Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 3897–3900

Detection of DNA hybridization on a liposome surface using ultrasound velocimetry and turbidimetry methods

Tibor Hianik,[a,*] Peter Rybar,[a] Sergej Yu. Andreev,[b] Tatiana S. Oretskaya[b] and Pankaj Vadgama[c]

[a]*Department of Biophysics and Chemical Physics, Comenius University, 842 48 Bratislava, Slovakia*
[b]*Chemistry Department, M.V.Lomonosov Moscow State University, Moscow 119992, Russia*
[c]*IRC in Biomedical Materials, Queen Mary & Westfield College, London E14NS, UK*

Received 16 March 2004; revised 21 May 2004; accepted 25 May 2004
Available online 19 June 2004

Abstract—19-mer oligonucleotides with oleylamine tethered at 3′ and 5′ terminal, respectively, were incorporated into unilamellar liposomes of dioleoylphosphatidylcholine (DOPC). Addition of complementary nucleotide resulted in hybridization with oligonucleotides located on different liposomes and caused liposome aggregation. Significant changes of sound velocimetry and turbidity were readily observed at 10 nM concentration of the complementary chain.

1. Introduction

Detection of oligonucleotide hybridization offers a convenient means of determining the presence of specific gene sequences in solution.[1] The various methods that have been proposed for this purpose include, amperometric,[2,3] gravimetric,[4–6] optical,[7] and surface plasmon resonance techniques.[8] In amperometric, gravimetric, and surface plasmon resonance methods, the probe, that is single stranded oligonucleotide (ssDNA), is usually anchored to the gold surface of a solid electrode or quartz crystal. Addition of complementary (target) oligonucleotide results in hybridization, that is formation of double stranded DNA (dsDNA), and as a consequence changes in the physical properties of the immobilized DNA film take place that are detected by above mentioned methods. Optical methods utilizing ssDNA modified by a fluorescent dye are applicable for detection of DNA hybridization at both solid surface or in a volume.

It has been shown that incorporation of target DNA into liposomes can be very useful for the amplification of amperometric detection of DNA hybridization.[9] Here the binding of liposomes loaded with target DNA to the probe DNA immobilized on a solid electrode resulted in changes of impedance of the system, that were much higher in comparison with the binding of free DNA, not attached to the liposomes. The use of unilamellar liposomes for the detection of DNA hybridization has been reported by Zhang et al.[10] They modified 20-mer probes of ssDNA using cholesterol at both 3′ and 5′ terminals, that allowed them to be incorporate into liposomes of dimyristoylphosphatidylcholine (DMPC). Addition of complementary oligonucleotides resulted in precipitation of liposomes or in changes in fluorescence intensity of dyes that were used as indicators of hybridization.

In this work we applied ultrasound velocimetry and turbidimetry for the detection of DNA hybridization at a liposome surface. We incorporated 19-mer ssDNA into a liposome by means of oleylamine. Thus, ssDNA was modified by oleylamine either at the 3′ or 5′ terminal. The colloid systems contained equimolar concentration of liposomes modified by DNA with oleylamine at 3′ and 5′ terminals, respectively. Addition of complementary oligonucleotide resulted in aggregation of the liposomes and changes of hydration of both the lipid bilayer and DNA, which were then detected by ultrasound velocity and turbidity.

Keywords: Oligonucleotides; Hybridization; Liposomes; Aggregation; Ultrasound velocimetry; Turbidity.
* Corresponding author. Tel.: +421-2-60295683; fax: +421-2-654267-74; e-mail: hianik@fmph.uniba.sk

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.05.057

2. Experimental section

2.1. Oligonucleotides

Synthesis of oligonucleotides (ODN) with oleyl-containing residues was carried out on an Applied Biosystems 380B automated DNA synthesizer (USA) by the phosphoramidite method as described elsewhere.[11] 2′-Deoxyribonucleoside 5′-*O*-dimethoxytrityl-3′-(*N*,*N*-diisopropylamido)-β-cyanoethyl-phosphites for oligonucleotide synthesis were purchased from Glen Research (USA); all other reagents for oligonucleotide chemistry were purchased from Fluka (Germany). The sequences of the synthesized oligonucleotides are presented in Table 1. The sequence of oligonucleotide (1) is characteristic of the fragment of the *Salmonella typhimurium* gene. An oligonucleotide is complementary to the oligonucleotides modified by oleylamine at 3′ (2) or 5′ (3) terminals. The oligonucleotide of the same composition as (2) and (3) but not modified with oleylamine (4) was used as noncomplementary ODN.

2.2. Oligonucleotide ligation

Ligation with T4 DNA Ligase (NewEnglandBiolabs, USA) was carried out at 4 °C overnight, with equimolar amounts of ODNs and threefold excess of matrix. The products were identified by relative mobility in PAGE (20%).

2.3. Liposomes

Large unilamellar liposomes (diameter ~ 100 nm) were prepared by an extrusion method according to MacDonald et al.[12] using LiposoFast (Avestin Inc., Canada). The liposomes were formed from dioleoylphosphatidylcholine (DOPC) (Fluka, Switzerland). The concentration of lipids was 2 mg/mL. The liposomes were prepared in 0.1 M NaCl + 10 mM Tris + 1 mM EDTA (pH 7.6). The modification of liposomes by oleylamine tethered to ssDNA was performed by addition of the respective oligonucleotides into the liposome suspension into a final concentration of 1 μM. All experiments were performed at the temperature $T = 20 \pm 1$ °C.

2.4. Turbidity measurement

Aggregation of vesicles following hybridization was determined turbidimetrically. This method of determination of the degree of aggregation is based on the dispersion of light by colloid particles in the system. The larger the particles, the better the dispersion of light and the higher the intensity recovered of light. The measurement cell contained the vesicle suspension, a reference cell was filled with buffer. Target complementary or noncomplementary ssDNA was added to both, sample and reference cells. The measurements were carried out at a wave length $\lambda = 555$ nm using a spectrofluorimeter SD2000 (Ocean Optics Inc., USA). Under experimental conditions no changes of intensity of light with the presence of only ssDNA or double stranded DNA, without liposomes occurred. Using the measured value of light intensity without (I_0) and with (I) complementary oligonucleotide, we determined decadic turbidity

$$\tau = -(1/L)\log(I/I_0), \quad (1)$$

where L is the optical path length. The quartz optical cells (Helma, Germany) were thermostated by Ultrathermostat U4 (Germany) with accuracy 20 ± 0.1 °C.

Table 1. Oligonucleotides and oleylamine conjugates

1	3′ d-TGG AAC GAC TTT AAA AGG G 5′
2	3′ d-C_{ole}CC TTT TAA AGT CGT TCC A 5′
3	3′ d-CCC TTT TAA AGT CGT TCC_{ole} A 5′
4	3′-d-CCC TTT TAA AGT CGT TCC A 5′

C_{ole}—Cytidines modified by oleyl residues.

2.5. Ultrasound velocimetry

The measurement of the velocity of ultrasound allows us to evaluate the elastic properties of aqueous media, such as liposome, protein or DNA suspensions. This evaluation is based on a simple relationship: $\beta = 1/(\rho u^2)$, where β is coefficient of adiabatic compressibility and ρ is the density. In the study of mechanical properties of solutions, measuring a relative change in physical characteristics per unit of solute concentration rather than its absolute value is often more important, precise and easier.[13] The so-called concentration increment of ultrasound velocity [u] is a convenient parameter that characterizes changes in compressibility of vesicles and can be easily determined experimentally. This value is defined by the relation

$$[u] = (u - u_0)/u_0 c, \quad (2)$$

where u and u_0 are the sound velocities in solution and buffer, respectively, c is the molar concentration of colloid particles, for example, liposomes. The determination of the [u] value is based on measurement of changes of resonance frequency of the acoustic wave propagated in a small cavity (0.7 mL) of the cell containing piezoelectric transducers. It has been shown, that changes of resonance frequency are proportional to changes of sound velocity.[13] In the experiments we used a two resonance cell configuration.[14] One cell was filled with sample—liposome modified by single stranded probe DNA, while the second with buffer. The target ssDNA was added both to the measured and reference cell, and the direct effect of ssDNA on the liposome suspension was measured at frequency ~ 7 MHz. Since the intensity of the sonic signal in the sample liquid was very small (the pressure amplitude in the ultrasonic wave was less than 10^3 Pa), any effect of the sound wave on a structural transition of the liposomes was avoided. Cells were thermostated at $T = 20 \pm 0.02$ °C with a Lauda RK 8 CS ultrathermostat.

3. Results and discussion

In a first series of experiments we studied changes of concentration increment of sound velocimetry [u] of liposome suspension following addition of complementary or noncomplementary oligonucleotide. The liposome suspension contained equimolar concentrations of liposomes modified by DNA with oleylamine at 3′ and 5′ terminal, respectively. Addition of complementary oligonucleotide resulted in an increase of the [u] value as shown in Figure 1 (curve 1), where the changes of the [u] value as a function of the concentration of target ssDNA are presented. Addition of noncomplementary oligonucleotide ((4), see Table 1) did not result in changes of sound velocity (Fig. 1, curve 2). We consider that addition of complementary oligonucleotide into the liposome suspension is accompanied by liposome aggregation as schematically presented in Figure 2. According to this figure, double stranded DNA is formed between the target oligonucleotide and the two probe oligonucleotides anchored to liposome monolayers by 3′ and 5′ terminals, respectively, depending on the location of oleylamine tether. In order to check this assumption we used turbidimetry, which is known to be sensitive to liposome aggregation. We found that turbidity decreases with increasing concentration of the target ssDNA (results not shown). Addition of noncomplementary ODN did not result in significant changes of turbidity (results are not shown). Thus, aggregation of liposomes does

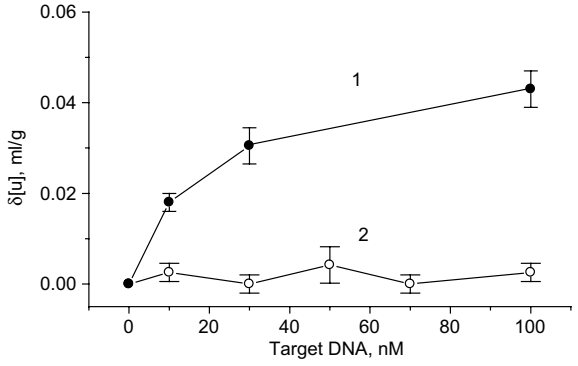

Figure 1. Dependence of concentration increment of sound velocity, [u] of liposome suspension composed of DMPC with incorporated ssDNA tethered with oleylamine at 3′ and 5′ terminal on concentration of 1—complementary, 2—noncomplementary oligonucleotide. The results represent mean ± SD obtained on three independent experiments.

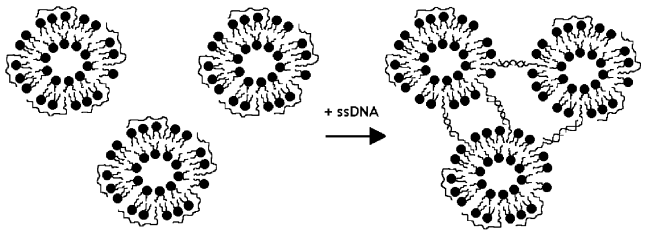

Figure 2. Schematic representation of liposomes modified by oleylamine and their aggregation following addition of complementary oligonucleotides.

Figure 3. Scheme of ligation DNA attached to the liposomes. Description is in the text. ODN 1, 2, and 3 are the oligonucleotides 1, 2, and 3, respectively—see Table 1.

appear to occur following addition of complementary oligonucleotide.

ODNs containing oleylamine moieties introduced into the surface of liposomes are likely to easily form duplexes in the presence of complementary DNA in solution. This fact was confirmed by the enzymatic ligation of two ODNs containing 5′- and 3′-terminal oleyl in the presence of complementary matrix (Fig. 3). For this purpose T4 DNA ligase was used. The ligated oligonucleotides were separated from liposomes and analyzed by gel electrophoresis. The data obtained show that the presence of liposomes has no negative influence on the efficiency of complementary interactions between ODNs containing oleyl residues. It was confirmed by the fact that yields of ligation products obtained in the presence of liposomes (65%) did not differ from those obtained in aqueous solutions (65–70%). This fact can be explained by the presence of large hydrophobic particles in the solution. It causes the effect of 'crowded' solution,[15] which had been shown to increase yields of ligation reactions among two duplexes with blunt ends. It means also that the presence of liposomes does not have negative influence on the efficiency of complementary interactions between oligonucleotides containing oleyl residues.

The mechanism for a decrease in turbidity following aggregation is known, and is connected with increased light scattering, that is increase of the intensity I, resulting from the formation of large aggregates of liposomes. The question, however, arises as to what the mechanism for the increase of the increment of sound velocity following addition of complementary ODN. The value [u] can be expressed as:

$$[u] = \varphi_V - \varphi_{KS}/2\beta_0 - 1/2\rho_0, \quad (3)$$

where φ_V—is the specific volume, $\varphi_{KS}/2\beta_0$ is the molar specific apparent compressibility, β_0 is the coefficient of adiabatic compressibility of the solvent (buffer) and ρ_0 is the density of the solvent.[13] Thus, the increase of [u], observed in the experiment can be related to increase of specific volume or to a decrease of specific compressibility of the lipid bilayer or DNA or both. In addition to intrinsic volume and compressibility of the membrane and DNA, the specific volume and compressibility of hydrated shell surrounding these structures should also be taken into account. Depending on temperature the hydrated shell of these structures revealed a different compressibility in comparison with the compressibility of free water molecules in buffer. For example, at 20 °C,

the compressibility of hydrated shell was lower then that for free water molecules.

Let us first discuss the possible changes of the volume. Recent experiments on DOPC monolayers at the air–water interface performed by the Langmuir–Blodgett technique showed that incorporation of 19-mer oligonucleotide with tethered oleylamine identical to that used in this study (ODN (2), see Table 1) resulted in an increase in the area per phospholipid molecule and in an increase of surface tension.[16] These changes have been connected with repulsive forces between the negatively charged sugar-phosphate backbone of DNA and the negatively charged phosphate groups of phospholipids. We have observed the increase of the area per phospholipid molecules following addition of ODN (2) by 16%. However, a slight decrease of area (2%) was observed after addition of complementary ODN (1). The increase of the area per phospholipid molecule at constant temperature, that is when the conformational state of the hydrocarbon chains of the phospholipids and hence also of their length should not change, could be connected to an increase of specific volume. However, in the case of hybridization, we observed a decrease of this value. We therefore assume, that the increase of $[u]$ is not connected significantly with changes of specific volume.

Let us now discuss the possible contribution of hydration effects. The contribution of hydration of nucleic acids and their building blocks to the value of $[u]$ has been analyzed by Buckin et al.[17] The contribution of hydration to the value $[u]$ for various bases was between 20 and 24 mL/mol. These values are comparable with the value of maximal increase of $[u]$ following addition of the complementary chain, that is $[u] = 0.043\,\text{mL/g} = 0.043\,\text{mL/g} \times 670\,\text{g/mol} = 28.8\,\text{mL/mol}$ (670 g/mol is molecular weight of DMPC). We therefore assume that the increase of $[u]$ is mostly due to the changes of hydration of both DNA and the membrane. The role of the membrane surface is crucial in these changes. Certainly, in the case of free DNA in a solution, we did not observed significant changes of $[u]$ following hybridization with complementary chain at comparable concentrations, that is 1–100 nM. We assume the following mechanism that could explain the role of the liposome surface in changes of hydration. Single stranded DNA interacts with the liposome surface as has been revealed by experiments with lipid monolayers even without presence of divalent cations, considered to be an important stimulator for the formation of so-called triple complexes, that is DNA–cation–membrane.[18] In the interaction of ssDNA with lipid monolayers both attractive and repulsive forces should be considered. The attractive forces are most probably due to the interaction of more hydrophobic bases with the membrane, while repulsive forces occur between the negatively charges sugar-phosphate backbone of DNA and negatively charged phosphate groups of phospholipids. ssDNA at the monolayer surface or at the surface of liposomes is randomly oriented and due to existence of above mentioned forces it is in close contact with the membrane surface. Addition of the complementary chain, however, disturbs the above system. The dsDNA has a different conformation, due to the existence of approximately 50 nm persistent length.[19] Considering that 19-mer DNA has a length of approximately $19 \times 0.36\,\text{nm} = 6.8\,\text{nm}$ (0.36 nm is the projection of one base to the DNA axis) it appears as a hard cylinder orientated perpendicularly or at a certain angle to the bilayer surface. The changes of orientation of the DNA cylinder can be provided only by a certain conformational mobility of the tethered oleylamine. The hybridization of DNA results in detachment of ssDNA from the membrane surface. Both the DNA molecules as well as the membrane surface previously covered by DNA immediately becomes hydrated. These additional hydration effects, in pure analytical terms, service to increase the velocimetry response, and contribute to the ability of the system to detect DNA hybridization at the liposome surface at very low concentration of the target, that is 10 nM.

Acknowledgements

This work was supported by the NATO SfP Programme (Project No. SfP—978003) and by the Slovak Grant Agency (Project No. 1/1015/04).

References and notes

1. Skogerboe, K. J. *Anal. Chem.* **1995**, *67*, 449R.
2. Millan, K. M.; Saraullo, A.; Mikkelsen, S. R. *Anal. Chem.* **1994**, *66*, 2943.
3. Wang, J.; Liu, G.; Polsky, R.; Merkoci, A. *Electrochem. Commun.* **2002**, *4*, 722.
4. Caruso, F.; Rodda, E.; Furlong, D.; Nikura, K.; Okahata, Y. *Anal. Chem.* **1997**, *69*, 2043.
5. Tombelli, S.; Mascini, M.; Sacco, C.; Turner, A. P. F. *Anal. Chim. Acta* **2000**, *418*, 1.
6. Hianik, T.; Gajdos, V.; Krivanek, R.; Oretskaya, T.; Metelev, V.; Volkov, E.; Vadgama, P. *Bioelectrochemistry* **2001**, *53*, 199.
7. Piunno, P. A.; Krull, U. J.; Hudson, R. H. E.; Damha, M. J.; Cohen, H. *Anal. Chim. Acta* **1994**, *288*, 205.
8. Caide, X.; Sui, S. F. *Eur. Biophys. J.* **1999**, *28*, 151.
9. Patolsky, F.; Weizmann, Y.; Willner, I. *J. Am. Chem. Soc.* **2002**, *124*, 770.
10. Zhang, G.; Farooqui, F.; Kinstler, O.; Letsinger, R. L. *Tetrahedron Lett.* **1996**, *37*, 6243.
11. Oretskaya, T. S.; Romanova, E. A.; Andreev, S. Yu.; Toth, C.; Gajdos, V.; Hianik, T. *Bioelectrochemistry* **2002**, *56*, 47.
12. MacDonald, R. C.; MacDonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L. *Biochim. Biophys. Acta* **1991**, *1061*, 297.
13. Sarvazyan, A. P. *Annu. Rev. Biophys. Biophys. Chem.* **1991**, *20*, 321.
14. Halstenberg, S.; Heimburg, T.; Hianik, T.; Kaatze, U.; Krivanek, R. *Biophys. J.* **1998**, *75*, 264.
15. Zimmerman, S.; Pheiffer, B. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5852.
16. Hianik, T.; Vitovic, P.; Humenik, D.; Andreev, S. Yu.; Oretskaya, T. S.; Hall, E. A. H.; Vadgama, P. *Bioelectrochemistry* **2003**, *59*, 35.
17. Buckin, V. A.; Kankiya, B. I.; Kazaryan, R. L. *Biophys. Chem.* **1989**, *34*, 211.
18. Kuvichkin, V. V. *Bioelectochemistry* **2002**, *58*, 3.
19. May, S.; Harries, D.; Ben-Shaul, A. *Biophys. J.* **2000**, *78*, 1681.