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Inclusion and fractionated release of nucleic acids using microcapsules made from plant cells

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ABSTRACT

The encapsulation and fractionated release of nucleic acids on vesicular packing (VP) materials have been investigated. The earlier described dependence of the permeation of nucleic acid molecules through the vesicle membranes on the salt concentration is a necessary precondition for both encapsulation and fractionation. Encapsulation is achieved by applying a suitable sample onto a VP column that has been equilibrated with a high-salt buffer. In that buffer the sample molecules are permeable. Immediately after sample application, elution is started with a low-salt buffer, from which the sample molecules are excluded. At the front between the two buffers the permeability changes, and some of the sample molecules distributed inside the vesicles cannot pass through the membranes. These encapsulated molecules can be released by increasing the salt concentration in the eluent. If the encapsulated nucleic acid sample is polydisperse, a stepwise or linear increase in the salt concentration leads to a fractionated release. The fractions obtained differ in their molecular size composition.

INTRODUCTION

A certain kind of microcapsule, a vesicular packing (VP) material consisting of clusters of empty plant cells, was recently shown to be suitable as a chromatographic support [1,2]. The use of the VP material for size-exclusion chromatography of proteins and dextranes has been described. The VP material differs from conventional materials used in gel permeation chromatography (GPC) both in structure and mechanism. Whereas GPC is based on size-dependent distribution within a gel matrix, vesicle chromatography (VC) is a chromatographic type of membrane separation, where the primary plant cell wall acts as an ultrafiltration membrane and encloses the stationary liquid phase. Depending on their size and charge, the sample molecules are either permeable or not permeable. For a suitable polydisperse sample this results in the occurrence of only two peaks, which are separated by a large difference in elution volume.

The chromatographic behaviour of nucleic acids was shown to be strongly dependent on environment factors such as pH and salt concentration. Divalent cations exert an especially strong influence [3], for example, ribonucleic acid (RNA) is excluded in a slightly buffered solution at pH 7.5, but is permeable on the addition of 20 m*M* magnesium chloride. Electrostatic interactions between the phosphate groups of the nucleic acid and the carboxyl groups of the cell wall are considered to be the main reason for this change. Thus size-exclusion and ion-exclusion effects influence the chromatographic behaviour of polyanions. Anionic molecules, which could permeate according to their size, are excluded at low salt concentrations due to an additional electrostatic barrier [4]. Electrostatic influences of carboxyl groups on the cell wall permeability have

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been discussed with respect to the kinetics of anion uptake by roots and plant tissues [5], but in these instances the assumed permeability changes were quantitative. Complete prevention of cell wall permeation of anions by the ion-exclusion effect has been seen with oligonucleotides [3].

Traditionally, microcapsules are of interest for the encapsulation of macromolecules, *e.g.* enzymes. Encapsulation is usually coupled with the formation or irreversible modification of the capsule membrane [6,7]. Reversible environment dependent changes in the effective pore size of an ultrafiltration membrane were also used for the inclusion of polymers in microcapsules. Sleytr and Sara [8] have described the use of vesicular membranes reassociated from bacterial cell wall proteins for the inclusion of certain proteins by a change of the salt concentration.

The VP material differs from these vesicular membranes by the complex structure and much higher mechanical stability of the ultrafiltration membrane (plant cell wall). This paper describes the use of the reversible change of permeability of the cell wall for the encapsulation of nucleic acids inside the microcapsules and the subsequent fractionated elution of these nucleic acids by 'release chromatography'.

EXPERIMENTAL

DNA from calf thymus and herring sperm, adenosine, 2′,3′-uridinemonophosphate and 2′,3′-cytidinemonophosphate were obtained from Serva. RNA (not further specified) and $tRNA^{val}$ were from Boehringer Mannheim.

Oligonucleotides were synthesized on an Applied Biosystems 380A oligonucleotide synthesizer and purified as described previously [3]. The oligonucleotides, nucleotides and nucleic acids were dissolved in the appropriate buffer to give an absorbance A_{260} of 1 unit in a 200-μl volume. All other reagents were of analytical-reagent grade and were obtained from Serva and Biomol.

The VP material described by Ehwald *et al.* [1], commercially available from Serva (Vesipor) and Permselekt (Permselekt) was used.

For the encapsulation of nucleic acids, the VP material was swollen for 5 min in distilled water. It was then titrated with tris(hydroxymethyl)aminomethane to pH 7.5. This step was followed by extensive washing using a high-salt buffer (5 m*M* Tris–HCl, pH 7.5; 200 m*M* $MgCl_2$, 500 m*M* NaCl) and finally with the appropriate buffer for chromatography. Short glass columns (injection syringes with an I.D. of 16 mm) were packed conventionally. The packing was covered with filter paper. After equilibration, at least 0.1 A_{260} units of the sample were loaded onto the column. The flow-rate was controlled by a peristaltic pump (Minipuls 2, Gilson).

Chromatograms were recorded using an LKB 2238 Uvicord S II recorder operating at 254 nm.

RESULTS AND DISCUSSION

As described previously [3], for a particular size range the permeation of nucleic acids through the vesicle membranes depends on the salt concentration due to the effect of the salt concentration on the electrostatic barrier [4]. At high salt concentrations the membranes are permeable, but the nucleic acids are excluded at low concentrations. If elution with a low-salt buffer is started immediately after sample application onto a column equilibrated at high salt concentrations, some of the sample molecules (about 20–25%) remain on the column. These mol-

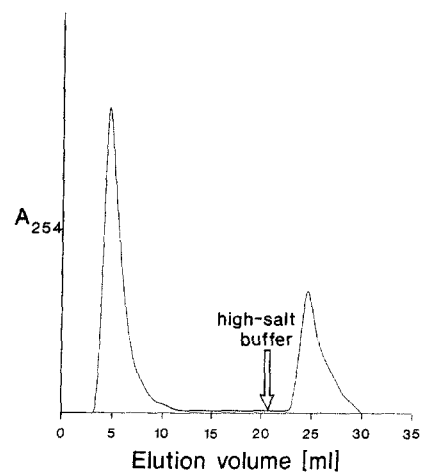

Fig. 1. Encapsulation and release of polydisperse RNA on VP material. Column dimensions, 25 × 16 mm; flow-rate 0.5 ml/min; column equilibration, high-salt buffer (1 m*M* Tris–HCl, pH 7.5; 20 m*M* magnesium chloride); sample, RNA in 50 μl low-salt buffer (1 m*M* Tris–HCl, pH 7.5); elution, first 21 ml with low-salt buffer, thereafter with high-salt buffer.

ecules are not eluted until the salt concentration in the eluent is increased (Fig. 1).

In earlier work we determined that the adsorption of nucleic acids onto VP materials does not occur at pH values above 7 [3]. Therefore, the behaviour illustrated in Fig. 1 with the salt-induced permeability changes are described as follows (Fig. 2). The column has been equilibrated with a high-salt buffer, the sample (for the sake of simplicity, a monodisperse sample in a negligibly small liquid volume) is applied and immediately thereafter elution with a low-salt buffer is started (Fig. 2, t_0). Exactly in the region where the sample is located, a steep salt gradient is formed (Fig. 2, t_1). Below a certain salt concentration, c_E, the considered sample molecule is excluded; above another (rather higher) concentration, c_P, it is completely permeable. The range between c_E and c_P corresponds to the fractionation range in VC, which is characterized by limited permeability. The molecules are partitioned between the extra-particular mobile phase and the intra-vesicular stationary phase. There is a residence time of the molecules inside the stationary phase while the mobile phase, and therefore the salt gradient, moves foreward. For this reason, the salt concentration at the vesicle membrane decreases, possibly falling below the value of c_E. In this instance the nucleic acid molecule is immobilized by inclusion in the VP material.

Some of the sample molecules pass into the zone of increasing salt concentration as they are moving faster than the eluent, as far as they do not permeate the membrane. Arriving in a region with a salt concentration $c = c_P$, the molecules become permeable and move as a peak with the same rate as the eluent. It is assumed by this model that this peak

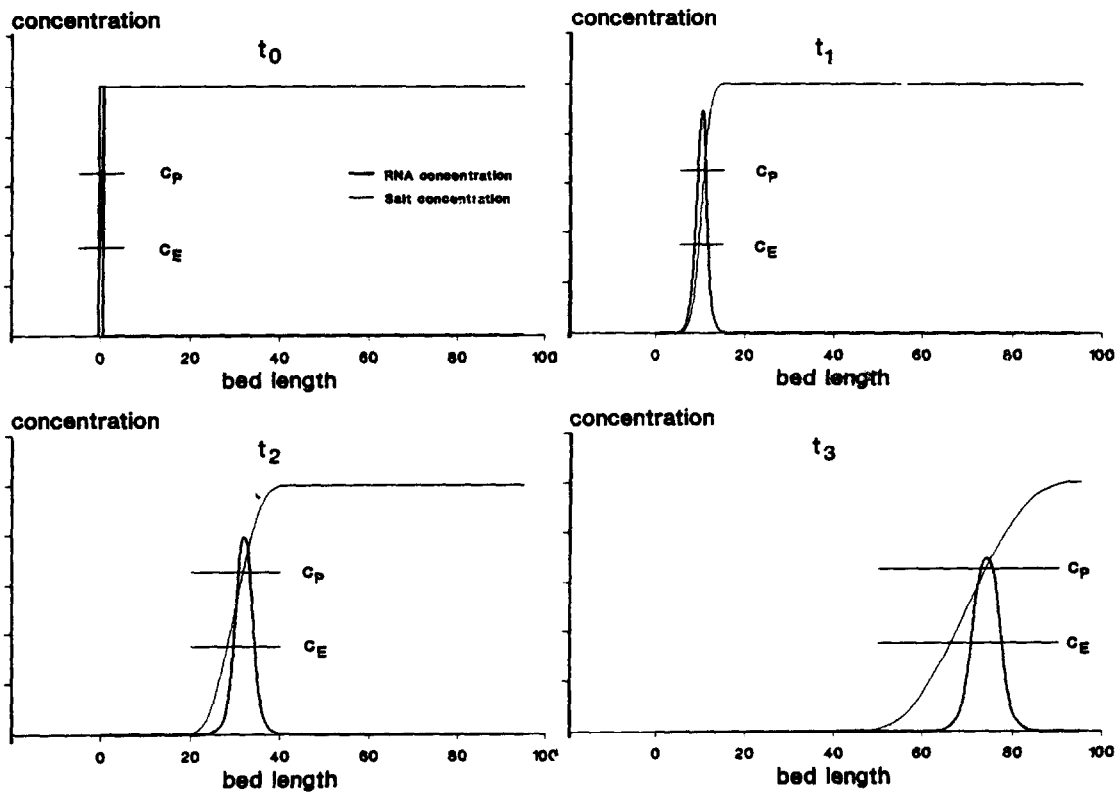

Fig. 2. Assumed concentration profiles during a chromatographic run of a monodisperse sample on a vesicle column at different times. Longitudinal section through the column: direction of the x-axis corresponds to the flow direction; RNA concentration and salt concentration are plotted as a function of the x-coordinate; x-coordinate as a percentage of the total bed length. Values for c_E and c_P chosen arbitrarily. Equilibration and elution performed in accordance with Fig. 1.

migrates ahead of the critical zone with a salt concentration of about c_E. As a result of peak dispersion, a few nucleic acid molecules may once again reach the critical zone. However, with increasing migration distance on the column the salt gradient flattens out and the conditions for inclusion become more unfavourable (Fig. 2, t_2 and t_3). The sample components not included leave the column with the salt gradient. Included molecules cannot leave the vesicles until the salt concentration in the eluent is increased above c_E.

For polydisperse samples with a suitable molecular size distribution the principle should be the same, but due to the different sizes of the molecules every species has different values for c_E and c_P.

The following results clearly show that the behaviour seen in Fig. 1 is in accordance with this explanation.

If column equilibration, sample dissolution and elution all are performed using the same buffer, nucleic acids were not held on the column either at low or high salt concentrations (Fig. 3). Obviously, not a certain salt concentration, but a change in the salt concentration is responsible for the observed immobilization.

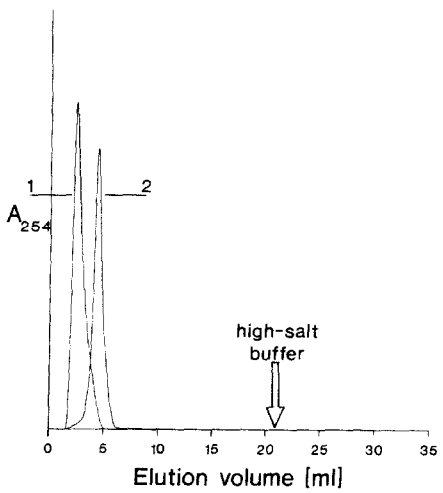

Fig. 3. Failure of immobilization at constant salt concentration. Curve 1, equilibration, sample dissolution and elution of the first 21 ml with 1 m*M* Tris–HCl, pH 7.5, then elution with 1 m*M* Tris–HCl, pH 7.5, 20 m*M* magnesium chloride. Curve 2, equilibration, sample dissolution and elution of the first 21 ml with 1 m*M* Tris–HCl, pH 7.5, 20 m*M* magnesium chloride, then elution with 1 m*M* Tris–HCl, pH 7.5, 200 m*M* magnesium chloride. Other conditions as in Fig. 1.

The retention on the column only occurs with samples, the molecules of which are excluded at low salt concentrations and which are permeable in high-salt buffer (Table I). Mononucleotides, which can permeate at high and low salt concentrations, were not encapsulated and the plasmides or ribosomal RNA which were always excluded showed no retention.

The vesicular structure of the packing material is essential for the retention. Mechanically destroyed cell wall vesicles show no retention of RNA (Fig. 4). As outlined previously [3], the destroyed material behaves like a conventional GPC material; the influence of the salt concentration on the elution volumes of nucleic acids is small. As the large isolated liquid volumes inside the VP material do not exist in the destroyed material, the sample molecules cannot be encapsulated.

The inclusion is not specific for magnesium chloride; it occurs with any other salt that influences the permeability of the cell wall for nucleic acid molecules. The effect has also been observed by using sodium chloride, potassium chloride, caesium chloride and calcium chloride; however, with monovalent cations a considerably higher concentration is necessary to include similar amounts of nucleic acids.

For small sample volumes, the bed length had no

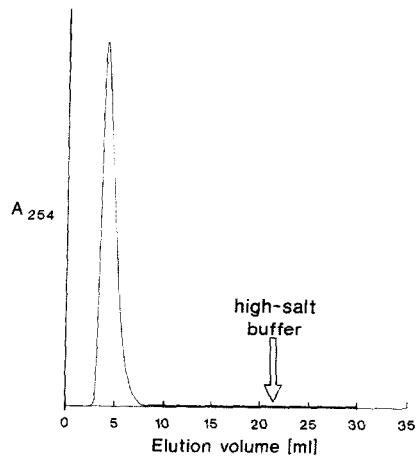

Fig. 4. Failure of immobilization with mechanically disintegrated VP material. All conditions as in Fig. 1. Destruction of the VP material was performed using a swinging mill until no intact cellular structures were detected under a microscope (magnification × 200).

TABLE I

ENCAPSULATION AND RELEASE OF NUCLEIC ACIDS, OLIGONUCLEOTIDES AND NUCLEOTIDES AS A FUNCTION OF THEIR PERMEATION BEHAVIOUR AT MAGNESIUM CHLORIDE CONCENTRATIONS OF 0 AND 20 m*M* IN 1 m*M* TRIS–HCl, pH 7.5.

Sample	Molecular weight	Permeation at 0 m*M* $MgCl_2$	Permeation at 20 m*M* $MgCl_2$	Encapsulation and release[a]
2′,3′-CMP	323	Permeating	Permeating	No
2′,3′-UMP	324	Permeating	Permeating	No
RNA from yeast[b]	< 15 000	Excluded	Permeating	Yes
DNA from herring sperm[b]	< 15 000	Excluded	Permeating	Yes
28mer oligonucleotide[c]	≈9000	Excluded	Permeating	Yes
$tRNA^{val}$ from *Escherichia coli*	≈30 000	Excluded	Excluded	No
Calf thymus DNA, native	≈5 000 000[d]	Excluded	Excluded	No
denatured[e]	≈2 500 000[d]	Excluded	Excluded	No
Plasmide pKK 161-8	≈3 000 000	Excluded	Excluded	No

[a] For evaluation of this parameter the experiment described in Fig. 1 was performed with the respective samples.
[b] Technical products, better referred to as crude mixtures of oligonucleotides.
[c] Sequence ATC TAG ATT GTG GGG GCG GCT CCC AAC A.
[d] Value represents an average of a broad molecular weight distribution.
[e] Denaturation was achieved by heating the sample for 10 min to 100°C and rapidly cooling down in an ice-bath.

significant influence on the amount of included molecules, at least in the range investigated. It is suggested that (*i*) with increasing migration distance the conditions for inclusion are less favourable due to the flattening of the gradient and (*ii*) after a few millimetres of migration all sample molecules have either been included or passed through the gradient. An efficient inclusion seems to occur only at the beginning of a chromatographic run.

If the sample is applied in a low-salt buffer and elution is performed as described earlier (see Fig. 1), an efficient inclusion can also be achieved by applying comparatively high sample volumes. As the sample molecules cannot permeate through the vesicle membranes in the sample buffer, they move faster than this buffer until they have reached the salt gradient. To obtain a high inclusion yield, the ratio between sample volume and packing volume should have a value such that the last applied sample molecules reach the zone with the critical salt concentration c_E just before this zone leaves the column. It was found experimentally that the sample volume can be as large as 60% of the packing volume for an efficient inclusion (Fig. 5). If the sample was loaded

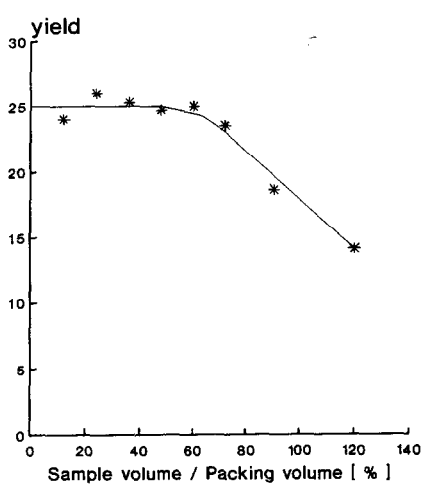

Fig. 5. Encapsulation yield as a function of the ratio between sample volume and packing volume. Yield as percentage of the released fraction compared with the total amount of RNA. Column dimensions 7.5 × 16 mm. Other conditions as in Fig. 1.

onto the column in a high-salt buffer and the above-described procedure was followed, only few molecules (about 3–5% of the RNA) were retained on the column. This is due to mixing between the high-salt sample buffer and the low-salt elution buffer at the rear side of the substance peak.

The inclusion did not lead to an absolutely durable encapsulation of all nucleic acid molecules of a polydispersed sample. The chromatograms presented here show, on closer examination, that the continuous elution of small amounts of included nucleic acids occurred at a low rate. The absorption A_{254} did not decrease to zero after inclusion, but to a value close to zero. An increase of the amount of loaded nucleic acid shows this more clearly (Fig. 6). A long elution time (20 h) with a low-salt buffer (50 packing volumes) resulted in a loss of about two-thirds of the included molecules. Thus the cell wall is not absolutely impermeable at low salt concentrations, but the permeability is decreased by some orders of magnitude.

The elution of the encapsulated molecules by permability changes can be used for the fractionation of polydisperse polymer preparations by a stepwise or continuous increase of the salt concentration. Fig. 7a shows the fractionation of polydisperse RNA in a sodium chloride step gradient. Four separated peaks were detected at the four different salt concentrations. The characterization of the collected peak fractions with respect their molecular size

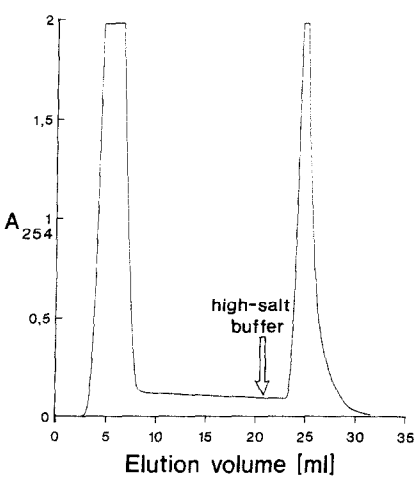

Fig. 6. Preparative encapsulation and release of polydisperse RNA. Sample volume, 1 ml. Other conditions as in Fig. 1.

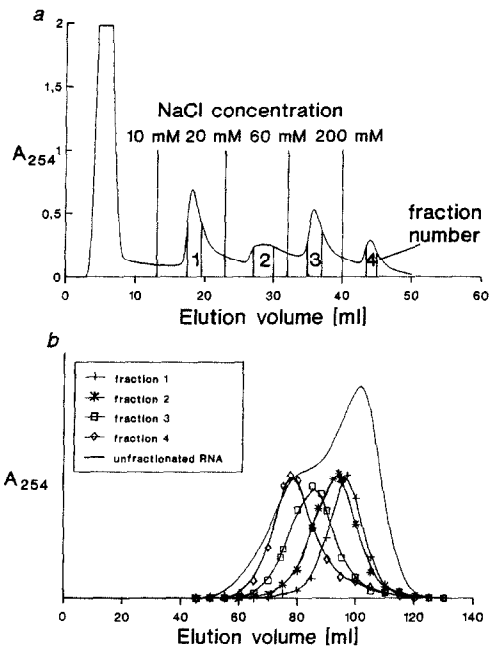

Fig. 7. (a) Preparative encapsulation and fractionated release of polydisperse RNA. Column equilibration with 5 m*M* Tris–HCl, pH 7.5, 200 m*M* sodium chloride. Sample, RNA in 1 ml of low-salt buffer (5 m*M* Tris–HCl, pH 7.5); elution, first with low-salt buffer, then stepwise increase of the NaCl concentration to the concentrations designated in the figure. All other conditions as in Fig. 1. (b) Investigation of the fractions collected in (a) using a HiLoad 16/60 Superdex 75 (preparation grade) column (600 × 16 mm) operated on a fast protein liquid chromatographic station (Pharmacia). Samples: collected fractions from (a) were concentrated by a factor of ten using a Speed Vac concentrator (Savant). Sample volume, 100 μl; eluent, 0.1 *M* Tris–HCl, pH 7.9, 0.2 *M* NaCl; flow-rate, 2 ml/min; chromatograms scaled to give an approximately equal A_{254} at peak maximum.

distribution by GPC of Superdex 75 (Fig. 7b) shows, compared with the non-fractionated RNA sample, that the elution from the VP column occurs in the order of increasing molecular size. The smaller included molecules become permeable first, while the larger molecules need higher salt concentrations for permeation. The elution of the included polydisperse RNA in a continuous linear sodium chloride gradient gave a single broad peak, whereby the mean molecular size was changing continously (chromatograms not shown).

Fractionated release similar to that shown in Fig. 7a was also observed with DNA from herring sperm and by using various salts. For the fraction-

ated release, large column lengths (high numbers of theoretical plates) are not necessary. This is a remarkable difference to GPC.

Inclusion in microcapsules and release by salt-induced permeability changes is a novel technique in nucleic acid fractionation. The method should not only apply to nucleic acids, but also to any other anionic polyelectrolyte with a suitable molecular size (distribution). Other factors influencing the permeability of cell walls for certain polymers, such as dehydration by organic solvent [9], can possibly also be used for the reported type of size fractionation. Therefore encapsulation and fractionated release might develop into a useful tool in polymer fractionation and characterization.

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