Acrylamide-based separation matrices

PhD thesis

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Introduction

This study focuses on the development of new acrylamyde-based matrices for the analysis of proteins. Tailor-made polymers can be prepared by co-polymerization of acrylamide and different acrylamide derivatives under specific conditions. The physical and chemical features of the polymers are varied according to the applied monomers and the polymerization conditions. A wide variety of the potential monomers used for the preparation and the chemical modifications make the preparation of hydrophilic, hydrophobic, charged or neutral polymers possible. One part of these polymers is visually transparent, i.e., there are no elements large enough to cause sufficient light scattering to make the matrix opalescent. Charged and non-charged polyacrylamide gels and polymer solutions are homogenous. On the other hand monoliths (continuous beds) are particulate media which consist of small particles that are covalently linked together. The various polyacrylamide polymers are applicable very well in the field of separation science.

Aims

The aims of the present study were as follows:

- Generally: to develop new types of acrylamide-based polymers for electrophoretic and chromatographic analysis of proteins.

- To develop a homogeneous gel (which contains positively charged groups) for on-line enrichment of a sample of negatively charged proteins.
- To design a renewable enzyme reactor based on the molecular imprinting approach.
- To synthesize artificial gel antibodies in the monolithic mode, which recognize and bind the protein that was present during the polymerization.

Materials and methods

Polyacrylamide gels for electrophoresis

Polyacrylamide gels for electrophoretic separations were prepared according to the protocol used in references. We used different types and amounts of monomers for the polymerization. The scheme of preparation was as follows:

The monomers (acrylamide, or acrylamide and 2-morpholino-ethylacrylamide (*Acrylamido buffer pK 6.2*)) and the cross-linker (*N,N'*-methylene-bisacrylamide) were dissolved in 0.02 M sodium phosphate buffer (pH 6.8). Following deaeration ammonium persulfate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were added to the monomer solution. This solution was pipetted into the glass column (i.d. 5 mm), closed at the bottom by dialysis membrane. Isopropyl alcohol was layered on the monomer solution to obtain a smooth gel surface for application of well-defined starting zone and to avoid contact with air (oxygen inhibits the polymerization, which proceeded overnight).

The electrophoresis was carried out in a vertical electrophoresis system connected with a power supply. By employing colored test proteins (R-phycoerythrin, albumin dyed with bromophenolblue) the efficiency of the method was visually illustrated by pictures.

Acrylamide-based polymers for selective recognition of proteins

For the synthesis of artificial antibodies we used non-charged monomers such as acrylamide, methacrylamide, and *N,N'*-methylene-bisacrylamide, piperazine diacrylamide as cross-linkers. The monomer solution contained the proteins of interest (β-galactosidase, hemoglobin). Following addition of ammonium persulfate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) to the monomer solution the polymerization started.

The selective polyacrylamide gel was prepared in glass tube, and then the gel was granulated by pressing it through a 60-mesh (0.25 mm) net and then a 100-mesh (0.15 mm) net.

The selective monolith was prepared *in situ* in the column tube (i.d. 6 mm) made from Plexiglas.

For the removal of the template protein from the gel granules, we used electrophoretic cleaning in the presence of sodium dodecylsulfate (SDS). The monolithic columns were washed with 10 % acetic acid containing SDS.

We used indirect methods such as cation-exchange chromatography and enzyme activity measurement to test for selectivity of the polymers.

Results and discussion

On-line enrichment of protein samples in homogeneous gel

In this study we described a method for on-line enrichment of a sample of negatively charged proteins. The gel column for enrichment and separation consists of 3 gel layer (preventing, concentration and separation gel).

The principle of the method for enrichment of the sample and sharpening of the starting zone is applied to analysis of proteins by polyacrylamide gel electrophoresis: upon the electrophoretic migration of the negatively charged proteins towards the anode, they are electrostatically captured in a thin layer of a polyacrylamide gel to which a weak base (2-morpholino-ethylacrylamide) is immobilized (this layer functions as a weak anion-exchanger). The pK-value of the base must be above the pI of the acidic proteins for their electrostatic interaction with the immobilized positive (amino) groups. The desorption of the adsorbed proteins is achieved by exchanging the buffer used in the enrichment step (where the pH of the buffer should be below the pK-value of the immobilized amino group) for buffer with pH at least one unit above the pK-value.

It should be stressed that desorption of proteins by increasing ionic strength of the buffer – a common approach in chromatography – cannot be employed in the method described herein or any other electrophoretic method because of the strong Joule heat generated, which cause parabolic zone deformations.

This zone-sharpening technique is also applicable to basic proteins if the thin layer for the enrichment of the proteins contains immobilized negative groups, which upon a decrease in pH loose their charge and thus release the proteins in a narrow zone (this layer has the property of a cation-exchanger).

This method for enrichment of a dilute protein solution and subsequent analysis can be applied in many modifications and for different classes of analytes, and may be used in the areas of proteomics.

Polymers for selective recognition

We synthesized polymers for selective recognition of proteins by molecular imprinting approach introduced by Hjertén and co-workers [Liao *et al*., 1996]. The method to synthesize a selective gel is based on polymerization of non-charged monomers (for example acrylamide and *N,N'*-methylene-bisacrylamide) in the present of the template protein. Following polymerization the template is removed. The cavity formed in the gel has a shape which corresponds to that of the template. If a mixture of proteins, one of them being the protein present during the polymerization, is added to the polymer, only this protein will be preferentially adsorbed. So called functional monomers are not used for the preparation of this gels because they often charged and, accordingly, give rise to strong electrostatic interactions and decrease the selectivity. The selective recognition is based on (1) the shape complementarities between the template molecule and its cavity, and (2) the special bonds formed between the template and the "wall" of the cavity in the polymer. This imprinting approach is universal, because it was primary developed for selective recognition of proteins, but with minor modifications it was used for recognition of bacteria [Bacskay *et* al., 2006] and viruses [Takátsy *et* al., 2006]. The synthesis of the polymers is performed in aqueous buffers, in contrast with other imprinting methods, which is unique and make them biocompatible.

The advantages of artificial antibodies are the easy of preparation and the stability of the polymers in harsh situations such as high temperature, pressure, extreme pH values. Molecularly imprinted polymers are considerably less costly to produce than natural antibodies. The approach also obviates the need for animals, which are used in making polyclonal antibodies. In addition, if natural antibody is difficult or impossible to create, artificially prepared mimics may serve as a useful alternative.

In the previous publications two potential application areas of the artificial gel antibodies were demonstrated. These beds can be employed to remove several proteins or bioparticles from a complex biological mixture and are suited to detect

extremely small differences in the chemical composition and the conformation by the electrophoretical analysis of the complex “antigen/gel antibody”.

In this study we showed two more application areas of the selective polymers.

1. Renewable enzyme reactor based on beds of artificial gel antibodies

Polyacrylamide gel antibodies were prepared from the monomers acrylamide and *N,N’*-methylene-bisacrylamide in the presence of β-galactosidase. Former molecular modeling experiments indicated that hydrogen bonds and induced dipole-dipole interactions dominate in the selective recognition of the polymers. The electrophoretic analysis of the complex “antigen/gel antibody” showed that the interaction between gel antibody and the antigen is extremely strong. We experimentally verified that the binding of the antigen (enzyme) is strong enough and in addition the activity of the enzyme is preserved. The polyacrylamide gel granules, prepared in the presence of β-galactosidase after incubation of this enzyme were tested with the solution of *o*-nitrophenyl-β-D galactopiranoside which immediately turn yellow upon contact with the gel bed, an indication of an active enzyme.

Mini reactors prepared in Pasteur pipettes can be designed, which has the advantage to be renewable if the enzyme activity decreases upon denaturation. The use of artificial gel antibodies for enzyme reactors has several advantages, such as, the selectivity is high, they are structurally more stable than conventional protein antibodies, due to the hydrophilic character of polyacrylamide gels and the absence of charged groups they do not exhibit non-specific adsorption of proteins, upon denaturation they can be regenerated by exchange for highly active enzyme and then used repeatedly, and for this regeneration a crude protein extract can be employed, because only the protein that was present during the synthesis of the gel antibodies will be captured.

As antigen we used β-galactosidase, but it is expected that this method is applicable to a great number of enzymes. The excellent properties make enzyme reactors based on artificial gel antibodies very attractive after improvements of its chromatographic properties.

2. Monolithic beds of artificial gel antibodies

The monolithic gel columns were synthesized from hydrophilic monomers to make them biocompatible. One column was prepared to be selective for hemoglobin by co-polymerization *in situ* in a chromatographic column tube of methacrylamide and

piperazine-diacrylamide in the presence of hemoglobin (and ammonium sulfate). The other column was prepared in the same way except that hemoglobin was not present during the polymerization. During the polymerization the polymer chains aggregates due to the hydrophobic interaction promoted by the salt, which results in the formation of channels between the aggregates large enough to permit hydrodynamic flow. The selectivity of the columns was tested after removal of the template protein. The sample, consist of hemoglobin, ribonuclease A and cytochrome *c* was pressed through both columns and the eluates were collected. For the analysis of the composition of the eluates we used chromatography on a cation-exchange column. The monolith prepared in the presence of hemoglobin adsorbed hemoglobin, but not the other proteins, i.e., this monolith was selective for hemoglobin, whereas, the blank (control) column had no selective properties. All three proteins appeared in the ion-exchange chromatogram, i.e., none of the three proteins applied were adsorbed to the monolithic blank column.

The amount of hemoglobin adsorbed by the selective monolithic column was low (~60-65 µg/mL Hb). The amount of protein adsorbed selectively by the monolithic column varies with the concentration of TEMED, APS and the monomers and with the pH and ionic strength of the monomer solution. The optimal experimental conditions are varied for any protein when the protein capacity is of interest.

As template we used hemoglobin, because of its easy accessibility and because it can be monitoring simply by eyes (hemoglobin is a colored protein). Our imagination that this imprinting approach is applicable to a great number of proteins, (for example transferrin, myoglobin) i.e., monolithic columns can be made in the form of artificial gel antibodies, which bind the protein that was present during the polymerization. An advantage of monolithic columns is that they can be synthesized in tubes of any diameter (for capillaries and microchips no frits are required to support the bed, since it can be attached covalently to the tube wall). The monoliths have a property that under certain conditions the resolution is virtually independent of the flow rate. Our selective monolithic column in combination with other types of monoliths (ion-exchange, hydrophobic, affinity chromatography columns) permits the complex separation/analysis of biological samples.

Conclusions

New acrylamyde-based polymers have been introduced for electrophoretic and chromatographic separation systems. The developed and used methods are based on selective and non-selective interactions between the target proteins and the matrices.

Polymers with charged or/and non-polar groups behave as ion-exchange or/and hydrophobic media. Proteins with many charged or/and non-polar groups bind strongly to bed which has a high density of ligands of the same types of groups by virtue of electrostatic or/and hydrophobic interactions. The adsorption is not selective because such a bed can strongly bind many proteins of varying structure.

- A polyacrylamide gel was developed to which a weak base (2-morpholino-ethylacrylamide) was immobilized. At low pH the negatively charged proteins electrostatically adsorbed to this gel, and thus enriched. Upon an increase of the pH the charged gel becomes neutral and releases the proteins. With the appropriate buffer system this homogeneous gel can be applied to enrich diluted protein samples in gel electrophoresis.

It is possible to create specific interactions between proteins and artificial polymers by molecular imprinting process. The selective recognition is based on the large number of weak bonds and the close fit between the protein and its imprint in the polymer.

- A novel type of bed was prepared and tested for enzyme reactor using β-galactosidase imprinted polyacrylamide gel. This type of enzyme reactor can be regenerated by exchange for a highly active enzyme and then used repeatedly. For the regeneration a crude protein extract can be employed because only the protein (enzyme) that was present during the synthesis of the gel antibodies will be recognized and captured.
- Monolithic column was synthesized in the form of selective gel, which binds hemoglobin that was present in the polymerization step. The column was prepared by co-polymerization of methacrylamide and piperazine diacrylamide *in situ* in a chromatographic column tube.

Observe that the objects of this study were to introduce novel types of polymers and to show potential application areas of special types of acrylamide-based polymers. I wanted to use simple devices for the demonstration. Many interesting experiments remain to be done in order to find the optimal experimental conditions.

List of papers

Rezeli M, Kilár F, Hjertén S, Monolithic beds of artificial gel antibodies. *J. Chromatogr. A* (2006) 1109: 100-102. **IF-2006: 3.554**

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Abstracts

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