Antibodies as Tailor-Made Chiral Selectors: An Interdisciplinary Approach to Enantiomer Separation and Detection

HOFSTETTER Heike, HOFSTETTER Oliver
Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, Illinois 60115, USA

Abstract: It has long been known that the configurational isomers of biologically active compounds, e.g. nutrients, pesticides, and drugs, may exhibit different activities in a chiral environment such as the human body. Although the majority of drugs presently in development are chiral, analytical and preparative methods for the quantitative determination and purification of stereoisomers still lag behind. One reason is that commonly used chiral selectors for the direct resolution of enantiomers are not tailor-made for a specific analyte. The identification of suitable selectors for a particular pair of enantiomers still requires considerable experimentation and is generally demanding with regard to material time and labor. The rational design of chiral host molecules therefore represents a challenge in facilitating enantiomer analysis. In this article we describe how a combination of techniques ranging from organic synthesis to molecular biology yields antibodies of predetermined specificity and stereoselectivity that can be used as tailor-made chiral selectors for the chromatographic separation of enantiomers and their sensitive detection in immunosensors.

Key words: chiral separation, chromatography, immunosensor, stereoselective antibodies

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Approximately two-thirds of all drugs presently in development and more than half of the 500 top-selling pharmaceuticals are chiral, i.e., they are optically active and can exist in nonsuperimposable stereoisomeric forms. Due to their distinct three-dimensional structure, however, such configurational isomers may differ in their interaction with, e.g., receptors, enzymes, and transport proteins, and have different pharmacodynamic and pharmacokinetic properties. Thus, one stereoisomer of a drug may exhibit a desired activity, while another may cause severe pharmacologic and toxicologic side effects or act as an antagonist. Increasing understanding of the stereoselectivity of drug actions has caused regulatory agencies such as the US Food and Drug Administration (FDA) to establish guidelines for the development of stereoisomeric drugs and to demand that manufacturers specify their stereochemical purity. Especially the analysis of enantiomers, though, may represent a challenging task as these mirror image isomers share most of their physical properties. Although a number of techniques have successfully been used to determine the enantiomeric composition of chiral compounds, direct separation and analysis utilizing chiral stationary phases (CSPs) in high performance liquid chromatography (HPLC) represents the most popular approach. CSPs are produced by immobilizing a so-called chiral selector onto a chromatographic support thus creating a solid phase that discriminates between the two mirror image forms of the analyte. Commonly used chiral selectors include oligo- and polysaccharides, macrocyclic antibiotics, synthetic polymers, π-donor/π-acceptor systems, alka- loids, crown ethers, ligand exchange selectors, and various proteins. Despite their success, however, these selectors are generally not tailor-made for a specific separation problem, which can make the identification of a suitable CSP a time-consuming trial-and-error exercise. While the availability of ever-growing databases in combination with novel screening techniques promises to facilitate the search for an appropriate selector, the utilization of rationally designed host molecules of predetermined specificity represents an intriguing alternative strategy for enantiomer analysis.

We have recently demonstrated that suitably raised antibodies can be used as tailor-made chiral selectors for enantiomer separation in HPLC and for the ultra-sensitive detection of enantiomeric impurities in immunoassays.
sensors. In this article we give an account of the interdisciplinary approach we are taking in our research to fully exploit the enormous potential of this class of proteins and to establish them as a new class of chiral host molecules for routine enantiomer analysis in chromatography and related techniques.

1 Antibody production

Nature’s combinatorial synthesis of specific host molecules

Over the past few years research at the interface of Chemistry and Biology has experienced a remarkable increase in popularity which has led to the proclamation and recognition of "new" areas such as Chemical Biology and Bionanotechnology. The application of antibodies as chiral selectors provides a good example of how multidisciplinary research can combine seemingly diverse fields like synthetic Organic Chemistry, Immunology, Cell- and Molecular Biology and Analytical Chemistry. Chemists who want to make use of the astounding characteristics of antibodies in their research need to have at least a basic understanding of the physicochemical properties of these biological macromolecules and how they can be raised. This section is intended to briefly introduce the reader to the most important immunological aspects of antibody production. For additional information cited references and more specialized immunological literature should be consulted.

Antibodies are glycosylated proteins glycoproteins which are produced by the immune system of vertebrates in response to invading pathogenic microorganisms and "non-self" biological material called antigens. In the first phase of an immune response membrane-bound antibodies on the cell-surface of B-lymphocytes so-called immunoglobulins M, IgM bind to the antigen this binding triggers proliferation of these cells and their differentiation into plasma cells. These secrete soluble antibodies which have the same overall specificity as the membrane-bound IgM and can bind to the invader thus marking it for destruction by other components of the immune system such as phagocytes and the complement system. The most abundant soluble antibodies in blood are immunoglobulins of the IgG class which are composed of four polypeptide chains namely two identical heavy chains and two identical light chains. IgGs have a molecular weight of approximately 150,000 and possess two binding sites at their amino-termini. According to the clonal selection theory only those B-cells whose membrane-bound IgM interact with the antigen or a portion thereof differentiate into plasma cells other B-cells which carry antibodies with different binding properties are not stimulated and therefore do not produce soluble antibody.

Fig. 1

Specific binding of antigen red circles to membrane-bound antibodies on the surface of a B-cell B2 triggers its proliferation and differentiation to daughter cells that secrete soluble antibodies.

Since there are millions of different B-cells each carrying only one type of antibody with a distinct specificity the immune system can be seen as a natural library from which only appropriate binders/host-molecules are selected and amplified. The vast number of antibodies that can be produced by the immune system is the result of somatic recombination of a limited number of gene loci and the random combination of heavy and light chains. In addition antibody diversity is further increased by the introduction of somatic mutations into the antibody-encoding genes during affinity maturation which leads to a fine-tuning of the antibody’s specificity and an increase in its affinity towards the antigen. As is the case for other types of protein-ligand interactions such as enzyme-substrate receptor-hormone or lectin-sugar systems the binding forces between an antibody and an antigen are of purely non-covalent nature and involve electrostatic interactions electron acceptor-electron donor forces hydrogen bonds and non-polar interactions
Lifshitz-van der Waals forces\cite{240}. Formation of attractive interactions is generally facilitated by a certain shape complementarity between the antibody’s binding site\cite{240} paratope\cite{240} and a corresponding target region on the antigen\cite{240} epitope\cite{240}.

As early as in 1917 Karl Landsteiner and colleagues impressively demonstrated that highly specific antibodies can readily be raised in animals against virtually any molecule\cite{250} including low-molecular weight compounds\cite{250}. Landsteiner also demonstrated that such antibodies may be stereoselective and distinguish between the enantiomers of chiral molecules\cite{250}\cite{250}. Surprisingly\cite{250} since then the stereoselectivity of antibodies has largely been ignored by chemists and has only rarely been used for the separation and detection of chiral compounds\cite{270} notably in radioimmunoassays for the determination of drugs and drug metabolites\cite{270}.

1.1 Production of stereoselective antibodies by classical immunological techniques

The general principles for the production of antibodies against small molecules\cite{250} haptens\cite{250} have not significantly changed since they were first described almost a century ago. Since compounds with a molecular weight under 5 000 generally do not elicit an immune response\cite{250} it is necessary to couple them to carriers that are recognized by the immune system as being non-self. Most widely used for this purpose are proteins such as keyhole limpet hemocyanin\cite{250} KLH\cite{250} bovine serum albumin\cite{250} BSA\cite{250} thyroglobulin\cite{250} and ovalbumin. A wide variety of established coupling reactions can be utilized to synthesize hapten-carrier conjugates\cite{250}\cite{250}\cite{250}\cite{250} depending on the presence of suitable functional groups on the hapten\cite{250} a procedure is preferred that maximizes presentation of the target structure on the surface of the conjugate. In the production of stereoselective antibodies\cite{250} care should be taken that the coupling reaction does not involve the stereogenic center and that\cite{250} ideally\cite{250} the conjugation is carried out via a group that is located on the opposite side of the hapten. If this is not possible with the original hapten\cite{250} the synthesis of appropriate analogs should be considered. The careful design and synthesis of suitable immunogens is probably the most important step in the production of antibodies of high specificity and stereoselectivity. However\cite{250} following these general rules and standard immunization protocols\cite{250}\cite{250} it is possible to obtain antibodies\cite{250} whose stereoselectivity is unrivalled. For example\cite{250} we raised stereoselective anti-amino acid antibodies that enable the detection of trace amounts of enantiomeric impurities in samples having an enantiomer excess\cite{250} ee\cite{250} of 99.998\cite{250}\cite{250}\cite{250}%. The antibodies were produced by linking the protein carrier KLH to an enantiomerically pure amino acid hapten distal to the \( \alpha \)-amino acid function\cite{250} the carboxylate/primary amino/hydrogen triad\cite{250} so as to expose this functional grouping for maximal antibody binding\cite{310}. Thus\cite{250} \( \beta \)-amino-D\cite{250} or \( \text{L-phenylalanine} \) was coupled to the protein via the \( \beta \)-amino group by diazotization\cite{250} and the resulting conjugates\cite{250} \( \beta \)-azo-D-phenylalanine-KLH and \( \beta \)-azo-L-phenylalanine-KLH\cite{250} respectively\cite{250} were separately used to immunize rabbits. Diazotization was especially suited for our purpose\cite{250} since it allowed the synthesis of protein-conjugates exposing the hapten’s stereogenic center without protection or deprotection of its functional groups. Conjugates of the pure enantiomers of \( \beta \)-amino phenylalanine and BSA\cite{250} also prepared by diazotization\cite{250} were used as solid phase coatings in an enzyme-linked immunosorbent assay\cite{250} ELISA\cite{250} to follow the immune response. It was found that both types of antibody only bind to the “correct” BSA-conjugates\cite{250} that is\cite{250} the anti-\( \beta \)-amino acid antibody only binds to the BSA-conjugated D-enantiomer\cite{250} while the anti-L-amino acid antibody only binds to the conjugated L-enantiomer. No significant binding to the opposite enantiomer was observed\cite{250} Fig. 2\cite{250}.

The specificity and relative affinity of the antibodies for the enantiomers of free\cite{250} that is\cite{250} undervatized amino acids was determined in competitive ELISAs. So far\cite{250} stereoselective binding of the anti-amino acid antibodies to more than 30 \( \alpha \)-amino acids has been demonstrated. The affinities are highest for structures that resemble the hapten\cite{250} i. e. \cite{250} bear aromatic side chains\cite{250} however\cite{250} amino acids with different side chains including aliphatic\cite{250} charged and non-charged residues\cite{250} are also stereoselectively recognized by the respective antibodies. Thus\cite{250} the antibodies can be considered to be” class-specific”.

The class-specificity of the anti-amino acid antibodies was recently further investigated by testing their ability to bind to structurally related
compounds and by comparing them to antibodies that have been raised against such molecules. For that purpose we produced stereoselective antibodies to α-hydroxy acids in which a hydroxyl group is substituted for the amino group of α-amino acids. Both antibodies recognizing either D-hydroxy acids or L-hydroxy acids were produced using p-azo-D- and -L-phenyllactic acid-KLH conjugates respectively as immunogens. A direct comparison of the anti-amino acid antibodies and anti-hydroxy acid antibodies revealed that both types of antibodies are not only highly stereoselective but also show perfect class-specificity for the group of compounds they were raised against. Thus anti-hydroxy acid antibodies only bind to the corresponding "correct" enantiomer of α-hydroxy acids but not to the opposite enantiomer of this class of compounds or to either enantiomer of α-amino acids. An analogous combination of stereoselectivity and class-specificity was also observed with the anti-amino acid antibodies. While the enormous degree of stereoselectivity displayed by these antibodies clearly exceeds what is generally observed with other chiral selectors also their class-specificity is by no means ordinary. It should be noted that many biological macromolecules have been found to be surprisingly indiscriminative with regard to their interaction with amino- or hydroxyl groups. In addition most other selectors used for enantiomer analysis of amino acids can also be applied to hydroxy acids.

The serum of immunized animals contains a heterogeneous population of antibodies that may differ with regard to specificity and affinity. Since they are produced by different plasma cells they are referred to as polyclonal antibodies. In order to obtain antibodies with homogenous binding properties monoclonal antibodies can be produced. This involves the immunization of mice or rats and the fusion of antibody-producing lymphocytes with myeloma cells. The resulting hybridoma cells combine the properties of antibody production and immortality and can readily be cultivated. Individual clones producing an antibody with desired characteristics can be identified utilizing a suitable screening procedure and expanded. Although in principle a wide variety of techniques can be applied in the screening process it is important to choose a method that can be used to conveniently test a large number of samples for distinct binding properties. In the production of monoclonal anti-amino acid antibodies and anti-hydroxy acid antibodies for example a high degree of stereoselectivity of hybridoma-secreted immunoglobulins was ensured by screening the culture supernatants in ELISA on three different coatings namely the BSA-conjugates of both the D- and L-enantiomers of the haptens and undervatized BSA. Several clones producing stereoselective antibodies against the corresponding amino or hydroxy acid enantiomers were successfully subcloned and expanded. In general the produced monoclonal antibodies did not deviate significantly from the respective polyclonal antibodies with regard to affinity and stereoselectivity as determined by ELISA. Moreover the monoclonal antibodies were also class-specific.

Fig. 2 Stereoselectivity of anti-D- and anti-L-amino acid antibodies as determined by non-competitive ELISA

Serial dilutions of antibody-containing rabbit serum were added to the BSA-conjugates of p-amino-p-phenylalanine and p-aminol-phenylalanine immobilized onto a microtiter plate. Bound antibody was detected using a secondary peroxidase-labeled goat anti-rabbit antibody and the substrate o-phenylenediamine. Absorbance was measured at 490 nm values obtained on non-derivatized BSA were subtracted.
and can therefore be used as chiral selectors for enantiomer discrimination of a large variety of analytes.

A major advantage of the hybridoma technology is that once monoclonal antibody-secreting cells have been established virtually unlimited amounts of a particular antibody can be produced. Small to medium quantities can be obtained from ascites fluid in mice or by cultivating hybridomas in small bioreactors. Production of large quantities of antibodies for a variety of "achiral" applications in industrial-size fermenters is already common practice and could easily be adopted for antibody-based chiral selectors.

1.2 Biotechnological production of stereoselective antibodies

The last two decades have seen a remarkable development in antibody engineering techniques. The application of such molecular biological methods for the production of stereoselective antibodies is particularly attractive since they offer the possibility for facilitated large-scale production in e. g. bacterial cell cultures and the manipulation of binding characteristics. In addition, biotechnology promises to significantly reduce or even bypass the use of animals. While in principle the whole repertoire of recombinant antibody technology can be applied to the design and construction of stereoselective antibodies we will focus here on just two strategies one that utilizes the genetic information of a particular monoclonal antibody and another that enables the production of stereoselective antibodies from a complex library.

If monoclonal antibody-producing hybridomas are available their mRNA can be isolated and used to amplify the genetic information that encodes the heavy and light chain variable regions of an antibody’s binding site. After combination with a so-called “linker” these sequences are cloned into a plasmid for transfection into suitable prokaryotic or eukaryotic host cells. The host’s protein synthesis machinery is then exploited to produce a monovalent antibody fragment whose heavy and light chain are covalently linked and which therefore is called a single-chain variable fragment scFv. Following this approach we have recently used the genetic information of an anti-amino acid antibody to produce a scFv that retains the stereoselectivity of the original antibody and preferentially binds L-amino acids. The scFv can now be produced in E. coli and be harvested from culture medium. Thus in theory there is no upper limit to the amounts of antibody that can be obtained this way. Once a reliable expression system has been established the antibody can be manipulated using genetic engineering techniques to e. g. vary its affinity towards a specific target molecule increase its stability or to completely alter its specificity. The potential value of genetic engineering techniques for the rational manipulation of the binding properties of stereoselective antibodies was recently demonstrated by Nevanen et al. who used site-directed mutagenesis to lower the affinity of enantioselective antibody-fragments for their targets.

An additional potential advantage of antibody-fragments is their reduced size compared to a whole antibody. With a molecular weight of approximately 30 000 scFvs for example are about five times smaller than IgGs. Consequently a higher density of binding sites can be realized for example upon immobilization of scFvs on a surface this should be especially beneficial in applications such as chromatography that call for high binding capacities.

While the biotechnological production of antibody fragments from monoclonals still requires immunization of animals another technique phage display provides the potential to bypass immunization and to produce complex antibody libraries from which antibodies with desired binding properties can be isolated within a relatively short time. This is achieved by fusing the antibody V regions to the amino-terminus of the coat protein pIII of filamentous bacteriophages. Upon infection of bacteria the fusion product is incorporated into the phage coat and the antibody is presented on the phage surface. The genetic material however which also contains the sequence of the fusion protein resides within the mature phage particle. In order to produce a complex library of antibody-presenting phages the genetic information of all antibody heavy and light chains of a donor for example a mouse is randomly combined to scFv sequences and cloned into plasmids. Such a library which can com-
prise billions of different antibodies can then be screened for phages having desired specificities using immobilized antigens in an appropriate affinity technique such as ELISA. Those phages displaying antibody directed against the chosen antigen will be bound on the antigen-coated surface while non-binding phages can be removed in a washing step. The bound phages can then be eluted from the surface by washing with a solvent that disrupts the antibody-antigen interaction and be used to re-infect bacteria.

Cultivation of the bacteria yields the desired antibody in large amounts. While antibodies to any chosen antigen are accessible from universal antigen-unbiased libraries that have been produced from non-immunized individuals it is more common to use the genetic material of immunized donors. Using the latter approach we demonstrated in a proof-of-concept experiment that stereoselective scFv can be obtained from an antibody-presenting phage display library. Phages displaying antibody directed against L-amino acids were isolated in a plastic vessel coated with p-azo-L-phenylalanine-BSA and used to re-infect E. coli. Soluble antibody was purified from the bacterial cell culture and tested for stereoselectivity in an ELISA. The expressed protein exhibited stereoselective binding to L-amino acids but did not interact with the D-enantiomer.

2 Applications of stereoselective antibodies in analytical techniques

To be attractive for widespread use in enantiomer separation and detection a potential chiral selector should possess a number of desired qualities. In addition to showing a high degree of stereoselectivity it should be reasonably stable be obtainable in large quantities at low cost and be widely applicable not only with regard to the possible number of target analytes but also to the type of methodologies this selector could be combined with. Considering this it may not immediately be obvious why antibodies should be suitable for routine enantiomer analysis in e.g. an industrial environment. The reason for that is that all proteins inherently share the following inherent disadvantages first of all proteins such as enzymes receptors and antibodies typically bind only to their natural ligands or structurally related compounds this greatly limits the potential number of binding partners and from an analytical chemist’s point of view the number of analytes. Proteins are also prone to denaturation and microbial degradation which can reduce their half-lives significantly. It is furthermore widely assumed that since proteins have to be isolated from biological sources and are not accessible by chemical synthesis they are difficult to obtain expensive and available only in small quantities.

As described above antibodies can be raised against virtually any molecule of interest even classes of compounds and can be obtained rather inexpensively in almost unlimited quantities by e.g. biotechnological production. The following examples from our research are intended to demonstrate that antibodies are furthermore surprisingly stable and can be conveniently used in an enormous variety of analytical techniques for enantiomer separation and detection ranging from liquid chromatography to high-throughput sensors.

2.1 Chiral immunoaffinity chromatography

Solid phase-immobilized antibodies have successfully been used for the chromatographic purification of high- and low-molecular weight antigens for almost four decades. The technique is known as immunoaffinity chromatography and is based on the principle that from a crude mixture only a suitable binding partner will be retained on a column from which it can be eluted in pure form Fig. 3.

While the binding step is carried out in a buffer that favors the association of antibody and antigen elution usually requires harsh conditions which may involve a drastic change in pH or the addition of organic solvents or chaotropic salts. The elution step usually causes protein denaturation which significantly shortens column lifetime. Although such a classical approach has occasionally been used for antibody-based separation of stereoisomers limited column reusability generally restricts it to special applications and makes it impractical for routine enantiomer separation.

In order to be able to compete with other commonly used CSPs a chromatographic system should permit thousands of separations under experimental conditions that pose no inconvenience
the immobilized antibody. In such cases, rapid elution of the retained enantiomer can still be achieved under mild isocratic buffer conditions by simply increasing the flow rate. Fig. 4 for example shows the separation of the enantiomers of phenylalanine at room temperature in less than two minutes using an analytical size stainless steel column 2.3 mm × 200 mm the CSP had been produced by derivatizing POROS with a monomodal anti-D-amino acid antibody. By applying a relatively high flow rate of 6 mL/min the whole chromatographic process can be carried out in phosphate buffered saline PBS at a neutral pH of 7.4. For comparison the application of a flow rate of 1 mL/min which is common for typical HPLC applications would result in an extremely broadened second peak making UV-detection of the retained enantiomer virtually impossible.

Fig. 3 General principle of immunoaffinity chromatography

From a mixture of compounds represented by circular sickle-shaped and triangular symbols that are applied to a column containing solid-phase immobilized antibody Y-shaped symbols only appropriate binding partners will be retained in the binding step which is performed in a neutral buffer. After washing the column with the same or another buffer that is favorable for the interaction between antibody and analyte a solvent is applied that disrupts this interaction. Eluted material can be detected by measuring UV-absorbance or another physical property.

to the scientist. We have recently demonstrated that antibody-based CSPs can meet these requirements if they are operated under mild isocratic conditions that do not compromise the protein’s structural integrity. Monoclonal antibodies directed against either D- or L-amino acids were immobilized on agarose or synthetic polymers or silica and used for enantiomer separation in analytical size microbore and capillary columns. Recently we also used anti-hydroxy acid antibodies for enantiomer separation in microbore columns. Using a synthetic high flow-through support material POROS which allows the use of flow rates up to 30 mL/min it was found that enantiomer separation is purely based on specific stereoselective interaction of the immobilized antibody with the analyte having the “correct” configuration while the opposite enantiomer is not retained and elutes with the void volume. This is in contrast to most other CSPs including other protein chiral stationary phases where typically considerable retention of the first-eluting enantiomer caused by non-specific or non-stereoselective interactions is observed. We found that the high flow-through material is particularly valuable for separation of analytes that show a relatively high affinity towards
similarly high\(^1\) whereas the affinity of the interaction is primarily determined by the dissociation rate constant. Thus\(^1\) a more strongly bound analyte dissociates slower from the CSP\(^1\) which results in an overall higher affinity towards the immobilized antibody. As a consequence\(^1\) the local concentration ratio of free versus bound analyte decreases at high flow rates due to dilution effects\(^1\) thus reducing the amount of dissociating analyte that is rebound. If both the association and dissociation kinetics are fast enough\(^1\) as is the case for more weakly bound analytes\(^1\) such a dilution effect will not be discernible. However\(^1\) if the dissociation step is slow and the flow rate high enough\(^1\) such an effect would result in a relatively lower retention at higher flow rates\(^1\) which would manifest itself in a decrease in \(k\). The \(k\) values for the first eluting enantiomer\(^1\) on the other hand\(^1\) were close to zero and independent of the flow rate\(^1\) which indicates that no noticeable specific or non-specific interactions between the CSP and this enantiomer occur\(^1\) this in turn\(^1\) implies that the retention of the second eluting enantiomer is solely based on specific interactions with the CSP.

As with other protein CSPs\(^2\) \(^54\) – \(^56\) optimization of chiral separations can also be achieved by varying other chromatographic parameters such as temperature\(^1\) ionic strength\(^1\) or pH of the mobile phase\(^1\) Fig. 5\(^1\).

The susceptibility of the antibody-antigen interaction towards changes in the environment\(^1\) here the mobile phase\(^1\) provides a convenient tool to\(^1\) e. g. \(\) increase the separation factor \(\alpha\) of a particular separation\(^1\) or to reduce separation time. Since we routinely operate our antibody columns under mild buffer conditions\(^1\) such as PBS\(^1\) that do not cause protein denaturation\(^1\) they are very stable and\(^1\) so far\(^1\) have been reused for thousands of separations over a period of more than four years. This is even more remarkable as the chromatographic system is routinely operated at room temperature\(^1\) and columns are stored under an azide-containing buffer at 4 °C only if not used for extended periods of time. Thus\(^1\) the antibody phases combine the advantages of tailor-made chiral selectors with the convenience of other commonly used CSPs. An advantage of chiral immunoaffinity chromatography\(^1\) however\(^1\) is that the stereoselectivity as well as separation conditions are predictable\(^1\) antibodies will preferentially bind to the enantiomer they were raised against\(^1\) and binding will occur under physiological buffer conditions.

The predictability of antibody-based CSPs is exemplified in Fig. 6\(^1\) which shows the inversion in elution order of the enantiomer separation of phenylalanine on two antibody columns with opposing stereoselectivity.

This example proves that the observed separations are undoubtedly the result of highly specific interactions between the antibodies’ binding sites and their corresponding binding partners. The belief that protein-based chiral separations are based on a protein’s “inherent chirality”\(^1\) since they are made from chiral building blocks\(^1\)

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namely L-amino acids[43] however is unsubstantial. It can be assumed that at least in most cases a particular three-dimensional arrangement of amino acid residues in a protein’s binding site is responsible for chiral discrimination.

Although the POROS material offers a high degree of experimental flexibility it has a lower capacity than for example silica. This may limit its utility for the separation of antibodies that have a relatively low affinity towards the immobilized antibodies. In the case of anti-amino acid antibody CSPs for example we were not able to use POROS as support for the separation of aliphatic amino acids. However such amino acids can be separated on phases that have been prepared by immobilizing stereoselective antibodies onto silica which has a much higher capacity[42].

We are currently investigating antibodies in a variety of different experimental setups and conditions to make full use of their extraordinary stereoselectivity. The development of miniaturized systems for example is especially attractive since they require only small amounts of analyte and chiral selector. Microbore and capillary systems can furthermore be interfaced with mass spectrometry[45] MS instruments for the sensitive detection of analytes that e.g. lack a chromophore. Since many buffers that are favorable for the antibody-antigen interaction such as phosphate buffers are incompatible with MS detection the identification of a suitable mobile phase can be a challenging task. However we found that our antibody-CSPs are stable in a low-ionic strength ammonium bicarbonate buffer which allows interfacing of miniaturized columns with an MS detector and separation of a number of amino acid enantiomers[42]. There appears to be no limit to the use of stereoselective antibodies for enantiomer separation as long as the operating conditions do not cause protein denaturation.

2.2 Chiral immunosensors

As discussed above in immunoaffinity chromatography the specific interaction between an analyte and an antibody results in a certain degree of retention with the extent of retention reflecting the strength of interaction between the binding partners. If this method is to be used for analytical purposes e.g. quantification the antibody-antigen bond has to be disrupted in order to elute the analyte and determine its concentration and amount respectively with the help of a suitable detection system. Thus analyte binding and detection usually are consecutive events. However a number of detection systems allow direct that is real-time monitoring of antibody-antigen or other receptor-ligand interactions. The combination of antibodies with such transducers results in the creation of immunosensors which may be employed for the qualitative or quantitative detection of target molecules. We have used a number of different physical transduction systems to design chiral immunosensors that allow enantiomer analysis in an automated or even high-throughput format. The first such sensor[57] was based on surface plasmon resonance SPR an optical phenomenon that allows detection of mass-changes on the surface of a gold-coated prism. We utilized a commercially available instrument BIAcore to design an SPR-based chiral immunosensor that enabled quantification of amino acid enantiomers up to an ee of 99.9%. Enantiomeric impurities contained in non-racemic samples were detected by their ability to inhibit binding of stereoselective antibody to a hapten-analogue immobilized on the sensor surface Fig. 7.

Despite the fact that the BIAcore instrument is amenable to automation it does not allow simultaneous analysis of multiple samples. However we have recently used other transduction elements that enable sensitive detection of enantiomeric impurities in a high-throughput format — a particularly challenging aim in enantiomer analysis.

Stereoselective anti-D- or anti-L-amino acid antibodies were covalently immobilized on the gold surface of nanostructured silicon microcantilevers to use analyte-induced nanomechanical surface stress for label-free detection[45]. When such surfaces were subjected to samples containing an appropriate binding partner i.e. corresponding amino acid enantiomers the antibody-antigen interaction caused nanoscale bending of the microcantilevers which could be detected with the help of a laser and a position-sensitive detector Fig. 8.

The temporary response of cantilever bending upon injection of analyte was found to be linearly proportional to the analyte concentration which allowed quantitative determination of enantiomer
Amino acid enantiomers were injected over the surface of microcantilevers derivatized with anti-L-aminoom acid antibody. Stereoselective interaction with analytes having the “correct” configuration here[3] L-Trp and L-Phe[] caused nanoscale bending of the cantilever. The kinetics of the deflection could be used for quantification[] not shown[].

recently demonstrated by combining stereoselective anti-amino acid antibodies with magnetic relaxation switches[] MRS[30]. MRS are dextran-coated superparamagnetic iron nanoparticles[] whose aggregation alters the T2 relaxation time of adjacent water protons. Such particles can conveniently be derivatized with a ligand or receptor using standard immobilization strategies. Addition of a bi- or multivalent binding partner to MRS induces self-assembly of the particles[] which can be monitored with a nuclear magnetic-resonance NMR[] instrument or magnetic resonance imaging[]. In order to employ MRS for antibody-based enantiomer analysis[] a hapten-analogue was covalently linked to the dextran-surface of the particles. Since IgG possesses two identical binding sites[] addition of antibodies recognizing the immobilized ligand induced aggregation of the particles. The presence of free amino acid analytes having the correct configuration[] however[] resulted in dispersion of the clusters[] which could be followed as an increase in the T2 relaxation time[] Fig. 9[].
Both the extent and rate of the measured signal were dependent on the concentration of the target enantiomer which allowed sensitive analyte quantification. Since the presence of the opposite enantiomer did not cause a significant change in the signal, trace amounts of amino acid enantiomers contained in non-racemic mixtures could be determined up to an ee of 99.998%. Moreover, the ability to measure thousands of samples simultaneously in a magnetic resonance imager within as little as two minutes can be considered a significant achievement in the field of enantiomer analysis.

3 Conclusions

Although stereoselective antibodies were first described almost eighty years ago their use as chiral selectors is still in its infancy. Recent work in this area has clearly demonstrated that these biological macromolecules offer an enormous degree of flexibility with regard to their production, manipulation, and application in analytical techniques. It is up to us scientists to recognize this potential and to exploit it to address present or future separation or detection problems.

While antibodies possess a number of properties that should make them attractive for widespread use, three particular qualities and their combination appear to distinguish them most evidently from other commonly used chiral selectors. The ability to raise antibodies against virtually any target molecule using either classical immunological or molecular biological techniques has the potential to considerably ease the future search for suitable separation systems for chiral compounds. The unsurpassed stereoselectivity of antibodies enables the detection of enantiomeric impurities at levels not achievable with any other selectors. Stereoselective antibodies can be utilized in an amazing variety of techniques ranging from immunoassays to sensors and chromatography and their combination with methods that have been established for "achiral" applications should be straightforward.

References

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Biographic information

Heike and Oliver Hofstetter received their diplomas in Chemistry and Biochemistry respectively from the Eberhard-Karls-University in Tübingen Germany before they joined Professor Volker Schurig’s group at the same institution as doctoral students. While Heike in her work focused on the use of peptides and proteins such as serum albumin as chiral selectors Oliver already then started to investigate antibody stereoselectivity. This project was part of a collaboration with Professor Bernard Green from the Hebrew University in Jerusalem and Professor Meinhold at the Weizmann Institute of Science in Rehovot Israel. After receiving their doctoral degrees Heike and Oliver continued to work with Meir Wilchek as postdoctoral students at the Weizmann Institute where they helped to develop a new protein labeling purification and detection system that is based on the interaction between 4-hydroxyazobenzenecarboxylic acid HABA and avidin and thus represents and extension of the avidin-biotin system. In August 2000 Oliver accepted a position as Assistant Professor of Biological Chemistry in the Department of Chemistry and Biochemistry at Northern Illinois University where he was appointed Adjunct Assistant Professor. Research in their group is multidisciplinary and ranges from immunogen synthesis and classical immunology to molecular and structural biology. A major focus of their work is the application of antibodies as chiral selectors in chromatography and immunosensors. Their research has been financially supported by various funding agencies including the German-Israeli Foundation Boehringer Ingelheim Fonds the Max-Planck-Society Research Corporation and the National Institutes of Health.