see* **Note 14**).

4. Notes

1. Phospholipids stored in organic solutions such as chloroform, should always be kept in a glass container layered with argon or nitrogen at -20 ºC and the closure of the vial should be sealed with teflon.
2. The choice of the fluorophores must be carefully considered. The fluorophore must be bright and photostable.
3. Lyophilized NAMs should be stored at -20 °C for 1 year (or as recommended by the manufacture). Exposure to direct light should be avoided in any step involving the manipulation of the fluorescently labeled oligonucleotides.
4. It is best to store it for a limited time of 2 weeks.
5. Concentrated HCl and NaOH solutions (2 M) can be used at first to narrow the gap from starting pH to the required pH. From then on it is recommended to use 1 M HCl and NaOH solutions to avoid a sudden drop or rise in pH, respectively.
6. After autoclaving the medium, let it cool until it reaches a safe temperature to manipulate the bottle (do not let it cool down completely, otherwise the media will start to solidify).

7. Be cautious using the probe sonicator, making sure it does not touch the walls of the microtube. After sonication the solution must be clear. An optimization should be performed to obtain the ideal size, as the number of cycles as well as the time of sonication are dependent on the chosen sonicator model. The amplitude must be as close as possible to 10% to prevent overheating (which can be minimized by placing the microtube in an ice bath, making sure the microtube is kept still and without touching the probe sonicator).
8. Make sure that air bubbles are not present in the capillary folded zeta cell after insertion of the sample solution.
9. Expected features: hydrodynamic diameter: 70-110 nm, polydispersion index (PDI): ≤ 0.3; zeta potential: 40-55 mV. If the obtained diameter and/or PDI is higher, it is probable that the sonication was not sufficiently effective and the preparation of liposomes should be repeated. In case the difference persists, the number and duration of the sonication cycles should be optimized. If the obtained zeta potential is outside the referred range, the pH of the HEPES buffer should be checked and if it is not correct a new buffer should be prepared and the liposomes preparation repeated. If the liposomes features are still different from expected, prepare new lipid stocks and repeat the liposomes preparation (it is also possible that incorrect storage/handling of the lipid stocks results in lipids oxidation).
10. Always add the diluted liposomes to the oligonucleotide solution. The ± charge ratio is calculated as the molar amount of positive charges on the DOTAP molecules (1 DOTAP molecule containing 1 cationic head group), divided by the molar amount of negative charges on the NAMs sequence (1 NAMs sequence containing 1 negatively charged phosphate group per NAM), as follows:

$$\pm \text{ charge ratio} = \frac{moles\ DOTAP\ in\ lipoplexes}{moles\ NAMs\ sequence\ in\ lipoplexes \times n^{o}\ mers}$$

For example, if you use a 10 NAMs sequence and add 2.4 µL of 20 µM NAMs to 9.6 µL of liposomes solution containing 750 µM DOTAP, it follows that

$$\pm \text{ charge ratio} = \frac{(9.6\ \mu L \text{ x } 750\ \mu M)}{(2.4\ \mu L \text{ x } 20\ \mu M) \text{ x } 10} = 15$$

11. The growth conditions are dependent on the chosen bacteria.
12. In case no pellet is visible, centrifuge for additional 5 min.
13. The washing step is pivotal to ensure that the NAMs are not attached to the bacterial envelope upon microscopy visualization.
14. For fluorescence quantification in image processing programs, analyze all samples using the same exposure time and the same excitation intensity.

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6. References

1. Dove A (2002) Antisense and sensibility. Nat Biotechnol 20 (2):121
2. Cerqueira L, Azevedo N, Almeida C, Jardim T, Keevil C, Vieira M (2008) DNA mimics for the rapid identification of microorganisms by fluorescence in situ hybridization (FISH). Int J Mol Sci 9 (10):1944-1960

3. Frickmann H, Zautner AE, Moter A, Kikhney J, Hagen RM, Stender H, Poppert SJCrim (2017) Fluorescence in situ hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. Crit Rev Microbiol 43 (3):263-293
4. Wiegant J, Brouwer AK, Tanke HJ, Dirks RW (2010) Visualizing nucleic acids in living cells by fluorescence in vivo hybridization. In: Fluorescence in situ Hybridization (FISH). Totowa, Humana Press, pp 239-246
5. Nicolosi D, Scalia M, Nicolosi VM, Pignatello R (2010) Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria. Int J Antimicrob Agents 35 (6):553-558
6. Drulis-Kawa Z, Dorotkiewicz-Jach, A (2010) Liposomes as delivery systems for antibiotics. Int J Pharm 387 (1-2):187-198
7. Dakwar GR, Braeckmans K, Ceelen W, De Smedt SC, Remaut K (2017) Exploring the HYDRAtion method for loading siRNA on liposomes: the interplay between stability and biological activity in human undiluted ascites fluid. Drug Deliv Transl Res 7 (2):241-251
8. Santos RS, Dakwar GR, Zagato E, Brans T, Figueiredo C, Raemdonck K, Azevedo NF, De Smedt SC, Braeckmans K (2017) Intracellular delivery of oligonucleotides in Helicobacter pylori by fusogenic liposomes in the presence of gastric mucus. Biomaterials 138:1-12
9. Taetz S, Bochot A, Surace C, Arpicco S, Renoir J-M, Schaefer UF, Marsaud V, Kerdine-Roemer S, Lehr C-M, Fattal EJO (2009) Hyaluronic acid-modified DOTAP/DOPE liposomes for the targeted delivery of anti-telomerase siRNA to CD44-expressing lung cancer cells. Oligonucleotides 19 (2):103-116
10. Remaut K, Lucas B, Braeckmans K, Sanders N, Demeester J, De Smedt SJB (2006) Delivery of phosphodiester oligonucleotides: can DOTAP/DOPE liposomes do the trick? Biochemistry 45 (6):1755-1764
11. Meng J, Wang H, Hou Z, Chen T, Fu J, Ma X, He G, Xue X, Jia M, Luo XJAa (2009) Novel anion liposome-encapsulated antisense oligonucleotide restores susceptibility of methicillin-resistant *Staphylococcus aureus* and rescues mice from lethal sepsis by targeting mecA. Antimicrob Agents Chemother 53 (7):2871-2878
12. Santos RS, Dakwar GR, Xiong R, Forier K, Remaut K, Stremersch S, Guimarães N, Fontenete S, Wengel J, Leite M (2015) Effect of native gastric mucus on *in vivo* hybridization therapies directed at Helicobacter pylori. Mol Ther Nucleic Acids 4:e269
13. Dakwar GR, Zagato E, Delanghe J, Hobel S, Aigner A, Denys H, Braeckmans K, Ceelen W, De Smedt SC, Remaut K (2014) Colloidal stability of nano-sized particles in the peritoneal fluid: towards optimizing drug delivery systems for intraperitoneal therapy. Acta Biomater 10 (7):2965-2975
14. Braeckmans K, Buyens K, Bouquet W, Vervaet C, Joye P, Vos FD, Plawinski L, Doeuvre Lc, Angles-Cano E, Sanders NNJNl (2010) Sizing nanomatter in biological fluids by fluorescence single particle tracking. Nano letters 10 (11):4435-4442
15. Semple SC, Akinc A, Chen J, Sandhu AP, Mui BL, Cho CK, Sah DW, Stebbing D, Crosley EJ, Yaworski E (2010) Rational design of cationic lipids for siRNA delivery. Nat Biotechnol 28 (2):172
16. Dos Santos N, Allen C, Doppen A-M, Anantha M, Cox KA, Gallagher RC, Karlsson G, Edwards K, Kenner G, Samuels L (2007) Influence of poly (ethylene glycol) grafting density and polymer length on liposomes: relating plasma circulation lifetimes to protein binding. Biochim Biophys Acta Biomembr 1768 (6):1367-1377
17. Remaut K, Lucas B, Braeckmans K, Demeester J, De Smedt S (2007) Pegylation of liposomes favours the endosomal degradation of the delivered phosphodiester oligonucleotides. J Control Release 117 (2):256-266

18. Peeters L, Sanders N, Jones A, Demeester J, De Smedt S (2007) Post-pegylated lipoplexes are promising vehicles for gene delivery in RPE cells. J Control Release 121 (3):208-217

Figure Captions

Fig. 1. Illustration of the intracellular delivery mechanism by liposomes able to fuse with bacterial membranes. In Gram-negative bacteria (top), liposomes fuse with the outer membrane. At the Gram-positive envelope (bottom), liposomes would have to cross the thick peptidoglycan layer to fuse with the cytoplasmic membrane, via an undetermined mechanism. Reproduced from *Advanced Drug Delivery Reviews*, **136-137**, R. S. Santos, C. Figueiredo, N. F. Azevedo, K. Braeckmans and S. C. De Smedt, Nanomaterials and molecular transporters to overcome the bacterial envelope barrier: Towards advanced delivery of antibiotics, 28-48 Copyright (2018) with permission from Elsevier.

Fig. 2 Schematic diagram of the preparation of lipoplexes via thin film hydration method. Sonication is performed after the formation of the vesicles to produce liposomes of smaller size. Complexation is achieved when negatively charged oligonucleotides encounter positively charged liposomes. Post-PEGylation is finally performed to increase the colloidal and association stability of the lipoplexes.