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ORIGINAL RESEARCH

Interactions of drugs and an oligonucleotide with charged membranes analyzed by immobilized liposome chromatography

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ABSTRACT: We studied the effect of charged lipids or detergent on the retention of drugs and an oligonucleotide by immobilized liposome chromatography to characterize solute–membrane interactions. This is a novel approach in analysis of oligonucleotide–liposome interactions. The charged lipids (phosphatidylserine or distearoyltrimethylammoniumpropane) or detergent (sodium dodecylsulfate) interacted electrostatically in a concentration-dependent matter with the solutes. The oligonucleotide ions presumably bound to the liposomes by multipoint interactions, which was saturable. Sodium dodecylsulfate seemed to affect the drug–membrane interactions more strongly than phosphatidylserine did, probably due to different positioning in the bilayer.

KEYWORDS: drug partitioning; electrostatic effects; immobilized liposome chromatography; liposomes; oligonucleotide–liposome complex; phospholipid bilayers; surfactants

INTRODUCTION

The ability of a pharmaceutical drug to reach its intracellular target is greatly dependent on the partitioning into and the permeability through the cell membrane. Phospholipids, the major membrane constituents, are used for drug–membrane interaction studies in, e.g., surface plasmon resonance analysis using liposome surfaces (Danelian *et al*., 2000; Baird *et al*., 2002; Abdiche and Myszka, 2004), affinity capillary electrophoresis with liposomes as pseudostationary phase (Zhang *et al*., 1995; Roberts *et al*., 1996), immobilized artificial membrane chromatography (Ong *et al*., 1996; Stewart and Chan, 1998; Taillardat-Bertschinger *et al*., 2002), liposome electrokinetic chromatography (Wiedmer *et al*., 2000, 2001; Burns and Khaledi, 2002) and immobilized liposome chromatography, also called drug partition chromatography (Beigi *et al*., 1998; Yang *et al*., 1999; Lagerquist *et al*., 2001; Österberg *et al*., 2001; Liu *et al*., 2002; Boija *et al*., 2004; Engvall and Lundahl, 2004). Other chromatographic methods use detergent in drug partition analysis, e.g., biopartitioning micellar chromatography (Molero-Monfort *et al*., 2000; Martínez-Pla *et al*., 2003) and electrokinetic chromatography with detergent vesicles (Hong *et al*., 1998; Razak *et al*., 2001). Phospholipid liposomes are also used for drug delivery, e.g., the use of cationic liposomes in treatment of cancer and viral diseases (Garcia-Chaumont *et al*., 2000). For therapeutic purposes, antisense oligonucleotides can be used for inhibition of specific gene expression through mRNA binding. One of the major obstacles in antisense therapy is the delivery of the oligonucleotide to the target cell. Efficient delivery systems, such as cationic liposome complexes, are often required to improve cellular uptake of the oligonucleotide (Garcia-Chaumont *et al*., 2000).

The present work is a part of a series of drug partition analyses by chromatography on immobilized natural and artificial membranes to characterize drug–membrane interactions. We analyzed the interaction of an oligonucleotide and several drugs with phosphatidylcholine (PC) or egg phospholipid (EPL) liposomes containing a positive synthetic lipid (1,2-distearoyl-3-trimethylammoniumpropane, DSTAP) or a negative

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Abbreviations used: DSTAP, 1,2-distearoyl-3-trimethylammoniumpropane; EPL, egg phospholipid; GLUT1, the human red cell glucose transporter; PC, phosphatidylcholine; PS, phosphatidylserine; SDS, sodium dodecylsulfate.

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natural phospholipid (phosphatidylserine, PS), or supplemented with a negative detergent (sodium dodecylsulfate, SDS). Comparisons were made with results determined on charged liposomes (Beigi *et al*., 1998) and on liposomes supplemented with detergents (Boija *et al*., 2004). Immobilized liposome chromatography is a novel approach to study the interaction between an oligonucleotide and positive liposomes, which can add important insights on liposomes as drug-delivery systems in antisense therapy.

EXPERIMENTAL

Materials. We purchased glass columns (HR 5, i.d. 5 mm) and Superdex 200 prep-grade gel from GE Healthcare (Uppsala, Sweden), DSTAP (>99%) and PC (hen's egg, >99%) from Avanti Polar Lipids (Alabaster, AL, USA), SDS (>99%) from Merck (Darmstadt, Germany) and alprenolol, corticosterone, cortisone, pindolol, propranolol and PS (bovine brain, approximately 98%) from Sigma (St Louis, MO, USA). Gemfibrozil and naproxen were gifts from Kirsti Gjellan (AstraZeneca, Södertälje, Sweden).

The oligonucleotide (5′-TTTTTTTTTTTTTTTTTC-3′, molecular weight 5789.66, sodium salt) was synthesized using the standard phosphiteamidite chemistry on solid phase (Caruthers, 1991; Beaucage and Iyer, 1992, 1993) and purified by HPLC to a single component, as evidenced by gel electrophoresis. EPLs were prepared from hen's egg yolk (Mascher and Lundahl, 1988) to consist of PC (70%), phosphatidylethanolamine (21%), other phospholipids and lysophospholipids (9%) and small amounts of cholesterol and other components (Yang and Lundahl, 1994). The buffer consisted of 10 mM Tris, 150 mM NaCl and 1 mM Na_2EDTA adjusted to pH 7.4 with HCl at 22°C. All chemicals used were of analytical grade.

Immobilized liposome chromatography. Liposomes were immobilized in gel beads according to Brekkan *et al*. (1997), and Lagerquist *et al*. (2001). Briefly, a lipid film consisting of EPL or PC alone or with addition of either DSTAP or PS was rehydrated with buffer to form multilamellar liposomes in suspension. Unilamellar liposomes were prepared by extrusion of the suspension more than 20 times through a polycarbonate filter (pore size 100 nm) on a LiposoFast extruder from Avestin (Mannheim, Germany). The multilamellar or unilamellar liposome suspensions were mixed with dried Superdex 200 gel beads, which immobilize small amounts of liposomes. The gel mixtures with multilamellar structures were freeze–thawed to entrap larger amounts of the lipid bilayers by fusion of the liposomes. Finally, the gel suspensions were washed by centrifugation, packed into HR glass columns and equilibrated with buffer. The unilamellar liposomes were stably immobilized, as shown by constant retention volumes of propranolol and pindolol (SD 0.09 and 0.01, respectively) during one month and after passage of 25 L of buffer at a flow rate of 1 mL/min (not shown). Multilamellar liposomes are stably immobilized for a period of several weeks (Beigi *et al*., 1998).

SDS was incorporated into the immobilized liposomes by use of detergent-supplemented buffer and pindolol was run to monitor the equilibration of SDS, similarly to Boija *et al*. (2004). Triplicates of oligonucleotide (0.5–4 mg/mL in buffer) and drugs (0.1 mg/mL in buffer, <5% ethanol) were run at 0.5 or 1 mL/min at 22°C and detected at 220 nm on an automatic HPLC system, LaChrom Elite, from Merck Hitachi (Darmstadt, Germany), or on Waters Tunable Absorbance Detector 484 or 486 from Millipore (Bedford, MA, USA) connected with an HPLC pump.

The drug partitioning was expressed as a K_S value (M^{-1}), i.e., the retention of a drug per amount of phospholipid in the gel bed [eq. (1)]:

$$K_S = \frac{V_E - V_0 - V_G}{A} \quad (1)$$

with V_E, the elution volume of the drug, V_0, the elution volume of a compound ($Cr_2O_7^{2-}$) that presumably does not interact with the liposomes or the gel,* and V_G, the retention volume of the drug on a liposome-free gel bed of the same type and size as the gel bed containing liposomes (Lagerquist *et al*., 2001; Boija *et al*., 2004). The phospholipid amount in the gel bed, A, was determined by phosphorus analysis (Bartlett, 1959) after the series of chromatographic runs. The weight fraction of DSTAP in the DSTAP/PC mixtures used to prepare columns was included in the A values in eq. (1).

RESULTS

Effect of DSTAP on oligonucleotide retention

The interaction of the oligonucleotide with multilamellar PC liposomes containing 0–33 mol% DSTAP was studied. The amount of DSTAP in the liposomal bilayers affected the retention of the oligonucleotide, as seen by increased retention with increasing DSTAP amount, although the oligonucleotide eluted before the chromate at 0 and 7 mol% DSTAP [Fig. 1(A)]. Upon increasing the oligonucleotide concentration, the elution volume decreased [Fig. 1(B)].

Effects of DSTAP, PS and SDS on drug partitioning

The partitioning of positive drugs into multilamellar DSTAP/PC liposomes was lower than into pure PC liposomes (Table 1). The partitioning of neutral and negative drugs also decreased (Table 1). The drug partitioning into multilamellar PC liposomes containing 0–16 mol% PS (Fig. 2) and unilamellar EPL liposomes containing 0–29 mol% PS (Table 1, 16 mol% not shown) increased for positive drugs and decreased for neutral and negative drugs as the PS concentration increased. Similarly, the partitioning of positive and negative drugs into unilamellar EPL liposomes

* Ethanol (2% v/v) elutes earlier than chromate and can be used as V_0. However, ethanol might elute lipids from the column.

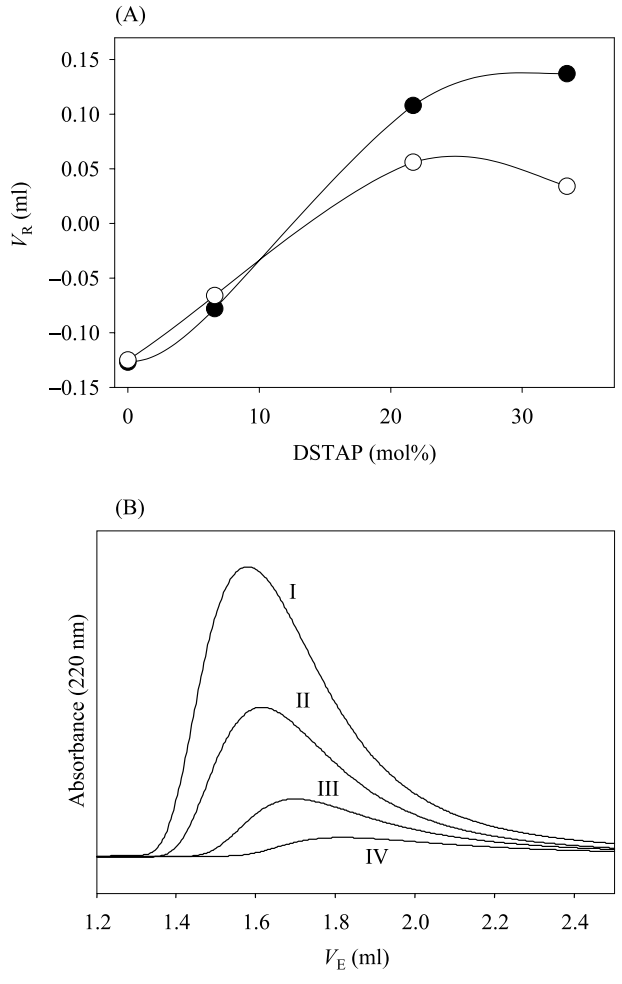

Figure 1. Oligonucleotide retention on multilamellar liposomes composed of 1,2-distearoyl-3-trimethylammoniumpropane (DSTAP) and phosphatidylcholine (PC). (A) Effect of the DSTAP amount (mol%) on the oligonucleotide retention volume, $V_R = V_E - V_0$ (mL). The oligonucleotide concentrations were 0.25 (●) and 0.5 (○) mg/mL and the lipid amounts were 5–25 μmol (four columns). The average SD of V_R was 0.01. (B) Effect of the oligonucleotide concentration on its elution volume, V_E (V_0 1.7 mL), determined on multilamellar PC liposomes with 33 mol% DSTAP. The oligonucleotide concentrations were 4 (I), 2 (II), 1 (III) and 0.5 (IV) mg/mL and the lipid amount was 6 μmol.

increased and decreased, respectively, with increasing concentrations of SDS (0–0.3 mM), whereas the neutral drug corticosterone showed a minor increase (Table 1, 0.05–0.2 mM SDS not shown). Drugs of opposite charges but with similar K_S values on neutral liposomes could be separated on both unilamellar and multilamellar liposomes containing DSTAP or PS, or supplemented with SDS (not shown).

DISCUSSION

The addition of a charged amphipathic molecule, DSTAP, to lipid bilayers increased the retention of the

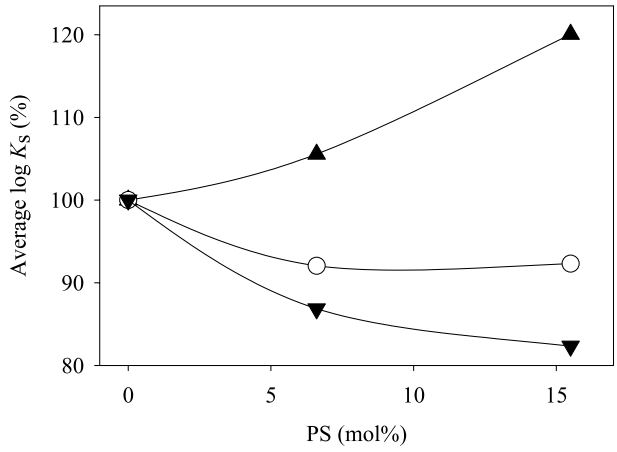

Figure 2. Effect of membrane charge on drug partitioning into multilamellar liposomes composed of phosphatidylserine (PS) and phosphatidylcholine (PC). The average log K_S (%) vs the amount of PS (mol%). The drugs were: alprenolol and pindolol (▲, positive); corticosterone and cortisone (○, neutral); and gemfibrozil and naproxen (▼, negative). The average SD of the log K_S values of the drugs was 0.01.

oligonucleotide [Fig. 1(A)] due to altered properties of the membrane. The repetitive charges (17 negative internucleotidic phosphates at physiological pH) on the backbone of the 18-mer oligonucleotide were attracted to the positive groups on DSTAP in the bilayer, which presumably enable multipoint interactions (Garcia-Chaumont *et al.*, 2000; Lu and Rhodes, 2002). At low amounts of DSTAP, the oligonucleotide eluted before chromate, indicating that the oligonucleotide does not partition into liposomes, in line with (Garcia-Chaumont *et al.*, 2000). Only limited interaction between the oligonucleotide and PC was expected when using a pure pyrimidine oligonucleotide, since binding weakens with an increasing pyrimidine content (Lu and Rhodes, 2002). The packing of the phospholipids in PC liposomes also affect the interaction, as shown by improved binding between the zwitterionic lipids and oligonucleotides below the transition temperature (Lu and Rhodes, 2002). Binding can, hence, be increased when the lipids are in gel phase, probably due to the more disrupted structure of the liposomes (Ickenstein *et al.*, 2003). In this study, the immobilized liposome chromatographic analyses were performed above the transition temperature for PC (10 ± 5°C; Cevc, 1993) with a pure pyrimidine oligonucleotide, which would give an estimate on the minimum retention for an oligonucleotide on the PC bilayer. However, when the positive lipid DSTAP was included in the liposomes, the retention of the oligonucleotide increased, due to electrostatic attraction.

The oligonucleotide retention was affected by its own concentration, and upon increasing the oligonucleotide concentration a saturation effect (Brekkan *et al.*, 1996) was observed (Fig. 1), similarly to Akhtar *et al.* (1991)

Table 1. Effect of charged lipids or detergent on the partitioning of drugs into liposomes

Drugs	Charge at pH 7.4	log K_S		Δ log K_S[a]		
		PC[b]	EPL[c]	DSTAP/PC[d]	PS/EPL[e]	SDS/EPL[f]
Alprenolol	+	2.48	2.49	−0.57	0.40	1.74
Pindolol	+	1.58	1.81	−0.22	0.16	1.06
Corticosterone	0	2.60	2.56	−0.28	−0.25	0.10
Cortisone	0	2.03	n.d.	−0.33	n.d.	n.d.
Gemfibrozil	−	2.51	2.46	−0.15	−0.53	−0.42
Naproxen	−	1.70	1.72	−0.01	−1.43	−0.47[g]

[a] The difference (Δ) in log K_S values between charged liposomes containing DSTAP, PS or SDS and neutral liposomes (PC or EPL). The average SD was 0.02.
[b] Multilamellar PC liposomes. The average SD was 0.003.
[c] Unilamellar EPL liposomes. The average SD was 0.01.
[d] Multilamellar PC liposomes containing 33 mol% DSTAP.
[e] Unilamellar EPL liposomes containing 1.5 mM (29 mol%) PS.
[f] Unilamellar EPL liposomes equilibrated with 0.3 mM SDS.
[g] Determined at 0.2 mM SDS.

and Lu and Rhodes (2002). The size and charge of the oligonucleotide led to sterical crowding effects and electrostatic repulsion between the oligonucleotide ions. These effects are presumably more pronounced than in a standard Langmuir case (Stankowski, 1983a,b; Sild *et al*., 1996). The sodium counterion of the oligonucleotide increased the ionic strength slightly, which reduced the electrostatic interactions. However, since the ionic strength of the buffer was very high in comparison with the change caused by the sodium counterion, this effect was probably neglible in comparison with the sterical crowding effect.

The partitioning of charged drugs was affected by the introduction of charged lipid or detergent, i.e., DSTAP, PS or SDS, into the liposomes, due to altered electrostatic properties of the membrane (Table 1, Fig. 2), similarly to Beigi *et al*. (1998), Österberg *et al*. (2001) and Boija *et al*. (2004). The same tendencies were observed on both unilamellar and multilamellar bilayers (Table 1, Fig. 2, Beigi *et al*., 1998; Boija *et al*., 2004). Furthermore, non-electrostatic effects were observed on the drug partitioning into charged liposomes, as shown by altered partition values of the neutral drugs (Table 1). The partitioning of the negative drugs into DSTAP/PC liposomes also decreased, despite the ionic attraction, which indicates that the non-electrostatic effect was larger than the electrostatic one for those drugs (Table 1). The non-electrostatic effect was possibly caused by altered membrane packing. Drug partitioning into the human red cell glucose transporter, GLUT1, proteoliposomes also showed non-electrostatic effects as well as electrostatic effects from charged amino acids (Lagerquist *et al*., 2001).

The detergent SDS affected the partitioning of charged drugs more than the phospholipid PS, as shown by large effects on the partitioning already at low concentrations of SDS (Table 1). This might be due to differences in positioning between SDS and PS ions in the bilayer. The detergent SDS, which was added in the buffer, probably had a shallower position in the bilayer with its charges more easily accessible to the drugs, whereas the phospholipid PS, which was added when preparing the liposomes, was located in the bilayer.

CONCLUSIONS

Immobilized liposome chromatography demonstrated how the oligonucleotide interacted with positive liposomes. This is of importance in the future studies on liposomal delivery of oligonucleotides. The retention of the oligonucleotide was affected by its concentration and the amount of DSTAP in the liposomes and probably bound by multipoint interactions. The positive DSTAP affected the charged drugs in an opposite manner to the negative PS. A higher PS concentration was needed to get a certain effect on the drug partitioning compared with the SDS concentration, probably caused by different positions of those molecules in the bilayer.

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