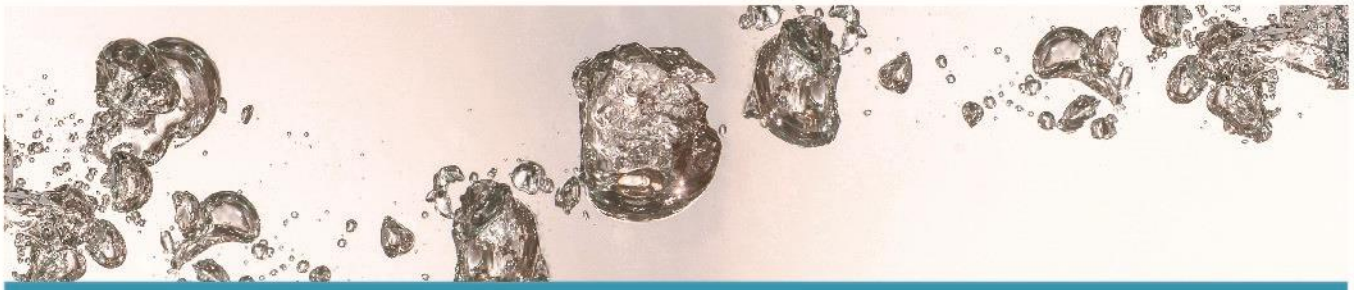
CIM Convective Interaction Media®

IMMOBILISATION PROCEDURE



For CIM® Epoxy Monolithic Columns

This procedure applies to CIMmultus™ Advanced Composite Columns (1 mL and larger) of Epoxy (XY) activation chemistry.

About CIM® Epoxy activated monoliths

Epoxy activated monolithic columns can be used for covalent immobilisation of amino- or thiol-containing compounds onto the monolithic surface to prepare custom chromatographic media. These columns are suited for coupling of both small organic compounds as well as larger biologically active molecules, such as peptides, proteins, and enzymes. The covalent nature of the bond between the ligand and matrix reduces leaching and improves stability and reusability. Immobilised supports enable a variety of customised affinity chromatography options with interactions specific to the target molecule.

Before you begin

Before you begin with the immobilisation procedure, read the product's documentation supplied in the package.

Due to the structure of the monolithic polymer, some of the conditions needed for immobilisation may be different than with traditional chromatographic supports. Please consider the following when preparing a specific immobilisation procedure:

- **pH stability of the ligand**

Coupling reactions with epoxy are performed at higher pH values (> 7, preferentially above pH 9) in suitable buffers. The upper pH range is limited by the ligand stability which should be considered when performing an immobilisation.

- **Thermal stability of the ligand**

The coupling of the ligand is achieved by a reaction between the epoxy groups of the support and amino or thiol groups on the ligand. Therefore, the coupling rate increases exponentially with temperature per the Arrhenius equation. From this point of view, an elevated temperature would be preferred for shortening the coupling time. On the other hand, some ligands may become unstable at higher temperatures and tend to agglomerate or even lose biological activity. The coupling temperature should be based on thermal stability of the ligand. For most ligands (especially those with higher molecular mass) the coupling is performed for several days at 4 °C (39 °F). if, however, the ligand is stable, room or even higher temperatures are recommended.

- **Composition of the coupling buffer**

Nucleophilic compounds such as Tris(hydroxymethyl)aminomethane and glycine should be avoided due to competing reactions with the epoxy groups. The ligand should be dissolved in a compatible coupling buffer for immobilisation. Suitable organic solvents may be added to assist in dissolving the ligand. Due to the slightly hydrophobic polymeric backbone of CIM® monoliths, higher ionic strength of a buffer (> 0.5 M) can enhance coupling, however, stronger buffers can cause limitations in industrial applications. Examples of buffers from

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literature include 20 mM sodium carbonate pH 9.3 and 0.1 M sodium borate pH 9.5 and 0.5 M phosphate buffer pH 8.0.

Immobilisation protocol

For proteins

The choice of coupling method depends on the ligand to be immobilized (the chemistry of its active groups, pH and temperature stability, reactivity etc.) The following general procedure can be used as a basis for the design of a ligand specific method.

It is recommended to use protein sample of concentration above 1 mg/mL in a suitable buffer. For an efficient immobilisation, between 3 and 10 mg of desired protein should be available per mL of monolith.

Prepare the system and the column per guidelines in the Instruction manual. With compatible columns, a syringe can be used to perform the immobilisation.

Use a low flow rate for the immobilisation procedure, up to half of the maximum flow rate for the type of column used (see the product documentation for more information).

1. Flush the column with at least 10 column volumes (CV) of deionised water.
2. Equilibrate the column by washing with at least 10 CV of suitable buffer (e.g. 0.5 M Na-Phosphate Buffer, pH 8).
3. Prepare the immobilisation solution by dissolving the protein in coupling buffer. Filter the solution through at least a 0.45 µm filter.
4. Flush the immobilisation solution through the column.

Note: Using a syringe instead of a pump is recommended when low volumes of immobilisation solution are used. A manual setup would have a syringe connected to either side of the column to collect the solution as it is pumped through. When all the solution is pumped and has collected in the outlet syringe, disconnect and reconnect the syringes to re-apply flow in the right direction. Pump the solution at regular intervals (every 15 minutes for example) to increase exposure of proteins to the monolithic surface.

Note: To connect a syringe to a 1 mL column, use a 10-32 UNF coned male to Luer adapter. For larger columns, a pump or HPLC system is recommended.

5. Disconnect the column and seal it with blind fittings.
6. Store at 4 – 25 °C (39 – 77 °F) for 2 – 3 days.

Note: Allow the immobilisation to occur at the highest possible temperature that the ligand withstands.

7. Wash the column with at least 10 CV of suitable buffer. Flush the column with at least 10 CV of deionized water or neutral buffer, such as 20 mM phosphate, pH 7.4.
8. A deactivation (blocking) of remaining epoxy groups is described in the chapter below.

For small organic molecules (organic amines, aminoacids, peptides)

Use a low flow rate for the immobilisation procedure, up to half of the maximum flow rate for the type of column used (see the product documentation for more information).

It is recommended to use a ligand sample of concentration > 10 mg/mL in a suitable buffer. Adjust to pH 8 – 11 using 2 M NaOH. For an efficient immobilisation, between 3 and 10 mg of desired protein should be available per mL of monolith and the volume of the ligand solution should be at least 15 CV.

Prepare the system and the column per guidelines in the supplied documentation. With compatible columns, a syringe can be used to perform the immobilisation.

1. Flush the column with at least 10 column volumes (CV) of deionised water.

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2. Equilibrate with at least 10 CV of coupling buffer which will be used for dissolving the ligand.

Note: To increase a solubility of hydrophobic ligands, an addition of organic solvent is suggested in aqueous solution, such as ethanol, acetonitrile, acetone, dioxane etc. In some cases, the immobilisation buffer is composed of only organic solvents.

3. Prepare the immobilisation solution by dissolving the ligand in coupling buffer. Filter the solution through at least a 0.45 µm filter.

Note: Higher ligand concentration in the immobilisation solution increases the reaction efficiency and is recommended. Simple amines with a concentration up to 5 mol/L can be used as an example.

4. Cycle the ligand solution through the column for 3 hours.

Note: A syringe can be used to cycle the immobilisation solution where applicable. See note under point 4 of the immobilisation protocol for proteins for detailed info.

5. Disconnect the column and seal it with the blind fittings.
6. Store at 4 – 80 °C (39 – 176 °F) for 2 to 48 hours.

Note: Allow the immobilisation to take place at the highest possible temperature accommodating ligand stability.

7. Wash the column with at least 10 CV of suitable buffer.
8. Equilibrate with at least 10 CV of suitable buffer (e.g. 20 mM TRIS, pH 7.4).

Deactivation of remaining epoxy groups:

Deactivation of the remaining epoxy groups to avoid non-specific interaction is recommended but not a requirement before operating the column. The epoxy groups on the monolith are very stable and require harsh conditions to successfully block them. Examples of suitable deactivation solutions are:

- Usually for proteinic ligands: 2 M ethanolamine, pH 10 for 24 hours at 4 – 37 °C (39 – 99 °F). **Note**: this agent will introduce secondary amino groups on the monolith and produce a positive charge on the surface.
- For sensitive ligands: 0.2 mercaptoethanol, pH 8 for 6 hours at room temperature
- For very stable ligands, such as small organic molecules: 0.5 M sulphuric acid for 6 hours at 50 °C (122 °F) or for 3 h at 65 °C (149 °F)

Note: These conditions may be too harsh for many chemically sensitive ligands. In such cases we recommend the use of alternative activated monoliths, such as CIM® Aldehyde and CIM® CDI.

To deactivate the epoxy groups:

1. Prepare the selected deactivation solution.
2. Flush the column with at least 20 CV of deactivation solution and keep it for the required time at the correct temperature (depending on the deactivation condition selected).

Note: The column can be disconnected from the system and sealed with blind fittings.

3. Flush the column with at least 10 CV of deionized water.
4. Wash the column with at least 20 CV of suitable buffer (e.g. 20 mM Na-Phosphate Buffer, pH 8) in order to thoroughly wash the deactivation solution.

Regeneration, cleaning in place and sanitisation procedures

Cleaning, regeneration, and sanitisation procedures are ligand and application specific. To ensure the reusability of the column, specific procedures should be prepared to care for the immobilised column.

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Storage

Column storage depends on the stability of the immobilised ligand. If the ligand allows, a 20 % ethanol solution is recommended. Alternatively, use phosphate buffered saline (PBS) pH 7.2 containing 0.2 g/L sodium azide (NaN_3). It is recommended to store the column between 2 °C (36 °F) and 8 °C (46 °F).

WARNING: Do not store the column below 0 °C (32 °F).
WARNING: Never let the monolith dry out!

Troubleshooting

1. **Quantity of immobilised ligand is small:**
   - Optimise the immobilisation procedure by considering the following parameters: buffer composition, time, pH, and temperature or ligand concentration.
   - Ensure that the monolithic column has been thoroughly flushed and equilibrated with the coupling buffer.
   - Prepare a new column and compare.

2. **Quantity of immobilised ligand is as expected, but affinity/capacity is low:**
   - Check ligand affinity before immobilisation. The ligand may be unstable, degraded, or old.
   - The chromatographic binding and eluting buffers may not be optimal. Using an unsuitable buffer can damage the column.
   - Residual particles in the sample may be causing fouling of the column or blockage of pores through non-specific binding. Consider developing a procedure to sanitise the column or a CIP procedure.
   - If the immobilisation site is in the vicinity of the active site of the ligand, the latter may be sterically hindered. Consider using a different immobilisation strategy.

3. **Loss of binding/capacity with time:**
   - Affinity columns have a limited lifetime, especially when not used regularly.
   - Eluting conditions may not be optimal, i.e. either too weak for complete elution of the target molecule, or too strong and therefore damaging or modifying the immobilised ligand. Both causes lead to a loss of binding capacity.

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Additional reading

For additional information you may consult the following articles and application notes:

- Nicoli, Raul, et al. "Trypsin immobilization on three monolithic disks for on-line protein digestion." Journal of pharmaceutical and biomedical analysis 48.2 (2008): 398-407.

- App note: A049 Immobilisation of proteins onto CIM – developing the most efficient affinity chromatographic monolith.

For any additional information please contact us:

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