GENERIC AFFINITY COLUMNS AS PRE-COLUMNS IN CAPILLARY LC

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Introduction

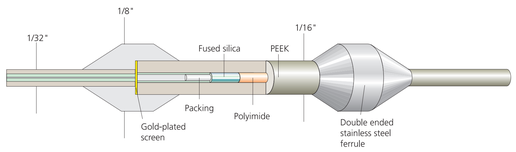
We have developed a column designed to be readily modified with ligands such as proteins or enzymes to function as affinity supports or as enzymatic reactors (ProteCol ™-CMD - patent pending).
The packing material is based on a macroporous silica coated with cross-linked carboxymethylated dextran.

Coating a large pore silica or a non-porous silica with a modified poly-saccharide such as carboxymethylated dextran (CMD), provides a surface with minimum interaction towards peptides, proteins, oligo-saccharides or poly-nucleotides. When activated with N-(3-dimethyl-amino propyl)-N'-ethyl-carbodiimide hydrochloride (EDC) and N-Hydroxy-succinimide (NHS) the support will react readily with any molecule containing a primary amine group such as peptides or proteins, a thiol group or an aldehyde group.

Applications for these columns could be the immobilisation of affinity ligands to either remove unwanted portions of a sample or to enrich the components of interest from a complex mix.

Furthermore, these columns can be derivatised with enzymes to perform in-line reactions such as tryptic digests.

ProteCol ™-CMD Column Design



Integrated connection tubing
Column/tubing material: PEEKsil ™
Packing material: carboxymethylated dextran silica
3µm particle size; 2000Å pore size
Dimensions: 75µm to 530µm ID; 5mm to 150mm length

Features Summary

- Dextran coating minimises non-specific binding of biological samples
- 2000Å pore packing material allows fast diffusion of proteins and peptides
- Immobilisation chemistry is compatible with the use of HPLC to derivatise the columns
- High degree of flexibility in the choice of ligands

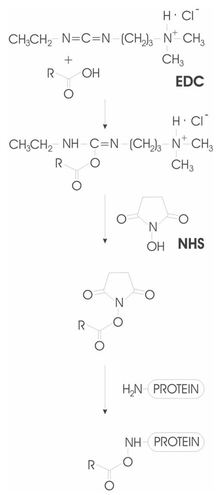
Immobilisation Chemistry

Carboxymethylated Dextran can be readily modified with any ligand containing a primary amine group, a thiol group or an aldehyde group.
The carboxylic acid groups are activated with N-(3-dimethyl-aminoproyl)-N'-ethyl-carbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS).

Benefits:
Reaction takes place at pH 6.5: compatible with silica
Reaction is fast: within the timeframe of an injection
Reaction is versatile: a large number of ligands can be immobilised.

Procedure

The activation of carboxymethylated dextran with EDC and NHS is a fast reaction. This allows the use of the HPLC and an autosampler as a delivery system for the reagents:



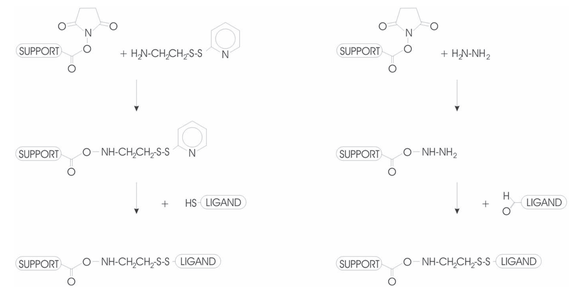
Mobile phase: 50mM phosphate buffer pH 6.5
Stock solutions:
- 4mg/ml EDC in mobile phase
- 6mg/ml NHS in mobile phase
- Ligand solution in mobile phase
- 10mg/ml ethanolamine in mobile phase.

Equilibrate column
Just prior to the immobilisation mix EDC and NHS stock 1:1
Inject 8µl of EDC/NHS at low flowrate (1µl/min for 300µm ID column)
Wash column
Inject ligand at low flow rate
Wash column
Inject ethanolamine at low flowrate
Wash column

Alternative Chemistries

Thiol coupling:
Activated CMD is further modified with 2-(2-pyrinidyl-dithio)ethaneamine

Hydrazine coupling:
Activated CMD is further modified with hydrazine



In-line Enzymatic Reactor Immobilised Trypsin

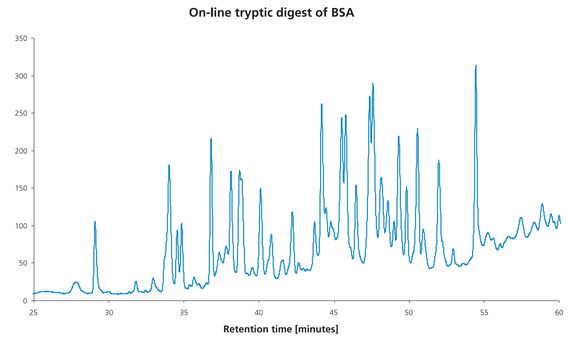
In-line tryptic digestion has some advantages over traditional methods:
- only small sample amounts are needed since the total digested sample is eluted onto the analytical column
- no autodigestion of trypsin since trypsin is covalently attached to the support surface
- when installed in-line with two switching valves digestion and consecutive analysis can be automated

Proteolytic digest of BSA using a trypsin derivatised CMD column

- Immobilize trypsin from porcine pancreas (EC 3.4.21.4) onto a 150mm x 300µm ID CMD column.
- Equilibrate column with a 50mM $NaHCO_3$ solution.
- Inject 5µl bovine serum albumin solution (10mg/ml in mobile phase) onto the column and stop flow.
- Keep column at 37°C for two hours
- Elute digest and collect in a 100µl sample vial
- Analyze digest by RP-HPLC

Reversed phase analysis of protein digest

Column: ProteCol-C18 100mm x 300µm ID
Packing: 3µm particle size; 300Å pore size; ODS
HPLC: Agilent 1100 CapLC with multi wavelength UV detector and 80nl flow cell
Mobile phase A: 0.1% TFA in water
Mobile phase B: 0.1% TFA in 80% acetonitrile/water
Gradient: 0 to 60 min: 0 to 100% B linear
60 to 70 min: 100% B
70 to 80 min: 100 to 0% B
80 to 100 min : 0% B
Flow rate: 4.0µl/min
Injection volume: 8µl
Temperature: ambient



Glycopeptide Enrichment Using Immobilised Concanavalin A

Concanavalin A (Con-A) is a lectin isolated from *Conavalis ensiformis* (Jack Bean) and has a selective binding affinity for terminal α-D-mannosyl and α-D-glucosyl groups. It can be used in affinity chromatography to selectively bind glycosylated proteins and peptides.
Con-A forms a number of oligomers depending on pH. The active dimeric form is stable at pH 5.6 and below. Between pH 5.8 and 7 Con-A forms tetramers; above pH 7 it forms higher aggregates. Because the higher oligomers are inactive, pHs above 5.6 should be avoided. Furthermore, Con-A requires Ca^{2+}, Mg^{2+} and Mn^{2+} to be active, which has to be added to all solutions.

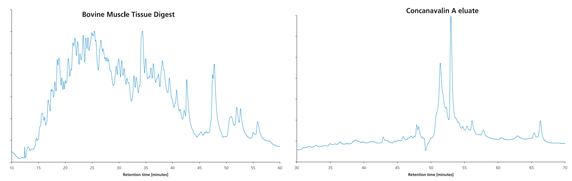
Bovine muscle tissue protein digest

- Freeze-dry tissue sample
- Grind and extract fat with iso-octane
- Dry and grind again
- Extract proteins with water
- Precipitate with TFA
- Wash with cold acetone, dry and weigh
- Re-dissolve with 50mM $NaHCO_3$ solution
- Add trypsin (protein to enzyme ratio of 20:1)
- Keep at 37°C over night
- Filter with 0.45µm filter

Trapping of Glyco-peptides using a Con-A derivatised CMD column

- The derivatisation with Con-A was performed analog to trypsin at pH 5.5 (dimeric form of Con-A).
- The column was equilibrated with 0.1% TFA in water
- The peptide mix from the protein digest was loaded onto the Con-A/CMD column.
- The non-retained portion of the peptide mix was washed off.
- The bound fraction was eluted with methyl- α-D-mannopyranoside.

Chromatographic conditions for the reversed phase analysis of the total protein digest and the eluate off the Con-A/CMD column were the same as for the analysis of the BSA tryptic digest.



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