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High-capacity composite adsorbents for nucleic acids

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ABSTRACT

Cytopore™ is a bead-shaped, macroporous and easily compressible cellulose-based anion-exchange material intended for cultivation of anchor-dependent animal cells. Reticulated vitreous carbon (RVC) is a strong, non-compressible, high voidage (97%) matrix material that can be cut to desired geometrical shapes. Cytopore and RVC were combined to cylindrical composites (25 mm × 10 mm) fitted inside chromatography columns. The composite combined the advantageous properties of both its constituents, making it suitable for column chromatography. The composite could withstand very high flow rates without compaction of the bed (>25 column volumes/min; 4000 cm h^{-1}). Chromatography runs with tracers showed a low HETP value (0.3 mm), suggesting that pore flow was in operation. The dynamic binding capacities (10% breakthrough) per gram of dry weight Cytopore were determined for several compounds including DNA and RNA and were found to be 240–370 mg/g. The composite was used to isolate pUC 18-type plasmids from a cleared alkaline lysate in a good yield. Confocal microscopy studies showed that plasmids were bound not only to the surface of the Cytopore material but also within the matrix walls, thus offering an explanation to the very high binding capacities observed. The concept of using a composite prepared from a mechanically weak, high-binding material and a strong scaffold material may be applied to other systems as well.

1. Introduction

There is a huge interest in gene therapy and DNA vaccination and in spite of some disappointing setbacks a large number of clinical trials are under way [1–4]. Once the first products reach the market a surge for suitable vectors are expected. Therefore, considerable efforts to design and develop new, effective separation methods and separation materials for gene vectors such as plasmids are ongoing. The usual starting point is an alkaline lysate followed by various combinations of methods e.g. precipitations, aqueous two-phase separations, membrane techniques and a plethora of chromatographic techniques [5–12]. A number of new separation agents with excellent properties have also appeared on the market, for example PlasmidSelect Xtra particles (GE Healthcare) [13], Mustang Q membranes (Pall Corporation) [14], Sartobind D membranes [15], Natrix Hydrogel membranes [16], Qiagen Plasmid Purification System [17], CIM DEAE monoliths (BIA Separation) [18] and Poros HQ perfusion beads (Applied Biosystems) [19]. The standard problem when developing adsorbents for plasmids has been to properly manage the size of plasmid molecules, being much larger than e.g. proteins, the traditional target for bioseparation materials. Understandably there has been an emphasis on adsorbents with very large pores having pore flow (e.g. monoliths, superporous particles, membranes) [14,20,21] to accommodate the plasmids and to diminish diffusion problems or on small-sized non-porous adsorbents to avoid pore diffusion and still get enough binding surface [22].

Ten years ago, Cabral et al. [23] published a patent that describes the use of Cytopore™ as an adsorbent for plasmids. Cytopore is a large-pore, cellulose-based support material (bead diameter 0.2–0.3 mm) intended for cultivation of anchorage dependent animal cells [24]. The material is carrying tertiary amino groups (DEAE), explaining its plasmid-binding properties, which were described as exceptional. However, all procedures in the patent related to static adsorption, one reason probably being that the Cytopore material has a spongy texture and is easily compressible. Thus, columns with the Cytopore material are difficult and impractical to handle.

In this report we have tried to make good use of the excellent binding properties of Cytopore and at the same time adapt it for column use, an often preferred format. The adaptation was possible especially by using reticulated vitreous carbon (RVC) [25] as a stabilizing matrix, preventing the Cytopore bed to collapse.

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This concept may have bearing also on other mechanically weak chromatography materials.

2. Materials and methods

2.1. Materials

Reticulated vitreous carbon slabs (10 cm × 10 cm × 2.5 cm) with a pore size of about 1 mm (20 ppi grade) were obtained from ERG Materials and Aerospace Corporation, Oakland, CA, USA. Cytopore 2 (1.8 meq. g^{-1} ion binding capacity) was obtained from GE Healthcare, Uppsala, Sweden, YOYO-1 (Yellow Orange-Yellow Orange) from Invitrogen, Stockholm, Sweden, and BSA, yeast RNA, bovine RNase and salmon sperm DNA from Sigma–Aldrich, Stockholm, Sweden. The DNA preparation was dialysed against 50 mM Tris–acetate buffer, pH 8.0, overnight to remove low molecular weight impurities. Agarose gel electrophoresis then showed that the preparation consisted of 100 bp DNA molecules. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. General procedures

Plasmid purification was followed by agarose gel electrophoresis and by analytical ion-exchange chromatography (Mini Q) as described earlier [21]. Plasmids were obtained from *E. coli* TG1 cultures harbouring a pUC 18 plasmid with a lactate dehydrogenase gene insert from *Bacillus stearothermophilus* (total plasmid size 4.0 kbp) [26]. An alkaline lysate containing 0.06 mg plasmid/ml was prepared using standard procedures [27].

2.2.2. Packing of Cytopore columns (no RVC)

Three milliliters of a homogenous Cytopore 2 suspension (10 mg dry weight/ml in 50 mM Tris–acetate, pH 8.0) was poured into a 5 mm I.D. and 5 cm long glass column provided with a flow adapter in one end and a column extension (10 cm) in the other end. The suspension was subsequently compressed into a bed by running tap water at high speed through the packing device. The column extension was removed and a second flow adapter was attached to the top of the column. The final bed height (15 mm) was achieved by compressing the bed additionally with aid of the top flow adapter.

2.2.3. Cutting of RVC matrix

The reticulated vitreous carbon (RVC) material was delivered as 25 mm thick slabs that easily could be cut into 10 mm diameter cylinders (25 mm × 10 mm), by using a cork bore-type cutting tool made of stainless steel. To improve the quality of the RVC cylinders it was beneficial to keep the cutting tool very sharp and have it supported by a frame to allow a steady and controlled boring through the RVC matrix.

2.2.4. Packing of composite column (with RVC)

The RVC cylinder (25 mm length × 10 mm width) was fitted into an empty chromatographic column (C10/20 column, GE Healthcare, 200 mm length × 10 mm I.D.) provided with an AC 10 flow adaptor at the bottom. The RVC cylinder was pushed into the glass column until it came into contact with the flow adaptor net. The top of the column was attached to a tap water faucet via PVC tubing and two T-connectors. The tap water had a maximum pressure of 4.8 bar. A pressure gauge was attached to one of the T-connectors and a 1 ml syringe was connected to the other via a tubing provided with a clamping device (Fig. 1).

The packing was initiated by opening the tap water faucet, filling the system with water running initially at several hundred milliliters per minute. The Cytopore was injected in form of a suspension containing 5 mg Cytopore 2 (dry weight) per ml of 2 M NaCl using a 1 ml Luerlock syringe. Totally eighteen 1-ml injections were made during 10 min, corresponding to 90 mg Cytopore 2, dry weight). At the end the water flow had decreased to about 80 ml/min. The water was run at this speed for additionally 15 min to stabilize the bed.

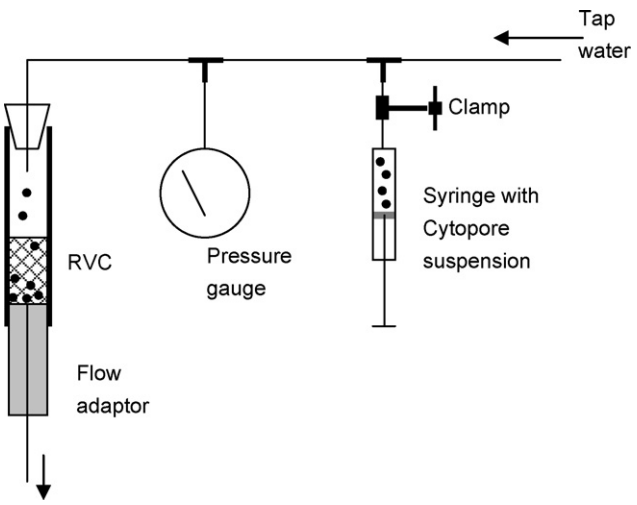

Fig. 1. Set-up for packing RVC–Cytopore composite columns. Details in the text.

The column was then removed from the packing set-up and an AC 10 flow adaptor was fitted on top of the composite bed. Before use the composite column was washed with 4 M NaCl to remove impurities that had been adsorbed from the tap water.

2.2.5. Basic chromatographic characterization of Cytopore columns

Columns as described above (with or without RVC) were attached to an HPLC system comprising of a pump (0–10 ml/min), injection valve with a 2 ml loop, bypass valve (to direct the flow through the column or via a bypass), a sensitive pressure transducer, a UV/vis detector and a recorder. HETP values were calculated from the elution profiles of injected pulses of a non-retained substance (usually 25 μl of 1% acetone).

To determine the adsorption capacity DNA, RNA, tatrazine or bovine serum albumin was pumped through the Cytopore columns until the recorder showed that a substantial breakthrough had occurred. All substances were dissolved in 50 mM Tris–acetate buffer pH 8.0. By briefly switching the bypass valve a rapid check of the absorbance at the column inlet could be obtained. Elution was carried out with 1 M NaCl dissolved in 50 mM Tris–acetate buffer, pH 8.0.

To ensure complete removal of adsorbed substances a cleaning in place (CIP) procedure was carried out between the runs. To that end the injection valve was used to consecutively inject three series of 2 ml of 0.5 M NaOH, 4 M NaCl and 3 M acetic acid at a pump speed of 1 ml/min. The running buffer was 50 mM Tris–acetate, pH 8.0.

2.2.6. Chromatographic isolation of plasmids from a cleared alkaline lysate

An ÄKTA Explorer 10 system was used (GE Healthcare). The alkaline lysate was diluted 3× with water and applied to the column via a loop (0–1 ml) or a superloop (0–10 ml or 0–150 ml; GE Healthcare). Running buffer was 50 mM Tris–acetate buffer pH 8.0, supplied with 1 mM EDTA and 0.25 M NaCl. Elution was carried out with a linear salt gradient (0.25–2 M NaCl). One milliliter fractions were collected and analysed with gel electrophoresis and analytical ion exchange chromatography to show the presence of protein, RNA and plasmids [21–22].

2.2.7. Confocal microscopy

Fluorescently labelled (YOYO-1) plasmids were first prepared from a cleared alkaline lysate. The cleared lysate containing about 0.06 mg/ml of plasmid was diluted three times with water. To break down interfering RNA the diluted lysate was provided with RNase (0.1 mg/ml of plasmid solution). After 2.5 h incubation at room temperature, YOYO-1 (Yellow Orange-Yellow Orange) was added. The addition was made in the following way to minimize uneven labelling: 10 μl of YOYO-1 stock solution (dissolved in DMSO) was mixed with 0.5 ml water and the diluted reagent was subsequently added to 2 ml of the RNase treated plasmid solution under intense mixing using a Vortex mixer.

One milliliter of a Cytopore suspension (0.1 mg suspended in 1 ml 0.25 M NaCl, 50 mM Tris–HCl, pH 8.0) was mixed with 0, 0.01, 0.10 or 1.0 ml of the above labelled plasmid preparation in four separate experiments. After 48 h on a rocking table at room temperature the Cytopore particles were spun down and the supernatant carefully removed. The particles were washed with buffer (2×) and with water (1×).

The cytopore particles with adsorbed, labelled plasmids were investigated with a Zeiss LMS 510 META confocal microscope using a 20× or 60× objective. A setup suitable for the YOYO-1 fluorophor was used (FITC channel; excitation at 488 nm, emission collected with a 505-550 filter). Optical slices (2 μm) were collected in the *z*-direction.

3. Results and discussion

3.1. Different sets of pores explain the high binding capacity of Cytopore

The patent by Cabral et al. [23] describes the adsorption of a 4.8 kb plasmid to a number of established adsorbents for biomolecules and to Cytopore: Q Sepharose High Performance (20 mg plasmid/g dry support); Q Separose Fast Flow (6 mg/g); Q Sepharose Big Beads (3.1 mg/g); Streamline QXL (6.2 mg/g) and Cytopore (1240 mg/g). Comparison of the beaded supports shows that small support particles have higher capacity, which make sense considering that smaller particles have a larger surface area available for plasmid binding (the outer surface). However, the most remarkable value is the binding of plasmids to Cytopore, 1240 mg/g. The explanation given in the patent for this exceptionally high binding capacity is the porous structure of Cytopore that should provide an excellent access for the plasmid to the binding sites, even those deep within the particles. As indicated in Fig. 2, this is indeed so. Cytopore particles are rather large and have an extremely spongy structure with very wide pores. According to the manufacturer and substantiated by the figure the pore size is around 30 μm. However, such pores are unnecessarily large considering the size of a 4.8 kbp plasmid, which has a hydrodynamic radius well below 0.5 μm. Thus, the available binding surface could be expected to be comparatively low with such excessively large pores, leading to a low binding capacity. Still, the moderate surface area (1.1 m^2/g according to the manufacturer), is obviously extremely efficient for plasmid binding. A quick calculation shows that the reported binding of 1240 mg/g corresponds to several layers of densely packed plasmids [10]. This is very remarkable but could perhaps at least partly be explained by the surface properties of the material. The wall surface of the large pores probably contains smaller pores not included in the 1.1 m^2/g value. If the plasmid could reach into such pores the high binding capacity is easier to understand.

To shed some light on this problem we labelled the plasmid with the intercalating dye YOYO-1 (Yellow Orange-Yellow Orange) Care was taken not to label the plasmids excessively, since this could possibly change their behavior on the ion exchanger. Confocal microscopy (Fig. 3) shows a dense binding of plasmids on the outer surface of the particle but also the inner surfaces (the walls of the very large pores) are densely covered with plasmids. Importantly, also inside the matrix itself a considerable binding is seen. In this part of the matrix the pores are most probably rather narrow, but still the plasmids manage to diffuse to these remote locations, at least when the incubation time is long. This is an important observation since it gives a ready explanation to why the binding is so exceptionally high with Cytopore. The explanation is simply that Cytopore offers a much larger binding area than one first assumes.

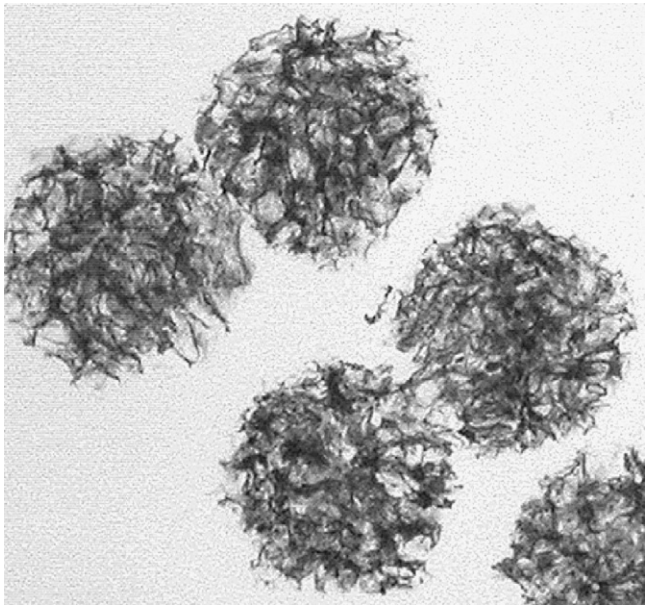

Fig. 2. Cytopore beads suspended in water. The bead diameter is approximately 250 μm.

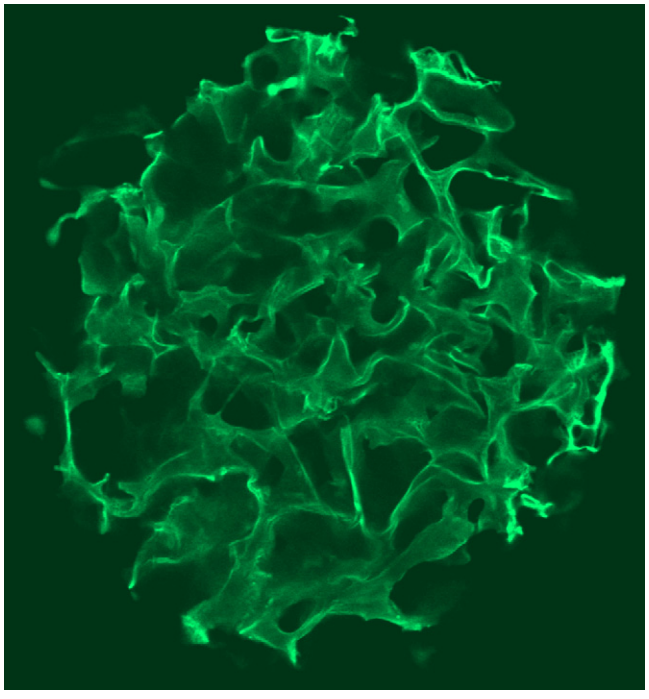

Fig. 3. Confocal microscopy of a Cytopore particle with adsorbed, fluorescently labelled (YOYO-1) plasmids. The image shows a 2-μm thick optical slice obtained from the upper part of a bead.

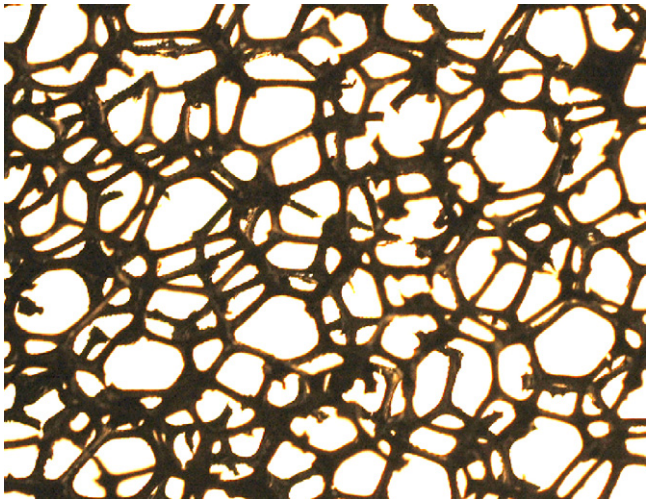

Fig. 4. Reticulated vitreous carbon (RVC). The picture shows an approximately 3 mm thick slice of RVC (20 ppi grade; pores per inch, i.e. pore size about 1 mm) and covers an area of 13 mm × 10 mm.

3.2. The spongy nature of Cytopore is problematic in chromatography

The binding capacity of the Cytopore material is attractive. However, the Cytopore material is inconvenient to handle in column chromatography procedures, due to its soft and spongy texture, as also pointed out earlier [23]. Our attempt to use neat Cytopore for column chromatography was only successful with narrow-bore columns under benign circumstances. Certainly, well-filtered cleared lysates could be pumped through 5 mm diameter columns at high speed (0.4 ml/min, 120 cm h^{-1}), which was very encouraging. However, when elution was attempted (at any flow rate) the column beds often collapsed. Presumably the elution conditions aggravated an already established clogging in the upper parts of the column leading to a high local pressure drop and a local bed collapse. Under the influence of the flow this local collapse translated through the bed causing a total bed collapse and loss of flow.

To relieve the Cytopore particles from excessive compression forces we combined Cytopore with a supporting structure present in the whole column volume. Any local clogging should now remain local, since the pressure drop and the forces generated by the clogging should be unburdened by the supporting structure and not transmitted further down the column. The supporting structure chosen here, reticulated vitreous carbon (RVC), is a strong but brittle material [25]. A prominent feature of RVC is its excess of void space, in fact, only 3% of the volume of RVC is glassy carbon material. The remaining 97% can thus be filled with soft adsorbents such as Cytopore. The RVC material is manufactured in a range of pore sizes. A thin slab of the quality used in this report (1 mm pore size) is shown in Fig. 4. Composites using RVC have been described earlier for separation purposes; RVC was combined with superporous agarose and polypyrrole in an attempt to achieve electrochemically controlled chromatography systems [28].

3.3. Packing of composite columns

The packing of RVC–Cytopore composites was carried out with the set-up schematically shown in Fig. 1. An important aspect of the packing was to fill the void spaces of the RVC matrix as completely as possible. Initial experiments showed that if only partial filling of the RVC structure was managed, a poor flow pattern was obtained in the chromatographic runs. To improve the filling, a high flow packing procedure was adopted. Although not extensively investigated, it seemed beneficial to use tap water instead of deionised water during the packing. Probably the presence of ions somehow influenced the behaviour of the Cytopore particles during the packing. It was also found important to use rather dilute Cytopore suspensions during packing to obtain densely packed composite without voids. When developing the procedure it was of considerable help to use a stereo microscope trained on the RVC structure inside the column. It made it easier to find conditions where the Cytopore particles travelled rather unhindered through the RVC matrix and settled as an even bed within the matrix. Fig. 5 shows a close-up of a packed column. Short segments of the RVC matrix close to the glass walls are visible.

Fig. 5. Close-up of a glass column with an RVC–Cytopore composite. The dimensions of the composite are 25 mm × 10 mm (l × I.D.). The RVC structure in immediate vicinity of the glass wall is clearly visible. The banded structures at the top and at the bottom of the column belong to the flow adaptors.

3.4. Flow properties of RVC–Cytopore composites

Fig. 6 shows a comparison between a 25 mm × 10 mm RVC–Cytopore composite column and a column packed with neat Cytopore. Both columns contained the same amount of Cytopore 2 matrix, 90 mg (dry weight). The RVC–Cytopore composite column behaved very well over a wide flow range and was completely dimensionally stable, at least up to 50 ml/min (equivalent to about 25 column volumes/min or 4000 cm h^{-1}). The pressure drop over the composite column was, however not completely linear, indicating that some particle rearrangements possibly occurred at high flow. However these rearrangements were reversible since the same pressure/flow curve was obtained when repeating the experiment. The reference column with neat Cytopore behaved dramatically different. Even at low flow rates a compression of the bed occurred and the back pressure increased. When the compression had reached about 25%, additional increase of the flow rate made the back pressure increase dramatically indicating a collapse of the bed.

The superior performance of the RVC–Cytopore is easily explained as the ability of the RVC to take up and bear the forces

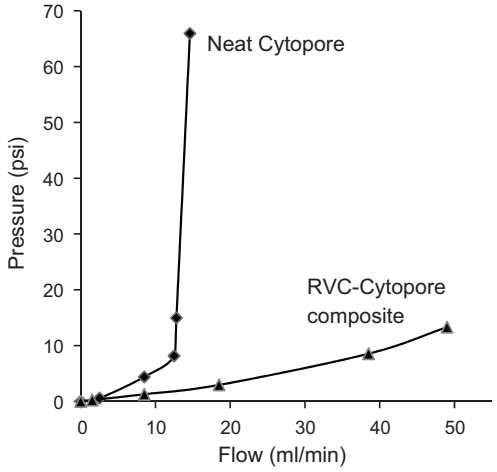

Fig. 6. Flow–pressure relationship for a column packed with an RVC–Cytopore composite or with neat Cytopore. Pressure drop in tubings and flow adaptors, etc. has been subtracted (reference run).

generated at high flow rates. One might consider the depth of each RVC pore (approximately 1 mm) as a unit packing cell for the Cytopore. With this view the unsupported height of the Cytopore matrix is of the order of only 1 mm. The pressure drop over such a short "column" is obviously very small, only 1/25 of the pressure drop over the whole column and low enough to prevent any significant compacting or bed collapse. The composite principle might be useful also with other adsorbents having interesting binding properties but unsatisfactory structural stability.

3.5. RVC–Cytopore performance

The RVC–Cytopore composite beds usually showed good bed properties as judged by the elution profiles of tracers, provided the packing procedures had been carried out properly. Thus, with acetone as tracer an HETP of 0.35 mm was measured for one column and the asymmetry factor was 0.9. These data are very satisfactory considering that the Cytopore particles have a diameter of 0.3 mm. A good packing of 0.3 mm particles would normally result in an HETP value of about 0.6 mm under ideal conditions (2× the particle diameter), which suggests that the Cytopore particles here behave as much smaller particles. The reason is most probably that the large pores of Cytopore allow particle perfusion, which is known to lower the HETP value [29]. The packing procedure (high speed packing) has probably also led to diminished interstitial pores, increasing the tendency to intraparticle flow. Observation of coloured tracers (tatrazine) showed, however, that the composites were not perfectly packed, in spite of the good HETP value observed. Thus, the front of the coloured substance was not perfectly even but showed some fingering, although not very severe. It was concluded that additional developing of the packing procedures might additionally improve the performance of the composites.

3.6. Adsorption of nucleic acid model substances

The intended use for the RVC–Cytopore composite is isolation of large molecules such as plasmids and viruses. However, due to the exceptional capacity of the material, the amount of available plasmids was far from sufficient to saturate even a single column. Therefore, model experiments were carried out with other nucleic acids (yeast RNA and salmon sperm DNA). Clearly, these nucleic acid preparations have properties that differ from those of plasmid DNA, making it difficult to draw precise conclusions regarding the merits of the composite for plasmid purification. Besides nucleic acids also bovine serum albumin (BSA) and tatrazine dye (trivalent) were used as model substances. The experiments were carried out as breakthrough experiments, where substances were pumped through the composite until a distinct breakthrough was observed. After a brief washing, elution was carried out with 1 M NaCl. Material balances indicated a recovery of 70–90% in case of nucleic acids and a 100% recovery in case of BSA and tatrazine. Additional elution with 4 M NaCl and cleaning-in-place (CIP) with 0.5 M NaOH, 4 M NaCl and 3 M acetic acid released only small amounts of material.

Table 1
Dynamic capacities for RVC–Cytopore composites[a].

Substance	Conc.	Flow rate	Dynamic capacity	
	(mg/ml)	(ml/min)	(mg/ml)	(mg/g)
DNA	0.2	0.4	13	290
RNA	0.2	0.4	16	370
BSA	0.2	1.0	3.5	80
Tatrazine	1.0 mM	2.0	0.017 mmol	0.50 mmol g^{-1}

[a] Composite size: 25 mm × 10 mm (l × I.D.). Composite volume: 2.0 ml. Cytopore 2 content: 90 mg (dry weight). Dynamic capacity refers to 10% breakthrough.

Table 1 summarises several breakthrough experiments and shows that the capacity of the composite is remarkably good. For nucleic acid it was 26–33 mg per composite column, which corresponds to 13–17 mg per ml bed volume or 240–370 mg/g dry Cytopore. This is clearly lower than the value (1240 mg/g) given by Cabral et al. [23]. However, the data in Table 1 refers to dynamic capacities at a 10% breakthrough, while the literature data [23] refer to static adsorption (>24 h adsorption). A considerably higher capacity would have been observed if the pumping had continued until the column had become saturated and the values had been calculated on the adsorption level at that stage. Thus, the data obtained here are reasonably in accord with the earlier data.

The binding of BSA and the low molecular weight tatrazine appears to be what could be expected. The tatrazine value (0.5 mmol g^{-1} corresponding to 1.5 meq. charges for a 3-valent compound) is in agreement with the reported ion exchange capacity of Cytopore 2 (1.8 meq. g^{-1}) [24] and the BSA value is in line with the capacity of many commercial ion exchangers [30].

3.7. Capture of plasmids from an alkaline lysate

A pUC 18-type plasmid (4.0 kbp) was isolated from a cleared lysate containing about 0.06 mg plasmid/ml. To improve the performance the cleared lysate was diluted 3× with water rendering a solution with an ionic strength that improves the plasmid binding capacity and diminishes competitive binding of low molecular weight RNA [21]. After washing the elution was carried out with a salt gradient and fractions were collected (Fig. 7) and analysed individually.

The broad peak between 2 and 15 ml contained protein and low molecular weight RNA going straight through the column. The peak between 21 and 25 ml contained not fully separated RNA and plasmids. Thus, between 23 and 25 ml 60% of the applied plasmids were eluted, contaminated with only minor amounts of high molecular weight RNA (Table 2). Between 21 and 23 ml the main part of the high molecular weight RNA appeared plus a minor part of the plasmids (10%), giving a total plasmid recovery of about 70%. This recovery was deemed quite satisfactory, considering the problems often encountered when releasing plasmids from ion exchange adsorbents [31]. By optimizing the elution conditions a better resolution could be expected.

In another set of experiments (data not shown) much larger volumes of diluted alkaline lysate was applied to a 25 mm × 10 mm composite column in order to demonstrate its capacity. Thus, 80 column volumes of lysate were processed and all plasmids added

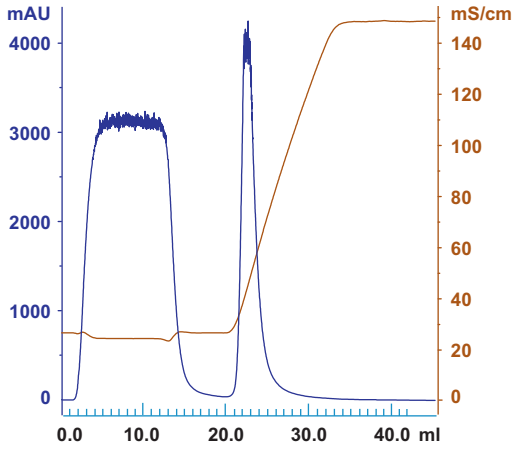

Fig. 7. Plasmid isolation from an alkaline lysate on an RVC–Cytopore column. Alkaline lysate (3.3 ml) was diluted 3× with water and applied to the column. When A_{280} had declined to near base line (at about 20 ml elution volume) a linear NaCl gradient (0.25–2 M) was applied as indicated by the conductivity curve.

Table 2
Fractionation of a cleared lysate on an RVC–Cytopore column. The data is obtained from the separation depicted in Fig. 7.

	Protein (mg)	RNA (mg)	Plasmid (mg)	Plasmid yield (%)
Alkaline lysate	14.98	9.6	0.19	100
Non-absorbed	11.47	7.3	0.00	0
HMW RNA peak[a]	0.03	1.5	0.022	10
Plasmid peak[b]	<0.010	0.19	0.12	60

[a] HMW RNA peak (high molecular weight RNA) is the front part (21–23 ml) of the peak eluting between 21 and 25 ml in Fig. 7.
[b] The plasmid peak is the last part (23–25 ml) of the peak eluting between 21 and 25 ml in Fig. 7.

were effectively bound. However, the recovery of the plasmids upon elution was low (about 20%). It was hypothesized that the reason was a rearrangement of the bound plasmids into a strong-binding state made possible by a long dwelling time on the column before elution was attempted.

4. Conclusions

We have shown that the Cytopore material can be used for column chromatography. It is fairly straightforward to prepare a composite adsorbent from reticulated vitreous carbon (RVC) and Cytopore. The composite has outstanding flow properties, a chromatographic performance (HETP) that suggests that intraparticle pore flow is operating and a very high capacity for nucleic acids. It is suggested that the composite concept may be used also in other systems involving structurally weak adsorbents.

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