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Application of Artificial Gel Antibodies for the Detection and Quantification of Proteins in Biological Fluids

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Abstract
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The molecular-imprinting method has previously been used for the synthesis of artificial gel antibodies, highly selective for various proteins. In present study, we have synthesized artificial gel antibodies against haemoglobin, albumin and different forms of growth hormone with the aim to develop a simple and rapid procedure to measure the concentration of these protein biomarkers in samples of clinical interest. A spectrophotometric method was developed to design a standard curve in the form of a straight line, whereby the true absorption (not the recorded "apparent" absorption) was plotted against a known protein concentration. The procedure, applied to quantitative analysis of albumin in human plasma and cerebrospinal fluid (CSF) from patients with ALS, indicated that the concentration of this protein was significantly enhanced in CSF from patients with amyotrophic lateral sclerosis (ALS), compared to control samples. A low level of albumin was observed in plasma from ALS patients compared to controls. Additionally, free zone electrophoresis was employed to detect human growth hormone (GH) activity in hormone preparations purified from human pituitaries. We have successfully synthesized antibodies capable of discriminating between dimeric and monomeric GH in samples of clinical origin. To quantify these proteins a calibration curve has been designed, i.e. a plot of the electrophoretic mobility of the complex GH/gel antibody against the protein concentration in the *sample*, for instance serum or CSF.

This method was also employed for qualitative and quantitative determinations of Somatropin, a non-glycosylated GH and glycosylated-GH in a body liquid.

Our results indicate that by this technique one can "fish out" with high accuracy various proteins from both body fluids containing a great number of other proteins. It might well apply also to biomarker proteins for other diseases.

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List of Papers

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals (I-IV). Papers I and II are reprinted with permission from John Wiley and Sons.

I **Ghasemzadeh, N.**, Nyberg, F., Hjertén, S. (2008) Highly selective artificial gel antibodies for detection and quantification of biomarkers in clinical samples. I. Spectrophotometric approach to design the calibration curve for the quantification. *J. Sep. Sci.*, 31:3945–3953

II **Ghasemzadeh, N.**, Nyberg, F., Hjertén, S. (2008) Highly selective artificial gel antibodies for detection and quantification of biomarkers in clinical samples. II. Albumin in body fluids of patients with neurological disorders. *J. Sep. Sci.*, 31:3954–3958

III **Ghasemzadeh, N.**, Rossbach, U. L., Johansson, B.M., Nyberg, F. (2010). Application of artificial gel antibodies for investigating molecular polymorphisms of human pituitary growth hormone. *In press.*

IV **Ghasemzadeh, N.**, Wilhelmsen, T.W., Nyberg, F., Hjertén, S. Precautions to improve the accuracy of quantitative determinations of biomarkers, for instance in clinical diagnostics (2010). *Submitted.*

Papers not included in this thesis

1. Sedzik J, Ghasemzadeh N, Nyberg F, Hjertén S. (2009), mm in: J. Sedzik and P. Riccio (eds.), Molecules: Nucleation, Aggregation and Crystallization. Beyond Medical and Other Implications, World Scientific Publishing, Singapore, Chapter 2, pp. 11-34.

2. Ghasemzadeh, N., Nyberg, F., Wilhelmsen, T. W., Ehrenberg, M., Fowler, R., Hjertèn, S. (2010) Highly selective artificial gel antibodies for detection and quantification of tRNA biomarkers in clinical samples. In progress.

Contents

Abbreviations

AD	Alzheimer´s disease
ALS	Amylotrophic lateral sclerosis
ANOVA	Analysis of variance
APS	Ammonium persulphate
BSA	Bovine serum albumin
CSF	Cerebrospinal fluid
DEAE	Diethylaminoethyl
ELISA	Enzyme-linked immunosorbent assay
GH	Growth hormone
GHRH	GH releasing hormone
GPC	Gel-permeation chromatography
Hb	Haemoglobin
CNS	Central nervous system
HMW	High molecular weight
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IEX	Ion exchange chromatography
Ig	Immunoglobulins
LMW	Low molecular weight
MI	Molecular imprinting
u	Electrophoretic mobility
m/v %	Mass/volume %
Oyster 650	A fluorophore
PD	Parkinson´s disease
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SS	Somatostatin
Tris	Tris(hydroxymethyl)aminomethane
TEMED	N,N,N',N'-tetramethylethylenediamine
T-unit	Tiselius-unit [10^{-5} cm^2/V·s]
UV	Ultra-violet
v/v %	Volume/volume %

Introduction

The scope of the thesis

Biological functions in all living organisms depend to a great extent on interactions between molecules. Complex biological structures, such as the DNA-duplex, membranes and whole cells, are formed through intermolecular binding mechanisms. These intermolecular formations are maintained by weaker binding forces, allowing dynamics in the formation and deformation of the complexes. Many biological processes are dependent on this dynamic property of such interactions. The rapid interactions that may occur between biological entities in a cell are responsible for the different reactions involved in the storage of the biological information flowing from DNA to RNA to protein. Furthermore, molecular interactions are responsible for processes consisting of receptor-ligand interactions, such as hormone responses and cell adhesions, are a result of "weak" interactions between molecules or groups of molecules.

The possibility to mimic the natural binding phenomena has attracted many researchers over a long period of time. There has been extensive research to develop methods based on artificial recognition technology on solid surfaces which can be used alternatively to biochemical system in the sense of structure and mechanism. One approach is the synthesis of selective recognition sites in a polymeric matrix using a molecule, a group of molecules, proteins, viruses or bacteria as templates. The template dissolved in a monomer solution selectively forms bonds with the monomers. Following addition of initiator a polymer containing recognition sites selective for the template is formed. In this field, many papers about recognition of small molecules have been published. However, synthesis of recognition sites selective for macromolecules, using artificial recognition technology, has been difficult. In 1996 Stellan Hjertén presented a novel method based on the synthesis of artificial gel antibodies for selective recognition of biopolymers. By this method, binding sites similar to those in native antibodies are created in a simple way, with the same or higher specificity and physical-chemical stability.

Biopolymers play an essential role within the field of medical sciences and drug discovery. Several diseases and health conditions can be traced by determining the concentration of proteins in body fluids. Furthermore, the detection and quantification of proteins are essential in food control, envi-

ronmental investigation and protein production. Therefore many researchers have tried to synthesize materials that selectively recognize proteins in a variety of complex biological media. So far the most commonly used recognition element for binding and recognition with high selectivity and affinity is the Nature´s own solution, the protein antibody. Although antibodies are an essential tool in protein detection and quantification and are widely employed in the diagnosis and prognosis of diseases, the need for new techniques with improved properties regarding for instance, selectivity, stability and accuracy, is gradually emerging. The access to highly selective, sensitive and reproducible techniques for detection of peptides and proteins is of fundamental importance in most areas of biochemical and biomedical research. Despite the fact that recent decades have seen an enormous development in biotechnological science the most common techniques for routine analysis of protein/peptides are still based on classical immunological approaches, such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assays (ELISA). Although new techniques based on modern mass spectrometry provide extremely high precision and sensitivity, they are still not available for routine screening of proteins/peptides in biological samples, particularly because of irreproducibility [Knowles, 2007]. Consequently, there is no instrumental set for fast screening of peptide/protein levels in routine samples.

Proteins have different physical-chemical properties and recognition properties which make them ideal tools for interaction with a specific surface. Therefore, this thesis concerns a search for a simple and fast screening technique to complement immunological methods, such as RIA and ELISA, for routine quantification of proteins/peptides in biological samples.

It focuses on the possibility for quantification of proteins by spectrophotometric measurements and by free zone electrophoresis in combination with the synthesis of artificial gel antibodies. The first part of this thesis concerns the synthesis of artificial gel antibodies against albumin and haemoglobin (biomarkers) and the development of a novel method based on absorption measurements for quantification of these proteins in a sample solution. This work continues with the application of artificial gel antibodies raised against albumin for quantification of this protein (a biomarker) in human plasma and cerebrospinal fluid (CSF) for diagnosis and prognosis of a neurological disease. The final part of this thesis is centered on quantification of different forms of growth hormone in samples containing this hormone.

The number of symposia on protein biomarkers and their use for diagnosis, prognosis and treatment of various diseases increases steadily. However, there are no or only very few novel biomarkers which are routinely employed in hospitals. The reasons may be many, for instance, weighing errors, lack of selectivity, strong adsorption of the biomarker to the vials, pipettes, and other surfaces it comes into contact with, the difficulty to determine μl-volumes accurately, etc. These problems seem to be over-looked and are, therefore, discussed in this study.

Molecular recognition

In 1940, Linus Pauling introduced the concept of molecular recognition [Pauling, 1940]. He discussed the process in which antibodies are produced in the presence of an antigen. The phenomenon, molecular recognition, has a fundamental role in biological systems and is responsible for the binding between two molecules, for instance, an antigen to an antibody, which can be employed for diagnosis, prognosis and treatment of different diseases, of DNA to a protein to stimulate the pathways of gene replication and protein production, of a substrate to an enzyme and of a protein to a ligand which is used in medicinal chemistry for drug design. When two macromolecules both geometrically and electrostatically "fit together" ("lock-and-key" theory) non-covalent interactions, such as hydrophobic interactions, electrostatic interactions and hydrogen bonds are responsible for holding them together [Fischer, 1894]. However, our knowledge about molecular recognition and protein-protein interaction is still limited. To achieve optimal binding of a template and recognition sites, several factors are essential: 1) the shape and size of the template and the recognition sites, 2) a large contact area for high specificity, 3) the physical and chemical properties of the protein, the sorbent surface, and the surrounding solution of the system. Some of these general parameters are discussed in the following chapter.

The chemical bonds

It is important to know what bonds are involved in order to understand how and why a molecule or groups of molecules interact with different surfaces. A chemical bond between two atoms or groups of atoms is formed when the forces acting between them are strong enough to lead to the formation of an aggregate with adequate stability to be considered as an independent molecular species [Pauling, 1967]. The bonds involved in biological systems take place with great specificity, which requires correct distances between the reacting groups. For instance, a target protein reacting with its receptor has a spatial arrangement of some negative, positive and hydrophobic groups, etc., in the protein molecule which fits together specifically with certain positive, negative and hydrophobic groups, etc.

Non-covalent bond

Non-covalent interactions are responsible for the 3-dimensional configuration that the biological polymers adopt. These interactions also play an essential role in the flexibility of the macromolecules, their interactions with each other and with other molecules in the cell. Non-covalent interactions between electrically charged particles include ionic interactions, van der Waals interactions, hydrogen bonds and hydrophobic interactions.

Ionic interactions include forces between permanent ions, between permanent dipole and ions, between randomly oriented dipoles, and between induced dipoles and ions.

A van der Waal´s interaction is caused by correlations in the fluctuating polarizations of nearby particles. Although this type of bond is very short-ranged and relatively weaker than other chemical bonds, it plays an essential role in fields as diverse as structural biology, supramolecular chemistry, polymer science, nanotechnology, surface science, and condensed matter physics.

Hydrogen bonds are responsible for the structure and properties of water, as well as the structure and properties of biological macromolecules such as proteins and nucleic acids. This bond-type is often described as an electrostatic dipole-dipole interaction and has been classified as weak or conventional, strong or low barrier and very strong or single-well [Frey *et al.,* 1994]. The strength of the hydrogen bond depends on the donor and the acceptor, as well as their environment. Since donors of biological interest are mostly confined to OH and NH groups and since acceptors vary much more in character, the strength of hydrogen bonds are mostly affected by the position of the acceptor atom in the periodic table, polarizability, field/inductive and resonance effects of substituents around the acceptor atom, and proximity effects, including steric hindrance of the acceptor site, intramolecular hydrogen bonding and lone-pair–lone-pair repulsions [Laurence *et al.,* 2000]. The strongest hydrogen bonds form between similar heteroatoms (oxygen or nitrogen) although hydrogen bonds formed between water molecules are relatively weak.

Hydrophobic interactions are responsible for many biologically relevant processes, such as the folding of globular proteins, the formation of protein–protein complexes, the assembly of micelles and double-layer membranes [Kauzmann*,* 1959; Tanford, 1980; Blokzijl and Engberts 1993; Chandler, 2005]. These interactions are the association of purely non-polar molecules in water. The strength of these interactions depends on the gain in entropy when water molecules with a "shell structure" around the lipophilic substance are released to be more flexible in the surrounding solution, i.e., the entropy of the system increases.

Solvent effects

Non-covalent interactions depend strongly on the pH and ionic strength of the solvent, as the pH affects the charge on both the macromolecules and surrounding surfaces wheras the ionic strength affects the distance over which electrostatic interactions are effective [Su *et al.*, 1998; Larsericsdotter *et al.,* 2001]. When the ionic strength of the solvent is increased, the electrostatic interaction between a negatively charged surface and a positive charged ligand decreases. However, when electrostatic interactions are re-

duced at high ionic strength, the structural changes are reduced as well [Buijs and Hlady 1997; Larsericsdotter *et al.,* 2001]. Changes in the pH of a solvent also has major effect on the electrostatic interaction between a ligand and binding site, either to enhance or to reduce it [Galisteo and Norde 1995].

Since proteins, native antibodies and/or receptors become denatured on exposure to extremes of temperature and pH and upon contact with organic solvents, development of other types of recognition species that can be used for similar purposes are continuing.

Molecular imprinting

Molecular-imprinting technology is based on the formation of recognition sites in a matrix by means of template polymerization (Fig. 1). The method is based on the same mechanism as that used by the native antibody and the antigen. A definition of 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 biparticles, such as 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 molecules being necessary for recognition to occur in the spaces vacated by the templating species.' [Alexander *et al.*, 2006]. Due to the expensive production and the complexity of protein antibodies, many researchers have tried to develop other techniques based on selective recognition of a foreign substance.

A short historical overview of molecular imprinting

The history of molecular imprinting started around 1930 when a theory was proposed for the variety of antibody production in the presence of xenobiotic antigens [Breinl and Haurowitz 1930; Mudd, 1932]. The high specificity of antibody-antigen complex was discovered in the chiral recognition of d- and l-tartaric acid causing the interaction between the antibody and an antigen [Landsteiner and van der Scheer, 1929]. The antibody would be oriented to fit the configuration of the antigen. In 1940, an intellectual breakthrough came from Pauling, who introduced the antibody diversity theory, based on the production of different antibodies in the presence of an antigen due to different three-dimensional configurations of the antibody polypeptide chain. Pauling´s theories were employed to synthesize selective recognition species for the template by imprinting of silica gels [Polyakov, 1931; Polyakov *et al.*, 1937] with small molecules (methylorange) [Dickey, 1949]. The silica gels interacted with "sensitizing" molecules (methyl-, ethyl-, propyl- and butyl orange) and were used to create specific sites or cavities. The template

was removed by drying and repeated washing with methanol. The selectivity of these recognition sites was tested by the addition of the template dye in a mixture of different dyes. The gel prepared in the absence of the template was used as control. These sensitized silica gels were used for the separation of substances by column or by thin-layer chromatography. However, the silica gels were instable and not reproducible and the template could not be removed completely. The selectivity of the silica gel was described by two mechanisms: the association mechanism, i.e. the selectivity is determined only by the template [Morrison *et al*., 1959] and the "footprint" mechanism, i.e., the selectivity was determined by the structure of the cavity, describing the specific adsorption of the template. Through this concept, we know today that there are two methods in the molecular-imprinting technique. The components are either kept in solution before the polymerization with the help of irreversible covalent bounds, or a previous arrangement between the imprinting molecules and the functional monomers through non-covalent and metal/coordinative bounds is established.

Covalent approach

In the early 1970's, Wulff and Klotz developed a basic type of molecular recognition: imprinting in organic polymers, also the called "covalent approach", [Wulff and Sarhan, 1972; Takagishi and Klotz, 1972]. This approach involves strong, reversible covalent complex formation between the template and the surrounding polymer. Ester bonds of carboxylic/boronic acids, boronate esters, ketals, imines (Schiff bases) are the most common linkage in the complex. Following polymerization, these covalent bonds must be cleaved, which is usually done by acidic hydrolysis. Resaturation of the polymer is performed by re-establishing the covalent bonds [Wulff, 1995].

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

There are also some approaches where a combination of covalent and non-covalent techniques is employed. Here, the template is covalently bound to the monomer during imprinting. However, following removal of the template the re-adsorption of the template takes place by non-covalent interactions [Sellergren and Andersson, 1990]. Such a polymer was also made for recognition of cholesterol [Whitcombe *et al.,* 1995].

Non-covalent approach

The synthesis of imprinted polymers by non-covalent interactions provides a great variety of possible interactions; namely, hydrogen bonds [Andersson and Mosbach, 1990; Nicholls *et. al.,* 1995; Chen *et. al.*, 2001], hydrophobic

14

interactions [Nicholls *et. al.*, 1995; Dauwe and Sellergren, 1996], ionic interactions [Sellergren *et. al.*, 1985] and π-π interactions [Dunkin *et. al.*, 1993]. The greater the variety of the interactions formed between the template and the polymer, the higher is the selectivity of the imprinted polymer. Since the polarity of the solvent has a major effect on non-covalent interactions, these imprints are usually prepared in organic solvents, such as chloroform or toluene [Mosbach and Ramström, 1996]. The complex between the template and the functional monomer is formed spontaneously by noncovalent self-assembled interactions, which are then sterically fixed during the polymerisation. Following removal of the template by diffusion, e.g., washing with the mobile phase, a macroporous matrix with specific recognition sites is synthesized. The adsorption capacity is determined by the arrangement of the functional groups and the shape of the recognition sites. This method has been employed to synthesize materials with adsorption capacities and selectivities similar to antibody-antigen interactions [Sellergren, 1997; Kriz *et al.*, 1997]

Synthesis of selective adsorbents using functional monomers

In non-covalent molecular imprinting the choice of appropriate monomers is very important, since they are involved in the complementary interaction with the template during the polymerization. The functional monomers may be vinyl, methaacrylate, acrylate and acrylamide monomers [Andersson *et al.*, 1984; Sellergren *et al.*, 1988; Dunkin *et al.*, 1993; Kempe *et. al.*, 1993]. Methacrylic acid (MAA) is often used as a functional monomer and ethyleneglycoldimethacrylate (EGDMA) as a cross-linker. Several other cross-linkers with different specificity, for instance, divinylbenzene, acrylic or methacrylic acids, ethylene glycol dimethacrylate, have been used. The template consists of several proton-accepting or hydrogen-bonding functional groups. Therefore, the selectivity and the adsorption capacity of the polymer are determined by the number of proton–or hydrogen-bonds accepting sites on the template, the acidity of the functional monomer [Dunkin *et. al.*, 1993], the basicity of these sites [Dauwe and Sellergren, 1996] and changes in the polymerisation conditions, for instance, polymerisation temperature [Sellergren and Shea, 1993; Lu *et al.*, 2004], pressure, optimum value of the functional monomer, monomer-template ratio and sample load [Andersson *et al.*, 1999] and the hydrogen-bond capacity of the solvent [Sellergren and Shea, 1993].

    The main requirements for successful protein adsorbents are: 1. They must be porous to maximize effective surface area. 2. Be rigid enough to allow good flow rates. 3. The bonds between the template and the adsorbents should be stable and should not be broken, for example, by washing with a buffer. 4. They should not interact non-specifically with the template. 5. The greater the number of bonds between the template and the recognition sites,

the stronger the adsorption the artificial binding site will show. To avoid non-selective electrostatic interactions with the template, acrylamide, a non-charged monomer is used in Paper I-IV since it fulfills all the criteria for an ideal imprinting species.

Molecular imprinting of macromolecules

The field of molecular imprinting has grown significantly in recent years and has been very successful for small molecules. Many small molecules are insoluble in water and therefore cannot be used in biological processes where all of the molecular recognitions occur in aqueous media. It is important to develop molecular imprinting techniques also for water-soluble bio-polymers, such as proteins, bio-particles, for instance, viruses and bacteria. However, there are difficulties associated with imprinting of macromole-cules … *"...macromolecules such as proteins are difficult to apply as templates..."* [Andersson, 1999].

During the past decade a new artificial antibody technology for selective recognition of protein structures [Hjertén and Liao, 1998; Liao *et al.,* 1996; Tong *et. al.*, 2001; Takátsy *et al.*, 2006a; 2007; Rezeli *et al.,* 2006], virus structures [Takátsy *et al.*, 2006b] and bacteria structures [Bacskay *et al*, 2006] has been introduced. Slow mass transfer and nonlinear adsorption isotherm can cause some problems when selective matrices are employed in chromatography [Sellergren and Shea, 1995]. However, using polyacryla-mide gels and electrophoresis [Liao *et al.,* 1996; Hjertén *et al.,* 1997; Tong *et al.*, 2001; Takátsy *et. al.*, 2006a; 2006b; 2007; Bacskay *et al*, 2006] or elec-trochromatography [Rezeli *et al.,* 2006], no such limitations appeared. A highly cross-linked polymer was formed when the template was dissolved in a monomer solution (Fig. 1). Following polymerization and removal of the template, highly selective cavities for the template were formed.
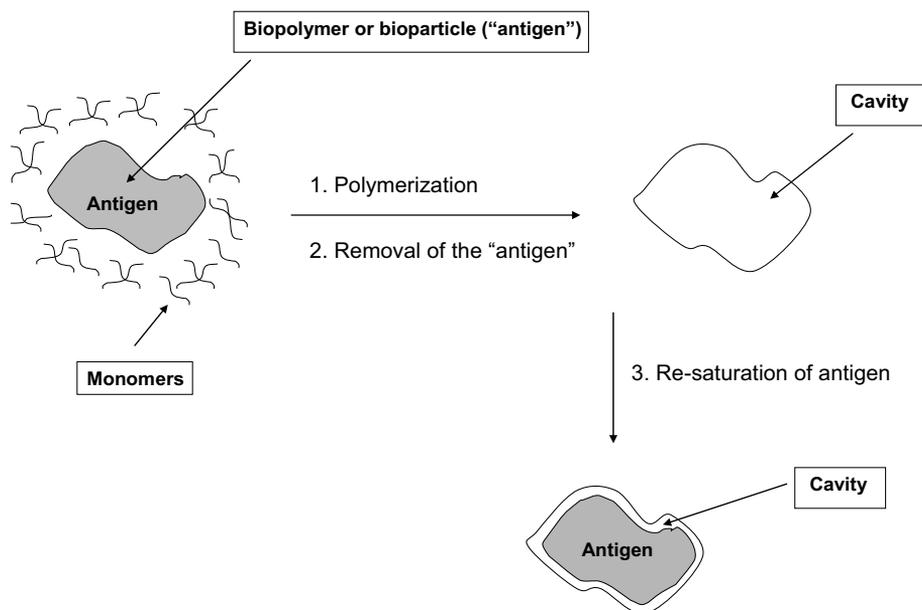
Figure 1. The principle of molecular imprinting of biopolymers (for instance proteins) and bioparticles (for instance viruses and bacteria).

Target proteins and the choice of biopolymers

Proteomics, studies of the protein structure and their function in a cell, tissue or organism, has provided essential information about these entities as potential biomarkers. An important task in this regard is to understand the relationship between the structure and the function of proteins. Such studies include the recognition and separation of proteins, as well as the determination of the interaction areas on their surfaces that have functional roles. Within medical sciences, the interest in peptides and proteins is growing. These compounds are found in cells, tissues and all body fluids and are studied in order to identify relevant biomarkers for diagnostic and prognostic purposes. Proteins have various important functions and have, therefore, a broad range of applications in drug discovery and as diagnostic biomarkers for detection, treatment and prediction of different complex diseases. Protein biomarkers can also be used to distinguish between different diseases showing similar symptoms, for instance, Alzheimer disease (AD) and vascular dementia [Jong *et al.*, 2006]. Other applications of biomarkers include early diagnostic of diseases, such as cancer, heart disease, Parkinsons disease (PD), etc., in their early stages, since early treatment is crucial for fast recovery and for long-term survival. Methods available today for the analysis of proteins from biological matrices such as plasma, urine, serum and different tissues are RIA, ELISA, MS, etc. A novel method based on molecular im-

printing technique for analysis and quantification of proteins is presented in this thesis. To study the concept of artificial antibody technology, imprinting of different circulating proteins (human albumin, haemoglobin, γ-globulin, growth hormone, transferrin) has been used. Bovine albumin and transferring used in some studies will not be discussed herein. The difficulty to determine accurately the concentration of a protein biomarker used in clinical samples and the reasons why so few protein biomarkers are not routinely used in hospitals are also discussed in this thesis.

Haemoglobin

Haemoglobin is a tetrameric protein mainly found in red blood cells and consists of four very similar subunits. Haemoglobin is a dimer of αβ subunits. Each subunit contains a heme group, which contains an iron atom making the binding and the transport of oxygen possible in the blood. The normal haemogobin concentrations in blood for adult male, adult female and children are 135-175 g/l, 122-150 g/l and 100-140 g/l, respectively.

Free zone electrophoresis combined with the synthesis of artificial gel antibodies has previously been used to study the selectivity of the gel antibodies against bovine and human haemoglobin [Takatsy *et al.*, 2007]. This study showed that the artificial gel antibodies "sense" the small difference in structure between these different forms of haemoglobin.

As a response to different conditions, for instance, mortality caused by iron deficiency, anemia (low haemoglobin concentration), hemoglobinopathies (genetic diseases), the level of haemoglobin is decreased 2-fold, which causes a decrease in oxygen carrying capacity [Yip, 2000]. Several clinical methods, for instance, hemoglobinometry, ELISA and colorimetry, have been employed to meet the great need for the determination of haemoglobin concentrations in blood. However, more rapid and accurate methods for the measurement of haemoglobin concentrations are desirable for diagnosis of various diseases associated with haemoglobin deficiency. A novel method to determine the concentration of human haemoglobin in a body fluid based on absorption measurements of artificial gel antibodies with selectively captured haemoglobin molecules will be described in this thesis.

Growth Hormones

The brain controls muscles and movement by production of hormones affecting organs, cell production and glands in humans [Strand, 2003]. Growth hormone (GH) is primarily recognized for its ability to promote longitudinal growth in children and adolescents, but is also affects various important metabolic functions throughout adult life. It is produced in the anterior pituitary gland by somatotrophs. The dry weight of the pituitary gland consists of around 10 % GH. Two hypothalamic peptides regulate the production of GH, somatostatin (SS) and GH releasing hormone (GHRH). GHRH stimulates differentiation and proliferation of somatotrophs [Tannenbaun, 1991;

Jansson *et al.*, 1985] and elicits release of GH, whereas SS antagonizes the GHRH-induced production of the hormone. Human growth hormone (hGH) is a member of a class of hematopoietins containing an antiparallel 4-helix bundle fold. The structure of hGH has been analysed by X-ray crystallography; it is a single-chain polypeptide of 191 amino acids and binds to a homodimeric receptor [Goeddel *et al.*, 1979; Pearlman and Bewley, 1993]. It mediates cell growth and differentiation by binding to specific transmembrane receptors. Growth hormone has a major effect on fat, carbohydrate and protein metabolism. Abnormal high secretion of hGH results in acromegaly, characterized by enlarged bones of the body. 25 % of patients with acromegaly develop type 2-diabetes [Sonksen *et al.*, 1991]. Metabolic effects of GH have been described as early insulin-like effects and late insulin antagonistic, diabetogenic, effects. Acute insulin-like effects include hypoglycaemia, increased lipogenesis and increased glucose and amino acid transport and metabolism. The late insulin-antagononistic or diabetogenic effects of GH include increased lipolysis, increased levels of free fatty acids, insulin resistance in animals and human, decreased glucose transport, hyperglycaemia and hyperinsulinaemia [Rizza *et al.*, 1982; Hansen *et al.*, 1986]. Long periods of GH treatment are required for treatment of these antagonistic effects. Furthermore, GH may have effects on the central nervous system (CNS) including memory, mental alertness, motivation, and working capacity [Nyberg, 2000]. Growth hormone is used as a drug in both humans and animals.

Growth hormone with different biological activities occur in the human body as several structural isoforms. It has been suggested that the number of GH forms that can be counted in plasma may exceed 100 [Baumann, 1991]. For instance, earlier studies have suggested that in addition to monomeric entities (molecular sizes around 20 kDa) the hormone may exist in covalently and noncovalently-linked dimeric forms (molecular sizes around 45 kDa) [Brostedt and Roos, 1988]. Additionally, other pituitary hGH isoforms of various sizes, from 5, 12 to 17, 22, 24, 27 and 35 kDa have been identified. Several studies by electrophoretic experiments have shown different migration velocities of mononeric GH in both in rodents [Roos et al. 1987] and human [Silberring *et al*. 1991]. The reason for charge or size heterogeneity of the hormone is the presence of glycosylated forms of GH. Earlier studies suggested the presence of N-glycosylated variants of GH in human pitutitary extracts [Garcia-Barros *et al.*, 2000] and by studies using lectin-binding techniques [Sinha and Lewis, 1986].

Other examples of the molecular heterogeneity of pituitary g caused by posttranslational modification are phosphorylation, acetylation, aggregation and deamidation. In 1956, the first hGH was purified [Li and Papkoff, 1956] and its structure was later characterized [Pearlman and Bewley, 1993]. However, in 1963, a process for the purification of growth hormone from whole frozen pituitaries was developed [Roos *et al.* 1963]. To understand how

growth hormone works is a huge challenge and still many of its functions are not identified. In clinical studies it is important to be able to discriminate between different forms of circulating proteins. For instance, studies have suggested that the ratio between size variants of GH and prolactin differ in patients with pituitary adenoma [Oosterom and Lamberts, 1985] and in some pathological conditions, such as galactorrhea, an alteration in the proportion of circulating monomeric and dimeric GH was reported [Wallis *et al*. 1982].

Furthermore, studies have also suggested that the level of larger forms of GH may vary in acromegalic patients compared to normal subjects [De Palo *et al*. 1990]. In clinical studies using radioimmunoassay (RIA) native antibodies do not discriminate between hormones of different size. Therefore it is considered that an estimated value of a hormone concentration as recorded by RIA measurements reflects the concentration of all immunoreactive components, including those differing in size, which is expected, considering the great surface similarity between the monomer and the dimer [Ochoa *et al*. 1993].

To overcome this difficulty and to design an assay that distinguishes between differently sized hormones in work presented in this thesis we have applied a recently developed technique based on artificial polyacrylamide antibodies prepared by a novel technique. We have employed these gel antibodies to detect growth hormone (GH) activity in fractions recovered and purified from fresh, frozen human pituitaries. This purification step yielded the growth hormone activity in two separate peaks in areas corresponding to those of proteins of the molecular weights of 22 and 45 kDa, respectively, of the monomer and the dimer. The hormones recovered as two separate entities of different molecular size was used for imprinting in polyacrylamide gels to obtain artificial antibodies recognizing these differently sized forms of the hormone.

Albumin

The most abundant protein by far in the circulatory system of human blood is human serum albumin (HSA) (constituting 55% of the total plasma proteins in blood). The concentration of albumin in blood is 35 to 50 g HSA/l of blood [Rowland and Tozer, 1995]. It is a single chain monomer with 583 amino acids, which include 35 Cys residues, 82 positively charged residues and 100 negatively charged residues [Peters, 1996]. Due to its high net surface charge and many disulphide bonds, HSA is a highly water-soluble and stable protein. HSA is a globular, non-glycosylated protein with a MW of approx. 67 kDa. HSA has different physiological functions, the main of which is to regulate the osmotic pressure to maintain the proper distribution of body fluids in the circulatory system (homeostasis) [Andersson *et al*, 1976; Valmet, 1969]. Albumin is also essential for the transport of hormones [Andersson *et al*, 1976], fatty acids, conjugated bilirubin and various drugs [The Green Cross Corp., 1995] in the blood stream. Its bovine analog,

BSA, is widely used as a model protein for chemical and physical studies, partly because of its abundance and availability in large amounts. Serum albumin is a marker of inflammatory states, since it is down-regulated in this state. It is also a biomarker of amyotrophic lateral sclerosis (ALS), a neurological disease characterized by a progressive loss of motor function. The rate of survival may vary among individual patients and is shown to range from a few months up to more than 10 years from the time-point of diagnosis. Proteomics studies have revealed the importance of biomarkers for diagnosis of patients with ALS and other motor neuron diseases from healthy individuals and from patients affected by other diseases [Dengler *et al*, 2005; Kolarcik and Bowser, 2006; Ranganathan *et al.,* 2005].

In work described in this thesis, we have analyzed albumin, which is not a unique CSF marker for ALS, although it has previously been shown that albumin in CSF samples from ALS patients is enhanced [Annunziata and Volpi, 1985; Apostolski *et al.,* 1991; Leonardi *et al.,* 1984; Meucci *et al.*, 1993].

Difficulties in disease detection and treatment, together with the lack of suitable diagnostic and prognosis tools, indicate a need for discovery or identification of biomarkers (proteins or peptides) for a certain disease. In this thesis, we describe the use of artificial gel antibodies against human albumin to quantify this constituent in human cerebrospinal fluid (CSF) and plasma collected from patients with amyotrophic lateral sclerosis (ALS). A standard curve was designed and levels of albumin could be determined and compared between the two body fluids (Paper II).

Both bovine (Paper I) and human albumin (paper II) were used to prepare artificial antibodies employed for studies described in this thesis.

Methods used in analysis and detection of antigens

Protein antibodies

The most commonly used construct for molecular recognition is the antibody. Antibodies, also called immunoglobulins (Ig), are highly abundant affinity molecules that play essential roles in the specific immune system of humans and other higher vertebrates. Antibodies protect our body from foreign invaders, such as virus and bacteria by activating the immune system to initiate destruction of the pathogen. They are found in human body fluids and in the blood of all other vertebrates as well. Antibodies produced by plasma cells have the same structure and differ only in the structure of the epitope-recognition site (also called hypervariable region) where the binding of the antigen occurs (Fig. 2). Because of extreme variations in the hypervariable region, millions of antibodies specific for different antigens exist with different binding sites. Based on the structure of their constant region, the immu-

noglobulins in humans are divided into five subclasses (IgA, IgD, IgE, IgG and IgM). All protein antibodies are Y-shaped and have four polypeptide chains, two identical light chains (23 kD) and two identical heavy chains (50-70 kD). Disulfide bonds and non-covalent interactions are responsible for the interaction between the heavy and light chains and between two heavy chains [Harris *et al.,* 1992]. The antigen binding site is mainly formed by six hypervariable loops in the variable regions (VH, VL), called the complementarity determining regions (CDRs) [Wu and Kabat, 1970]. Native antibodies are produced by immunizing experimental animals with an antigen, thereby activating their immune response to produce antibodies against different epitopes of that specific antigen. Polyclonal antibodies produced by immunization have different amino acid sequences binding to different epitopes on the same antigen. However, monoclonal antibodies recognizing only a single epitope are more desirable for therapeutic use.
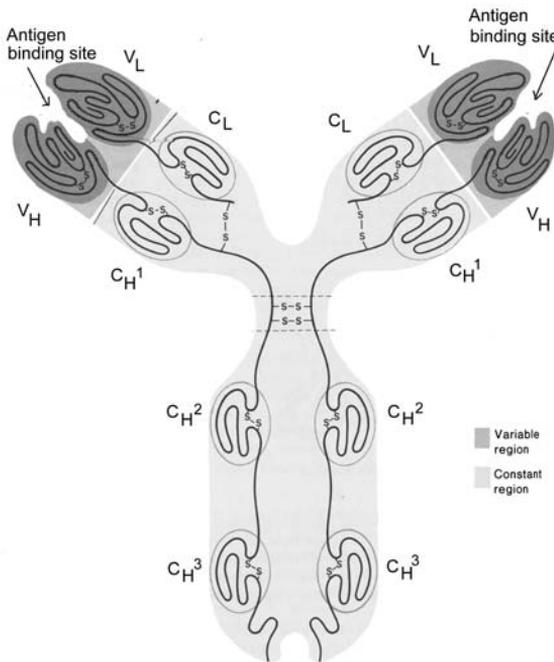
Figure 2. Schematic presentation of protein antibody [Zubay, 1983].

Antibodies are used in several immunodiagnostic tests for diagnosis of infectious diseases, such as multiple sclerosis and hepatitis [Nowinski *et al.,* 1983]. The antigens (for instance, a protein) are often investigated by ELISA, two-dimensional gel electrophoresis (2D-GE), RIA and mass spectrometry (MS). This leads to a growing need for new improved methods for quantification and identification of different proteins from biological sam-

22

ples, toxins and drugs. The antibodies are proteins and therefore are influenced by sample handling and time. This puts great demand on the method to be used to avoid fast degradation of antibodies. Due to the major difficulties and the high costs of production of human antibodies, alternative methods with similar recognition properties and affinity capacity are essential in clinics.

In this thesis, a molecular imprinting-method for detection and quantification of different proteins is described.

Artificial polyacrylamide gel antibodies

The synthesis of these gel antibodies

Artificial gel antibodies are synthesized from a solution of acrylamide and bis-acrylamide in the presence of an antigen (the template). Following polymerization the gel is granulated and the antigen (for instance a protein, a virus or a bacterium) is removed. The resulting MIPs have cavities, the shape of which corresponds to the shape of the antigen. This imprinted polymer (after removal of the imprinting template/antigen) has the unique property to capture the template/antigen from, for instance a body fluid, with very high selectivity.

The structure and porosity of the gel is influenced by the monomer and cross-linker concentrations, and their ratio (Fig. 3), by the concentration of the catalyst and by the pH of the monomer solution.
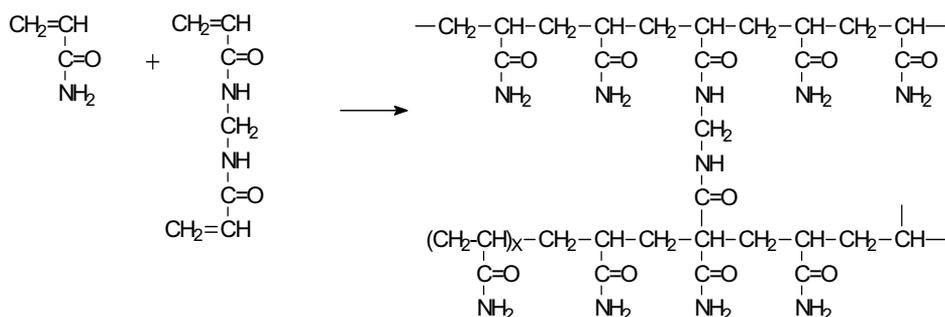
Figure 3. The synthesis of the artificial gel antibodies from a solution of acrylamide and N, N´-methylenebisacrylamide.

The following two methods have been used for the detection and analysis of an antigen selectively recognized by artificial gel antibodies.

23

The chromatographic method

The sample containing a mixture of different proteins is applied to a column packed with gel granules selective for a certain antigen. The same volume of the sample is then applied to another column packed with non-selective gel granules synthesized in the absence of the antigen (the blank column). All substances present in the sample should appear in the eluate from this column at the initial concentrations.

This experiment is then performed on the column packed with the selective gel granules synthesized in the presence of the antigen. In the ideal case, all substances present in the sample appear in the eluate from this column except the antigen because it has been adsorbed to the bed.

This method was employed to test the selectivity of artificial gel antibodies against proteins with somewhat different structures (see Fig. 4). A comparison of the two chromatogram traces shows that myoglobin from horse, but not that from whale, was adsorbed onto the column prepared in the presence of the former protein, indicating a high degree of selective recognition, although the amino acids sequences of these two myoglobin species differ by only three amino acids (Fig. 5) [Sedzik *et al.*, 2009].
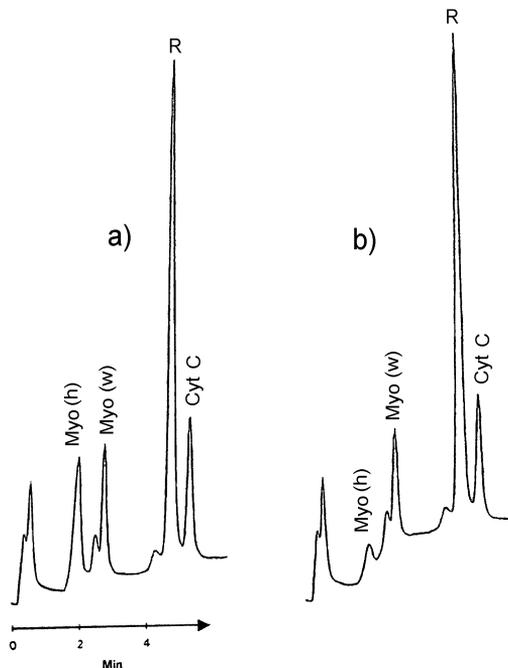
Figure 4. Ion-exchange chromatography of the eluate from a column packed with non-imprinted gel granules (a) and gel antibodies selective for myoglobin from horse (b). The bed in column (b) does not recognize myoglobin from whale, although the structural differences are very small [Hjertén *et al.*, 1997]; see Fig. 5.
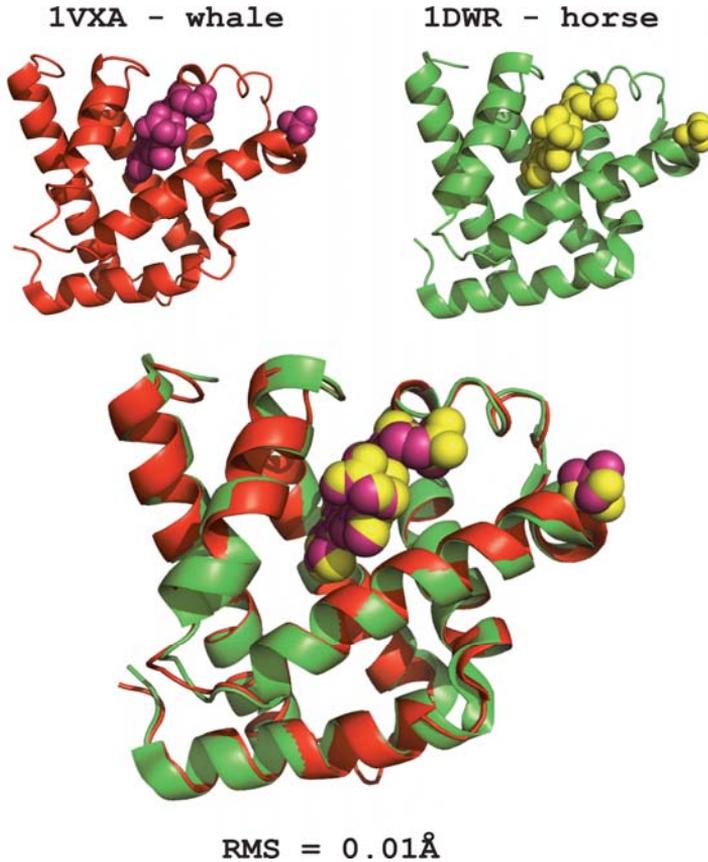
Figure 5. The structure of whale myoglobin (1VXA) and horse myoglobin (1DWR). Both proteins can be fitted with RMS=0.01 Å [Sedzik *et al*., 2009].

The artificial gel antibodies used in this study are in the form of granules. However, they can also be prepared as continuous beds (monoliths) [Rezeli *et al.,* 2006]. These beds can selectively capture a protein for determination of its concentration in a body fluid.

The electrophoretic method

Electrophoresis is the migration of charged particles and molecules in a solution under the influence of an applied electric field [Kohlrausch, 1897]. For our electrophoretic analyses of gel granules with captured protein biomarkers we used the free zone electrophoresis approach designed in 1958; [Hjertén, 1958]; see Fig. 6. All of the many theoretical and practical problems caused by convection, adsorption to the tube wall, electroosmosis, detection of the analytes by UV-scanning of the electrophoresis tube, including indirect detection were solved by Hjertén (1967). This scanning technique

has the great advantage to give the true separation pattern, whereas in the recent alternative used in capillary electrophoresis, with a stationary detector an apparent separation pattern is obtained. The width of a peak, which is obtained in time units, is not proportional to the width of the zone in length units; nor is the peak area proportional to the amount of the analyte in the zone. In the presence of electroosmotic flow only apparent plate numbers can be calculated. Hjertén has shown how these apparent plate numbers can be transformed to true plate numbers [Hjertén, 1958], which, unfortunately, are seldom reported. The narrow widths of the peaks obtained in the presence of a high electrophoretic flow are very often taken as an indication of narrow zones in the capillary-a completely erroneous interpretation.

Free zone electrophoresis performed in a rotating narrow bore quartz tube is the precursor of capillary electrophoresis, a method which is widely used for identification and separation of many important biological molecules, for instance, amino acids, nucleotides, nucleic acids and proteins. Recently, free zone electrophoresis was used to study the selectivity of artificial gel antibodies for both biopolymers, such as (proteins) and bio-particles, such as viruses and bacteria. These experiments showed that artificial gel antibodies can sense differences in structure between strains of bacteria, between wild type and a mutant of Semliki Forest Virus, between iron-free and iron-saturated human serum transferrin and between human and bovine hemoglobin, although the structures of all these templates are very similar.
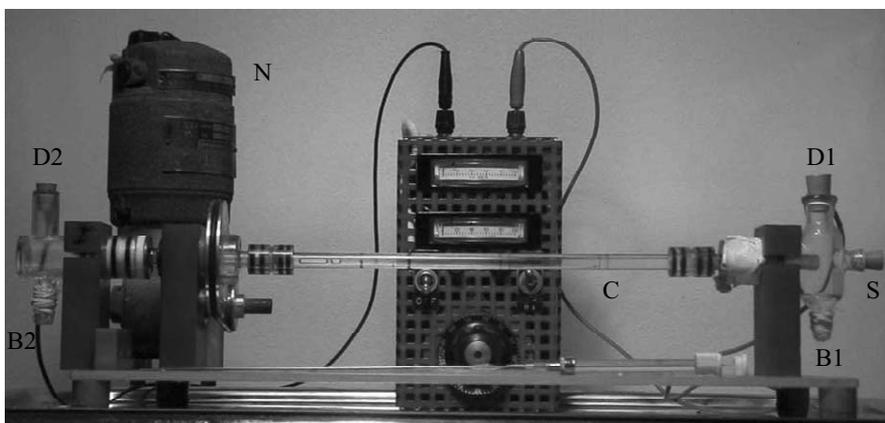
Figure 6. The original, 1958-version of the apparatus for free zone electrophoresis, used in this study. C, the glass narrow-bore separation tube length 245 mm, OD 9.6 mm, ID: 2.5 mm. This tube is rotated at a speed of 40 rpm with the help of a motor (N), to suppress convective disturbances of the zones. D1 and D2 are electrode vessels. The sample (in our experiments granules) was injected into the rotating tube (C) by a syringe after removal of stopper (S). Dialysis membranes (B1 and B2) counteract hydrodynamic flow in tube C.

This simple, easy-to-handle, inexpensive electrophoresis technique can with advantage be employed not only to study the selectivity of the imprinted gel granules, but also to quantify a template (for instance a biomarker) in a sample solution (see Paper III and IV).

Some methods for purification and analysis of proteins

Short history

The concept of chromatography can be traced to the Old Testament (Exodus 15:25; where Moses purifies water using a piece of wood). The Russian botanist M. S. Tswett (1872-1919) demonstrated by simple experiments that glass columns packed with calcium carbonate could be used to separate different plant pigments. Since then many researchers have tried to repeat the work of Tswett, but failed. In 1940 liquid chromatography and ion-exchange chromatography for purification of several rare earth element oxides were introduced which was a breakthrough for the development of various chromatographic methods.

Gel permeation chromatography

Gel permeation chromatography (GPC), also known as size-exclusion chromatography or gel filtration, is based on the separation of molecules based on their molecular sizes. Large molecules elute earlier than do smaller molecules [Porath and Flodin, 1959].

Ion-exchange chromatography

Ion-exchange chromatography (IEX) was developed in the 1960s and has since then been used frequently for separation of macromolecules. In IEX, the charged ions in the stationary phase are loaded with a counter-ion of opposite charge ion during regeneration and equilibration. IEX can be run either in the cationic or in the anionic form. In cation-exchange chromatography the stationary phase is loaded with negatively charged ions which adsorb positively charged molecules, whereas in anion-exchange chromatography the phase has positively charged groups which adsorb negatively charged molecules [Fritz, 2004]. In IEX, the separation of the proteins depends on their net surface charge, which is affected by the composition and the concentration of the mobile phase. A protein will adsorb to an anion exchanger at a pH above the isoelectric point of the protein and below the isoelectric point to a cation exchanger. Various proteins can be separated by altering the ionic concentration or the pH of the mobile phase.

The potential of spectrophotometry in protein analysis

The most widely used method to determine the concentration of a protein is based on absorbance measurements at 280 nm (aromatic band) and 205-220 nm (peptide band). According to Beers´ law, the absorbance is directly proportional to the concentration of the analyte. The extinction coefficient, expressed $E^{1\,mg\,ml^{-1}}$ varies from one protein to another, since the quantity of the UV-absorbing amino acids differs significantly. Absorption measurements at 280 nm gives an accurate concentration determination of a purified protein, provided a proper blank is employed. Absorption at shorter wavelengths is used as a far more sensitive method. Due to absorption by oxygen at short wavelengths, peptide absorption measurements at 192 nm cannot be recommended. The determination of the concentration of a protein is affected by the presence of compounds such as salts and buffers, and by pH, temperature, ionic strength and, in some cases, by interactions with other proteins and adsorption to surfaces of vials, cuvettes, etc. To increase the accuracy of absorption measurements, buffers with minimal light absorption must be employed. Most salts absorb light below 215 nm with the exception of phosphate buffers, buffers based on the ammonium ion, borate buffers, pyrrolidine and triethylamine. In order to achieve good reproducibility in quantification of proteins by absorption measurements, buffers with good buffer capacity should be used: a 20 mM phosphate buffer, pH 6.8 is used in Papers I and II of this thesis.

The granular, artificial gel antibodies cause disturbing light scattering in spectrophotometric measurements. The assay was therefore modified to get accurate absorption values for the determination of the protein concentration in these granules (see Papers I and II).

However, a novel assay based on accurate absorption measurements of the stained protein at longer wavelengths, around 500-800 nm to determine the concentration of a protein in a sample solution is presented in this thesis. At these wavelengths the light scattering is strongly reduced.

The potential of fluorescence measurements in protein analyses

Fluorescence is widely used in biochemical, biological and biophysical sciences to detect protein interactions and conformational deformations. The advantage of this technique is its high sensitivity and easy labeling of proteins with dyes (fluorophores). Fluorophores are often aromatic components where absorption of light causes a molecule to become fluorescent, i.e., to emit photons. The emitted photons have less energy and longer wavelengths than the exciting photons. The intensity of the fluorescence depends on both the types of fluorophore and its environment, such as pH and chemical composition. Fluorophores can be attached to various functional groups in proteins including, amino groups, carboxylic groups, thiols and amides.

Aims of the thesis

Artificial gel antibodies have shown high selectivity for different antigens. Therefore, the selectivity need not to be improved. This thesis deals particularly with the quantification of a protein, for instance, in a body fluid, using these gel antibodies. They may be a complement to protein antibodies raised in animals, or in some cases a substitute. The advantage of this approach is that it may increase the precision of the assay and avoid the use of animals for raising antibodies.

1. Albumin and haemoglobin are used as biomarkers of several diseases. A first aim of this work was to develop a method based on spectrophotometric measurements to determine the concentrations of albumin and haemoglobin in body fluids.

2. To determine the concentration of HSA in CSF and plasma from patients with ALS.

3. To examine the effect of the molecular structures of proteins on the molecular recognition in the imprinting technique upon recognizing macroassemblies, like growth hormone (*monomeric, dimeric, non-glycosylated and glycosylated*) applying the electrophoretic method.

4. To develop a universal electrophoresis technique to determine the concentration of proteins in a body fluid based on their selective capture by artificial gel antibodies

5. To investigate whether the combination of synthesis of artificial gel antibodies and electrophoretic analysis of the complex gel antibody/antigen could be used to design standard curves to facilitate the determination of the concentration of biosynthetic GH (Somatropin) and glycosylated hGH in a sample solution.

6. By free zone electrophoresis detect the activity of monomeric and dimeric GH in fractions purified and obtained from human pituitaries.

Materials and methods

Proteins

Haemoglobin and albumin

Haemoglobin at a concentration 0.28 mg/ml was prepared from human blood [Molteni *et al.,* 1994]. Bovine and human albumin embedded in the artificial gel antibodies was stained with 0.5% w/v Coomassie Brialliant Blue G-250 (CBB) in 7% acetic acid. The determination of the concentration of these proteins, captured by their gel antibodies, can be determined at wavelengths in the visible part of the spectrum, where light scattering is much less pronounced.

Growth hormone

Somatropin (Somatropin CRS batch 2) was purchased from the European Directorate for Quality of Medicines & HealthCare (EDQM), Strasbourg, France, and glycosylated- hGH (1st International Standard of hGH) was obtained from the National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom); these two GH variants were purified as described in [Somatropin monograph 0951, 2001].

Monomeric and dimeric growth hormones samples were prepared from fresh, frozen human pituitaries according to the method of Roos et al. (1963). The human pituitaries were homogenized and extracted at pH 5.5. The homogenate was centrifuged, yielding a supernatant subjected to ammonium sulfate precipitation. The precipitate was dissolved in Tris buffer and filtered through a column of Sephadex G-100. The purified active material obtained from column yielded in two peak fractions, one of which contained proteins of molecular sizes ranging from approximately 25 to 50 kdalton [Roos *et al.* 1963]. Prior to further purification by chromatography on DEAE-Sepharose-CL6B and Sephadex G-100, the frozen fraction was dialyzed overnight in 20 mM Tris-HCl buffer, pH 7.5.

For the DEAE-Sepharose-CL-6B separation the dialyzed material was diluted with water (1:1) and then adsorbed to the column (5 cm x 15 cm), equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was further washed with one volume of this buffer and the residue was eluted with the

Tris-HCl buffer containing 0.5 M NaCl. Fractions of 5 ml were collected at a flow rate of 60 ml/h. The protein fractions 40-48 were collected in a pool of 40 ml and taken for further purification on a Sephadex G-100 column (2.5 cm x 80 cm) equilibrated with 0.04 M ammonium bicarbonate and operated at a flow rate of 50 ml/h. Fractions of 2 ml were collected and this purification step yielded the growth hormone activity in two separate peaks in areas corresponding to those of proteins of the molecular weights of 22 and 45 kD, respectively.

The fractions 50-58 and 66-75 were collected separately in two pools designated the dimeric and monomeric GH fraction, respectively. For further purification, these two pools were applied on HPLC gel filtration column and subsequently analysed by HPLC ion-exchange chromatography. The protein content in each fraction was determined by light absorption measurements, assuming that the absorption in a cuvette with a 1-cm light path length at 280 nm corresponds to 1 mg of protein/ml. The HPLC gel filtration was carried out using the ÄKTA System equipped with a pre-packed Superdex 75 HR10/30 column.

Around 3 mg of lyophilized monomeric or dimeric GH material was applied to the column equilibrated with 0.05 M NH_4HCO_3 buffer and fractions of 1 ml were collected at a flow rate of 0.75 ml/min. In order to study the GH activity, the artificial gel antibody techniques combined with zone electrophoresis was used. Ion-exchange chromatography (IEC) was then conducted on the ÄKTA-purifier for a selection of fractions. Resource Q, a pre-packed strong cation exchange HPLC-column with a column volume (CV) of 6 ml was used. One ml of the preceding SEC fraction was diluted with an equal volume of 20 mM Tris-HCl (pH 8.8) and loaded onto the IEC column. Unbound sample was washed out before starting the elution of the bound sample compounds by a linear gradient of 0 - 0.5 M potassium chloride in 20 mM Tris-HCl (pH 8.8). The length of the gradient was set to 14 CV at a flow rate of 4 ml/min. The volume of the saved fraction was 1 ml.

Selective gels, molecular recognition of 'antigens' by molecularly imprinted matrices

Papers I-IV

The selective gels were prepared according to the procedure used in refs. [Liao *et al.*, 1996; Hjertén *et al.*, 1997; Tong *et al.*, 2001]. This method includes mixing of the template protein 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 templates were different proteins (bovine and human albumin, haemoglobin or different forms of growth hormone). Non-charged granules were

also prepared in the absence of any template substance to be used as a *blank*. The experimental conditions were as follows:

I. The concentrations of the monomer and the cross-linker, their ratio, the amount of the catalyst and pH have major effects on the structure of the gel. Polyacrylamide gels having a total monomer concentration (T) of 6 % (m/v) and a crosslinking concentration (C) of 5 % (w/w) [Hjertén, 1962] were synthesized in the presence of a template (human and bovine albumin, haemoglobin). Acrylamide (142 mg) as monomer and N,N'-methylene-bis-acrylamide (7.5 mg) as cross-linker were dissolved in 1.4 ml of 50 mM Tris-HCl buffer, pH 8.5. Bovine albumin (4 mg), dissolved in 1 mL of this buffer, was mixed with the monomer solution. Prior to de-aeration, 10 µl of a 5 % (v/v) TEMED solution was added. The polymerization, initiated with 10 µl of a 10 % (m/v) ammonium persulphate solution, proceeded overnight at room temperature. Deaeration is required for the synthesis of reproducible poyacrylamide gels because oxygen inhibits the polymerization.

The concentrations of acrylamide and N,N´-methylenebiscryalmide were the same as that employed for bovine albumin for preparing haemoglobin-imprinted gel granules. Prior to polymerization, 200 µl of haemoglobin was dissolved with the monomer solution.

Two procedures were used for the removal of the template adsorbed to the gel antibodies to obtain gel granules called, *control gel*s or protein-depleted granules.

In *method I* the imprinted albumin gel granules were treated with trypsin leaving albumin molecule-shaped cavities in the gel granules. To achieve this, 0.75 mg of trypsin dissolved in 2.5 ml of 25 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$, was added to 2.5 ml of settled, albumin imprinted gel granules. The enzyme treatment proceeded for 4 h at 37^oC.

In *method II* the haemoglobin-imprinted molecules were also removed by repeated decantation with 50 mM SDS in 50 mM Tris-HCl buffer, pH 8.5 and 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 KCl (K-ions precipitate dodecyl sulphate).

II. The gel selective for human albumin was prepared using the same procedure as that employed for bovine albumin (see Paper I).

III. The synthesis of the artificial gel antibodies against the monomertic and dimeric GH fractions was carried out using the same procedure as that employed for Somatropin and glycosylated growth hormone (see Paper IV).

IV. The concentrations of acrylamide and N,N´ -methylenebisacryalmide were the same as those given in paper I for preparing haemoglobin and albumin-imprinted gel granules. 12.28 mg of a dry powder in ampoules containing 2% glycine, 0.2% mannitol, 0.2 % lactose and 0.25% of a lyophilized solution of pH 7.3, were added to the above acrylamide monomer solution to a final concentration of 1.735 mg glycosylated-hGH/ml of the solution. For removal of the imprinted GH the gel granules were treated with trypsin as described in Paper I.

Artificial gel antibodies selective for Somatropin were prepared using the same procedure as that described above for glycosylated hGH. 15 mg of a powder containing 5 % Somatropin was used in the polymerization step.

## Resaturation of depleted selective gel granules

Re-establishment of artificial gel antibodies/protein complexes from gel granules depleted of the template protein was made by incubation of the gel granules in the protein solution for 2-3 h. Following adsorption/capture of the antigen the non-bound protein molecules were removed by repeated decantations using 50 mM Tris-HCl buffer, pH 8.5, or 20 mM phosphate buffer, pH 6.8. Blank granules were treated in the same way.

Determination of the concentration of a biomarker

Spectrophotometric approach to design the calibration curve for the quantification (Paper I-II)

Artificial gel antibodies selective for albumin were prepared as described in Paper I. A spectrophotometric method for the determination of the light absorption of the proteins (antigens), captured in the gel antibodies was developed. 900 µl of granulated gel antibodies, depleted of the protein antigen, was transferred to six test tubes. 1800 µl of 50 mM Tris-HCl, pH 8.5, was added to the reference test tube (to be employed for the reference cuvette) containing albumin-depleted albumin-selective artificial gel antibody granules (control gel). Five samples for the sample cuvette were prepared by the addition of 39, 64, 129, 193 and 257 µl of a solution of albumin (28 mg of albumin dissolved in 10 ml of the 50 mM Tris-HCl buffer) to each of the five test tubes for the albumin solutions (corresponding to concentrations of albumin of 60–400 µg/ml) containing 900 µl of settled gel granules, pre-treated with trypsin, suspended in different volumes of the Tris-HCl buffer (Fig. 7). The treatment with albumin proceeded for 3 h. Following re-adsorption of the protein and staining with CBB, the gel granules were quickly transferred to the sample cuvette. When a protein has no chromophore (albumin in this case) its concentration in a sample can be determined

following staining of the protein with CBB. For this procedure, 90 μl of the CBB solution was mixed with 1.8 ml of 0.05 M Tris-HCl, pH 8.5, and 900 μl of settled gel granules containing different amounts of protein. The staining proceeded for 30 min. The excess stain was removed by repeated decantations until the solution above the gel granules was colourless. The control gel granules were transferred to the reference cuvette following the same procedure. The light absorbed by the blue-stained proteins in the gel granules at 588 nm was determined spectrophotometrically and plotted against the concentrations of albumin in the sample solutions for the design of the calibration curve.
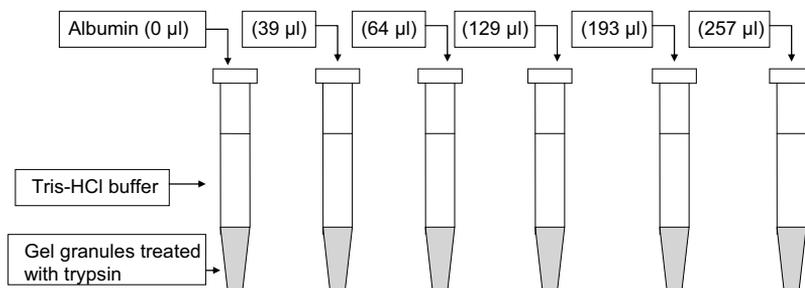
Figure 7. Schematic description of the procedure used to design the standard curve, using absorption measurements.

The calibration curve for human albumin (Paper II) was prepared using the same procedure as that employed for bovine albumin (see Paper I).

The calibration curve for haemoglobin was prepared using the same procedure as that employed for albumin (see Papers I-II). The sample cuvette contained granular, artificial gel antibodies selective for haemoglobin prepared by adding 11.5, 22.5, 45.0, 90.0 and 180 μl of haemoglobin to 900 μl of the haemoglobin-selective SDS-free gel granules, suspended in 1990, 1980, 1955, 1910 and 1820 μl of the phosphate buffer (the total volume of each sample was 2900 μl). This re-adsorption of haemoglobin proceeded for 2 h. The reference cuvette contained a suspension of Hb-selective gel granules depleted of haemoglobin (control gel).

Haemoglobin has a chromophore with an absorption maximum in the visible part of the spectrum (at 416 nm). Therefore the standard curve can be prepared without staining of the protein.

Determination of the albumin concentration in CSF and plasma from the standard curve (Paper II)

The calibration curve for human albumin described above was employed to determine the albumin concentration in CSF and plasma from six patients diagnosed for ALS. Control CSF and plasma samples were obtained from patients who had normal concentrations of albumin. Control CSF from three different pools of this fluid and control plasma from three separate pools of plasma were studied. Each pool of control CSF and plasma originates from around ten subjects. The thawed plasma was centrifuged for 5 min at 1200 rpm and was then immediately added to gel granules for selective adsorption of albumin. 1 ml of CSF was added to 900 μl of sedimented gel granules selective for human albumin, suspended in 800 μl of 50 mM Tris-HCl buffer, pH 8.5. 1800 μl of a solution of plasma (49 μl of plasma mixed with 12.6 ml of the Tris-HCl buffer), corresponding to concentrations of albumin of about 200 μg/ml, was added to 900 μl of settled gel granules, pretreated with trypsin. The treatment with CSF and plasma proceeded for 3 h. Following adsorption and staining of albumin, the gel granules were packed in the sample cuvette. The reference cuvette contained stained control gel granules selective for albumin. The absorption of the stained human albumin selectively adsorbed to the gel granules was recorded at 588 nm.

The free zone electrophoresis approach to design the calibration curve for the quantification of biomarkers (papers III-IV)

Free zone electrophoresis has been used advantageously to monitor selective interactions between a protein (the antigen) and an artificial gel antibody synthesized by the molecular-imprinting approach. The technique was used to design standard curves to determine the concentration of different forms of growth hormone in a sample solution.

The standard curves of Somatropin (non-glycosylated GH) and glycosylated GH in paper IV were prepared by the same procedure as that above used for albumin (Papers I-II), except that in this case free zone electrophoresis (instead of a light absorption measurement) was employed to quantify different forms of growth hormone selectively captured by the artificial gel antibodies. The migration distance of the gel granules treated with different concentrations of GH was measured during electrophoresis for 40 min.

Electrophoretic experiments were performed in the original apparatus of free zone electrophoresis [Hjertén, 1958]. A small suspension of the artificial antibodies was injected into the rotating 245 mm long glass tube (inner diameter: 2.5 mm, outer diameter: 9.6 mm), coated with polyacryalmide to eliminate EOF and non-specific adsorption [Hjertén, 1985]. The rotational speed was 40 rpm to suppress sedimentation of the sample. The running voltage was set at 500 V, yielding a current of about 0.2 mA. The charged gel

particles migrated towards the anode. Often two sample zones were applied in the rotating electrophoresis tube: gel granules saturated with different forms of GH (Somatropin or glycosylated hGH) and a control (gel granules, depleted of growth hormone by trypsin). By substraction, the true electrophoretic migration distance of GH selectively adsorbed to the artificial gel antibodies was obtained. The mobilities were calculated in Tiselius units (10^{-5} cm^2 V^{-1} s^{-1}) [Catsimpoolas *et al.*, 1976] and plotted in the standard curves. The mobility of the artificial gel antibody granules treated with a sample of different forms of GH (of unknown GH concentration), inserted into the calibration curve gives on the x-axis the concentration of the monomer in this sample solution, provided that there are no dimers in the sample. Dimers in the sample will probably also be adsorbed but it is difficult to predict to what extent, which decreases the accuracy in the determination of the concentration of GH. An interesting question is whether GH exists in both the monomeric and dimeric form in the pituitary. If so, what is the ratio of the concentrations of the monomers and the dimers. We cannot exclude that dimers form upon storage.

The standard curves designed above were used to determine the concentration of the monomer and dimer GH in the eluates from the gel filtration experiments (Paper III). Briefly, 450 μl from each test tube was added to 450 μl of a settled suspension of the gel antibodies and 450 μl of 50 mM Tris-HCl buffer, pH 8.5. Following incubation and washing of the granules their electrophoretic mobility was determined by free zone electrophoresis.

Results and Discussion

A test to find out whether a gel is appropriate as a matrix for an artificial gel antibody

Selective gels (also called artificial antibodies) are used in molecular recognition experiments. The gels are synthesized from acrylamide with N,N'-methylene-bis-acrylamide, as a crosslinker (Fig. 3) in the presence of the template (the 'antigen'), e.g., a protein [Liao *et al.*, 1996; Hjertén *et al*., 1997; Tong *et al.*, 2001; Takátsy *et al.*, 2006a; 2007], a virus [Takátsy *et al.,* 2006b] or a bacterium [Bacskay *et al.*, 2006]. However, imprinted species prepared to have good molecular-imprinting properties should not be synthesized from charged or hydrophobic monomers, since there is a risk that they may cause a decrease in the selectivity of the gel antibodies. One may also come into a situation where the protein cannot be released, since at high (low) ionic strengths hydrophobic interactions are strong (weak), whereas electrostatic interactions are weak (strong).

Polyacrylamide gels polymerized in the presence of a protein as a template, gives these a special conformation. The interaction between the high-molecular weight antigen (protein) and the cavity in the gel antibody should have the character of short-range forces, such as hydrogen bonds, van der Waal´s forces and induced dipole-dipole interactions; hydrophobic and electrostatic bonds should, thus, be avoided. A simple experimental test for this property is to take two absorption spectra of the protein antigen, one prior to and one following the polymerization [Tong *et al.,* 2001]. A shift in the absorption maximum (around 280 nm) indicates that short range forces have developed between the protein and the cavities in the gel, i.e., the gel is a candidate for a gel antibody.

In contrast to polyacrylamide gels agarose gels are not composed of free draining polymers, (they are in the form of helices) and cannot therefore clasp a protein as heartily as can polyacrylamide polymers. Therefore, no shift in absorption maximum of albumin was observed for agarose gels [Hjertén *et al*., 1997].

Fluorescence imaging of protein selectively captured in granular, artificial gel antibodies

Fig. 8 shows an experiment where this technique was used. A. Non-imprinted blank gel granules treated with Oyster 645; B. γ-globulin depleted gel granules (control); C. γ-globulin was added to the same type granules as those used in B) and non-adsorbed γ-globulin was removed, Oyster 645 was added and then non-reacting Oyster was sucked off. These experiments show that fluorescence imaging is a complement to absorption measurements in the UV-region. Unfortunately, for *quantitative* determination of the protein captured the method was inaccurate.
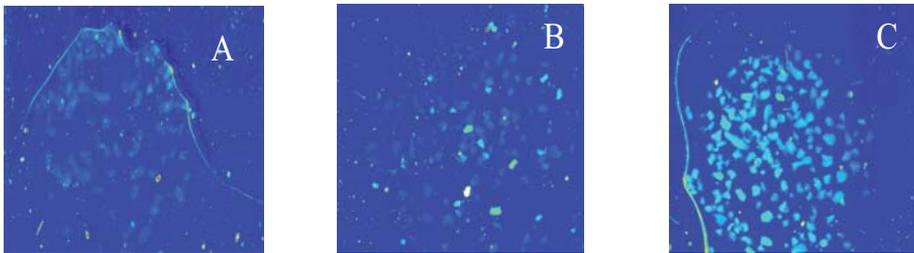

Figure 8. Fluorescence imaging of artificial antibodies selective for γ-globulin. The fluorescence intensity of A. non-imprinted gel granules (blank gel) labelled by Oyster 645; B. γ-globulin imprinted gel granules treated by pepsin, washed with buffer and then treated with the same fluorophore as in A; C. the same gel as in B with the exception that the γ-globulin was added to the gel particles (the gel granules were labelled with Oyster 650). The difference in the fluorescence intensity of A, B and C shows that the γ-globulin can be recognized by the gel particles synthesized to be selective for γ-globulin.

Determination of the concentration of high-molecular weight bio-markers in a body fluid

Determination of the concentration of albumin in CSF and plasma

Paper I deals primarily with the development of a spectrophotometric method for the determination of the concentration of a biomarker (in this paper albumin and haemoglobin) in a body fluid. However, the development of this method was not straight-forward because of the disturbing background in the absorption measurements caused particularly by light scattering from the gel granules. The absorption as recorded by the spectrophotometer is, therefore, not proportional to the concentration of the protein. The parallel

light beams, 1-3 entering the cuvette with protein-free or protein-containing gel granules in a spectrophotometer change their directions by refraction (1), reflection (2) and scattering (3), respectively, when they leave the cuvette (see Fig. 9) and, therefore, they will be falsely recorded as 100% absorbed. Only beam 0 does not change its direction upon the passage through the cuvettes and, therefore, strikes the photosensitive area of the detector, as any beam should do in the ideal case.
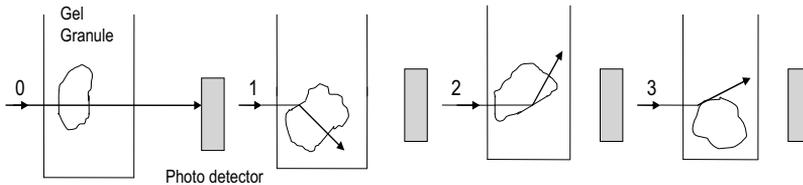
Figure 9. A schematic illustration how the incident parallel light beam becomes divergent when it passes the cuvette packed with irregular protein-free and protein-containing gel particles. Light beam 0 does not change its direction upon the passage through the cuvette. Light beams 1–3 change their directions by refraction, total reflection and scattering, respectively, and, therefore, they are not recorded.

Scattering from the polyacrylamide gel granules in the visible part of the spectrum is small (Fig. 10A) and staining of proteins with CBB with an absorption maximum at 588 nm (Fig. 10B) can, therefore, with advantage be used for the determination of the concentration of proteins, for instance, for the design of a standard curve (Fig. 11). For coloured proteins, for instance, haemoglobin with an absorption maximum at 416 nm, no staining is required (data not shown). The concentration of an antigen in a sample (for instance albumin) can be determined from the corrected experimental absorbance value of the protein-containing selective gel granules. The true absorbance-value is, therefore, the recorded absorbance value at the maximum minus the absorbance value (+0.017 in Figure 10B) at the base of the spectrum.
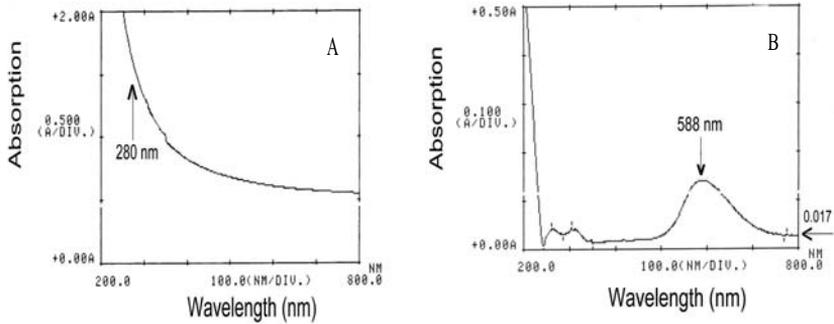
Figure 10. A. Absorption spectra of non-imprinted (blank) gel granules. The high absorbance values at short wavelengths are probably caused by true absorption by the gel granules and light scattering. B. Absorption spectra of CBB-stained artificial gel antibodies with selectively adsorbed albumin.

In conclusion, the spectrum should always be recorded, not only the apparent absorbance value at the absorption maximum, since this value includes "absorption" originating from losses of light caused by refraction, reflection and scattering.

Paper II is centered around a technique to capture albumin (a biomarker) from body fluids (in this case CSF and plasma) by artificial gel antibodies and then determine the concentration of the biomarker with the aid of a standard curve (Fig. 11).

The measured absorbance value at 588 nm of the CBB-stained CSF- and plasma-albumin, selectively adsorbed to the gel granules, was inserted into the standard curve (Fig. 11) to determine its concentration in CSF and plasma. The plasma and CSF were diluted to concentrations of albumin around 200 μg/ml before they were added to the selective gel granules. The artificial gel antibodies could detect albumin with high precision in both human plasma and cerebrospinal fluid (CSF). Quantitative analysis of albumin in CSF and plasma in control subjects and in ALS patients are given in Fig. 12. The mean level of albumin in CSF from (ALS) patients is more than two-fold higher than that from control subjects (Fig. 12). This finding is in agreement with previous reports for albumin in CSF from ALS patients [Annunziata and Volpi, 1985; Apostolski *et al.,* 1991; Leonardi *et al.,* 1984], which confirms the validity of our analysis technique and indicates that the barrier permeability may be affected in ALS. In several other disorders related to CNS, for instance, in patients with multiple sclerosis, an impairment of the blood-brain-barrier (BBB) leads to leakage of several plasma proteins into the brain parenchyma [Vos *et al.,* 2005]. Furthermore, disruption of the BBB in areas of motor neuron degeneration in the brain and spinal cord also occurs in mice-modeling ALS [Garbuzova-Davis *et al.*, 2007]. The plasma from ALS patients exhibits a large decrease in albumin concentration com-

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pared to the albumin concentration in plasma from control subjects (Fig. 12). This finding is somewhat surprising, since there are no literature reports about any differences in albumin concentration between plasma from ALS patients and healthy controls.
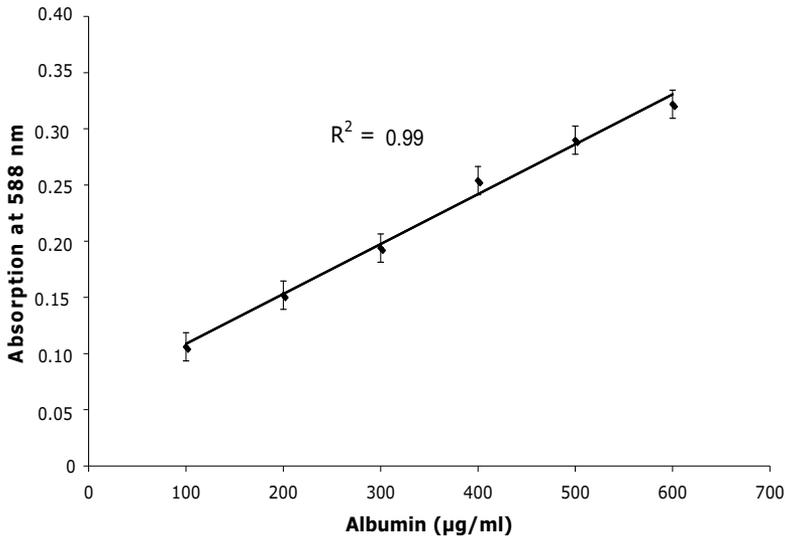
Figure 11. Standard curve for the determination of the concentration of human albumin following staining with CBB in a body fluid (in this case CSF and plasma).
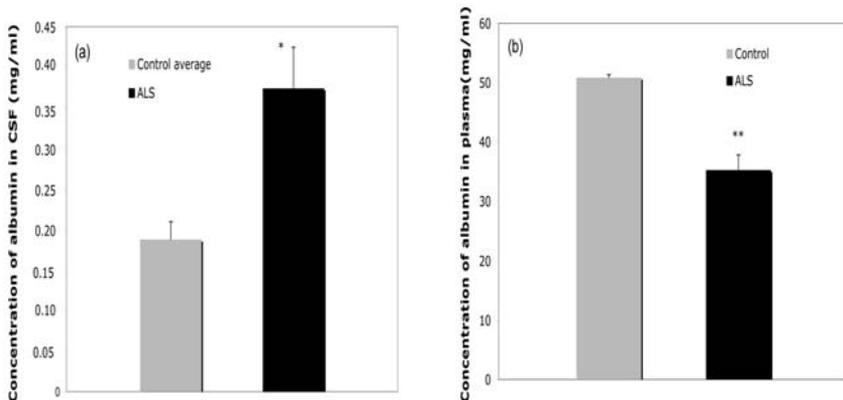
Figure 12. Quantitative analysis of albumin in CSF (a) and plasma (b) in control subjects and in ALS patients. The standard curve in Fig. 11 was used for the determination of the concentration of albumin. Data are expressed as mean values SEM. a) *P < 0.05 (0.037); b) **P < 0.01 (0.0016).

The principle of the method for the determination of the concentration of different forms of growth hormone by free zone electrophoresis

Part of our research is centered around GH and its biochemical and pharmacological effects and around separation techniques in general. In Paper III artificial gel-antibodies were used to probe human growth hormone (GH) activity in hormone fractions purified from human pituitaries. A partially purified fraction containing differently sized forms of the hormone (Fig. 13A) was further processed to yield two separate entities of different molecular sizes (22 and 45 kD) (Fig. 13B). These two forms of GH antibodies were used for imprinting in polyacrylamide gels to obtain artificial antibodies recognizing these differently sized forms of the hormone in experiments analyzing the two forms of the hormone by HPLC gel-permeation. The dimeric fraction analyzed by electrophoresis in a SDS/ polyacrylamide gel was resolved into several components (data not shown). However, the fact that the dimeric fraction also contains noncovalent-linked monomeric entities of sizes 20, 22 and 24 kdalton, opens for the possibility that the dimers of GH may be split into these entities in the presence of SDS.
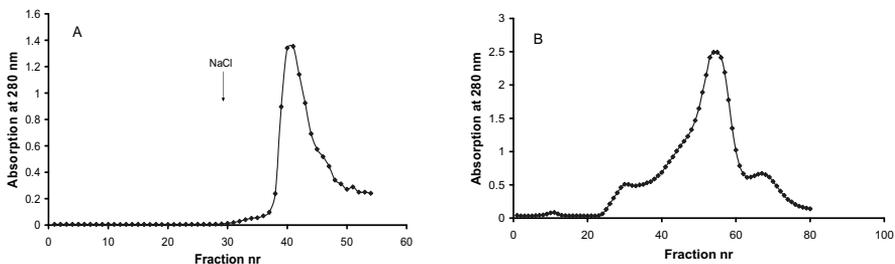
Figure 13. A. Enrichment of hGH isoforms from a human pituitary extract partially purified by DEAE anion-exchange chromatography. B. Separation of hGH isoforms from partially purified human pituitary extract by gel filtration chromatography. The growth hormone activity was found in two separate fractions, designed as dimeric and monomeric GH.

The artificial gel antibodies raised against the dimeric and monomeric fractions were used for the preparation of standard curves (Fig. 14) and then applied to probe the hormone fractions collected after separation on the HPLC gel permeation column (Fig. 15). The selective recognition of the template entity was studied by means of a very recently developed method based on free zone electrophoresis of the granular artificial gel antibodies (Paper IV).
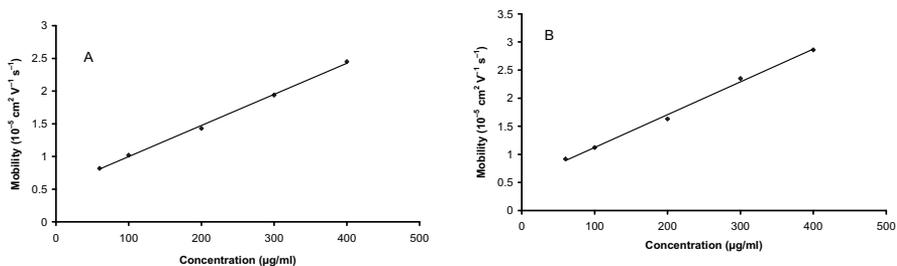
Figure 14. Standard curves for the determination of the concentration of A. monomer (antigen) and B. dimer (antigen) based on free zone electrophoresis experiments.

The experiment shows that the artificial monomeric antibodies were more potent to recognize the monomeric GH (Fig. 15A), whereas the antibodies against dimeric GH were more effective to capture the dimeric hormone fraction with much higher affinity than for the monomeric hormone material (Fig. 15B). By this method artificial gel antibodies may be used to discriminate between GH monomer and dimeric forms in assays of the GH level in the circulation. Artificial gel antibodies have much higher selectivity than native antibodies in their ability to detect the differences in structure between proteins (in this case monomeric and dimeric GH) since all segments of the surface of a protein are imprinted in the *gel* antibodies, but not in the *protein* antibodies, although the interaction forces are the same. Furthermore, in the detection of dimeric forms of the hormone by native antibodies raised against monomeric GH the antigenic area of the hormone may be hidden in the dimeric complex, i.e., the site for immunological interaction between the antibody and the hormone may more or less coincide with the interaction site between the hormone units forming the dimeric entity. In the case of artificial gel antibodies there are interactions sites enough for both monomers and dimers of GH to be recognized, since the selectivity of these antibodies depends on the whole surface area of the template used in the polymerization step. This conclusion is supported by an experimental test: The complex GH *monomer*/ artificial gel antibody against the monomer, as well as the complex GH *dimer*/artificial gel antibody against the monomer migrate in free zone electrophoresis experiments, i.e., the mobility of the latter complex is not zero. Although the antigens did not represent 100% pure protein hormones it was possible to use the generated antibodies to establish standard curves for assays of the hormone in biological samples.
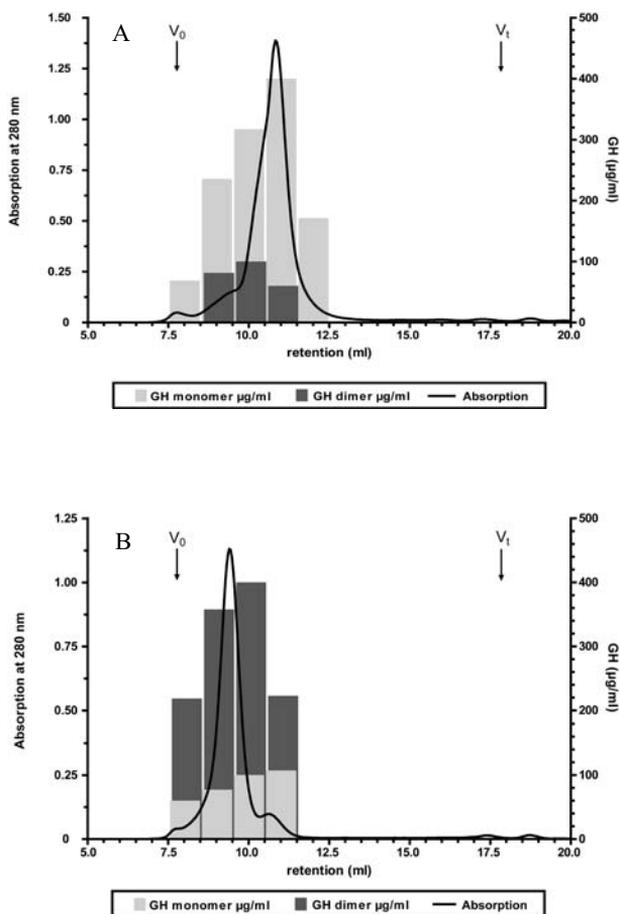
Figure 15. Analysis of the concentration of monomeric and dimeric GH by HPLC gel filtration. For determination of the concentration of selectively adsorbed monomeric and dimeric GH to artificial gel antibodies, the standard curves in Fig. 14 was used. Artificial gel antibodies could easily differ between the two forms of GH, since in A. the complex monomeric selective gel granules/monomer shows a higher electrophoretic mobility than the complex monomeric selective gel granules/dimer and in B. the complex dimeric selective gel granules/dimer shows a higher electrophoretic mobility than the complex dimeric selective gel granules/monomer.

In paper IV a combination of the synthesis of artificial gel antibodies and electrophoretic analysis was used to design standard curves for quantification of biosynthetic, non-human, non-glycosylated GH (Somatropin) and human, glycosylated hGH in a sample solution. Selective gels prepared by polymerization of the monomers in the presence of a template can be cut into small pieces (granules). Therefore, they can be subjected to an electrophoretic analysis by free zone electrophoresis (Fig. 6). Upon the removal of the template from the gel granules (control) they move only slightly in the elec-

trical field since most, but not all protein molecules have been removed by the enzyme treatment. These gel granules can, therefore, be used as reference gels for the determination of true mobility of gel antibodies with captured charged proteins. The true migration velocity is, therefore, the difference in the migration velocity of the selective gel granules containing the antigen at a certain concentration and the velocity of the control gel granules. The complex gel antibody/antigen is charged only in virtue of the charge of the template, e.g., a protein (at a pH which differs from the pI of the template substance; Figs. 16 and 17). During an electrophoresis experiment the antigen does not leave the cavity and the plot is, therefore, a straight line. The migration velocity of the complex Somatropin/artificial gel antibodies and the complex glycosylated GH/artificial gel antibodies increased with an increase in the concentration of GH in the sample solution, as expected (Figs. 16 and 17). The mobility of these gel antibodies treated with a sample of GH, inserted into the standard curve (Figs. 16 and 17) gives on the x-axis the concentration of the GH (for instance, as a biomarker) in the sample solution (for instance a body fluid). It should be noted the method of 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 method described, based on selective biomarkers, has the potential to become an important tool for diagnosis, prognosis and treatment of various diseases.
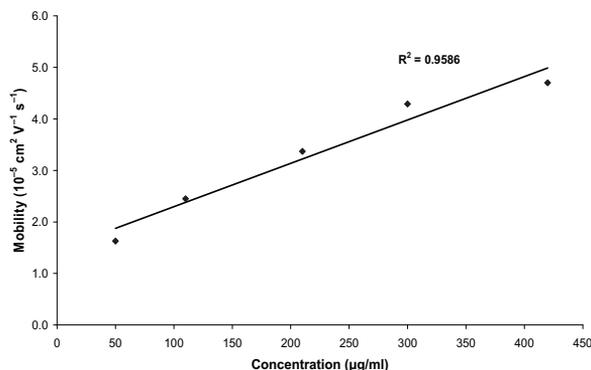

Figure 16. Standard curve for the determination of the concentration of the monomer of Somatropin in a sample solution by free zone electrophoresis. The sample tubes consisted of artificial gel antibodies selective for Somatropin, treated with different concentrations of Somatropin.
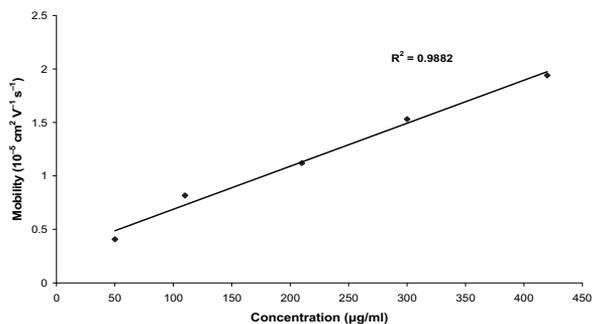
Figure 17. Standard curve for the determination of the concentration of glycosylated GH in a (body fluid) by free zone electrophoresis. The sample cuvette contained granular, artificial gel antibodies selective for glycosylated GH resaturated with different concentrations of glycosylated GH. The control tube contained a suspension of selective gel granules, enzymatically depleted of glycosylated GH (control gel granules).

The purity tests by electrophoresis show that the impurities in Somatropin were below 1 %, as shown in Fig. 18. Similar electrophoretic or chromatographic purity tests should routinely be done prior to the free zone electrophoresis experiments for the determination of the mobilities in the standard curves. The higher the purity of the sample, the higher is the accuracy in the determination of the concentration of the biomarker and, thereby, also the higher is the probability of a correct diagnosis of a disease. Other important factors for an accurate determination of this concentration are described in the following section.
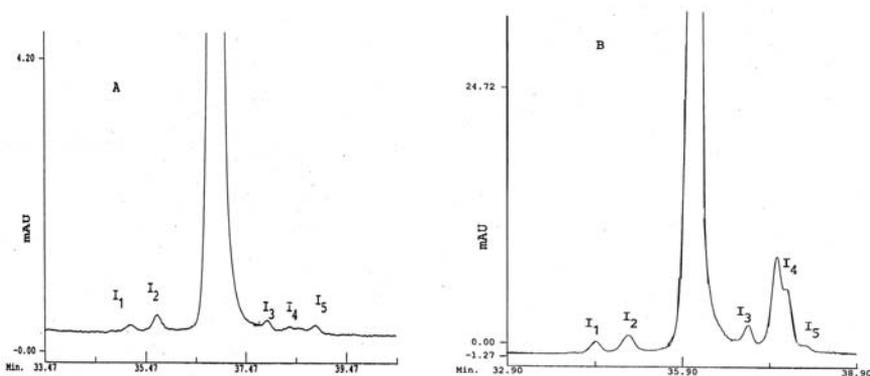
Figure 18. Analysis by capillary electrophoresis of Somatropin, 1.0 mg/ ml. (A) Initial test solution. (B) Initial test solution after 285 days. [Somatropin, monograph 0951, 2001]. This figure illustrates the importance of analyzing a potential biomarker for its purity. A purity of x percent at the determination of the concentration of the biomarker in the body fluid. The total error is this error plus errors caused by adsorption to vials and pipettes, weighing error, volume error at pipetting, etc.

The experimental difficulties to determine the concentration of high-molecular weight bio-markers in a body fluid

Many different analysis techniques are available to analyze and purify proteins in tissues and cell cultures. There are problems with biomarker assays which cannot be solved by designing a calibration curve. For instance, for GH assays 200 % variability in determination of the concentration of this biomarker from different immunoassays has been reported by different laboratories participating in national external quality assessments [Bidlingmaier, 2008]. The heterogeneity of these and other assays depend on the heterogeneity of the protein itself. To achieve optimal accuracy in quantitative analysis of biomarkers, the purity of the proteins is of fundamental importance.If possible, the prepared sample should be free from interfering analytes. One should also investigate whether the protein occur in polymeric forms. If so, one should also investigate whether these forms appears in a fresh sample, because it is known that some proteins self-polymerize. Therefore, a pre-purification may be required. Human serum albumin in powder form self-polymerised upon storage in a cold-room into dimmers and polymers up to heptamers [Hjertén *et al.,* 1994]. Frozen samples growth hormone also contained dimers (see paper III). One should also investigate whether dimers, trimers, etc., of the protein do exist in fresh samples or are storage artifacts. Neglecting this precaution may be one of the many reasons why so few protein biomarkers are routinely used in hospitals. Another reason may be the presence of other proteins than the biomarker in the sample employed for the design of the standard curve. Other probable reasons may be adsorption to vials, pipettes, etc., and inaccuracy in the determination of sample volumes, as well as weighing errors due to moisture in the sample. The variation in the volume of the liquid delivered by the pipette is often around 6 % according to the manufacture [Rumery, 2008]. Similar tests performed in Paper I gave the same result. The probable maximum error, caused by these parameters is (see Paper IV) the sum of the maximum errors caused by weighing (5 %), pipetting (6 %) , adsorption (between a few and 40 %) and the determination of the concentration of the protein biomarker if it appears in polymerized forms (5 %). The probable maximum error, cannot be below 5 + 6 + a % + 5 = 16+ a % (a is the error caused by adsorption, which may vary between a few and 40 %.

The loss by adsorption of a protein biomarker to pipettes, vials, etc. may be small or very large, depending on the degree of hydrophobicity of the walls of the pipettes, vials, etc. In a CE-experiment where the capillary was coated with dimethyldiallylammonium chloride and the sample contained positively charged proteins, the loss of proteins was 39-54 % in spite of the relatively low hydrophobicity of the coating and the strong electrostatic re-

pulsion between the proteins and the wall coating. Errors caused by adsorption (particularly of proteins) to pipettes and cuvettes are often neglected. However, at low protein concentration the losses of the analyte can be very large. For pipettes and cuvettes of glass we recommend a polyacrylamide coating [Hjertén, 1973]. Under the title "A coming trap the acrylamide´s……negligible propensity for nonspecific interactions with proteins is emphasized" [Dorman et al, 2010]. Methyl cellulose in the buffer decreases the adsorption to plastic surfaces [Hjertén, 1967].

The influence of these parameters and also how to minimize their negative effects are discussed more thoroughly in Paper IV.

Some advantages of artificial gel antibodies compared to native protein antibodies

The synthetic gel antibodies have some distinct advantages over protein antibodies; for instance, they are easier to synthesize, no experimental animals are used for the synthesis, they are more stable and are probably more selective, since gel antibodies have a larger contact area with the antigen and contain more binding sites for the antigen. For a quantitative estimation of all forms of a protein immunological tests based on *protein antibodies* might be an alternative, but experiments indicate that such tests are not accurate. For instance, the dimer of GH, one of the proteins discussed in paper III, could not be detected accurately in a RIA test based on protein antibody. Accordingly, not only monomeric, but also dimeric GH interacts with synthetic *gel* antibodies against the monomer, in sharp contrast to *protein* antibodies generated in animals (see the discussion above and Paper III). However, the RIA test may be applicable in studies where only the concentration of the monomer of GH is of interest, and not that of the dimer. The reason for the extremely high over-all selectivity of gel antibodies 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 most part of the 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 bonds between the antigen and the "wall" of the cavity, for instance, hydrogen bonds and induced dipole-dipole interactions (i.e., short range forces are developed) [Tong *et al*., 2001]. (c) the charge of the complex antigen/artificial gel antibody, quantitatively expressed in the form of electrophoretic mobility, a technique recently introduced (see Papers III-IV). Therefore, artificial gel antibodies fulfill the criteria to be an ideal tool for quantification of the biomarkers. Using artificial gel antibody technology, the loss of the biomarker due to non-specific ad-

sorption to vials, pipettes etc., are minimized, since the whole surface of the biomarker is surrounded by the non-charged polyacrylamide gel granules.

Conclusion

This thesis describes new methods for the determination of the concentration of a protein biomarker in body fluids. The main outcomes of the studies included in this thesis are:

- For quantitative determinations of the biomarker we have developed a method based on absorption measurements of the gel granules. We designed standard curves in the form of a straight line to determine the concentration of haemoglobin and albumin (two biomarkers) in a sample solution.

- Artificial gel antibodies were employed to extract biomarkers from plasma and cerebrospinal fluid for diagnosis of neurological disorders.

- For the synthesis of artificial gel antibodies against GH we have purified the hormone from frozen fractions obtained from earlier partially purified pituitary material. We have successfully obtained antibodies capable of discriminating between dimeric and monomeric GH in samples of clinical origin.

- The method based on the combination free zone electrophoresis/artificial gel antibodies was employed for a quantitative determination of Somatropin and glycosylated-GH in a body liquid.

The standard curve established in the present study allows detection of biomarkers in the microgram range. However, the true levels of biomarkers in body fluids are often in the nanogram range. Artificial gel antibodies will "fish out" biomarker proteins even when their concentration in the body fluid is that low. However, at low protein biomarker concentrations the adsorption of the biomarker to vials, cuvettes, etc., during handling can be very large and must, therefore, be minimized. To attain optimal accuracy in quantitative analysis of biomarkers, their purity should always be analyzed, for instance, by capillary electrophoresis (see Fig. 18).

Future perspectives

The selectivity of the artificial gel antibodies is high, very likely higher than that of conventional protein antibodies. This high selectivity can be taken advantage of in many applications. Here follow a few suggestions on the application range of artificial antibodies.

1. To employ our new techniques to determine the concentration of growth hormone (GH) in plasma and CSF samples in patients with GH deficiency for instance, fibromyalgia, which is a chronic pain condition connected with lowered levels of circulating GH [Nyberg, 2000]. The standard curve established in the present study allows detection of GH amounts around 60 µg/ml. The true levels of the hormone in plasma are in the range from a few ng/ml up to 100 ng/ml. The great challenge for future studies is to further refine the procedure, including purification, to reach detection levels in the range of those that are relevant for routine analysis in the clinic. Also, future studies need to take into consideration that the pattern of GH polymorphism in plasma may be more complicated than to discriminate between monomeric and dimeric forms of the hormone. It has been suggested that the number of GH forms that can be counted in plasma may exceed 100 [Baumann 1991]. Furthermore, apply this technique to probe GH levels in cell cultures [Svensson et al., 2008] or in experimental animals [LeGrevés et al., 2006] in our attempt to study the beneficial effect of GH on memory and cognition in animals treated with opiates.

2. Design of biosensors for quantitative detection of proteins, viruses, bacteria, spores, etc. For example, it should be very simple to synthesize a gel antibody against erythropoietin for doping tests, for detection of Anthrax, HIV or any pathogen.

3. We will reduce the size of the artificial gel antibodies from the present 0.1 mm-granules down to the µm and nanomolar scale.

4. A new type of enzyme reactors. These reactors have the great advantage that they rapidly and cost-effectively can be regenerated when the enzyme activity becomes low by removing the enzyme and applying a fresh, *non-purified* enzyme extract. Due to the selectivity of the enzyme reactor *only* the

enzyme will be adsorbed and all other proteins will pass through unretained. This is in sharp contrast to conventional enzyme reactors based on covalently attached purified enzymes, where the inactivated bed must be replaced by a fresh bed.

5. To purify dipeptidyl prolyl endopeptidase (DP-IV), a major enzyme responsible for the degradation of substance P and the opioid peptides endomorphins. DP-IV inhibitors have recently been shown to be useful in the treatment of inflammation and type-2 diabetes [Demuth et al., 2005]. Therefore, it is of clinical relevance to have access to techniques to detect and recover this enzyme.

6. Assay of activity in neuropeptide systems.
Within this project we plan to focus on the development of methods for measuring activity in various neuropeptide systems. Our present procedure does not allow synthesis of artificial gel antibodies for low-molecular weight peptides. However, we plan to overcome this difficulty by focusing on neuropeptide prestages and precursors, which also are released and present in tissues and body fluids. In fact, many high-molecular weight prestages of tachykinins [Nyberg et al., 1985] and opioid peptides are present at comparatively high concentrations in tissues and various body fluids, such as cerebrospinal fluid (CSF). Therefore, if not commercially available we plan to isolate precursor peptides (MW > 10.000 dalton) using conventional antibody techniques to get enough protein/peptide material to synthesize artificial gel antibodies. In assays using these artificial antibodies we also plan to use iodine-labeled peptide/proteins to establish sensitive assays, in analogy to RIAs. However, it should be mentioned that we have prepared gel antibodies against insulin, which has a molecular weight of 5600.

7. Assay for quantification of tRNA.
Preliminary experiments with tRNA as antigen are very promising [Ghasemzadeh et al, in progress]. The analyses of tRNA are based on free zone electrophoresis of gel antibodies saturated with tRNA of different concentrations. Astandard curve for the determination of the concentration of tRNA in a sample solution has been designed. The method has the potential to become an important tool for diagnosis, prognosis and treatment of various diseases by analyses based on tRNA biomarkers, provided that such exist.

Populärvetenskaplig sammanfattning

Konventionella antikroppar är proteiner, vilka produceras hos människa och djur för att inaktivera en rad skadliga substanser, t.ex. gifter, sjukdomsalstrande virus och bakterier. De framställs i försöksdjur ofta för medicinsk användning, men ytterst sällan i människa, eftersom sådana försök ofta är plågsamma och hälsovådliga. Man vill ju också minimera djurförsök av samma skäl.

Av dessa och många andra orsaker är det förståligt att forskare sedan lång tid tillbaka har *försökt* syntetisera *artificiella* antikroppar, men framgången har varit mycket begränsad. 1996 publicerades den första artikeln, där man påpekade orsakerna och samtidigt visade hur man *generellt* och mycket *enkelt* kan framställa antikroppar mot proteiner i form av *stabila gelkorn*.

Metoden är applicerbar också på virus och bakterier (konventionella protein-antikroppar mot dessa biopartiklar i nativ form kan ej framställas i försöksdjur, eftersom de där degraderas metaboliskt). Syntesen av artificiella antikroppar är enkel. Man startar med att lösa vissa substanser (s.k. monomerer) i vatten och sedan tillsätta det protein (eller det virus eller den bakterie) man är intresserad av och sedan låta monomererna reagera (polymerisera). Gelen, som bildas pressas genom ett metallnät för att få gelkorn 0.1-0.2 mm stora. Proteinmolekylerna, som ligger spridda i gelkornen, tvättas bort med proteaser, d.v.s., enzymer som bryter ned proteiner i mindre molekyler, som sedan tvättas bort med vatten. Det hålrum som då bildas har en form som överensstämmer exakt med proteinets form. Om man sätter en vattenlösning av detta protein och andra proteiner till dessa gelkorn passar *endast* det protein, som var närvarande under gelbildningen att passa in i hålrummet, de andra inte. Så snart proteinet kommit in i hålrummet fixeras det till dess väggar genom olika typer av svaga bindningar. Eftersom antalet av dessa bindningar är stort, kommer proteinet att fixeras mycket starkt till hålrummets väggar.Med samma teknik kan man fixera virus och bakterier till gelantikropparna, d.v.s applikationsområdet är enormt. Endast fantasin skapar begränsningar.

Syftet med studierna i denna avhandling har varit att undersöka infångning av s.k. biomarkörer, ofta proteiner vilkas förekomst och koncentrationer ger information om en patient har en viss sjukdom, t. ex. cancer, kronisk smärta

eller en beroendesjukdom. Biomarkörer kan användas inte bara för denna diagnos av en sjukdom, men också för dess prognos och behandling. I avhandlingens första del har vi utvecklat en optisk metod som är tänkt att användas för att bestämma koncentrationen av albumin, som är en biomarkör för ALS (en neurologisk sjukdom) i ryggmärgsvätska och plasma från patienter med denna sjukdom. Albuminhalten hos friska personer och patienter med ALS har vi jämnfört. Studien visade en ökning av albuminhalten i ryggmärgsvätskan hos patienter med ALS. Detta resultat får stöd av andra studier, som visat att halten av albumin miskar i ryggmärgvätskan hos patienter med ALS. Vid många neurologiska sjukdomar relaterade till centrala nervsystemet sker det läckage av flera plasmaprotiner p.g.a skada på blood-hjärnbarriären d.v.s väggarna i hjärnans blodkärl. Vidare visade studien att albuminhalten i plasma hos patienter med ALS är mycket lägre än hos friska personer. Resultatet är en överaskning för oss eftersom, litteraturen rapporterar ingen förändring av albumin halten hos ALS patienter jämnfört med friska personer.

Den andra delen av avhandlingen bygger på att infånga olika former av tillväxthormon med gelantikroppar för att kunna bestämma deras halter i hypofysen, som är en körtel, belägen under hjärnan. Denna studie är särskilt intressant eftersom konventionella antikroppar inte kan särskilja mellan olika former av tillväxthormonet. Vår studie har visat att gelantikroppar kan känna skillnaden i stukturen mellan olika former av tillväxthormonet, vilket har stor betydelse för diagnostik av sjukdomar, t. ex. cancer i hypofysen. Vi har dessutom utvecklat en laddningskänslig metod för att kunna bestämma halten av tillväxthormonet i olika storlekar och former. Sista delen av avhandlingen har givit svar på många frågor, t.ex. varför det är så få protein biomarörer som används på sjukhus för diagnostik, prognos och behandling av olika sjukdomar.

Sammanfattningsvis verkar gelantikroppar ha större specificitet för att binda proteiner än konventionella antikroppar. Gelantikroppar binder till hela ytan av proteinet men konventionella antikroppar binder bara till en del av proteinets yta, vilket ger lägre specificitet.

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