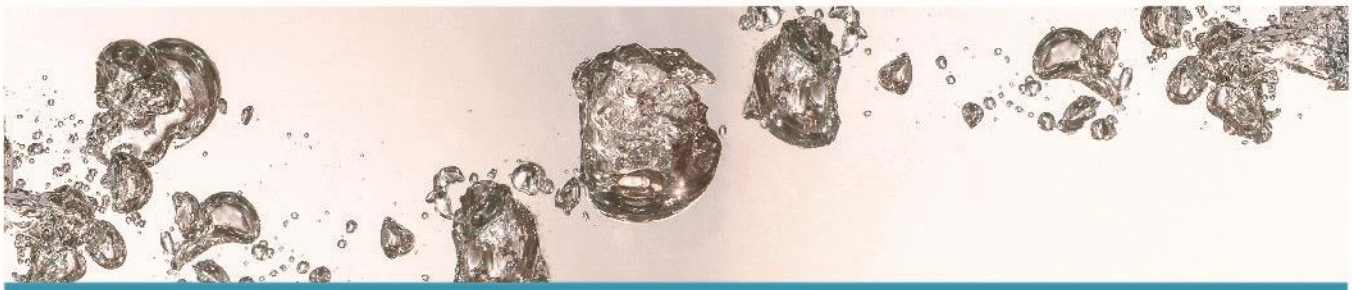
IMMOBILISATION PROCEDURE



For CIM® CDI Monolithic Columns

This procedure applies to CIMac™ Analytical Columns (0.1 mL), CIMmic™, and CIMmultus™ Advanced Composite Columns (1 mL and larger) of carboxyimidazole (CDI) activation chemistry.

About CIM® CDI columns

CIM® CDI Monolithic Columns are composed of a solid highly cross-linked monolithic carboxyimidazole (CDI) methacrylate-co-ethylene-dimethacrylate polymer and an appropriate housing. They can be used to immobilise ligands (e.g. proteins, peptides, or other amine or thiol-containing molecules) to perform specific separations of large and small biomolecules. The CIM® CDI Monolithic Columns operate at high flow rates, low back pressures, and are extremely easy to use.

Before you begin

Before you begin with the immobilisation procedure, please read the documentation supplied with the product.

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 is performed at a higher pH (> 7) where carboxyimidazole (CDI) groups are more reactive. The maximum pH value is limited by ligand stability and should be thoroughly checked and optimised in each case. Coupling of proteins between pH 7 and 9 is a good compromise. Depending on the stability of the coupling ligand, the immobilisation can be performed at pH 11.

• Thermal stability of the ligand

The coupling of the ligand to the CIM® CDI Monolith is achieved by a reaction between CDI groups of the support and the amino or thiol groups of the ligand. Therefore, the coupling rate increases exponentially with temperature according to 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 become unstable at higher temperatures and tend to agglomerate or even to lose biological activity. So, the coupling temperature should be based on thermal stability of the ligand. For most ligands (especially those with high molecular mass) optimal results are achieved when the reaction takes place for several days at 4 °C (39 °F).

• Composition of the coupling buffer

The coupling buffer type does not significantly influence the coupling efficiency. But, in cases where the ligand stability depends on the environment the selection of the buffer is of utmost importance. Recommended buffers are phosphates, 2-(N-morpholino) ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), borate, carbonate. Tris(hydroxymethyl)aminomethane (TRIS), glycine or other nucleophilic substances should be avoided as they may compete with the ligand for the CDI groups, thus lowering the coupling efficiency. For coupling, the ligand should be dissolved in a coupling buffer. Sometimes, addition of a small amount of organic solvent is necessary to completely dissolve the ligand. The polymeric support is slightly hydrophobic, and coupling can be enhanced by using

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buffers with higher ionic strength (e.g. 0.5 M buffers or buffers with the addition of appropriate salts, such as NaCl or Na_2SO_4 with concentrations up to 1.5 M). Such buffers enable the ligand to come closer to the surface facilitating the coupling reaction. Care should be taken with certain ligands which are susceptible to agglomeration and precipitation in high ionic strength buffers. The agglomerates and precipitates will hinder the coupling and eventually clog the column.

• Immobilisation protocol

The choice of the coupling method depends on the properties of the ligand to be immobilised (the chemistry of its active groups, pH and temperature stability, reactivity, etc.). The following two general procedures for proteins and small organic molecules can be used as a basis for the design of a ligand specific coupling method.

The immobilisation steps should be performed at a flow rate of 2 – 10 CV/min on CIMac™ Analytical columns (0.1 mL) and 0.5 – 1 CV/min for CIM®/CIMmultus™ Columns.

General immobilisation procedure for proteins

It is recommended to use protein sample of concentration above 0.5 mg/mL in a suitable buffer (e.g. 0.5 M Na-Phosphate, pH 8.0 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 supplied documentation. 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. Prepare the immobilisation solution by dissolving the protein in a suitable buffer, as specified above.
3. Equilibrate the column with at least 10 CV of the buffer (e.g. 0.5 M Na-Phosphate Buffer, pH 8.0).
4. Cycle the ligand solution through the column for 3 hours.

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, compress the outlet syringe to reverse the flow direction (applies to CIM® Disk, CIMmic™ and CIMac™) or disconnect and reconnect the syringes to re-apply flow in the correct direction (applies to CIM® Tube and CIMmultus™ columns). 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 column, use a 10-32 UNF coned male to Luer adapter.

5. Disconnect the column and seal it with the blind fittings.
6. Store at 4 – 25 °C (39 – 77 °F) for 2 to 48 hours.

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

7. Re-connect the column to LC/HPLC or use syringes.
8. Wash the column with at least 10 CV of the buffer (e.g. 0.5 M Na-Phosphate Buffer, pH 8).
9. Deactivate residual CDI groups with 5 CV of 2 M ethanolamine pH 9.0.

Note: If the ligand is not stable in 2 M ethanolamine pH 9.0, the pH can be lowered to 8.0, but deactivation will be less efficient.

10. Disconnect the column and seal it with the blind fittings.
11. Store at room temperature for 24 hours.
12. Flush the column with at least 10 CV of deionised water.
13. Equilibrate with at least 10 CV of suitable buffer (e.g. 20 mM TRIS, pH 7.4).
14. The column is now ready for use.

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General immobilisation procedure for small organic molecules (organic amines, amino acids, peptides)

Prepare the system and the column per guidelines in the supplied documentation. 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 with at least 10 CV of solvent which will be used for dissolving the ligand.
3. Dissolve the ligand in a suitable solution (typically water). Ensure a concentration between 1 and 100 mg of ligand per mL of solution (concentrations ≥ 10 mg/mL are preferable). Adjust the pH to 8-11 (depending on ligand stability) with 2 M NaOH. To improve ligand solubility and to prevent precipitation, partially replace the water with a non-nucleophilic organic solvent (e.g. ethanol, THF, dioxane, DMF). Total volume of the ligand solution should be at least 15 CVs.
4. Cycle the ligand solution through the column for 3 hours.
5. Disconnect the column and seal it with the blind fittings.
6. Store between 4 °C (39 °F) and 25 °C (77 °F) for 2 to 48 hours.

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

7. Re-connect the column to LC/HPLC or use syringes.
8. Wash the column with at least 10 CV of suitable buffer (e.g. 0.5 M Na-Phosphate Buffer, pH 8).
9. Deactivate residual CDI groups with 5 CV of 0.1 M NaOH. Alternatively, use 2 M ethanolamine pH 9.0 if the ligand is not stable in NaOH.
10. Disconnect the column and seal it with the blind fittings.
11. Store at room temperature for 4 to 20 hours.
12. Flush the column with at least 10 CV of deionised water.
13. Equilibrate with at least 10 CV of suitable buffer (e.g. 20 mM TRIS, pH 7.4).
14. The column is now ready for use.

Caring for the affinity CIM® column

To extend the life span of the column with immobilised ligand, please observe the following:

- Always use freshly prepared mobile phases (buffers).
- Always filter the mobile phases (buffers) and samples through a 0.22 µm filter.

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.

Storage

Storage conditions should be determined based on the ligand stability. It is recommended to store the column between 2 °C (36 °F) and 8 °C (46 °F) in a suitable storage solution.

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

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TROUBLESHOOTING

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.

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.

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.

For any additional information please contact us:

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