Effect of chemical structure on molecular recognition

Ph. D. Thesis

Anikó Takátsy

Supervisors

Prof. Ferenc Kilár
Prof. Stellan Hjertén

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Content

Abbreviations

CE	capillary electrophoresis
CEC	capillary electrochromatography
CZE	capillary zone electrophoresis
E. coli	Escherichia coli
EDTA	ethylendiammintetraacetate
EGDMA	etylenglycoldimethacrylate
EMIT	enzyme multiplied immunoassay technique
EOF	electroendosmotic flow
Fig.	figure
Hb	haemoglobin
HIV	Human Immunodeficiency Virus
HPLC	high performance liquid chromatography
IMAC	Immobilised Metal Affinity Chromatography
M	mol/dm^3
MAA	methacrylicacid
MES	2-(*N*-morpholino)ethanesulfonic acid
MIP	molecularly imprinted matrixes
pI	isoelectric point
psi*s	1 pound per square inch*sec =6894.75729 $m^{-1}*kg*s^{-1}$
refs.	references
rpm	revolutions per minute
SDS	sodium-dodecylsulphate
SDS-PAGE	sodium-dodecylsulphate -polyacrylamide gel electrophoresis
SFV	Semliki Forest Virus
SPE	solid phase extraction
TBE	tryptophan butylester
TEE	tryptophan ethylester
TEMED	N,N,N',N' Tetramethylethylenediamine
Tf	human serum transferrin
TME	tryptophan methylester
Tris-HCl	Tris(hydroxymethyl)-aminomethan buffer pH adjusted with HCl
TLC	thin layer chromatography
T-units	Tiselius unit
%v/v	volume/volume%
%w/v	mass/volume%
3D	three dimensional

1. Introduction

After mapping the human genome, proteomics came into the main focus of the biological and biochemical research. An important task is to understand the relationship between the structure and function of proteins. The studies include the recognition and separation of proteins, as well as the determination of the interaction areas on their surfaces which have functional roles.

Among the separation techniques affinity chromatography is one of the newest and most selective methods. In this technique the separation is based on specific interactions between the molecules of a stationary and a through this migrating mobile phase (eluent). Usually enzyme inhibitors or antibodies are bound to the surface of the stationary phase, binding components by specific interactions, while other components are eluted. By changing the composition of the mobile phase the specific interactions (between the enzyme and inhibitor or between the antigen and antibody) can be eliminated, causing the release of the selectively bound component. Affinity chromatography is a kind of molecular recognition process.

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

1.1 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]

The history of molecular imprinting began with the idea of preparation of antibodies, which originates form Pauling [Pauling, 1940]. The first problems were specific recognition and reproducibility to solve. Parallel to the antibody biosynthesis theory, the imprinting of silica gels [Polyakov, 1931; Polyakov *et. al.*, 1937] with small molecules (methylorange) [Dickey, 1949] was reported.

After the initial imprints in 1950's with poor specific recognition and reproducibility, the immobilization of enzymes and cells was managed by entrapment in polyacrylamide gel networks [Bernfeld and Wan, 1963; Mosbach and Mosbach, 1966]. Prearrangement of acrylic monomers with different functionalities gave good results even for smaller molecules. The biological interactions are mainly based on noncovalent forces as it is already known and used in affinity chromatography, one main stream of the molecular recognition processes follow this way [Arshady and Mosbach, 1981; Ekberg and Mosbach, 1989; Mosbach, 1994].

The affinity chromatography and its complementary electrophoretic methods are the most often used separations techniques in biomolecular recognition research [Bernfeld and Wa, 1963]. The high selectivity of the process comes first of all from the usage of immobilized antibodies. A polymer with 'chemical memory' was already synthesized in 1931 [Polyakov, 1931; Polyakov, *et. al.*, 1937; Dickey, 1949], but that could be used only for recognition of small molecules.

The molecular imprinting can be achieved by polymerization of functional monomers in the presence of one or more template molecules [Wulff, Sarhan, 1972; Takagishi and Klotz, 1972; Liao *et. al.*, 1996; Schweitz *et. al.*, 1998]. After removal of the template, cavities remain in the polymer, which corresponds to the original molecule [Vlatakis *et. al.*, 1993]. This process is often used for chiral separation of drugs [Fischer *et. al.*, 1991; Kempe and Moschbach, 1994;], aminoacid derivatives [Kempe and Moschbach, 1995; Wulff, 1995] and carbohydrates, with the use of charged monomers.

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.

Hjertén and coworkers have worked out a new procedure for selective recognition of proteins in 1996 [Hjertén and Liao, 1998]. Selective gels were made against human hemoglobin, cytochrome C, human serum transferrin [Liao *et. al.,* 1996], human growth hormone, ribonuclease and myoglobine from horse [Hjertén *et. al.*, 1997] from non-ionic monomers, to decrease the non-specific electrostatic interactions [Liao *et. al.,* 1996; Hjertén *et. al.*, 1997; Hjertén and Liao, 1998, Tong *et. al.*, 2001]. The selectivity was tested with similar proteins (horse and whale myoglobine) [Hjertén *et. al.*, 1997]. Selective gels were made also against more than one template proteins in the same polymerization [Hjertén *et. al.*, 1997].

1.1.1 Covalent approach

Parallel to that the principles of host-guest chemistry were set out by Cram, Lehn and Pedersen and have been developed for low molecular weight ring or cage systems such as crown ethers [Cram, 1988a; 1988b], cryptates [Lehn, 1988], cyclodextrins [Wenz, 1994], cyclophanes [Pedersen, 1988; Schneider, 1990] and other synthetic or semisynthetic systems. The additional incorporation of catalytically active groups can lead to enzymelike catalysts. Wulff and Klotz have settled independently a basic type of molecular recognition system: imprinting in organic polymers, also called „covalent approach", in the early 1970's [Wulff and Sarhan, 1972; Takagishi and Klotz; 1972]. This molecular imprinting approach involves strong, reversible covalent complex formation between the print molecule and the surrounding polymer. The most common types of linkages are esterbonds of carboxylic/boronic acids, boronate esters, ketals, imines (Schiff bases). These covalent bonds must be cleaved after the polymerisation, which is usually done by acidic hydrolysis. Resaturation of the polymer is achieved through re-establishing of the covalent bonds [Wulff, 1995].
Shea and coworkers have developed reversible ketal formation based systems. The intramolecular functional group separations [Shea and Dougherty, 1986], the influence of the

template (diketone) shape [Shea and Sasaki, 1989] and the site isolation and polymer chain dynamics [Shea and Sasaki, 1991] were well discussed.

Another possibility for covalent imprinting approaches to use metal complexation between the template and the matrix [Dahl and Arnold, 1991; Mallik, *et. al.,* 1994]. This was also used in immobilised metal affinity chromatography (IMAC) for the recognition of proteins containing surface localized histidines.

There are also some applications in which the combination of covalent and noncovalent techniques is used. The template is covalently bound to the monomer during imprinting but the rebinding of the template after the cleavage takes place by noncovalent interactions [Sellergren and Andersson, 1990]. Such polymer was also made for recognition of cholesterol [Whitcombe, *et. al.,* 1995].

1.1.2 Noncovalent approach

The advantage in noncovalent type of imprinted polymer preparation is, that it gives a great variety of possible interactions, namely ionic interactions [Sellergren *et. al.*, 1985], hydrogen bonds [Andersson and Mosbach, 1990; Nicholls *et. al.,* 1995; Chen *et. al.*, 2001], π-π interactions [Dunkin *et. al.,* 1993] and hydrophobic interactions [Nicholls *et. al.*, 1995; Dauwe and Sellergren, 1996]. The greater the variety of the interactions formed between the template and the polymer, the bigger the selectivity of the imprinted polymer. Since the noncovalent interactions are strongly dependent of the polarity of the solvent, these approaches are usually used in organic solvents, such as chloroform or toluene [Mosbach and Ramström, 1996]. Aqueous eluents can improve column efficiency but lower the sample load capacity [Sellergren and Shea, 1995].

For this approach methacrylic acid (MAA) is very often used as functional monomer and ethyleneglycoldimethacrylate (EGDMA) as cross linker. The template has several proton-accepting or hydrogen-bonding functional groups, therefore the rebinding selectivity and affinity are strongly dependent on the number of proton – or hydrogen-bond accepting sites on the template, the basicity of these sites [Dauwe and Sellergren, 1996], the acidity of the functional monomer [Dunkin *et. al.*, 1993] and changes in polymerisation conditions, i.e. polymerisation temperature [Sellergren and Shea, 1993; Lu *et. al.,* 2004], where the optimal value low enough for stabilizing of the monomer-template complex but high enough for the polymerisation process should be found, lower hydrogen-bond capacity of the solvent

[Sellergren and Shea, 1993], pressure, optimum value of the functional monomer, and monomer-template ratio and sample load on [Andersson *et. al.*, 1999].

From the template and functional monomer molecules noncovalent self-assembled complexes formed spontaneously, which are then sterically fixed during the polymerisation. After removal of the imprinting molecules, for what in this case no cleavage is needed (the template simply diffuses out of the polymer by washing with the mobile phase), a macroporous matrix is remained with specific recognition sites (Figure 1).

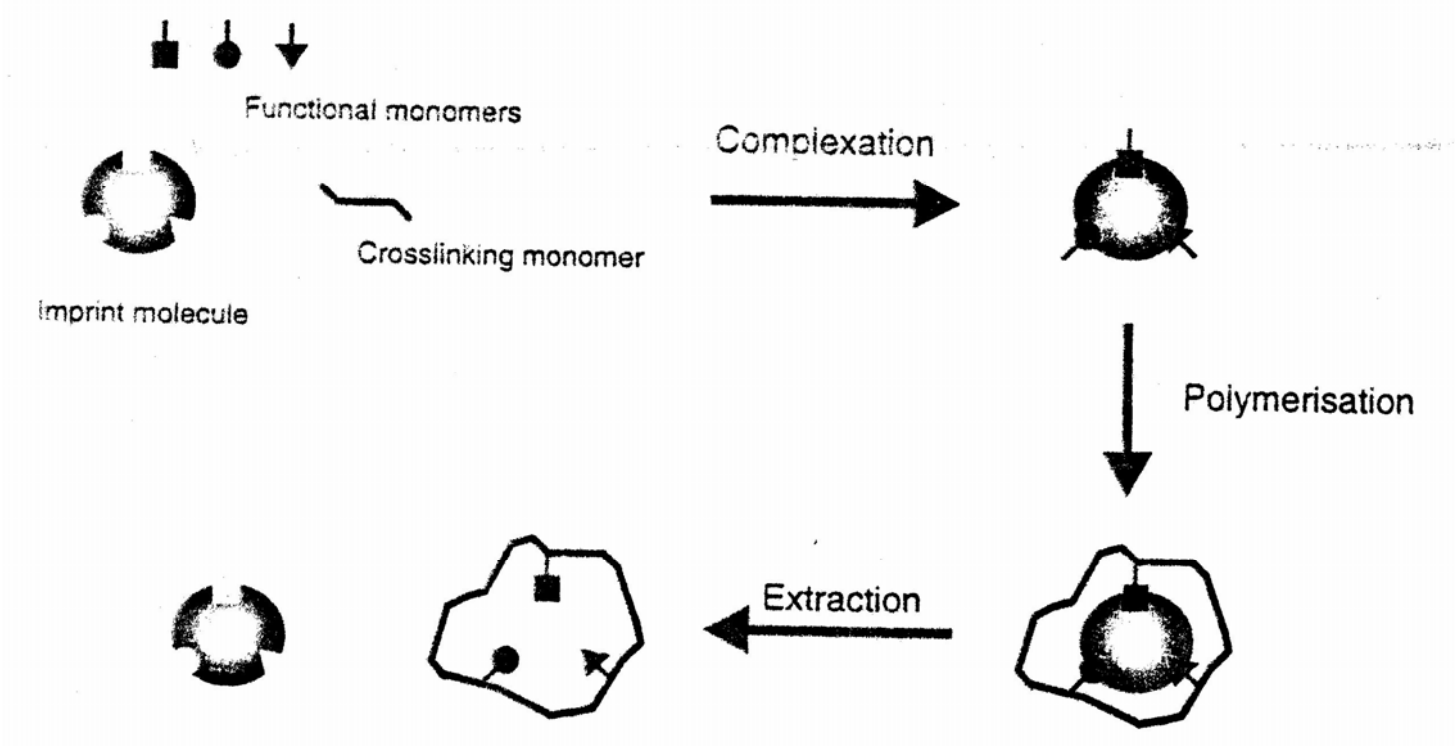


Figure 1. The scheme of the imprinting process using functional monomers

The affinity for the analyte is resulted in by the shape of the sites, maintained by polymer backbone, and the arrangement of the functional groups in the recognition sites. In many cases this technique has proven to be capable of producing materials with rebinding affinities and selectivity in the same order as commonly observed for antibody-antigen interactions [Sellergren, 1997; Kriz *et. al.*, 1997].

1.1.3 Selective gels prepared with polyacrylamide

Specific, imprinted structure can be prepared if the polymerization of acrylamide is performed in the presence of a template substance (e.g. protein, virus or bacteria). After removal of the template the polyacrylamide gel is capable of selective recognition of the template structures from complex mixtures. The structure and porosity of the gel depends on the ratio of the functional monomer and the crosslinker (Figure 2).

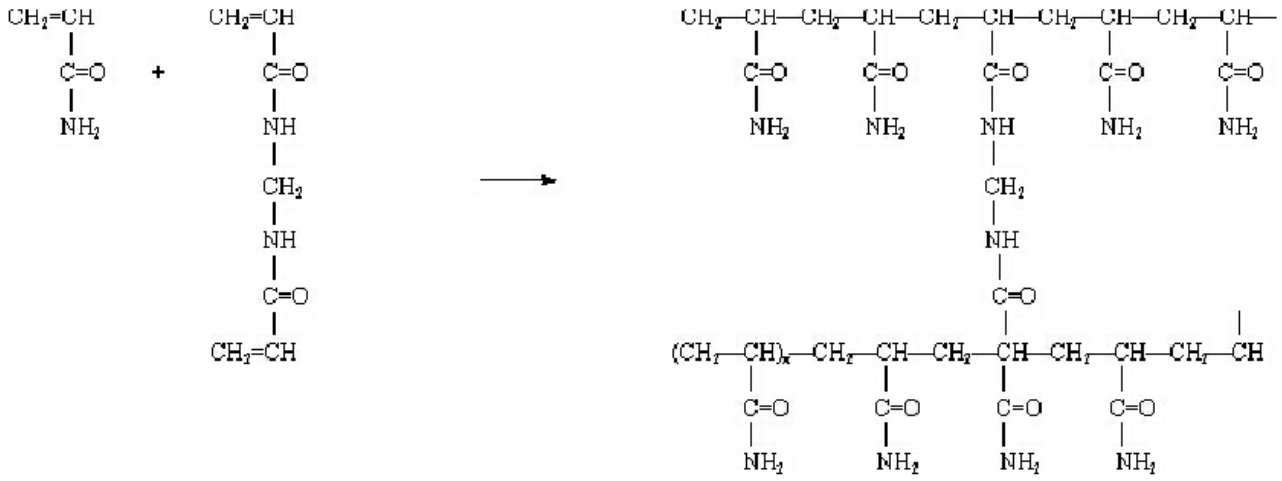


Figure 2. Polymerization of polyacrylamide from acrylamide as monomer and N, N'-methylene-bisacrylamide as crosslinker with the help of TEMED and ammonium-persulphate.

The most common procedure is the synthesis of a bulk polymer that is fragmentised after the polymerisation [Vlatakis *et. al.*, 1993; Kriz *et. al.*, 1995; Liao *et. al.*, 1996, Hjertén *et. al.*, 2003; 2003b; Takátsy *et. al.*, 2002; 2006b; 2006c; 2007; Bacskay *et. al.*, 2006]. Polymers can be prepared also in situ in chromatographic columns [Matsui *et. al.,* 1993] and also in capillaries for electrophoretic systems [Vlatakis *et. al.,* 1993; Schewitz *et. al.*, 1997; 1998; Rezeli *et. al.* 2006]. Since the particle size and shape has a crucial influence on flow properties in chromatography, the homology of the imprinted polymers is very important. Both imprinted coatings have been prepared on silica and trimethylopropane trimethacrylate particle [Norrlöw *et. al.*,1984] and preparation of beads through suspension or emulsion polymerisation have been made [Sellergren, 1994; Hosoya *et. al.*, 1996]. For sensor-devices thin layer or polymer membranes have been developed [Piletsky *et. al.*, 1995; Kriz *et. al.*,

1996]. The polymer can be directly cast as thin layer on the surface of a chip, or glued on the surface of a coated glass plate [Kriz *et. al.*, 1994].

1.2 Characteristics of the molecularly imprinted polymers

There are many benefits and a few drawbacks of using the imprinted polymers preferably to native antibodies. As it was already mentioned the affinity and selectivity of the synthetic entities are comparable to the native molecules. One of the main advantages of the molecularly imprinted particles (MIPs) is that imprints and thus selective recognition system can be made against compounds or particles, against which it is difficult or impossible to raise antibodies (*e.g.*, virus, bacteria) [Takátsy *et. al.,* 2006c; Bacskay *et. al.,* 2006].

Beside that the use of animals for experiments or for production of antibody is avoided, the synthetic polymers are easy to prepare even in the preparative scale. Beside the ethical and economical aspects, other practical points of view are: their long-term stability and resistance to physically [Takátsy *et. al.*¸ 2007] or chemically harsh conditions [Kriz *et. al.,* 1995], in non-aqueous and extreme pH solutions also [Wulff, 1995]. The preparation and purification of antibodies is time-consuming, involves several steps. Immunoaffinity phases are sometimes difficult to regenerate and reuse and their stability is limited. Additionally to the easy preparation of the imprinted polymers, they can be regenerated and reused without any apparent loss of performance even after prolonged use. When a common imprinting process is used, with small adjustment of the recipe (*e.g.*, amount of the cross linker), they may be used for widely different compounds with very high selectivity [Takátsy *et. al.,* 2006b; 2006c; 2007; Bacskay *et. al.*, 2006].

The polymer systems have mainly been synthesized on the base of polyacrylate-/acrylamide [Arshady and Mosbach, 1981; Norrlöw *et. al.*, 1984; Liao *et. al.,* 1996; Ou *et. al.*¸ 2004; Takátsy *et. al.*, 2006c; Bacskay *et. al.*, 2006; Rezeli *et. al.*, 2006] or polystyrene [Andersson *et. al.*, 1984]. Typical functional monomers used are carboxylic acids (acrylic acid, methacrylic acid, vinylbenzoic acid) [Andersson *et. al.*, 1984; Plunkett *et. al.*, 1995; Kempe, 1996], sulphonic acids (acrylamido-methylpropanesulphonic acid) [Dunkin *et. al.*, 1993] and heteroaromatic bases (vinylpyridin, vinylimidazole) [Ramström *et. al.*, 1993; Kempe *et. al.*, 1993]. For metal chelating approaches iminodiaceticacid derivates are often used [Dahl and Arnold, 1991]. The most efficient crosslinking agents are acrylate esters of diols and triols, such as the ethylene glycol dimethacrylate (EGDME) and the trimethylolpropane trimethacrylate [Andersson *et. al.*, 1984; Kempe *et. al.*, 1993; Kempe and Mosbach, 1995].

For styrene systems isomers of divinylbenzene are used. Polysiloxane based polymers are also known [Glad *et. al.*, 1985].

Most of the molecularly imprinted polymers are polymerised in the presence of small molecules as template under organic conditions, but biological systems are aqueous based and macromolecules have a great importance. Hjertén and coworkers have solved these limitations [Hjertén and Liao, 1998] with the preparation of imprinted polyacrylamide gels against different proteins [Liao *et. al.,* 1996; Hjertén *et. al.,* 1996; 1997; 2003a Tong *et. al.*, 2001; Takátsy *et. al.*, 2002; 2006b; 2007; Rezeli *et. al.,* 2006], virus [Takátsy *et. al.*, 2006c] and bacteria [Bacskay *et. al*, 2006]. Although, nonlinear adsorption isotherm and slow mass transfer can cause some problems when imprinted matrices are used in chromatography [Sellergren and Shea, 1995], by the use of the polyacrylamide gels and electrophoresis [Liao *et. al.,* 1996; Hjertén *et. al.,* 1997; Tong *et. al.*, 2001; Takátsy *et. al.*, 2006b; 2006c; 2007; Bacskay *et. al*, 2006] or electrochromatography [Rezeli *et. al.,* 2006], no such limitations appeared.

1.3 Possible application areas of molecularly imprinted matrixes

A large number of various substances have been imprinted for various practical applications [Wulff, 1995; Mosbach and Ramstöm, 1996; Sellergren, 1997]. The main applications can be collected in four big groups. Molecularly imprinted polymer matrixes can be used as:

→ tailor-made separation materials
→ enzyme mimics for catalytic applications
→ antibody mimics in recognition and assay systems
→ recognition elements in biosensors

1.3.1 Separations

There are several separation techniques that can be used in combination with the molecular imprinting approaches. The first to mention is high performance liquid chromatography (HPLC). As HPLC stationary phase monodisperse, spherical particles are needed [Mayes and Mosbach, 1996]. The band broadening and asymmetry effects due to the nonlinear isotherm and the distribution of the binding sites from small amount with high affinity and selectivity, to large amount with worse binding parameters are problems to mention [Sellergren and Shea, 1995]. Beside column chromatography imprinted polymers have been used also in thin layer

chromatography (TLC) [Kriz *et. al.*, 1994]. In solid phase extraction (SPE) the load capacity and the recovery are the most important things [Berrueta *et. al.*, 1995]. The *in situ* polymerisation of the imprinted polymer in capillary electrophoresis (CE) or capillary electrochromatography (CEC) gives certain advantages [Baba and Tsuhako, 1992; Nilsson *et. al.*¸ 1994, Schweitz *et. al.,* 1997; 1998; Rezeli *et. al.*, 2006]. The membrane or thin layer format of the MIPs are usable in sensor applications [Kriz *et. al.*, 1997].

In the field of separations concerning molecular imprinting processes, the chiral separation of racemic mixtures is extensively studied. Chiral separation of drugs [Kempe and Mosbach, 1994], aminoacid derivatives [Kempe, 1995], peptides [Kempe and Mosbach, 1995], proteins [Kempe and Mosbach, 1995; Kempe *et. al.*, 1995], carbohydrates [Wulff and Haare, 1991] and miscellaneous [Ramström *et. al.,* 1993] was examined.

1.3.2 Catalysis and artificial enzymes

The molecularly imprinted polymers in four ways can be used as enzyme mimics in catalysis.

- The use of transition state analogues as templates
- The use of coenzyme analogues
- The use of coordination compounds for mediation of catalytic reactions
- The use of bait-and switch strategies for organization of catalytic groups in the sites.

1.3.3 Antibody mimics

Molecularly imprinted polymers can be used alternatively to natural antibodies, as recognition elements in immunoassays. The first trial for such an assay has been based on competitive radioligand binding protocol [Vlatakis *et. al.*¸1993]. In these promising experiments artificial antibodies were prepared from methacrylic acid (MAA) as functional monomer and ethyleneglycol dimethacrylate (EGDMA) as crosslinker, in the presence of theophylline and diazepam as imprinting template. Through ionic interactions with the carboxyl-group of the functional monomer, hydrogen bonds, dypole-dypole interactions and hydrophobic interactions so high selectivity was achieved that the anti-theophylline polymers were able to distinguish between theophylline and the closely related caffeine. The cross-reactivity of the imprinted synthetic polymers was practically identical to those reported for monoclonal antibodies against these drugs [Vlatakis *et. al.,* 1993]. With the help of the anti-theophylline

polymer and commercial immunoassay techniques (EMIT) significant drug concentrations were able to prove from human patients' serum samples.

1.3.4 Biosensor-like devices

In biosensors a recognizing entity, such as enzyme, antibody or receptor is immobilized at the interface between the sensor and the analyte sample. The binding of the analyte to that recognizing element generates a chemical signal that is subsequently transduced into an electrical signal that is amplified and monitored.

The stability of the molecularly imprinted polymers even under harsh conditions, the possibility of preparation of MIPs in thin layer or membrane format are such unique properties that make them especially suitable for sensor technology.

Different types of sensors have been used in combination with the molecularly imprinted polymers [Kriz *et. al.*, 1995; 1997]. Potentiometric measurements with flow through column electrode for distinguishing of enantiomers of amino acid derivatives [Andersson *et. al.,* 1990], permeation measurements through imprinted membranes [Piletsky *et. al.*, 1995] biomimetic sensor based on capacitance measurement on field-effect transistor, conductometric measurements [Kriz *et. al.*, 1996] have been made in this field.

1.4 Bio-imprinting

The use of the idea of making molecular imprinting is not limited only to synthetic polymers. Natural polymers, such as proteins can also be used as basic element of preparation of imprint. This approach can open new way in biotechnology with e.g. creation of new function for an enzyme. In experiments of Mosbach and coworkers, after enzyme inhibitor complex had formed, the conformation of the enzyme was fixed with organic solvents. After removal of the inhibitor, the enzyme was able to synthesize D-amino acid esters in additional to the natural L enantiomers [Stål *et. al.*, 1991]. Changing the substrate of an enzyme is also possible [Johansson *et. al.*, 1995], or creation of new binding sites for small molecules [Dabulis and Klibanov, 1992].

1.5 Chiral recognition

The optical isomers are asymmetric compounds, which differ from their mirror image. For enlightening this quality often our left and right hands are mentioned as model system. The name comes also in chemistry from the Greek word kheir (hand) (Kelvin, 1884). The isomers, differing only in how their solutions rotate the plane of the planar polarized light, are called enantiomers. Originally, the terms D (dextro, for right) and L (laevo, for left) were meant to indicate the direction of rotation of plane of polarization of polarized light. Even though it is true for glyceraldehyde, that the solution of D- glyceraldehyde (what was the model for this nomenclature) rotates the plane of polarization to the right and the solution of L-glyceraldehydes to left, as many other monosaccharides also do, but this correspondence does not hold in all cases. Additionally for this system always some reference (e.g. glyceraldehydes) molecules are needed. There for the so-called R-S system was described, to give the absolute configuration for stereoselective compounds (Chan, Ingold and Prelog, 1956). In this system each functional has a certain priority ($OR>OH>NH_2>CO_2H>CHO>CH_2OH>CH_3>H$). If the molecules is viewed with the group of the lowest priority away from us and according to the above described priority row the priority decreases clockwise in the remaining three groups the absolute configuration is called R (from the Latin word rectus=right), if it decreases counterclockwise, the absolute configuration is S (sinister=left). According to this nomenclature, D-glyceraldehide has R absolute configuration and L-glyceraldehide has S.

About 40 % of the drugs are chiral compounds [Gübitz and Schmid, 2000]. 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 [De Boer *et. al.*, 2000; Fanali, 2000; Amini, 2001; Rizzi, 2001] or antibiotics [Gübitz and Schmid, 2000], but also many enantioselective interactions with carbohydrates [Sutton *et. al.,* 1997] and proteins (e.g. bovine and human serum albumin, avidin, α-glycoprotein, cellobiohydrolase, casein and human serum transferrin are described [Kilár and Fanali, 1995; Kilár, 1996; Schmid *et. al.*, 1998; Gübitz and Schmid, 2000; Haginaka, 2000, Visegrády *et. al.*, 2000a; 2000b; 2000c; 2000d; Kilár and Visegrády, 2002].

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) [Fanali *et. al.*, 2001].
The capillary electrophoresis is a widely used very efficient separation technique, which allows a fast analysis of small amount of the sample also in the examination of interaction of chiral compounds.

1.6 Theoretical background of electrophoresis

Electrophoresis is the migration of electrically charged particles in electric field [Kohlrausch, 1897]. This method is widely used for separation and identification of many, important large biological molecules for example amino acids, peptides, proteins, nucleotides and nucleic acids.
The diffusion and flow caused by the heat in free solution decrease the efficiency of the separation. To minimize the influence of these unwished effects the electrophoretic experiments are mostly carried out in polyacrylamide or agarose gel. Although the size-depending technique of the electrophoresis in viscose matrix is widely used in the separation of biomolecules, there are still a few questions to answer. The long analysis time, small efficiency, problems with the detection and automatization are problems to solve.
Hjertén published the first results on "capillary electrophoresis" that was a break through in the solution of the mentioned problems [Hjertén, 1958]. He used a narrow bore glass tube instead of the usual slab format that must not be filled with the viscose matrix (causes heating and therefore hinders the flow). With that step the electrophoresis in free solution became possible. The narrow bore tube was rotated around its longitudinal axis, eliminating the settle down of the migrating components that could have been a problem with such large biomolecules as proteins.
At the beginning of the 80's Jorgenson and Lukacs used the first real capillary with 75 μm inner diameter. Jorgenson clarified connections between the acting parameters and the quality of the separation. The use of a capillary brings many advantages. The solution (electrolyte) in the narrow bore tube has a high resistance; this makes possible the application of high electric field, what shorten the analysis time, while the dissipated achievement decrease with its square root ($P = I^2R$). The big surface/area ratio results good distribution of the generated heat. This technique makes possible quick separations with big efficiencies and resolutions with the necessity only a small amount of the sample and solvents. The theoretical plate

number can achieve the 10^5 range that result narrow peaks on the electropherograms and therefore complex mixtures with a huge number of different components can be separated. As long as the laminar flow of the chromatographic systems as a parabolic flow profile, in the electrophoretic systems, the big value of the theoretical plate number is caused by plug-like flow profile. Also an advantage of the electroendosmotic flow, that either charged and non-charged, chemically different components can be examined in these systems. The charged components migrating because of their own charges, as long as either complexes can be made from the non-charged components, or they can be brought into micelles, or migrating with the endosmosis.

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.

1.6.1 Stereoselective recognition in capillary electrophoresis systems

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.

In a noncrosslinked polyacrylamide coated capillary [Hjertén, 1985] interactions between the capillary wall and the sample could be eliminated. When human serum transferrin is injected partially into the capillary and the pH of the background electrolyte is adjusted to a pH (pH 6), which is around the isoelectric point of this protein (it is a mixture of isoforms with slightly differing isoelectric points), no observable movement of the protein zone can be found. The protein thus forms a continuous zone at the injection side of the capillary and the charged enantiomers migrate through this protein plug toward the detector, interacting with the recognition sites on the protein surface (Figure 3).

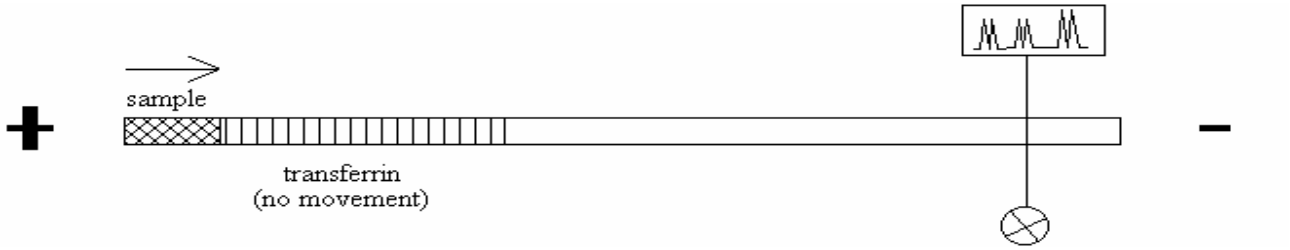


Figure 3. Experimental setup of chiral separation by zone electrophoresis through a pseudostationary transferrin plug. Polyacrylamide (PAA) coated capillary, pI (Tf) = 6, background electrolyte (BGE): 100 mM MES, pH 6

Since the enantiomers are recognized with different binding strength by the chiral protein surface, the migration velocity of the enantiomers will be different. In this way the stereoselective recognition results in separation during the electrophoretic movement, the components arrive at different migration times to the detector.

2. Aims

Experiments to characterize molecular recognition were designed.

1. Stereoselective recognition by proteins has high importance in both the biological effect of *e.g.,* drugs and mapping of protein surface binding sites. The transferrin is an effective chiral selector. The role of the molecular structure in stereoselective interactions was studied in enantioseparations of tryptophan methyl-, ethyl- and butyl esters with human serum transferrin.
   The pH dependence of this stereoselective interaction was also 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*., hemoglobins and transferrins.
3. To define the specificity of selective gels a universal electrophoresis based technique was developed.
4. The effectivity and selectivity of imprinted gels were studied upon recognizing macroassemblies, like viruses (*Semliki Forest Virus*) and bacteria (*E. coli*) applying the electrophoretic method.

3. Materials and Methods

The chemicals and the methods are described in details in the articles published [Takátsy, *et. al*., 2006a; b; c; 2007; Bacskay, *et. al*., 2006]

3.1 Proteins and bioparticles

3.1.1 Preparation of human hemoglobin

Human blood (5 ml) was delivered in an EDTA containing test tube from the University Hospital in Uppsala and centrifuged at 3600 rpm (radius 9.5 cm) for 10 minutes. The supernatant was decanted and the pellet, containing the red blood cells, was suspended in 0.9 % NaCl solution and centrifuged at 3600 rpm for 5 minutes. This washing procedure with NaCl solution was repeated three times. A 200-µl suspension of red blood cells was mixed in an Eppendorf tube with 800 µl of deionised water to lyse the cells. The tubes were centrifuged at 11000 rpm for 3 minutes to spin down cell debris and different types of organelles. The supernatant (containing hemoglobin at a concentration of approximately 300 mg/ml, determined with photometry) was removed and stored in a freezer in Eppendorf tubes.

3.1.2 Viruses and bacteria

Semliki Forest Virus (SFV, *Togaviridae*, diameter: 70 nm, particle weight: 7 x 10^6), a mutant of SFV, differing from wild type only by 3 amino acids in one of the capsid proteins, and BK-4 virus like structure (*Papovaviridae*, diameter: 55 nm) were obtained as suspensions at a concentration of 5 mg/ml (kind gifts of Holland Cheng, and Josephina Nilsson, Karolinska Institute, Stockholm, Sweden). The structures of SFV (wild type and mutant) [Acheson and Tamm, 1967; Kenney *et al.*, 1994, Forsell *et al.,* 2000, Mancini *et al.*, 2000; Lescar *et al.*, 2001; Garoff and Cheng, 2001; Sedzik *et al.*, 2001; Roussel *et al.*, 2006] and BK-4 virus like particles [Belnap *et al.*, 1996; Frisque, 1999;Twarock, 2005] are described in the literature.
Suspensions of *Escherichia coli* MRE-600 and *Escherichia coli* BL21 (Gram-negative bacterial strains), and *Lactococcus lactis* (a species of non-sporulating, non-motile, Gram-positive bacteria) were obtained from Centre for Surface Biotechnology, Uppsala University (Uppsala, Sweden). The concentration of the bacterial suspensions was adjusted by dilution in

order to have a suspension having 200-500 cells in the view field of a microscope (Photomicroscope III, Zeiss, Germany). Moeller-Hinton broth/agar was used for cultivation and the strains were identified by biochemical tests [Farmer, 2003; Bopp *et al.*, 2003]. *Escherichia coli* cells are elongated (rod-shaped), 1-2 μm in length and 0.1-0.5 μm in diameter.) *Lactococcus lactis* are cocci that group in pairs and short chains, typically 0.5 - 1.5 μm in length.

3.2 Selective gels (gel antibodies)

3.2.1 Synthesis of selective gels and neutral, non-interacting blank gels

The selective gels were prepared according to the protocol used in refs. [Liao *et al.*, 1996; Hjertén *et al*., 1996 1997; Tong *et al.*, 2001; Takátsy *et al.*, 2006b, 2006c, 2007; Bacskay *et al.*, 2006]. This scheme includes mixing of the template protein, virus or bacterium with the monomer solution, polymerization, gel-granulation, removal of template molecules (with various methods), and – if required – a re-establishment of the template-gel complex. The detailed experimental conditions were as follows.

Polyacrylamide gels with the total concentration T = 6 % and the cross-linking concentration C = 5 % [Hjertén, 1962] were synthesized in the presence of a template from acrylamide (57 mg), N,N'-methylene-bis-acrylamide (3 mg), 10 μl of a 10 % (w/v) ammonium persulphate solution and 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).

The templates are proteins, virus or bacteria, often named with the collecting term: *antigen*.

10 mg of bovine hemoglobin or 10 mg of human serum transferrin (iron-free or iron-containing) were dissolved in the buffer (980 μl) when a **protein** was the template. In the case human hemoglobin, 80 μl hemoglobin solution was mixed with 20 mM sodium phosphate (pH 6.8) to a total volume of 980 μl.

The same method was employed for the synthesis of artificial gel antibodies against the wild type and mutant of Semliki Forest **Virus**. In this procedure 5 μl of the virus suspension at a

concentration of 5 mg/ml of wild type or mutant SFV was added with stirring to 975 µl of 20 mM sodium-phosphate buffer, pH 6.8.

Following deaeration the solution was supplemented with 10 µl of a 5 % (v/v) TEMED solution.

Selective gels against **bacteria** were prepared similarly. The detailed experimental conditions were as follows. Acrylamide (171 mg), N,N'-methylene-bis-acrylamide (9 mg), 30 µl of a 10 % (w/v) ammonium persulphate solution and a suspension of the bacteria (240 µl) were mixed with 20 mM sodium phosphate (pH 6.8) to a total volume of 2970 µl. Following deaeration the solution was supplemented with 30 µl of a 5 % (v/v) TEMED solution.

The polymerization was allowed to proceed overnight (although it started within 10 min). *Blank* gels were prepared in a similar way in a phosphate buffer in the absence of the template and are, accordingly, electrically neutral. The gels were granulated by pressing them twice through a 60-mesh (0.25 mm) net and then through a 100-mesh (0.15 mm) net.

3.2.2 Washing procedure to remove the templates from the selective gel antibodies

To remove the template **protein** from the gel granules they were washed first with a detergent solution (50 mM SDS in 50 mM Tris-HCl buffer, pH 8.5), because SDS causes denaturation and desorption of the proteins from the gel and then with buffer alone (50 mM Tris-HCl, pH 8.5, a Tris-solution was titrated by HCl to the desired pH). The procedure took place overnight (or over the day). Two methods were used to accomplish the template removal.

1. In the simple washing procedure SDS and part of the protein molecules were washed out during stirring with the Tris-buffer. The absence of SDS was controlled by the addition of potassium chloride.

Alternatively, the protein-containing selective gel granules were packed in a Pasteur pipette with a 3 mm high bed of glass wool at the constriction of the pipette [Liao *et al*., 1996] and washed with the SDS solution. Desorption of the protein proceeded overnight in both cases or during the day. The granules were freed 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). If the washing of these selective gel antibodies (called ***control*** granules) was incomplete not only the protein capacity, but also the

selectivity was lower (the gel granules may get cation-exchange properties). Following the washing a subsequent electrophoretic step can remove possible residues of SDS and protein.

2. In the electrophoretic cleaning procedure the granules were transferred to a vertical glass tube (covered at the bottom with a dialysis membrane). Both electrode vessels contained 50 mM Tris-HCl buffer (pH 8.5) (Figure 4). Electrophoresis proceeded overnight at room temperature.

Figure 4. Removing of the imprinting molecules with the help of electrophoresis. The removal was carried out in two steps: first 50 mM SDS in 50 mM Tris-HCl buffer pH 8.5 was used, than 50 mM Tris-HCl buffer pH 8.5 was used to remove the SDS.

In some experiments enzymatic degradation of the protein (the "antigen") were used. Gel granules prepared in the absence of protein are called ***blank*** granules. The **virus-containing** selective gel granules were treated with one of the above-mentioned procedures. To remove the **bacteria**, used as templates (antigens), the gel granules were treated with lysozyme and then washed with 50 mM SDS in 50 mM Tris-Cl, pH 8.5. Finally SDS was removed by washing with buffer alone.

3.2.3 Resaturation of depleted selective gel granules

Re-establishment of complexes from gel granules depleted of the template **protein** was made by incubation with rotation of the gel granules in the protein solution for 30 minutes. After the saturation the nonbound protein molecules were removed by washing procedure repeated three times. In this step the granules were rotated for 20 minutes with 50 mM Tris-HCl, pH 8.5 buffer, and then the suspensions were centrifuged and decanted. Blank granules were treated in the same way.

The saturation of the depleted **virus** selective gels was done by repeated incubations with virus suspensions and intermittent washings with buffer. In details: 100-µl of the suspension of the gel antibodies in 50 mM Tris-HCl, pH 8.5 was mixed in a test tube with 112 µl of the virus suspension (final virus concentration, 1.7 µg/ml). For efficient equilibration of the gel antibodies the test tube was rotated with virus particles for 30 min at room temperature and then centrifuged for 30 min. The supernatant was decanted and the sedimented gel antibodies were washed with the buffer to remove free virus particles. This washing procedure was repeated four times. Granules of a blank gel were treated similarly.

Resaturation of the gel granules depleted of the template **bacteria** was made by incubation of the gel granules in the bacterial suspension.

3.3 Electrophoretic methods to study (molecular) recognition

3.3.1 Chiral recognition by transferrin in a capillary electrophoretic system

As chiral selector, iron-free human serum transferrin solution (100 mg/ml) was introduced into a non-crosslinked polyacrylamide coated [Hjertén, 1985] fused silica capillary (Figure 3), with a total length of 35.0 – 47.0 cm, an effective length of 31.5 - 42.5 cm and 50 µm as inner diameter. The protein was injected into the capillary at the anodic side, with 10-150 psi*s injection factor. This resulted in about 1-15 cm long protein plug (marked by *z*). 10 psi*s injection corresponds to 1 cm [Kilár and Fanali, 1995].

Tryptophan-methyl-, -ethyl- and –butyl-esters were dissolved in water at a concentration of $3*10^{-5}$ M. Pressure injection was used with 5-25 psi * s injection factor.

The capillary electrophoresis experiments were performed with a BioFocus 3000 equipment (Bio-Rad Laboratories, Hercules, CA, USA), at 20°C. 100 mM MES buffer, pH 6 was used as background electrolyte in the experiments. 15 kV Voltage was applied. The current was *ca*. 25 µA. The detection was made with UV light at 206 or at 215 nm.

The protein zone behaved as a pseudostationary zone and the samples interacted with the protein molecules, while migrated through this zone.

The migration times were measured, and migration velocities, mobilities and resolutions were calculated.

The resolutions were calculated according to the following equation:

$$R=2(t_R-t_S)/(w_S+w_R), \qquad [1]$$

where t is the migration time, w is the peak width at the baseline; *R* and *S* refer to the R (slow migrating isomer) and S (fast migrating isomer) enantiomers, respectively.

The samples had two different migration velocities, since they were migrating through the protein zone (v_z - lower velocity value due to the interaction with the protein) and after leaving this zone they migrated in free solution (v_{zo}).

The migration velocity out of the transferrin plug was also determined, as

$$v_{zo}=le/t_0, \qquad [2]$$

where t_0 is the migration time of the tryptophan derivatives in capillary free zone electrophoresis (determined in separate runs in the absence of transferrin) .

The average velocities of the enantiomers were calculated for the characterization of the migration with the

$$v=l_e/t \qquad [3]$$

formula, where l_e the effective length of the capillary and t is the total migration time.

To calculate the migration times of the enantiomers in the protein zone, the following equation was used

$$t_z=t-[(l_e-z)/v_{zo}], \qquad [4]$$

where t_z is the migration time in the transferrin zone, v_{zo} is the migration velocity in free solution (out of the protein zone), l_e the effective length of the capillary, and z the length of the transferrin zone.

The migration velocities of the enantiomers in the protein zone can be specified as

$$v_z=z/t_z \qquad [5]$$

This was used to calculate the mobility of samples in the transferrin zone.

3.3.2 Electrophoretic analysis to study the selectivity of the gel antibodies

3.3.2.1 Instrumentation

All 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 [Hjertén, 1958] to prevent disturbing sedimentation of the sample zones [Hjertén, 1967]. Although, Hjertén was aware of the possibility of doing free zone electrophoresis experiments in stationary, very narrow capillaries (see Introduction in his thesis [Hjertén, 1967]), the diameters of commercial quartz capillaries in the fifties-sixties were not small enough to prevent sedimentation of the solute zones. In fact, Hjertén's approach is still the only one for carrier-free zone electrophoresis of particles as large as the artificial gel antibodies. Therefore, for this project we borrowed the apparatus, depicted in Figure 5 from the Museum of Medical History in Uppsala.

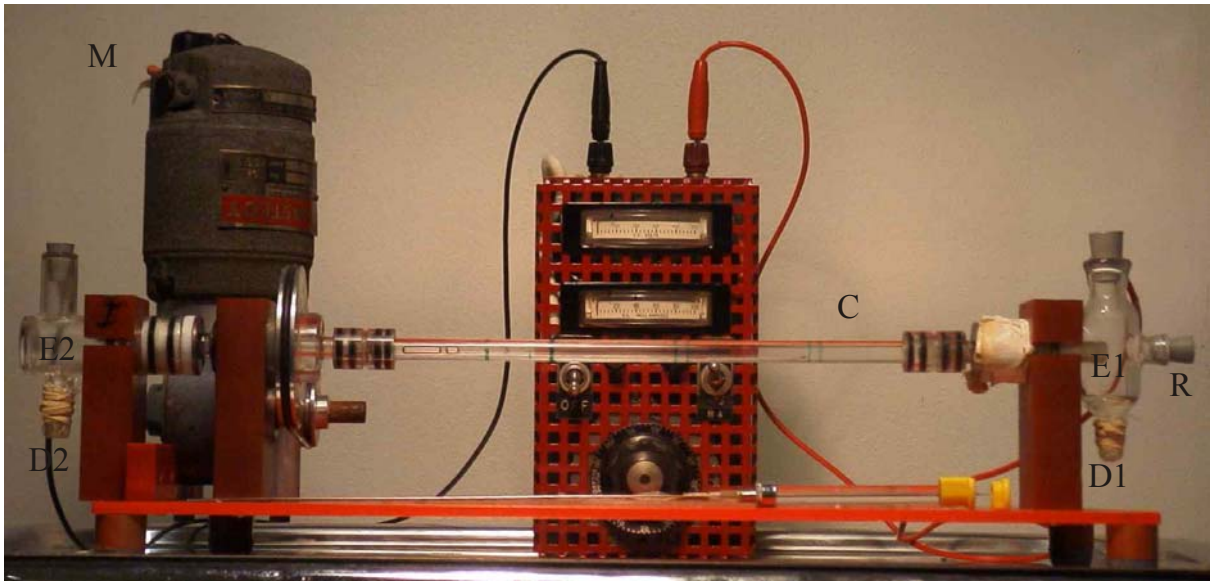


Figure 5. The first "capillary electrophoresis" instrument (Hjertén, 1958)
A glass narrow bore tube length 245 mm, OD 9.6 mm, ID: 2.5 mm) is rotated at a speed of 40 rpm with the help of a motor (M). E1 and E2 are electrode vessels. The sample is injected to the rotated narrow bore tube (C) on the right side after removing the stopper (R). Dialysis membranes (D1 and D2 usually in water) help to get rid of the hydrodynamic flow. Suspension of the gel granules were injected to the narrow bore tube with a syringe and voltage was applied.
The migration was followed with eyes; marks were made on the surface of the tube.

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 [Hjertén, 1985] to minimize adsorption and electroosmotic flow (alternatively, the coating can consist of cross-linked methyl cellulose [Hjertén and Liao, 1998]). 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. Compared to very narrow capillaries, wider capillaries (tubes) have the advantage that the samples can be applied by a syringe, i.e. several sample zones can be introduced into the tube and analyzed in the same run which increases the accuracy in the determination of relative migration velocities (and mobilities) and reduces the number of runs required. We took advantage of this possibility and often applied three samples; for instance, one consisting of gel granules selective for transferrin, one of control granules, and one of blank granules (definition see above).

3.3.2.2 Application of sample and calculation of mobilities

A suspension (20-25 μl) of the artificial antibodies (5-10 gel granules) in the running buffer was sucked into a 245 mm long, thin metal tube (i.d: 0.5 mm, o.d: 1.5 mm) attached to a 100-μl syringe, and then 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) [Catsimpoolas *et al.*, 1976].

3.3.2.3 Preparation of sample and electrophoretic conditions

Each series of experiments was preceded by a test of the efficiency of the above described cleaning method of the gel granules, *i.e.,* protein-depleted, selective ***control*** gel granules and non-selective ***blank*** gel granules were applied as two separate zones, while the buffer-filled narrow bore tube rotated. The cleaning was considered satisfactory when the migration velocities of these two types of granules were similar (Figure 14).
Two procedures were used for the equilibration of the selective gel antibodies with protein.
In *Method I* the entire electrophoresis tube was filled with **protein** (antigen) solution (e.g. buffer containing approximately 0.3 mg/ml of human hemoglobin or 5 mg/ml of bovine

hemoglobin). 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 (the removal of the antigen is described in Section 3.2.2 *Washing procedure to remove the templates from the selective gel antibodies*) and the blank granules were each separately transferred to a protein solution (*e.g.*, 500 μl of a 1 mg/ml iron-free transferrin solution was added to 100 μl of a granule suspension) 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. Following this procedure the granules contained only selectively bound protein.

A zone of blank granules (gels polymerized in the absence of the protein) was also injected into the revolving capillary in order to obtain a higher accuracy in the determination of the true migration distances and, thus, the mobility of selective gel granules.

The electrophoretic migration of the granules in the rotating tube was visually followed. The migration distances were measured at different times by a ruler. The '*true'* mobility of gel antibody granules (with selectively attached proteins) were calculated following the subtraction of the migration distances of blank gel granules or antigen-depleted gel granules (also called *control gels*) from the measured migration distances. The application of the blank (or antigen-depleted) gel granules is necessary to control the possible incidental effects of slight electroosmosis or/and a hydrodynamic flow in the capillary (caused by buffer leakage).

The slope of the straight line fitted in a plot of the true migration distance against migration time provided the migration velocity of the selective protein-containing granules. The effective mobilities were calculated not only for the granules by the above procedure, but also for free iron-containing and iron-depleted transferrin, using capillary free zone electrophoresis in a 50-μm capillary, coated with polyacrylamide [Hjertén, 1985] (BioFocus 3000, Bio-Rad Laboratories, Hercules, CA, USA). All free zone electrophoresis runs were performed at 22°C.

Granules of gel antibodies synthesized in the absence or presence of **viruses** were analyzed in the same run. For instance a suspension (20-25 μl) of the artificial antibodies (5-10 gel granules) in the running buffer was injected into the rotating "capillary" (narrow bore tube) similarly, a suspension of gel granules, prepared in the absence of the "antigen" was injected. The widths of the starting zones were 3-4 mm. The voltage was applied and the electrophoretic migration of the granules towards the cathode was visually followed. Migration distances were determined with the aid of a ruler at time intervals of about two minutes. The migration velocity, *v*, of the gel particles is obtained from the slope of the straight line obtained in a plot of migration distance against time. The mobility is obtained by division of the migration time by the field strength (V/cm). In a typical experiment three zones were applied in the narrow bore tube, one consisting of granules of the complex selective gel antibody/virus (wild type), one consisting of the complex this gel antibody/a mutant of this virus, and one of a blank, *i.e.*, gel granules prepared in the absence of virus. In some experiments a fourth sample was applied: control gel granules, i.e. selective gel granules, washed with SDS and buffer to remove the antigen (the virus). The gel antibody has a high selectivity if the mobility of the first zone is higher than the mobility of the second zone. The mobility of the third zone should be zero, and that of the fourth zone zero or very low in the absence of a hydrodynamic flow caused by leakage or electroosmosis.

The experiments with **bacteria** were performed similarly, as those with viruses in the rotating narrow-bore tube. Usually three sample zones were applied: gel antibodies saturated with bacteria (the antigen), control granules (gel antibodies freed of the bacteria), and blank gel granules (synthesized by the same method as the selective gels, but in the absence of bacteria). The starting position of the different sample zones were marked with a colour pen. The migration distances were determined with a ruler (the zones can be visually detected) and the values were plotted against the migration time.

4. Results

[Visegrády *et. al.* 2000a; 2000b; 2000 c; 2000d; Kilár *et. al.*, 2002; Hjertén *et. al.* 2002; 2003a; 2003b; Takátsy, *et. al.,* 2002; 2006a;b;c;d; 2007; Bacskay, *et. al.*, 2006]

4.1 Chiral recognition of tryptophan-esters by human serum transferrin

Capillary electrophoretic separations of three tryptophan derivatives were performed in the absence and in the presence of a human serum transferrin zone in the capillary. In the absence of transferrin the tryptophan methyl- (TME), ethyl- (TEE) and butyl-ester (TBE) enantiomers appeared as single peaks in zone electrophoresis at each pH (Figure 6).

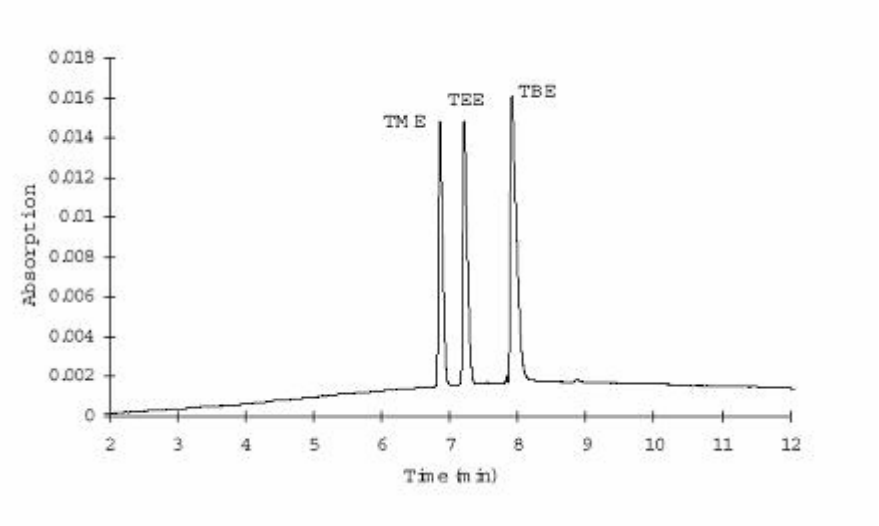


Figure 6. Free capillary zone electrophoresis (CZE)
Conditions of free zone capillary electrophoresis: coated capillary, total length 35 cm, effective length 31.5 cm, ID 50 mm, UV detection at 206nm. Background electrolyte 100mM MES, pH 6. Voltage 15 kV, current 25 μA, temperature 20ºC. Sample concentration 10^{-4} M in water, injected by 10 psi * s.

The mobilities at pH 6 were 18.5±0.5, 17.0±0.5 and 15.5±0.5 Tiselius-units, respectively. In the presence of transferrin, however, enantiomeric separations were observed when the compounds migrated through the protein zone (Figure 7).

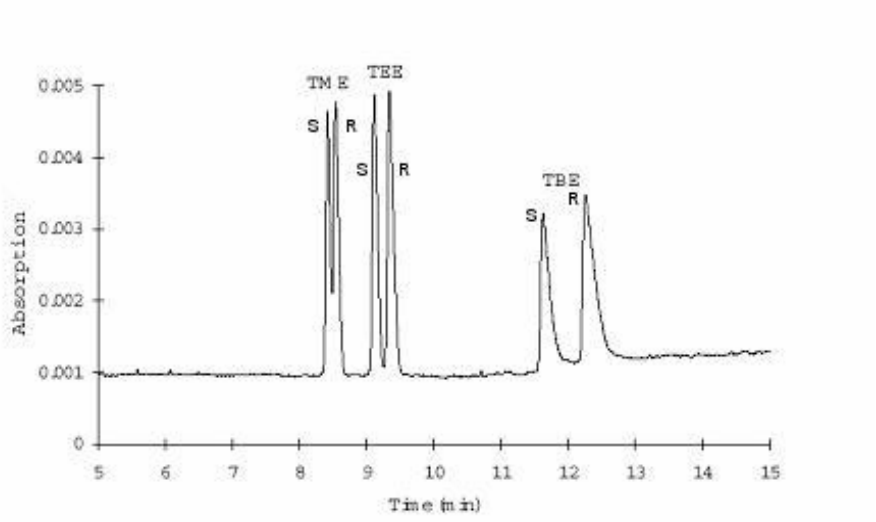


Figure 7. Free zone electrophoresis of the tryptophan derivatives through an iron-free transferrin zone. Experimental conditions: 200 mg/ml Tf in 100 mM MES, pH 6 (100 psi*s), sample concentration 10^{-4} M (10 psi*s) 15 kV, 25 µA, 215 nm, 20ºC.

This technique, now called "partial filling" was introduced by Hjertén [Valtcheva *et al.*, 1993]. Since iron-free transferrin has an isoelectric point around 6 [Kilár, 1991] the protein molecules will form a "pseudostationary" zone in the background electrolyte (100 mM MES, pH 6) and the compounds migrate trough this zone. The resolution of the enantiomers depends on the length of the protein zone: the longer the transferrin zone the higher the resolution of the enantiomers (Figure 8).

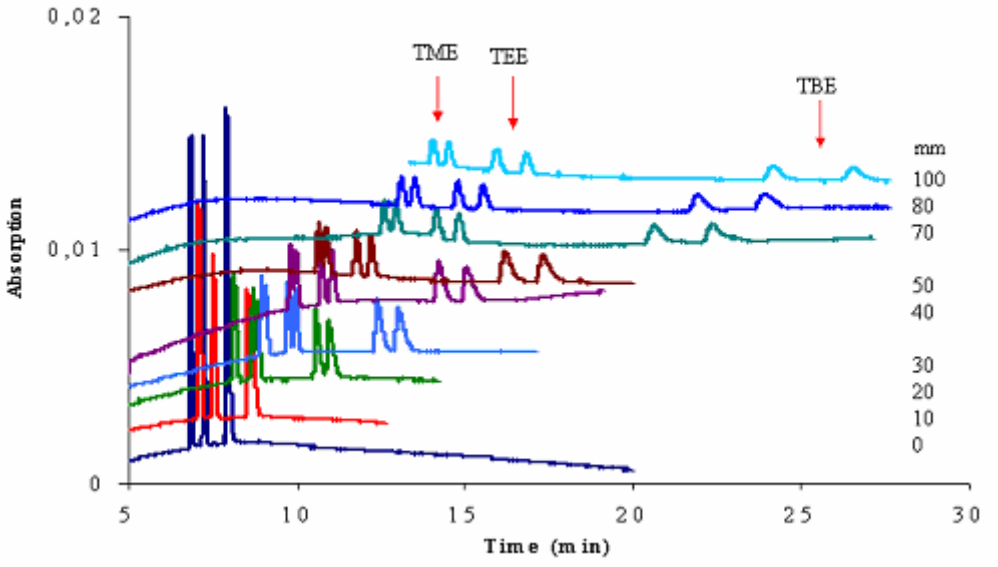


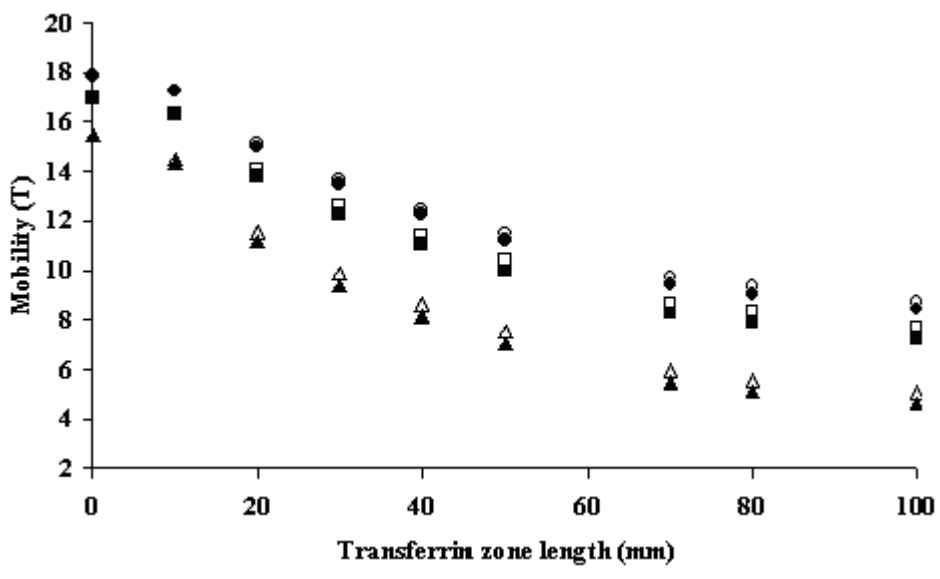
Figure 8. Separation of tryptophan esters applying different transferrin zone-length
Injection of 200 mg/ml transferrin sample (0-100 psi*s) into a coated capillary with 35 cm total length, 31.5 cm effective length, 50µm ID. UV detection at 206 nm. Background electrolyte 100 mM MESH, pH 6. Voltage 15 kV, current 25 µA, temperature 20ºC.

The TBE enantiomers show the highest resolution, the TME the lowest resolution and TEE in between in experiments, when these compounds were applied in a mixture. In order to elucidate the differences between the behaviour of the compounds interacting with the protein surface during the electrophoretic migration the velocity and mobility data should be calculated and compared in a fair way. We used the following approach. If the capillary is not filled totally with the chiral separator (protein), migration of the compounds in a capillary consists of two steps: *a)* migration within the pseudostationary transferrin zone and *b)* migration in background electrolyte alone in the absence of transferrin to the detection window. Within this movement, two migration time elements and two migration mobilities should be calculated and compared for the enantiomers of the three compounds. To calculate the mobility of the different compounds (enantiomers) within the transferrin zone we made an assumption, *i.e.,* 10 psi*s injection results in a 10 mm transferrin zone. This was necessary, since the viscosity of the transferrin solution is not known, and without that the precise length of the injection zone cannot be done. To confirm the assumption we calculated the migration times of each enantiomers within the zone (see Table 1.) and in the rest of the capillary (till the detection window) as it is described in the *Materials and Methods*. If the calculation shows the same mobility within the transferrin zone for runs with different protein zone-length, our assumption is correct.

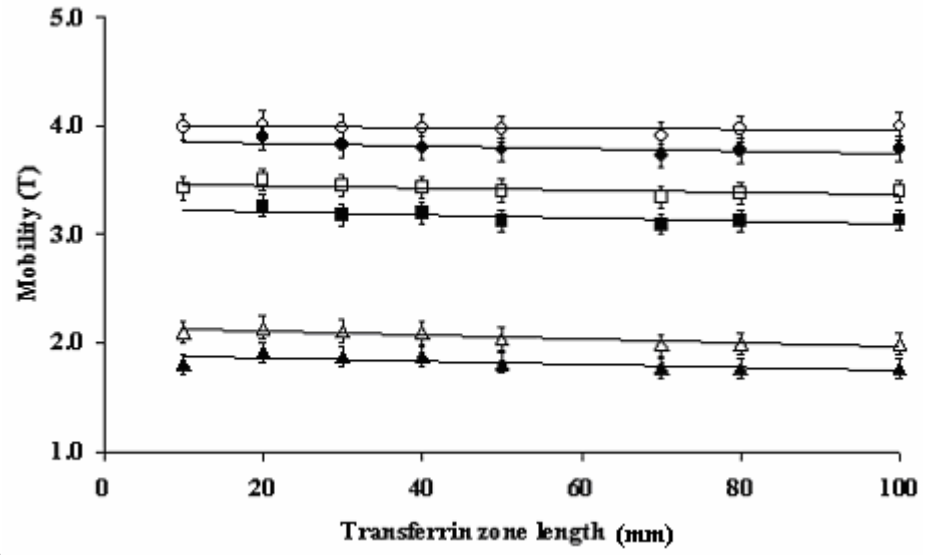
Table 1. Migration times of the enantiomers in the transferrin zone
Injection of 200 mg/ml transferrin sample (0-100 psi*s) into a coated capillary with 35 cm total length, 31.5 cm effective length, 50µm ID. UV detection at 206 nm. Background electrolyte 100 mM MESH, pH 6. Voltage 15 kV, current 25 µA, temperature 20ºC.

Injection factor (psi*s)	TME		TEE		TEE	
	t (min)		t (min)		t (min)	
	S	R	S	R	S	R
0	0.00	0.00	0.00	0.00	0.00	0.00
10	0.36	0.36	0.39	0.39	0.76	0.76
20	1.49	1.54	1.71	1.85	**2.92**	**3.28**
30	2.45	2.56	2.84	3.11	4.85	5.49
40	3.41	3.58	**3.97**	**4.30**	6.78	7.62
70	6.52	6.88	7.71	8.40	13.56	15.31
80	**7.14**	**7.55**	8.47	9.23	15.01	17.01
100	8.27	8.76	9.86	10.77	17.49	19.85

Figure 9 shows the average mobilities (9a) and mobilities within the transferrin zone (9b) for the enantiomers depending on the transferrin zone injected (the slight decrease in mobilities depending on the transferrin zone length is within the experimental error).



a)



b)

Figure 9. Mobilities of tryptophan methyl-ester (○,●), tryptophan ethyl-ester (□,■) and tryptophan butyl-ester (△,▲) enantiomers in capillary zone electrophoresis. The enantiomers are migrating through a transferrin zone of different lengths. The average mobilities (a) of the compounds depend on the length of the transferrin zone length, but no significant change of the mobilities (b) can be seen within the protein zone. Calculation of the mobility values is described in *Materials and Methods*. Experimental conditions: Injection of 200 mg/ml transferrin (10 psi*s – 100 psi*s) into the coated capillary, total length 35 cm, effective length 31.5cm, ID 50 μm, as chiral separator protein plug. UV detection at 206 nm. Background electrolyte 100 mM MES, pH 6. Voltage 15 kV, current 25 μA, temperature 20°C.

In this calculation we assumed that the mobilities of the enantiomers outside the transferrin zone are the values determined by zone electrophoresis experiments in the absence of transferrin. Upon these calculations we may now deduce the dependence of chiral resolution on time spent by the enantiomers in the transferrin zone during their electrophoretic migration (Table 2., Figure 10.). The resulting curves of the compounds with the longer alkyl chains have the same course, while the curve for TME differs significantly from the others.

Table 2. The measured migration times and the calculated resolution of TME, TEE and TBE enantiomers in terms of the injection factors (transferrin zone length).
Experimental conditions: coted capillary, total length 35 cm, effective length 31.5 cm, ID 50 µm, UV detection at 206 nm. Background electrolyte 100 mM MESH, pH 6. Voltage: 15 kV, current 25 µA, temperature 20 ºC.

Injection factor (psi*s)	TME			TEE			TEE		
	t (min)		Resolution	t (min)		Resolution	t (min)		Resolution
	S	R		S	R		S	R	
0	6.85	6.85	**0.00**	7.23	7.23	**0.00**	7.92	7.92	**0.00**
10	7.10	7.10	**0.00**	7.51	7.51	**0.00**	8.55	8.67	**0.57**
20	8.13	8.18	**0.37**	8.71	8.85	**0.72**	10.59	10.95	**0.97**
30	8.98	9.09	**0.52**	9.73	10.00	**0.96**	12.39	13.03	**1.51**
40	9.82	10.00	**0.77**	10.74	11.07	**1.04**	14.20	15.04	**1.64**
70	12.61	12.97	**0.98**	14.14	14.83	**1.63**	20.60	22.35	**2.13**
80	13.12	13.53	**1.00**	14.78	15.54	**1.80**	21.92	23.92	**2.44**
100	14.04	14.53	**1.16**	15.94	16.85	**1.84**	24.15	26.51	**2.68**

The pH dependence of resolution applying a 10 cm long, 100 mg/ml transferrin zone in the capillary (in 100 mM MES) is presented in Figure 11. The pH dependence shows a maximum for the TEE and TBE enantiomers, while the TME enantiomers are not resolved under this experimental condition.

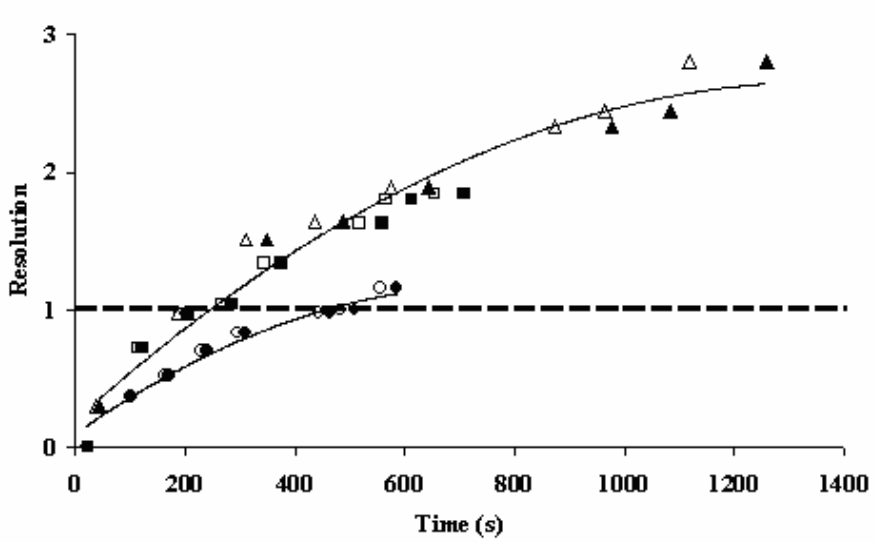


Figure 10. The resolution of tryptophan methyl-ester (○,●), tryptophan ethyl-ester (□,■) and tryptophan butyl-ester (△,▲) enantiomers vs. the sojourn of the enantiomers within the transferrin zone in capillary electrophoresis. The points for each compound were obtained from experiments with different transferrin zones in the capillary (see Fig. 9). The longer the compounds stay in the zone, the higher the resolution of the enantiomer pairs. Calculation of the time values is described in the Materials and Methods. Experimental conditions: coated capillary, total length 35 cm, effective length 31.5 cm, ID 50 µm, UV detection at 206 nm. Background electrolyte 100 mM MESH, pH 6. Voltage: 15 kV, current 25 µA, temperature 20 ºC.

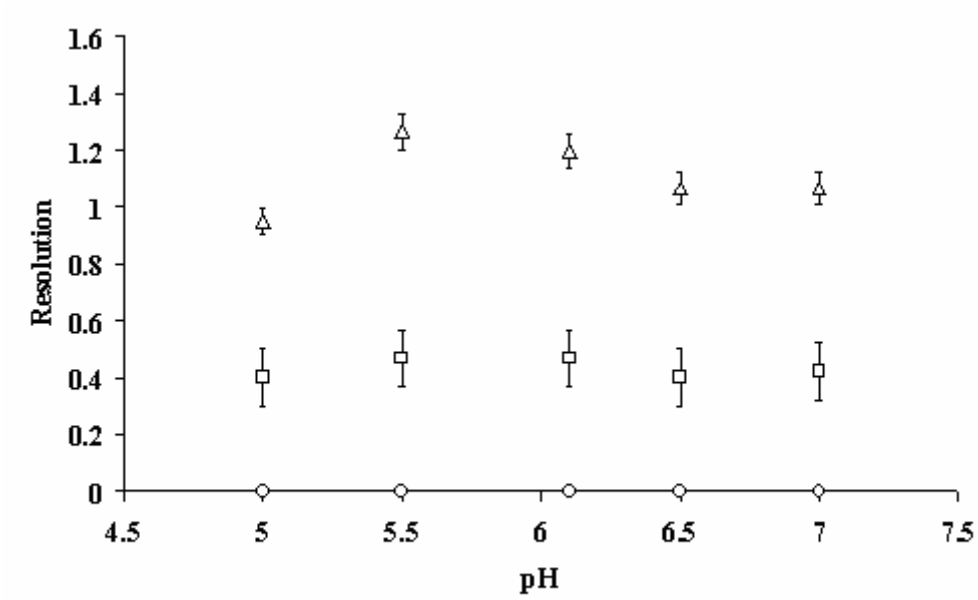


Figure11. pH dependence of resolution (R) of tryptophan methyl-ester (○), tryptophan ethyl-ester (□) and tryptophan butyl-ester (△) enantiomers in capillary electrophoresis through a transferrin zone. A 100 mg/ml transferrin solution was injected (100 psi*s) prior to sample injection. Experimental conditions: coated capillary, total length 35 cm, effective length 31.5 cm, ID 50 µm, UV detection at 206 nm. Background electrolyte 100 mM MESH, pH 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5. Voltage: 15 kV, current 25 µA, temperature 20 ºC.
The tryptophan methyl-ester enantiomers were not resolved under the given experimental conditions. A maximum in resolution of TEE and TBE enantiomers was obtained at pH 5.5.

4.2 Molecular recognition of 'antigens' with molecularly imprinted matrices

Molecularly imprinted selective gels were prepared to obtain information about recognition of proteins (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) [Takátsy, *et. al.*, 2006c] and bacteria (Escherichia coli and Lactococcus lactis) [Bacskay, *et. al.*, 2006].

Selective gel granules were synthesized from acrylamide and methylenebisacrylamide monomers in the presence of different template proteins, viruses, virus like particle and bacteria as described in *Materials and Methods* (Section 3.). Non-charged granules were also prepared in the absence of any template substance to be used as ***blank***. The templates were removed by one of the cleaning procedures described in *Washing procedure to remove the templates from the selective gel antibodies* (Section 3.2.2). These cleaned granules are called ***control gels*** or protein-depleted granules.

See the experiments in Figure 12, where the coloured gel shows the presence of the bound hemoglobin, and the non-coloured gel is a blank gel.

Figure12. Picture of polyacrylamide gels. On the left human hemoglobin ***selective gel*** (polymerization took place in the presence of human hemoglobin), on the right T_6C_5 polyacrylamide gel, used as ***blank***.

The success of the cleaning process was tested before the experiments. Control and blank granules were injected into the narrow bore tube filled with background electrolyte (50 mM Trish-HCl, pH 8.5) and zone electrophoresis of these granules were performed. Since the polyacrylamide gel is neutral, no movement of the cleaned granules is expected. The efficiency of the washing procedure was accepted when no movement of the granules took place, or when the granules migrated with the same velocity caused by the possible incidental effects due to slight electroosmosis and/or hydrodynamic flow in the capillary (caused by buffer leakage) (Figure 13).

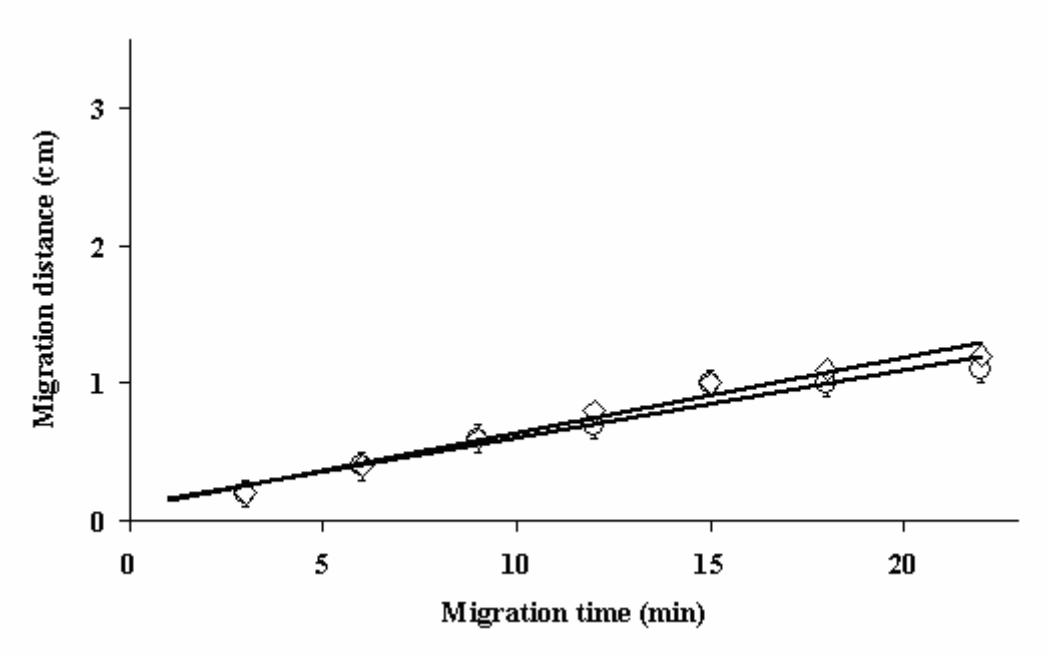


Figure 13. Testing of the washing procedure
Capillary free zone electrophoresis of gel granules synthesized to be selective for iron-free transferrin and then washed with SDS to remove transferrin and finally with buffer to remove SDS (◇) and blank gel granules (O) (see Section *Preparation of gel antibodies*). The experiment illustrates that iron-free transferrin, selectively adsorbed to gel antibodies against this protein, can be removed by washing with buffer containing SDS. The migration of the gel granules is not caused by EOF, but a hydrodynamic flow due to buffer leakage.

4.2.1 Experiments with hemoglobins (Testing the specificity of the selective gels)

The chemical structures of the human and bovine hemoglobin are similar; therefore they were used as model compounds to test the specificity of the selective gels.

Human hemoglobin-selective gel granules, depleted of this protein, were run in zone electrophoresis in a buffer containing human hemoglobin at a concentration of about 0.3 mg/ml (*Method I.* see Section 3.3.2.3). Figure 14 shows the true migration distances from the injection position of the gel granules (*i.e.*, the migration distances corrected for buffer flow and EOF) as a function of migration time. The selective gel granules migrated toward the anode (ca. 4 cm within 20 min), indicating that they were negatively charged by interaction with hemoglobin (pI about 7). The mobility of the selective gel granules was estimated at 13.6 ± 0.3 T-units. Using BioFocus 3000 (BioRad Laboratories, Hercules, USA) a similar value was obtained for *free* hemoglobin under the same experimental conditions (μ_{Hb} = 14.7 T-units).

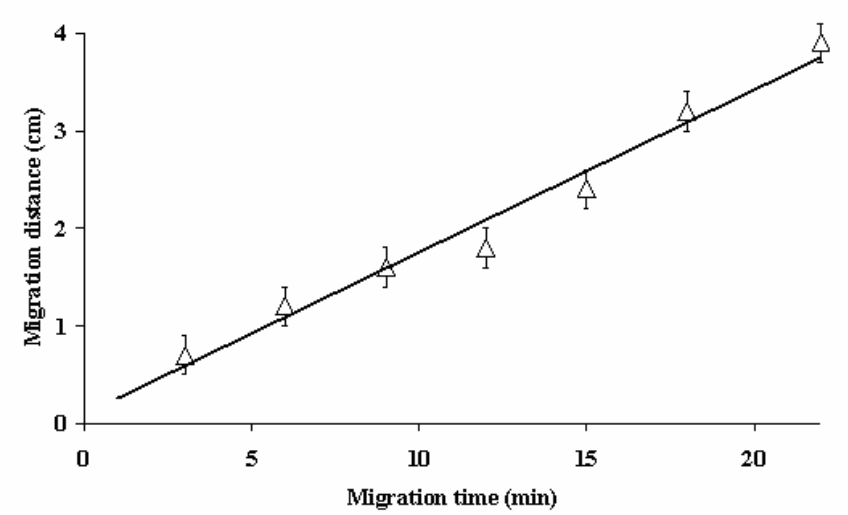


Figure14. Free zone electrophoresis of gel granules selective for human hemoglobin in a rotating narrow-bore tube ("capillary") filled with background electrolyte (50 mM Tris-HCl, pH 8.5), containing human hemoglobin (0.3 mg/ml). The coated tube had the length 245 mm, outer diameter 9 mm, and inner diameter 2.5 mm. Experimental conditions: voltage, 500 V; current, ca. 50 μA; room temperature. The plotted migration distance was obtained by subtracting the measured migration distance of blank granules from that of the selective gel-granules.

Figure 15 shows the time course of the migration of bovine hemoglobin selective gel granules in a background electrolyte containing 5 mg/ml of this protein. The mobility of these granules was 12.4 T-units.

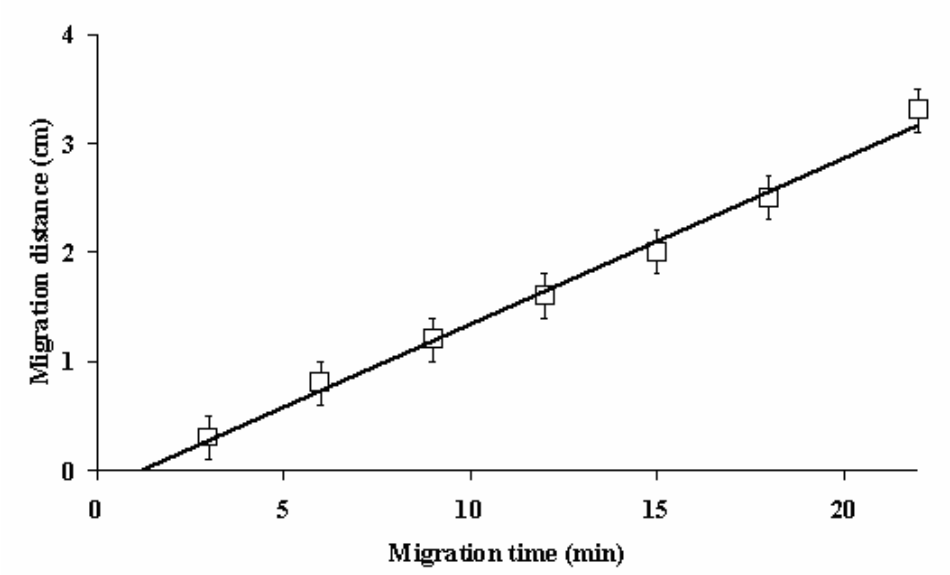


Figure 15. Free zone electrophoresis of granules selective for bovine-hemoglobin in a rotating narrow-bore tube ("capillary") filled with background electrolyte (50 mM Tris-HCl, pH 8.5), containing bovine hemoglobin (5 mg/ml). Experimental conditions: voltage, 500 V; current, ca. 50 μA; room temperature. The plotted migration distance was obtained by subtracting the measured migration distance of blank granules from that of the selective gel-granules.

The selective interaction between gel and proteins was also studied with cleaned human hemoglobin selective gel granules (antigen-depleted granules) following *incubation* in human hemoglobin solution (*Method II.* see Section 3.3.2.3). Non-bound proteins were then removed by washing the granules three times with background electrolyte. Figure 16 shows zone electrophoresis of human hemoglobin-treated and not human hemoglobin-treated selective gel granules, as well as of blank granules treated with hemoglobin and then washed three times. The true mobility of the human hemoglobin-saturated selective granules was 12.7 T-units. The figure illustrates (1) that human hemoglobin is adsorbed by gel granules synthesized to become selective for this protein, and (2) that a washing with SDS- containing buffer efficiently releases the antigen.

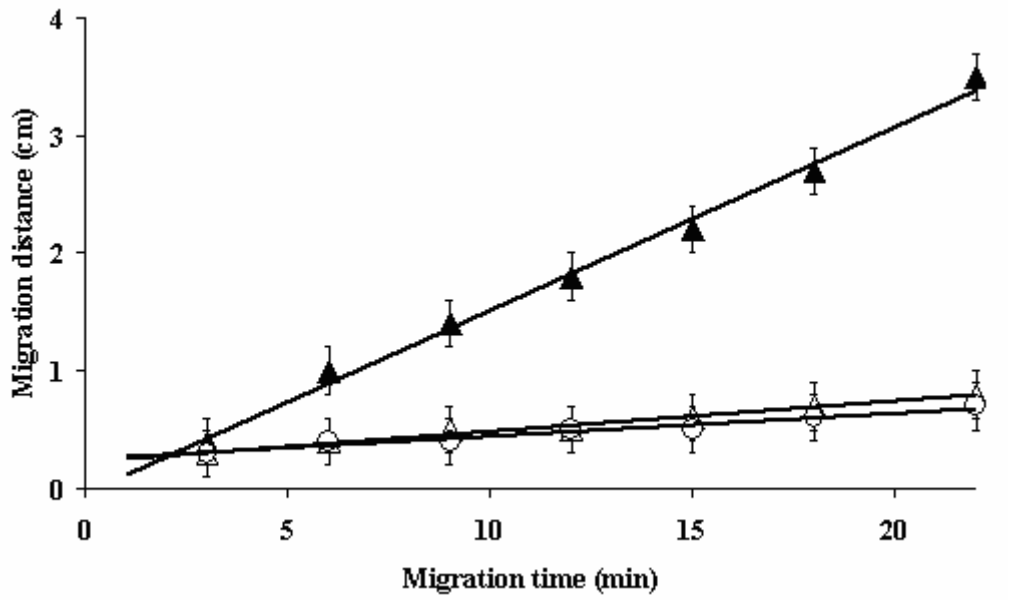


Figure 16. Free zone electrophoresis of gel granules selective for human-hemoglobin incubated (saturated) with human hemoglobin prior to electrophoresis (▲), human hemoglobin-selective gel granules depleted of this protein (Δ) and blank granules (O) in a rotating narrow-bore tube ("capillary") filled with background electrolyte (50 mM Tris-HCl, pH 8.5). Hemoglobin was not added to the background solution.). Experimental conditions: voltage, 500 V; current, ca. 50 μA; room temperature.
Saturation process: 250 μl hemoglobin was added to 100 μl granules of hemoglobin selective- and blank ones, after the cleaning of the particles with SDS and buffer (both cleaning method were used and tested: washing with stirring and decantation, and electrophoretic cleaning whit 50 mM SDS in 50 mM Tris-HCl, pH 8.5 and 50 mM Tris-HCl, pH 8.5). To get interaction between the gel granules and the protein solution, the test tubes were rotated 30 min at room temperature. After the incubation centrifugation was used to get the particles sediment. The solution was decanted and 3 times washing were used to get rid of the free hemoglobin. The test tubes were filled up with buffer (50 mM Tris-HCl pH 8.5) and were rotated for about 20 min, followed by centrifugation and decantation. Then the granules were introduced into a glass tube filled with buffer (50 mM Tris-HCl pH 8.5) and voltage was applied.

Figure 17 displays the plot of the true migration distance versus migration time for human hemoglobin selective gel granules, cleaned by SDS-washing or by electrophoresis. Both samples (depleted from the template) were treated (saturated) with human hemoglobin. The unbound protein was removed by washing three times with buffer. The figure shows that the efficiency of the SDS-washing is equivalent to the cleaning by electrophoresis. Incomplete removal of the protein or SDS would decrease the selectivity. The true mobility of the gel antibodies, saturated with human hemoglobin, was about 10.7 ± 0.3 T-units.

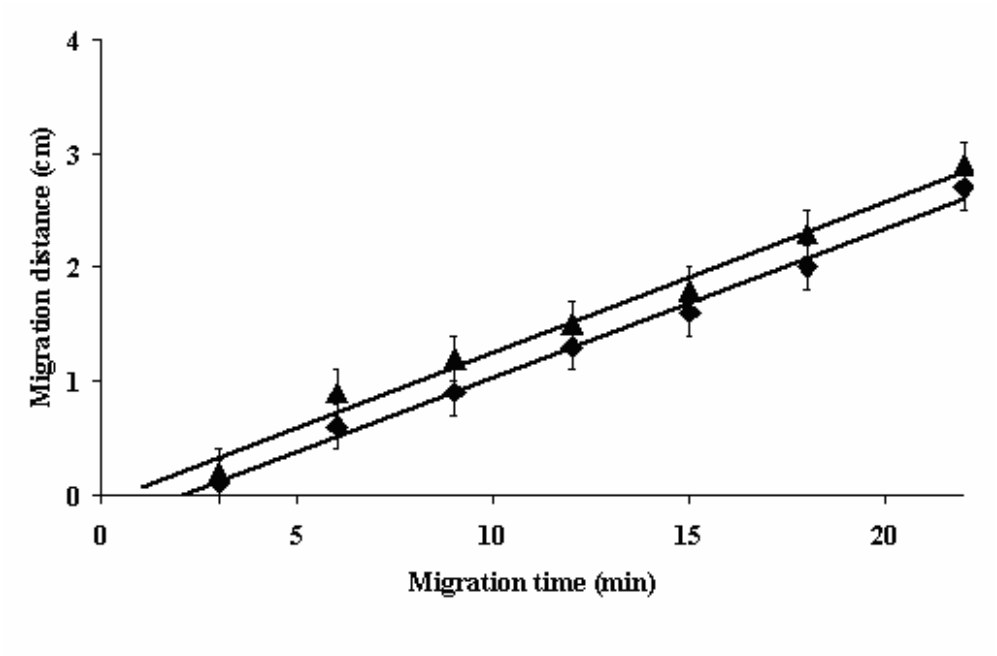


Figure17. Free zone electrophoresis of saturated gel granules selective for human hemoglobin in a rotating narrow-bore tube filled with background electrolyte (50 mM Tris-HCl, pH 8.5). The selective granules were first depleted with SDS-washing (◆) and electrophoretic cleaning (▲), then the granules were saturated again by incubation with human hemoglobin. Hemoglobin was not added to the background solution. Experimental conditions: voltage, 500 V; current, ca. 50 μA; room temperature. The plotted migration distance was obtained by subtracting the measured migration distance of blank granules from that of the selective gel-granules.

The selectivity of the human hemoglobin and bovine selective gel granules was studied by electrophoresis in a bovine hemoglobin-containing electrolyte (Figure 18). Evidently, the gel antibodies against bovine hemoglobin interact strongly with this protein. However, no significant difference in the migration distances of human hemoglobin-selective gel granules, compared to those of the blank granules, was observed, *i.e.*, the gel granules prepared in the presence of human hemoglobin did not interact with bovine hemoglobin. The error bars represent the standard deviations of the respective migration times, obtained in different runs. Experiments with the human hemoglobin selective gel and blank gel granules in an albumin

solution resulted in no migration differences showing that albumin does not interact with these hemoglobin-selective gels (not shown here).

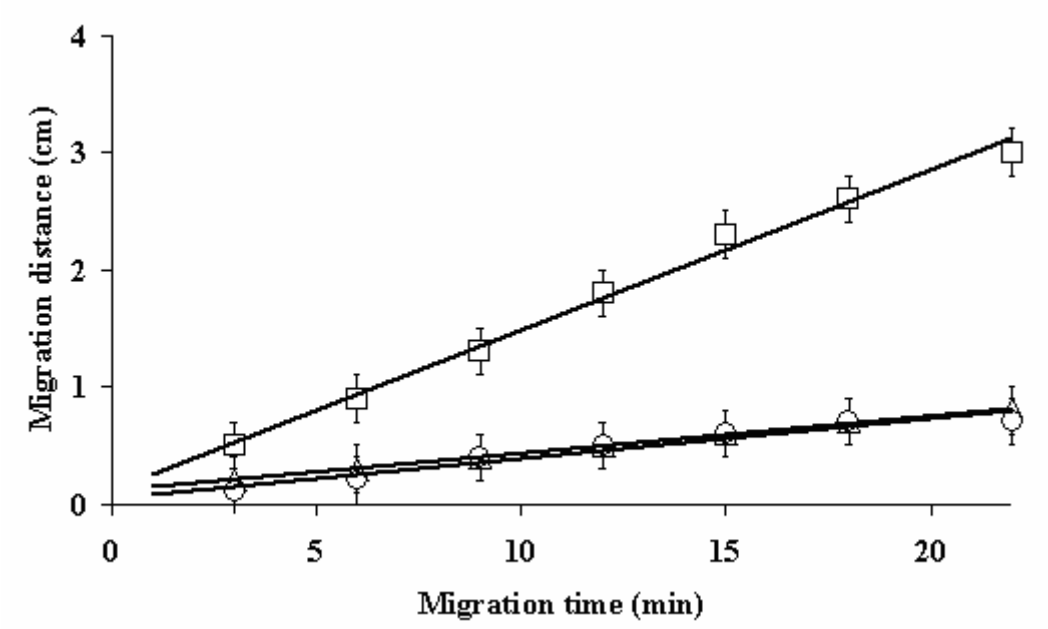


Figure 18. Free zone electrophoresis of gel antibodies selective for bovine hemoglobin (□) and human hemoglobin (Δ) in a rotating narrow-bore tube filled with background electrolyte, containing bovine hemoglobin (5 mg/ml). Experimental conditions: voltage, 500 V; current, ca. 50 μA; room temperature. The plot shows that the gel antibodies selective for bovine hemoglobin do not recognize human hemoglobin and are, accordingly, highly selective. The migration distances of blank gel granules (O) - prepared in the absence of protein - have not been subtracted from the measured migration distances of the protein selective gels.

4.2.2 Experiments with human serum transferrin

Experiments with iron-free and iron-saturated transferring give the opportunity to test the selectivity of gels with very high specificity. The conformational changes of transferring upon binding of iron is known [Kilár and Simon, 1985], therefore selective gels were prepared in the presence of human serum iron-free and iron-saturated transferrins as templates.

Figure 19 shows that gel granules synthesized to be selective for iron-free transferrin interact with more iron-free protein molecules than do gel granules synthesized to be selective for iron-containing transferrin. In analogy with this observation Figure 20 indicates that gel granules synthesized to be selective for iron-containing transferrin adsorb more of this transferrin compared to iron-free transferrin.

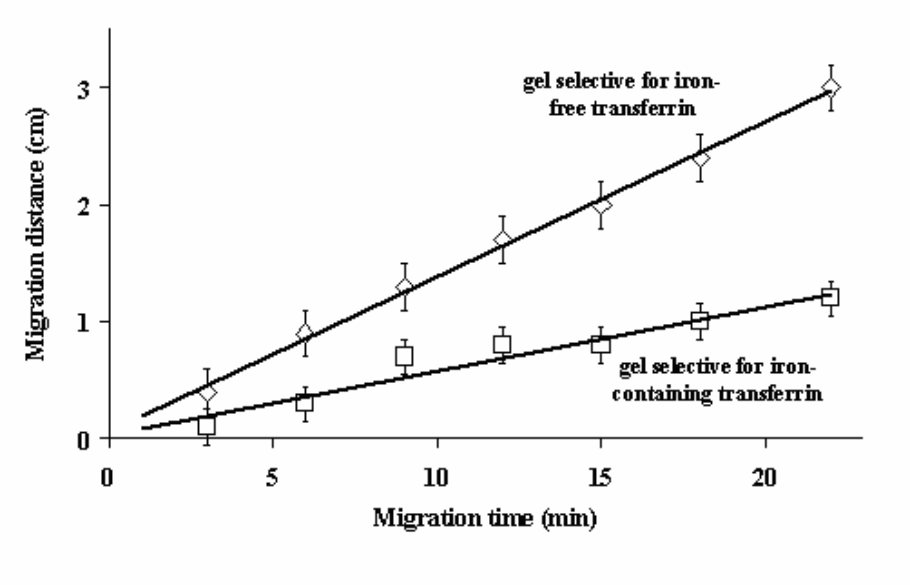


Figure 19. Free zone electrophoresis of gel antibodies selective for iron-free transferrin (◇), and gel antibodies selective for iron-containing transferrin (□) in a rotating narrow bore tube ("capillary") filled with background electrolyte (50 mM Tris-HCl, pH 8.5) containing iron-free transferrin (5 mg/ml). The experiment shows that the gel selective for iron-free transferrin cross-reacted with iron-containing transferrin, but to a less extent, as expected, since these two proteins differ in their conformation (but not in their amino acid sequence); The plotted migration distance was obtained by subtracting the measured migration distance of blank granules from that of the selective gel-granules.

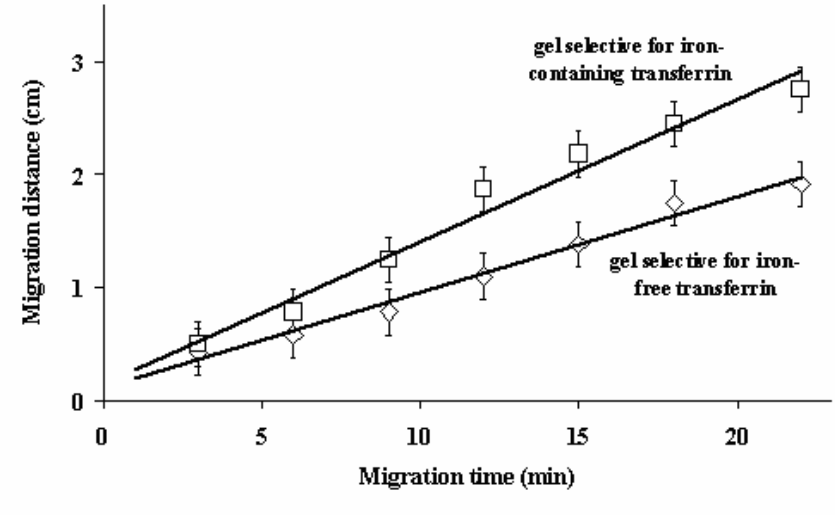


Figure 20. Free zone electrophoresis of gel antibodies selective for iron-free transferrin (◇), and gel antibodies selective for iron-containing transferrin (□) in a rotating narrow bore tube ("capillary") filled with background electrolyte (50 mM Tris-HCl, pH 8.5), containing iron-containing transferrin (5 mg/ml). Evidently, the gel synthesized to be selective for iron-containing transferrin adsorbed more of this protein than did the gel synthesized to be selective for iron-free transferrin and can thus sense the small difference in the conformation of these two proteins (as could the gel antibodies against iron-free transferrin; see Fig. 19) - observe that the amino acid sequences are identical. Experimental conditions: voltage, 500 V; current, ca. 50 μA; room temperature. The plotted migration distance was obtained by subtracting the measured migration distance of blank granules from that of the selective gel-granules.

The mobility data for the gel granules at different concentrations of transferrin in the background electrolyte are summarized in Table 3.

Table3. Mobilities of the selective granules in the presence of the imprinting molecule
The mobilities of iron-free and iron-saturated transferrin were estimated at 9.3 and 9.5 Tiselius units, respectively

Concentration of iron free transferrin in the BGE (mg/ml)	Concentration of iron saturated transferrin in the BGE (mg/ml)	Mobility of the iron free transferrin selective gel granules (Tiselius Unit)	Mobility of the iron saturated transferrin selective gel granules (Tiselius Unit)
2		8.5	3.9
5		10.8	4.5
	2	3.1	7.4
	5	6.9	10.3

The experiment displayed in Figure 21 differs from those in Figures 19 and 20 in (*1*) that the selective gel granules were in contact with the antigen solution (iron-free transferrin) during a relatively long time (30 min) *prior to* the electrophoresis step (see *Method II* in Section 3.3.2.3 *Preparation of sample and electrophoretic conditions*), (*2*) and that the background electrolyte did not contain transferrin.

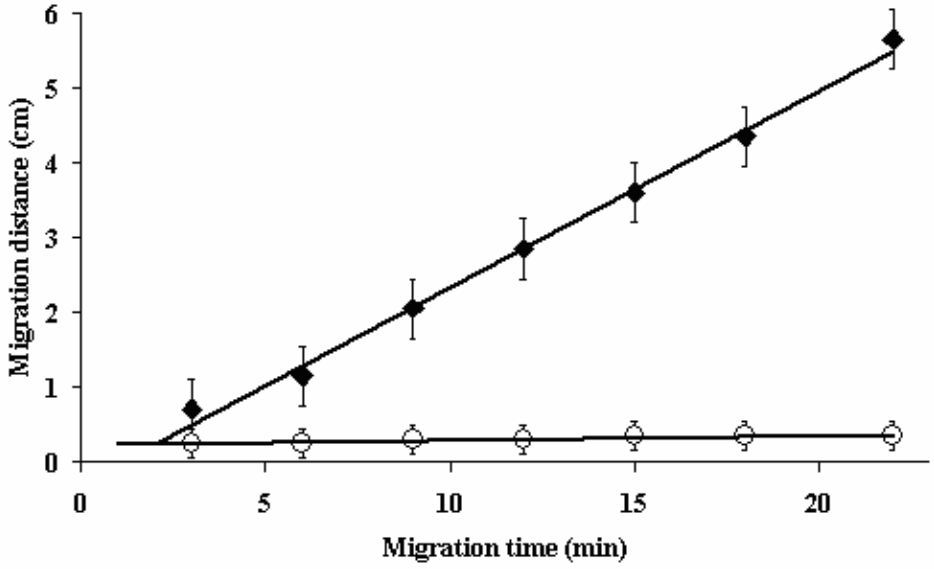


Figure 22. Capillary free zone electrophoresis of gel antibodies against iron-free transferrin saturated with this protein (◆). To 100 □l of SDS and buffer washed (50 mM SDS in 50 mM Tris-HCl, pH 8.5 and 50 mM Tris-HCl, pH 8.5) transferrin selective- and blank granules 500 □l transferrin solution was given (1mg/ml concentration). To get interaction between the gel granules and the protein solution, the test tubes were rotated 30 min at room temperature. After the incubation centrifugation was made to

get the particles sediment. The solution was decanted and 3 times washing were used to get rid of the free transferrin. The test tubes were filled up with buffer (50 mM Tris-HCl pH 8.5) the tubes were rotated for about 20 min, followed by centrifugation and decantation. Then the granules were introduced into a glass tube filled with buffer (50 mM Tris-HCl pH 8.5) and voltage was applied.
The background electrolyte was the same as in the experiments shown in Figs. 19 and 20, but it did not contain transferrin. The constant migration velocity (= the slope) indicates that the transferrin is not released during the run, i.e. the protein is strongly bound to the gel antibody. The open circles correspond to a zone of blank gel granules (O). The migration distances of the selective gel granules in this plot were not corrected for the migration distances of the blank granules.

From the fact that the migration velocity is constant (the line in Fig. 21 is straight) one can conclude that transferrin is not released during the run, *i.e.*, the protein is strongly bound to the artificial gel antibody – a feature it shares with conventional protein antibodies (the mobility of the complex gel antibody against iron-free transferrin/iron-free transferrin was 20.9 Tiselius units). This finding also means that the antigen need not be present in the background electrolyte, *i.e.*, the total amount of the antigen required for an analysis can be very small. An important conclusion is that the artificial gel antibodies have the potential to "fish out" a particular protein, for instance from serum or other biological liquids, even when the concentration of the protein is low. This property in combination with the high sensitivity makes the gel antibodies important tools for clinical diagnosis of diseases via protein biomarkers, e.g., for early detection of cancer.
The mobilities of iron-free and iron-saturated transferrin, 9.3 and 9.5 Tiselius units, respectively, were determined by free zone electrophoresis, using the BioFocus 3000 instrument (Bio-Rad, Hercules, CA, USA).

4.2.3 Experiments with virus-like particles and viruses

Artificial gel antibodies were prepared against *Semliki Forest Virus* wild type, a mutant of this virus, and BK-4 virus like particles. Their selectivity was studied by free zone electrophoresis. Each series of these experiments was preceded by a test of the quality of the cleaning procedure: i.e., virus-depleted, selective gel granules and non-selective blank gel granules were applied as two separate zones in the rotating, buffer-filled tube. The cleaning was satisfactory when the electrophoretic migration velocities of these two types of granules were similar and negligible.
Gel antibodies (granules) against *Semliki Forest Virus* (wild type) and blank gel granules synthesized in the absence of SFV were studied by electrophoresis in a background electrolyte

containing this virus. The selective granules were freed from the template by washing with buffer containing a detergent before electrophoresis, as described in *Material and Methods*. Figure 22 shows that the gel antibodies migrate at a constant speed, which is much higher than the speed of the neutral blank granules, indicating that the gel antibodies have formed a strong complex with the virus particles. The blank granules migrated slightly because of some leakage of buffer or non-efficient removal of SDS.

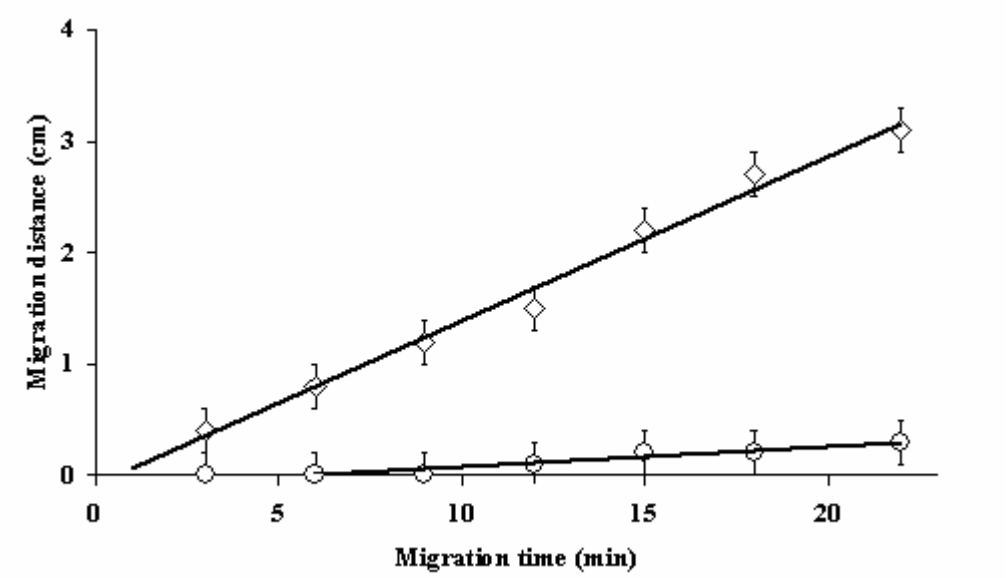


Figure 22. Free zone electrophoresis of gel granules selective for Semliki Forest Virus (wild type) (◇) and blank gel granules (O) in a rotating narrow bore tube ("capillary") filled with background electrolyte supplemented with the virus. The blank granules slightly migrate due to some leakage of buffer or non-efficient removal of SDS. Background electrolyte: 50 mM Tris-HCl, pH 8.5, containing 1.7 µg/ml SFV. Voltage: 500 V. The charged particles migrated towards the anode, current: about 50 µA. Length of the narrow bore tube: 24.5 cm, i.d: 2.5 mm, o.d: 9.6 mm. Rotational speed of the tube: 40 rpm. The plot shows that the neutral gel antibodies interact with the charged virus particles and, therefore, migrate in the electrical field.

Figure 23 shows the electrophoretic analysis of gel granules synthesized in the presence of SFV or in the presence of a protein (hemoglobin) in a background electrolyte containing BK-4 virus-like structure (0.1 mg/ml). Three samples were applied: granules selective for SFV (depleted of the virus), blank granules and granules selective for human hemoglobin (depleted of hemoglobin). The three lines in the figure have approximately the same slope (they almost coincide), i.e; the gel granules selective for SFV (wild type) do not interact with the BK-4 virus-like particles, nor does the gel granules selective for hemoglobin.

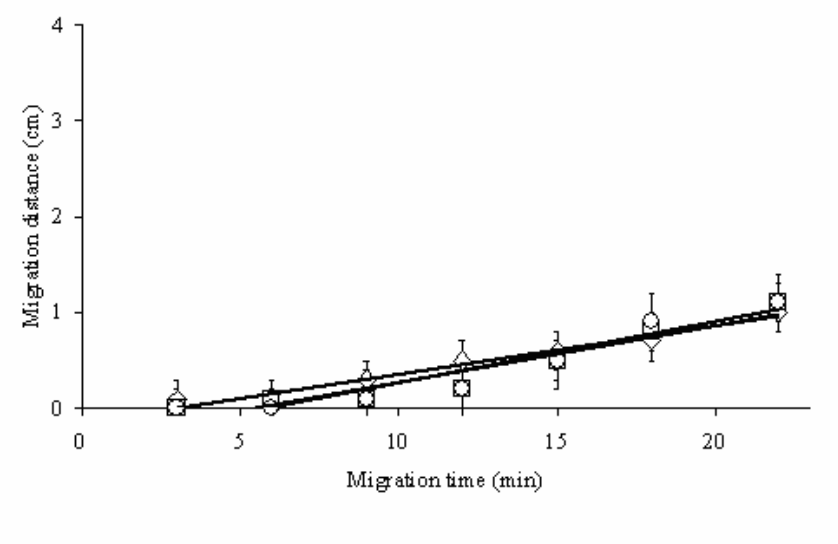


Figure 23. Free zone electrophoresis of gel granules selective for Semliki Forest Virus (wild type) (◇), blank gel granules (O) and gel granules, selective for human hemoglobin (□) to test whether these three types of granules interact with BK-4 virus-like structure. Background electrolyte: 50 mM Tris-HCl, pH 8.5, containing 0.1 mg/ml of BK-4 virus-like structure. Voltage: 500 V. The charged particles migrated towards the anode, current: about 50 μA. Evidently, within the experimental errors the three types of gel particles have the same migration rate, indicating that they do not exhibit unspecific interaction with the BK-4 virus-like structure.

Free zone electrophoresis of gel antibodies against Semliki Forest Virus (wild type) saturated with SFV was also carried out accordingly *Method II* (see Section 3.3.2.3) described in Materials and Methods. In these experiments no virus added to the background electrolyte. The blank granules were treated with SFV prior to electrophoresis and the background electrolyte did not contain virus. Electrophoresis for about 25 min at 500 V gave the diagram in Figure 24.

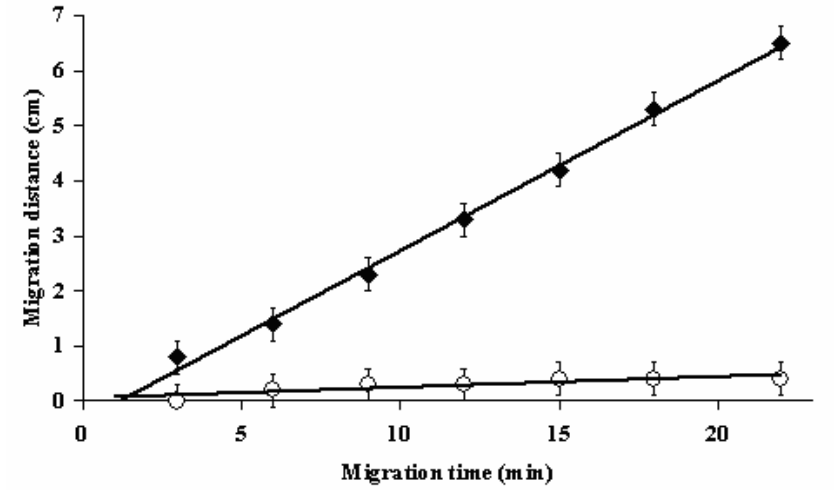


Figure 24. Free zone electrophoresis of gel granules selective for Semliki Forest Virus (wild type) and saturated with this virus (◆) and blank gel granules (O). The background electrolyte did not contain virus. The migration distance is plotted against time. Voltage: 500 V. The charged particles migrated

towards the anode, current: about 50 μA. The finding that the plot gives a straight line shows that the charge is constant during the experiments, i.e., the selective bond between the gel antibody and the virus particle is strong.

The selectivity of gel antibodies against the wild type of Semliki Forest Virus was also tested. Four samples were applied: (1) gel granules selective for Semliki Forest Virus (wild type) saturated with this virus, (2) the same selective gel, but saturated with Semliki Forest Virus (mutant), (3) the same selective gel, depleted of this virus and (4) blank granules treated with SFV (wild type). From Figure 25 one can conclude that granules selective for Semliki Forest Virus (wild type) interact with Semliki Forest Virus (mutant), although to a much less extent than does the wild type, since the mobilities of the two gel-virus complexes differ by about 50 %.

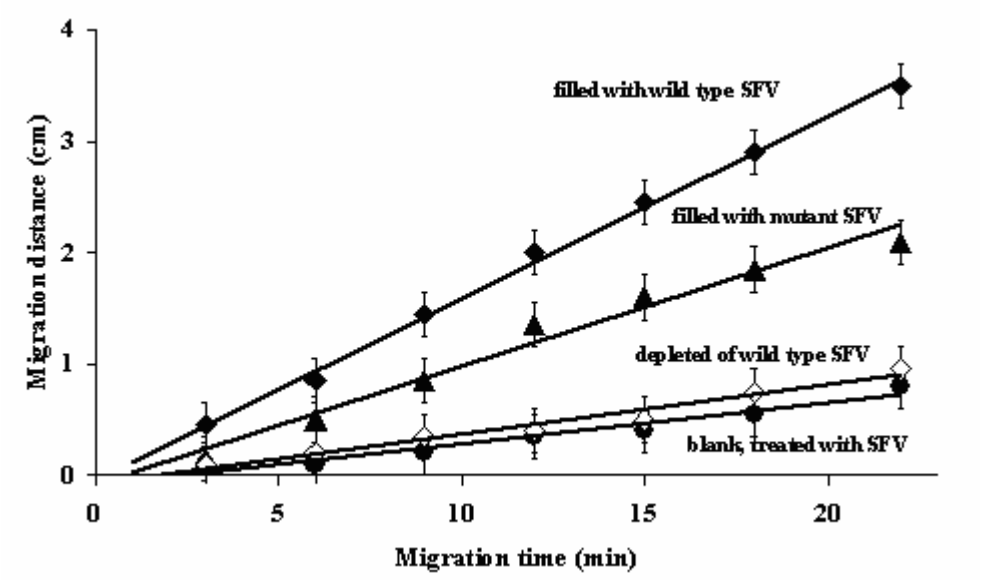
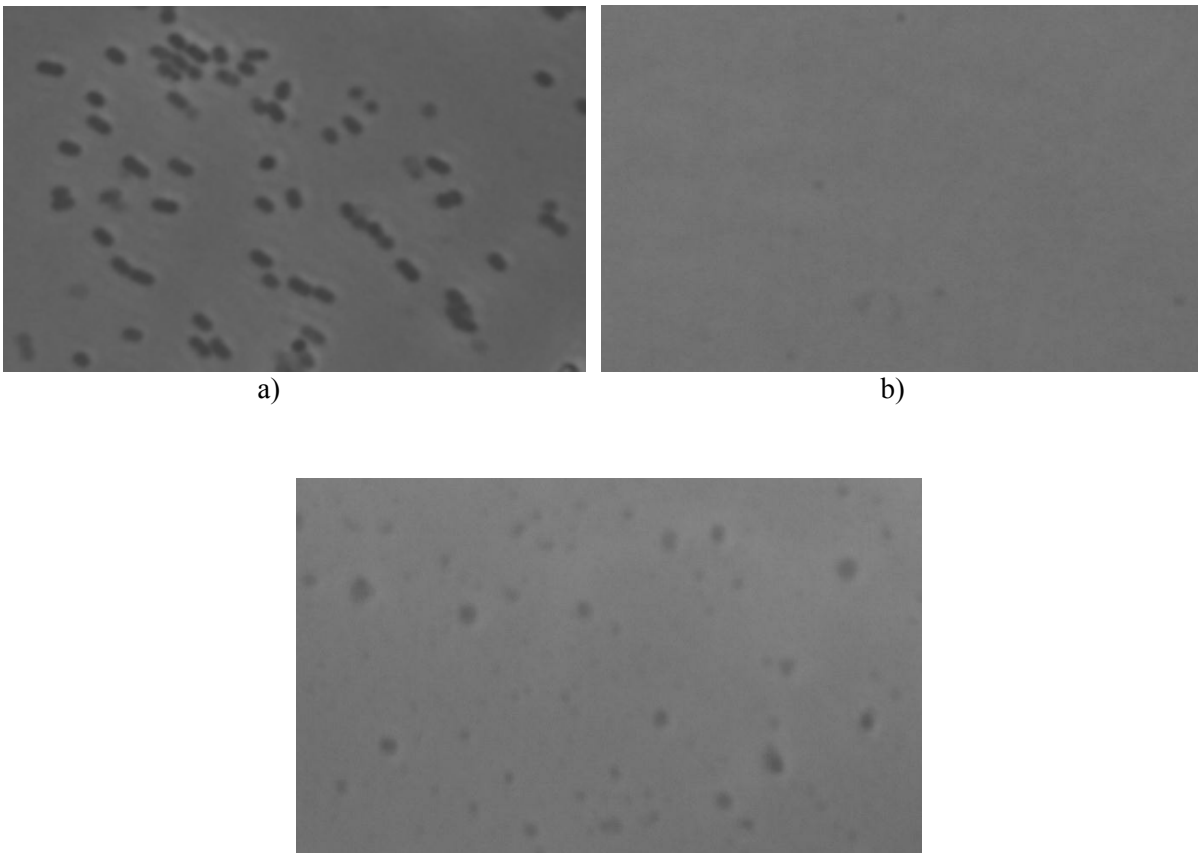


Figure 25. Free zone electrophoresis of gel granules, selective for Semliki Forest Virus (wild type) filled with this virus (◆) and the same type of gel granules filled with SFV (mutant) (▲), SFV-selective gel granules depleted of SFV (◇) and blank granules treated with SFV (wild type) (●). Voltage: 500 V. The charged particles migrated towards the anode, current: about 50 μA. The background electrolyte (50 mM Tris-HCl, pH8.5) did not contain SFV.

4.2.4 Experiments with bacteria

Selective gels were prepared in the presence of *Escherichia coli MRE-600*. The selectivity was tested after the granulation both with *Method 1* and *Method 2* (described in Section 3.3.2.3). The selective gels gave good results and high selectivity. (For more details see Bacskay *et. al.*, 2006).

The recognition process in the imprinted selective gels was controlled by a microscopic method. Thin layers of blank and selective T_6C_5 polyacrylamide gels were polymerized without any template and in the presence of *Escherichia coli MRE-600* under argon atmosphere. After the enzymatic treatment and the washing process both gel layers were treated ('resaturated') with the bacteria suspension. Microscopic pictures were taken to follow the recognition. On the blank gel no bacteria were seen. On the selective gel bacteria were recognized as 'black' spots (Figure 26a). After enzymatic treatment and washing with SDS containing buffer, and finally with buffer no bacteria were seen on the gel, *i.e.* the bacteria have been removed (Figure 26b). The incubation with the bacteria caused a resaturation of the gel (Figure 26c), where selective cavities could bind the bacteria.



a) b)

c)

Figure 26. Microscopic pictures of the *E. coli* selective gel. Bacteria are bound to the *E. coli* selective thin flat gel (a), the gel after enzymatic cleaning and washing did not contain particles (b), but after incubation with the bacteria cells were recognized on the surface again (c).

5. Discussion

5.1 The effect of chemical structure on chiral recognition

Separation of chiral compounds in the presence of a protein might elucidate the mechanism of molecular recognition by a macromolecular surface. The influence of amino acid side chains and the spatial structure of the interacting compounds are of importance in the recognition phenomenon. It was already known that iron-saturated transferrin is not able to resolve tryptophan ester enantiomers [Kilár and Fanali, 1995]. Also model calculations have been made in this field [Visegrády *et al.,* 2000a, 2000b, 2000c, 2000d; Kilár *et al,* 2002; Kilár and Visegrády, 2002]. The calculations for docking the enantiomers at the iron binding site of transferrin provided no significant differences in the positioning of the tryptophan enantiomers and in the binding energies for the protein-enantiomer complexes, while the electrophoretic runs show "high" resolution of the TBE enantiomers compared to the others (Fig. 7, 8). When interaction of different compounds with a protein is compared the interaction time should be equal. In our chiral separation approach the resolution of enantiomers by a separator (transferrin in this case), depends on the time available for interaction. Therefore, supposing that the transferrin zone is stationary, the mobility of the compound might significantly influence the resolution of the enantiomers, because during a slower movement (longer time available for interaction) the recognition of the different enantiomers is more efficient than it is during a faster migration through the protein zone. By this idea the true differences in the interaction of the three tryptophan derivatives could be compared. Since the mobilities of TME, TEE and TBE are different the comparison of the resolution should be based on the same time for interaction. In doing so different runs (with different transferrin zone lengths) can correctly be compared. The line at R=1 (Figure 10) shows that different transferrin zone widths of transferrin were required to obtain baseline resolution of the different compounds. A much longer migration through the transferrin zone was necessary to obtain complete recognition of TME enantiomers compared to TEE and TBE. However, interestingly the points for the TEE and TBE in this R vs. time curves follow the same course. The possible explanation of this phenomenon may be that the long ethyl and butyl chains provide the same difference in the positioning of one of the enantiomers compared to the others on the protein surface (note that *S* enantiomers have a weaker binding than *R*-ones [Kilár and Visegrády, 2002]), while, seemingly, the spatial recognition of the

small methyl groups in the TME enantiomers by the transferrin surface is more difficult. Although, the model calculations showed that the differences in the binding energies are similar for the three compounds [Kilár and Visegrády, 2002], the experimental results are not contradicting to this, since the strength of the binding is not reflected in the electrophoretic migration changes.

The experiments performed between pH 5 to 7 show a maximum resolution for TEE and TBE, whereas TME enantiomers are not resolved under the applied experimental conditions. This observation is not surprising, since TME enantiomers need higher ionic strength and higher protein concentration for stereoselective recognition. Transferrin releases iron at pH 5 or lower [Princiotto and Zapolski, 1975; Evans and Williams, 1978] due to a conformational change in the structure [Jeffrey *et al*., 1998], which can explain the maximum resolution at pH 5.5-6. Above this pH another effect could influence the effectivity: the change in the charge of the tryptophan compounds. Above pH 7, we were not able to perform experiments, although the transferrin molecules, having negative surface charge, migrate towards the anode, but the migration of the enantiomers was very slow or even they did not migrate at all.

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. The obvious statement that the chemical structure affects chiral recognition by a protein was demonstrated by the differences between compounds with long alkyl chains (TEE and TBE) and TME 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 TME enantiomers. Further studies will, however, be necessary to clarify the precise role of the structural components in molecular recognition by proteins [Gagyi *et al*., 2006]**.**

5.2 The effect of chemical structure on (macro) molecular recognition

5.2.1 Test for the efficiency of the selective gel granules

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 (Figure 1, 2), in the presence of different template particles ('antigen'), e.g. proteins [Liao *et al.*, 1996; Hjertén *et al.*, 1996; 1997; Tong *et al.*, 2001; Takátsy *et al.*, 2006b; 2007], viruses [Takátsy *et al.,* 2006c] and bacteria [Bacskay *et al.*, 2006] (Figure 27).

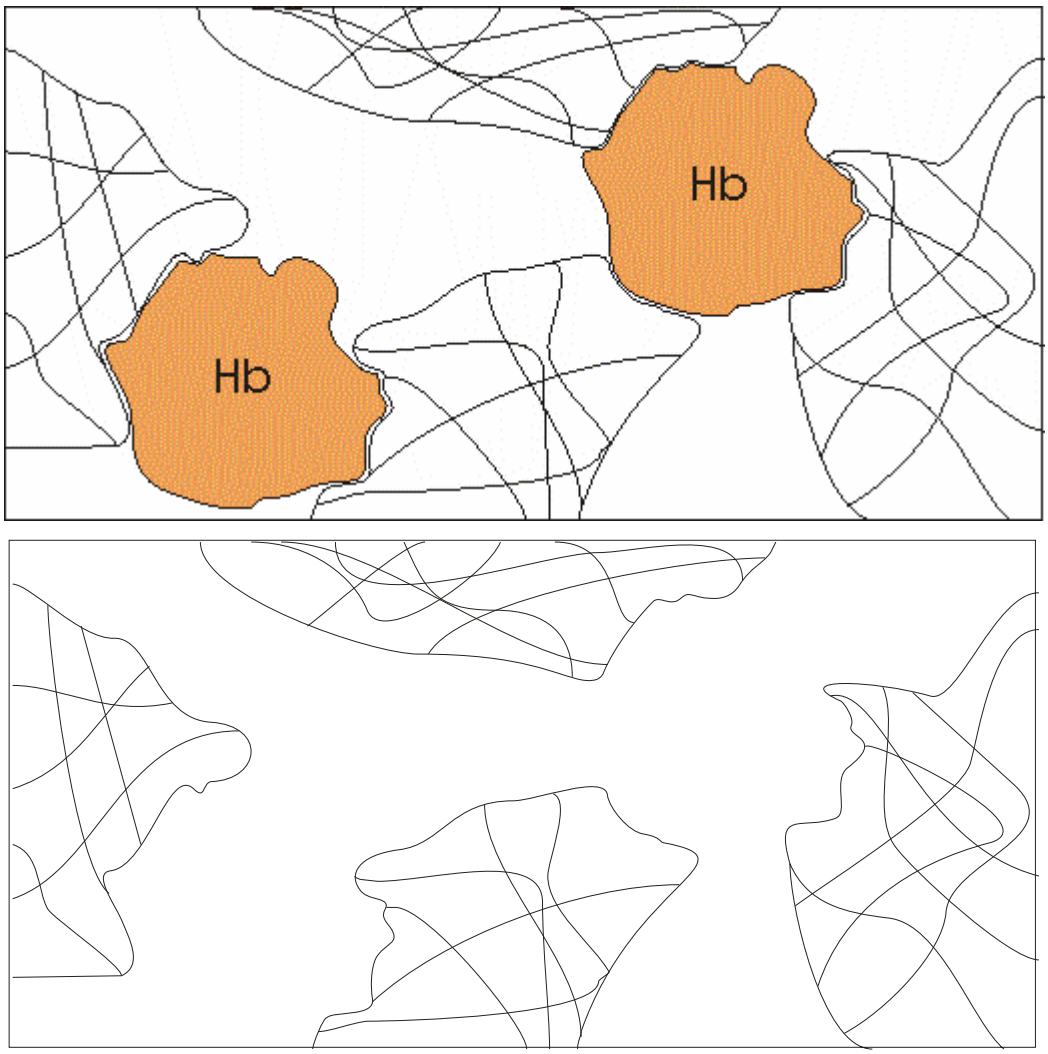


Figure 27. The working scheme of the molecular recognition of selective gels

a) Structure of the selective gel with hemoglobin (Hb) as template, b) structure of the selective gel, after the removal of the template (chemical memory: recognition take place in the cavities, which have the shape of the template).

The gels can be cut into small pieces (granules), what makes possible an easy electrophoretic detection of the recognition processes in a rotating narrow bore electrophoresis system (Figure 5). After removing the template from the gel, we receive a non-charged polymer, with cavities, which have the shape of the imprinted particle (Figure 31). This property was utilized, to investigate the selectivity of the artificial gel antibodies, because the polyacrylamide gel is neutral and, therefore, the cleaned, unsaturated, and/or blank granules of this gel do not move in an electrical field. The complex gel antibody/antigen is, thus, charged only in virtue of the charge of the template, e.g. protein (at a pH which differs from the pI of the template substance; Figs. 15 and 16). If SDS (the detergent used for the cleaning, see Section *3. Materials and Methods*) and the templates are completely removed from the gel, the granules become non-charged again (Fig. 16), and the mobility is going to be zero. The selective recognition of the template molecule can, thus, be studied by electrophoresis of the granular artificial gel antibody. This is also a simple test for the efficiency of the washing of the granules (see Fig. 13).

To compensate for a possible hydrodynamic flow in the rotating tube, caused by buffer leakage, a zone of non-charged blank granules (prepared in the absence of protein) should be injected. The 'true' migration distance is then the difference between the migration distances of the sample granules and those of these non-charged granules. Observe that in the absence of leakage an electroosmotic flow in the closed system in the free zone electrophoresis apparatus causes a parabolically distorted zone, but only a small displacement [Hjertén, 1967].

By simple washing with buffer the template selectively interacting with the gel antibody could not be removed. At pH 8.5, where the experiments were made all kind of the template-gel antibody complexes were negatively charged (pH higher than the pI values of the model proteins) and therefore migrated toward the positive pole. A similar washing of the blank granules removed the template completely. Their migration toward the positive pole is caused by leakage or/and incomplete removal of SDS. The fact that the plot of migration distance against time gives a *straight* line also in the absence of template in the background electrolyte shows that the complex is very strong (Fig. 21); if the interaction is week/reversible the template (in the Figure, the transferrin) molecules would successively be released and the migration velocity (the slope) would decrease.

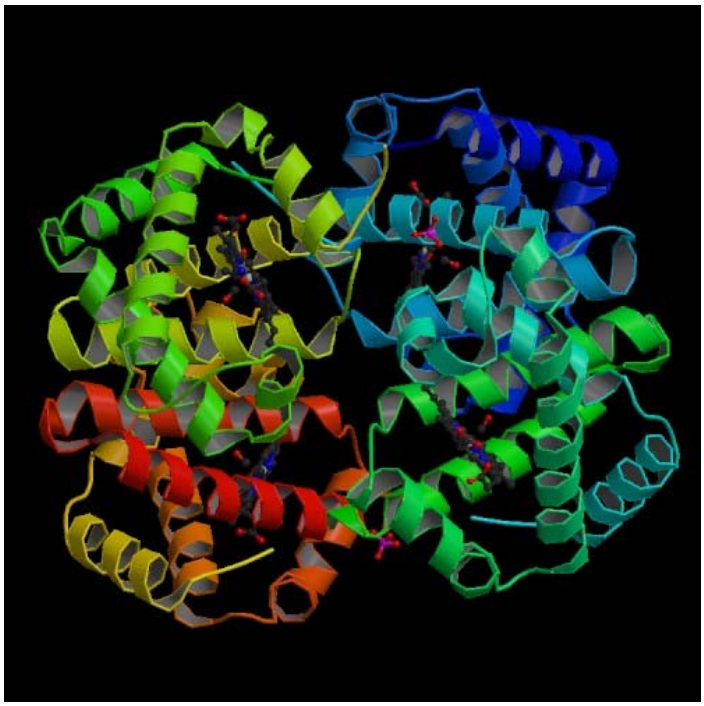
For an accurate measurement of the electrophoretic migration distance and mobility, the washing of the selective granular gel antibodies and the blank granules for removal of SDS should be done at the same time and in the same way until the selective granules migrate at

the same speed as do the blank granules (Fig. 13). One can then expect also the selective gel granules to be free from SDS and, thus, migrate in subsequent runs only by virtue of the charge of the selectively adsorbed protein.

5.2.2 Effect of three dimensional structure

The results with two different hemoglobin species (human and bovine) show, that the selectivity of the gel granules is high (Figs. 16 and 17). Both gels recognize the template protein since upon interaction with the negative protein the granules migrate in the electrical field (Figs. 15 and 16). However, the granules remained uncharged in a solution containing a protein, which is "unknown" to them: Albumin was not recognized by any of the two hemoglobin-selective gels, indicating no nonspecific 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.

No migration of human hemoglobin selective gel was observed in a bovine hemoglobin solution (Fig. 18), although the 3D structure and the amino acid composition are similar for these related proteins (Fig. 28).

a) 

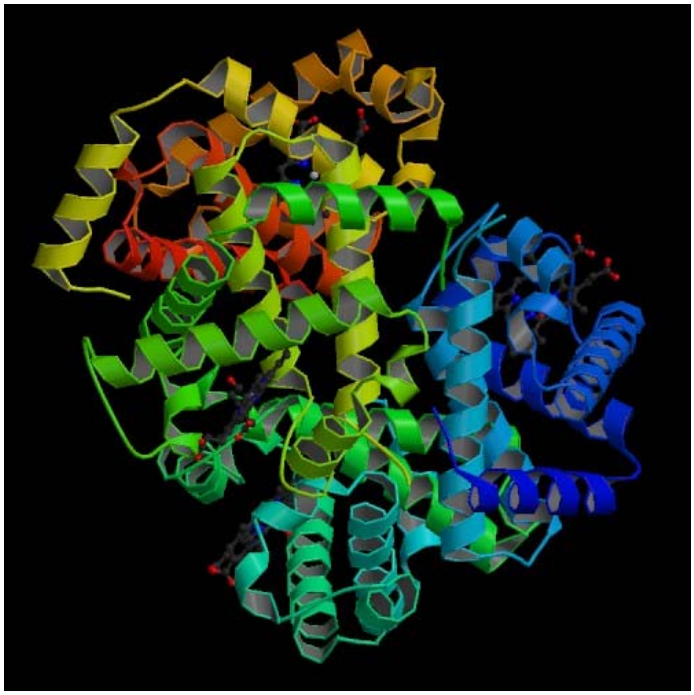
b) 

Figure 28. Structural differences between human and bovine hemoglobin

a) Ribbon structure of human hemoglobin (from RCSB Protein Databank 1MKO)
b) Ribbon structure of bovine hemoglobin (from RCSB Protein Databank)

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 (Figs. 15 and 16). In other words: the interaction is virtually irreversible under normal experimental conditions. 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) and run in a protein-free background electrolyte was somewhat lower (Fig. 16) compared to the mobility of granules, submitted to electrophoresis in the protein solution (Fig. 14): 12.7 and 13.6 T-units, respectively. The mobility of free human hemoglobin is 14.7±0.3 T-units, thus slightly higher than the mobility of the complex gel antibody/human hemoglobin.

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. However, this constant is high, since all migration points fall on the straight line drawn in the plots.

The fact that all measuring points in the plots fall on the same straight line means that no hemoglobin molecules or only a negligible number are lost during the run, *i.e.*, the interaction between gel antibody and the "antigen" is extremely strong. This high selectivity, characteristic of gel antibodies, prepared as described herein, in the other topics of this study [Takátsy *et. al.,* 2006b, 2006c, 2007; Bacskay, *et. al.,* 2006] and in refs. [Liao, *et. al.,* 1996; Hjertén, *et. al.,* 1997; Hjertén and Liao, 1998; Tong, *et. al.,* 2001] can, of course, be used for several practical applications, for instance to "fish out" a particular protein, virus or bacterium, which makes them attractive for a great number of experiments, including many of those typical of conventional protein antibodies. However, 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.

Surprisingly, freeze-dried granules, selective for human hemoglobin, regain their selectivity upon rehydration (results not shown here). The selectivity of the gel granules was similar for different batches of the artificial antibodies (the reproducibility is satisfactory). For example, the electropherogram shown in Figure 18 could be repeated with several batches of gels without loss of selectivity, *i.e.* bovine hemoglobin was (in all experiments) not recognized by the gel granules selective for human hemoglobin. 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 [Hjertén *et. al.*, 1997; Takátsy *et. al.* 2006b, 2006c, 2007] It is also possible to use the selective gels, in a form of a chromatographic column [Rezeli *et. al.,* 2006]. The successful application of the gel for chromatography proves the robustness of this imprinting methodology.

5.2.3 Effect of conformational changes

To go further the specificity for so tiny differences like conformational changes in a protein was tested, with the help of iron-free and iron-saturated human serum transferrin.

The data in Table 3 show that the mobility of the complex (iron-free transferrin)/(gel antibody, selective for iron-free transferrin) is higher (8.5 and 10.8 Tiselius units) than the mobility of the complex (iron-free transferrin/gel antibody, selective for iron-saturated transferrin) (3.9 and 4.5 Tiselius units).

Analogously, the mobility of the complex (iron-saturated transferrin/gel antibody, selective for iron-saturated transferrin) is higher (7.4 and 10.3 Tiselius units) than the mobility of the

complex (iron-saturated transferrin)/(gel antibody, selective for iron-free transferrin) (3.1 and 6.9 T-units). These findings are in agreement with the data presented in Figs. 19 and 20. Observe that the mobility is higher, the higher the concentration of transferrin in the background solution. At a concentration of 5 mg/ml the mobility (10.8 and 10.3 T-units) is even higher than that of free transferrin (9.3 and 9.5 T-units).

The Tiselius values (9.3 and 9.5) are within the experimental errors, since a difference of 1°C in temperature (which is a realistic assumption) gives a 2.7 % change in the mobility values. Therefore, it is not surprising that free zone electrophoresis cannot differentiate between these native forms (only in the presence of a denaturant, urea [Kilár and Hjertén, 1993]. However, the electrophoresis method described herein applied on the complex (protein/gel antibody against this protein) can easily distinguish these proteins in their native state (Table 3) – another example of the high resolution of this analytical technique.

The results in Figures 19 and 20 demonstrate that the binding of the proteins (used as templates for the preparation of the selective gel) is time-dependent. Granules, which bind the template protein during the electrophoretic analysis (*i.e.*, the protein is dissolved in the background electrolyte), migrate slower (with lower mobilities) than those granules (Fig. 21), which are saturated (incubation for 30 min) with the template protein before electrophoresis (observe that in this case no template protein is dissolved in the background electrolyte).

The above observations that the binding of proteins to the gel antibodies is both time and concentration-dependent should be kept in mind since it facilitates the interpretation of many experimental details.

5.2.4 Bioparticles: Effect of surface structure

Gel antibodies synthesized in the presence of Semliki Forest Virus (wild type) interact with this virus, as displayed in Figs 22 and 24, but not with BK-4 virus-like particle (see Fig 23). Figure 23 also shows that 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 (23.6 Tiselius units, Fig. 24), compared to the gel-granules, which interacted, with the virus only during the time for the electrophoretic run (10.7 Tiselius units, Figs 22). A similar time dependence was observed for transferrin interacting with the selective gel antibodies [Takátsy, et. al., 2006b].

A striking example of the extremely high selectivity is the experiment presented in Figure 25. The gel antibody synthesized in the presence of SFV (wild type) interacts with this virus (effective mobility of the virus-gel complex is 10.3 Tiselius units), although also with SFV (mutant), but to a less degree (mobility: 5.7 Tiselius units). 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 clasps the entire surface of the antigen. It should be emphasized that the latter type of antibodies cannot be produced against viruses and cells since these and other bioparticles are degraded metabolically in the experimental animal.

The straight lines (slopes) in *e.g.,* Figs 22 and 24, show that 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, for removal of virus from the circulation (a potential method for treatment of virus infections), *etc.*

Experiments with bacteria

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. Due to the size of the bacteria and the size of the gel granules the electrophoresis was performed in a narrow-bore tube, since capillaries with smaller inner diameter cannot accomodate these particles. [Bacskay *et. al.*, 2006]. As an additional method to prove this imprinting technique, thin layers of blank and E. coli selective gels were also prepared and microscopic technique was used to follow the recognition.

Figure 26 shows that 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.

5.3 Some advantages of artificial gel antibodies compared to native protein antibodies

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, probably more selective since gel antibodies have a larger contact area with the antigen (protein antibodies clasp only part of the antigen). The potential applications are numerous: they can be valuable tools in both human and veterinary medicine, for instance for detection of biomarkers for clinical diagnosis, for detection of HIV, West Nile Fever and Hepatitis viruses in blood (such tests are required in several countries prior to a blood transfusion), as sensing elements in biosensors, for rapid detection of air-borne anthrax, for rapid detection of bacteria in urine, perhaps for injection into the blood circulation system to catch HIV particles, *etc.*, *etc.* It should be added that native antibodies against viruses and bacteria cannot be raised in animals since these bioparticles are metabolically degraded. This is in sharp contrast to artificial antibodies, which with very high selectivity can easily be synthesized against bioparticles [Takátsy, 2006c; Bacskay, 2006].

Artificial gel antibodies have a high selectivity for antigens (templates), such as proteins [Liao, *et. al*., 1996; Hjertén, *et. al*., 1996; 1997; Hjertén and Liao, 1998; Tong, *et. al*., 2001; Takátsy, *et. al*., 2006b; 2007] virus [Takátsy, *et. al.*, 2006c] and bacteria [Hjertén *et. al*. 2002; Takátsy *et. al*., 2002; Hjertén, *et. al.*, 2003a; 2003b; 2005; Bacskay et. al. 2006]. The results from the experiments discussed before verify the efficiency of the following procedure to detect minor differences in the conformation and the structure of any two proteins, viruses, bacteria, etc.: 1. preparation of an artificial antibody against one of the two antigens; 2. analysis of the two complexes between this artificial antibody and the two antigens by free zone electrophoresis in a revolving narrow bore tube. 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. The selectivity can be compared with that obtained when three high-resolving separation methods, each utilizing a unique separation parameter, are consecutively applied. If the two plots of migration distance against migration time differ there is a difference in structure between the two antigens (see Fig. 19, 21, 26, 29).

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.

5.4 New electrophoretic technique to follow the molecular recognition processes

In the 1996 paper [Liao, *et. al.,* 1996] and 1997 paper [Hjertén, *et. al.,* 1997] it was proved that artificial gel antibodies can be synthesized and showed that ion-exchange chromatography on a small monolithic column with advantage can be used to study the selectivity of the gel antibodies. To this end one column packed with granules synthesized in the presence of a certain protein was prepared and another column in the absence of this protein (blank column). A sample of several proteins, one of them being the protein present during the synthesis of the selective gel granules, was applied on both columns and the eluents were analysed by ion-exchange chromatography. The column bed prepared in the presence of a certain protein is selective if the peak corresponding to that protein is absent in the chromatogram for this column, but present in the chromatogram for the blank column. This chromatographic analysis method is very robust and has the advantage to be so simple that the experiment can be performed in any laboratory. However, it requires relatively large samples, and does not permit analysis of bacteria and other large particles (viruses).

The capillary electrophoresis method, described herein, does not have these disadvantages. In fact, it permits, in principle, analysis of one single gel granule. The accuracy in the determination of the migration distance with the aid of a ruler is, of course, not very high and the fact that all steps are manual is not in line with the requirements of a modern analysis technique.

However, the capillary electrophoresis apparatus described in 1967 is fully automated [Hjertén, 1967]. Following the application of the sample the capillary is scanned automatically at predetermined times of internal, i.e. one can follow the course of a separation – a very attractive feature which even the most sophisticated, modern conventional CE-instruments lack. The many electropherograms thus obtained give a very high accuracy in the determination of peak areas and mobilities. The method is micropreparative since the zones can easily be sucked out of the rotating capillary by the same syringe as is employed for the application of the sample.

6. Conclusions and future

The chemical structure affects chiral recognition by a protein was demonstrated by the differences between compounds with long alkyl chains (TEE and TBE) and TME 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 TME enantiomers [Takátsy, *et. al.*, 2006a]. Further studies will, however, be necessary to clarify the precise role of the structural components in molecular recognition by proteins [Gagyi et al., 2006].

For better understanding of the molecular recognition a special imprinting technique for large biomolecules was used. The same protein (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 used 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 [Takátsy, *et. al.*, 2006b].

The same detection technique can be employed for selective gel antibodies against bioparticles, such as viruses [Takátsy; *et. al.,* 2006c] and bacteria [Bacskay, *et. al.*, 2006]. The combination of synthesis of selective gels 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.

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. To use this process a selective gel with a very high capacity is needed.

7. References

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