ENTRAPMENT OF RIBONUCLEIC ACIDS IN LIPOSOMES

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1. Introduction

The introduction of biologically active macromolecules into cells and the study of their interaction has always been a difficult problem because of the failure of cells to incorporate them. The versatile role of liposomes for the introduction of such molecules into cells is becoming increasingly apparent [1,2]. Thus, liposomes have been used as carriers for enzymes [3,4], anti-tumour drugs [5] and small molecules [6,7]. Synthetic double-stranded ribonucleic acid, poly (I): poly (C), has been incorporated in liposomes and used as inducers of interferon.

The trapping of a native, biologically-active (translatable), messenger RNA (coding for globin synthesis) in large unilamellar liposomes is reported here. The entrapped RNA resistant to ribonuclease treatment, could be re-isolated intact from the liposomes and, as determined by protein synthesis stimulation in a cell-free system, it remains biologically active.

2. Materials and methods

Beef brain phosphatidylserine was obtained from Lipid Products, Redhill, Surrey. $Na^{125}I$ carrier-free (300 mCi/ml) was from the Radiochemical Centre, Amersham, Bucks. Pancreatic ribonuclease, pronase and diethylpyrocarbonate were purchased from Sigma. Oligo(dT)-cellulose was from Collaborative Res., MA and Sepharose-4B from Pharmacia Fine Chem. All other reagents were from BDH Chemicals Ltd. All glassware and solutions were treated with diethylpyrocarbonate and autoclaved to destroy any ribonuclease activity.

Rabbit reticulocytes and reticulocyte polysomes were prepared as in [11]. Poly(A)$^+$ RNA (globin messenger RNA) was prepared by affinity chromatography on oligo(dT)-cellulose [12] and its iodination was carried out as in [13]. The radioactivity was measured with a Packard-5230 Auto gamma scintillation spectrometer. Radio-labelled RNA spec. act. was 2×10^8 cpm/μg.

Large unilamellar liposomes containing globin mRNA were prepared as in [14]. Beef brain phosphatidylserine was freed of divalent cations by washing with EDTA. Phosphatidylserine, 10 μmol/ml in NHTE buffer, pH 7.4 (0.1 M NaCl, 2 mM histidine, 2 mM TES, 0.4 mM EDTA, pH 7.4) was sonicated in a bath type sonicator at 30°C, until the suspension was optically clear. Ca^{2+}, 0.02 mmol, was added and the mixture was incubated for 1 h at 37°C and centrifuged at 3000 × g for 10 min. The pellet was suspended in 0.1 ml RNA solution, which had been previously dialysed against NHTE buffer, pH 7.4. The mixture was vortexed, 0.02 mmol EDTA was added and incubated at 37°C for 30 min. The liposomes were recovered by centrifugation (30 000 × g, 20 min at 20°C) and were washed with NHTE buffer, pH 7.4.

The entrapped RNA was extracted as follows: liposomes containing RNA were incubated with ribonuclease, 60 μg/ml, for 60 min at room temperature and the whole mixture was applied to Sepharose-4B column. Liposome-containing fractions were pooled and centrifuged (30 000 × g, 20 min at 20°C). The pellet was suspended in PBS, incubated for 60 min at room temperature with predigested pronase, 2 mg/ml, and then centrifuged as above. RNA extraction buffer (0.1 M NaCl, 1 mM EDTA, 0.5% SDS,

0.2 M Tris, pH 7.5) 2 ml, containing 10 μl/ml diethyl pyrocarbonate and 0.1 mg/ml *E. coli* t-RNA as carrier, were added to the pellet and the mixture was vortexed. An equal volume of phenol–chloroform (1:1, v/v) was added, and the mixture was shaken vigorously. The aqueous phase was extracted one more time with phenol– chloroform and the RNA was precipitated by the addition of 2 vol. ethanol at –20°C. Electrophoresis of RNA was carried out on 4% acrylamide gels in 99% formamide buffered with 0.02 M barbitone, at pH 9, as in [15]. After electrophoresis, the gels were washed to remove formamide, sliced and the radioactivity was measured as in [16]. The modification of the reticulocyte lysates system [17] was adapted for cell-free translation of globin mRNA. The cell-free system products were analysed on 9–12% gradient polyacrylamide gel as in [18].

3. Results and discussion

3.1. *Entrapment of RNA in liposomes*

Electron microscopy showed that the prepared liposomes formed a very heterogeneous population of unilamellar vesicles of diameters 0.1–1.0 μm (fig.1). Trapping of globin mRNA was assayed radioisotopically and varied from 2–3.5% of the input RNA, in different experiments (table 1). To demonstrate that globin mRNA was in fact entrapped and not adhering to the surface, liposomes were treated with ribonuclease and chromatographed on Sepharose-4B (section 2). Figure 2A shows the elution pattern of liposomes before treatment with ribonuclease. There are three peaks of radioactivity, one in exactly the same position as the liposomes (void volume), a second broad one which represents the free RNA and a third which represents

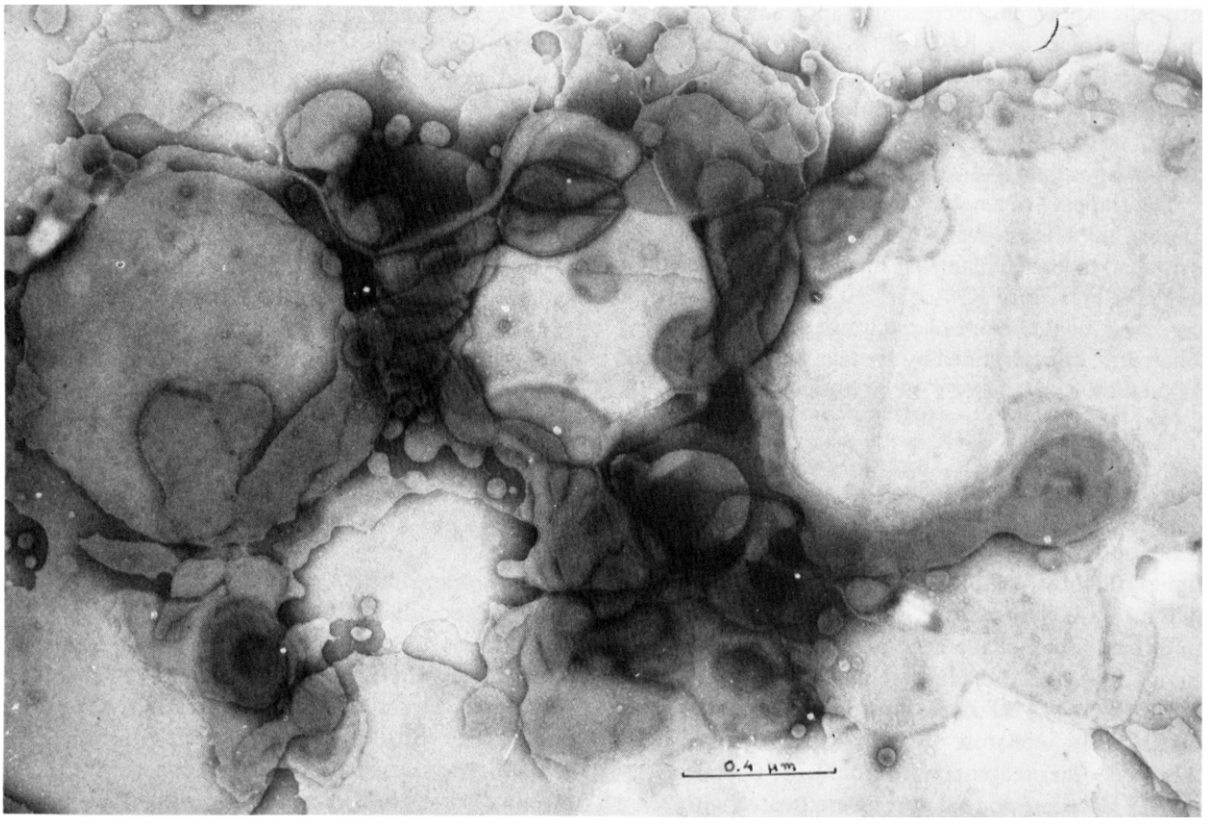

Fig.1. Electron micrograph of phosphatidylserine liposomes negatively stained with 1% uranyl acetate.

Table 1
Entrapment of RNA in liposomes

Expt.	Incubated ^{125}I-labelled RNA (cpm × 10^{-3})	Entrapped ^{125}I-labelled RNA (cpm)	Entrapment (%)
1	13 088	470 634	3.59
2	1937	43 086	2.22
3	1233	36 753	2.98

The entrapment of ^{125}I-labelled RNA in liposomes was assayed after treatment of liposomes with ribonuclease and chromatography on Sepharose-4B. The liposome fractions were pooled and the radioactivity of trichloroacetic acid insoluble material was measured

small nucleotides (not precipitable with trichloroacetic acid, data not shown). After treatment of liposome-entrapped RNA with ribonuclease the first peak of radioactivity remains intact but the second one is moved to the position of small nucleotides (fig.2B).

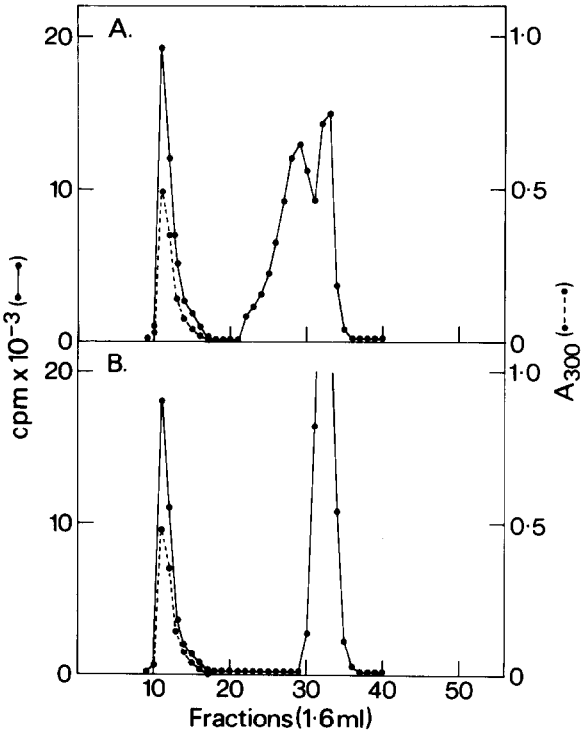

Fig.2. Sepharose-4B chromatography of liposomes containing ^{125}I-labelled RNA. Washed liposomes were suspended in 1 ml PBS (phosphate buffered saline) and applied to a 1.2 × 42 cm Sepharose-4B column which had been equilibrated with the same buffer. Fractions (1.6 ml) were collected and A_{300} (•- - -•) and radioactivity (•——•) read. (A) Before, (B) after, ribonuclease treatment.

To exclude any possibility of non-specific binding of RNA to liposomes, a sample in which the globin mRNA was added after the formation of liposomes was run on the Sepharose-4B column. Figure 3A shows that there is no radioactivity in the position of liposomes. After treatment with ribonuclease all the radioactivity moves again to the last peak, fig.3B.

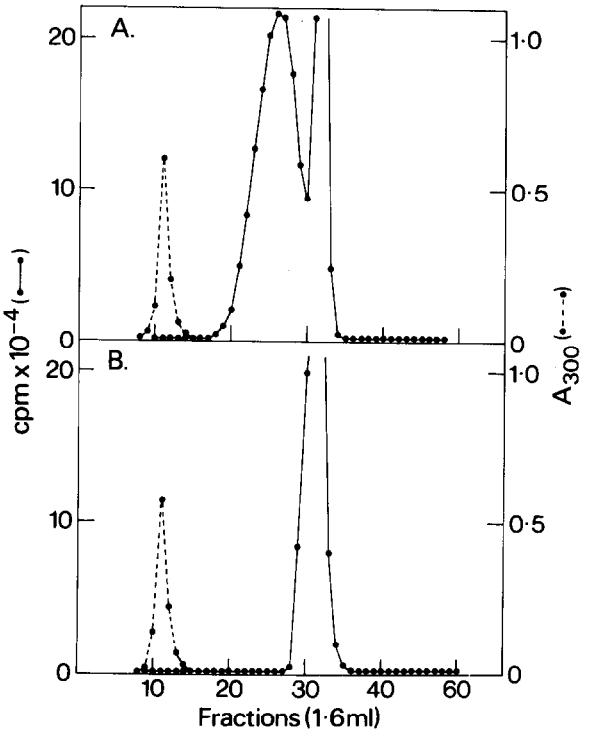

Fig.3. Sepharose-4B chromatography of liposomes incubated with exogeneous ^{125}I-labelled RNA. For details, see fig.2 legend. (A) Before, (B) after, ribonuclease treatment. A_{300} (•- - -•); radioactivity (•——•).

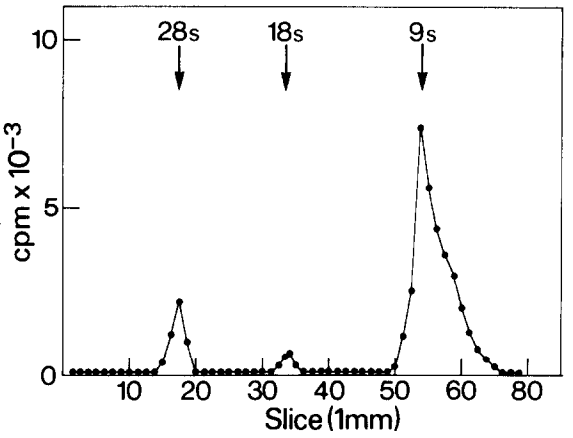

Fig.4. Polyacrylamide gel electrophoresis of RNA extracted from liposomes. Total reticulocyte RNA was used as marker.

3.2. *Integrity and biological activity of liposome-entrapped RNA*

After entrapment of RNA in liposomes, the suspension was treated with ribonuclease and pronase and the entrapped RNA was extracted and electrophoresed as described in section 2. Figure 4 shows that most of the RNA extracted from liposomes migrates in the same position as the marker 9 S RNA. The other two minor peaks corresponded to 28 S and 18 S ribosomal RNA, which contaminates the poly(A)$^+$ RNA preparation.

Also, the RNA extracted from liposomes was used for stimulation of protein synthesis in reticulocyte lysates. Figure 5 shows that the incorporation of [^{35}S]methionine in acid-insoluble material depends on the amount of added RNA. When the same amount of globin mRNA was used but from the original preparation, negligible differences were observed. Figure 6 shows that the RNA extracted from liposomes directed the synthesis of globin when translated in a cell-free system.

4. Discussion

The present data demonstrate for the first time the entrapment of native, biologically active RNA (globin mRNA) in liposomes. The choice of large unilamellar liposomes for the entrapment of nucleic acids has a number of advantages. First, they have a significantly

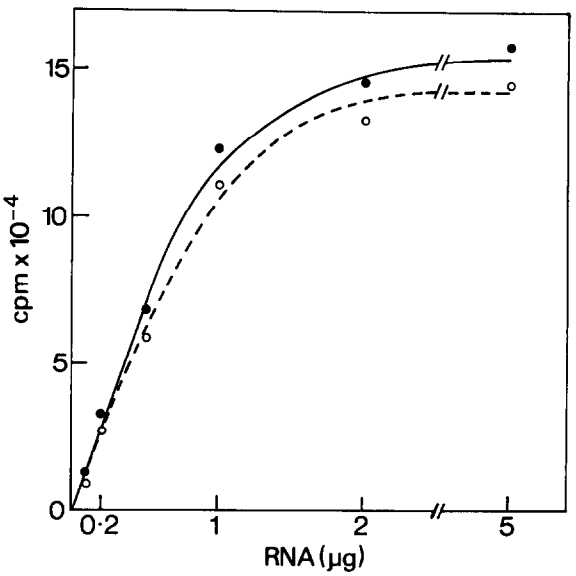

Fig.5. Incorporation of [^{35}S]methionine by reticulocyte lysate system. [^{35}S]Methionine 20 μCi/tube in total vol. 100 μl was added. After incubation for 90 min at 28°C the reaction was stopped by placing the samples on ice. NP-40 1% 400 μl was added and a 10 μl portion taken for estimation of acid insoluble material. Original globin mRNA (•——•). Globin mRNA extracted from liposomes (○- - -○).

larger internal aqueous space (0.1–1.0 μm) than small unilamellar liposomes (0.02–0.09 μm), a fact that permits the entrapment of a wide range of large macromolecules or macromolecular complexes. Secondly, the sonication step which is likely to in-

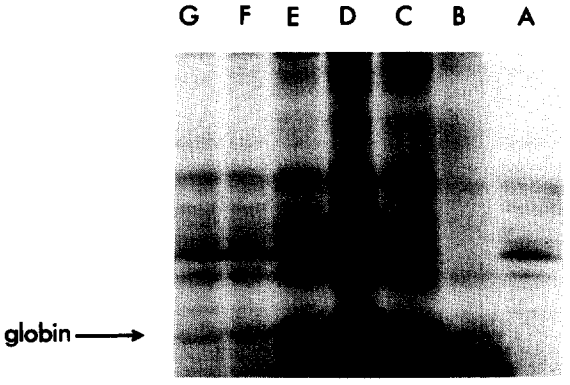

Fig.6. Polyacrylamide gel electrophoresis of cell-free system products: (A) No RNA. (B–D) 0.2, 2.0 and 5 μg globin mRNA. (E–G) 2, 1 and 0.2 μg RNA extracted from liposomes.

activate nucleic acids takes place place before the entrapment, and thirdly the use of negatively charged phosphatidylserine in preparing liposomes minimised the possibility of nucleic acid binding to the surface of the lipid bilayers by electrostatic interactions. Also as fig.2, shows entrapped RNA was protedted from ribonuclease activity and it remains biologically active, as determined by the stimulation of protein synthesis in cell-free system (fig.5,6). Figure 4 proves that in addition to the 9 S RNA (mol. wt 300 000), 18 S (mol. wt 700 000) and 28 S (mol. wt 1 700 000) can be entrapped in liposomes. Such liposomes can also fuse with cells, a fact that permits the direct introduction of nucleic acids into the cytoplasm and provides a new method for ultra-microinjection of nucleic acids with many advantages over the techniques developed until now.

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