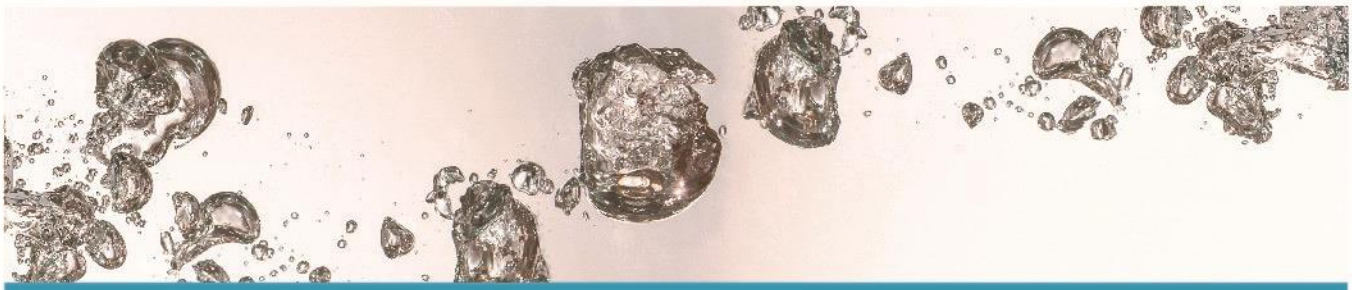
IMMOBILISATION PROCEDURE



For CIM® ALD (Aldehyde) Monolithic Columns

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

About CIM® ALD activated monoliths

Aldehyde activated monolithic columns can be used to immobilise proteins or other aminated organic compounds utilising a reductive amination reaction. In this way a stable covalent amino linkage between a ligand and chromatographic support is achieved. . The resulting columns provide high purification efficiency and low leaching of the immobilised ligand. The unique characteristics of aldehyde activated monoliths compared to CIM® CDI or CIM® EDA are neutral surface of chromatographic support together with high hydrophilicity due to high hydroxylation.

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

Aldehyde activated monoliths are stable in storage solution (20% ethanol) at 2 – 8 °C (36 – 46 °F) for over 2 years. Coupling reactions with aldehyde are performed at broad pH values (4 – 10) in suitable buffers employing different reducing agents. The pH range is limited by the ligand stability in alkaline conditions (above pH 10) which should be considered when performing an immobilisation. Proteins are usually most stable at pH close to neutral, therefore coupling between 5.5 and 7.5 is recommended.

• Thermal stability of the ligand

Coupling of the ligand to the CIM® ALD Monolith is achieved by a reaction between ALD groups of the support and amino groups on the ligand. The coupling temperature should be based on thermal stability of the ligand. For protein immobilisation, satisfactory results are achieved when the reaction takes place overnight between 4 (39 °F) and 20 °C (68 °F).

• Composition of the coupling buffer

The coupling buffer type does not significantly influence the coupling efficiency. When the ligand stability depends on the environment, 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 buffers containing amino functional groups should be avoided as they may compete with the ligand for the aldehyde group on the chromatographic support, lowering the coupling efficiency.

• Immobilisation protocol

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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 immobilisation procedure is an example of a coupling method for protein and can be used as a starting point for protein binding optimisation. Other organic compounds containing amino groups can also be immobilised on the column. Specific protocols need to be prepared based on the ligand properties.

Immobilisation protocol

It is recommended to use protein sample of concentration above 0.5 mg/mL in 100 mM phosphate, pH 7.2 buffer. A phosphate buffered saline (PBS buffer) is also accepted. 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 PSIM for more information).

Note: The following procedure uses sodium cyanoborohydride ($NaCNBH_3$) as reducing agent. $NaCNBH_3$ is toxic and should be handled in a fume hood. Less toxic reducing agents may be used, such as 2-picolin borane complex or $NaBH_4$. Coupling efficiency, optimal buffer composition and pH are not the same for different reducing agents.

To immobilise the protein of interest, follow these steps:

1. Prepare the solution of protein in an appropriate buffer (for example, 100 mM phosphate, pH 7.2). The concentration should be between 0.5 and 2 mg/mL. Note: The reductive amination using $NaCNBH_3$ is usually more efficient at lower pH, for example in 100 mM MES buffer, pH 5.6. If the ligand stability allows use of such pH, consider the coupling reaction at such conditions.
2. Filter the solution through 0.45 µm filter.
3. Flush the aldehyde column with at least 10 column volumes (CV) of deionised water, followed by 10 CV of 100 mM phosphate buffer, pH 7.2.
4. Dissolve the reducing agent (typically sodium cyanoborohydride, $NaCNBH_3$) into the protein solution to a final concentration of 3 mg/mL. For example, 6 mg of NaCNBH3 should be dissolved in 2 mL of protein solution. If precipitation of protein occurs with the addition of reducing agent, filter the immobilisation solution before applying to the column.

Note: A freshly prepared stock solution of NaCNBH3 in immobilisation buffer (30 mg/mL) can be used instead. Combine this stock solution with the solution of protein in a volumetric ratio of 1:9.

5. Recirculate the immobilisation solution through the monolithic column at a low flow rate (see PSIM for details) for at least 3 h at room temperature.

Note: If more convenient, the reaction may be performed overnight at 4°C.

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.

6. After immobilisation, wash the column with 10 CV of 100 mM phosphate buffer, pH 7.2, then continue with the deactivation of remaining aldehyde groups.

Deactivation of remaining aldehyde groups:

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1. Prepare the deactivation solution: dissolve $NaCNBH_3$ in 300 mM phosphate buffer, pH 7.0 (5 mg/mL), then add ethanolamine (3 µL/mL). Check the pH of the deactivation solution does not exceed pH 8.
2. Wash the column with at least 10 CV of 300 mM phosphate, pH 7.0.
3. Flush the column with 20 CV of deactivation solution.
4. Seal the column with blind stoppers and keep at 4 – 25 °C (37 – 77 °F) for 15-24 hours.
5. Wash the column with at least 10 CV of 300 mM phosphate, pH 7.0.
6. Wash the column with at least 10 CV of PBS or other neutral buffer containing between 0.5 and 1 M NaCl.
7. Store the column in PBS, pH 7.2 containing 0.2 g/L sodium azide (NaN_3).

Note: If the immobilised compound is stable, store the column in 20% ethanol.

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

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.

Additional reading

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

- App note: A049 Immobilisation of proteins onto CIM – developing the most efficient affinity chromatographic monolith.
- App note: Versatility of CIM monoliths for covalent immobilisation of antibodies
- Naldi, Marina, et al. "Towards automation in protein digestion: Development of a monolithic trypsin immobilized reactor for highly efficient on-line digestion and analysis." Talanta 167 (2017): 143-157.
- Chen, Wei-Qiang, et al. "Immobilized monolithic enzymatic reactors and its application on analysis of In-Vitro Fertilization media samples." Electrophoresis (2017).

For any additional information please contact us:

Tel.: +386 5 9699 500 | sales@biaseparations.com | www.biaseparations.com