Effect of chemical structure on molecular recognition

Ph. D. Thesis

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Introduction

Molecular recognition and selective gels

Molecular recognition is the phenomenon responsible for, for instance, binding of an enzyme to a substrate, of a medicinal drug to a biological target, and of two complementary DNA strands together (among many others). It occurs when two molecules are both geometrically and electrostatically complementary, in other words, when they can both "fit together", as well as bind to each other using non-covalent forces ("lock-and-key", Fisher, 1984). These non-covalent forces include hydrogen bonds, electrostatic interactions, hydrophobic interactions and metal coordination. The study of molecular recognition is the major focus of supramolecular chemistry, and is extremely important for the pharmaceutical industry in drug design.

A definition of the molecular imprinting is: ‘The construction of ligand selective recognition sites in synthetic polymers where a template (Atom, ion, molecule, complex or a molecular, ionic, macromolecular assembly, including micro-organisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species.’ [Alexander *et. al.*, 2006]

There are two basic methods in imprinting techniques. The components are either kept in solution before the polymerization with the help of reversible covalent bounds (Wulff and his coworker), or a previous arrangement between the imprinting molecules and the functional monomers through non-covalent and metal-coordinative bounds are established (Mosbach and coworker).

In the latest years many experiments verified, that polymers containing molecular imprints can serve as synthetic binding sites for native antibodies and can be used for recognition in immunoassay based analysis.

A new procedure for selective recognition of proteins was investigated by Hjertén and coworkers in 1996. Selective gels were made against human hemoglobin, cytochrome C, human serum transferrin, human growth hormone, ribonuclease and myoglobine from horse from non-ionic monomers, to decrease the non-specific electrostatic interactions. The selectivity was tested with similar proteins (horse and whale myoglobine). Selective gels were made also against more than one template proteins in the same polymerization.

Chiral recognition

About 40 % of the drugs are chiral compounds. It is known that in most cases the pharmacological activity is connected to one of the enantiomers (eutomer). In many instances unwanted side effects and/or toxicity is coupled to the other optical isomer (distomer). Therefore the separation and detection of the enantiomers even in small amount is very important. For the separation of the optical isomers chiral selectors are used. These can be big, cyclic organic compounds (e.g. crown ethers or cyclodextrines or antibiotics, but also many enantioselective interactions with carbohydrates and proteins (e.g. bovine and human serum albumin, avidin, α-glycoprotein, cellobiohydrolase, casein and human serum transferrin are described.

The stereoselective interactions formed with the enantiomers can be observed with many different separation techniques (most often chromatography and electrophoresis, capillary electrochromatography or coupled techniques). The capillary electrophoresis is a widely used, very efficient separation technique, which makes possible a fast analysis also in the case of analysis of chiral recognition.

In my work two different molecular recognition processes were studied. Chiral compounds were separated with the help of a protein and selective gel granules were used for identification of proteins and bioparticles.

Aims

Experiments to characterize molecular recognition were designed.

Stereoselective recognition by proteins has high importance in both the biological effect of *e.g.*, drugs and mapping of protein surface binding sites.

1. The transferrin is an effective chiral selector. Study the role of the molecular structure in stereoselective interactions through enantioseparations of tryptophan methyl-, ethyl- and butyl esters with human serum transferrin.
   Among the intermolecular forces the electrostatical interactions and the hydrogen bonds playing the most important role in the recognition processes. The pH dependence of this stereoselective interaction should be studied.

2. The effect of the molecular structures of proteins on the molecular recognition in the imprinting technique was studied by different proteins, *e. g.*, human – and bovine hemoglobins, and human serum transferrin.

3. To define the specificity of selective gels a universal electrophoresis based technique was developed.

The previously widely used molecular imprinting techniques were used for selective recognition of small molecules, but there was no such effective and simple tool for viruses and cells (like bacteria).

4. The effectivity and selectivity of imprinted gels were studied upon recognizing macroassemblies, like viruses (*Semliki Forest Virus*) and bacteria (*Escherichia coli*) applying the electrophoretic method.

Methods

Chiral separation with human serum transferrin as pseudo-stationary phase in capillary electrophoresis

Electrophoretic techniques were used to study molecular recognition processes.

Electrophoresis is the migration of electrically charged particles in electric field (Kohlrausch, 1897). The efficiency of the separation in free solution electrophoresis (Tiselius, 1937) is not always sufficient because of the diffusion and flow. To overcome this problem experiments in rotating narrow bore quartz tube were performed in 1958 by Stellan Hjertén. The capillary electrophoresis, what is the successor of this method at present is widely used for separation and identification of many, important biological molecules for example amino acids, peptides, proteins, nucleotides and nucleic acids. A large number of detection techniques can be combined with capillary electrophoresis that also enlarges the number of the possible applications. Beside these advantages the system is very easy to automatize and its setup is very simple.

The capillary electrophoresis allows a fast analysis of small amount of the sample also in the examination of interactions of chiral compounds. One possibility for the chiral selection of small molecules is the use of a protein as chiral selector. The interactions formed between the enantiomers and the stereoselective recognition sites of the protein are differing in their strengths. The two optical isomers interact with the protein for different time (the stronger the interaction, the longer the isomer stays in contact with the recognition site) what cause the separation of the raceme mixture.

Chiral separation of tryptophan derivatives (tryptophan methyl-, ethyl- and buthyl- esters) were made with human serum transferrin as chiral separator. The transferrin was injected with different ‘pressures’ (10 to 100 psi*s) producing partially filled capillary, and electrophoretic experiments were made through the ‘pseudo-stationary’ protein plug at pH 6 that is an average isoelectric point of the human serum transferrin. At this pH no movement of the protein plug was detected and the migration of the tryptophan derivatives through the different plug length (according to the injection; 10 psi*s is equal about 10 mm plug length) can be observed.

Experiments were performed at different pHs, from pH 5 to pH 7, around the isoelectric point (pH 6) of the human serum transferrin to monitorize the influence of the pH on the chiral

separation of the tryptophan derivatives. The exact pH value that accords the isoelectric point of the human serum transferrin (that is a mixture of isoforms) depending on the sialic acid, and iron content of the protein, therefore just an average isoelectric point can be given for the protein. In the above mentioned range transferrin can be used as a pseudo-stationary phase.

Selective gels - Molecular recognition of 'antigens' with molecularly imprinted matrices

Acrylamide gel polymerized in the presence of protein or virus as a template, gives a specific structure. This imprinted polymer after removal of the imprinting template makes possible the selective recognition and the removal of the template from complex mixtures.

The structure of the gel is influenced by the monomer and cross-linker concentrations, and their ratio, by the amount of the catalyst and by the pH. Polyacrylamide gels with the total concentration T = 6 % and the cross-linking concentration C = 5 % were synthesized in the presence of a template. The gel was polymerized from acrylamide (57 mg) as monomer, with N,N'-methylene-bis-acrylamide (3 mg) as cross-linker, dissolved in 980 µl of a 20 mM sodium phosphate buffer, pH 6.8 (prepared by mixing appropriate amounts of 20 mM sodium-di-hydrogen phosphate and 20 mM disodium-hydrogen phosphate, or alternatively, titrating an aqueous solution containing 20 mM of phosphoric acid by NaOH to the desired pH) with 10 µl of a 5 % (v/v) TEMED as catalyst and 10 µl of a 10 % (w/v) ammonium persulphate solution. Degassing of the mixture is very important, because the oxygen inhibits the polimerization.

Selective gels were synthesized in the presence of different template proteins (human and bovine hemoglobin, iron free and iron saturated human serum transferrin), viruses, virus like particle (Semliki Forest virus, wild type and mutant, BK-4 virus like structure) and bacteria (Escherichia coli and Lactococcus lactis). Non-charged granules were also prepared in the absence of any template substance to be used as *blank*. The templates were removed by washing with 50 mM SDS in 50 mM Tris-HCl buffer, pH 8.5 and the granules were cleaned from SDS by washing with buffer (50 mM Tris-HCl buffer, pH 8.5) until no precipitate of potassium dodecyl sulphate in the eluent or the washing solution could be detected upon the addition of potassium chloride (potassium dodecyl sulphate is only slightly soluble in an aqueous solution). These cleaned granules are called *control gel*s or protein-depleted granules. The polyacrylamide is a non-charged polymer, therefore the gel granules moving only after the formation of interactions with a charged entity (e.g. protein, virus). From their movement in electrophoretic field one can conclude whether the interactions are formed or not.

Two procedures were used for the equilibration of the selective gel antibodies with template.

In *Method I* the entire electrophoresis tube was filled with template (antigen) solution. Usually two or three zones, each consisting of 5-10 granules suspended in the buffer, were injected into the rotating tube: for instance, one zone with cleaned, selective gel antibodies (control gel, *i.e.*, gel antibodies depleted of the antigen), one zone with non-selective, blank gel granules and one zone with granules of gel antibodies selective for another antigen than that present in the background solution. The positions of the starting zones were marked by a pen.

In *Method II* the equilibration was accomplished by repeated decantations, *i.e.,* the antigen-free, selective gel antibodies and the blank granules were each separately transferred to a template solution and for equilibration the two test tubes with this suspension were rotated for 30 min at room temperature and then centrifuged. To get rid off non-adsorbed protein the test tubes were filled with buffer (50 mM Tris-HCl buffer, pH 8.5), rotated for 20 minutes at room temperature, centrifuged and decanted. Following addition of buffer this procedure was repeated three times. After this procedure the granules contained only selectively bound protein.

Electrophoretic experiments were performed in the original apparatus of 1958 for free zone electrophoresis in which a narrow bore tube ("capillary") is rotated around its long axis at a speed of about 40 rpm to prevent disturbing sedimentation of the sample zones. The runs were conducted in a 245 mm long glass tube (inner diameter: 2.5 mm, outer diameter: 9.6 mm) coated with covalently attached polyacrylamide to minimize adsorption and electroosmotic flow. The experiments were performed at room temperature, at a running voltage of 500 V. The charged particles migrated towards the anode, and the current was about 50 μA.

A suspension (20-25 μl) of the artificial antibodies (5-10 gel granules) was injected into the rotating "capillary". The voltage was applied and the electrophoretic migration of the granules was visually followed. The mobilities of the particles were calculated from the migration data and given in Tiselius units (10^{-5} cm^2/Vs).

Results

Role of chemical structure in molecular recognition by transferrin

Chiral capillary electrophoretic separations of tryptophan derivatives with different alkylchain-length (methyl-, ethyl- and buthyl-esters) were performed in the presence of human serum transferrin, as pseudo-stationary chiral selector phase. In all experiments the tryptophan-buthylester enantiomers showed the highest resolution and the methyl-ester the smallest whereas the ethyl was in between. The capillary was not totally filled up with protein used as a chiral selector; therefore the mobilities were calculated in a 'fair way', from migration in the absence of the protein and migration in the transferrin plug. Different plug lengths were applied in the experiments, but the calculations gave the same mobilities in transferrin zone. The obvious statement that the chemical structure affects chiral recognition by a protein was demonstrated by the differences between compounds with long alkyl chains (the tryptophan ethyl-, and buthyl-esters showed similar behavior) and the methyl-ester, having only a methyl group and hydrogen, needed higher ionic strength and transferrin concentration for the stereoselective recognition.

We can conclude that a correct, meaningful comparison of chiral recognition of compounds with different structures should be/can be done by applying (considering) identical experimental conditions. For example, where the migration velocities of separands and separators, are different the time for interaction should be equal.

Universal Method for Synthesis of Artificial Gel Antibodies by the Imprinting Approach Combined with a Unique Electrophoresis Technique for Detection of Minute Structural Differences of Proteins, Viruses and Cells (Bacteria)

Selective gels (also called as artificial antibodies) can be prepared and used in molecular recognition processes. The gels are polymerised from acrylamide and N,N'-methylene-bis-acrylamide, as crosslinker in the presence of different template particles ('antigen') The templates can be proteins (e.g human and bovine hemoglobin, iron free and iron saturated

human serum transferrin), viruses (Semliki Forest virus, wild type and mutant, BK-4 virus like structure) and bacteria (Escherichia coli and Lactococcus lactis).

A polyacrylamide gel is neutral and, therefore, granules of this gel do not move in an electrical field – this property was utilized to investigate the selectivity of the artificial gel antibodies synthesized in the presence of the antigen (the template protein). The complex gel antibody/antigen is, thus, charged only in virtue of the charge of the template (e.g. in the case of a protein at a pH which differs from the pI of the protein). When the antigen is completely removed from the gel, it becomes non-charged again. The selective recognition of the template entity can, thus, be studied by electrophoresis of the granular artificial gel antibody.

Previously it was showed with ion-exchange chromatography of the eluate from a column packed with gel antibodies against myoglobin from horse that this protein was adsorbed, but myoglobin from whale not, although the structures of these two myoglobin species are very similar.

In this study it was experimentally verified that a simple, inexpensive and easy-to-handle electrophoresis-based technique also can advantageously be used to monitor selective interactions between a protein and an artificial gel antibody, synthesized by the molecular-imprinting approach. The granules are selective for the protein used as a template during the polymerization. The differences in the 3-D structure between iron-depleted and iron-saturated human serum transferrin is reflected in large differences in the electrophoretic mobilities of the gel antibody – protein complexes, in spite of the facts that there are no or only a very small difference in the mobilities of the free transferrins and that the amino acid sequences are the same.

The same detection technique can be employed for gel antibodies against bioparticles, such as viruses and bacteria.

The combination of synthesis of artificial gel antibodies and electrophoretic analysis of the complex gel antibody/antigen is unique in the sense that - despite its simplicity – it can reveal extremely small differences in the chemical composition and the conformation, not only of molecules, but also of particles.

The results with two different hemoglobin species (human and bovine) show, that the selectivity of the gel granules is high. Both gels recognize the template protein since upon interaction with the negative protein the granules migrate in the electrical field. However, the granules remained uncharged in a solution containing a protein, which is “unknown” to them. No migration of human hemoglobin selective gel was observed in a bovine hemoglobin

solution, although the 3D structure and the amino acid composition are similar for these related proteins. (Experiments are made according to *Method I*)

The interaction between the "empty" 'selective' gel granules and the antigen molecules is fast and strong, since these granules started to migrate immediately after they were injected into the electrophoresis tube and voltage was applied, and in all plots a straight line was obtained, i.e. the hemoglobin molecules do not leave the gel antibody during the electrophoresis run. In other words: the interaction is virtually irreversible under normal experimental conditions (room temperature, atmospheric pressure). It is, however, a question whether the kinetics for some interaction sites is slow, since the mobility of gel granules saturated with protein (*i.e.*, incubated in protein solution – *Method II*) and run in a protein-free background electrolyte was somewhat lower compared to the mobility of granules, submitted to electrophoresis in the protein solution. It should be noted the granulation of the gel makes the granules and the surface of the granules non-uniform. Therefore, only an average binding constant can be determined for the interaction between protein and selective artificial gel antibody granules.

The gel antibodies are very stable: freeze-dried granules, selective for human hemoglobin, regain their selectivity upon rehydration. The selectivity of the gel granules was similar for different batches of the artificial antibodies (the reproducibility is satisfactory). A strong indication that the reproducibility is very high is that we can easily differentiate between minute differences in the structure of the antigens by these gel antibodies. Albumin was not recognized by any of the two hemoglobin-selective gels, indicating no non-specific interaction between albumin and the artificial gel antibody. In fact, one reason why cross-linked polyacrylamide was chosen as matrix was that this gel is highly bio-compatible, i.e., the non-specific interaction with proteins is negligible, which is manifested in narrow and symmetrical zones in SDS-PAGE experiments of proteins.

The synthetic gel antibodies have some distinct advantages over (commercial) protein antibodies; for instance, they are simpler to synthesize and more stable, experimental animals are not required for the synthesis and their high selective since gel antibodies have a larger contact area with the antigen (protein antibodies clasp only part of the antigen). There are also potential application areas, where the artificial antibodies are the only alternative, since protein antibodies against native viruses and bacteria cannot be produced in an experimental animal, because these antigens are metabolically degraded. This is in sharp contrast to artificial antibodies, which with very high selectivity can easily be synthesized against bioparticles.

Gel antibodies synthesized in the presence of Semliki Forest Virus (wild type) interact with this virus, but not with BK-4 virus-like. There is no interaction between the BK-4 virus like structure and the gel antibodies against hemoglobin. The interaction with the virus is time-dependent, since a higher mobility of the gel-virus complex was obtained when the gel was saturated prior to the experiment, compared to the gel-granules, which interacted, with the virus only during the time for the electrophoretic run

A striking example of the extremely high selectivity is the gel antibody synthesized in the presence of Semliki Forest Virus (wild type) interacts with this virus, although also with Semliki Forest Virus (mutant), but to a less degree. The method for synthesis of artificial antibodies gives, accordingly, a very high selectivity since the wild type and the mutant differ only by three amino acids in one of the three proteins on the surface of the virus particle! The high selectivity may in part be explained by the plausible assumption that the entire surface of the antigen is clasped by the gel antibody in sharp contrast to conventional protein antibodies whose contact area with the antigen is much smaller.

The net charge density of the gel antibodies is constant during the experiments, which indicates that the virus particles are strongly attached to the gel antibodies. This is an important finding since it means that one can “fish out” viruses from a highly dilute suspension, which is a prerequisite for many potential applications, for instance, for analysis of blood for HIV, West Nile Fever and hepatitis prior to a blood transfusion.

With the help of the of the universal method described in this study, it is also possible to produce selective gels against macromolecules and bioparticles, such as bacteria. The recognition of the bacteria by the selective gel antibodies was followed by electrophoresis of gel granules becoming charged upon binding the bacteria. As an additional method to prove this imprinting technique, thin layers of blank and Escerichia coli selective gels were also prepared and microscopic technique was used to follow the recognition.

Gels synthesized in the presence of *E. coli MRE-600*, washed with Tris-buffer and then granulated, were charged, indicating that the bacteria were entrapped by the granules. Upon treatment with lysozyme, washing with buffer containing SDS and finally with SDS-free buffer, the selective gel layers showed the same picture as the blank ones. This means that bacteria were enzymatically removed from the gel layer. Observe that bacteria are too large to penetrate into the gel layer, therefore, fewer spot were seen after resaturation. Bacteria attached only to the surface of the gel layer. This microscopical technique can be used for an easy visualization for the control of recognition.

The combination of synthesis of artificial gel antibodies and electrophoretic analysis of the complex gel antibody/antigen is unique in the sense that - despite its simplicity – it can reveal extremely small differences in the chemical composition and the conformation, not only of molecules, but also of particles.

The reason for the extremely high over-all selectivity might be that it is based on three different independent selectivities, originating from (a) the close fit between the antigen and its imprint in the gel (and the possibility that the entire surface of the antigen is clasped by the gel antibody in sharp contrast to conventional protein antibodies, which have only a limited area in contact with the antigens), (b) the nature and the number of bonds between the antigen and the cavity (the imprint) in the gel and (c) the charge (zeta potential) of the gel antibody/antigen complex.

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Conclusions and future

The chemical structure affects chiral recognition by a protein was demonstrated by the differences between compounds with long alkyl chains (tryptophan ethylester and -buthylester) and tryptophan methylester having only a methyl group and hydrogen to be differentiated in stereoselective recognition. Higher transferrin concentration and ionic strength, as well as longer residence time in the protein zone was necessary for successful stereoselective differentiation of tryptophan methylester enantiomers. Further studies will, however, be necessary to clarify the precise role of the structural components in molecular recognition by proteins.

For better understanding of the molecular recognition a special imprinting technique for large biomolecules was used. Human serum transferrin (among other proteins) was used as a template to prepare imprinted polyacrylamide selective gels. A simple, inexpensive and easy-to-handle electrophoresis-based technique was investigated to monitor interactions between the template and the gel selective, for the protein used as a template during the polymerization. The differences in the 3-D structure between iron-depleted and iron-saturated human serum transferrin is reflected in large differences in the electrophoretic mobilities of the gel antibody – protein complexes, in spite of the facts that there are no or only a very small difference in the mobilities of the free transferrins and that the amino acid sequences are the same.

The same detection technique can be employed for selective gel antibodies against bioparticles, such as viruses and bacteria. This technique makes the identification of a given virus or bacterium from complex mixtures (like human blood or urine) very simple. The presence of the template bioparticle can be proved also in a kit-format. The template particles after the selective recognition caged by the imprinted gels can be stained for example, with Coomassie Brilliant Blue or fluorescent stainings. (Results are not discussed here).

Spectrophotometric measurements can be made with the solution at a given wavelength, if selective interactions occur between the selective gel and the sample, after the incubation with the selective gel; the supernatant has a different absorbance value. For this purpose we need selective gels with improved capacity.

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