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Application Note

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Phenyl Membrane Adsorber for Bioprocessing

Sartobind® Hydrophobic Interaction Membrane Chromatography

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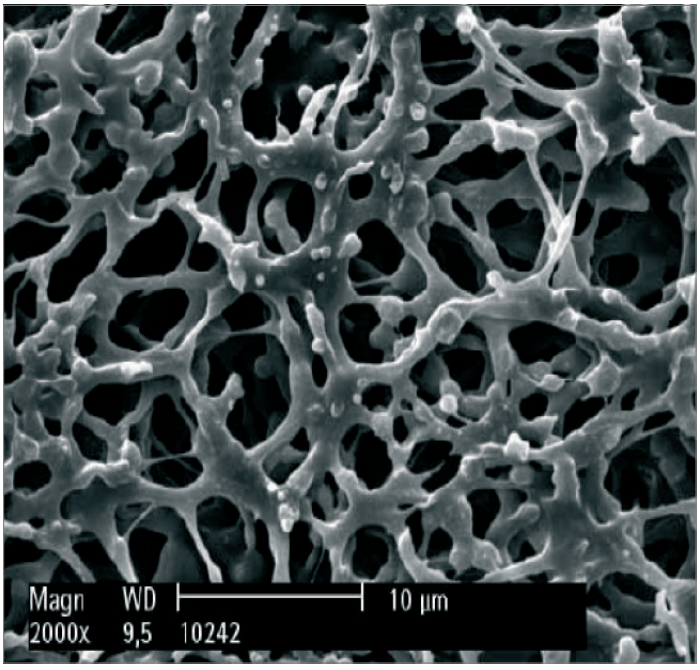
Abstract

Sartobind Phenyl capsules and cassettes with 8 mm bed height are hydrophobic interaction chromatography (HIC) devices with macroporous membranes. They can be used for chromatographic separations during downstream processing of viruses and proteins. HIC separates and purifies biomolecules based on differences in their hydrophobicity. Buffers with high concentrations of salt promote the adsorption of proteins on the hydrophobic membrane matrix. Due to the large pore size, membrane adsorber chromatography devices can be operated at high flow rate.

This application note on HIC membrane adsorbers describes the following:
- influences of various process conditions on binding behavior
- membrane adsorber performance in comparison to resins
- aggregate removal
- scale up performance
- further applications of hydrophobic interaction membrane chromatography.

Introduction

Sartobind Phenyl membranes combine the advantages of macroporous 3 µm membranes with a hydrophobic ligand and can be considered as a alternative of the conventional chromatography resins. Sartobind Phenyl capsules are used in downstream processes for the removal of hydrophobic protein aggregates and for purification of large biomolecules or viruses.



The different device sizes are shown in Fig. 1. For screening purposes 96-well plates (not shown) are also available.

Hydrophobic interaction membrane chromatography separates and purifies biomolecules based on differences in their hydrophobicity. On average 50 % of a protein or peptide surface is accessible for such interaction.
The strength of the interaction depends on the sufficient number of exposed hydrophobic groups of the sample and on membrane ligand type and density. Sample properties, temperature, type and concentration of salt and pH as well as additives influence the binding process as well.

The low substitution of the phenyl ligand on the membrane allows for mild elution of biomolecules such as peptides, proteins, viruses, phages or other biomolecules. Thus preserving the biological functions of the molecules.

The use of chaotropic (salting-in) agents such as urea, guanidinium chloride or organic solvents such as ethylene glycol, dimethyl sulphoxide, dimethyl formamide or propanol will compete very effectively with bound proteins and displace them. Such additives can help during membrane cleaning as the phenyl membrane has been developed not only for single use flow-through but also for bind and elute and reuse.

Fig. 1: Sartobind Phenyl capsules: pico (left, 0.08 mL 4 mm bed height) nano 3 mL, 150 mL, 400 mL, 800 mL, 1.2 L, 5 L Jumbo and 1.6 L Cassette (8 mm bed height)

1. Influence of salt type and concentration

The protein binding capability of phenyl membrane has been analyzed with different types of salt.

1.1 Materials

Test proteins	Lysozyme, ovalbumin and IgG
Test device	Sartobind Phenyl 96 well plate (3 membrane layers, 1 cm²/well)
Protein concentration	1 mg/mL
Loading	0.8 mg protein per cm² membrane, 2 × 0.4 mL per well
Buffer	0.1 M sodium phosphate pH 6 - 7, different salt types at varying concentrations
Equilibration	2 × 0.5 mL sulfate buffer | well
Salt types	Ammonium sulfate, sodium citrate and sodium chloride

1.2 Method

Each well was equipped with three layers of phenyl membrane (in total 1 cm² area) to screen the influence of the salt on binding capability. The amount of bound protein was calculated from the difference of the protein concentrations between protein loading solution and flow-through fraction.

1.3 Results

The degree of protein retention correlates with the increase in salt concentration. In some cases however, the binding did not increase even with higher salt concentration. This can be due to the precipitation of proteins and demonstrates that mobile phase composition in regard to type of salt and concentration has to be chosen carefully. The results do not necessarily reflect the maximum binding capacity. The amount of the loaded protein was limited.

NaCl: Higher salt concentration of up to 3 M is necessary to achieve the binding level of the other salts. In the case of ovalbumin (see Fig. 3), NaCl is even not appropriate to achieve sufficient binding.

Na_3Citrate: Sodium citrate needed the lowest salt concentration. In the case of lysozyme (see Fig. 2) the use of high concentrations resulted even in diminished protein binding. This can be precipitation or protein stability effects resulting in lower binding capacity.

$(NH_4)_2SO_4$: Ammonium sulfate gives the favourable results as concentration can be chosen in moderate range while precipitation tendency is not extensive. (Fig. 2, 3 and 4)

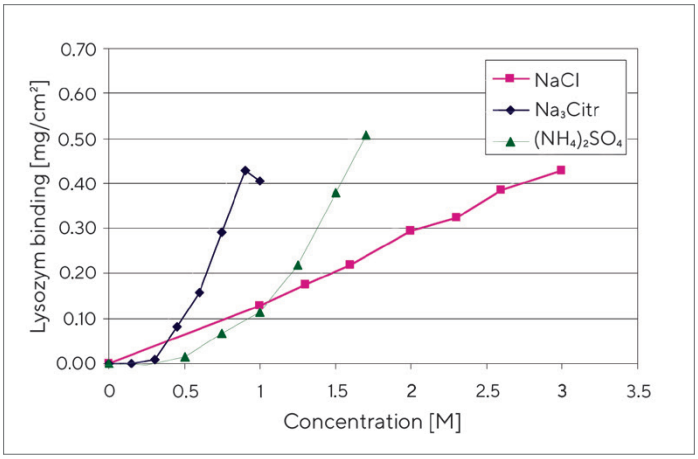


Fig. 2: Lysozyme bound per cm² phenyl membrane as a dependence on type of salt and salt concentration

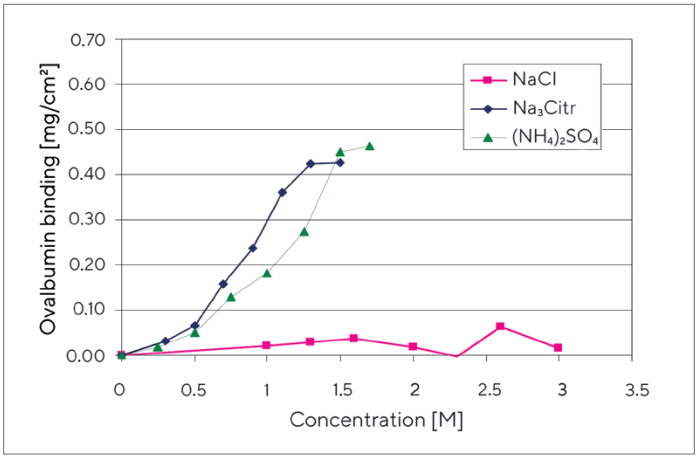


Fig. 3: Ovalbumin bound per cm² phenyl membrane as a dependence on type of salt and salt concentration

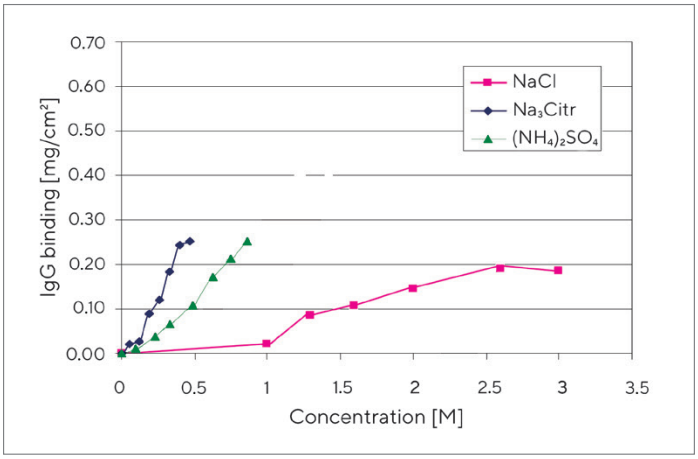


Fig. 4: IgG bound per cm² phenyl membrane as a dependence on type of salt and salt concentration

* Bed volume = membrane volume

2. Mab binding on Sartobind Phenyl and on a conventional phenyl column

2.1 Materials

Load samples	
Mab1 and Mab2:	each IgG 1 mg/mL and 0.8 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate pH 7.0
BSA, lysozyme:	each 2 mg/mL and 2 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate pH 7.0
Elution buffer:	50 mM potassium phosphate pH 7.0

2.2 Method

Two monoclonal antibodies (Mab1 and 2) were loaded onto a membrane device and on a column at specified flow rates. The bed height and bed volume were 30 mm/1 mL for the column and 4 mm/2 mL for the membrane.

2.3 Results

Dynamic binding measurements showed that the phenyl membrane had comparable binding capacity to a conventional resin (Tab.1, Fig. 5). A major difference is the reduced process time with the adsorber by a factor of 15 due to the high flow rate

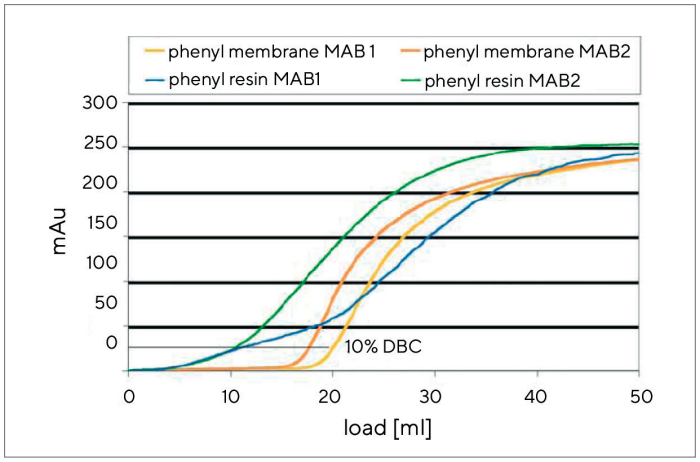


Fig. 5: Dynamic binding capacity of Mab (10% breakthrough) on membrane and resin

	Sartobind Phenyl	Phenyl resin
Number of layers	15	-
Bed height	4 mm	30 mm
Bed volume*	2 mL	1 mL
Flow rate	10 mL/min	1 mL/min
Flow rate	5 BV/min	0.33 BV/min
Binding capacity 10% breakthrough with:		
Mab1	8.9 mg/mL	10.5 mg/mL
Mab2	12.5 mg/mL	10.8 mg/mL
BSA	10 mg/mL	n.d.
Lysozyme	23 mg/mL	n.d.

Table 1: Mab binding on phenyl membrane and resin

3. Lab scale screening: Binding of an antibody in 0.1 – 1 M ammonium sulfate

The membrane was incorporated into 96-well plates for high throughput screening (HTS).

400 µl of a 1 mg/mL IgG sample in 50 mM potassium phosphate buffer pH 7 with different ammonium sulfate concentrations were loaded on a phenyl 96-well plate (4 mm bed height, 0.15 mL bed volume). The amount of IgG in the flow-through, wash and elution was determined by measuring absorbance at 280 nm.

In the subsequent experiments, IgG was loaded at 1 M salt in the same buffer.

3.1 Results

IgG was almost completely bound at 1 M ammonium sulfate (Fig. 6a). Recoveries greater than 95 % were found in the samples loaded at a salt concentration of 1 M in binding buffer (Fig. 6b).

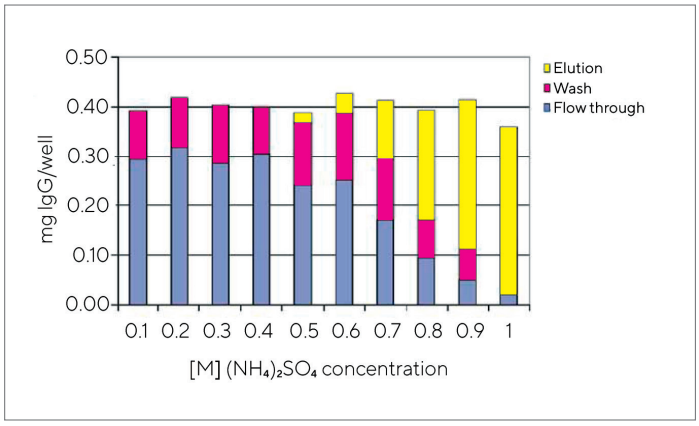


Fig. 6a: Amount of IgG bound on phenyl membrane at increasing $(NH_4)_2SO_4$ concentrations

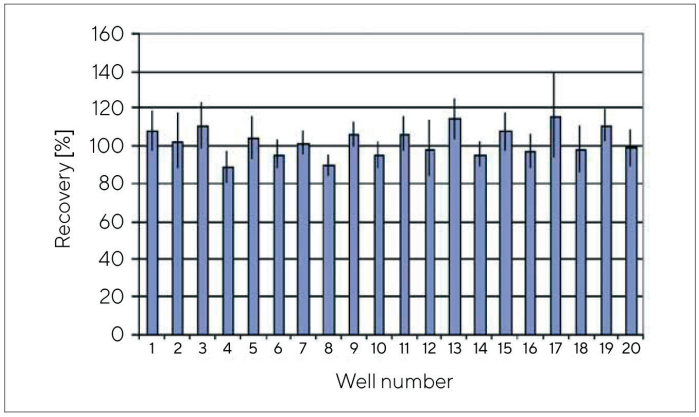


Fig. 6b: IgG recovery eluted from Sartobind Phenyl

4. Sartobind Phenyl and a phenyl resin challenged with a mixture of cytochrome c, trypsinogen and polyclonal antibody

The proteins were eluted by linear gradient: 1.5 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate pH 7 to 50 mM potassium phosphate.

4.1 Results

The elution from Sartobind phenyl was better resolved as compared to phenyl sepharose (Fig. 7).

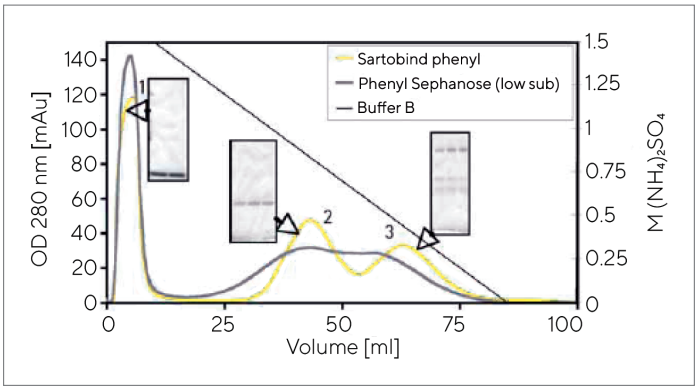


Fig. 7: Gradient elution of cytochrome c (1), trypsinogen (2) and IgG (3) bound on membrane and resin

5. Separation of cytochrome c, myoglobin, lysozyme and α-chymotrypsinogen

Proteins were dissolved in 1.7 M $(NH_4)_2SO_4$, 50 mM potassium phosphate at pH 7.0. Elution was done by step gradient with low salt buffer in 50 mM potassium phosphate pH 7.0 at a flow rate of 10 mL/min.

5.1 Results

The proteins listed above were base-line separated at room temperature with a simple step gradient (Fig. 8).

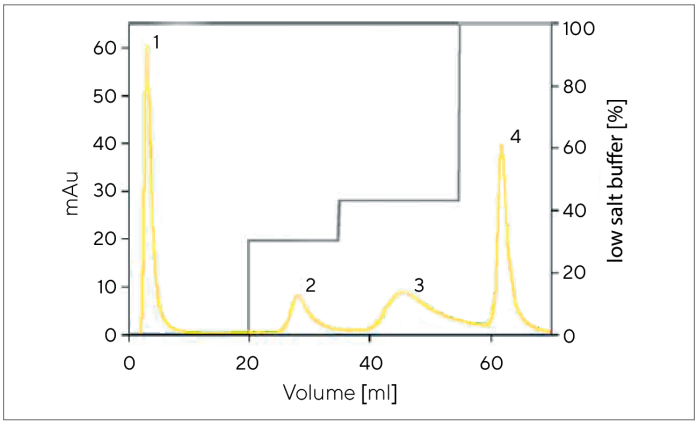


Fig. 8: Step elution of cytochrome c (1), myoglobin (2), lysozyme (3) and α-chymotrypsinogen (4) bound on the membrane

6. Aggregate removal

Sartobind phenyl membrane was used for aggregate removal in flow-through mode in a purification process for a recombinant protein. The loading conditions were chosen to selectively retain the aggregates while allowing the target protein monomers to flow through the membrane. Elution was done by washing the membrane with pure H_2O.

6.1 Results

Fig. 9 shows that the amount of aggregates bound to the membrane increased as the concentration of ammonium sulfate in the load increased.

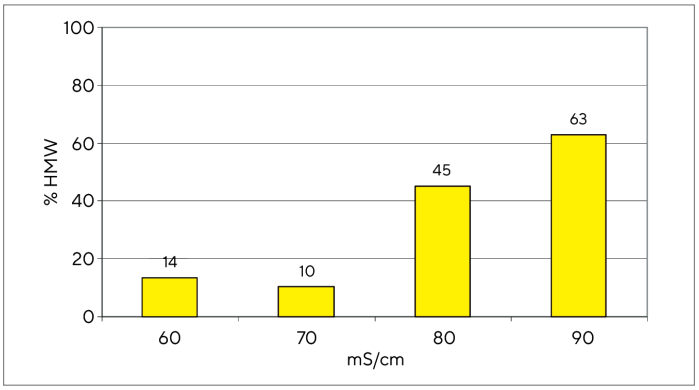


Fig. 9: Aggregates (%) eluted from Sartobind Phenyl.
The conductivity represents the different salt concentrations at loading.

7. Influence of flow rate

7.1 Results

Fig. 10 shows the breakthrough curves with Sartobind Phenyl nano (3 mL) at different flow rates of 5, 10 and 20 mL/min. The sample was a human monoclonal antibody at a concentration of 1 mg/mL.

A higher flow rate had only minor influence on breakthrough.

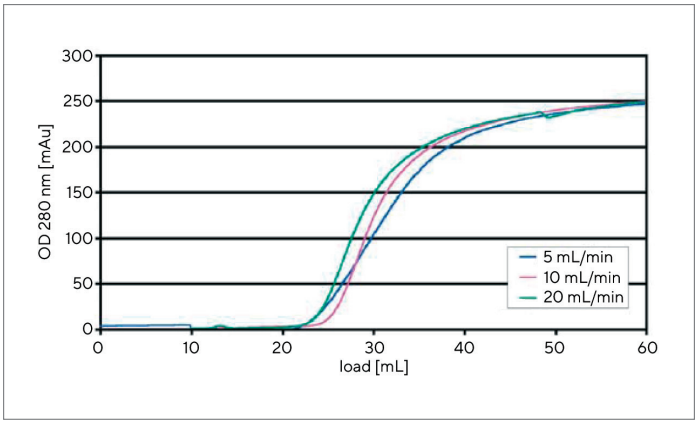


Fig. 10: Mab binding on the membrane at different flow rates

8. Scale up

Conventional applications from hydrophobic columns can be transmitted to hydrophobic membranes, especially applications with <1 kg biomolecules per hour due to the limitation of capsule size. Parallel and serial connections of capsules are possible for increased capacity, although use of one module in recycling mode is preferable because it is the simplest set up. The high flow rates can allow for 4 to 6 complete chromatographic cycles per hour. A calculation of binding capacity per mL and hour results in a productivity of 50 to 100 mg IgG per mL per hour.

In a scale up experiment with 3 mL, 150 mL, 400 mL, 1.2 L and 5 L capsules, globuline (~1 g/L) in a 1 M ammonium sulfate pH 7.0 was loaded at 5 MV/min.

8.1 Results

Breakthrough curves of Sartobind Phenyl nano 3 mL up to 5 liter membrane volume (Jumbo) capsules in Fig. 11 represent a successful 1700 fold scale up.

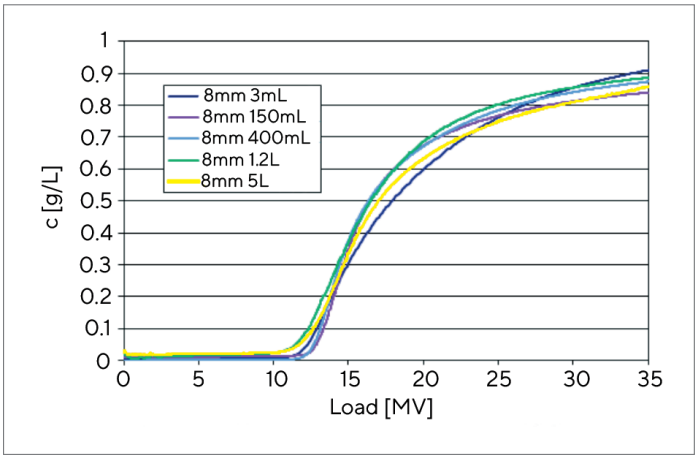


Fig. 11: Scale up performance of Sartobind Phenyl capsules

Applications of hydrophobic interaction membrane chromatography

- Hydrophobic interaction chromatography (HIC) on membrane and beads is comparable. High salt concentrations in the loading buffer promote the binding of the target molecule to the HIC adsorbent.
  By decreasing the salt concentration in a linear or step gradient the bound molecule is eluted. In all applications it is advisable to optimize by moderating temperature, pH, salt concentration and type of salt.

- One of the preferred applications of membranes is their use in flow-through steps. Such conditions need certainly less binding capacity and reduce consumption of salt.
  The ready to use capsules or modular cassettes in a holder does not require packing or testing and allows for a "plug and play" approach in downstream processing.

- Misfolded proteins, aggregates, dimers, trimers, tetramers of biomolecules as well as leached chromatographic ligands can display higher hydrophobicity than the protein of interest. A process can be developed to specifically retain impurities while allowing desired product to flow through.

- Directly after the protein A column, a HIC membrane flow-through mode can be implemented as a polishing step to remove host cell proteins from a monoclonal antibody fragment for example. This step reduces also leached protein A and misfolded proteins.

- The HIC membrane is applicable for removal of endotoxins from target proteins produced e.g. from inclusion bodies of Escherichia coli when a denaturing step is required to solubilize the target molecule.

- Hydrophobic membranes have been successfully applied in bind and elute applications. The large pore size of > 3 µm makes them especially suitable for the separation of large biomolecules above 100 kDa including vaccines, conjugated vaccines, virus particles and phages. There is almost no visible size exclusion effect detectable.

- A HIC step can be ideally used as an initial step after a precipitation or ion exchange chromatography step when the conductivity is already high and addition of salt for high binding of the proteins fits perfectly into the bioprocess scheme.

- Double stranded DNA displays higher hydrophobicity than single stranded DNA. This enables separation of such products during plasmid DNA purification. Hydrophobic membranes can also be applied to separate oligonucleotides from drivetrains oligos when a difference in structure and|or chemistry results also in a difference of hydrophobicity.

Summary

The Sartobind Phenyl membrane can be considered as a replacement to columns for polishing (flow-through) operations and a number of bind and elute applications as they work at much higher flow rates, reduced complexity and without size exclusion effects when purifying large biomolecules.

Cost savings can be anticipated by time savings per high flow rates and elimination of cleaning validation requirements when used single use.

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