Prepare a reversible and measurable sulfhydryl-reactive affinity column

TR0026.2

Introduction

A variety of activated resins are available for covalent and irreversible attachment of proteins, peptides and other ligands for use in affinity purification techniques. However, researchers may occasionally wish to use a reversible chemistry to immobilize a ligand, allowing for its recovery during or after the affinity purification experiment. The following simple protocol uses two Thermo Scientific Pierce Protein Research Products to prepare a reversible sulfhydryl-reactive affinity support. One unique feature of the coupling chemistry is its production of UV-absorbing byproduct that can be measured to determine the amount of ligand immobilized.

The activation protocol involves conjugation of Sulfo-LC-SPDP (Product No. 21650) to the amine-derivatized DADPA Agarose Resin (Product No. 20266) (Figure 1, Reaction 1). Subsequent coupling of sulfhydryl-containing molecules occurs by disulfide exchange with the pyridyldithiol group (Figure 1, Reaction 2). Efficiency of large-scale coupling reactions can be estimated using absorbance at 343 nm to determine the amount of pyridine-2-thione released. Once prepared, the support allows for affinity purification and recovery of the coupled ligand by reduction of the disulfide bond with dithiothreitol (DTT) or similar reagent.

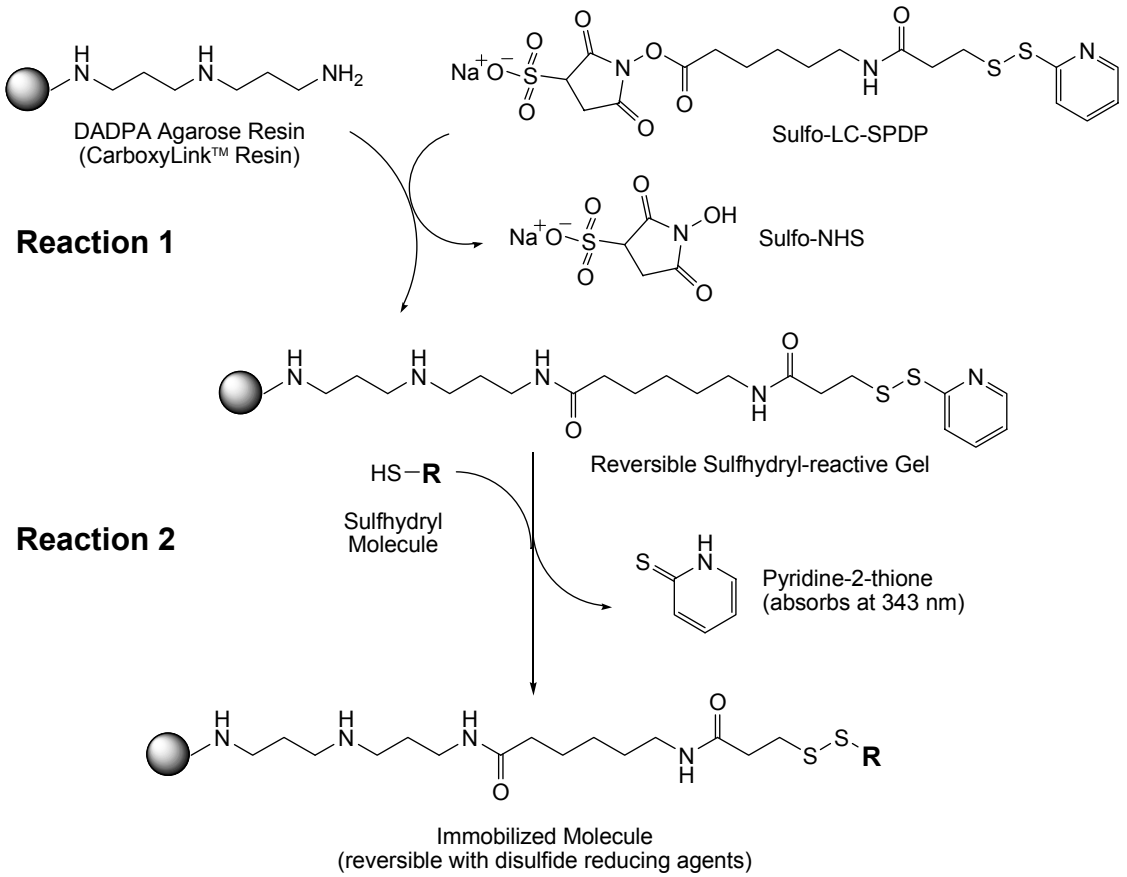

Figure 1. Reaction scheme for reversibly immobilizing sulfhydryl-containing molecules to affinity supports.

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Preparation of Pyridyldithiol-activated Agarose

Scale the activation protocol to suit the intended application (e.g., batch or spin-cup immunoprecipitation, or packed gravity-flow column chromatography). Alternatively, activate a large amount of support and then store it for later small-scale immobilization experiments. Pyridyldithiol-activation involves modifying amino groups of the DADPA support with a molar excess of Sulfo-LC-SPDP. Use reaction conditions (e.g., pH and buffers) consistent with the product instructions for Sulfo-LC-SPDP (Product No. 21650). Perform the coupling reaction using reagents equilibrated to the same temperature (either room temperature or 4° C).

A. Materials Required

- Columns: Pierce Spin Columns (Product No. 69705 or 69725) or Pierce Centrifuge Columns (e.g., Product No. 89807)
- DADPA Agarose Resin: Immobilized Diaminodipropylamine, also called CarboxyLink™ Resin (Product No. 20266)
- Sulfo-LC-SPDP (Product No. 21650)
- PBS (0.1 M phosphate, 0.15 M sodium chloride, pH 7.2, Product No. 28372) or other amine-free buffer at pH 7.2-8.0

B. Procedure

1. Fill an empty column with desired amount of DADPA Resin slurry (2 ml of resin slurry makes a 1 ml resin bed).
2. Wash and equilibrate the support by passing five resin-bed volumes of PBS through the column.
3. Replace bottom column cap and add one resin-bed volume of PBS.
4. For every milliliter of resin bed used, weigh and dissolve 8-10 mg of Sulfo-LC-SPDP in 0.5 ml of ultrapure water.
5. Immediately add the Sulfo-LC-SPDP solution to the column containing the PBS/resin slurry.
6. Cap the column and mix reaction slurry by gentle inversion for one hour.
7. Place the column upright and allow resin to settle (pack) as a bed.
8. Uncap the column, drain the Sulfo-LC-SPDP reaction solution down to the top of resin bed, and replace the bottom cap. Do not completely drain column.
9. Wash column with 10 resin-bed volumes of PBS. Cap column while resin is still immersed in buffer.
10. Proceed to the next section (immobilization) or store column at 4°C. For long-term storage add 0.05% sodium azide.

Immobilize Sulfhydryl-containing Molecules

The immobilization protocol involves replacing the pyridyldithiol groups on the activated affinity support with sulfhydryl-containing molecules such as proteins or peptides. The resulting linkage between the resin and molecule is a disulfide bond. For maximum immobilization, perform the reaction with at least a two-fold molar excess of sulfhydryl-containing molecule over the pyridyldithiol groups on the resin (= amino groups on the original DADPA agarose).

The molecule for immobilization must have free (reduced) sulfhydryls. Reduce peptide disulfide bonds with TCEP Disulfide Reducing Resin (Product No. 77712). Reduce disulfide bonds in high molecular weight proteins using 5 mM TCEP (1:100 dilution of Thermo Scientific Bond-Breaker® TCEP Solution, Product No. 77720) for 30 minutes at room temperature, followed by two passes through an appropriate desalting column (e.g., Thermo Scientific Zeba™ Desalt Spin Columns, Product No. 89882, 89890, 89892, 89894). Be aware that proteins (e.g., antibodies) might be inactivated by complete reduction of their disulfide bonds. To selectively reducte hinge-region disulfide bonds in IgG use 2-Mercaptoethylamine•HCl (2-MEA, Product No. 20408).

A. Materials Required

- Pyridyldithiol-activated Affinity Column (prepared in previous section)
- Sulfhydryl-containing molecule
- PBS containing 1 mM EDTA, which minimizes metal-catalyzed oxidation of sulfhydryls (i.e., disulfide bond formation)

B. Procedure

1. Equilibrate the prepared pyridyldithiol-activated affinity column with three resin-bed volumes of PBS/EDTA. Replace the bottom cap when solution drains down to the top of resin bed. Do not allow the column to drain completely.
2. Dissolve sulfhydryl-containing molecule in one or two resin-bed volumes of PBS/EDTA and immediately add to column.
3. Replace top cap and mix column contents by gentle inversion for 15 minutes.
4. Incubate column upright without mixing for 30 minutes.
5. Uncap column and collect (save) the reaction solution that drains from the column. Replace the bottom cap when solution drains down to the top of resin bed. Do not allow the column to drain completely.
6. Wash column with five resin-bed volumes of PBS/EDTA, collecting (saving) the solution that drains and combining it with that collected in step 5.

   Note: Estimate the coupling efficiency by measuring the absorbance and calculating the concentration of pyridine-2-thione collected in the flow-through/wash fraction, which reflects the amount of molecule immobilized. Measure the absorbance at 343 nm in a 1 cm-wide cuvette; if the absorbance is > 2.0, dilute the sample by a factor sufficient to make it < 2.0.

   The extinction coefficient of pyridine-2-thione at 343 nm is $8.08 \times 10^3\ M^{-1}\ cm^{-1}$, such that its concentration is described by the following equation:

$$\text{Molar concentration} = \frac{\text{Absorbance at 343 nm}}{\text{Molar extinction coefficient} \times \text{Path length}} = \frac{A_{343}}{8080}$$

   Therefore, the amount of molecule immobilized is described by the following equation:

$$\text{moles of immobilized ligand} = D \times V\ \text{ml} \times \frac{1\,\text{L}}{1000\ \text{ml}} \times \frac{A_{343}}{8080}$$

   where D is the dilution factor required to make absorbance measurement < 2, and V is the volume of the flow-through/wash fraction containing the pyridine-2-thione sample.
7. Proceed to affinity purification experiment or store column at 4° C. For long-term storage add 0.05% sodium azide.

Affinity Purifying Samples Using the Reversible Affinity Column

The prepared affinity column may be used in different ways and scales for affinity purification. The immobilized ligand will remain covalently attached to the beaded agarose support as long as disulfide reducing agents (DTT, 2-ME, TCEP, etc.) are excluded from the binding and elution buffers. Alternatively, the immobilized ligand can be eluted together with the captured molecular target using disulfide reducing agents; this strategy is especially useful when the binding interaction is either covalent or of such strong affinity that it cannot be dissociated efficiently with known elution buffers.

Related Thermo Scientific Products

44894	**AminoLink® Plus Immobilization Kit**, reagents and columns for immobilization of amine molecules
44895	**SulfoLink®Immobilization Kit for Proteins**, for irreversible immobilization of sulfhydryl proteins
44899	**SulfoLink®Immobilization Kit for Peptides**, for irreversible immobilization of sulfhydryl peptides
44900	**CarboLink® Immobilization Kit**, for irreversible immobilization of carbohydrate molecules
44899	**CarboxyLink™ Immobilization Kit**, for irreversible immobilization of carboxyl molecules

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.