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Chromatographic fractionation of nucleic acids using microcapsules made from plant cells

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ABSTRACT

The chromatographic fractionation of nucleic acids and oligodeoxyribonucleotides on a vesicular packing (VP) material has been investigated. As the VP material consists of microcapsules with a negatively charged ultrafiltration membrane, the experiments were focused on permeation chromatography in aqueous buffers of different ionic compositions. The adsorption of oligonucleotides and nucleic acids by the VP material occurring at pH values below 7 and especially in the presence of divalent cations, was negligible in neutral or weakly alkaline buffers. In such buffers the separation principle is based only on the permeation of polyanions into the stationary liquid within the vesicles. Polydisperse samples may be separated into an excluded peak fraction (at 40% of bed volume), fractions with intermediate elution volumes and a permeating peak fraction (at 95% of bed volume). The ratio between these fractions can be varied gradually by changing the salt concentration. Using defined oligonucleotides the maximum chain length for permeation into the whole stationary phase was smaller than the minimum chain length for complete exclusion. Both values were dependent on the salt concentration and pH. They could be varied gradually and reversibly over a wide range by altering the magnesium chloride concentration from 0 to 10 m*M*. In contrast to ion-exclusion effects known from gel permeation chromatography, in vesicle permeation chromatography there is no salt effect on the elution volume of the permeable fraction. Preparative applications for the fractionation of oligodeoxyribonucleotides and nucleic acids are presented.

INTRODUCTION

Microcapsules are widely used for the immobilization of enzymes, microorganisms and mammalian tissues as well as for the encapsulation of drugs. In addition, there are a variety of potential applications as chromatographic supports. Microcapsules consisting of the intact primary cell wall of higher plants have been successfully applied as a chromatographic material [1]. This vesicular packing (VP) material was shown to be suitable for a new type of exclusion chromatography. In contrast to gel permeation chromatography (GPC), the separation mechanism is not based on a size-dependent distribution within a matrix, but on permeation through a thin ultrafiltration membrane into a large stationary liquid volume. Hence, vesicle chromatography (VC) is a chromatographic form of membrane separation rather than a subtype of GPC. Whereas in GPC the fractionation range is extended to more than one order of magnitude in terms of molecular weight, a very small fractionation range was observed in VC. Molecules with small differences in molecular size may be separated by a large difference in elution volume. The maximum Stokes' diameter for permeation into the whole stationary liquid volume of standard VP materials was determined using calibrated dextrans to be 5.6 nm [2]. Not only the size and shape of a molecule, but also its charge governs the permeability through membranes. Negatively charged proteins with Stokes' diameters normally allowing permeation (pepsin and ovalbumin) were excluded from the VP material [2]. It is assumed that electrostatic interactions between the proteins and

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TABLE I

OLIGODEOXYRIBONUCLEOTIDE SEQUENCES (DIRECTION 5′ → 3′)

Chain length	Sequence
2	CC
3	AAC
4	GAT G
5	AAC TC
6	CCG AAC
7	ACA CAT C
8	ACA CAT GC
9	CGC GTT CCA
11	CTA GAC ATA TG
14	CTT TCA AGA TCC CC
17	TAG CAT CAA CCG CAG CC
20	GTT GAA GGA TCA ACA TTT TG
23	GTC GAC CAA TGG GGT GGC TTT GC
25	CCG GTC TGA GAG GAT GGC CGG CCA C
28	ATC TAG AAT GTG GGG GCG GCT CCC AAC A
33	GGA GGA GGC CCC TTA GGA GCT TGT CGG GGT TGA
36	CGT CAG CTC GTG CCG TGA GGT GTT GGG TTA AGT CCC
40	CGA CCG TTC CGG GTT CCA ATA TCA TGG TCG TGT CCA GGC A
45	AGC TTA GAA CTG AAG GCC AGC TTC ACG GGC AGC ATC TGC CAG TGC
50	CTG GGC GCA ACT CGC CTG GTG GTA CAT CGT ACC CCG CGT CAC ATT TAT GC
59	GCT GTG GGT AAA GCT GTC GCT GAA CGC GCT CTG GAA AAA GGC ATC AAA GAT GTA TCC TT
70	GTC ACC TGC AGT CTA GAT CTA ACA CCG TGC GTC TTG ACT ATT TTA CCT CTG GCG GTC ATA ATG GTT GCA G

the negatively charged cell wall matrix cause this exclusion. Compared with proteins, nucleic acids and oligonucleotides have an extremely high charge density. Therefore, electrostatic interactions with the cell wall should be more important than for proteins. Thus, nucleic acids and oligonucleotides promised to be suitable for systematic studies of the effect of charge in VC. This work considers the influence of salt concentration and pH value on separation.

EXPERIMENTAL

DNA from calf thymus and from herring sperm, adenosine, 2′,3′-uridinemonophosphate (2′,3′-UMP) and 2′,3′-cytidinemonophosphate (2′,3′-CMP) were obtained from Serva. RNA (not further specified) and $tRNA^{val}$ were from Boehringer Mannheim. Plasmid pKK 161-8 [3] was a gift from Dr. R. K. Hartmann (Freie Universität Berlin, Berlin, Germany).

Oligonucleotides were synthesized on an Applied Biosystems 380A oligonucleotide synthesizer following the phosphoramidite strategy. The sequences are listed in Table I. The raw products were purified by reversed-phase high-performance liquid chromatography on ODS Hypersil. The dimethoxytrityl groups were removed by treatment with 80% acetic acid. Oligonucleotides with a chain length greater than ten were desalted on NAP 10 columns (Pharmacia) and shorter oligonucleotides on self-packed Sephadex G 10 columns. Water was removed by using a Speed Vac concentrator (Savant). 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 from Permselekt ("Permselekt"), was used. Furthermore, alkali-treated VP material with an increased separation limit was tested.

For chromatographic use, the VP material was swollen for 5 min in distilled water. It was then titrated with one buffer component to the desired pH. The pH value should be equal to that of the buffer used in the chromatographic separation. This step was followed by extensive washing using a high salt buffer (desired pH, 200 m*M* magnesium chloride, 500 m*M* sodium chloride) and finally with the appropriate buffer for chromatography. Short glass columns (injection syringes with an I.D. of 16 mm) were packed conventionally. For the preparative fractionation, an Econo column (Bio-Rad) was

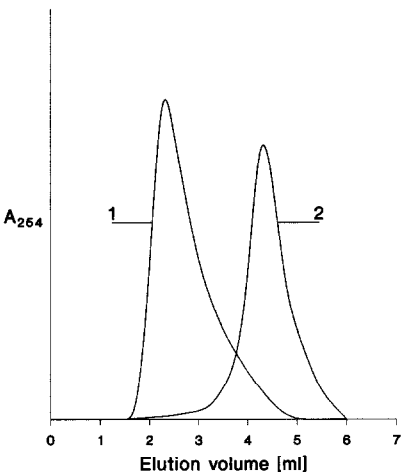

Fig. 1. Vesicle chromatography of RNA at 0 and 20 m*M* magnesium chloride. Column dimensions, 25 × 16 mm; sample volume, 50 μl; flow-rate, 0.8 ml/min. Column equilibration, sample dissolution and elution with: (1) 1 m*M* Tris–HCl, pH 7.5; and (2) 1 m*M* Tris–HCl, pH 7.5, 20 m*M* magnesium chloride.

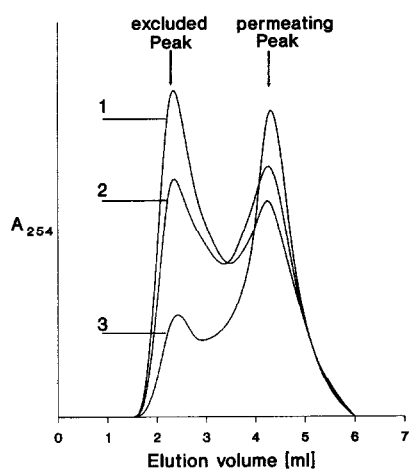

Fig. 2. Vesicle chromatography of RNA at different concentrations of magnesium chloride. Conditions as in Fig. 1. Buffer composition: (1) 1 m*M* Tris–HCl, pH 7.5, 0.2 m*M* magnesium chloride; (2) 1 m*M* Tris–HCl, pH 7.5, 1 m*M* magnesium chloride; and (3) 1 m*M* Tris–HCl, pH 7.5, 3 m*M* magnesium chloride.

used. The packing material was covered with filter paper. After equilibration, at least 0.04 A_{260} units of the sample were loaded onto the column. The flow-rate was gravity-controlled and maintained in the range 0.2–0.5 ml cm^{-2} min^{-1}. For the chromatographic separation of oligonucleotides, a peristaltic pump (Minipuls 2, Gilson) was used running at a constant flow-rate. Unless otherwise indicated, the experiments were carried out at pH 7.5.

The chromatograms were recorded using an LKB 2238 Uvicord S II spectrometer operating at 254 nm.

RESULTS

At pH 7.5, peak elution volumes of nucleotides or nucleic acids larger than one packing volume have not been observed, indicating that the elution behaviour was determined only by permeation and not by adsorption.

Ribonucleic acid from yeast, a technical product with a broad molecular weight distribution, showed an elution behaviour which was remarkably dependent on the magnesium chloride concentration (Figs. 1 and 2). At low salt concentration (Fig. 1, line 1) one peak was observed leaving the column with the void volume (about 40% of the total packing volume). Most RNA molecules were obviously excluded. If magnesium chloride was present at a concentration of 20 m*M* in the buffer and the sample, the peak appeared with the total permeation volume (90–95% of the packing volume, Fig. 1, curve 2). Fig. 2 shows the chromatograms of the same RNA preparation at magnesium chloride concentrations of 0.2, 1 and 3 m*M*. The addition of 0.2 m*M* magnesium chloride led to the appearance of a minor permeating peak (Fig. 2, curve 1). About 70% of the sample components were still excluded.

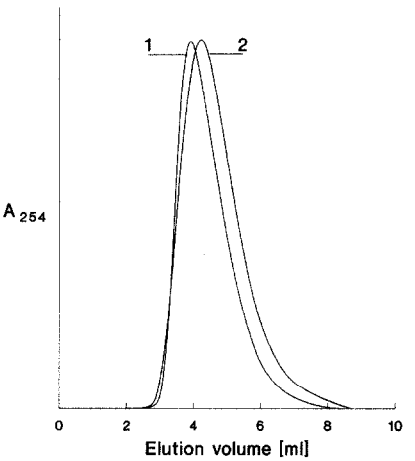

Fig. 3. Chromatography of RNA using destroyed VP material. Conditions as in Fig. 1. The material was destroyed using a swinging mill, until no intact cellular structures were detected under a microscope (magnification 200 ×). Buffers: (1) 1 m*M* Tris–HCl, pH 7.5; and (2) 1 m*M* Tris–HCl, pH 7.5, 20 m*M* magnesium chloride.

TABLE II

PERMEATION BEHAVIOUR OF NUCLEIC ACIDS, OLIGONUCLEOTIDES AND NUCLEOTIDES 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* $MgCl_2$
2′,3′-CMP	323	Permeating[c]	Permeating
2′,3′-UMP	324	Permeating	Permeating
RNA from yeast[a]	<15 000	Excluded[d] (Fig. 1)	Permeating (Fig. 1)
DNA from herring sperm[a]	<15 000	Excluded	Permeating
$tRNA^{val}$ from *Escherichia coli*	≈30 000	Excluded	Excluded
DNA from calf thymus	≈5 000 000[b]	Excluded	Excluded
Plasmide pKK 161-8	≈3 000 000	Excluded	Excluded

[a] Technical products, best referred to as crude mixtures of oligonucleotides.
[b] Value represents an average of a broad molecular weight distribution.
[c] "Permeating" refers to an elution volume at peak maximum of 90–95% of the packing volume.
[d] "Excluded" refers to an elution volume of about 40%.

Increasing the magnesium chloride concentration to 1 m*M* (line 2) resulted in an increase in the permeating fraction (about 55% of the sample) and a decrease in the excluded fraction. Only a small part of the sample was excluded at a concentration of 3 m*M* (line 3).

After mechanical rupture of its vesicular structure, the cell wall preparation showed a totally different chromatographic behaviour (Fig. 3). With the same RNA as used in Figs. 1 and 2, one peak was observed, the location of which was influenced slightly by the magnesium chloride concentration. A group separation into excluded and permeating molecules did not occur.

Chromatography with intact VP material at different magnesium chloride concentrations was carried out using a variety of nucleotides and nucleic acids. Table II gives a summary of the results obtained.

The nucleotides 2′,3′-CMP and 2′,3′-UMP were too small to be excluded and permeated through the membranes into the whole stationary volume at all salt concentrations. Similar to RNA from yeast, (technical) DNA from herring sperm exhibited a chromatographic behaviour influenced by the salt concentration. Larger molecules such as tRNA, plasmides or highly polymerized DNA from calf thymus were excluded in all instances. The ionic influence on the permeability is not specific for magnesium salts, as shown with sodium and calcium chlorides. The concentration of monovalent cations

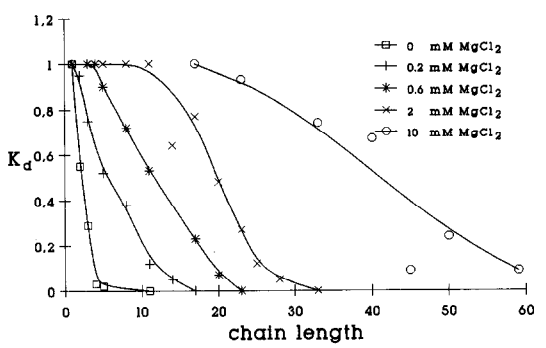

Fig. 4. Relationship between K_d value and chain length at different magnesium chloride concentrations. Column, 60 × 16 mm, standard VP titrated to pH 7.5; Sample, 0.04 A_{260} unit each; flow-rate, 0.3 ml/min; buffer, 1 m*M* Tris–HCl, pH 7.5; magnesium chloride concentrations as indicated in the figure.

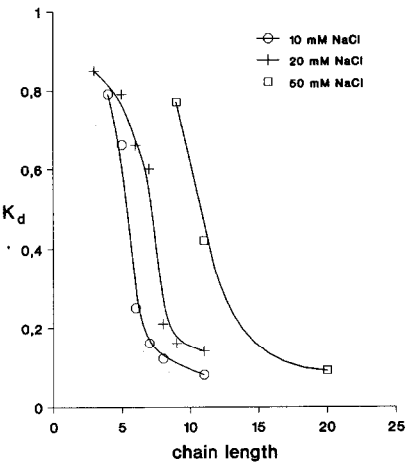

Fig. 5. Relationship between K_d value and chain length at different sodium chloride concentrations. Conditions as in Fig. 4; sodium chloride concentrations as indicated in the figure.

necessary for permeation is much higher; 3 m*M* magnesium chloride led to permeation of most RNA molecules (see Fig. 2), whereas 50 m*M* sodium chloride was required for a similar chromatogram in the absence of magnesium chloride (data not shown).

To obtain a more precise knowledge of the relationships between molecular size and permeability, purified monodisperse synthetic oligonucleotides were chromatographed at different magnesium chloride concentrations (for sequences, see Table I). At certain salt concentrations, oligonucleotide peaks appeared between the exclusion and the permeation volume, *i.e.* within the fractionation range. Fig. 4 shows the relationships between the chain length of the oligonucleotides and the apparent volumetric distribution coefficients (K_d values) at pH 7.5.

The permeation is strongly dependent on the magnesium chloride concentration. In the absence of magnesium chloride only adenosine was eluted with the whole packing volume (apparent K_d value close to unity), whereas the K_d values of the di- and trinucleotide were in the range 0–1. Even oligonucleotides with a chain length greater than 3 were excluded. With increasing magnesium chloride concentrations the exclusion limit rose and the frac-

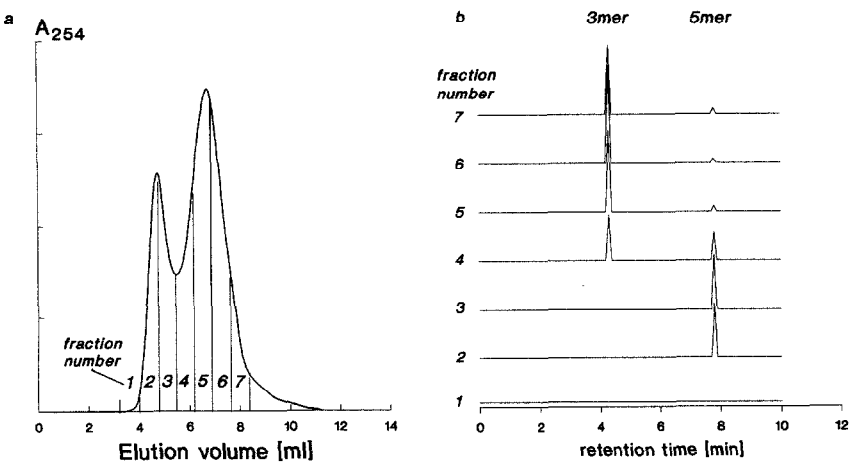

Fig. 6. Preparative fractionation of a mixture of the trinucleotide d(AAC) and the pentanucleotide d(AAC TC). (a) Chromatogram. Column, 115 × 10 mm standard VP material, titrated to pH 7.5; sample, 2 A_{260} units of the mixture; flow-rate, 80 µl/min; buffer, 1 m*M* Tris–HCl, pH 7.5, 0.1 m*M* magnesium chloride. (b) Anion-exchange chromatograms of the fractions collected in (a). Column, Mono Q; gradients, 0–100% B in 25 min (A = 10 m*M* sodium hydroxide; B = 1.2 *M* sodium chloride in A); flow-rate, 1 ml/min.

tionation range became more extended. In the presence of 10 m*M* magnesium chloride, a comparatively broad fractionation range was found from a chain length of 25–60 nucleotides. Similar curves were found using calcium chloride instead of magnesium chloride (not shown). With the monovalent cations Na^+ and K^+ a dependence of permeation limits and K_d values on the salt concentration was also observed, but much higher concentrations were required to give comparable effects to divalent cations. Fig. 5 shows the relationships between K_d values and sodium chloride concentration.

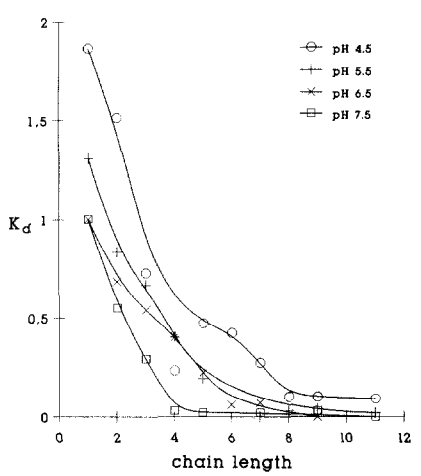

Fig. 7. Relationships between K_d value and chain length at different pH values. Column, 60 × 16 mm, standard VP material, titrated to the appropriate pH; sample, 0.04 A_{260} units each; flow-rate, 0.3 ml/min. Buffers, pH 7.5: 1 m*M* Tris–HCl; pH 6.5: 1 m*M* 2-(N-morpholino)ethanesulphonic acid buffer; pH 5.5: 1 m*M* phosphate buffer; and pH 4.5: 1 m*M* acetate buffer.

Generally, intermediate K_d values were dependent on flow-rate and bed length. They increased with the residence time of the sample on the bed. Peaks of oligonucleotides with intermediate K_d values were broader than those with K_d values of 0 or 1.

The counterion-controlled permeability of oligonucleotides can be used for preparative fractionations. Fig. 6a shows the separation of a trimer from a pentamer. The eluted fractions were collected and analysed for their composition by anion-exchange chromatography; the chromatograms are presented in Fig. 6b. The fractions of the first peak were free from trinucleotide. Owing to tailing of the excluded peak, the trimer peak was contaminated with small amounts of the pentamer.

Fig. 7 shows the relationships between chain length and K_d value at different pH values in the absence of magnesium chloride. Apparently, at lower pH values, larger molecules permeate into the inner volumes of the vesicles. However, at pH 4.5 and 5.5 some smaller molecules, which were obviously retarded by adsorption, exhibited K_d values

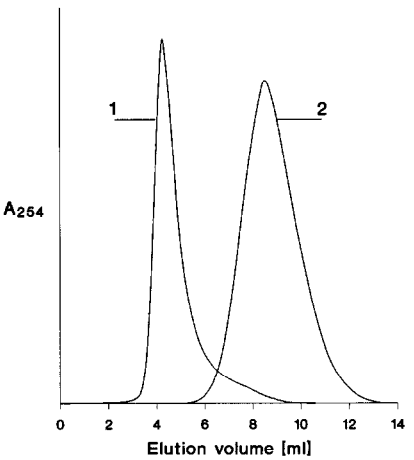

Fig. 8. Chromatography of $tRNA^{val}$ on a VP material with a size limit for permeation of 10 nm. Column, 50 × 16 mm, sodium carbonate-treated VP material, titrated to pH 7.5; sample, 0.01 A_{260} unit of $tRNA^{val}$; flow-rate, 0.3 ml/min. Buffers: (1) 1 m*M* Tris–HCl, pH 7.5; and (2) 1 m*M* Tris–HCl, pH 7.5, 20 m*M* magnesium chloride.

significantly greater than 1.0. In the presence of 5 m*M* magnesium chloride a decrease of the pH to values below 7 resulted in strong or even total adsorption of the oligonucleotides. At pH 6.5 a broad peak over more than one bed volume appeared, whereas the oligonucleotides were totally adsorbed at pH 4.5 and 5.5, respectively.

The counterion-dependent barrier function of the vesicle membrane for RNA was also observed using a VP material with increased separation limits. This VP material was obtained by treatment of the vesicular plant cell wall preparation with sodium carbonate [2], leading to de-esterification and controlled partial decomposition of polygalacturonic acid (unpublished results). As an example, a VP material with a size limit for the permeation of neutral macromolecules close to 10 nm (determined as described by Ehwald *et al.* [2]) was used for the investigation of the elution behaviour of $tRNA^{val}$. This molecule was too large for permeation with standard VP materials (see Table II). With the modified VP material a dependence of the permeability of this tRNA on the salt concentration was found. The tRNA was completely excluded at pH 7.5 in the absence of magnesium chloride, but was totally permeable with 20 m*M* magnesium chloride (Fig. 8).

DISCUSSION

The stationary phase built by the packing particles investigated here consists of thin vesicle membranes (negatively charged purified plant cell walls) and liquid enclosed within the vesicles. The vesicle membranes are considered to be cation-exchange membranes with a high charge density. Non-esterified carboxyl groups are present at high concentrations (0.7 mval/g dry material) in the pectin component. This component (a network of polygalacturonans) is responsible for sizing the neutral macromolecules [2] and also determines the size- and environment-dependent permeability of polyanions. The vesicular structure of the VP material is essential for efficient group separations and for the dependence of the elution volumes on the salt concentration. Comparison of Figs. 1, 2 and 3 shows the importance of the free stationary liquid inside the VP material for the fractionation result.

Permeation chromatography with this material is dominated by membrane permeation between the mobile phase and the enclosed liquid. As the enclosed liquid comprises about 95% of the volume of the packing particle, the partial exclusion of polyanions from the negatively charged cell wall matrix has an insignificant effect on the elution behaviour, as long as it does not prevent the fast equilibration of the enclosed liquid volume with the mobile phase. Indeed, polyanions were eluted with a K_d value of 1 below a critical chain length (Fig. 4). This is a remarkable difference compared with the GPC of anions on negatively charged porous or gel-like support materials, where the K_d values of all anions are reduced at low ionic strength due to the Donnan equilibrium.

The results of this work show that the permeability of the vesicle membrane for nucleic acids depends on both their chain length and ion environment factors. The most important of these factors are salt concentration and the type of cations. Weakening of the electrostatic repulsion by Mg^{2+} ions may be traced back to (1) the well known Debye–Hückel screening interactions which reduce the field strength in the surroundings of the polyanion proportional to the ionic strength and (2) charge reduction by localized binding of the cations to the polyanion (counterion condensation) [4]. Generally, the latter effect is more efficient with divalent cations

than with monovalent ions. This might explain the need for the much higher sodium chloride concentrations to give comparable effects to magnesium chloride (compare Figs. 4 and 5).

In addition, permeation through the microcapsule membranes may be influenced by enlargement of the molecular dimensions of the nucleic acids at low salt concentrations as a result of intramolecular phosphate repulsion [5].

Considering the size limit of permeation of the investigated VP material for neutral dextran molecules (Stokes' diameter of 5.6 nm) or proteins (molecular weight 35 000 dalton) [2], the effective exclusion of a nucleotide tetramer by the VP material in the absence of Mg^{2+} ions (Fig. 4) is a striking result. The electrostatic repulsion between the polyanion and the vesicle membrane is obviously strong enough to prevent diffusional exchange of the oligonucleotide with the enclosed liquid phase, at least for the given separation time. The combined effect of size and electrostatic exclusion of polyanions from the charged membrane matrix may be described by the concept of Dubin *et al.* [6], who analysed the thickness, X_E, of an electrical barrier around a charged particle in pores with equally charged surfaces. X_E may be added to the Stokes' radius of the molecule or subtracted from the radius of the pore to describe the liquid space fraction accessible for diffusion of the charged molecule within the charged particle. Values of X_E have been determined empirically by comparison of the molecular radii estimated by size-exclusion chromatography of polyanions on porous glass with those derived from the intrinsic viscosity at different salt concentrations. Concerning this concept, the critical thickness of the electrical barrier X_E which prevents the permeation of a polyanion through the cell wall is the difference between the critical Stokes' radius for the exclusion of neutral polymers and the Stokes' radius of the polyanion. In addition to the qualitative aspect of polyanion exclusion from the matrix (impermeability of the membrane) there is also, at X_E values below the critical value, a quantitative aspect, *i.e.* decreased permeability. The latter is a consequence of the decreased concentration and mobility of permeable polyanions in the negatively charged matrix. This might explain why intermediate K_d values were dependent on flow-rate and bed length. As the separation time and bed length were not sufficient to reach the diffusional equilibrium, the intermediate K_d values refer to the given experimental conditions. They do not only reflect an excluded portion of the stationary liquid volume, but also (or mainly?) the limited permeability of the vesicle membrane.

Peak broadening by limited diffusion is the most serious problem for the application of counterion-dependent size fractionation of oligonucleotides and nucleic acids by VC. Between the excluded and permeable fraction a kinetically influenced fractionation range has to be taken into account. The investigations with synthetic oligonucleotides show its dependence on the salt concentration. The correlation between K_d and the chain length is not ideal, as the permeability is influenced by the size, shape and charge of the oligonucleotide. The net charge does depend not only on the chain length, but also on the base composition. In addition, base pairing may lead to secondary structures with different hydrodynamic properties, *e.g.*, the investigated 14mer oligonucleotide seems to behave differently from its neighbours (Fig. 4).

As the dissociation of the pectin carboxyl groups depends on the pH value, the permeability of polyanions through the cell walls should also be influenced by the proton concentration. Furthermore, owing to the protonation of adenine and cytosine (pK_a around 4.5), the influence of the sequence-dependent charge might be of functional relevance. Fig. 7 shows that the K_d values of the oligonucleotides are varied by the pH value, but to a different extent.

Whereas all the effects described at pH 7.5 can be discussed as pure permeability effects, additional adsorption phenomena must be considered at pH values below 7. K_d values far above 1 clearly indicate an additional retention (Fig. 7). Therefore, it is not possible to decide unequivocally whether the influence illustrated in Fig. 7 derives from increased permeability or from a retention due to adsorption phenomena.

The polydisperse RNA and DNA samples which were excluded in one instance and permeable in the other, undergo systematic changes in their chromatographic behaviour between magnesium chloride concentrations of 0 and 20 mM (Fig. 2). In contrast to conventional gel chromatography media, it is not the location of the peak maximum, but the ratio

between the excluded and permeating peak that is varied by the salt concentration. The opportunity to vary the exclusion limit of the VP material over a wide range allows the adaption of the separation conditions to the respective separation problem. Alkali-treated VP materials with irreversibly increased separation limits are also susceptible to reversible adjustment of their permeability for larger polyanions by the salt concentration. This is of special interest for the fractionation of naturally occurring nucleic acids.

The dependence of the fractionation result in VC on the kind and concentration of salts and the pH value should not only apply to nucleic acids. Any other anionic polyelectrolyte should be influenced in a similar way. Therefore, aqueous VC might develop into a useful tool for polyelectrolyte fractionation and characterization.

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