CNBr-activated **Sepharose** 4 Fast Flow

AFFINITY CHROMATOGRAPHY

- Rapid, efficient coupling and maintained biological activity of the ligand
- Multipoint attachment of many protein ligands, resulting in a chemically stable product
- Fast Flow matrix gives high productivity and is easy to scale up
- Comprehensive technical and regulatory support for pharmaceutical production simplifies validation
- Over twenty successful years of using CNBr-activated Sepharose media documented with many references

Introduction

The preparation and use of affinity chromatography media by coupling biospecific ligands to CNBr-activated matrices is a widely used, successful, and well-documented technique.

CNBr-activated Sepharose™ 4 Fast Flow (Fig 1) is a preactivated affinity matrix that combines the advantages of CNBr coupling with the high flow and stability characteristics of Sepharose 4 Fast Flow. In our experience, the CNBr coupling technique has a well-proven track record for the purification of therapeutic proteins. This, plus the performance of the matrix at large scale, makes the use of CNBr-activated Sepharose 4 Fast Flow particularly attractive for manufacturing applications in the pharmaceutical industry. Furthermore, the medium is a member of the BioProcess™ media family and carries comprehensive technical and regulatory support for production applications.

Characteristics

Product description and use

CNBr-activated Sepharose 4 Fast Flow is a bead-formed, highly cross-linked preactivated matrix produced by reacting Sepharose 4 Fast Flow with cyanogen bromide (CNBr). Proteins and other molecules containing primary amino groups can be coupled directly to the preactivated gel.

Fig 1. Ligands are coupled to CNBr-activated Sepharose 4 Fast Flow using a rapid and efficient process. The coupled ligand maintains a high level of biological activity.

Multipoint attachment of proteins provides the immobilized product with good chemical stability. The resulting affinity medium can isolate a specific substance from a complex mixture, often achieving very high yield and purity in a single step. Many references demonstrate that binding affinity is frequently well maintained after CNBr coupling.

A typical application of preactivated affinity media like CNBr-activated Sepharose 4 Fast Flow is based on antigen-antibody reactions with immobilized monoclonal antibodies as ligands. In such cases, purification factors of 2000 to 20 000 can be obtained.

Table 1 summarizes the main characteristics of CNBr-activated Sepharose 4 Fast Flow.

Table 1. Characteristics of CNBr-activated Sepharose 4 Fast Flow

Sepharose 4 Fast Flow Matrix	
Mean particle size	90 µm
Particle size range	45–165 µm
Bead structure	Highly cross-linked 4% agarose, spherical
Linear flow*	150 cm/h at 100 kPa
CNBr-activated Sepharose 4 Fast Flow	
Swelling factor	4–5 ml drained gel/g
Coupling capacity	13-26 mg -chymotrypsinogen/ml drained gel
pH stability**	
long term	3–11
short term (CIP)	2–11

* At 25°C in water in an XK 50/60 column, 25 cm bed height. The flow properties are normally slightly better after the CNBr activation. The flow rate after coupling may differ depending on the ligand.

** Depends largely on the ligand stability.

Companion product

A companion product to CNBr-activated Sepharose 4 Fast Flow is NHS-activated Sepharose 4 Fast Flow, which has been activated to form active N-hydroxysuccinimide (NHS) esters. NHS coupling forms a chemically stable amide bond with ligands containing primary amino groups. Compared with CNBr-activated Sepharose 4 Fast Flow, NHS-activated Sepharose 4 Fast Flow is in many cases more suited to coupling smaller proteins and peptides.

We recommend users of affinity chromatography, especially those developing purifications for scaling up to production, to evaluate both products. NHS-activated Sepharose 4 Fast Flow is described separately (refer to Data file 18-1113-53).

Sepharose 4 Fast Flow matrix

Sepharose 4 Fast Flow is a highly cross-linked agarose matrix. In its preactivated CNBr form, it offers much improved performance when compared with the well-established CNBr-activated Sepharose 4B. The Fast Flow matrix has a higher rigidity and can thus be run at high flow rates (see Table 1). As the available capacities for proteins are similar in both cases, the Fast Flow matrix offers greater productivity.

The higher mechanical strength of the cross-linked matrix makes it well-suited for use in large columns. Scaling up a purification developed on CNBr-activated Sepharose 4 Fast Flow is therefore simple and more predictable. The coupled product is stable at low pH, which is often required for elution from some immunoadsorbents.

Note: For applications that require operation at high pH, note that the amide bond formed when using the companion product NHS-activated Sepharose 4 Fast Flow is stable up to pH 13 for normal use.

Storage

CNBr-activated Sepharose 4 Fast Flow is supplied freeze dried. Additives are included to preserve the bead form of the gel. When stored below 8°C, the shelf life is at least 18 months.

Long term stability studies (up to 91 weeks) show that freeze dried CNBr-activated Sepharose 4 Fast Flow is very stable when stored under recommended conditions. The degree of reswelling showed only a slight decrease and the coupling yield (when tested with soybean trypsin inhibitor) was maintained at a high level. Note, however, that the stability of the coupled gel is dependent on the attached ligand.

The coupling reaction

The coupling reaction, which is rapid and spontaneous, is easy to carry out and requires no special chemicals or equipment.

CNBr-activated Sepharose 4 Fast Flow is supplied as a freeze-dried powder stabilized with additives. Coupling a ligand to the activated matrix involves first swelling and washing* the gel followed by coupling. Instructions included with the product describe methods for coupling ligands and the effect of different conditions on the coupling efficiency. Users should develop a specific procedure for each individual application.

* Washing removes additives included in the freeze dried product to maintain its activity. Current literature may recommend using 200 ml (50 gel volumes) cold 1 mM HCl per gram freeze dried gel. This amount may be difficult to handle for process scale users of CNBr-activated Sepharose 4 Fast Flow. Recent studies have shown, however, that by increasing the contact time between gel and HCl (B-method above), the amount of cold 1 mM HCl required to wash out these additives can be reduced to one third of this recommendation, 10–15 gel volumes of cold 1 mM HCl per gram freeze dried gel (Fig 1), without affecting the coupling reaction. Further details are available from Cytiva.

Cleaning-in-place and sanitization

Cleaning-in-place (CIP) is a cleaning procedure that removes contaminants that may remain in the packed column after regeneration. Regular CIP also prevents the build-up of these contaminants in the CNBr coupled product and helps maintain the capacity, flow properties, and general performance of the medium.

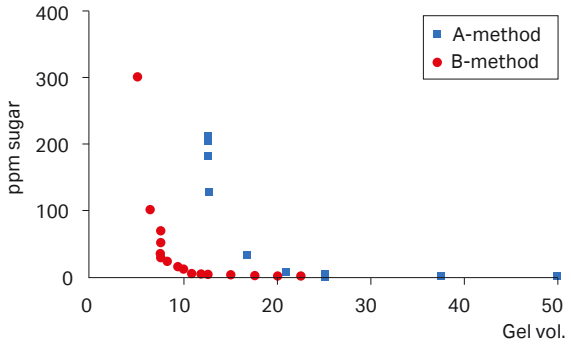

Fig. 1. The content of sugar in the filtrate after washing with different gel volumes of cold 1 mM HCl.

Sanitization inactivates microbial contaminants in the packed column and related equipment.

A specific CIP and sanitization protocol should be designed for each process according to the type of contaminants present and the stability of the coupled ligand. Experience has shown that NaCl, sterile-filtered buffer or non-ionic detergent are all effective cleaning agents.

One generally recommended procedure is to wash alternately with high and low pH buffers (0.1 M Tris HCl containing 0.5 M NaCl, pH 8.5, and 0.1 M sodium acetate containing 0.5 M NaCl, pH 4.5).

A slightly harsher treatment that may help remove strongly bound proteins is to wash the column with a non-ionic detergent included in the pH 8.5 buffer named above or applied separately.

Washing with several column volumes of 20% to 70% ethanol may also be effective on strongly bound proteins.

In all cases, we recommend you test washing procedures at small scale first, especially for higher concentrations of ethanol.

The frequency of CIP depends on the nature and condition of the starting material, but one CIP cycle is generally recommended every 5 separation cycles.

Applications

The laboratory scale use of CNBr-activated coupling media based on Sepharose is very well-documented in the literature. However, for reasons of commercial secrecy, detailed information about process scale use and manufacturing applications of CNBr-activated Sepharose 4 Fast Flow is generally not available.

Nevertheless, the use of this medium in the development of a process to purify native gp120 from HIV-1 infected T-cells has recently been described (1). Here, the authors coupled *Galanthus nivalis* agglutinin (GNA), a lectin from the bulb of the snowdrop, to CNBr-activated Sepharose 4 Fast Flow and then used the coupled gel to help purify the outer envelope glycoprotein gp120 of HIV-1, which is a major target for immunotherapy.

Coupling procedure

The procedure used for coupling GNA to CNBr-activated Sepharose 4 Fast Flow is summarized below.

1. Suspend the preactivated gel in 1 mM HCl for 30 min and allow to swell.
2. Wash with 15 gel volumes of cold 1 mM HCl.
3. Dissolve the GNA in coupling buffer and adjust to pH 8.3.
4. Add the washed gel to the GNA solution and incubate overnight at +4°C. (The coupling can also be performed at room temperature for 3–4 h).
5. Wash and resuspend the coupled gel in 1 M ethanolamine for 2–4 hours at room temperature to block unused activated sites.
6. Wash the gel eight times with alternating 50 mM Tris, 1 M NaCl pH 8.0 and 50 mM glycine, 1 M NaCl pH 3.5 buffers.
7. Wash the gel with 10 gel volumes of PBS.

Chromatographic purification

Following cell growth and detergent treatment to solubilize the gp120 from the viral particles and infected cells, the glycoprotein was partially purified in a cation exchange expanded bed adsorption step on STREAMLINE™ SP in a STREAMLINE 50 column.

The partially purified sample was loaded at flow rates of up to 300 cm/hr onto a 1.6 cm diameter column containing 10 ml GNA-coupled Sepharose 4 Fast Flow (1 mg GNA/ml gel). Gp120 was then eluted by reverse flow with 1 M methyl-α-D-mannopyranoside at flow rates up to 60 cm/hr. Samples were collected and analyzed by gp120 ELISA, silver stained SDS-PAGE and Western blot.

Figure 2 shows the chromatogram of the affinity separation.

Figure 3 A and B shows a silver stained SDS-PAGE gel and a Western blot.

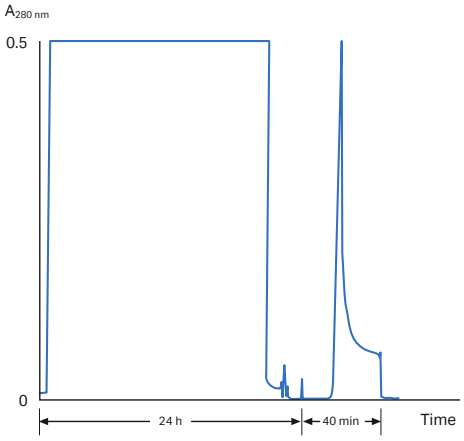

Fig. 2. Affinity separation of native gp120 protein on GNA-coupled Sepharose 4 Fast Flow.

Ninety four percent of the gp120 bound to the GNA-coupled Sepharose 4 Fast Flow and about 53% eluted under the conditions used. The total concentration of gp120 was 28.8 µg/ml, but this increased to between 100–150 µg/ml in the peaks.

Gp120 was recovered at high purity and specificity, as shown by Lane 6 in Figure 3 A and 3 B.

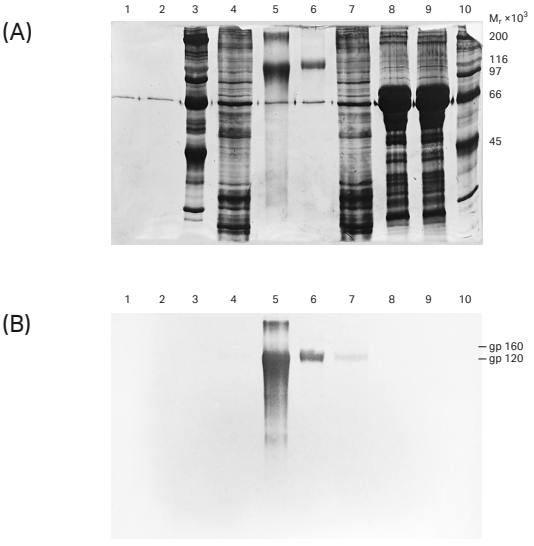

Fig. 3. (A) Silver stained SDS-PAGE gel and (B) a Western blot. Lane 1: Sample buffer. Lane 2: Sample buffer. Lane 3: Standards (LMW Calibration Kit, Cytiva). Lane 4: Flow through from GNA-coupled Sepharose 4 Fast Flow. Lane 5: gp120. Lane 6: Eluate from GNA-coupled Sepharose 4 Fast Flow. Lane 7: Eluate from the STREAMLINE cation exchange step. Lane 8: Flow through from the STREAMLINE cation exchange step. Lane 9: Starting material. Lane 10: Standards.

Reference

1. Gilljam, G., *et al.* Purification of native gp120 from HIV-1 infected T-cells, Poster presented at Recovery of Biological Products VII, San Diego, CA, USA (1994).

Ordering information

Product	Size	Code no.
CNBr-activated Sepharose 4 Fast Flow	10 g	17-0981-01
	250 g	17-0981-03
	2 kg	17-0981-05

cytiva.com/bioprocess

CY14620-03Sep20-DF