Comparative study of the protection of modified and unmodified dsDNA by cationic and non-cationic lipids and liposomes to digestion by DNase I

Marta Legido and Chris Abell*

Department of Chemistry, Cambridge University, Lensfield Road, Cambridge, UK CB2 1EW

A comparative study of the protection of dsDNA by cationic and non-cationic lipids and liposomes from degradation by DNase I reveals formulation-dependent differences in protection.

Introduction

The potential of gene therapy will not be realised until major problems associated with delivery of small, synthetic antisense oligonucleotides into the cell have been overcome more effectively.[1] Several important strategies have evolved to address this issue, including modification of the oligonucleotides by conjugation with molecules that confer lipophilicity onto the oligonucleotide.[2,3] This kind of modification has been reported to increase nuclease resistance of the DNA.[4] Another major approach to improving cellular uptake and metabolic stability of oligonucleotides has been to use liposomes to deliver DNA into cells where they are then internalised by endocytosis.[5] Uncharged or negatively-charged liposomes do not encapsulate the DNA efficiently.[6] Encapsulation was improved if the DNA was modified to make it more lipophilic.[3] Cationic liposomes have proved more successful and have been used for the delivery of the DNA and RNA into cells.[7] The DNA is usually complexed on the positively charged outer surface of the liposome, but has also been entrapped inside the vesicle.[8]

The relative merits of the different liposome preparations are difficult to assess as the many studies reported are not always directly comparable. In this paper we report a comparative study of the stability to digestion by DNase I of short dsDNA, both unmodified and with a lipophilic modification, in the presence of cationic and non-cationic lipids and liposomes. The degradation of dsDNA was measured by following the decay of the enhanced fluorescence of the complex formed between dsDNA and PicoGreen.[9]

Results and discussion

Two short oligonucleotides (17mers) were prepared to form duplex **1**: 5′-GGAGCTCCAGCTTTTGT-3′ and 5′-ACAAAAGC**T**GGAGCTCC-3′. The modified duplex **2** was identical except that the base in **T** was replaced by a modified 2′-deoxyuridine substituted at the 5-position with -SCH_2-$CH_2NHCOC_{11}H_{23}$.[10] The decrease of fluorescence due to the degradation of the PicoGreen complex with dsDNA was monitored under a range of conditions, the graphs in Figs. 1–3 show the percentage of fluorescence remaining as a function of time.

It has previously been shown that DNA inside liposomes is protected against degradation by nucleases.[11] Our first objective was to repeat this result to establish the validity of our experimental approach. dsDNA **1** was encapsulated in the cationic liposomes by sonicating the mixture of cholesterol–L-α-phosphatidylcholine–dipalmitoyl-L-α-phosphatidylethanolamidolysine (Cho–PtdCho–Lys-Pam_2Gro*P*Etn) in the presence of the DNA and PicoGreen. The fluorescence observed immediately after sonication and centrifugation of the sample was taken as the starting point (100% in Fig. 1). Treatment of this sample

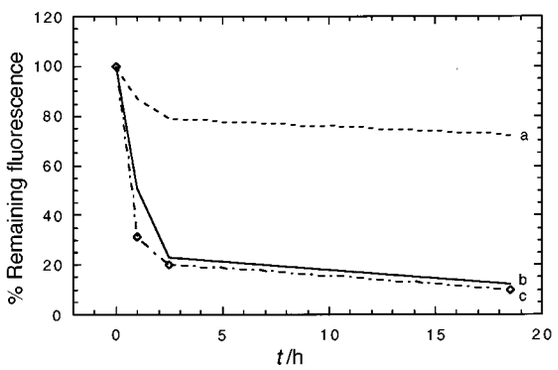

Fig. 1 Degradation of dsDNA **1** entrapped in cationic liposomes. Timecourses of the decrease in fluorescence of the PicoGreen dsDNA complex as it is degraded by DNase I. (a) **1** Entrapped in cationic liposomes; (b) as for (a) but treated with Triton (0.5%) for 1 h at 37 °C prior to addition of DNase I; (c) a control with **1**, PicoGreen and DNase I in TE buffer.

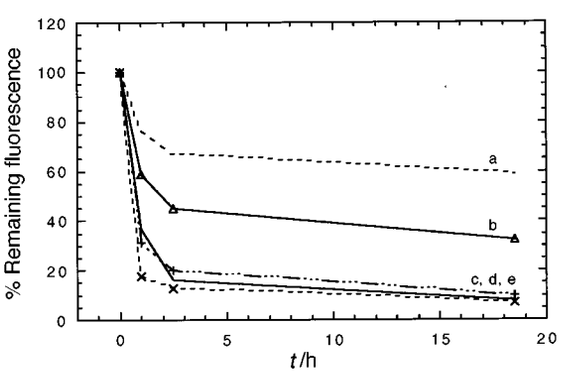

Fig. 2 Degradation of dsDNA **1** in the presence of cationic and non-cationic lipids and liposomes. Timecourses of the decrease in fluorescence of the PicoGreen dsDNA complex as it is degraded by DNase I. (a) **1** Complexed with a mixture of cationic lipids; (b) **1** complexed with empty cationic liposomes; (c) a control with **1**, PicoGreen and DNase I in TE buffer; (d) **1** complexed with non-cationic lipids; (e) **1** complexed with empty non-cationic liposomes.

with DNase I showed that the DNA inside the liposome is well-protected from degradation. The initial fluorescence decreased to about 80% over three hours and then slowly thereafter. The initial decrease in fluorescence may be due to degradation of dsDNA that has not been entrapped into the liposomes, as entrapment efficiencies of about 80% have been reported for this kind of cationic liposomes.[8] The subsequent small slow decrease in fluorescence may be due to decomposition of PicoGreen. It is not affected by addition of more DNase I. The fluorescence of the control sample containing only **1** and PicoGreen decayed to less than 15% of its initial value. This residual fluorescence is due to unassociated PicoGreen and lipids and does not indicate any residual dsDNA. To prove that the dsDNA really was sequestered in the liposomes, an aliquot of the loaded liposome preparation was treated with Triton

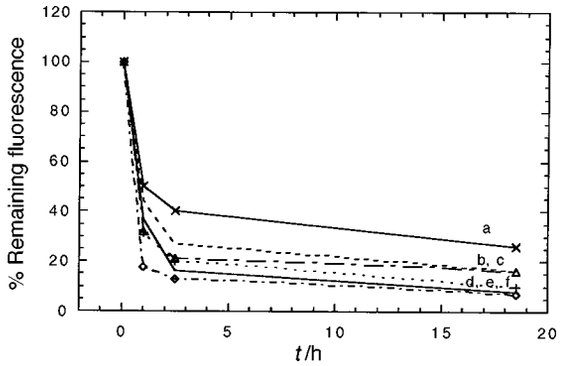

Fig. 3 Degradation of dsDNA **1** and **2** in the presence of non-cationic lipids and liposomes. Timecourses of the decrease in fluorescence of the PicoGreen dsDNA complex as it is degraded by DNase I. (a) **2** Complexed with non-cationic lipids; (b) **2** complexed with empty non-cationic liposomes; (c) a control with **2**, PicoGreen and DNase I in TE buffer; (d) **1** complexed with non-cationic lipids; (e) **1** complexed with empty non-cationic liposomes; (f) a control with **1**, PicoGreen and DNase I in TE buffer.

X-100 prior to addition of DNase I. The fluorescence decreased in the same way that the control sample did containing only the dsDNA complex, showing that there is no complexation between DNA and lipids in the presence of Triton. All the above experiments were also carried out using the modified duplex **2**. The results (not shown) were essentially the same as seen with **1**.

Fig. 2 compares the protection afforded by mixing dsDNA with lipids (without sonication) and liposomes, both cationic and non-cationic. Empty small cationic liposomes were formed by sonication in the absence of dsDNA, which was added subsequently with PicoGreen. The sample was then treated with DNase I. By the time that the control sample, only containing **1** and PicoGreen was completely digested, the fluorescence of the cationic liposome-protected sample had decreased to 45%. Similarly, **1** and PicoGreen were added to the same mixture of lipids as had been used to prepare the liposomes, but now without any sonication. Upon treatment with DNase the fluorescence decreased, but this time to only 65% of its original value.

These experiments were also performed using the non-cationic mixture of lipids with and without sonication. In both cases the initial fluorescence decreased rapidly during treatment with DNase I showing that the dsDNA was completely degraded.

Using the lipophilic-modified duplex **2** and cationic mixtures of lipids or liposomes the results (not shown) were similar to those for **1** albeit the decrease in fluorescence was marginally smaller.

A more marked difference in stability between **1** and **2** was observed when the non-cationic mixture of lipids (Cho–PtdCho 3 : 7) was used (Fig. 3). Using the unmodified DNA **1** the duplex was rapidly destroyed. The decrease in fluorescence was very similar in experiments with lipids or liposomes and in the control lacking either. Using the modified DNA **2** the presence of lipids afforded some protection, as the fluorescence dropped to only 40% after two hours. The presence of liposomes also afforded some protection to **2** relative to the corresponding control over the first few hours.

It has previously been shown that this mixture of non-cationic lipids does not entrap DNA efficiently when sonicated in the presence of dsDNA,[8] and our results show that there is no protection by mixing with preformed liposomes or the unsonicated lipid mixture. The modification of the oligonucleotide did afford some protection of the dsDNA, most significantly in the presence of the unsonicated lipids. This suggests that there is some interaction between the lipophilic sidechain and the lipids in solution.

It is a possibly surprising result of these studies that unsonicated lipid mixtures are more efficient at protecting against degradation of the duplex in the presence of DNase I than liposomes.

The general trends that emerge from this study are that the cationic lipids afford more protection than non-cationic lipids. Enclosure within a liposome provides most protection, but depends upon having a good entrapment efficiency. Both these conclusions are apparent from previous studies. What is perhaps surprising is that a simple mixture of lipids affords significant protection, more than mixing the dsDNA with preformed liposomes. The unsonicated lipid mixture forms some kind of large liposome structures[12] which could interact more efficiently with the dsDNA than the small liposomes which are formed by sonication. The significant point is that sonication is time consuming and may indeed be counterproductive. The other conclusion from this work is that the simple experimental system we have used, employing PicoGreen to study duplex degradation has successfully replicated the general trends noted in published work.

Experimental

Materials

Bovine pancreatic DNase I (2000 units mg^{-1}), Triton X-100, cholesterol (Cho) and L-α-phosphatidylcholine (PtdCho) were purchased from Sigma. PicoGreen was purchased from Molecular Probes. Dipalmitoyl-L-α-phosphatidylethanolamido-lysine (Lys-Pam_2Gro*P*Etn) was synthesised as described by Puyal.[8] Oligonucleotides were synthesised by Oswel DNA Service (University of Southampton) on an Applied Biosystems ABI 394 DNA synthesiser, using phosphoramidite chemistry.

Liposome preparation

Cationic liposomes were prepared using Cho–PtdCho–Lys-Pam_2Gro*P*Etn in a proportion of 30 : 55 : 15. The non-cationic liposome was prepared using a mixture of Cho–PtdCho 3 : 7.[8] The mixtures of lipids were dissolved in chloroform and the solvent was removed under reduced pressure. The lipid mixtures were resuspended in TE buffer pH 7.4 (10 mM Tris, 1 mM EDTA) at a final concentration of 4 mg ml^{-1} and cooled over ice. The mixtures were sonicated (30 s on, 60 s off) until the turbidity cleared, using a probe sonicator (DAVE, type 7530 A). After sonication, the liposome suspensions were centrifuged at 7000 *g* for 10 min.

Liposome encapsulated dsDNA

The dry lipid mixtures were resuspended in TE buffer pH 7.4 at a final concentration of 4 mg ml^{-1} and mixed with the duplex DNA (1 μg ml^{-1}) and PicoGreen (5 μl ml^{-1}). The mixtures were then sonicated as described above.

Preparation of DNA–liposome and DNA–lipid mixtures

The duplex DNA (**1** or **2**) (1 μg) and PicoGreen (5 μl) were mixed with the lipids mixture in TE buffer (1 ml) or with a suspension of empty small liposomes (1 ml) prepared as described above.

Fluorescence studies

Fluorescence measurements were carried out on a Shimadzu RF-5001 PC spectrofluorophotometer, using a 1 cm light-path cell. Fluorescence was measured immediately after addition of PicoGreen (dsDNA–PicoGreen complex: $\lambda_{ex} = 480$ nm, $\lambda_{em} = 520$ nm) to the various DNA–liposomes or DNA–lipids mixtures, and at several times during the DNase treatment.

DNase digestion

A solution of DNase I (0.1 mg in 400 μl) in NaCl (0.1 M) was freshly prepared before treatment. Samples containing dsDNA,

PicoGreen and different solutions of liposomes or lipids were treated with DNase I (2.5 μg ml^{-1}) and incubated at 37 °C after addition of $MgCl_2$ (1 M, 25 μl ml^{-1}) and NaCl (5 M, 10 μl ml^{-1}). Control samples containing a solution of dsDNA and PicoGreen complex in TE buffer were treated in the same way to follow the degradation of free DNA by the DNase I.

Acknowledgements

We thank Justin Tomkins for preparing the modified oligonucleotide. A grant from the Basque Government to M. L. is gratefully acknowledged.

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Paper 8/02212G
Received 20*th March* 1998
Accepted 8*th April* 1998