protein immobilization

Supports that Couple a Variety of Functional Groups

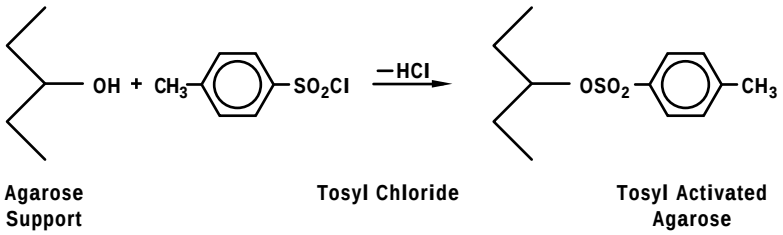
Tosyl Activated Agarose

Ligands, such as antibodies or antigens, can be covalently coupled to affinity supports by many different chemical methods. The availability of primary amines on the surface of proteins makes amine coupling popular for affinity chromatography supports. Tosyl Activated Agarose (Product #20415) can be used to couple primary amines. Hydroxyls on the surface of cross-linked beaded agarose are reacted with *p*-toluenesulfonyl chloride (tosyl chloride) to yield a sulfonated support (see Figure 20). These sulfonates can couple to nucleophiles, such as primary amines or thiols, to yield a stable affinity support.[43] The tosylated support also couples to imidazole, or tyrosine hydroxyl groups.[43]

Tosyl Activated Agarose couples more rapidly and more efficiently to sulfhydryl groups than to primary amines. Sulfhydryl coupling supports are convenient when accessible free sulfhydryls exist in the protein, or when one can be easily generated through reduction of a disulfide bond. Coupling of antibodies through reduced disulfide bonds in the hinge region is a popular way to couple without interfering with the antigen binding region.

Tosyl Activated Agarose couples to primary amines at an optimal pH range of 9-10.5.[43] Reducing agents, primary amines and other strong nucleophiles must be avoided in the coupling buffer because they will compete in the coupling reaction.

Figure 20



Phosphate, HEPES, bicarbonate and borate buffers are recommended. Reducing agents or primary amine containing buffers may be used to inactivate any unreacted sulfonyl groups after the ligand has been immobilized.

Pierce Tosyl Activated Agarose is supplied as 25 gm of powder, and 1 gm of powder will swell to approximately 2 ml of settled gel. Pierce Tosyl Activated Agarose is supplied with a Column Trial Kit, which can also be purchased separately as Product #29925. This kit is included to aid in the column packing. The trial kit includes two columns each of 2, 5 and 10 ml gel volume plastic columns, along with appropriate porous discs and serum separators that may be used for placing discs in the columns. A 25 ml column extender (Product #29923) is also included to provide a reservoir that allows the user to add larger volumes of sample or buffer than the column itself will hold.

References

43. Nilsson, K. and Mosbach, K. (1984). Immobilization of ligands with organic sulfonyl chlorides. *Meth. Enzymol.* **104,** 56-69.

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Tresyl Activated Agarose

Another support that can be used for primary amine coupling is Tresyl Activated Agarose (Product #20417). Hydroxyls on the surface of agarose are reacted with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) to yield a sulfonated support (Figure 21). This sulfonated support is approximately 100-fold more reactive than a Tosyl agarose support. Tresyl Activated Agarose can couple to nucleophiles, such as primary amines or thiols, to yield a stable affinity matrix.[43] The tresylated support also couples to imidazole and tyrosine hydroxyl groups.[43]

Tresyl Activated Agarose couples more rapidly and more efficiently to sulfhydryl groups than to primary amines. Sulfhydryl coupling supports are convenient when a free sulfhydryl exists on the protein, or when a sulfhydryl can be easily generated through reduction of a disulfide bond.

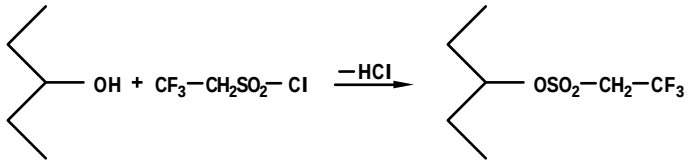
Tresyl Activated Agarose couples to primary amines and sulfhydryls at physiological pH and above.[43] Reducing agents, primary amines and other strong nucleophiles must be avoided in the coupling buffer because they will compete in the coupling. Phosphate, HEPES, bicarbonate and borate buffers are recommended as coupling buffers. Reducing agents or primary amine containing buffers may be used to inactivate any unreacted sulfonyl groups after the ligand has been immobilized.

The incubation time and temperature will vary from protein to protein. Proteins with available sulfhydryl groups will couple faster than those with only primary amines. Coupling to sulfhydryls is very fast at room temperature (1-2 hours), but the reaction time at 4°C is still quite good (5 hours).

The amount of ligand coupled is important for producing a functional affinity support. If the protein loading is too low, non-specific binding may become a problem. If the protein loading is too high, steric hindrance may result. Generally, 1-10 mg of immobilized protein per ml of gel is used to make an agarose affinity support, with values of 2-6 mg of immobilized protein per ml of gel being the most common.

Pierce Tresyl Activated Agarose is supplied as 25 g of powder, along with a column sample kit that contains a variety of columns and accessories to aid in column packing. The support is 4% beaded agarose, and 1 gm of powder will swell to approximately 5 ml of settled gel.

Figure 21



Agarose Support **Tresyl Chloride** **Tresyl Activated Agarose**

References

43. Nilsson, K. and Mosbach, K. (1984). Immobilization of ligands with organic sulfonyl chlorides. *Meth. Enzymol.* **104,** 56-69.

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ImmunoPure® Epoxy-Activated Agarose

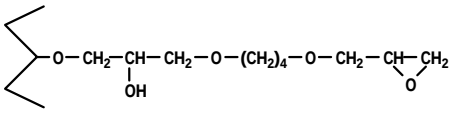
Epoxide chemistry is a useful way to immobilize ligands containing nucleophiles, such as amino, thiol and hydroxyl (including phenolic) functional groups. Epoxide-activated supports are produced by the immobilization of bifunctional oxiranes such as 1,4-butanediol diglycidyl ether onto agarose supports. These activated supports have limited stability in aqueous media, so it is necessary to use them quickly after they are generated or rehydrated. Proteins, carbohydrates, peptides and amino acids are among the ligands that can be coupled to Epoxy-Activated Agarose (Product #'s 20242, 20242). Figure 22 illustrates the reaction scheme for coupling a primary amine to Epoxy-Activated Agarose.

1,4-Butanediol diglycidyl ether-activated agarose forms stable covalent bonds to the immobilized ligand, while introducing a hydrophilic spacer arm between the support and the ligand. Agarose activated with this compound is partially cross-linked during the coupling. Consequently, activation reduces the porosity of the gel to a small extent and increases the rigidity of the support. Ligands are coupled to Epoxy-Activated Agarose at alkaline pH (9-13), usually at 25°C or higher. Coupling to hydroxyls is favored at a higher pH of 11-13. The conditions selected to couple ligands to Epoxy-Activated Agarose will be determined by the stability of the ligand.

A detailed study to determine conditions for coupling proteins, peptides, carbohydrates and amino acids was performed by Sundberg and Porath.[44] Raising pH and increasing temperature and reaction time are methods to improve coupling efficiency.[44] The stability of the ligand will determine which of these factors is the best way to improve coupling. Proteins are coupled at pH 8.5-10 at 25°C in 15-48 hours.[44] Recommended coupling conditions for amino acids, peptides and carbohydrates are pH 9-11 at temperatures ranging from 25-75°C for 4-15 hours.[44] Thiol-containing ligands can be coupled at pH 7.5-8.5.

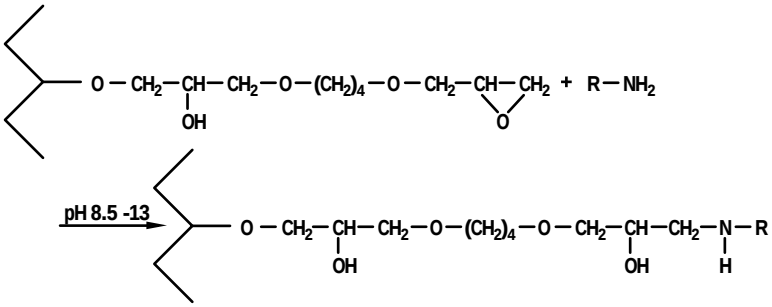
Cyclohepta-amylose immobilized onto Epoxy-Activated Agarose was used to affinity purify cereal α-amylase.[45] The α-amylase was recovered from the support by elution with cyclohepta-amylose.[45] Epoxy-Activated Agarose is an effective means of immobilizing non-reducing sugars for the purification of lectins. The lectins were recovered by competitive elution with carbohydrates. For most of the non-reducing sugars tested, the C6 position is the main site of immobilization.[46] Soybean trypsin inhibitor was immobilized on Epoxy-Activated Agarose and used to bind bovine trypsin at pH 7.8.[44] Trypsin was eluted using low pH and/or high salt, or with competitive binding with benzamidine.

Pierce ImmunoPure® Epoxy-Activated Agarose is available in two package sizes—2 gm of gel (Product #20241) and 10 gm of gel (Product #20242). The support is 6% cross-linked beaded agarose. Upon rehydration, 1 gm of gel will swell to 3-5 ml.



Epoxy-Activated Agarose

Figure 22: Reaction scheme for coupling a primary amine (R—NH)2 to Epoxy-Activated Agarose.



Reaction scheme for coupling a primary amine $(R–NH)_2$ to Epoxy-Activated Agarose

References

44. Sundberg, L. and Porath, J. (1974). Preparation of adsorbents for biospecific affinity chromatography. I. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. *J. Chromatogr.* **90,** 87-98.

45. Silvanovich, M. and Hill, R. (1976). Affinity chromatography of cereal α-amylase. *Anal. Biochem.* **73,** 430-433.

46. Uy, R. and Wold, F. (1977). 1,4-Butanediol diglycidyl ether coupling of carbohydrates to sepharose: Affinity adsorbents for lectins and glycosidases. *Anal. Biochem.* **81,** 98-107.

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Alkylamine Beads

Many different techniques can be used to couple proteins to alkylamine derivatized beads. Coupling with glutaraldehyde is the simplest method. However, it is critical to remove all excess glutaraldehyde before adding protein, so that protein-protein cross-linking does not occur. Preparing acrylamine supports is also possible by reacting Alkylamine Beads with *p*-nitrobenzyl chloride, then binding protein through an azo linkage. A third example of coupling that has been reported is the use of succinylation, followed by EDC (Product #22980).

Adding Sulfo-NHS (Product #24510) to the EDC reaction is reported to increase the efficiency of coupling at a more neutral pH.[47] An example of coupling a carbonyl-containing peptide to Alkylamine Beads using EDC and Sulfo-NHS is illustrated in Figure 23. Conjugating an amine-containing ligand to an amine-containing support can be done in a one-step reaction using dimethylpimelimidate, or DMP (Product #20666).[25] This reaction is shown in Figure 24. A sulfhydryl-containing peptide can also be coupled using the heterobifunctional cross-linker, Sulfo-SMCC (Product #22322), as illustrated in Figure 25.[48]

Pierce Alkylamine Beads (Product #21202) are hexylamine derivatized uniform nonporous spherical polystyrene beads. The bead diameter is 1/4 inch, and the loading is approximately 3 µmoles of amine function per bead.

Figure 23: Coupling a carbonyl-containing peptide to alkylamine beads using EDC and Sulfo-NHS.

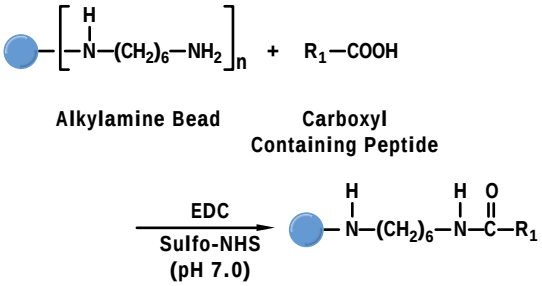


Figure 24: One-step coupling of an amine-containing peptide.

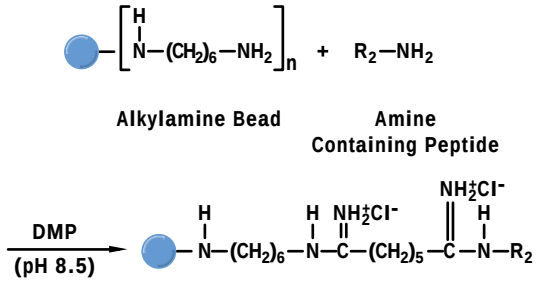
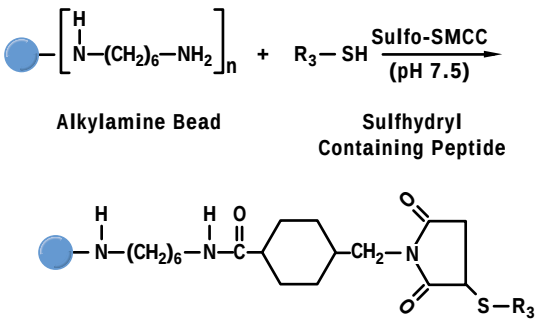


Figure 25: Coupling a sulfhydryl-containing peptide using Sulfo-SMCC.



References

25. Schneider, C., *et al.* (1982). A one-step purification of membrane proteins using a high efficiency immunomatrix. *J. Biol. Chem.* **257,** 10766-10769.

47. Staros, J.V. (1986). *Anal. Biochem.* **156,** 220-222.

48. Hashida, S., *et al.* (1984). *J. Applied Biochem.* **6,** 56-63.

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Underivatized Polystyrene Beads

Polystyrene beads are ideal for a variety of immobilization applications. They are gaining popularity for use in RIA and immunoassay systems. Pierce Polystyrene Beads, Underivatized (Product #23804) are spherical and non-porous, and they have a special finish that greatly increases the surface area of the bead. This makes it possible to load high protein amounts. Proteins can be loaded onto the bead surface by way of hydrophobic adsorption. Hydrophobic interactions occur at alkaline pH values in the range of 8.5 to 10, within 1-2 hours at room temperature. Under these conditions a 1/4-inch bead is capable of adsorbing 1 µg of protein per bead from a 2 µg per bead solution of protein.[49]

References

49. Foster, P.A., *et al.* (1987). *J. Biol. Chem.* **262,** 8443-8446.

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Columns and Accessories

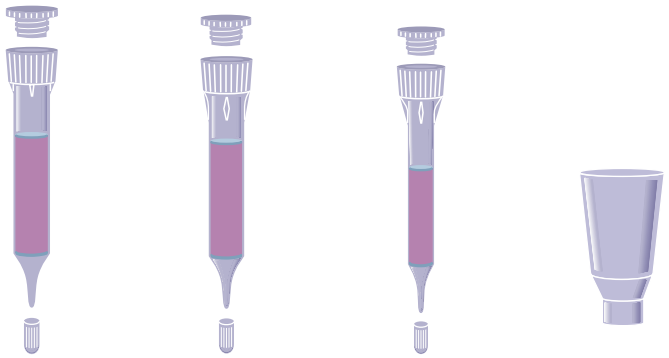
Pierce also offers a variety of columns and accessories. Pierce Reusable Glass Columns (Product #20055) are supplied in a convenient six-pack, complete with an all polyethylene porous disc, end fitting and end cap. Because these columns are made of borosilicate glass, they can be used with both aqueous and organic eluents. These Reusable Glass Columns are designed for compatibility with all types of chromatography. A total volume of 8 ml allows the use of a variety of bed volumes, with sufficient room left above the packed bed to deliver eluents.

Pierce Disposable Plastic Columns eliminate the problem of dried out column gel beds. This is accomplished using porous polyethylene discs. One disc is placed at the bottom of the gel bed and another disc is placed on top of the gel bed. Buffers applied to the packed column will flow through until the meniscus reaches the top disc.

Pierce Disposable Columns are supplied complete with porous polyethylene discs, stoppers and end caps. Stoppers and end caps permit easy storage of packed columns. Columns can be prepacked and stored until ready for use. In addition, these columns are compatible with most types of aqueous buffer eluents that are commonly used in chromatography. Columns are available in a choice of sizes as shown in Figure 26. For convenience, there are also specially designed Disposable Polypropylene Funnels (Product #29923), which fit all three Disposable Columns. Pierce's Disposable Columns Trial Pack (Product #29925) includes 2 of each size of Disposable Column and 2 Disposable Polypropylene Funnels.

Serum Separators (Product #44886) are also available from Pierce. These Serum Separators make column packing easier by helping to correctly place the frit on top of the column gel bed.

Figure 26: Disposable plastic columns.



29924
(16 mm I.D. x 107 mm)
10 ml bed volume

29922
(12 mm I.D. x 107 mm)
5 ml bed volume

29920
(8 mm I.D. x 102 mm)
2 ml bed volume

29923
Funnel fits 29920, 29922
and 29924