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Informational Liposomes: Complexes Derived from Cholesteryl-conjugated Oligonucleotides and Liposomes

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Abstract: Oligonucleotides with cholesteryl groups tethered near one or both termini bind efficiently to liposomes. Liposomes tagged with such oligonucleotides are recognized by complementary oligonucleotides free in solution or bound to a membrane, as shown by experiments in which the liposomes are precipitated or are immobilized on a membrane as a consequence of hybridization.

Liposomes serve as models of cellular membranes,[1-3] as agents for transferring biologically active molecules into cells,[4,5] and as prototypes of self assembling systems.[6,7] We describe here a convenient method for embedding information in and extracting information from liposomes. The information input is effected by mixing a defined oligonucleotide bearing a conjugated hydrophobic tail (a cholesteryl group) with a preformed liposome in aqueous medium,[8] and the information is extracted by hybridization of the anchored oligomer with a complementary oligonucleotide. The hybridization event can be readily detected visually, either as precipitation of the liposome complex or by immobilization of a fluorescently tagged liposome complex on a membrane spotted with the target oligonucleotide.

The structures of the oligonucleotides used for this study are listed in Table 1. In these compounds cholesterol is linked through the oxygen atom to an internucleotide phosphorus atom by a -$CONHCH_2CH_2NH$- tether.

Table 1. Oligonucleotides and Cholesteryl Conjugates

1 5' d-ACACCCAATTCTGAAAATGG	**6** 5' d-C_{ch}TCGCACCCATCTCTCTCC$_{ch}$T
2 5' d-ACACCCAATTCTGAAAATG$_{ch}$G	**7** 3' d-GAGCGTGGGTAGAGAGAGGA
3 5' d-A$_{ch}$CACCCAATTCTGAAAATG$_{ch}$G	**8** 5' d-TTTTTTTTTTTTTTTTTT$_{ch}$T
4 3' d-TGTGGGTTAAGACTTTTACC	**9** 5' d-T$_{ch}$TTTTTTTTTTTTTTTT$_{ch}$T
5 3' d-T$_{ch}$GTGGGTTAAGACTTTTACC	

EXPERIMENTAL SECTION

Oligonucleotides. Oligonucleotides were synthesized by phosphoramidite chemistry and characterized by reversed phase HPLC, gel electrophoresis, and melting curves as previously described.[9] For the step involving introduction of an internucleoside cholesteryl substituent, coupling of the nucleoside was effected via hydrogen phosphonate chemistry, and the cholesteryl group was attached by oxidative coupling of β-(cholesteryloxycarbonylamino)ethylamine.[9]

Liposomes. A chloroform solution (308 μL) containing dimyristoylphosphotidylcholine (DMPC) (5 mg, 7.4 μmol) and cholesterol (0.58 mg, 1.5 μmol) was evaporated under nitrogen, warmed under vacuum for 2 h

at 37 °C, and hydrated with 1 mL of aq. buffer (pH 7.2, 145 mM NaCl, 10 mM Na-HEPES). The mixture was frozen (Dry Ice-acetone bath) and thawed 10 times (37 °C) and then pass through a 0.1 μm polycarbonate membrane (Nucleopore) 20 times. Aliquots of this standard solution were used for the experiments with the oligonucleotides. Electron microscopy of the vesicles stained with potassium phosphotungstate showed relatively uniform spheres with an average diameter of about 100 nm.

Hybridization accompanied by precipitation of the liposomes. A representative precipitation reaction is described. Compound **3** (0.43 A_{260} units, 2.6 nmol, in 31 μL of water) and an aliquot (100 μL) of the liposome solution were mixed in 765 μL of buffer (pH 7.0; 10 mM Tris.HCl, 0.1 M NaCl). On addition of compound **4** (1.14 A_{260} units, 5.3 nmol, in 104 μL of water) a precipitate formed immediately. After centrifugation for 15 min in a Brinkman Model 5414 centrifuge, a UV spectrum of the supernatant revealed 0.61 A_{260} units of oligonucleotide in solution. RP HPLC showed that this oligomer was exclusively compound **4** (2.8 nmol). This amount corresponds well with the amount expected (2.7 nm) if one equivalent (2.6 nm) had precipitated with the liposome-**3** complex. The precipitate was taken up in 1 mL of water. Reversed phase HPLC of this solution showed two peaks of approximately equal area, one at 12.7 min (**4**) and one at 58.1 min (**3**), as expected for the oligomers brought down by the liposome.

Fluorescein labelled liposomes. A solution of fluorescein isothiocyanate (20 mg, 50 μmol) and β-(cholesteryloxycarbonylamino)ethylamine (25 mg, 50 μmol) in DMF:$CHCl_3$ (3:1) was stirred for 2 h at room temperature. Evaporation of the solvents under vacuum and recrystallization of the solid residue afforded the fluorescein-cholesteryl conjugate (40 mg, 91%). Fluorescently tagged liposomes were obtained by mixing DMPC (5 mg, 7.4 μmol) in 250 μL $CHCl_3$ with the fluorescein-cholesteryl conjugate (1.16 mg, 1.4 μmol) in 116 μL $CHCl_3$. After evaporation of the solvent under nitrogen, the residue was hydrated at 37 °C with 1.00 mL of buffer (pH 7.2, 145 mM NaCl, 10 mM Na-HEPES) and liposomes were generated by freeze-thaw and extrusion through a Nucleopore membrane as described for the unlabelled liposomes.

Hybridization and detection with fluorescein labelled liposomes. A nitrocellulose membrane was spotted at three positions with a 1 μL solution containing the target oligonucleotide. The membrane was air-dried for 30 min, baked at 80 °C in a vacuum oven for 2 h, soaked in 6xSSC[10] for 2 min, and placed in a plastic bag along with 50 μL of the labelled liposome solution, 0.2 A_{260} units of the probe cholesteryl-oligonucleotide conjugate, 10 μL denatured salmon sperm DNA (100 μg), NaCl (58 mg), and 940 μL of 5xDenhardt's solution.[10] The bag was sealed and warmed at 37 °C for 2 h; then the membrane was removed and washed with 6xSSc for 10 min. After drying the fluorescent spots were observed under uv light.

RESULTS AND DISCUSSION

Control experiments showed that the liposomes did not interfere significantly with the hybridization of complementary unmodified oligomers or hybridization of an unmodified oligomer with a complementary oligomer bearing a single cholesteryl group. Thus the dissociation temperature, Tm, for **1**+**4** and also for **2**+**4**

(each oligomer 2.5 μM) was 60 °C when determined in a standard buffer (0.1 M NaCl, 10 mM Tris.HCl, pH 7.2) and 58 °C when measured in the buffer in the presence of liposomes (0.27 mM in lipid). Also, individual liposome solutions containing the cholesteryl conjugates **2** and **3** (2.5 μM in 0.9 mL buffer and 100 μL of the liposome solution) were found to be stable with respect to precipitation. The stability of the liposome solution containing the oligomer with two cholesteryl substituents is somewhat surprising since one might expect that the cholesteryl groups would anchor in different liposomes, leading to cross-linking, aggregation, and precipitation. Absence of precipitation therefore suggests that in this system the oligonucleotide conjugate, as a relatively flexible single stranded oligomer, prefers to fold back to the liposome surface so that both cholesteryl groups are anchored in the same liposome.

In contrast to the behavior of **2**, when an unmodified complementary oligonucleotide (**4**) was added to a liposome solution containing the bis-cholesterol derivative, **3**, in the standard buffer solution, an immediate precipitate appeared. Further investigation revealed several significant aspects of this transformation. (1) Formation of the precipitate depends on the sequence of the added oligonucleotide. Addition of a non-complementary oligonucleotide is ineffective; no precipitate is formed. (2) No precipitate appears in the absence of salt, which favors hybridization of the oligonucleotides. (3) The aggregation is reversible. After collection of the precipitate by centrifugation, the precipitate will redissolve in water. (4) When an excess of a complementary oligonucleotide (**4**) is added, all of the cholesteryl oligonucleotide separates with the precipitate, along with an equivalent of the complementary oligonucleotide; excess **4** remains in the aqueous layer.

These observations were confirmed by study of another set of oligonucleotides, **6** and **7**. In addition, we found that mixing a liposome solution containing **2** with a liposome solution containing the complementary oligonucleotide anchored at the 5' end, **5**, similarly led to precipitation. On the basis of these data we believe that cholesteryl-oligonucleotide conjugates bind efficiently to the liposomes, that the bound oligomers hybridize to complementary oligonucleotides in solution, and that hybridization triggers a conformational change leading to precipitation of the liposomes. A plausible explanation for precipitation is that hybridization converts the flexible immobilized oligomer to a relatively stiff, extended duplex, which favors a structure with the terminal cholesteryl groups anchored in different vesicles. This change would afford the cross-links responsible for aggregation. For the system **2** + **4**, hybridization directly affords the cross-links between the liposomes.

To explore further the properties of oligonucleotides anchored to liposome surfaces we examined immobilization of doubly tagged liposomes on a nitrocellulose membrane. In this experiment liposomes were prepared with fluorescein anchored at the membrane through a linker joined to a cholesteryl group; then they were further labelled with an oligonucleotide, by mixing with a cholesteryl-oligonucleotide conjugate in solution, and used as probes for oligonucleotides immobilized on the membrane. The results are shown in Table 2. In each case spots containing 10^{-3}, 10^{-4}, and 10^{-5} A_{260} units, respectively, of the target oligonucleotide were tested. A plus sign indicates that a fluorescent spot was observed for each sample, and a minus sign, that no fluorescence was observed for any of the spots. Fluorescence spots appeared when, and only when, the oligonucleotide at the

surface of the liposome was complementary to the oligonucleotide on the membrane. The intensity of the fluorescence depended on the amount of target oligonucleotide spotted on the membrane, the highest concentration showing the strongest fluorescence. In principle, the intensity of the signal could also be controlled by varying the structure and amount of the chromophore imbedded in the liposomes. These results demonstrate that the oligonucleotides anchored via a cholesteryl group at the surface of the liposomes serve to immobilize the liposome on a membrane containing the complementary oligonucleotide. It may be noted that probe oligonucleotides conjugated at both ends as well as at one end are effective.

Table 2. Hybridization with Oligonucleotide Conjugates Associated with Fluorescent Liposomes

Exp.	Probe	Target	Signal
1.	5' d-T_{ch}TTTTTTTTTTTTTTTTTT$_{ch}$T[a]	Poly(dA)	+
2.	5' d-T_{ch}TTTTTTTTTTTTTTTTTT$_{ch}$T	Poly(dT)	-
3.	5' d-TTTTTTTTTTTTTTTTTT$_{ch}$T	Poly(dA)	+
4.	5' d-C_{ch}TCGCACCCATCTCTCTCC$_{ch}$T	3' d-GAGCGTGGGTAGAGAGAGGA	+
5.	5' d-C_{ch}TCGCACCCATCTCTCTCC$_{ch}$T	Poly(dA)	-

a. *ch* represents a cholesteryl group linked by an oxycarbonylaminoethylamino group to an internucleotide phosphorus atom.

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