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Journal of Chromatography A, 878 (2000) 17–25

JOURNAL OF CHROMATOGRAPHY A

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Pellicular expanded bed matrix suitable for high flow rates ☆

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Received 27 October 1999; received in revised form 31 January 2000; accepted 10 February 2000

Abstract

A new type of expanded bed matrix with a heavy core of stainless steel covered with an agarose layer was prepared. Two bead size fractions, the smaller one (32–75 μm ∅) having a single particle core and the larger (75–180 μm ∅) with an agglomerate of stainless steel particles constituting the core, were chosen for further characterisation. The dispersion behaviour was determined both in packed bed and expanded bed modes by the retention time distribution method (RTD) and compared with the Streamline matrix (Amersham Pharmacia Biotech). The comparison turned out in favour of the new matrix. Flow rates as high as 3000 cm/h were used with the larger fraction, giving stable expanded beds with good mass transfer properties. The matrices were mechanically stable without any tendency to crack or peal, even after prolonged use.
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Keywords: Pellicillar expanded bed matrix; Flow-rate; Retention time distibution method; Expanded bed; Absorbants; Agarose-stainless steel composite; Fluidised bed

1. Introduction

Chromatography is probably the most ubiquitously used separation method for proteins. Usually it is performed in a packed bed, which limits its usage to solutions free from particulate matter. To obtain a particle free solution, a filtration and/or a centrifugation step precedes the chromatography step. Expanded bed (EB, or classified fluidised bed as it also is called), is a new chromatography mode that allows for particulate matter in the liquid phase, and partially maintains the good separation features of a packed bed [1–3].

In EB, the chromatographic flow enters the separation column from the bottom, causing the bed to expand, usually with a factor 2–4, dependent on flow rate, viscosity and particle properties. Significantly, the matrix particles have an increased density and show a size distribution, which leads to a classification of the expanded bed — the heaviest particles at the bottom and the lightest particles at the top. Ideally, each particle will find its own place of equilibration in the expanded bed and will reside there during the separation process. The expanded mode will allow particles such as cells and cell debris to pass freely through the bed.

Important applications suitable for expanded beds are the isolation and concentration of recombinant proteins from fermentation broth [4]. These samples could be quite viscous and contain cells and cell debris. The viscosity is still a problem with today's

☆This work was carried out within the Swedish Center for Bioseparation.

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PII: S0021-9673(00)00223-5

matrices [3], and often makes it necessary to dilute samples prior to the EB step. With heavier matrices this should not be necessary. The number of available matrices specially designed for EB chromatography is limited and a demand for an improved matrix with high density and fast equilibrium between liquid and solid face has been presented [3].

Available matrices suitable for EB are prepared using a number of different concepts. The increased weight necessary at moderate to high flow rates is gained either by using a high-density porous material or by combining a non-porous heavy material with a porous lighter material. The use of porous silica beads [5] is an example of the first design, and the incorporation of small quartz particles in agarose beads, as in Streamline (Amersham Pharmacia Biotech) matrices, is an example of the second strategy. By increasing the bead size the weight of the particles can be further increased to allow higher flow rates. However, this has the disadvantage of increasing the diffusion distance within the particles. To avoid this drawback, materials of even higher density have been used, such as porous zirconium oxide particles instead of porous silica [6] and stainless steel incorporations instead of quartz as in some Streamline matrices [7].

If construction of matrices for even higher flow rates is attempted, not only the high density should be considered but also the short diffusion distances, to ensure that the intra-particle mass transport will match the high flow rate. An old concept for achieving short diffusion distances is to make pellicular matrices with an inert core and a porous surface layer [8,9]. For HPLC this concept was mainly abandoned when very small silica beads (~5 μm) came into use. In EB there are some limitations in bead size determined not only by the density range of suitable materials but by the fact that the beads must be held in the column while contaminating particles in the feedstock should be allowed to freely pass through. The pellicular concept is therefore attractive, giving the opportunity to choose a suitable diffusion distance regardless of bead size and allowing the adjustment of the density of the core material to fit the desired flow rate.

Obviously, capacity will suffer to some extent in a pellicular particle compared to a homogenous porous particle. With a dense core material however the capacity reduction can be kept at acceptable levels and other solutions for increasing the weight are not necessarily beneficial. Another benefit from this pellicular approach is the option to choose a coating material with low non-specific adsorption and well-known properties.

In this article we describe how such a matrix can be prepared from stainless steel beads by covering them with a layer of agarose. The beads are characterised with respect to expansion and dispersion behaviour.

2. Experimental

2.1. Materials

Agarose powder (Sepharose quality) was a gift from Amersham Pharmacia Biotech (Uppsala, Sweden). Sorbitane trioleate (Span 85) was purchased from Aldrich Chemical Company, Inc (Milwaukee, WI, USA) and paraffin oil from Struers Kebo lab A/S (Albertslund, Denmark). Stainless steel powder (density 8 g/cm^3) produced by Anval (Torshälla, Sweden) was sieved into a 32–50 μm fraction. Bovine serum albumin (BSA), heat shock fractionation, 98% purity, was purchased from Sigma Chemical Co (St. Louis, MO, USA). Latex particles (310 nm ∅ hydroxyl modified) were purchased from Seradyn, Mitsubishi Kasei Corporation (Indianapolis, IN, USA). Dextrane T 70 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to increase the viscosity of the buffer in some experiments. Other chemicals were of analytical grade.

2.2. Equipment

The matrices were produced in a cylindrical glass reactor (23 cm high, 6 cm ∅) provided with three (7 mm wide) baffles, inserted into the reactor vertically. The reactor was stirred with a flat paddle (13.5 cm high, 3.3 cm wide) divided into four tightly aligned sections with the corners of each section slightly bent, to promote vertical mixing. The paddle was adjusted to 0.5 cm above the bottom of the reactor.

Chromatography experiments were run using FPLC equipment consisting of a LCC-500, a P-500 pump, detector UV-M (with detection set to 254 nm),

motor valve Mv 7 (Amersham Pharmacia Biotech, Uppsala, Sweden) and a two-channel recorder. A C10 column (10 mm ∅; Amersham Pharmacia Biotech) with a sintered plastic filter inserted on top of the lower adapter and a prolonged top adapter (modified in-house) was used. A specially designed stand for keeping the column in a correct vertical alignment was produced in-house and used in all experiments. Bed height was measured with a ruler.

2.3. Preparation of the matrix

The pellicular agarose/stainless steel matrix was prepared according to the following general protocol inspired by an earlier procedure [10]. By varying some parameters the output of different fractions could be altered.

An agarose solution (15 ml; 4% w/v) was prepared by heating a suspension of agarose in water to 95–100°C in a microwave oven and keeping it at that temperature for 1 min. During the warm-up period, care was taken to keep the agarose powder well suspended. The solution was thermostated (60°C) in a water bath. Fifty gram preheated stainless steel beads (32–50 μm ∅) were added and thoroughly mixed into the agarose solution. The mixture was poured into a stirred thermostated glass reactor (55°C, 1000 rpm) containing 300 ml paraffin oil and 2.4 g Span 85. After 30 s the reactor was cooled to below 15°C. The stirring continued for an additional 2 min. The composite beads consisting of a stainless steel core covered with an agarose layer formed was allowed to settle. The bulk of the oil was removed and the beads washed with lukewarm water with plenty of detergent until no oil remained (checked by microscopy). The resulting beads were examined in microscope and classified into suitable fractions using stainless steel sieves (Retsch 75, 180 and 300 μm, Haan, Germany). Before using the matrix, it was elutriated i.e. transferred into a column without top adapter and flushed with water (at 3500 cm/h for the ''heavy'' matrix and at 760 cm/h for the ''light'' matrix). This removed beads with significantly different fluidisation behaviour than those of the majority, e.g. lacking stainless steel cores. Only very little material was removed with this procedure. The minimum fluidisation velocity was determined for the ''heavy'' matrix by slowly increasing the flow rate until the whole column was expanded. The boarder between sedimented and fluidised matrix beads was easy to detect as the beads started to move when they became fluidised. An additional help to observe the local behaviour of the bed was to use a magnifying lens (microscope eyepiece giving 10× magnification).

In some batches the stirring speed was increased to speeds of up to 2000 rpm and the amount of detergent increased up to 7.8 g. Both measures favoured the production of small beads but increased the risk of getting non-coated metal particles.

2.4. Characterisation

The general appearance of different matrix fractions was evaluated under the microscope. Selected fractions (''heavy'' and ''light'') were characterised by packed bed chromatography with respect to gel content and bed porosity by comparing the residence time of 24 μl pulses of latex particles (excluded) and azide (assumed to have full pore access). The Richardson–Zaki parameter (n) [11] was determined from the equation:

$$U = U_t \cdot \epsilon^n$$

where U is the fluidising velocity, U_t the terminal settling velocity and ϵ the void of the bed.

Expansion, dispersion and diffusion behaviour at different flow rates were evaluated in expanded mode using Retention Time Distribution (RTD) measurements [12] of pulses (24 μl) of latex particles and BSA. The tubings from the injection valve to the column and column to the detector were kept short. The extra column dispersion was measured and corrected for (using the standard set-up, with the exception that the flow adapters were pushed together). Sodium phosphate buffer (0.1 M), pH 7.0 was used in all the above experiments. The concentration of azide and BSA in the samples was 1.0 mg/ml. Latex was diluted to give a similar detector signal to the azide at 254 nm. Viscous samples, with an 8 times increase in viscosity (8.0 cP), were created by the addition of 9.4% (w/w) dextrane (Mw 70 kD) to both the buffer and the samples [13].

3. Results and discussion

3.1. Preparation

We have developed a new, pellicular, EB matrix, using a procedure very similar to the one employed in the manufacturing of standard agarose matrices. The melted agarose, or agarose stainless steel mixture in this case, was emulsified into droplets of suitable size in a water-immiscible phase. The process was optimised to obtain a core of stainless steel covered with a relatively thin agarose layer. The readily accessible outer agarose layer was expected to provide a speedy interaction with target molecules, and the solid high-density core should ensure a reasonable expansion, even at high flow rates. Two fractions of the material produced were chosen for characterisation. One had a diameter of 32–75 μm and a density of about 4.4 g/cm^3 with a single particle core (''light''). The other had a diameter of 75–180 μm and a density of about 3.3 g/cm^3 with a cluster of metal particles in the centre (''heavy'') (Fig. 1a, b). Particle fractions with even higher densities (6 g/cm^3) were also obtained, but are not described here.

One typical batch gave about 1.8 ml of the ''light'' fraction, with an agarose layer of up to 10 μm, and about 16.5 ml of 75–300 μm ∅ beads. The 16.5 ml were further sieved at 180 μm, where the fraction below 180 μm predominated. A fraction of large particles (>300 μm) was also obtained (7 ml).

The main factors affecting the formation of different fractions were the stirring speed and detergent concentration. Increasing one or both of these factors favoured the formation of smaller beads with thinner agarose coatings.

3.2. Basic characterisation

In Fig. 1, the general appearance of the matrices is shown. No tendency for cracking or peeling of the agarose layer was observed even after prolonged use. The porosity of the beads (determined by size exclusion chromatography experiments with azide and latex particles) was found to be 51% for the light matrix and 67% for the heavy matrix, which means that the solid core was about 49% and 33% of the bead volume. This would give them a density of 4.4 and 3.3 g/cm^3 respectively. The column void with sedimented matrices (not packed by pressure) was found to be 0.36 for the ''light'' matrix and 0.39 for the ''heavy''. A Richardson–Zaki evaluation of the expansion at varying flow rates was also carried out. The constant (n), calculated by linear regression, gave a value of 5 for the ''light'' matrix and 3 for the ''heavy'' matrix. This means that the expansion of the ''light'' matrix increased more rapidly with increased flow than in the case for the ''heavy'' matrix. The terminal settling velocities were found to be about 5900 cm/h for the ''heavy'' matrix and 1500 cm/h for the ''light'' matrix according to the Richardson–Zaki evaluation. In practice, the upper limit in flow rate (above which the top surface of the expanded matrix became less distinct) was found to be 610 and 3050 cm/h (8 and 40 ml/min in a 10 mm ∅ column) in water.

The inertness of the matrices to non-specific adsorption was not explicitly measured. The very small surface area of the core as compared to the internal area of the agarose matrix and the fact that the core is embedded in agarose where the agarose polymer could be expected to neutralise possible interaction-sites on the core, makes any significant non-specific adsorption less likely. Furthermore, in experiments with immunoglobulin adsorption to protein A-derivatised pellicular beads [14], no non-specific adsorption was noticed.

3.3. Dispersion behaviour

The dispersion behaviour at different flow rates, using pulses of latex and BSA, was measured and compared with that of a commercially available matrix. The general features of the compared matrices were:

- Streamline-protein A ∅ 80–165 μm (commercial), average density ~1.3 g/cm^3
- light pellicular gel ∅ 32–75 μm, average density~4.4 g/cm^3
- heavy semi-pellicular gel (agarose beads with a cluster of heavy material in the middle ∅ 75–180 μm, average density ~3.3 g/cm^3

All matrices contained stainless steel as a density increaser in 4% agarose gel.

In the case of BSA the term ''dispersion coefficient'' must be understood in a wider sense than

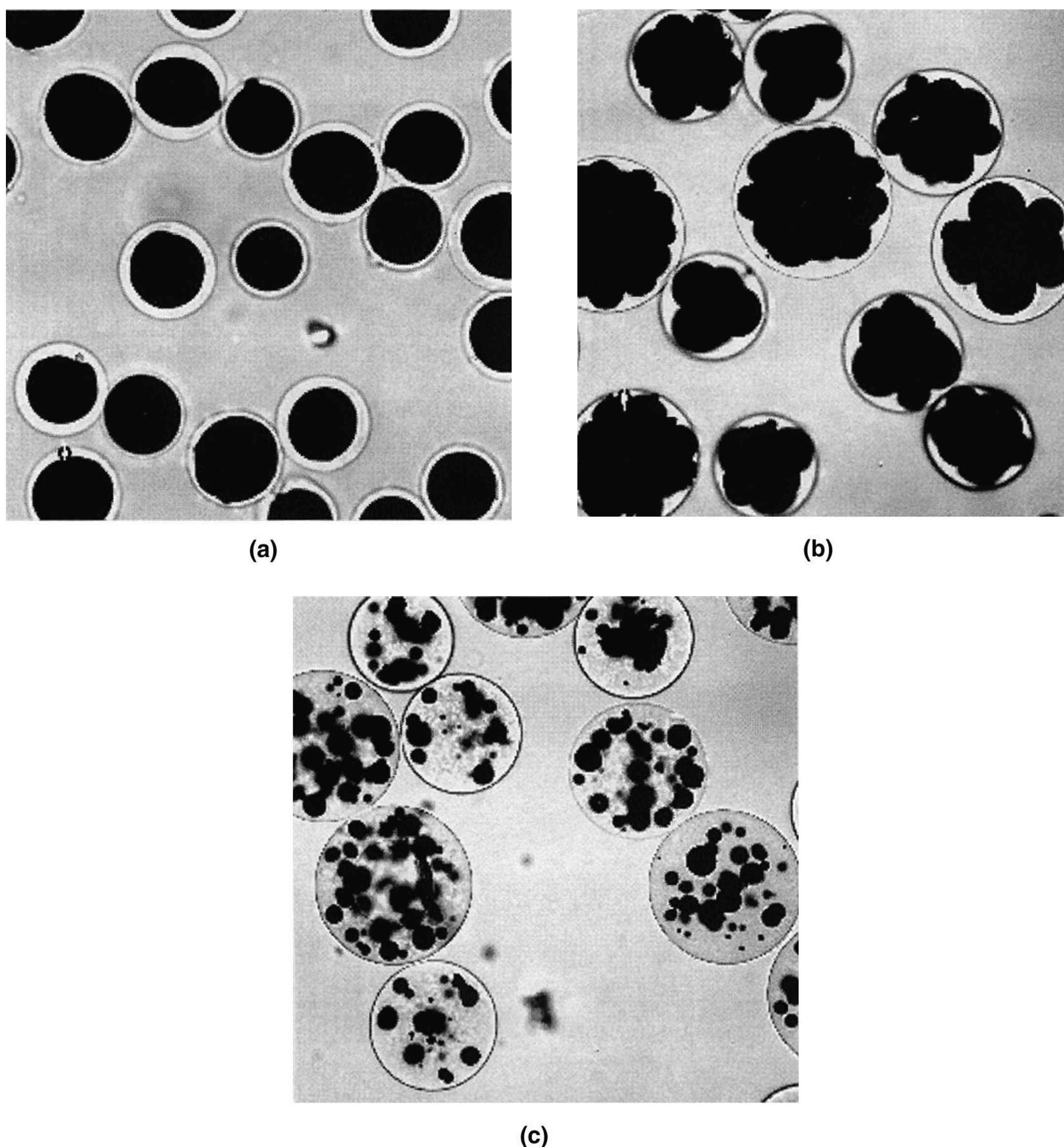

Fig. 1. Photographs of (a) ''light'' matrix (32–75 μm ∅); (b) ''heavy'' matrix (75–180 μm ∅) and (c) Streamline protein A (80–165 μm ∅), taken through a microscope (40× magnification) using a CCD camera.

commonly described in engineering because it contains the effect of diffusion within the porous matrices. The accessible gel volume was 15% less for BSA than for azide in Streamline and 12% in the other two, according to residence time measurements in packed beds.

On the whole, the dispersion was found to be low throughout the study, showing that the column and flow distributor were suitable for expanded beds. In Table 1 it can be seen that the dispersion coefficient increased with increased flow rate/degree of expansion and that it was generally higher for BSA than for latex. In a packed bed the dispersion coefficient for non-penetrating solutes usually increases proportionally to the flow rate [15] i.e. the dispersion coefficient doubles when the flow rate is doubled.

Table 1
Dispersion behaviour of three EB matrices: Streamline, ''light'' pellicular matrix and ''heavy'' pellicular matrix. The measurements were made in a 1 cm ∅ column and the bed height before expansion was 6 cm

Matrix	Flow cm/h	Equivalent flow cm/h	Expansion H/H_0	Latex			BSA		
				Dax $m^2/s \cdot 10^6$	Bo	Peak volume ml	Dax[b] $m^2/s \cdot 10^6$	Bo[b]	Peak volume ml
Expanded mode									
''Light''	248		2.0	1.6	78	4.2	1.6	76	4.9
	382		2.5	2.1	104	5.0	2.6	90	6.4
	610		4.1	2.3	220	6.3	3.5	143	8.3
Streamline	264		2.0	2.0	61	4.8	3.1	37	7.5
	382		2.6	3.7	54	6.8	6.1	32	10.3
''Heavy''	611		1.4	4.1	64	2.6	7.1	37	4.3
	76[a]	608	1.4	0.86	38	3.5	1.2	27	5.9
	191[a]	1528	1.9	2.6	35	5.7	3.6	25	8.5
	382[a]	3056	2.8	5.8	39	9.5	7.9	28	11.4
Packed mode									
''Light''	38		0.95	0.099	183	0.65	0.090	202	1.1
Streamline	38		0.95	0.14	110	0.94	0.49	31	3.9
''Heavy''	38		1.0	0.074	211	0.73	0.22	70	2.2

[a] A viscous buffer was used.
[b] Effective quantities including effects of pore diffusion.

The dispersion coefficient for the ''light'' matrix, according to this limited study, increased less whereas for the other two it increased somewhat more with increased flow in expanded mode (Fig. 2, Table 1). For an empty column, the increase is indicated to be dependent on the square of the flow rate [16].

3.3.1. The influence of matrix

It was a positive surprise to find that the dispersion for latex was lower for the new ''light'' matrix than for Streamline at the same flow rates. A reliable comparison between Streamline and the ''heavy'' matrix is difficult to do since they could not be run at

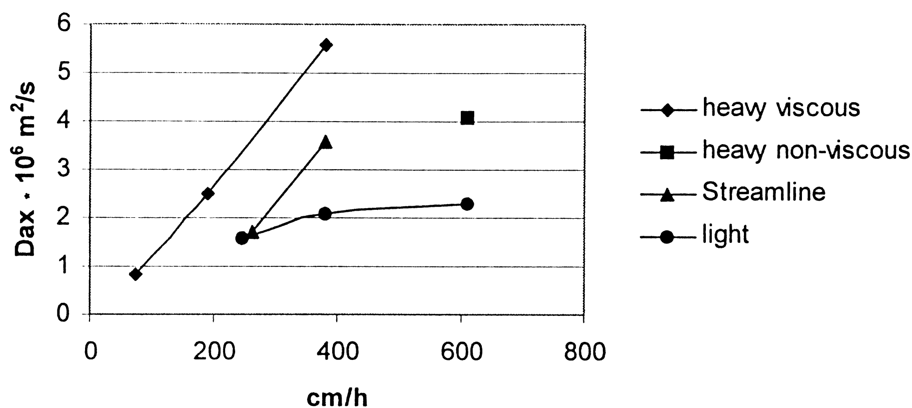

Fig. 2. The diagram shows the dispersion coefficient for latex particles as a function of flow rate for the ''light'', ''heavy'' and Streamline matrix. A viscous mobile phase was used with the ''heavy'' matrix to make it expand at eight times lower flow rate than would be the case for a non-viscous mobile phase. One data point with the non-viscous buffer is also included for the ''heavy'' matrix.

the same flow rate under comparable conditions i.e. with a buffer of the same viscosity. The minimum fluidising velocity for the "heavy" matrix in water was 500 cm/h. If the Streamline curve was extended towards higher flow rates in Fig. 2, it would indicate a higher dispersion coefficient than for the "heavy" matrix. In trying to find a reason for this, one could argue that a low dispersion was likely to be due to a more stable bed. The bed stability is affected by the range of size and density of the matrix [17,18]. If the distribution is too small, the bed will not be classified. Instead the beads will move freely throughout the bed. A comparably narrow density distribution would be expected in the Streamline protein A matrix, since the metal particle inclusions are small but numerous and randomly distributed in the agarose. The pellicular matrices on the other hand have one or a few metal particles in each bead covered with an agarose layer of varying thickness. This gives them a large range of densities as well as a size distribution, stabilising the bed. Another reason for the low dispersion could be the actual bead size, which is lower for the "light" matrix than for the commercial one. In packed beds the dispersion is approximately proportional to the bead size [15]. This relation has not been clearly demonstrated for expanded beds. In our packed bed experiments the D_{ax} values did not seem to obey this rule either. This can partly be explained by the difference in column void, giving rise to differences in interstitial flow velocities. The packing of the columns also differs from ordinary packed beds in that the beads were allowed to sediment and hence fractionate in the column.

3.3.2. The influence of diffusion distance

Latex particles passing the column are dispersed because of eddies created in the flow stream and variations in flow rates in the different interstitial channels in the bed. For pore-penetrating solutes like BSA, the variations in time spent inside the matrix pores, for the different BSA molecules, is a source of additional dispersion. (The lower the diffusion rate, and the longer the diffusion distance, the more dispersed the solute will be.) If the molecular diffusion inside the matrix is included in the dispersion, the effective dispersion can be described by an effective dispersion coefficient. The effective dispersion coefficient can be interpreted as the extent of dispersion per time unit. Hence, if the solute molecules spend a long time inside the pores (i.e. large accessible pore volume), and if the variation in the time spent is small (short diffusion distances or fast diffusion rate), the effective dispersion coefficient can be even lower for a pore penetrating solute than for a non-penetrating solute. On the other hand, if the variation of the time spent inside the pores is large, the effective dispersion coefficient will be large.

In this study, in most cases the dispersion coefficient was found to increase for BSA as compared to latex, as expected for a slow diffusing molecule. However, for the light matrix the coefficient was unchanged at 248 cm/h (two times expansion) and even slightly lower at 38 cm/h (packed bed mode). For the Streamline matrix the dispersion coefficient was greater with BSA than with latex particles at all flow rates. This was also the case for the "heavy" matrix. This difference between the matrices was not surprising considering the large differences in diffusion distances. A rough estimate of the average agarose layer on the light matrix would be 5.5 μm, the heavy 12 μm and Streamline 65 μm (radius).

3.3.3. The effect of viscosity

High viscosity samples are frequently encountered in downstream processing of fermentation broths. To test if the "heavy" matrix had the potential to handle such solutions, a highly viscous buffer (8.0 cP i.e. eight times the viscosity of water) was used in most of the measurements carried out with this matrix (Table 1). The results showed that it behaved very well with respect to bed expansion and bed stability.

Increased viscosity affects the dispersion behaviour of the column. In Table 1, it can be seen that at the flow rate of 382 cm/h the viscous buffer already gave a higher dispersion than the non-viscous at 611 cm/h. Similar results have also been obtained by others [19]. Hence, the dispersion coefficient for the heavy matrix at 382 cm/h can not be directly compared with the dispersion of the other two matrices at the same flow rate. A comparison of the dispersion at the same flow rate for both viscous and non-viscous conditions was not possible due to bed expansion. Only one flow rate with the non-viscous buffer was obtained because of the upper speed limit of the pump. The expansion behaviour of the matrix

in water was studied using another chromatography set up, capable of higher flow rates, but not suitable for dispersion measurements.

3.3.4. The total column dispersion

In Table 1 the dilution of the 24 μl tracer pulses injected into the column is shown by giving the volume they had upon exiting the column (the values were obtained from the standard deviation (σ) of the peaks as $4^*\sigma$). This is a good practical measure of the total mixing taking place during the passage through the column. These values highlight the fact that a more expanded column (light 610 cm/h) with a low dispersion coefficient might expose the pulse to more mixing (6.3 ml peak width) than a less expanded column (heavy 611 cm/h) with higher dispersion coefficient (2.6 ml peak width). The Bodenstein number is often used to show the degree of mixing in a column. This number takes the flow rate, the column height and the dispersion coefficient into account. The Bodenstein numbers for the above cases are 220 and 64 (a high number meaning a low degree of dispersion). Hence, the Bodenstein number gives a false impression of the efficiency of an expanded bed, the reason being that with increased expansion not only the pulse width is increased but also the residence time.

3.4. Which one of the matrices is the best?

Both of the new matrices had some advantages. Attractive features of the light matrix were the low dispersion and fast mass transport properties. Advantages of the heavy matrix were its low degree of expansion, possibility for extremely high flow rates and potentially higher binding capacity (due to the higher percentage of agarose). A potential disadvantage of the heavy matrix was its lack of a single solid core, since an agglomerate of core particles also has some diffusive regions in the central part of the particle, where solutes can get delayed. A good compromise combining the best features of both matrices could be a single dense core that is somewhat larger than in the light matrix, maybe 60–80 μm, covered with an intermediately thick layer of agarose (about 10 μm). Such a matrix would consist of 53% agarose and have an average density of 4.3 g/cm^3 with intermediate expansion behaviour in comparison with the two matrices described in this paper. The agarose content, and hence the potential total capacity, of the Streamline matrix is higher than for the new matrices by a factor of very roughly 2. In Ref. [14], the dynamic capacity of Streamline protein A and a pellicular matrix, of the kind described here, were compared. It was shown that with short columns and high flow rates the pellicular matrix had equal or higher dynamic capacity than Streamline, even though this matrix had an even thinner agarose layer than the ones described here.

4. Conclusions

A new type of expanded bed matrix is described based on agarose-covered stainless steel particles. It showed better fluidisation, dispersion and diffusion properties than the commercial Streamline protein A matrix and allowed flow rates up to 3000 cm/h. The new material should be useful with viscous media.

Acknowledgements

This work was financially supported by The Swedish Center for BioSeparation. Advice on suitable materials for incorporation in the matrix was given by Hans Berg, Amersham Pharmacia Biotech and advice on dispersion calculations by Anders Axelsson and Bernt Nilsson, Department of Chemical Engineering. E.P wants to express her gratitude to Jesus for his inspiration and guidance throughout this work.

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