Phospholipid membrane permeability of peptide nucleic acid

Pernilla Wittung[a,*], Johan Kajanus[a,**], Katarina Edwards[b], Peter Nielsen[c], Bengt Nordén[a], Bo G. Malmström[d]

[a]*Department of Physical Chemistry, Chalmers University of Technology, S-412 96 Göteborg, Sweden*
[b]*Department of Physical Chemistry, Uppsala University, PO Box 532, S-751 21 Uppsala, Sweden*
[c]*Center for Biomolecular Recognition, The Panum Institute, Department of Biochemistry B, Blegdamsvej 3c, DK-2200 N Copenhagen, Denmark*
[d]*Department of Biochemistry and Biophysics, Göteborg University, Medicinaregatan 9C, S-413 90 Göteborg, Sweden*

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Abstract Phospholipid vesicles (liposomes) as membrane models have been used to study the penetration properties of peptide nucleic acid (PNA), a new DNA analog in which the nucleobases are attached to a pseudo-peptide backbone. The liposomes were characterised by carboxyfluorescein efflux, light-scattering and cryogenic transmission electron microscopy. The liposome structure was found not to be affected by the incorporation of PNA or an oligonucleotide. Two 10-mer fluorescein-labelled PNAs were found to have low efflux rates (half-times of 5.5 and 11 days), comparable to a 10-mer oligonucleotide (half-time of 7 days). We conclude that passive diffusion of unmodified PNA is not an effective way of transport into biological cells.

Key words: Liposome; Peptide nucleic acid; Cryogenic transmission electron microscopy; Fluorescence

1. Introduction

Oligonucleotides, and their analogs, are of great interest as potential tools for sequence-specific modification of DNA and gene-targeted drugs [1–3]. Natural oligonucleotides are rapidly degraded by enzymes in living organisms, and for this reason oligonucleotide analogs are potentially more useful as drugs. One such analog is peptide nucleic acid, PNA [4–6]. In PNA the entire deoxyribose backbone is replaced by a structurally homomorphous pseudo-peptide backbone, composed of *N*-(2-aminoethyl)glycine units, as shown in Fig. 1. PNA has been found to be a very potent DNA mimic in terms of sequence-specific binding to DNA and RNA [7], and it is resistant to biological degradation [8]. Thus, PNA is considered promising as a pharmaceutical agent [9].

For PNA to be useful as an antigene or antisense drug, it must, however, be able to enter into the cells. We have, therefore, investigated the membrane permeabilities of PNA molecules, and compared them to the permeability of an oligonucleotide, by the use of intermediate-sized liposomes as a model system. The liposomes were shown to be largely unilamellar with an average size around 100 nm in diameter by cryogenic transmission electron microscopy. We find the efflux rate of fluorescein-labelled 10-mer PNA molecules incorporated in such liposomes to be slow (half time of the order of one week), similar to the efflux rate of an oligonucleotide of the same size.

*Corresponding author. Fax: (46) (31) 772-3858.

***Present address:* Department of Organic Chemistry, Chalmers University of Technology, S-412 96 Göteborg, Sweden.

2. Materials and methods

2.1. Chemicals

The PNA and DNA oligonucleotides were synthesised as described elsewhere [5,6]. Soybean phospholipids (containing ca. 15% L-α-phosphatidyl choline) were obtained from Sigma (type II-S; Sigma P5638), as was also Triton X-100. 5-Carboxyfluorescein (and 6-) was obtained from Molecular Probes (C-194). All other chemicals were of analytical grade.

2.2. Preparation and characterisation of liposomes

Liposomes were prepared by an extrusion method [10]. A suspension of 50 mg of phospholipids in 1 ml of 5 mM Na-phosphate buffer, pH 7.0, also containing the substance to be entrapped (carboxyfluorescein, PNAs or oligonucleotide), was vortexed for 5 min and then subjected to five freeze (liquid N_2) and thaw (room temperature) cycles. The suspension was, thereafter, passed 21 times through a 100-nm polycarbonate filter in a commercial, small volume (0.5 ml) extrusion apparatus (LipsoFast-Basic). The lipsomes were separated from non-entrapped substance by gel filtration on a Sephadex G-100 column.

The size of the liposomes was determined by quasi-elastic light scattering (Malvern Instruments) with a helium–neon laser light source. Size and quality was observed by cryogenic transmission electron microscopy [11,12]. The surface charge of the liposomes was measured with a Malvern Zetasizer 2c. The quality of the liposomes was, in addition, checked by measurements of the leakage of carboxyfluorescein. A solution of carboxyfluorescein (80 mM) was included in the buffer when preparing the liposomes. In this case, a Sephadex G-25 column was used to separate the liposomes from the surrounding solution. The fluorescence of the separated liposome suspension was measured at 520 nm with an excitation wavelength of 470 nm on an Aminco SPF-500 Quantum Corrected spectrofluorimeter. The total concentration of carboxyfluorescein was measured after disruption of the liposomes with Triton X-100.

2.3. Efflux measurements

A solution of fluorescein-labelled PNA or oligonucleotide (at a concentration of approx. 50 μM) was entrapped in liposomes as described. From the separated liposome suspension, incubated at 20 °C, small aliquots were taken at different times. The aliquots were diluted 6-fold with buffer and then centrifuged through a Micron filter (Microcon 50, Amincon Inc.) at $2000 \times g$ for 10 min. The liposome-free filtrate was then further diluted with buffer, and the fluoresecence measured in the wavelength interval 500–600 nm with excitation at 497 nm. Again, the total concentration of entrapped substance was obtained by disrupting the liposomes with Triton X-100; even if the labelled oligomers fluoresce, this was necessary to avoid errors introduced from a non-linear concentration dependence of the fluorescence with liposome suspensions as a result of absorption statistics [13,14].

2.4. Partition coefficients

The partition coefficients of the PNAs and an oligonucleotide between octanol and water solution were determined. Equal volumes of octanol and the oligomer solution were shaken together mechanically for 20 min and then allowed to separate. The octanol phase was then transferred to a new water phase and shaken. After separation, the fluorescence of the two water phases was measured, and with the aid of the measured fluorescence and the volumes used, the partition coefficients (water/octanol) were calculated.

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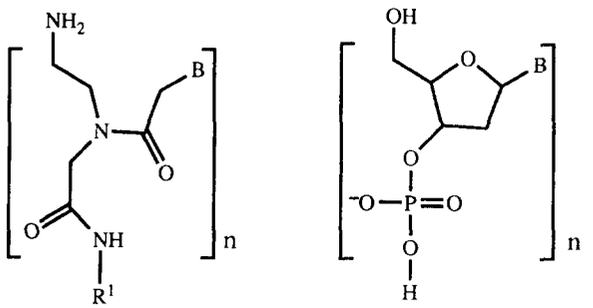

PNA **DNA**

Fig. 1. Chemical structure of PNA and DNA.

Table 1
Half-time for the efflux from liposomes and partition coefficients (water/octanol) for two 10-mer PNAs and a 10-mer oligonucleotide

Compound	Efflux half-time (days)	Partition coefficient (water/octanol)
Fl-CTCTTTTTTT-Glu_{10}-NH_2	11.0 ± 1	15 ± 5
Fl-TTTTCCTCTC-Lys-NH_2	5.5 ± 1	107 ± 10
5′-Fl-GTAGATCACT-3′	7.0 ± 1	495 ± 15
Carboxyfluorescein	2.0 ± 0.1	–

3. Results and discussion

Light-scattering demonstrated an average diameter of the liposomes of 70 ± 20 nm, but the cryogenic transmission electron microscopy revealed a polydispersity with a dominating size around 100 nm (Fig. 2). The micrographs show, in addition, that the liposomes were mainly unilamellar but with some proportion of bilamellarity. The surface of the liposomes had a negative charge, corresponding to an effective zeta potential of about −115 mV at pH 7.0, as determined from the electrophoretic mobility.

The quality of the liposomes was checked by measuring the escape of entrapped carboxyfluorescein into the surrounding solution. At the concentrations used, the fluorescence of the entrapped carboxyfluorescein is self-quenched, whereas the escaped molecules fluoresce. The liposomes prepared here were found to have a half-time for the carboxyfluorescein leakage of 48 h (Table 1), which is characteristic for a very tight phospholipid bilayer [15]. The same efflux rate was found with a centrifugation method (see section 2.3). The lipsomes were also characterised after incorporation of PNAs or oligonucleotides and found to be intact both by light-scattering and cryogenic transmission electron microscopy, for up to 3 weeks.

The efflux of fluorescein-labelled PNAs was studied by fluorescence spectroscopy. Two 10-mer PNA molecules and, for comparison, a 10-mer oligonucleotide were investigated (see Table 1 for sequences). One PNA had 10 glutamic acid residues attached to the carboxy-terminal. The PNAs were found to traverse the lipsome membrane slowly. As shown in Fig. 3 and Table 1, the two PNA molecules studied had efflux half-times comparable to the efflux half-time of the oligonucleotide. The half-time for the oligonucleotide (7 days) is comparable to that found for the escape from multilamellar vesicles of a 15-mer methylphosphonate oligonucleotide labelled by rhodamine

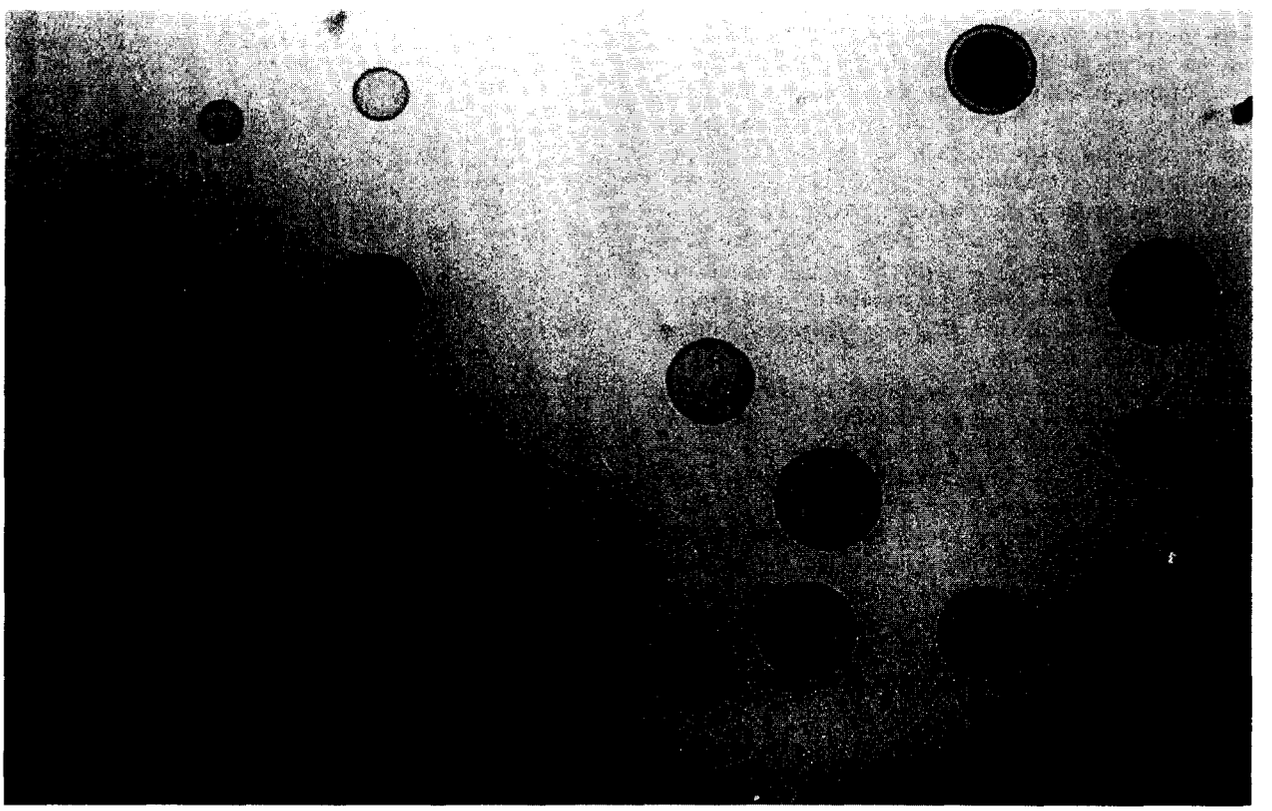

Fig. 2. PNA-containing liposomes depicted by cryogenic transmission electron microscopy. The magnification was 120,000 ×.

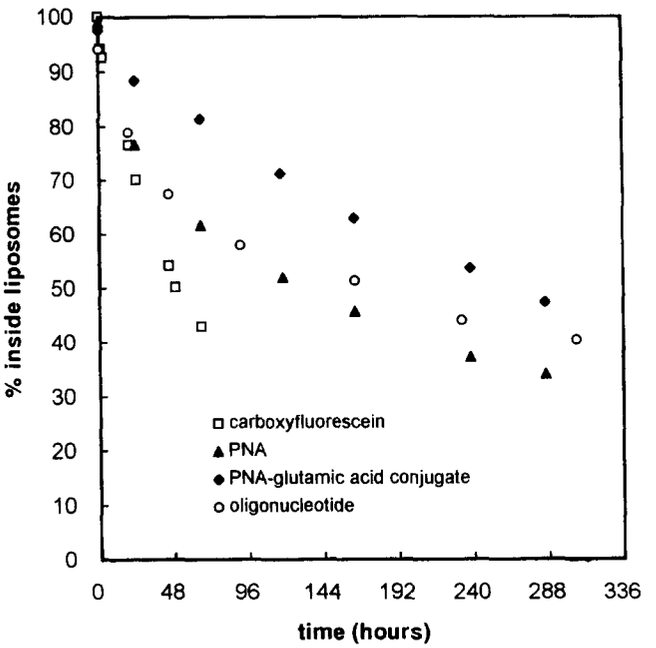

Fig. 3. Release of PNAs, DNA and carboxyfluorescein from liposomes with time. The calculated efflux half-times are listed in Table 1.

[16]. It was also shown that unmodified oligonucleotides, 14-, 7- and 4-mers, have efflux half-times of 7–9 days, as probed by radioactivity. In another report, a 10-mer phosphothioate oligonucleotide labelled with fluorescein showed an efflux half-time of 6.3 days from multilamellar vesicles [17].

The partition coefficients (water/octanol) for the PNAs and the oligonucleotide are listed in Table 1. Both PNAs molecules have partition coefficients much smaller than what is found for the oligonucleotide. This shows that the PNAs are more lipophilic than the oligonucleotide. They are still, however, more soluble in water than in octanol. The results suggest that the entrapped PNA is situated in the aqueous compartments of the lipsomes rather than being dissolved in the lipid membrane. For comparison, the partition coefficient (water/octanol) for a 12-mer methylphosphonate oligonucleotide was found to be 24 [16] and for a 16-mer unmodified oligonucleotide 57000 [18]. Lipophilic modifications of phosphothioate oligonucleotides, in order to increase the permeability, showed only modest effects on penetration rates [17], suggesting that simple considerations, based on Overton's rule [19] of the relationship between lipophilicity and membrane permeation, may not apply to the permeation of large, complex compounds, such as PNA or oligonuclotides. It is also possible that the liposome structure is dynamic, and that this allows the slow escape of the large molecules. This possibility gets some support from the fact that the observed leakage rates of the entrapped molecules are all around 7 days, despite differences in size, polarity and charge.

Our results indicate that the entry of PNA into living cells by passive diffusion through the membranes will be very slow. Thus, other mechanisms of delivery must be found for cellular uptake of PNA. The slow efflux of PNA from liposomes may, however, be useful for delivery, if liposomes are used as drug carriers, releasing its content into cells upon fusing with the cell membrane. Modifying PNA by groups specific for receptor proteins on certain cell membranes is another attractive possibility to achieve cell-type specific directed delivery.

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