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Review

Non-porous hydrophilic resin-based packings for the separation of biopolymers

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ABSTRACT

Commercial non-porous hydrophilic resin-based packings are reviewed. Characteristics of the packings and their applications to the separation of biopolymers are described briefly. Proteins, peptides, oligonucleotides and DNA fragments can be separated rapidly, typically in 5–15 min, with high resolution by ion-exchange, reversed-phase and hydrophobic interaction chromatography on these materials.

CONTENTS

1. INTRODUCTION

Although mainly totally porous packings have been employed in high-performance liquid chromatography (HPLC), non-porous packings, which were once explored in the early stages of HPLC [1,2] but dit not come into general use, have attracted attention in recent years, especially in the separation of biopolymers. Since Unger and co-workers showed that non-porous packings of small particle diameter (1.5 μm) are very useful for rapid separations of proteins [3,4], various types of non-porous packings based on silicas [5–12], synthetic hydrophilic resins [13–24], cross-linked polystyrenes [25,26] and agarose [27,28] have been prepared and examined for the separation of biopolymers. We have also been investigating non-porous packings based on hydrophilic resins, which are now commercially

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available. Separations of proteins, peptides, oligonucleotides and DNA fragments by ion-exchange chromatography (IEC), reversed-phase chromatography (RPC) and hydrophobic interaction chromatography (HIC) on these packings are briefly reviewed.

2. CHARACTERISTICS OF NON-POROUS HYDROPHILIC RESIN-BASED PACKINGS

Four types of packings, TSKgel DEAE-NPR, SP-NPR, octadecyl-NPR and butyl-NPR, are now commercially available (Tosoh, Tokyo, Japan). They were prepared by chemically bonding diethylaminoethyl, sulphopropyl, octadecyl and butyl groups on the surface of non-porous spherical synthetic hydrophilic resin of particle diameter 2.5 μm. The exact chemical composition of the base material is not available, but it contains many primary hydroxyl groups. The ligands described above were introduced by utilizing the hydroxyl groups. They are chemically very stable and can be operatad over a wide pH range (2–12). They can also be exposed to more extreme pH values for short period. There is no problem in washing them with 0.5 *M* sodium hydroxide solution. The ion-exchange capacities of DEAE-NPR and SP-NPR are *ca.* 0.15 and 0.10 mequiv./ml and the pK_a values of the ionic groups are *ca.* 11.2 and 2.3, respectively. The octadecyl and butyl group contents of octadecyl-NPR and butyl-NPR have not been determined. The proteins adsorption capacities, determined with bovine serum albumin (DEA-NPR) and haemoglobin (SP-NPR), are *ca.* 5 mg/ml.

These materials are commercially available in prepacked columns of 35 mm × 4.6 mm I.D. Because they are very rigid owing to the non-porous structure, although they are resin-based, particles as small as 2.5 μm in diameter can be used without problems. The columns can be operated at flow-rates up to 1.6 ml/min. Pressure drops are *ca.* 100 bar at a flow-rate of 1 ml/min with typical eluents.

3. APPLICATIONS TO THE SEPARATION OF BIOPOLYMERS

3.1. Proteins [17,22,23]

Fig. 1 shows a separation of a protein mixture obtained by IEC on DEAE-NPR. Four acidic proteins and their impurities were separated very rapidly, in about 5 min, and the resolution attained were very high. More rapid separations were also possible with a slight decrease in resolution by changing the elution conditions, *e.g.*, by decreasing the gradient time. The resolution in Fig. 1 seems almost comparable to or even higher than that attainable by gel electrophoresis, which has been widely employed to separate proteins on the basis of ionic properties.

Fig. 2 is another example of a protein separation. A mixture of seven proteins was separated by RPC on octadecyl-NPR. The peak of ovalbumin in Fig. 2 is broader than the peaks of other proteins and some shoulders are observed. Although the reason for this is not clear, one possibility is that different components were present in the ovalbumin sample and were partially separated. If this is so, it is an indication of the very high resolution of RPC on octadecyl-NPR for protein separation, because such a partial separation of ovalbumin components by RPC has not been reported previously.

Rapid separations of proteins with high resolution were also possible by HIC on

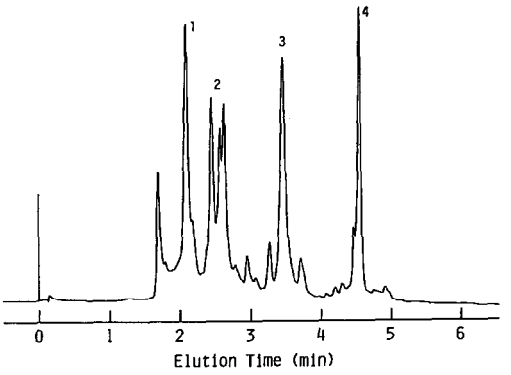

Fig. 1. Separation of a protein mixture by IEC on a TSKgel DEAE-NPR column. A mixture of (1) conalbumin, (2) transferrin, (3) ovalbumin and (4) trypsin inhibitor was separated at 25°C with a 10-min linear gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 1.5 ml/min and monitored with a UV detector at 280 nm. (From ref. 17.)

butyl-NPR. Fig. 3 is an example of the application of HIC to the separation of a commercial sample of trypsin. Several peaks were observed. The largest and the second largest peaks were identified as β-trypsin and α-trypsin, respectively, by an enzymatic activity test and sodium dodecyl sulphate polyacrylamide gel electrophoresis. β-Trypsin is converted into α-trypsin by cleaving the molecular chain at a single position. However, the two chains of α-trypsin, having molecular weights of *ca.* 10 000 and 13 000, are connected with six disulphide bonds and the structures of α- and β-trypsins are similar. Therefore, these two types of trypsins differ only slightly in their chromatographic behaviour. Nevertheless, it was possible to separate them completely in about 6 min. The same trypsin sample was also separated by RPC on octadecyl-NPR but the two main components were separated better by HIC than by RPC. The

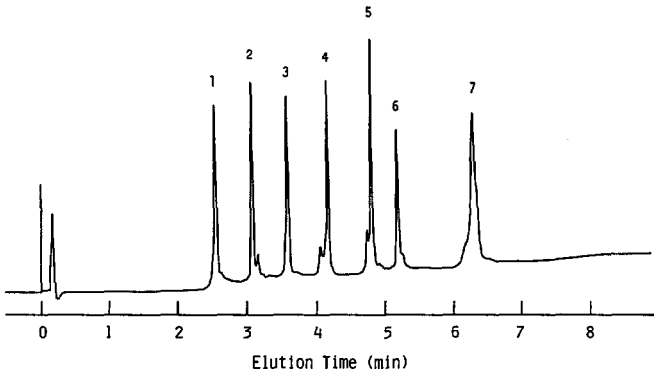

Fig. 2. Separation of a protein mixture by RPC on a TSKgel octadecyl-NPR column. A mixture of (1) ribonuclease, (2) insulin, (3) cytochrome *c*, (4) lysozyme, (5) α-lactalbumin, (6) myoglobin and (7) ovalbumin was separated at 25°C with a 10-min linear gradient of acetonitrile from 15 to 80% in 0.05% trifluoroacetic acid (TFA) at a flow-rate of 1.5 ml/min and monitored with a UV detector at 220 nm. (From ref. 23.)

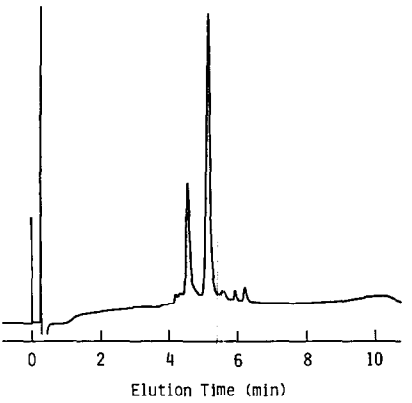

Fig. 3. Separation of trypsin by HIC on a TSKgel butyl-NPR column. A commercial sample of trypsin was separated at 25°C with a 10-min linear gradient of ammonium sulphate from 2 to 0 *M* in 20 m*M* Tris–HCl buffer (pH 7.5) at a flow-rate of 1.0 ml/min and monitored with a UV detector at 280 nm. (From ref. 22.)

short separation time should be particularly advantageous in HIC because some proteins tend to be easily denatured during HIC separation [29].

The non-porous packings were compared with totally porous packings in the separation of proteins. The same protein mixtures as in Figs. 1–3 were separated on totally porous packings, TSKgel DEAE-5PW, Phenyl-5PW RP and Phenyl-5PW. A higher resolution was attained in much shorter time (one fifth to one sixth) on the non-porous packings than the totally porous packings.

The recovery of proteins from the non-porous packing columns was usually almost quantitative. Proteins were recovered in high yield even with sample injection of very small amounts (low- and sub-microgram levels) and yet in small volumes (0.05–0.2 ml) under typical elution conditions as in Figs. 1–3. The recovery of enzymatic activity was also high, usually more than 80%, in IEC and HIC. These high recoveries are assumed to be due to the small surface area of the non-porous packings. This is one of the advantages of non-porous packings.

On the other hand, the loading capacity was low owing to the small surface areas of the non-porous packings. The maximum sample load resulting in the highest resolution was only 0.5–5 μg for pure samples. In the separation of crude samples, amounts up to 10–200 μg, depending on the purity of the sample, could be applied with little decrease in resolution. These maximum sample loads are significantly lower (*ca.* 1–5%) than those on totally porous packings. This is the greatest disadvantage of non-porous packings.

3.2. Peptides [23,24]

Figs. 4 and 5 show separations of peptide mixtures obtained by IEC on SP-NPR and by RPC on octadecyl-NPR. As shown, it was possible to separate peptides of relatively high molecular weight rapidly with high resolution. However, small peptides with molecular weights below *ca.* 1000 were usually eluted as slightly broad and tailing peaks. The reason why such small peptides elute as broad tailing peaks is probably because the resin-based non-porous packings have very small pores, although they are

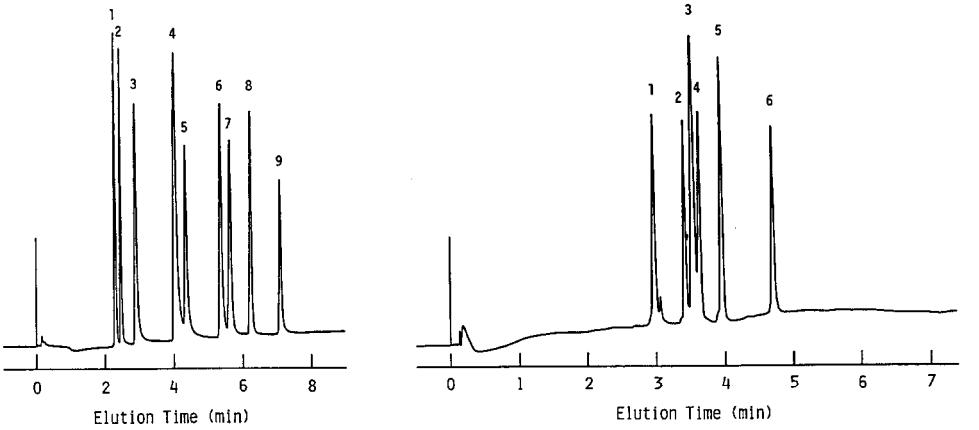

Fig. 4. Separation of a peptide mixture by IEC on a TSKgel SP-NPR column. A mixture of (1) γ-endorphin, (2) bombesin, (3) luteinizing hormone-releasing hormone, (4) somatostatine, (5) α-melanophore-stimulating hormone, (6) substance P, (7) glucagon, (8) insulin and (9) β-endorphin was separated at 25°C with a 30-min linear gradient of sodium sulphate from 0 to 0.25 *M* in 20 m*M* acetate buffer (pH 3.5)–acetonitrile (60:40, v/v) at a flow-rate of 1.5 ml/min and monitored with a UV detector at 215 nm. (From ref. 24.)

Fig. 5. Separation of a peptide mixture by RPC on a TSKgel octadecyl-NPR column. A mixture of (1) α-endorphin, (2) bombesin, (3) γ-endorphin, (4) angiotensin, (5) somatostatin and (6) calcitonin was separated at 25°C with a 10-min linear gradient of acetonitrile from 0 to 80% in 0.2% TFA at a flow-rate of 1.5 ml/min and monitored with a UV detector at 215 nm. (From ref. 23.)

stated to be non-porous. If small molecules enter such very small pores, the diffusion rate there should be slow, which results in broad and tailing peaks.

The recovery of peptides was also high and the loading capacity was low, as in the separation of proteins.

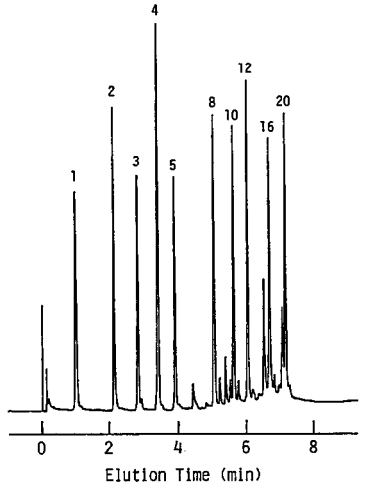

Fig. 6. Separation of a mixture of oligoadenylic acids with chain lengths of 1, 2, 3, 4, 5, 8, 10, 12, 16 and 20 nucleotides by IEC on a TSKgel DEAE-NPR column. The mixture was separated at 25°C with a 20-min linear gradient from 0 to 0.4 *M* sodium perchlorate in 20 m*M* Tris–$HClO_4$ buffer (pH 9.0) at a flow-rate of 1.5 ml/min and monitored with a UV detector at 260 nm. (From ref. 19.)

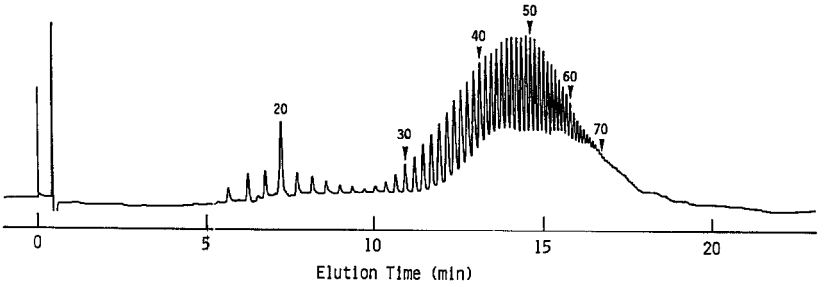

Fig. 7. Separation of a hydrolysate of polyadenylic acid containing from the 17-mer to *ca.* the 100-mer by IEC on two TSKgel DEAE-NPR columns connected in series. The separation was performed at 25°C with a 60-min linear gradient from 0.25 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0) at a flow-rate of 1.0 ml/min and monitored with a UV detector at 260 nm. (From ref. 19.)

3.3. Oligonucleotides [19]

Fig. 6 shows a separation of a mixture of oligoadenylic acids with chain lengths of 1, 2, 3, 4, 5, 8, 10, 12, 16 and 20 nucleotides, obtained by IEC on DEAE-NPR. These comparatively small oligonucleotides could be separated well in as short a time as 8 min.

Fig. 7 shows a separation of a hydrolysate of polyadenylic acid containing from the 17-mer to *ca.* the 100-mer. Baseline separations were obtained for up to the 32-mer and peaks appeared for up to about the 70-mer, and yet the separation was completed in less than 20 min. Although a similar separation to that in Fig. 7 has been reported by others, the separation time was *ca.* 20 h [30]. As far as is known, this was the first rapid separation of large oligonucleotides with high resolution.

Although the separations in Figs. 6 and 7 are based on chain length, it was also possible to separate oligonucleotides according to base composition by IEC on DEAE-NPR. It has been demonstrated that IEC on DEAE-NPR is very useful for the purification and purity analysis of synthetic oligonucleotides, which are very important in the syntheses of DNA or RNA.

3.4. DNA fragments [21]

Fig. 8 shows a chromatogram of pBR322 DNA–Hae III digest, which contains 22 fragments with base pairs of 7–587, obtained by IEC on DEAE-NPR. Small DNA fragments (less than 600 base pairs) could be separated almost completely when they differed in chain length by 5–10%.

Fig. 9 shows a chromatogram of λDNA–Hind III digest containing eight fragments with base pairs of 125–23 130, which suggests that IEC on DEAE-NPR is also very effective even for large DNA fragments over 1000 base pairs. It is possible to achieve almost baseline separations in a very short time, *e.g.*, 5 min, for large fragments differing in chain length by more than 50%.

Hecker *et al.* [31] and Westman *et al.* [32] reported good separations of DNA restriction fragments using totally porous anion exchangers having large pores. However, their applications were limited to small fragments, mostly smaller than 1000 base pairs in chain length, and much longer times (1–8 h, with some exceptions) were required than in Figs. 8 and 9. Stowers *et al.* [33] and Merion *et al.* [34] reported

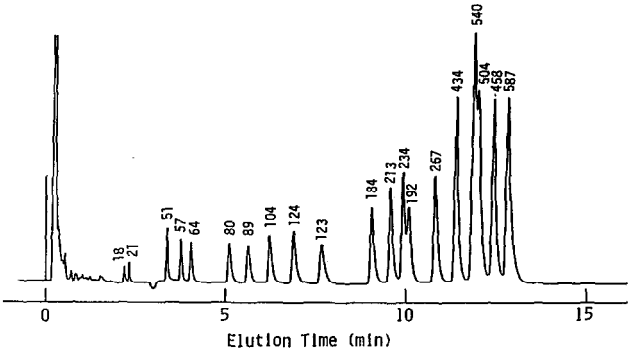

Fig. 8. Separation of pBR322 DNA–Hae III digest by IEC on a TSKgel DEAE-NPR column. The separation was performed at 25°C with a 0.1-min linear gradient from 0.25 to 0.45 *M* sodium chloride followed by a 2.9-min linear gradient from 0.45 to 0.5 *M* and a 57-min linear gradient from 0.5 to 1.0 *M* in 20 m*M* Tris–HCl buffer (pH 9.0) at a flow-rate of 1.5 ml/min and monitored with a UV detector at 260 nm. The numbers on the peaks are chain lengths of the DNA fragments in base pairs. (From ref. 21.)

efficient separations of a wide range of nucleic acids including large DNA restriction fragments. The resolution that they attained and those in Figs. 8 and 9 seem to be equivalent, but the separation times in Figs. 8 and 9 are shorter.

Although agarose or polyacrylamide gel electrophoresis has been the most common technique for separating DNA fragments owing to its high resolution, some problems occur in quantitative measurements of the components, scaling-up, recovery

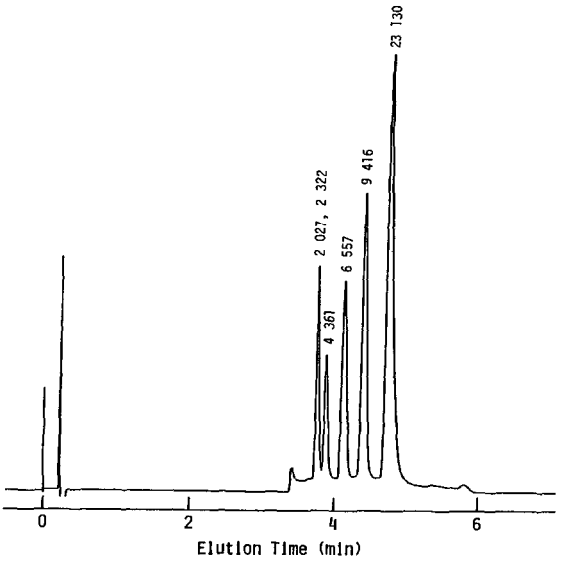

Fig. 9. Separation of λDNA–Hind III digest by IEC on a TSKgel DEAE-NPR column. The separation was performed at 25°C with a 10-min linear gradient from 0.5 to 1.0 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0) at a flow-rate of 1.0 ml/min and monitored with a UV detector at 260 nm. The numbers on the peaks are chain lengths of DNA fragments in base pairs. (From ref. 21.)

of the separated components, etc. IEC on DEAE-NPR does not show such problems. Accordingly, IEC on DEAE-NPR should be a good alternative to gel electrophoresis for the analysis and purification of DNA fragments.

4. CONCLUSION

Commercial non-porous resin-based packings, TSKgel DEAE-NPR, SP-NPR, octadecyl-NPR and butyl-NPR, are very useful for separating a wide range of biopolymers, proteins, peptides, oligonucleotides and DNA fragments rapidly and with high resolution. The separation time is typically 5–15 min. Consequently, they should be useful in particular for applications that require rapid separations such as in clinical analysis and monitoring of purification or reaction processes. They are also useful for general analyses such as laboratory purity tests. In addition, they should be applicable effectively to very large molecules owing to the lack of diffusion into and out of their pores. However, their loading capacities are low because their surface areas are small. Therefore, they do not seem suitable for large-scale separations. In contrast, the samples are recovered in high yield even with sample injections of very small amounts (low- and sub-microgram levels) owing to the small surface area. Accordingly, they are useful for micropreparative separations.

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