Desalting and buffer exchange with Sephadex® G-25

Application Note

Introduction

Gel filtration has successfully been employed for size based separations of macromolecules since the late 1950s. The separation technique was rapidly established as a complementary technique to adsorption chromatography by the introduction of the first commercially available media, Sephadex®, in 1959 [1]. Gel filtration, or size exclusion chromatography, may be performed in three principally different modes, depending on the size differences of the solutes to be separated [2, 3]

(i) group separation
(ii) fractionation
(iii) determination of molecular mass (distributions)

When the size difference is large, i.e. a factor of more than 10, we talk about group separation (e.g. desalting, buffer exchange) and when the size difference is small, i.e. a factor of 2 to 5, we talk about fractionation. The third mode is analytical gel filtration, e.g. determination of molecular mass distributions, where an array of molecular masses is to be separated. These different modes put different requirements on the equipment and media used and will yield widely different productivity in terms of information or material processed. This note will focus on the use of gel filtration for group separation with special reference to Sephadex G-25 of different particle sizes.

Principle for group separation

Desalting and buffer exchange of protein samples are two examples of group separation. The large size difference between the protein and the low molecular solute makes it possible to select a gel filtration medium that will exclude the protein from the porous network while allowing full permeation of the low molecular weight solute.

This is the most productive mode of preparative gel filtration (in terms of mass of product per volume medium and unit time) and will, as we shall see below, allow large freedom for choosing experimental conditions that otherwise would be detrimental to the separation of more closely sized molecules.

In gel filtration the separation takes place due to the, for steric reasons, different access to the pore volume of molecules of different sizes. The separation thus takes place over a volume equal to the pore volume [3]. In desalting (which from now on is used for describing group separation) the gel filtration medium is used to selectively exclude the large molecular weight solute, e.g. protein or DNA from entering the porous gel phase (Fig. 1). Thus, a rational choice of matrix will allow the protein to be eluted in the void, or interstitial, volume of the column. As a result the protein zone will be diluted to a minimum, as caused by eddy dispersion only (e.g. non-equilibrium effects are eliminated). This also means that the zone broadening, or dilution of the protein zone, is rather insensitive to fluid velocity and high velocities (e.g. exceeding 500 cm/h) may be employed for fast desalting (since the zone broadening of low molecular weight solutes is also generally small unless extreme velocities are used [3]). Low molecular weight impurities, e.g. salt, will permeate the pore volume and will have a retention volume equal to the total liquid volume of the column (if provided the size of the molecule is small as compared to the pore size of the gel filtration medium). If the impurities have an intermediate size they will only permeate part of the pore volume giving a less favorable situation (see optimization).

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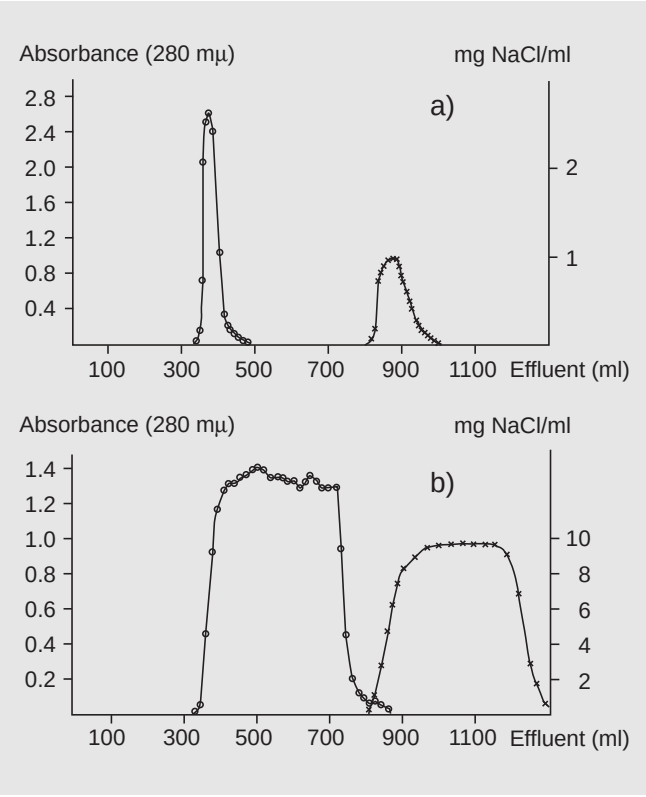

Fig. 1. Principle of desalting of proteins by gel filtration.

Sample; ○ = hemoglobin, × = NaCl. Sample volume; a) = 10 ml, b) = 400 ml. Hemoglobin is eluted in the void volume of the column (i.e. 370 ml) while sodium chloride is eluted with the total liquid volume (i.e. 860 ml). Almost the entire pore volume is used for desalting! Column: 85 × 4 cm i.d. packed with Sephadex® G-25 M. Reproduced from Ref. 4 with permission. ©1961 Elsevier.

Fig. 1 illustrates the principle of group separation. A solution of hemoglobin is desalted using Sephadex G-25 Medium [4]. Fig. 1a shows that hemoglobin is eluted in the void volume, V_o, and the salt at the total volume, V_t. This leaves the entire pore volume ($V_i = V_t - V_o$) to be used for applying a large sample volume. This is illustrated in Fig. 1b where 400 ml is successfully desalted. This is close to the pore volume of the bed, i.e. 490 ml. The dispersion effects cause a slight zone broadening which is the reason why the entire pore volume may not be employed for desalting, i.e. as seen by Fig. 1b the sample zones are close to, but not identical with, square shaped zones. It may also be noted that the dilution of the hemoglobin fraction is very low, e.g. the major part of the protein is eluted in 450 ml giving a dilution of roughly 10%.

Important characteristics of a desalting gel filtration medium

From the principles of group separation we may conclude that the following characteristics of the gel filtration medium are important

(i) pore size
(ii) pore volume
(iii) particle size
(iv) matrix rigidity

Pore Size

The pore size is chosen such that the large molecular weight solute is excluded from the gel matrix. Sephadex G-25 has a nominal molecular mass exclusion limit of 5000 for proteins [5]. However, the pore size must not be too small since the low molecular weight impurities will then not elute at the total volume and this will limit the applicable sample volume.

Pore Volume

The pore volume is a very important characteristic of the gel filtration medium and will directly influence the sample volume that is applicable. Therefore the matrix volume of a desalting gel filtration medium used for preparative use should be kept as low as possible. However, this is contradictory to the demand of high matrix rigidity which generally is increased with increased matrix volume. A small pore volume may of course be compensated for by using a larger bed volume but this will be at the expense of lower productivity (see below).

Particle Size

The particle size will influence the sample dispersion in the bed. However, since the large molecular mass solute will not enter the porous phase, the main cause of zone broadening in gel filtration (i.e. due to non-equilibrium) is not present and only eddy dispersion contributes. This effect may be small compared to dispersion from sample application and column dead volumes (e.g. at inlet and outlet). The calculated influence of the mean particle size of different grades of Sephadex G-25 on the column dispersion is shown in Fig. 2. Data for the particle size of Sephadex G-25 is given in Table 1 and was taken from Ref. 5. It may be

Table 1. Data for Sephadex® G-25 [5].

Sephadex G-25	Dry particle diameter (µm)	Mean wet particle diameter (µm)	Maximum recommended fluid velocity (cm/h), L=15 cm
Superfine	20–50	52	50
Fine	20–80	88	65
Medium	50–150	140	100
Coarse	100–300	320	200

concluded that the influence of particle size in the range 50 to 300 µm on the bed dispersion is rather small for column lengths above 15 cm. However, for shorter columns bed dispersion reduces the sample volume that can be applicable, especially for media of large particle size.

In addition to the effect from the dispersion of the bed as illustrated by Fig. 2, a contribution from column dead volumes may be anticipated, the extent being dependent upon the column design. This will reduce the relative influence of the particle size to some degree. The effect will be largest for small particles since these yield lower dispersion than larger particles. If the sample volume is very small a column of small size is recommended. If dilution of the sample must be kept to a minimum a small particle size gel filtration medium should be selected (e.g. Sephadex G-25 Fine or Sephadex G-25 Superfine).

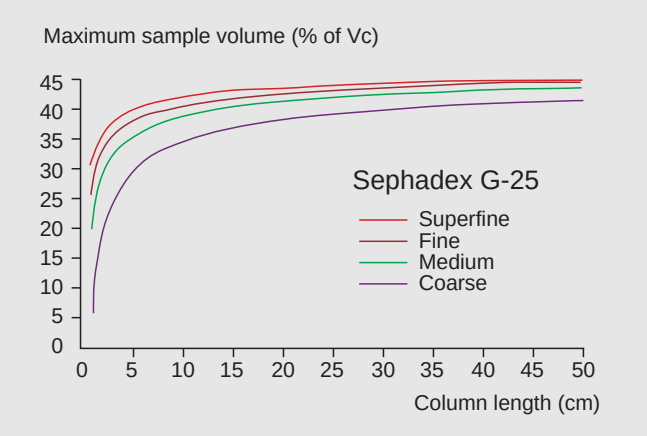

Fig. 2. The influence of bed dispersion for different particle sizes on the available separation volume in desalting. The maximum sample volume that may be applied is reduced due to eddy dispersion effects. The influence of dispersion is larger for larger particle sizes and short columns. Column dead volumes will add to the total dispersion noted. Vc is the total bed volume.

Pressure/Flow

The particle size has a large influence on the pressure drop over the packed bed. In principle the pressure drop is inversely proportional to the particle size squared [3]. This is due to that smaller intraparticle channels are formed by smaller particles. The higher liquid velocities in these smaller channels will result in higher friction forces acting on the particles yielding higher pressure drops but also leading to higher stress which may result in compaction of the bead [3]. Therefore the applicable flow rate is reduced with decreasing particle size of Sephadex G-25, as seen from Table 1. The reduced size of the flow channels with lower particle size will also lead to a higher sensitivity to viscous fingering effects, i.e. the distortion of highly concentrated sample zones due to hydrodynamic instability of the rear part of the zone [6].

A high matrix rigidity will allow high flow rates to be employed for fast desalting but this may be at the expense of the maximum sample volume that can be applied and these factors need to be considered simultaneously. If large sample volumes are to be quickly desalted it may be better to use a larger particle of low matrix volume, e.g. Sephadex G-25 Coarse which will allow high flow rates and high sample volumes.

Productivity of desalting

The productivity is expressed as the amount of product purified per unit time and unit bed volume [7]. The amount of purified product is equal to the concentration times the sample volume times the yield. The yield for desalting (and gel filtration in general) is very high and may be set to 100%. The maximum concentration of proteins that may be applied is restricted by the relative viscosity of the sample compared to eluent and a sample concentration of 50 mg/ml of a protein having a molecular mass of 50 000 seems to be the upper limit in simple aqueous eluents [3]. The maximum volume of sample that may be applied on Sephadex G-25 is illustrated by Fig. 2 and the maximum fluid velocity is taken from Table 1. From this information the maximum productivity using different column lengths and particle sizes may be calculated (in order not to be too close to the upper concentration limit a protein concentration of 25 mg/ml is recommended). The productivity of desalting using these presumptions is illustrated in

Fig. 3, showing that Sephadex G-25 Coarse will always yield the highest productivity and that this may, in favorable situations approach 150 grams per hour and liter gel filtration medium. This is in good agreement with experimental results where the productivity for purifying human serum albumin from ethanol was calculated to be 50 grams per hour and litre gel filtration medium at a load of 16% of the bed volume [8]. It may be noted that this productivity is comparable with and sometimes exceeds that of adsorptive modes of chromatography.

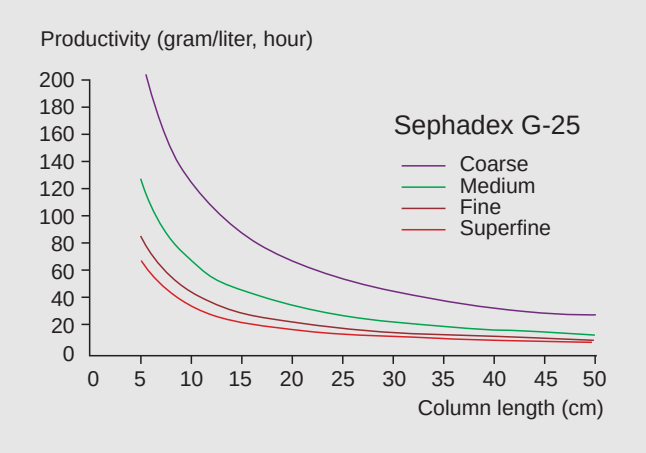

Fig. 3. Theoretical productivity of different grades of Sephadex® G-25 at maximum recommended flow rates (i.e. taken from Table 1) and for a protein concentration of 25 mg/ml. The decreased productivity with increased column length illustrates that, for a given column volume, it is optimal to use this volume in a short column with large diameter (i.e. as long as system effects such as sample application dispersion and dead volumes are negligible).

Optimization of desalting

In order to utilize the entire pore volume for desalting it is important that the pore size is not too small since this will lead to low molecular weight impurities being eluted too early. A gel filtration medium of larger pore size which still excludes the protein of interest should be used, or the sample volume must be reduced to allow for separation (the sample volume may not exceed the difference in elution volume of the impurity and the void volume).

As seen from Fig. 3 the productivity decreases with increasing column length. Thus it is advantageous to use "short and fat" columns as long as the column construction minimizes column dead volumes. In Fig. 3 it is anticipated that all columns may be run at the maximum recommended flow rate. However, this is not the case in practice and for longer columns (i.e. longer than 15 cm) the flow rate needs to be reduced in order to not compact the bed. This is due to the cumulative drag forces acting on particles which increase with bed length [3]. Therefore the productivity will be lower as depicted in Fig. 3, unless the bed is segmented, i.e. packed in sections (e.g. using Process Stack Columns) which allows the maximum flow rate to be used. Some extra dispersion will take place, however, as a result of the increase of column dead volumes.

Applications

The use of Sephadex G-25 for desalting covers all scales of preparative work.

One example of micropreparative desalting was given by Hellman and co-workers [9] who desalted 50 µl of a reduction/alkylation mixture on a 10×0.32 cm i.d. column of Sephadex G-25 Superfine prior to sequence analysis. Fast desalting on a laboratory scale of 1.4 ml sample is illustrated by Fig. 4. As shown in Fig. 1, desalting of up to several hundreds of ml sample may be performed in standard gel filtration columns prepacked with Sephadex G-25.

Desalting at larger scale may be exemplified by the intermediate group separation of allergen extract from low molecular weight impurities using a BPG 450 column packed with Sephadex G-25 Superfine [10]. The bed height was 15 cm, giving a bed volume of 25 liters. The sample volume was up to 6 liters (24% load) and the flow corresponded to 1.1 cm/min giving a cycle time of 17 minutes. The dilution factor was 1.4. A classical example of "desalting" was given by the deethanolization of human serum albumin where 12 liters of 9% protein solution was purified in a cycle time of 17.4 minutes using a 60×40 cm i.d. column of Sephadex G-25 Coarse [8]. This corresponds to a productivity of 50 g/hour and liter.

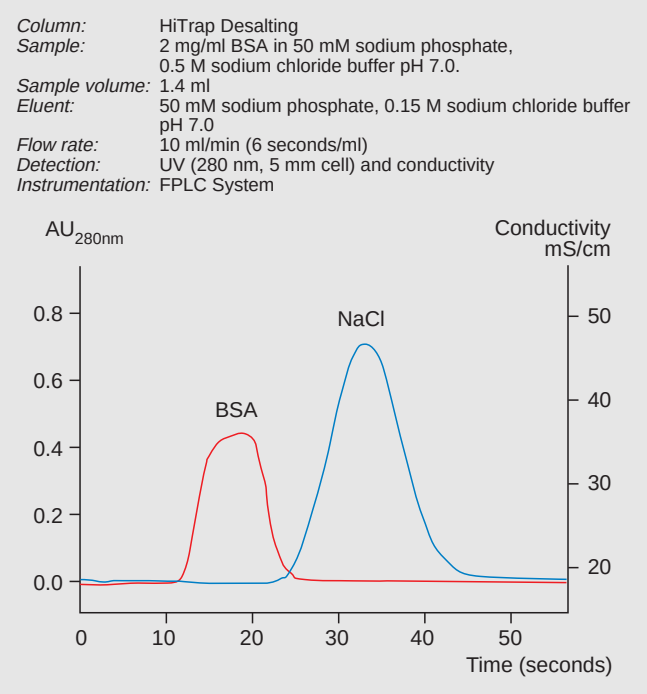

Fig. 4. Fast desalting in laboratory scale.

Horton [11] described the application of a 2500 liter column of Sephadex G-25 Coarse for desalting a crude enzyme preparation [11]. The column used was a stainless steel GF 18–10 (100 × 180 cm i.d.), the sample volume was 875 liters (i.e. 35% of the column volume) and the flow rate was 62.5 liters/min giving a cycle time of 1 hour. The paper also provided a comparison between the productivity of Sephadex G-25 Coarse and Sephadex G-25 Medium, see Table 2. The conclusion is that Sephadex G-25 Coarse provides the higher productivity of these two gels. The figures for productivity are in agreement with Fig. 3, taking into consideration that fluid velocity and relative sample volume are lower and sample concentration is higher than in the theoretical case.

Gel filtration can offer a very robust purification step as noted for the use of Sephadex G-25 Coarse for initial buffer exchange of raw plasma in a large scale albumin fractionation plant [12]. The column, containing 75 liters of gel was used for more than 6 years and processed 70 000 liters of material.

Thus, Sephadex G-25 has been used for desalting in a variety of applications and configurations. Some selected large scale applications of Sephadex G-25 for desalting are compiled in Table 3.

Table 2. Comparison of productivity between different grades of Sephadex® G-25 for desalting of albumin from ammonium sulfate [11].

Parameter	Sephadex G-25 Coarse	Sephadex G-25 Medium
Sample volume (L)	31	37
Relative sample volume (%)	25	30
Sample capacity (L/h)	70	18
Salt concentration in product (%)	0.4	0.04
% salt removed	98.0	99.9
Albumin conc. in product (%)	4.0	5.0
Dilution factor of albumin	1.5	1.2
Albumin processed (g/h)	4125	1170
Productivity (g/h, L)	33	9
Cost per kg of albumin (USD)	0.64	2.20

Note: Albumin concentration in feed is 6%, salt concentration in feed is 24%. Column is a GF 04-10, 100 × 40 cm i.d., giving a bed volume of 125 liters.

One column format that is suitable for large scale desalting is the BPSS (BioProcess Stainless Steel). A configuration where three BPSS columns are connected in series for large scale group fractionation for final polishing is given in Fig. 5 [13]. Other formats than those mentioned in Table 3 that have been tested for desalting using Sephadex G-25 include laboratory columns, e.g. XK columns, Process Stack Columns, and CHROMAFLOW columns. The optimal packing procedure differs between different types of columns and packing instructions can be obtained from your local supplier.

Fig. 5. Industrial fractionation by gel filtration using three BPSS columns (30x140 cm i.d.) in series giving a total bed volume of 1500 liters. By courtesy of R. Hershberg.

Table 3. Examples of large scale desalting using Sephadex® G-25.

Bed volume (L)	Column type	Bed dimensions (length × i.d. cm)	Sephadex grade	Application	Sample load (%)	Cycle time (min)	Ref.
25	BPG 450	15 × 45	Superfine	Intermediate purification of allergen extract	24	17	10
75	BPSS 400	60 × 40	Coarse	Buffer exchange of hybridoma cell culture supernatant	20	54	14
125	GF 04-10	100 × 40	Coarse	Desalting of albumin	25	27	11
170	BPSS 600	60 × 60	Coarse	Initial desalting of plasma	15	20	15
2500	GF 18-10	100 × 180	Coarse	Initial enzyme purification	35	60	11
2500	GF 18-10	100 × 180	Coarse	Desalting of whey	22	32	16

Notes: BPG = BioProcess Glass columns, BPSS = BioProcess Stainless Steel columns, GF = Gel Filter.

Conclusions

Sephadex G-25 is very suitable for desalting protein and DNA preparations. Sephadex G-25 Coarse will generally be the gel filtration medium of choice for industrial scale. It is especially suitable for rapid processing of large volumes of feed where the requirement for productivity is high. In cases where sample dilution must be minimized the higher resolution of a smaller particle size, e.g. Sephadex G-25 Medium may be required.

For laboratory scale desalting where less effort needs to be spent on optimization it is common to use more conservative sample volumes (i.e. a load below 20%) and Sephadex G-25 Medium or Sephadex G-25 Fine can be recommended to ensure a low dilution factor.

For micro-preparative work using very small sample volumes, Sephadex G-25 Superfine will be the gel of choice.

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Ordering information

Product	Pack size	Code No.
Sephadex G-25 Superfine	100 g	17-0031-01
	500 g	17-0031-02
	5 kg	17-0031-03
Sephadex G-25 Fine	100 g	17-0032-01
	500 g	17-0032-02
	5 kg	17-0032-03
Sephadex G-25 Medium	25 g	17-0033-10
	100 g	17-0033-01
	500 g	17-0033-02
	5 kg	17-0033-03
Sephadex G-25 Coarse	100 g	17-0034-01
	500 g	17-0034-02
	5 kg	17-0034-03
	40 kg	17-0034-07

Pre-packed columns

Product	Quantity	Code No.
Pre-packed Disposable Columns PD 10 (Sephadex G-25 Medium)	30	17-0851-01
HiTrap Desalting (Sephadex G-25 Superfine)	5 x 5 ml	17-1408-01
Fast Desalting Columns HR 10/10 (Sephadex G-25 Superfine)	1	17-0591-01
Fast Desalting PC 3.2/10 (Sephadex G-25 Superfine)	1	17-0774-01

Large scale columns

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