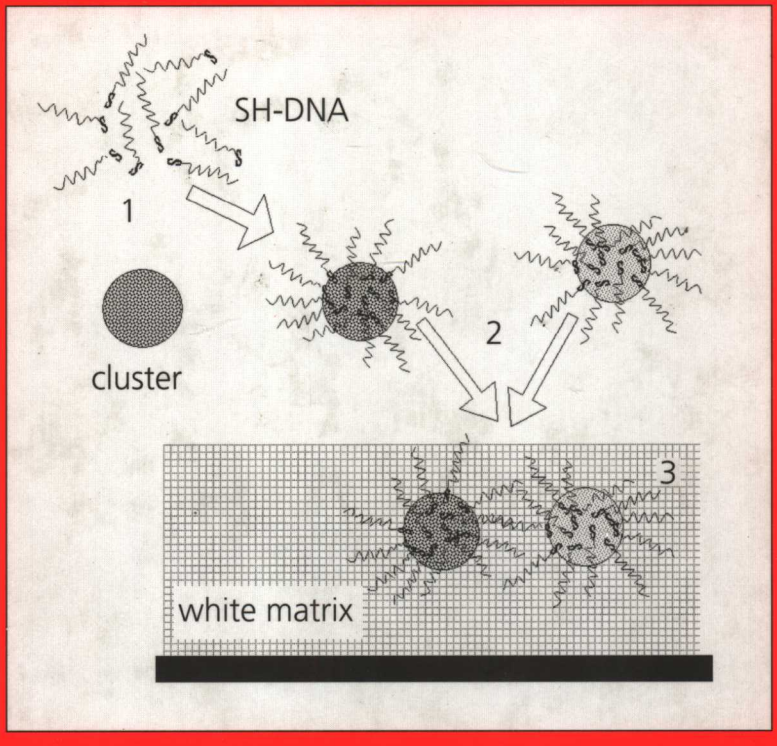
Methods and Tools in Biosciences and Medicine

Analytical Biotechnology

Edited by:
Thomas G. M. Schalkhammer



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Preface

Modern analytical biotechnology is focused on the use of a set of enabling platform technologies that provide contemporary, state-of-the-art tools for genomics, proteomics, metabolomics, drug discovery, screening, and analysis of natural product molecules. Thus, analytical biotechnology covers all areas of bioanalysis from biochips and nano-chemistry to biology and high throughput screening. Moreover, it aims to apply advanced automation and micro fabrication technology to the development of robotic and fluidic devices as well as integrated systems.

This book focuses on enhancement technology development by promoting cross-disciplinary approaches directed toward solving key problems in biology and medicine. The scope thus brings under one umbrella many different techniques in allied areas. The purpose is to support and teach the fundamental principles and practical uses of major instrumental techniques. Major platforms are the use of immobilized molecules in biotechnology and bioanalysis, immunological techniques, immunological strip tests, fluorescence detection and confocal techniques, optical and electrochemical biosensors, biochips, micro dotting, novel transducers such as nano clusters, atomic force microscopy based techniques and analysis in complex media such as fermentation broth, plasma and serum. Techniques related to HPLC, capillary electrophoresis, gel electrophoresis, and mass spectrometry have not been included in this book but will be covered by further publications.

Fundamentals in analytical biotechnology include basic and practical aspects of characterizing and analyzing DNA, proteins, and small metabolites. The structure of this book should provide a clear sense of where each technology is used and how to implement most effectively each technology in developing and using effective, efficient analytical methods for characterizing and analyzing biomolecules. The protocols included as a workshop will be of value to all students, scientists, or regulatory, technical, and quality-insurance personnel.

Additional modules are included that address basic experimental procedures, acquisition of transferable skills, security concerns, and awareness of the commercial and ethical considerations of scientific research. It is clear that not all readers will have practical experience in biotechnology, although a basic understanding of chemistry and physics (optics and electro) is assumed. Coupled with this knowledge the book starts with familiar techniques providing the chemical basis and understanding of how to bind molecules to surfaces and how to analyze chemical surface groups as a basis for further reading. Novices in the field should read chapter by chapter, whereas experts can use any chapter without needing information from others (except where cited). Student readers' initial exposure is simply one of orientation, such that they are able to recognize the equipment and become familiar with some of the basic concepts and further on to operate them routinely. Thereafter, specialized chapters such as AFM or nano-particles introduce novel techniques in a "hands-on" way. A

variety of techniques, which may be used for the identification and characterization of cells and biomolecules down to single-molecule detection, provide a tool-kit for modern bioanalysis. Aside from the instruction to a variety of practical techniques, the add-on-modules also ensure that the reader is equipped with the skills that are essential to the training of good scientists. Risk management and experimental planning are all dealt with if hazardous chemicals are to be used. Further more specialized guidance is offered with respect to focus of the chapters, resources available for the literature review, and practical hurdles that must be overcome.

The central idea is that the book should not be prescriptive but should offer the greatest flexibility. All chapters offer the opportunity to undertake related experiments. The practical information at the application stage is outlined in protocols, associated with helpful information. A wide span of the "level of choice" is offered, starting from simple readout of fluorescence and moving up to single-molecule handling with atomic force microscopes. Detailed protocols ensure that a number of selected experiments may be used as a basis for practicals and courses. Our experience is that basic knowledge combined with protocols permits a greater depth of investigation and a more fruitful application.

Thomas G. M. Schalkhammer März 2002

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Immobilized Biomolecules in Bioanalysis

Fritz Pittner

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1 Introduction

For analytical purpose, immobilized biomolecules may be employed in various areas. Because of their specificity, enzymes have been used for several decades for the assay of analytes in complex samples to avoid laborious purification procedures. Beyond that, immobilized enzymes offer further advantages because they are more stable, reusable, and may be easily attached to microtiter plates, chips, test strips, electrodes, and bioreactors connected to bioanalytical devices.

To isolate or enrich analytes followed by qualitative or quantitative determination, affinity chromatography techniques employing specific and selective biorecognition may be used. For this purpose, antigens, antibodies, receptors, hormones, or other biomolecules exhibiting strong biorecognitive affinities may be immobilized to – in most cases – solid supports. Also, such bio-components may be immobilized to construct new types of ELISAs, gene probes, etc., connected to analytical biochips, microelectrodes, and so on.

In order to reach these aims, it is of vital interest to have good and reliable immobilization techniques at hand, which will be reported in this chapter. Techniques to immobilize biomolecules comprise micro-encapsulation, entrapment in polymeric networks, adsorption, covalent binding, and cross-linking with bi-functional reagents. For our purposes, in most cases these last two techniques turned out to be the most practical and will be treated preferentially, to provide you with recommended, simple, and efficient immobilization techniques to reach your aims.

To attach a biomolecule to a matrix, either reactive groups of this biomolecule or groups of the respective matrix have to be employed. If there are no reactive groups available, they must be introduced. Sometimes cross-linking reagents are useful for coupling, and sometimes spacers are necessary to make the biological ligand more accessible, especially if it is a small molecule.

However, most coupling reactions can be carried out with comparatively few reactive groups, which will be introduced now, together with an appropriate choice of coupling techniques.

Reactive residues of biomolecules recommended for coupling are:

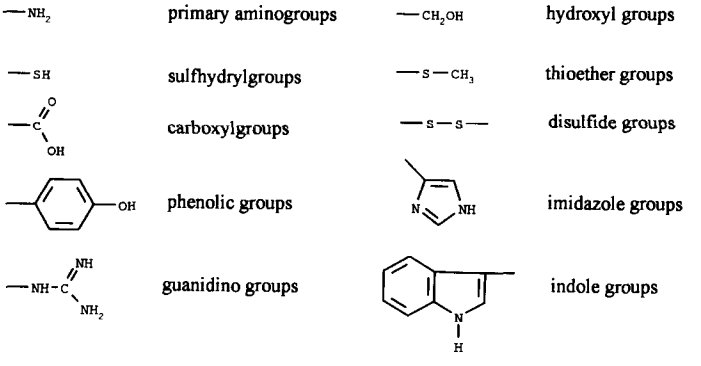

Figure 1

As carrier or support for the biomolecules nearly every nontoxic substance may be used that possesses reactive groups or is capable of introducing them.

2 Methods

2.1 Basic activating and coupling techniques:

Zero cross-linking

The smallest reagents available for bioconjugation are the so-called zero-cross-linkers. They act as activators, mediating the conjugation of two reactive groups of different molecules by forming a bond containing no additional atoms. In this case no intervening linker or spacer will be incorporated.

This is necessary, e. g., in the preparation of hapten-carrier conjugates. The intention here is to generate only an immune response to the attached hapten. Therefore, additional epitopes created by cross-linkers or spacers must be avoided. In this case zero-length cross-linking agents eliminate the potential for this type of cross-activity, since the only mediate is a direct linkage between two substances.

The reactions presented in the following may be performed in aqueous or nonaqueous environments, depending on the desired application.

The types of bonds involved here are:

- amide linkages (i. e. condensation of a primary amine with a carboxyl group)
- phosphoramidate linkages (i. e., reaction of an organic phosphate group with a primary amine)
- secondary or tertiary amine linkage (i. e., reductive amination of primary or secondary amines with aldehyde groups)

Carbodiimides

These compounds are employed to mediate the formation of amide linkages between an organophosphate and an amine [1, 2].

Protocol 1 EDC [or EDAC; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride]

This reagent is very soluble in water as well as the isourea formed as a byproduct of the cross-linking reaction. Both may be removed easily from the desired products by dialysis or gel filtration [3–11]. Caution, the presence of both carboxyl groups and amines on one of the molecules to be conjugated with EDC may lead to partial self-polymerization.

1. The protein to be modified may be dissolved (about 10 mg/ml if possible) in one of the following media:
   * water
   * 0.1 M MES, pH 4.7–6.0
   * 0.1 M sodium phosphate pH 7.3
2. Dissolve the substance to be coupled in the same solution used in step 1. Small molecules should be added in more than 10 molar excess to the protein amount present.
3. Add the solution prepared in step 2 to the protein solution
4. Add EDC to the above solution to obtain a 10-fold molar excess – or more, if possible – of EDC to the protein. Avoid precipitation and scale back to added amount until a soluble incubation mixture is obtained.
5. React for 2 h at room temperature.
6. Purify the resulting conjugate by gel filtration or dialysis. Recommended buffer for this procedure: 0.01 M sodium phosphate, 0.15 M NaCl pH 7.4 works in most cases. Unwanted turbidity may be removed by centrifugation. In case of immunogen conjugates this is not necessary, since precipitated immunogens are often more immunogenic than the soluble ones.

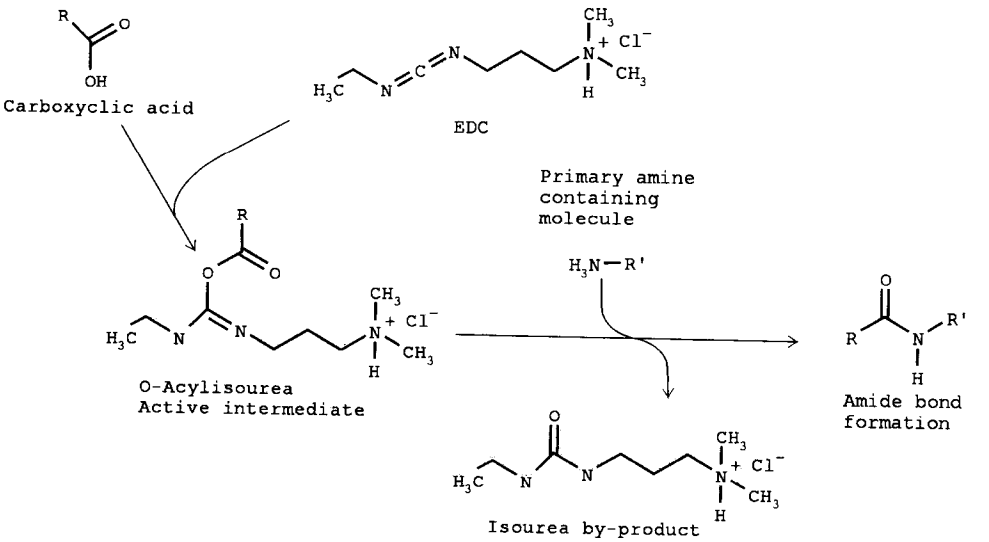

Figure 2

Protocol 2 EDC combined with sulfo-NHS

Sulfo-NHS is able to increase the efficiency of EDC-mediated reactions by forming sulfo-NHS ester intermediates. These intermediates are more stable in aqueous solutions than are those formed from the reaction of EDC alone with carboxylate which results in higher yields of desired amide bond formation [12]. This technique is very useful to create activated proteins [13]. Problems to solve: In addition to potential side reactions of EDC, the high efficiency of the sulfo-NHS mediated reaction may result in insoluble conjugates, particularly when coupling peptides to carrier proteins. In such cases scale back the amount of EDC/sulfo-NHS added to the reaction. Sometimes sulfo-NHS has to be omitted completely.

Protocol

1. Dissolve the protein to be modified (1–10 mg/ml) in 0.1 M sodium phosphate buffer pH 7.4. (To increase the solubility of some proteins; addition of an appropriate amount of NaCl is recommended).
2. Dissolve substance to be coupled in the same buffer in at least a 10-fold molar excess, if solubility allows increase the excess even further.
3. Combine the two solutions to obtain an at least 10-fold molar excess of small molecule to protein if possible.

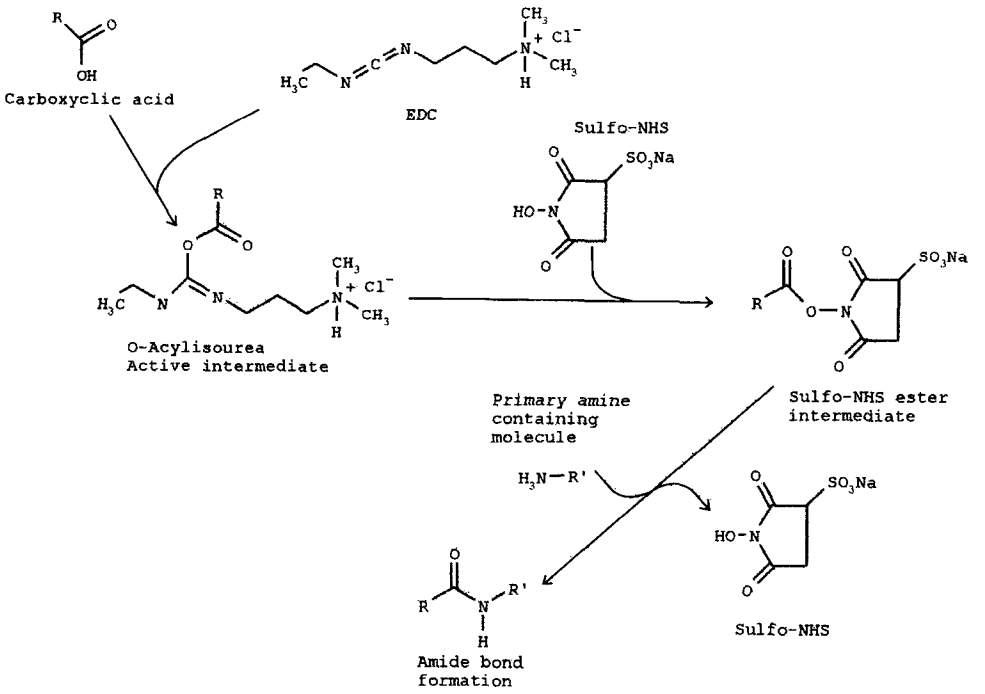

Figure 3

4. Add EDC (at least 10-fold molar excess to the protein) and sulfo-NHS (final concentration 5 mM). Use of highly concentrated stock solutions is recommended. Mix well. If undesired precipitation occurs, scale back the amount of addition until conjugate remains soluble.
5. Incubate for 2 h at room temperature.
6. Purify the product by dialysis or gel filtration.

Schiff base formation followed by reductive amination

Primary and secondary amines can react with aldehydes or ketones to form Schiff bases. Formation of Schiff bases is enhanced at alkaline pH values, but they are not completely stable unless reduced to secondary or tertiary amine linkages by $NaBH_4$ or $NaCNBH_3$[14]. $NaBH_4$ also converts the remaining aldehyde groups into nonreactive hydroxyls, whereas $NaCNBH_3$ will not affect the original aldehyde groups or the activity of some sensitive proteins.

For the following procedure it is assumed that the requisite groups are present either on the two molecules to be conjugated or on the carrier and the biomolecule to be immobilized.

Protocol 3 Schiff base formation followed by reductive amination

1. Dissolve the amine containing compound to be conjugated at a concentration of 1–10 mg/ml in a buffer having a pH between 7 and 10. (The higher the pH, the more conjugate will be formed.) Buffers to be recommended: 0.1 M sodium phosphate buffer or 0.1 M sodium citrate buffer. Do not use amine-containing buffers.
2. Add an amount of the aldehyde-containing compound to the mixture in step 1 to obtain the desired molar ratio for conjugation.
3. Add about 10 ml 5 M cyanoborohydride in 1 M NaOH/ml of the conjugation mixture. Cyanoborohydride is highly toxic: Avoid skin contact, do not inhale, and use a fume hood when carrying out this procedure!!
4. Incubate for 2 h at room temperature.
5. Add 20 ml 3 M ethanolamine (pH adjusted to the desired value with HCl) per ml of the volume of the reaction mixture. Allow to react for 15 min. at r. t.
6. The conjugate may be purified by gel filtration or dialysis using an appropriate buffer. In the case of immobilized biocompounds, the immobilisate may be washed with buffer, 0.1 M NaCl solution, ice cold distilled water followed by buffer.

Homobifunctional cross-linkers

These cross-linkers act as a molecular rope, tying amino group-containing biomolecules together by reacting covalently with the same common groups on both molecules. Also, carriers containing amino groups may be activated with excess of cross-linker to immobilize biomolecules containing primary amino or hydrazide groups.

Activation of hydrazide carriers with glutaraldehyde
This is a good method to immobilize biomolecules to inorganic or organic insoluble carriers, providing spacers long enough to make immobilized ligands or proteins sterically accessible to the bulk solution [15].

Protocol 4 The cyanogen bromide method of coupling

Activation with cyanogen bromide is a good means to activate carriers with plenty of –OH groups for immobilization of biomolecules with sterically accessible aminogroups.
CAUTION: Cyanogen bromide is highly toxic and explosive in the solid state upon friction. It must be kept under alkaline conditions; otherwise, HCN or dicyan will be formed.
Various procedures for cyanogen bromide activation are given in the literature [15]. One of the most convenient is the following one.
Protocol

1. Wash wet packed polysaccaride carriers (10 g) (e. g., Sepharose 4B or Cl-4B (Pharmacia)) with water.
2. Suspend it in 20 ml 2 M K_2CO_3 solution, cooled to 0 °C under gentle stirring or shaking.
3. Activate with 1 ml cyanogen bromide solution in N,N-dimethylformamide (DMF) (1 g/ml) for 90 s.
4. Filter and wash with 50% aqueous DMF followed by ice water and 0.2 M aqueous $NaHCO_3$ solution.
5. Add the substance to be coupled (50–500 mg) dissolved in about 20 ml of appropriate buffer pH 7.6–9.0 (avoid buffers containing primary amino groups) and incubate the mixture by rotating slowly in the cold for 24 h. Decrease in pH may result in a decrease of the amount of ligand coupled but sometimes may be compensated for by a better survival of biological activity. Below pH 6, the coupling efficiency is usually too low to be of practical use. Higher pH may increase the coupling yield, but also may damage the ligand and cause unwanted cross-linking.
6. Remove unbound ligand by washing with buffer.
7. Store wet in the cold before use.

Bisoxirane coupling
This technique is useful for introducing low molecular weight ligands through amino or hydroxyl groups. The reaction proceeds in two steps: (1) activation in which one oxirane group of the reagent reacts with hydroxyl in the polymer matrix, leaving the other group free; (2) the coupling itself, where the remaining oxirane ether is allowed to react with the ligand forming substance containing either amino groups (or other nucleophils) or OH groups. Agarose is one of the best carriers to be activated with this technique. It is recommended to synthesize such a highly activated gel on a small scale to obtain optimal results [16].

Protocol 5 Bisoxirane coupling

1. Wash about 7 ml wet, packed Agarose gel (Pharmacia) with distilled water on a glass filter; remove water by gentle suction.
2. Suspend the gel in 5 ml 1 M NaOH containing 2 mg $NaBH_4$/ml and 5 ml 1,4-butanediol-diglycidylether and incubate the mixture at r. t. for 10 h under slow stirring or gentle agitation.
3. Wash the activated gel thoroughly with distilled water and store until used in distilled water at 5 °C. The gel may be kept about 1 week without loosing its coupling properties.
4. For coupling of the ligand, suspend the gel in 5 ml 0.2 M Na_2CO_3 solution at pH 9, containing up to 600 mg of the ligand. Keep in suspension by stirring gently for 2 days at room temperature. (If the stability of the ligand allows, keep the mixture at higher temperature.)
5. Wash the products sequentially with water, 1 M NaCl solution and the buffer to be used.

Divinylsulfone coupling

This coupling procedure is useful for the activation of hydroxylic polymer carriers for coupling of a ligand:

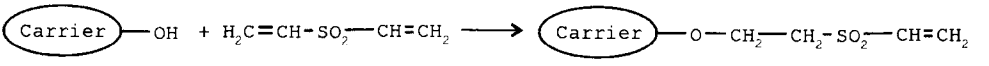

Figure 4

The use of an excess of the reagent is recommended to minimize unwanted cross-linking side reactions. The vinyl groups thus introduced into the matrix are more reactive than the oxirane groups described above. They are able to couple amines alcohols and phenols under very mild conditions [16].

Caution: The products are unstable under alkaline conditions (pH 8–10). Only alkali-stable proteins and other biocomponents may be immobilized by this method.

Protocol 6 Divinylsulfone coupling

1. For activation, 10 ml of packed hydroxyl-containing carrier (e. g., Sepharose) is suspended in 10 ml 1 M Na_2CO_3 solution (pH 11); divinylsulfone (about 2 ml) is added.
2. The suspension is kept for 70 min. on a shaker at r.t.
3. Stop the reaction by adjusting the pH to 7.0 and wash the gel on a glass filter funnel with excess water (about 500 ml) in small portions.
4. For coupling, the activated gel is suspended in 10 ml 0.3 M Na_2CO_3 solution (pH 10) containing about 200 mg of the biocomponent to be immobilized. The suspension is agitated gently on a shaker for 2 h at r.t.

5. Wash the product excessively with the following solutions:
   a) 0.3 M Na_2CO_3 solution (pH 10) containing 1 M NaCl
   b) 0.3 M glycine (pH 3) containing 1 M NaCl
   c) 0.05 M Tris/Cl buffer (pH 7.5) containing 0.5 M NaCl
6. Store the gel in buffer (5 c) at 5 °C
7. Treatment with 1 M glycine (pH 7–8) is recommended to ensure complete blocking.

Immobilization of biomolecules on aldehyde-containing gels

A very good means of obtaining various carriers containing reactive aldehyde groups for coupling of biomolecules is use of spacers containing hydrazide groups followed by glutaraldehyde [15]. Such spacers are useful with low-molecular-weight ligands to increase steric accessibility to the bulk solution. On the other hand, covalent multiattachment of macromolecules (like proteins) with the help of spacers offers the opportunity to stabilize the ligand, but keeps it flexible enough to maintain the biorecognitive properties to a high extent. The following recipes are given to synthesize various types of hydrazido-carriers.

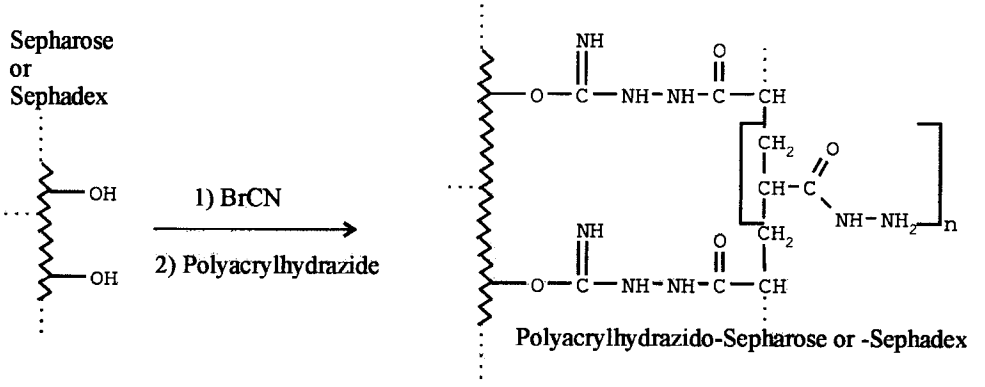

Figure 5 Polyacrylhydrazido-Sepharose

Protocol 7 Polyacrylhydrazido-Sepharose

1. Wash packed Sepharose 4B or Cl-4B (10g) with water, suspend in 20 ml 2 M K_2CO_3 solution, cool to 0 °C and activate with 1 ml cyanogen bromide solution in DMF (N,N-dimethylformamide) (1 g/ml) for 90 s.
2. Filter and wash with 50% aqueous DMF, followed by ice water.
3. Supend the activated gel immediately in 30 ml 0.2 M aqueous $NaHCO_3$ solution containing 0.1–0.6 g water-soluble polyacrylhydrazide and agitate the mixture on a shaker for 16 h at 4 °C.
4. Filter off the gel and wash with 0.1 M NaCl solution until samples of the solution show no color when tested with 2,4,6-trinitrobenzene-sulfonic acid (TNBS).