PROTEIN-PAK EPOXY-ACTIVATED AFFINITY PRODUCTS

I. INTRODUCTION

Waters Protein-Pak™ Epoxy-Activated Affinity products are silica-based materials with nominal 40 μm particle diameter and nominal 500 Å pore size. The packing is epoxy-activated for ligand coupling. It is capable of rapid purification of large sample volumes at high flow rates.

a. Chemistry

Waters Protein-Pak Epoxy Activated Affinity packing is prepared by bonding an epoxide functionality to silica. The resulting epoxy groups covalently bond amino, sulfhydryl, and hydroxyl moieties on small and large ligand molecules including proteins and peptides. The order of reactivity of the ligand nucleophiles is: \( RS^- > RO^- > RSH > R_1R_2NH > ROH. \)

b. Theory of Affinity Chromatography

Affinity chromatography is the only chromatographic method in which specific molecules are isolated on the basis of their bioselective interaction with an immobilized ligand. Other chromatographic methods rely on reversible adsorption due to physical and/or chemical properties of the biomolecule and the support.

Epoxy-activated affinity chromatography can be performed through the following six steps, as shown in Figure 1. Refer to Section IV, b Operation, to perform an affinity chromatography separation as illustrated.

Step 1: Ligand coupling or immobilization
Step 2: Loading sample mixture
Step 3: Adsorbing target material
Step 4: Washing out impurities
Step 5: Eluting target material
Step 6: Re-equilibrating the packing material

II. USE OF EPOXY-ACTIVATED AFFINITY PACKINGS

a. Coupling Buffers
b. Coupling Conditions
c. Blocking Procedure
d. Washing Procedure
e. Storage

III. USING THE MICRO-COLUMN

a. Preparing the Micro-Column
b. Isolation of the Selected Product
c. Description of the Products

Epoxy-activated packings are available in bulk and in Micro-Column configurations. Bulk material, the Micro-Column, and conditions for coupling the ligand are described below.

**Bulk Material**

The bulk packing material is available in 25 and 100 gram amounts. Larger quantities are available on request. The material is shipped as a dry white powder and should be stored dry at 4 °C. The bulk density of the material is about 0.5 g/mL, so about 0.5 grams of material is required per mL of column volume. The epoxide content is greater than 100 μmoles/gram.

**Micro-Column**

The Micro-Column contains 0.5 mL of Protein-Pak Epoxy-Activated Affinity packing with 50 to 100 μmoles of available activated sites. The Micro-Column volume is 3 mL, resulting in a 2.5 mL reservoir. The outlet has a filter. The top of the bed does not have a filter. The Micro-Column housing is polypropylene which is stable to all normal coupling temperatures (4 °C to 45 °C) and pHs (pH 2 to 9.5).

**Ligand Coupling**

Couple ligands to the epoxy-activated packing by following the procedures for Coupling Conditions covered in Section II. Unreacted epoxy groups on the coupled material may interfere with subsequent use in a chromatographic separation. After the affinity ligand is immobilized, the remaining epoxy groups can be blocked by reaction with amino compounds of low molecular weight (for example, glycine, tris buffer, or ethanolamine). The derivatized material is ideal for use in either batch processes or in packing columns.

**II. USE OF EPOXY-ACTIVATED AFFINITY PACKINGS**

**a. Coupling Buffers**

Prepare buffers and solvents using the following general guidelines.

- Carbonate, borate, and phosphate buffers are preferred. Sodium chloride or other non-nucleophillic salts may be added to achieve a desired ionic strength.
- Do not carry out the coupling in tris, glycerine or other nucleophillic buffers. These buffers will react with the epoxy groups.

**b. Coupling Conditions**

This section outlines general procedures for coupling a ligand to the material. Table 1 lists general conditions for the covalent binding of ligands based on different functional groups. The temperature limitation of the coupling reaction depends on the stability of the ligand; generally ligands can be coupled to the support between 4 °C and 55 °C.

**CAUTION:** Bonded phases on silica are susceptible to hydrolysis at extreme pHs. When using the material at pH 2 or pH > 9.5, bonded phase hydrolysis may occur and result in non-specific chromatographic effects.
Table 1: Guidelines for Coupling to an Epoxy-Activated Packing Material

<table>
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<th>Functional Group on the Ligand</th>
<th>Coupling Conditions</th>
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| Primary, secondary, and aromatic amines and sulphydryl | Any of the following at 20 °C to 55 °C, 17-24 hours:  
  • 0.1-1.0M phosphate pH 6-7.5  
  • 0.1 M borate, pH 8-9.5  
  • Organic solvents |
| Hydroxyl | Any of the following at 4 °C-45 °C, 17-72 hours:  
  • 0.1-1.0M phosphate pH 3-6.5  
  • Organic solvents such as dimethyl sulfoxide, dimethyl formamide, acetonitrile  
  • Alkali pH 11-13 (no more than 18 hours) |
| Proteins including enzymes and antibodies | Any of the following at 4 °C-45 °C, 24-72 hours:  
  • 0.1-1.0M phosphate pH 3-7.5  
  • 0.1-0.5M carbonate or borate, pH ≥ 8.0 with or without 0.05 to 1.0 M NaCl |

* For insoluble or partially soluble ligands, an organic solvent may be added to any of the above buffers.

To couple the ligand

1. Weigh out the amount of material required to pack the column. The bulk density of the material is about 0.5 g/mL, so about 0.5 grams of material is required per mL of column volume.

2. Place the material in a tube or bottle with a screw cap that fits securely. A screw cap is required so that no liquid is lost when the container is rotated on a laboratory wheel or shaken.

3. Add three parts coupling buffer or solvent to one part packing material, slurry or suspend, and rotate on a laboratory wheel or shaker for 5 minutes. Let the material settle.

4. Decant the supernatant liquid and then wash the packing material three times with fresh coupling buffer or solvent, using part buffer or solvent, using three parts buffer or solvent to one part packing material.

5. Dissolve the ligand in the coupling buffer or solvent in a test tube. Keep the concentration of the ligand solution as high as possible.

Note: During the coupling step, the final volume of buffer mass of affinity material should be 3 to 4 mL solution per gram of material.

6. Following the guidelines shown in Table 1, mix the ligand solution with the material using a rotating wheel. Alternatively, when carrying out couplings at temperature above room temperature, use a shaker in a water bath. Do not use a magnetic stirrer because the silica beads may fragment or the ligand may be damaged.

c. Blocking Procedure

1. The blocking solution may be added after the coupling solution is removed or added directly to the reaction mixture. Block unreacted epoxy groups with a final concentration of 1 M ethanolamine solution at pH 9.5 in coupling buffer (2 mL/g) by rotating for 18 to 24 hours at 4 °C.

2. Filter off the coupling/blocking supernatant using a coarse-porosity, sintered glass funnel. Save the solution to quantitate the amount of unbound ligand.

d. Washing Procedure

Perform the following sequence of washes to remove excess ligand and blocking solution. Use 6 mL of solution per gram of packing material for each wash.

1. Coupling buffer or solvent (one wash)  

Note: If coupling is performed in an organic solvent, initial washes should be of an increasing polarity, for example toluene, acetone, and then water.

2. 1 M NaCl solution (four washes).

3. Use one of the following buffers (one wash):  
   Loading buffer based on the application  
   Storage buffer (see Section II, e below)
The coupling supernatant and sodium chloride washes may be saved to quantitate the unbound ligand.

**e. Storage**

Store the material containing immobilized ligand at 4 °C (do not freeze) in a solution of:

- 0.01 to 0.1M sodium phosphate (pH 7.4) or
- 0.1 to 0.5 M sodium chloride or
- 0.01 % sodium azide

**III. USING THE MICRO-COLUMN**

Prepare the Micro-Column containing the epoxy-activated packing and isolate the selected product as follows.

**a. Preparing the Micro-Column**

1. Allow the Micro-Column to warm to room temperature.
2. Remove the cap at the bottom first and then the stopper at the top. Removing the stopper and cap in this order prevents the dry powder from scattering.
3. Place the Micro-Column in a ring stand or rack to allow test tubes or beakers to be put underneath the cartridge.
4. If vacuum will be used to remove the liquid from the Micro-Column, attach the vacuum source to the bottom of the Luer outlet.
5. Wash the packing material in the Micro-Column by putting 1.5 mL of HPLC grade water in the reservoir and allow it to flow through by gravity or vacuum.
6. Use the guidelines in Table 1, Section II to select coupling buffer for your ligand. Take 1.5 mL of coupling buffer to wash the packing material in the Micro-Column. Add the buffer to the reservoir and allow it to flow through by the gravity or vacuum.
7. Put the cap on the bottom of the Micro-Column. Add an additional 1.5 mL of coupling buffer, and stopper the top.
8. Place on a laboratory rotator for five minutes to thoroughly equilibrate the material in the coupling buffer.
9. Remove the stopper and cap and let the buffer drain off by gravity or vacuum. Put the cap back on the bottom of the Micro-Column.
10. See Sections II, a and II, b for recommended coupling buffers and conditions.
11. Add the ligand to be immobilized in 1.5 mL of the coupling buffer (for optimum mixing).
12. Stopper the Micro-Column and place it on the laboratory rotator at the desired temperature and time period. See Sections II, a and II, e for recommended coupling, blocking, washing, and storage conditions.

**b. Isolation of the Selected Product**

1. Wash the coupled packing with the elution buffer until the eluent collected reaches steady or zero absorbance value at the desired wavelength (use and UV-Visible spectrophotometer).
2. Wash the coupled packing with loading buffer at least 10 column volumes (5 mL).
3. Apply the crude, prefiltered sample (preferably in loading buffer) to the Micro-Column.
4. Wash the unbound material off the Micro-Column until low or zero absorbance is read for the eluent. Then wash the Micro-Column with the elution buffer to low or zero absorbance.
5. Alternatively, the Micro-Column can be capped and the biomolecule to be isolated can be applied to the column in 2.5 mL loading buffer; the Micro-Column can then be stoppered and rotated for 10 minutes or longer to assure maximum binding of the biomolecule to the ligand. After the stopper and cap are removed, continue as outlined in Step 4 above.
6. Re-equilibrate in 5 mL of loading buffer if a second purification is planned at this time.
7. While these Micro-Columns are recommended for single use only, the Micro-Column may be reused at a later date; reuse is dependent on the application, including degree of cleanliness of packing material needed, stability of ligand and time elapsed between uses.
V. STORAGE AND CARE OF THE AFFINITY COLUMN

This section contains information on column life, storage, and regeneration. Liquid chromatography columns have a finite life which is directly related to the care and use they receive. Store the column under the conditions which prevent loss of ligand function. A general rule is to store columns with protein ligands at 4 °C in pH 6 to 7.4 buffer containing a preservative such as 0.01% sodium azide. The column cleaning procedure depends on the ligand and the application. If removing debris, a typical procedure is to wash the columns with 10 column volumes of the following solutions in the following order:

1. Low pH wash, 5% to 10% acetic acid.
2. HPLC grade water or equivalent
3. 1 M sodium chloride
4. HPLC grade water or equivalent
5. Re-equilibrate the column in the chromatography starting buffer.

IV. CHROMATOGRAPHY GUIDELINES

a. Column Packing

Pack the column with the affinity material containing an immobilized ligand prepared as specified in the section above. One gram of bulk material nominally provides 2.0 mL of column volume. The bulk material may be slurry packed into any column. Following the column packing guidelines from the care and use manual for the column being used. The material can be slurry packed into an eluent compatible with the ligand.

b. Operation

To perform an affinity chromatographic separation:

1. Prior to equilibrating with loading buffer, equilibrate the column by washing with elution buffer until a steady baseline is obtained. Then switch to a loading buffer for at least 5 column-volumes.
2. Load the prefiltered sample containing the selected material to be purified. See Steps 2 and 3 in Figure 1. When the affinity interaction is weak or the kinetics are poor, reduce the flow rate or recycle the sample though the column until a steady state is achieved. The amount of solute remaining in the eluent will reach a constant value.
3. Wash the column with loading buffer until a steady baseline is obtained. See Step 4 in Figure 1.
4. Change the eluent to remove the bound sample. The limits on the pH range and ionic strength of the elution buffer or solvent are determined by the nature of the affinity ligand, the solute and the column matrix for the specific application. See Step 5 in Figure 1.
5. Re-equilibrate the column in the loading buffer.

c. Storage

- 0.01 to 0.1 M sodium phosphate (pH 7.4)
- 0.01 to 0.5 M sodium chloride
- 0.01% sodium azide
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