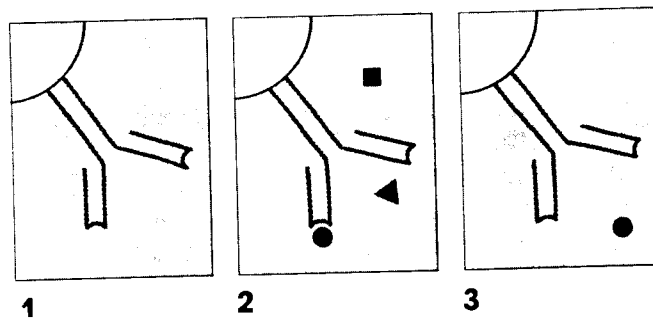


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Immunoaffinity purification is one of the most powerful techniques for the isolation of proteins. Under the proper conditions, purifications of 1,000- to 10,000-fold can be achieved routinely in a single step. Purifications of greater than 10,000-fold have been reported, but are possible only with particularly good antibodies or under particularly unique elution conditions.

Immunoaffinity purification can be divided into three steps:

- (1) preparation of the antibody-matrix
- (2) binding the antigen to the antibody-matrix
- (3) elution of the antigen



In the first step, either monoclonal antibodies or affinity-purified polyclonal antibodies are covalently attached to a solid-phase matrix. There are a large number of different protocols for covalently binding antibodies to a solid phase, but probably the easiest is linking antibodies to protein A beads. After the preparation of the antibody-bead matrix, the antigen is bound to the antibodies, and contaminating macromolecules are removed by washing. In the third step, the antibody-antigen interaction is broken by treating the immune complexes with strong elution conditions, and the antigen is released into the eluate.

## MAJOR CONSTRAINTS

There are several factors that contribute to the success of an immunoaffinity purification. The three most crucial are the starting purity of the antigen, the affinity of the antibody for the antigen, and the ease with which the antibody-antigen bond can be broken.

The relative purity of the starting antigen is the single most important factor in determining the purity of the final product using immunoaffinity techniques. Because of the unique properties of the antigen-antibody interaction, no other type of chromatographic technique is likely to yield greater purification in a single step. However, the degree of purification is not unlimited, as the use of affinity columns has certain inherent background problems. Commonly, 1000-fold purifications are routine, and in most applications 10,000-fold purifications are possible. If the protein antigen of interest is rarer than this, immunoaffinity purification must be combined with other methods to achieve a homogeneous product. This may be accomplished by the use of other purification steps either before or after the immunoaffinity column.

The affinity of the antibody for the antigen is the most worrisome of the problems encountered with immunoaffinity purification. The affinity of the antibody will determine both the total amount of antigen that can be removed from the antigen-containing solution. For antibodies with high affinities ( $>10^8 \text{ mol}^{-1}$ ), quantitative removal can be achieved in less than 1 hr. Even at high antibody concentrations, low-affinity antibodies ( $10^6 \text{ mol}^{-1}$ ) will never bind all of the antigen in the solution. Note that all of the times for reaching equilibrium will be considerably longer for immunoaffinity purifications than for techniques such as immunoprecipitation, because the antibodies are bound to a solid support, thus drastically slowing the kinetics of binding. Tricks that can be used to trap antigen more efficiently, such as using multiple antibodies (p. 31), are not appropriate for immunoaffinity purification. Increasing the avidity by these methods will lead to higher antigen binding, but will also increase the problems encountered when eluting the antigen.

The third factor that will influence the success of an immunoaffinity purification is the relative ease with which the antigen can be eluted. This is determined solely by the type and number of bonds that form the antibody-antigen interaction, and is, therefore, related to the antibody affinity. However, the affinity does not determine whether the antigen will be easy to elute. The ideal antibody for an immunoaffinity purification is one that has a high affinity for the antigen and whose binding can be reversed by a simple but gentle change in an easily manipulatable variable such as pH. The methods for designing these manipulations are described on p. 551. A common mistake in designing immunoaffinity purification experiments is to equate low affinity with easy elution. Although this may prove to be the case for some low-affinity antibodies, often it is not true. The bonds that hold the antigen to the antibody are of the same basic types for both high-affinity and low-affinity antibodies (see Chapter 3). For example, both high-affinity and low-affinity antibodies may bind an antigen through a salt bridge and a hydrophobic interaction. Eluting the antigen in both cases requires both bonds to be disrupted and, hence, poses similar problems for the choice of elution conditions. In addition, low-affinity antibodies introduce new problems, including difficult binding (see above), continual leaching from the column during washing, and gradual, rather than sharp, elution profiles.

## CHOICE OF ANTIBODY

Most immunoaffinity purifications are done with monoclonal antibodies or affinity-purified polyclonal antibodies. In some cases, total polyclonal antibodies may be appropriate, but almost never will pooled monoclonal antibodies be useful.

### Immunoaffinity Purification Using Polyclonal Antibodies

Immunoaffinity purification using polyclonal antibodies has limited applications. Because polyclonal antibodies usually bind to numerous sites on an antigen and therefore bind with high avidity, they are difficult to elute. The harsh conditions needed to elute an antigen when it is bound by several different antibodies usually will damage the antibody column and at least partially denature the antigen. Even when the number of binding sites between the antigen and a polyclonal antibody–matrix is kept low by using saturating amounts of antigen, successful elution will be difficult. In this case, the elution conditions for each antibody–antigen interaction will be different, so efficient elution cannot be established. Another disadvantage when using polyclonal antibodies is that they will contain a number of spurious activities against unrelated antigens. These will include all the antibodies in the animal's serum at the time of collection.

Two types of polyclonal antibodies that are useful for immunoaffinity columns are those that have been raised against synthetic peptides or defined regions of an antigen. In both of these cases, because the epitopes for binding the antigen to the column are located in one small region, it may be possible to achieve efficient elution.

One method to make immunoaffinity purifications possible using polyclonal antibodies is to select specific antibodies on an antigen-affinity column (p. 313). This use is limited to cases where sufficient quantities of antigen are available to prepare these columns. However, in some cases, such as purifying a protein from one species with an antibody against an analogous protein from another species, this may be helpful. Affinity-purifying the polyclonal antibodies solves several problems. Only the antibodies that bind to the antigen are collected, and because they have been eluted from the antigen, the exact conditions for releasing the antigen from the immunoaffinity column already have been determined.

### **■ Immunoaffinity Purification Using Monoclonal Antibodies**

Using monoclonal antibodies for immunoaffinity purifications has a number of advantages compared with other sources of antibodies. Monoclonal antibodies are available in an essentially unlimited supply, and high-affinity monoclonal antibodies can bind to a large proportion of the available antigen. Because all the antibodies are identical and bind to the same epitope, all of the antigen interactions can be broken under similar conditions. Because there are only a limited number of bonds that will hold the antigen-antibody complexes together, the conditions to release the antigen will normally be gentler than those needed for polyclonal interactions.

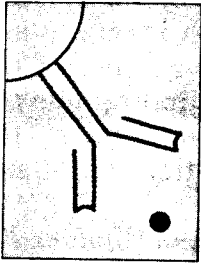
The problems that are found when using monoclonal antibodies for immunoaffinity purification normally concern the properties of antigen interaction, commonly low-affinity reactions or cross-reactions. Low-affinity interactions are a problem common to all immunoaffinity purifications and are discussed above. Cross-reactions are particular to monoclonal antibody purifications and are only seen for a subset of antibodies. In these cases, the antibodies bind to other antigens through shared epitopes. These epitopes may be part of a more extensive homology, in which case the monoclonal antibodies may be useful in studying the related proteins but not for purifying the original antigen. On the other hand, the cross-reactions may be limited to the epitope itself. In this case, using other monoclonal antibodies may eliminate the cross-reaction. Another solution would be to purify the antigen away from the spurious protein by some other chromatographic or extraction technique.

### **■ Immunoaffinity Purification Using Pooled Monoclonal Antibodies**

Except in unusual circumstances, there is no reason to pool monoclonal antibodies for preparing immunoaffinity columns. All of the problems of elution discussed in the section on polyclonal antibody columns apply to pooled monoclonal antibodies. The only common use of these reagents is in the purification of antigens that will be denatured before use or in preparing an antigen-antibody-bead complex for immunizations (p. 135).

**SUMMARY****Immunoaffinity Purification**

Purification factor is 1000- to 10,000-fold



- Rapid antigen purification, columns often reusable
- Yields purified antigen
- Not useful for quantitation
- Efficiency depends on antigen concentration and antibody affinity
- Needs moderate affinity antibody

	<b>Polyclonal Antibodies</b>	<b>Monoclonal Antibodies</b>	<b>Pooled Monoclonal Antibodies</b>
<b>Signal Strength</b>	Good	Antibody dependent, (poor to excellent)	Excellent
<b>Specificity</b>	Good, but high background	Excellent, but some cross-reactions	Excellent
<b>Good Features</b>	Availability	Precise and often gentle elution conditions Specificity Unlimited supply	No advantages
<b>Bad Features</b>	Elution often impossible	Finding suitable antibody	Elution often impossible

## I PROTOCOLS FOR IMMUNOAFFINITY PURIFICATION

Immunoaffinity purification can be divided conveniently into three steps: (1) the preparation of the antibody column, (2) the binding of the antigen to the antibody-bead matrix, and (3) the elution of the antigen from the column (affinity chromatography was first introduced by Cuatrecasas 1968. See *Methods Enzymol.* vol. 34 and Wilchek et al. 1984, for general considerations).

### I Preparing Antibody Affinity Columns

There are a number of methods that can be used for covalent attachment of antibodies to solid-phase matrices. Their advantages and disadvantages are summarized in Table 13.1 along with possible coupling methods. The methods for coupling antibodies to solid-phase matrices are divided into three classes: (1) protein A/G beads, (2) activated beads, and (3) activated antibodies. The most useful of these coupling methods makes use of previously prepared matrices that have been modified to contain secondary reagents binding specifically to antibodies. The most commonly used of these matrices is the protein A beads. Protein A binds specifically to the Fc domain of antibodies, and after the antibody is bound the interaction is stabilized by cross-linking with a bifunctional coupling reagent. The second class of coupling method directly couples the antibody to an activated bead. Beads are activated chemically to contain reactive groups. The beads are mixed and coupled with purified antibodies. The third method of coupling antibodies to beads is to activate the antibody first, placing the reactive group on the soluble antibody. The activated antibody is then mixed and coupled with beads. A variation of this third technique that also combines portions of the activated-bead methods is the biotin/streptavidin approach. For this method, antibodies are modified to contain biotin groups. The antibodies are then bound to streptavidin beads.

For most purposes the matrices that require the least preparation are the best ones to try first. If no background information argues against their use, the protein A bead columns should be tried first. If these present problems, either for reasons of cost or difficulties in the purification, then other methods can be substituted.



**TABLE 13.1**  
**Methods for Coupling Antibodies to Beads**

Method	Variations	Antibody oriented?	Coupling group on antibody	Advantages	Disadvantages
Protein A	Direct coupling	Yes	-NH <sub>2</sub>	Good antibody orientation	Expensive Protein A still available for antibody binding
	Coupling through anti-Ig antibody	Yes	-NH <sub>2</sub>	Easy	
Activated Beads	Carbonyldiimidazole	No	-NH <sub>2</sub>	Cheap Activated beads often commercially available	Antibody often damaged by coupling Multiple steps for activating and coupling
	Cyanogen bromide	No	-NH <sub>2</sub>		
	Glutaraldehyde	No	-NH <sub>2</sub>		
	Hydroxysuccinimide	No	-NH <sub>2</sub>		
	Tosyl chloride	No	-NH <sub>2</sub> , -SH		
Activated Antibody	Carbodiimides	No	-NH <sub>2</sub> , -COOH	Cheap	Antibody may be damaged by coupling Multiple steps for activating and coupling
	Condensing agents	No	-NH <sub>2</sub>		
	Glutaraldehyde	No	-NH <sub>2</sub>		
	Periodate	Yes	Sugar		
Biotin Antibody Streptavidin Beads	—	No	-NH <sub>2</sub>	Bind in solution	Expensive

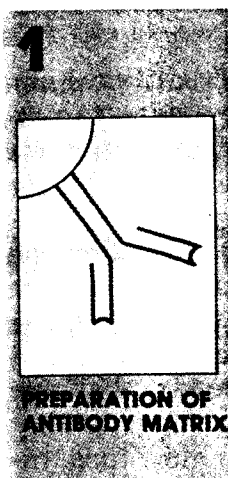
### ***Coupling Antibodies to Protein A Beads***

Protein A bead–antibody columns are one of the most versatile column matrices used for affinity purification. The columns are easy to prepare, and because the antibody molecules are bound to the matrix via the Fc domain, the antigen binding site is oriented correctly for maximal interaction with the antigens.

Antibodies can be bound directly to protein A or can be linked via an intermediate anti-immunoglobulin antibody. Antibodies that have an Fc domain with high affinity for protein A can be linked directly; those with a lower affinity may need the intermediate layer (pp. 615 and 622). Once the antibodies are bound to the protein A beads, they are cross-linked to the protein A via a bifunctional coupling reagent. Any bifunctional reagent can be used, but most workers now use dimethylpimelimidate (DMP), which is cheap and easy to handle. Both binding groups of DMP bind to free amino groups. Because the carbon backbone has a great deal of flexibility, most antibody–protein A pairs will have reactive sites within a suitable distance to allow efficient coupling. In the rare cases where this is not true, other cross-linkers with carbon spacers of different lengths can be used. Antibodies that have free amino groups as a key portion of the antigen binding site cannot be linked using these reagents. Other bifunctional reagents can be used and can be tested easily for coupling antibodies to protein A in small-scale procedures that resemble the techniques discussed below.

Because protein A has different affinities for antibodies from different species, classes, and subclasses (see Table 15.1, p. 617, and Table 15.2, p. 618), it may not be possible to couple a high concentration of every antibody to protein A beads. This can be circumvented by three different approaches. First, the antibodies can be bound using protein G beads. Protein G has a different spectrum of binding affinities than protein A (see Table 15.1, p. 617, and Table 15.2, p. 618), and protein G beads are now available commercially. Second, antibodies with a low affinity for protein A can be bound to an intermediate layer of anti-immunoglobulin antibodies that do have a high affinity for protein A. The most commonly used intermediate antibodies are prepared in rabbits. Third, with some antibodies, particularly mouse IgG<sub>1</sub>s, the affinity with protein A can be increased by adjusting the binding conditions. Normally this is done using high salt concentrations that favor the hydrophobic bonds found in the protein A–Fc binding. Protein G affinity columns are prepared identically to protein A bead columns. The other two methods are discussed below.

One caution that should be included when considering the use of these types of affinity columns is that the protein A molecules on the columns that are not coupled to antibodies will be available for interaction with proteins in the antigen preparation. Therefore, when these columns are used with mammalian antigen sources, the columns will also purify antibodies that are found in the antigen preparation.




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## PREPARING PROTEIN A BEAD-ANTIBODY AFFINITY COLUMNS—DIRECT COUPLING\*

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This method can be used for coupling mouse monoclonal antibodies from the IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>3</sub> subclasses and polyclonal antibodies from mouse, rabbit, human, horse, donkey, pig, guinea pig, dog, or cow.

1. Bind the antibody to protein A beads. Antibodies with high affinity for protein A can be added from any source including serum, tissue culture supernatant, ascites, or purified solutions. If it is essential to know the exact concentration of antibodies on the beads, the antibody should be purified prior to binding to the column (p. 288).

For general-purpose columns, bind approximately 2 mg of antibody per milliliter of wet beads. When using low-affinity antibodies or in other specialized cases, higher concentrations of antibodies may be appropriate.

Mix the antibodies and protein A beads. Incubate at room temperature for 1 hr with gentle rocking. Passing the antibody through a column containing the beads will make a gradient of antibody concentrations. The top of the column will have a high concentration, while the bottom of the column will have a low concentration. To avoid this, the antibodies should be bound by mixing in a slurry.

2. Wash the beads twice with 10 volumes 0.2 M sodium borate (pH 9.0) by centrifugation at 3000g for 5 min or 10,000g for 30 sec.
3. Resuspend the beads in 10 volumes of 0.2 M sodium borate (pH 9.0) and remove the equivalent of 10  $\mu$ l of beads. Add enough dimethylpimelimidate (solid) to bring the final concentration to 20 mM.
4. Mix for 30 min at room temperature on a rocker or shaker. Remove the equivalent of 10  $\mu$ l of the coupled beads.
5. Stop the reaction by washing the beads once in 0.2 M ethanolamine (pH 8.0) and then incubate for 2 hr at room temperature in 0.2 M ethanolamine with gentle mixing.

\*After Gersten and Marchalonis (1978); Schneider et al. (1982); Simanis and Lane (1985).

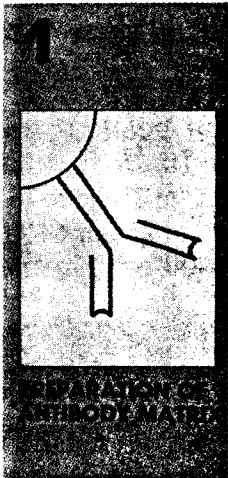
6. After the final wash, resuspend the beads in PBS with 0.01% merthiolate.
7. Check the efficiency of coupling by boiling samples of beads taken before and after coupling in Laemmli sample buffer (p. 684). Run the equivalent of 1  $\mu$ l and 9  $\mu$ l of both samples on a 10% SDS-polyacrylamide gel (p. 636) and stain with Coomassie blue (p. 649). Good coupling is indicated by heavy-chain bands (55,000 mw) in the "before" but not in the "after" lanes.

The affinity columns are stable for over 1 year when stored at 4°C in buffer containing merthiolate.

The beads are now ready for binding of the antigen (p. 541).

## NOTES

- i. If there are small amounts of heavy chains in the coupling check (step 7), prewash the coupled beads with 100 mM glycine (pH 3.0) to remove any antibodies that are bound only by noncovalent binding to the protein A molecules.
- ii. Coupling with dimethylpimelimidate must be performed above pH 8.3. If coupling is inefficient, check that the pH is above 8.3 after the addition of the dimethylpimelimidate.




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**PREPARING PROTEIN A BEAD-ANTIBODY AFFINITY  
COLUMNS—DIRECT COUPLING IN HIGH SALT\***

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This method can be used for coupling mouse monoclonal antibodies from the IgG<sub>1</sub> subclass.

1. Adjust the pH of the antibody solution to pH 9.0. Check the volume and add NaCl to raise the concentration to 3 M. Check the pH and readjust to pH 9.0, if necessary.
2. Bind the antibody to protein A beads. This coupling procedure is normally used for mouse IgG<sub>1</sub> antibodies, and under these salt and pH conditions, protein A beads will bind approximately 2–5 mg of antibody per milliliter of wet beads. Therefore, the binding can be done in antibody excess without worrying about high concentrations of antibody.
3. Mix the antibodies and protein A beads. Incubate at room temperature for 1 hr with gentle rocking. Passing the antibody through a column containing the beads will make a gradient of antibody concentrations. The top of the column will have a high concentration, while the bottom of the column will have a low concentration. To avoid this, the antibodies should be bound by mixing in a slurry.
4. Wash the beads twice with 10 volumes of 3 M NaCl, 50 mM sodium borate (pH 9.0) by centrifugation and aspiration.
5. Resuspend the beads in 10 volumes of 3 M NaCl, 0.2 M sodium borate (pH 9.0) and remove the equivalent of 10  $\mu$ l of beads. Add enough dimethylpimelimidate (solid) to bring the final concentration to 20 mM.
6. Mix for 30 min at room temperature on a rocker or shaker. Remove the equivalent of 10  $\mu$ l of the coupled beads.
7. Stop the reaction by washing the beads once in 0.2 M ethanolamine (pH 8.0) and incubating in 0.2 M ethanolamine (pH 8.0) at room temperature for 2 hr with gentle mixing.

\*After Gersten and Marchalonis (1978); Schneider et al. (1982); Simanis and Lane (1985).

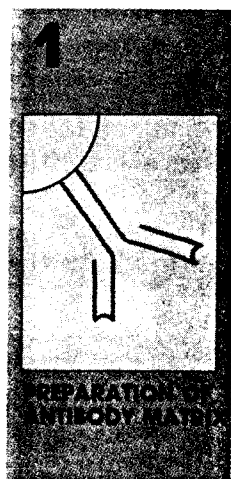
8. After the final wash resuspend the beads in PBS with 0.01% merthiolate.
9. Check the efficiency of coupling by boiling samples of beads taken before and after coupling in Laemmli sample buffer. Run the equivalent of 1  $\mu$ l and 9  $\mu$ l of both samples on a 10% SDS-polyacrylamide gel (p. 636) and stain with Coomassie blue (p. 649). Good coupling is indicated by heavy-chain bands (55,000 mw) in the "before" but not in the "after" lanes.

The affinity columns are stable for over 1 year when stored at 4°C in buffer containing merthiolate.

The beads are now ready for binding of the antigen (p. 541).

### NOTES

- i. If there are small amounts of heavy chains in the coupling check (step 9), prewash the coupled beads with 100 mM glycine (pH 3.0) to remove any antibodies that are bound only by the noncovalent binding to the protein A molecules.
- ii. Coupling with dimethylpimelimidate must be performed above pH 8.3. If coupling is inefficient, check that the pH is above 8.3 after the addition of the dimethylpimelimidate.




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**PREPARING PROTEIN A BEAD–ANTIBODY AFFINITY  
COLUMNS—COUPLING THROUGH AN ANTI-IMMUNOGLOBULIN  
ANTIBODY LAYER\***

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Antibodies from all sources can be coupled to protein A using this technique.

1. Bind the anti-immunoglobulin antibodies to the protein A column (p. 310). Normally these antibodies will be prepared in rabbits. If unfractionated polyclonal antibodies will be used, saturate the protein A beads with antibodies (approximately 20 mg/ml of beads). If affinity purified anti-immunoglobulin antibodies are used, bind approximately 2 mg/ml.
2. Bind the antibody to be used for the immunoaffinity column to the anti-immunoglobulin antibody–protein A beads. Antibodies can be added from any source including serum, tissue culture supernatant, ascites, or purified solutions. The concentration of antibodies on the beads will be determined by the amount of anti-immunoglobulin antibodies, so an excess of monoclonal antibodies can be added to the anti-immunoglobulin antibody–protein A beads.
3. Mix the antibodies and anti-immunoglobulin antibody–protein A beads. Incubate at room temperature for 1 hr with gentle rocking. Passing the antibody through a column containing the beads is fine as long as a saturating amount of antibodies is used (approximately 2 mg/ml of beads).
4. Wash the beads twice with 10 volumes 0.2 M sodium borate (pH 9.0) by centrifugation at 3000g for 5 min.
5. Resuspend the beads in 10 volumes of 0.2 M sodium borate (pH 9.0) and remove the equivalent of 10  $\mu$ l of beads. Add enough dimethylpimelimidate (solid) to bring the final concentration to 20 mM.
6. Mix for 30 min at room temperature on a rocker or shaker. Remove the equivalent of 10  $\mu$ l of the coupled beads.
7. Stop the reaction by washing the beads in 0.2 M ethanolamine (pH 8.0).

\*After Gersten and Marchalonis (1978); Schneider et al. (1982); Simanis and Lane (1985).

8. After final wash, resuspend the beads in PBS with 0.01% merthiolate.
9. Check the efficiency of coupling by boiling samples of beads taken before and after coupling in Laemmli sample buffer. Run the equivalent of 1  $\mu$ l and 9  $\mu$ l of both samples on a 10% SDS-polyacrylamide gel (p. 636) and stain with Coomassie blue (p. 649). Good coupling is indicated by heavy-chain bands (55,000 mw) in the "before" but not in the "after" lanes.

The affinity columns are stable for over 1 year when stored at 4°C in buffer containing merthiolate.

The beads are now ready for binding of the antigen (p. 541).

## NOTES

- i. If there are small amounts of heavy chains in the coupling check (step 9), prewash the coupled beads with 100 mM glycine (pH 3.0) to remove any antibodies that are bound only by the noncovalent binding to the protein A molecules. Check coupling again.
- ii. Coupling with dimethylpimelimidate must be performed above pH 8.3. If coupling is inefficient, check that the pH is above 8.3 after the addition of the dimethylpimelimidate.



### *Coupling Antibodies to Activated Beads*

The most common method of covalently binding antibodies to a solid-phase matrix is to activate beads using any of a number of chemical agents and then bind purified antibodies to the beads (for general reviews, see Porath and Axén 1976; Scouten 1987). This approach offers several advantages. The beads normally can be activated in much harsher conditions than proteins can sustain, thus allowing the use of a range of activating protocols. Preparing activated beads is relatively cheap, and many of the coupling methods yield a linkage that is stable to a wide range of denaturing conditions. Another advantage is that there are a number of activated beads available commercially. Table 13.2 lists five of the commonly used activating agents and recommended commercial products. In general, the commercial beads provide a good source of activated beads for the initial stages of an immunoaffinity purification. For large-scale preparations or for limited budgets, the activation procedures for several of the common methods are given below.

There are a wide variety of beads available for preparing an activated support. Table 13.3 summarizes the properties of several of the most common. Important variations exist in resistance to pH and temperature or nonspecific binding between the various beads. Most beads are more resistant to pH and temperature changes after coupling with antibodies, as this acts to cross-link the beads and may greatly stabilize them.

**TABLE 13.2**  
**Activated Beads**

Reagent	Binding group on matrix	Ligand attachment	Stability of final linkage	Recommended matrix	Commercial examples
Carbonyldiimidazole	-OH	-NH <sub>2</sub>	Avoid pH > 10	Agarose Cross-linked agarose Mixed agarose/polyacrylamide Polyacrylic	Reacti-Gel 6X (Pierce)  Reacti-Gel GF-2000 (Pierce) CNBr-activated Sepharose (Pharmacia)
Cyanogen Bromide	-OH	-NH <sub>2</sub>	Good, some leaching	Agarose Cross-linked agarose Mixed agarose/polyacrylamide Polyacrylic	
Glutaraldehyde	-NH <sub>2</sub>	-NH <sub>2</sub>	Excellent	Polyacrylamide Mixed agarose/polyacrylamide	Act-Ultrigel AcA 22 (IBF) Affigel 10 (BioRad)
Hydroxysuccinimide	-COOH	-NH <sub>2</sub>	Excellent	Cross-linked agarose Modified to contain -COOH	
Tosyl Chloride	-OH	-NH <sub>2</sub> -SH	Excellent	Cross-linked agarose Polyacrylic	Activated Microspheres (KPL)

**TABLE 13.3**  
**Beads for Activation**

Matrix	Chemical stability	pH range	Temperature range	Groups available for coupling	Activators	Commercial examples
Agarose Beads	Fair Avoid chaotropic agents, extended exposure to urea or guanidine	4–10	4–30°C	–OH	Cyanogen bromide Carbonyldiimidazole	Bio-Gel A (BioRad) Sephacrose (Pharmacia) Ultragel A (IBF)
Cross-linked Agarose Beads	Good Avoid extended exposure to chaotropic agents	3–14	4–120°C	–OH	Cyanogen bromide Carbonyldiimidazole Tosyl chloride	Sephacrose CL (Pharmacia)
Polyacrylamide Beads	Excellent Strong alkali on uncoupled beads converts amide to carboxylic	2–10	4–120°C	–NH <sub>2</sub>	Glutaraldehyde	Bio-Gel P (BioRad)
Copolymers of Polyacrylamide and Agarose	Fair Avoid chaotropic agents, extended exposure to urea or guanidine	4–10	4–30°C	–OH –NH <sub>2</sub>	Cyanogen bromide Carbonyldiimidazole Glutaraldehyde	Ultragel AcA (IBF)
Polyacrylic Beads	Excellent Strong alkali on uncoupled beads converts amide to carboxylic	<1–11	–20–120°C	–OH	Cyanogen bromide Carbonyldiimidazole Tosyl chloride	Trisacryl (IBF)

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**PREPARING CARBONYLDIIMIDAZOLE-ACTIVATED BEADS\***

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Antibodies bound to carbonyldiimidazole-activated beads provide a stable complex that is useful for a wide range of elution procedures, but coupled beads should not be treated with strong alkali. Because the activation is done in anhydrous conditions, only beads that are resistant to these conditions should be used. Both cross-linked agarose and polyacrylic beads are good supports. The activating reaction is easy, and the activated beads are stable for several weeks when stored in dry acetone or dioxane.

1. Transfer 10 ml of wet beads to a sintered glass filter. Sequentially wash with 30% dioxane in water, 70% dioxane in water, and 100% dioxane.
2. Resuspend the beads in 10 ml of dioxane and transfer to a dry beaker.
3. Add 0.35 gram of carbonyldiimidazole. Incubate at room temperature for 15 min with mixing on a shaker or rocker.
4. Return the beads to the sintered glass filter. Wash with dioxane, and then remove the dioxane by suction.

The activated beads can be stored for several days in dry dioxane. Store at 4°C.

Use for coupling (p. 536).

**NOTE**

- i. Carbonyldiimidazole-activated cross-linked agarose and polyacrylic beads are available commercially from Pierce.

\*After Bethell et al. (1979); Hearn et al. (1981); Hearn (1987).

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**PREPARING CYANOGEN BROMIDE-ACTIVATED BEADS\***

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Cyanogen bromide-activation is the most commonly used method for preparing antibody-affinity columns. Two advantages of cyanogen bromide (CNBr) coupling are the high capacity and the extensive literature on these types of preparations. Two disadvantages of this method are the highly toxic activating reagent and the isourea bond that couples the antibody to the solid phase. This bond is not as stable as other linkages (Lasch and Koelsch 1978) and adds an extra charged group to the column. The stability will be a major problem only when repeated use of the columns or extended exposure to high temperature, extremes of pH, or nucleophiles (no Tris, azide, etc.) is planned. Because of the additional charge group added by the isourea linkage, all buffers for antigen binding and elution should contain at least 100 mM salt. Agarose, cross-linked agarose, and polyacrylic beads can be activated by CNBr.

**Caution** Cyanogen bromide is extremely toxic. It is volatile and should only be used in a fume hood.

1. Transfer 10 ml of wet beads to a sintered glass filter. Wash with distilled water. Wash with 1 M sodium carbonate buffer (pH 11.0).
2. Add 10 ml of 1 M sodium bicarbonate (pH 11.0) and transfer the beads to a suitable beaker.
3. Move to a fume hood. Weigh out 1 gram of cyanogen bromide (CNBr) and dissolve in 1 ml of acetonitrile. Add the CNBr to the beads.
4. Incubate at room temperature for 10 min with constant agitation. Monitor the pH. Adjust as necessary to keep between 10.5 and 11.0 by adding 4 N NaOH.
5. Transfer the beads to a sintered glass filter. Use suction to draw the CNBr buffer into a vacuum flask containing 100 mM ferrous sulfate to inactivate the CNBr.
6. Sequentially wash the beads with water, several milliliters of 95% acetone, and several changes of 100 mM sodium phosphate (pH 7.5) (or an alternative binding buffer).

Use immediately for coupling (p. 536).

**NOTE**

- i. CNBr-activated beads are commercially available from Pharmacia.

\*Axén et al. (1967); March et al. (1974); Kohn and Wilchek (1984).

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**PREPARING GLUTARALDEHYDE-ACTIVATED BEADS\***

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Glutaraldehyde can be used to activate beads through  $-NH_2$  groups. The glutaraldehyde linkage is very stable with the glutaraldehyde backbone forming a spacer arm between the bead and the antibody in the final product. Polyacrylamide and polyacrylamide-agarose beads provide a suitable support for glutaraldehyde activation.

1. Transfer 10 ml of wet beads to a sintered glass filter. Wash with distilled water and then with several volumes of 0.5 M sodium phosphate (pH 7.5).
2. Transfer the gel to a suitable flask or beaker. In a fume hood add an equal volume of 25% glutaraldehyde (electron microscope grade). Check the pH. It should be between 7.2 and 7.6; adjust if necessary.
3. Gently mix the slurry overnight at 37°C using a shaker or rocker.
4. Transfer the beads to a sintered glass filter and wash extensively with 0.5 M sodium phosphate buffer.

If the gel will not be used immediately, store in phosphate buffer at 4°C. It is stable for several weeks to months.

The gel is now ready for coupling (p. 536).

**NOTE**

- i. Glutaraldehyde-activated beads are commercially available from IBF.

\*Ternynck and Avrameas (1972); Korn et al. (1972); Guesdon and Avrameas (1976).

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**HYDROXYSUCCINIMIDE-ACTIVATED BEADS**

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Of the methods for activating beads, hydroxysuccinimide is one of the least often used, because the beads must be modified to contain carboxylic acids prior to reacting with the *N*-hydroxysuccinimide. Although these carboxylic acid beads can be purchased from several suppliers, there is little or no savings over purchasing the final activated beads. Therefore, a protocol is not included for preparing hydroxysuccinimide-activated beads. Two commercial sources offer hydroxysuccinimide-activated beads. Pharmacia offers a resin activated CH-Sepharose 4B, in which the spacer arm is linked to the beads via an isourea bond (p. 532). Because the isourea bond is less stable than other bonds, BioRad's Affi-Gel, which has linkage through an ester, is recommended.