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Advanced Analytical Methods for Pharmaceutical and Diagnostic Applications

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Abstract. Driving forces for the development of novel analytical technologies in the life-science industry are described. Technologies which either were developed in Bio-Analytical Research or brought to a reliability required for routine applications will be elucidated and, on the basis of practical examples, the impact of modern analytical technologies on the industrial research and development will be discussed: Optical biosensors based on evanescent excitation of luminescence allow for real-time monitoring of the binding of active compounds to specific biomolecular recognition sites. Molecular imaging technologies have the potential to gain rapid access to physical maps of genomic materials. Capillary electrophoresis or affinity gel electrophoresis are well suited for the fast determination of oligonucleotide mixtures in nl amounts of samples. Integrated capillary electrophoresis on chips will allow to multiplex capillary systems at low costs and results in high separation efficiencies. MALDI-TOF MS is an easy to operate non-scanning mass spectrometric instrumentation for the analysis of high molecular weight biopolymers such as immunoglobulins.

Introduction

Analytical technologies play a key role in the research and development process of pharmaceutical products: in the research phase they are indispensable for the identification of the molecular targets, the selection of a substance with a suitable binding affinity to the target or the characterization of the molecular structure. In the development phase analytical tasks are the identification of metabolites, the parameters of drug-protein interactions, and the determination of pharmacokinetic parameters. There typically large numbers of animal and human samples have to be analyzed. In close context with pharmaceutical applications is the development of analytical technologies for human diagnostics. The enormous progress made in molecular biology within the past years together with advanced analytical technologies allows not only the efficient identification of molecular drug targets but also opens novel ways for diagnosing infectious and genetic diseases. In the last years activities in our department were focused on evaluating, adapting and further developing advanced analytical technologies and methodologies in those areas. Examples below shall identify the major driving forces for such developments as well as their technical implementations.

- Novel, more potent drug candidates which are administered at lower doses and, therefore, result in much lower blood concentration levels set new standards in terms of the detection limit of analytical methods used for monitoring their metabolic and pharmacokinetic behavior. For example, the daily dose of the new aromatase inhibitor Letrozole (*Femara*®) is only 2 mg compared to 250 mg for aminogluteth-imide (*Orimeten*®) which results in blood concentrations in the nanomolar range for *Femara* compared to micromolar for *Orimeten*.
- Novel chemical classes of drug compounds often require more powerful separation and detection methods. The introduction of antisense oligonucleotides, typically oligonucleotides with 20 to 25 bases which hybridize to specific sites on mRNA and, therefore, block the expression of cell proteins, required novel methods in order to allow a fast detection of the parent compounds and the enzymatic degradation products. Capillary Electrophoresis and Optical Biosensors have the potential to address these issues.
- The discovery of new molecular markers to diagnose specific diseases such as Fatty Acid Binding Protein (FABP) for acute myocardial infarction, or the identification of RNA and DNA sequences to identify viral infections or genetic diseases require complicated assay procedures in order to achieve limits of quantitation in the range of 10 to 1000 molecules. With signal or target amplification methods such as Polymerase Chain Reaction and use of labeled branched DNA such limits of quantitation can be achieved today. However, many of these amplification methods are not suitable for fast diagnostic point of care applications. Optical biosensors based on evanescent excitation of fluoresence are considered by us as a potential technology for ultrasensitive and fast detection of oligonucleotides and DNA/RNA strands.
- The investigation of bioaffinity interactions is not only an important task for the chemical optimization of the potency of drug interaction with a receptor but also for the detection of binding properties of drugs to blood proteins. This type of analytical information can be obtained in an easy and accurate way by the application of evanescent wave biosensors.
- Biotechnology and molecular biology in general, the human genome project in particular had a tremendous impact on the development of advanced analytical techniques. The high molecular weight of many biopolymers, the complex mixtures in biotechnological processes as well as the very small volumes in which minute concentrations of analytes have to be determined, required a reevaluation of existing analytical methods. For the determination of biopolymers in complex samples it was Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) which did allow a fast analysis without a tedious sample preparation method. We will demonstrate how MALDI-TOF MS in combination with a simple sample preparation method was applied to monitor the antibody production during a cell cultivation. Approaches to establish functional physical maps, *e.g.*, to in-

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vestigate the localization of genes and to monitor the expression of mRNA are currently under development.
- Novel approaches in drug research such as combinatorial chemistry, random screening or the assessment of genetic information pose increasing demands on analytical technologies in terms of small sample volumes and high sample throughput. Miniaturization, multiplexing and automation allow to reach these goals. We will introduce the concept of Integrated Capillary Electrophoresis, demonstrate how such chips can be obtained at low costs and – using lambda phage digests – how DNA fragments can be separated at picomolar concentrations on 20 mm short channels.

Optical Biosensors

Outline of Technology

The demand in life sciences and human diagnostics for analytical methods adequate to measure biospecific interactions at extremely low concentrations, in small sample volumes and with a minimum of sample preparation is the driving force for the development of a novel generation of biochemical sensors [1]. FOBIA (Fast Optical Biospecific Interaction Analysis) is a planar waveguide based optical sensor system dedicated to selectively measure minute amounts of analytes in complex media, such as, *e.g.*, blood serum, plasma or tissue extracts, on a real time basis [2]. To achieve the ultimate sensitivity, a threefold selectivity approach was incorporated:

1) The evanescent wave sensing principle for spatial selectivity,
2) the immobilized biological recognition elements for (bio)chemical selectivity, and
3) the luminescent labeling for amplification and specific signal generation.

The evanescent field penetrates only some 100 nm from the sensor surface into the solution and thus allows a real-time monitoring of binding kinetics. As in the well accepted and widely used solid-phase bioassays, the immobilized biorecognition elements on the transducer surface can be used to selectively capture the analytes. The fluorescent label of the tracer, added at known concentrations, allows for an easier differentiation between specific and nonspecific binding events, provides superior sensitivity and makes the sensors signal independant of the molecular weight of the analytes.

Transducer Layout and Optical Configuration

The transducer consists of a thin waveguiding layer (< 200 nm) with a high refractive index (> 2.2) on a glass or plastic substrate. Excitation light is coupled into the waveguide by means of a grating structure in the waveguiding layer at one end of the sensing area. The light propagates a few mm in the waveguide generating an evanescent field. All fluorophores bound to the surface and thus located within the evanescent field are selectively excited.

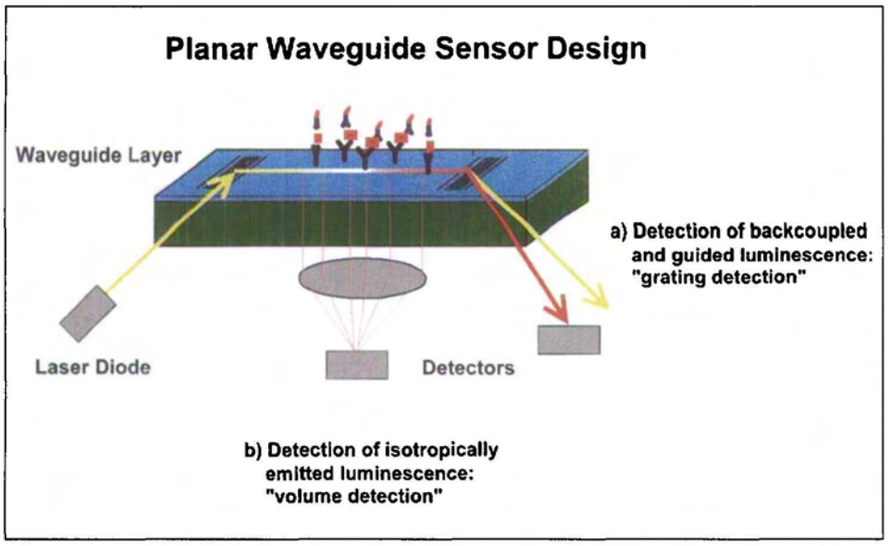

Fig. 1. *Fundamental detection configurations available for planar waveguide biosensor systems.* Both the isotropically emitted fluorescence that was excited within the penetration depth of the planar waveguide ('volume detection') and that part of the fluorescence, that was coupled back and propagated as a guided mode in the wave-guiding film can be detected, using a second grating for outcoupling the emission ('grating detection').

Planar waveguides provide different possibilities of detection configurations. Both the fluorescence that is excited in the evanescent field, but isotropically emitted ('volume detection') and the portion of fluorescence light, that is coupled back into the waveguide, can be detected. With a second grating the guided emission light can be coupled out of the waveguide ('grating detection') and directed towards the detection system (*Fig. 1*).

Immuno and Oligonucleotide Assays

Target analyte classes, in general, can be any molecules specifically binding to their immobilized affinity partners by (bio)specific recognition or leading to a specific change of a physical-chemical property of the recognition element. Until now, the list of affinity systems adapted to the FOBIA system reflects a large part of the spectrum of known biochemical affinity interactions (antigen-antibody, receptor-ligand, complementary DNA/RNA, oligonucleotide-strands, protein-drug interaction) and could be extended to synthetic affinity systems as well. Due to the inherent extremely high sensitivity and real-time measuring capability of FOBIA, its application in new target areas, like tracing RNA/DNA targets in biological samples (*e.g.* genetic diagnostics, genotyping), or for kinetic studies of biospecific interactions (*e.g.* drug-protein) could be beneficial.

Multiplexed sensor systems allow to analyze many samples without compromising sensitivity, in parallel thus opening the perspective for new applications requesting a high throughput.

The feasibility of using optical sensor technology for studying sequence specific DNA hybridization, including quantitative hybridization assays and real-time kinetic investigation of DNA hybridization have already been demonstrated [3]. The example below shows results of the FOBIA antisense oligonucleotide hybridization assay designed and developed for quantitative analysis of human serum samples in preclinical studies.

To achieve maximum sensitivity, preference was given to the so-called adapter assay which is schematically depicted in *Fig.* 2, using 3'-amino-substituted oligonucleotides covalently attached to epoxy-silanized planar waveguide chips. The adapter assay format involves the competition between the Antisense Target Molecule (ATS) and the tracer (Cy5-labeled ATS) for hybridization to a longer oligonucleotide (adapter). The adapter mediates the binding of the complex to the capture oligonucleotide immobilized on

the surface of the sensor chip. Modified oligodeoxynucleotides with higher stability in serum were used throughout.

FOBIA provides a unique opportunity for fast assay optimization. The kinetic feature of the evanescent field sensor technology (*i.e.* determination of association and dissociation rate constants of molecular interactions) allows modeling of a binding assay by means of using kinetic and thermodynamic constants of the given affinity partner and varying assay parameters, such as tracer and binder concentration and assay time for optimizing assay performance [4][5]. Investigating binding kinetics of various Cy5-labeled ATS's (tracer) to the immobilized RNA (binder) with the FOBIA system, the equilibrium dissociation constant determined for a P = S-ATS-2'-MeO-RNA tracer – capture combination was $5 \cdot 10^{-10}$ M (k_{ass} = $1 \cdot 10^{-5}$ 1/Ms, $k_{diss} = 5 \cdot 10^{-5}$ 1/s). According to the theory of competitive binding assays, sensitivity of the hybridization assays should be in the range of ~$2–5 \cdot 10^{-11}$ M, using 0.5–1/K concentrations of the tracer as well as the binder (note, that lowering the concentration of the tracer and/or binder would not result in further sensitivity improvement of the assay).

Measurements of the adsorption of ATS to plasma proteins in 10% human plasma pool (HPP) in dependence of the addition of proteinase K, an enzyme with high proteolytic and no nuclease activity, resulted in dose-response curves almost equivalent to the dose-response curves observed in buffer. Very good signal-to-signal reproducibility was obtained using a sample matrix containing 10% human plasma with sensor lifetimes longer than eight weeks.

The performance of the adapter assay on the planar waveguide biosensor system was tested by measuring five consecutive dose-response curves in buffer at a concentration of adapter and tracer of 0.1 and 0.5 nM, respectively. The resulting data are shown in *Fig. 3*. Sensitivity achieved with this assay matches perfectly with the value theoretically obtained from the kinetic measurement (LOD = $2 \cdot 10^{-10}$ M, LOQ = $7 \cdot 10^{-10}$ M). The precision profile shows coefficients of variation of less than 6.2% in the ATS concentration range of 1 nM to 200 nM.

Whereas the above assay, performed in a competitive format, was an example for an application of high pharmacological relevance, the principle system sensitivity is demonstrated for a noncompetitive assay. An example of a direct binding assay using the binding of Cy5-labeled immunoglobulin (IgG) to immobilized

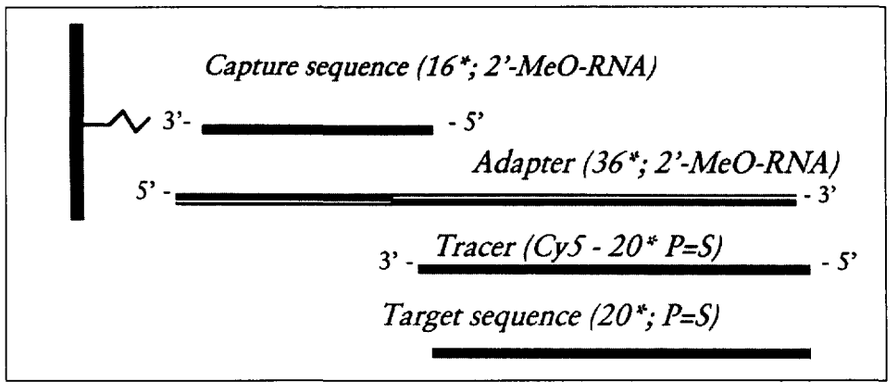

Fig. 2. *FOBIA Adapter assay format for quantitation of antisense oligonucleotides.* The length and the modification for each oligonucleotide is given in parentheses.

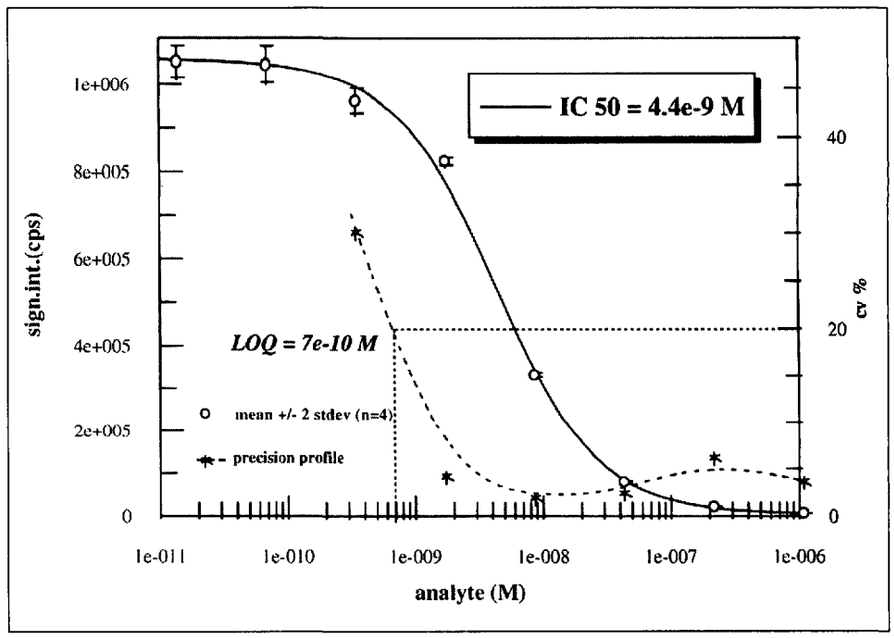

Fig. 3. *Dose-response curve and precision profile of the FOBIA antisense oligonucleotide assay from human plasma* (the figure shows true analyte concentrations; samples were 10 times diluted prior measurements)

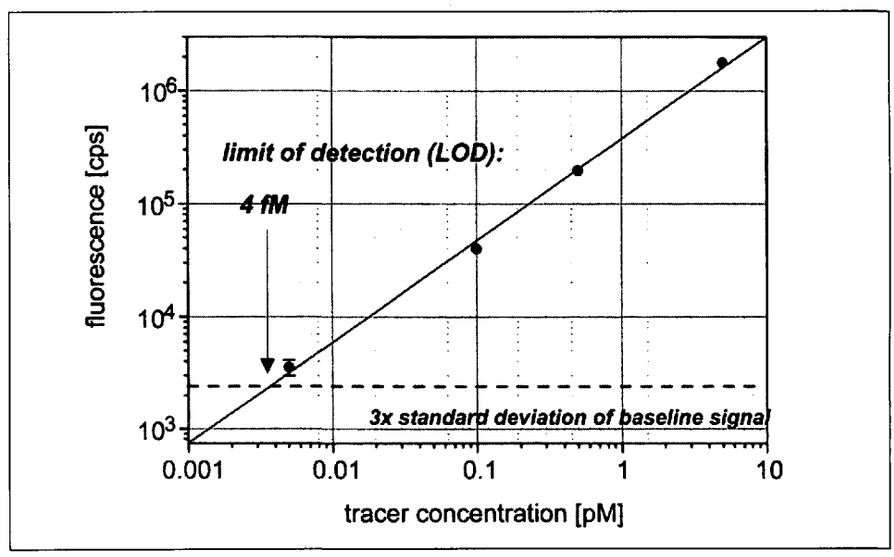

Fig. 4. *Sensitivity of planar waveguide sensor system in model assay: Dose-response curve for the binding of Cy-5 labeled immunoglobulin to immobilized protein A.* The detection limit was determined from the threefold signal noise, when the baseline was recorded.

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protein A is given in *Fig. 4*. For this model bioaffinity system, the detection limit was reached, after a binding period of only 4 min, at a 4-femtomolar concentration of labeled IgG. A total amount of 4 attomoles (2.4×10^6 molecules in 1 ml of sample solution) had been applied to the sensor.

Molecular Imaging Technologies

To gain simple and fast access to physical maps of genomic material, processes like Optical Mapping Technology (OMT) [6][7] and Fiber Fluorescence *in situ* Hybridization (Fiber FISH) [8][9] approaches are evaluated and further developed. Both approaches are based on the singularization, linearization, orientation, and controlled adsorption of DNA molecules on specially treated surfaces. In OMT markers are introduced by (enzymatic) endonuclease digestion resulting in the insertion of physical gaps into the molecule. The resulting fragments stay localized on the surface and are stained by intercalating fluorescence dyes. When visualized by epifluorescence microscopy physical genomic maps can be directly 'read'.

In the Fiber FISH approach the elongated and adsorbed DNA molecules are thermally denatured and subsequently hybridized with single stranded – labeled – complementary DNA fragments. The hybridization event can be detected by epifluorescence microscopy after a signal amplification, *e.g.*, by either applying a cascade of fluorescently labeled antibodies or by alternately adding biotinylated anti-avidin and fluorescently labeled biotin to the sample. As cDNA or fragments of genomic DNA are used for the hybridization process 'functional' positions can be marked and visualized on the DNA strand.

Whereas OMT can be considered an '*ab initio*' method requiring little information of the target to establish physical maps and to allow for a differential display of, *e.g.*, wild type and mutant DNA, in Fiber FISH a higher level of information is required to select or create the appropriate labels to be used for the more specific interrogation tasks. *Fig. 5, a* and *b* give a short schematic overview and comparison of both approaches.

The experience gained during the initial phase of OMT development was applied to define and establish a building

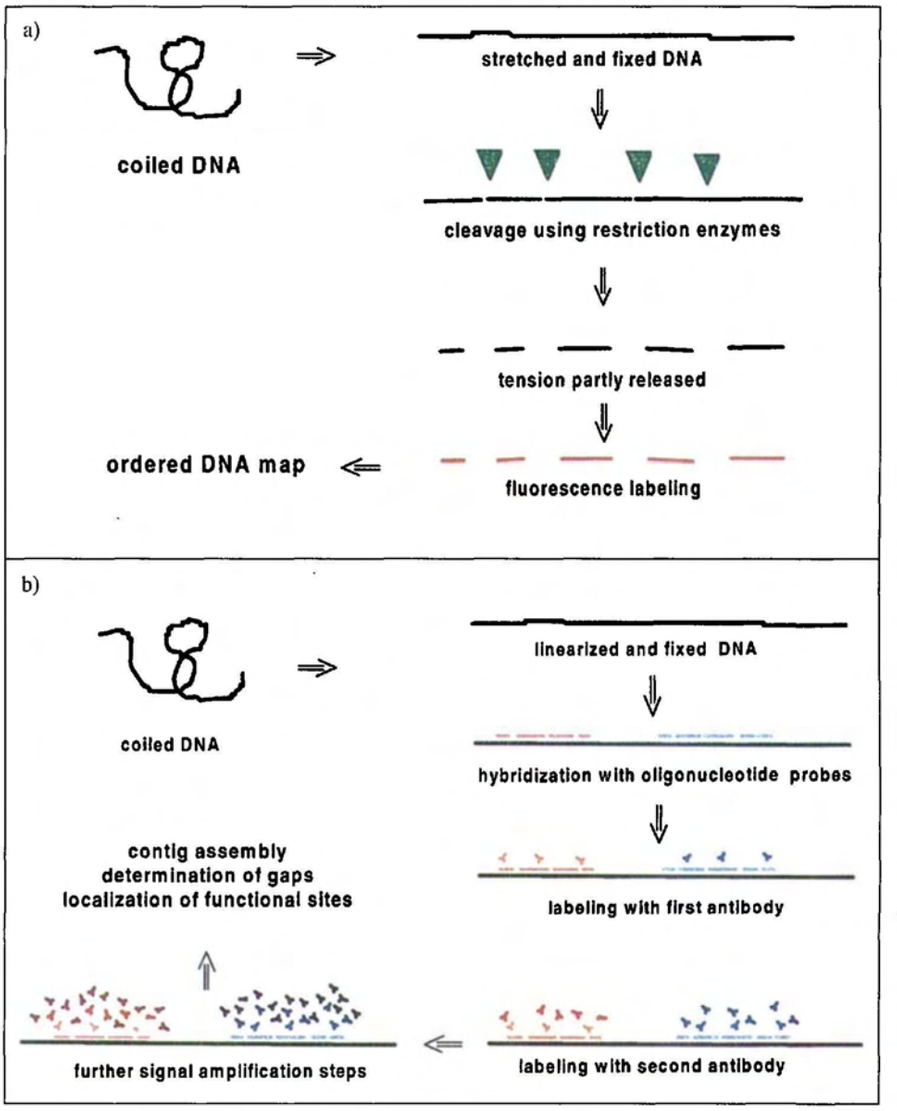

Fig. 5. a) *Schematic visualization of the OMT process;* b) *schematic visualization of the Fiber FISH process*

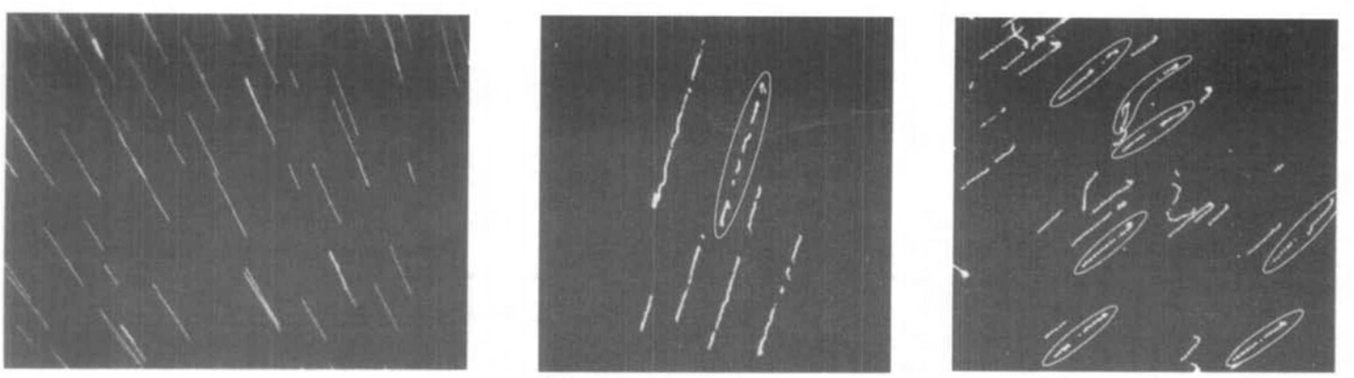

Fig. 6. *Lambda DNA* (48 500 base pairs, equivalent to *ca.* 16 μm in length) *stretched on silanized surfaces.* Left: plain stretching and staining; center: additionally Bam HI digestion; left: Kpm I digestion performed.

block approach to guide the development of surfaces better suited for the OMT, Fiber FISH, and mRNA expression arrays. This model is based on the assumption that different factors contribute to the DNA – surface interaction, that – in an ideal case – these factors can be influenced individually, and that the correct balance of functionality, density of functional groups, and background polarity is responsible for the successful use in the different processes.

Based on this model several alternative surface modification approaches are being developed. Starting from simple to produce – but hard to tune and stabilize – aminopropyltriethoxysilane coated glass better suitable surface modification methods were introduced. More complex silane and polymer based coatings allowed for controlled manipulations of large strands of DNA on surfaces. Using the same model a self-assembled monolayer approach currently is being pursued. To facilitate, control, optimize and potentially automate the processes involved in OMT/Fiber FISH appropriate microfluidics currently is being developed.

Know-how developed for the imaging of genomic DNA – ranging from surface chemistry, microfluidics, to epifluorescence detection – currently is being applied for the development of fluorescence based technologies in an array format suitable, *e.g.*, for quick development of oligonucleotide hybridization assays and especially for monitoring of mRNA expression.

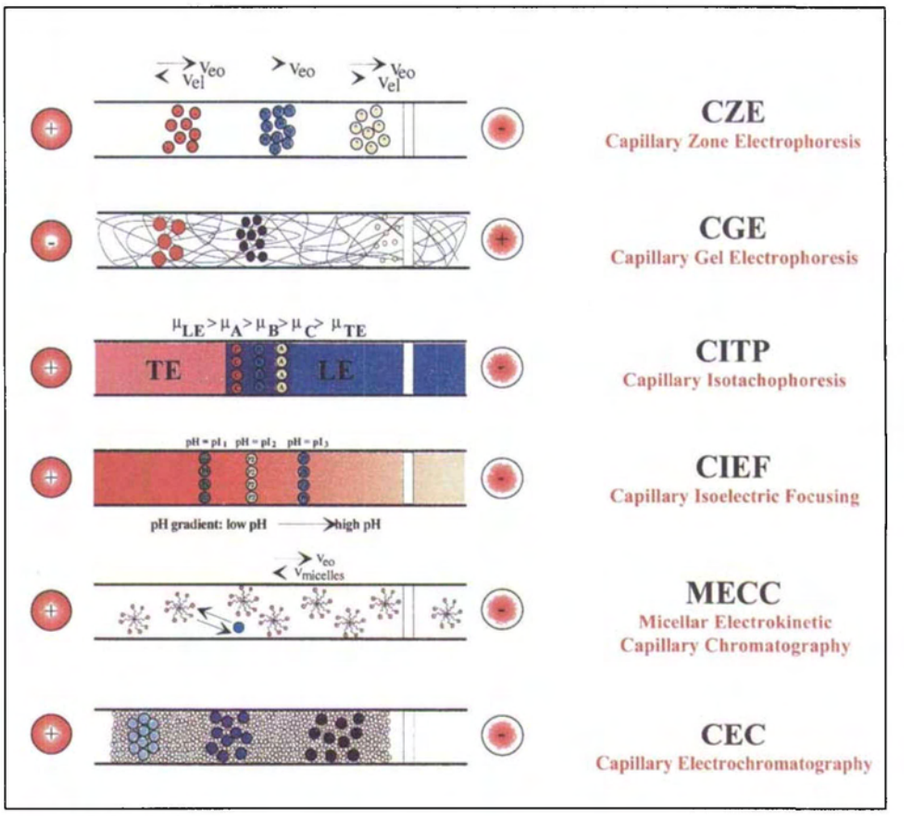

Fig. 7. *CE Separation modes*

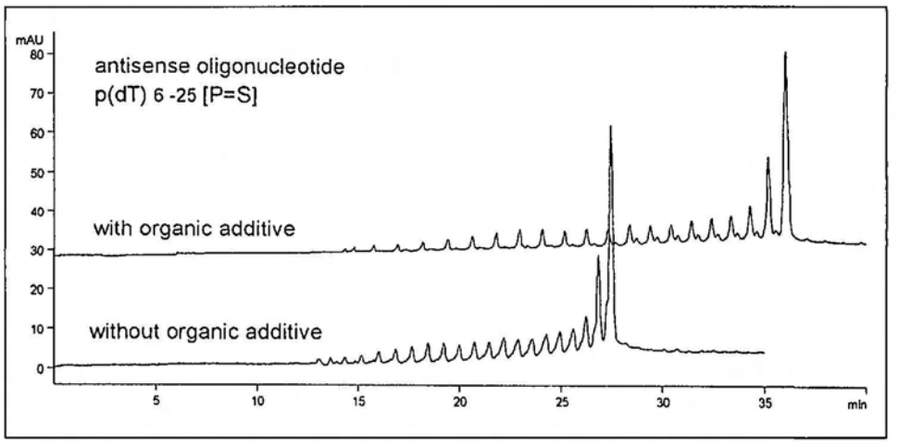

Fig. 8. *CGE of phosphorothiate oligonucleotides pd(T)$_{6-25}$ with a replaceble polymer sieving matrix, with* (upper trace) *and without* (lower trace) *20% acetonitrile in 200 mM Tris, 100 boric acid, pH 8.5 buffer; capillary: 100 µm ID; effective length: 25 cm, total length: 35 cm*

Capillary Electrophoresis

Outline of Technology

Capillary Electrophoresis (CE) [10] is an automated analysis technique, combining features of high performance liquid chromatography (HPLC) and electrophoresis. Contrary to HPLC, an electric field of several hundred V/cm is used to mobilize the solutes and, in free solution applications, an electroosmotic solvent flow (EOF). The typically 50–100 µm diameter of the separation capillary, the small injected sample volumes of just a few nl and the plug shaped flow profile of the EOF allow fast and high efficient CE separations. Separation performance is often at least one order of magnitude better than in HPLC typically, theoretical plates in the order of several 100000 to a few million per capillary can be obtained.

In electrophoretic separations the analytes are typically separated according to their *electrophoretic mobilities*, which depend on both its charge and size. Similar to HPLC, a multitude of techniques were developed to adapt to a specific separation problem as shown in *Fig. 7*. These different modes allow to emphasize the *charge* or *size* aspect in the more electrophoretic modes or the sample hydrophobicity in the chromatographic modes. In Capillary Zone Electrophoresis, *e.g.*, small charged compounds are separated in a free buffer solution in untreated fused silica capillaries. Typical applications range from peptide analysis to the analysis of charged low molecular weight compounds including pharmaceutical active substances and their chiral resolution. Proteins, which tend to easily adsorb to surfaces can be separated in capillaries with a hydrophilic coating according to their charge to mass ratio.

Since most nucleic acids have pronounced size differences but similar charge-to-mass ratios, sieving media such as agarose and polyacrylamide gels have been very popular for their analysis. If these sieving media are filled into a capillary, their porous nature will decrease the

electrophoretic mobility as a function of solute size. Capillary Gel Electrophoresis (CGE) [11] is used predominately for the analysis of double-stranded DNA fragments and single-stranded oligonucleotides with smaller fragments moving faster through the polymer network.

The separation mechanism of Capillary Isotachophoresis (CITP or ITP) [12] is again based on the charge-to-mass ratio. In contrast to CZE, the sample is sandwiched between a leading and a terminating buffer. Upon focusing, sample components are stacked according to their electrophoretic mobility. ITP is often used as a preconcentration technique. If one is only interested in the charge differences, the addition of ampholines can create a pH-gradient for *capillary isoelectric focusing (CIEF)* [13]. For the separation of neutral solutes *Micellar Electrokinetic Capillary Chromatography (MECC)* [14] or *Capillary Electrochromatography (CEC)* [15] are the methods of choice. The interaction of the non-charged analyte in MECC with a charged surfactant such as sodium dodecyl sulfate (SDS) allows their partition between micellar and buffer phase similar to chromatography. CEC is a true chromatographic technique with electric field solvent pumping. MECC and CEC have a similar broad application range as HPLC with the additional benefit of higher separation performance.

We have focused our attention to the separation of nucleic acids of CE. With the antisense approach and gene therapy as potential drug development programs in the pharmaceutical industry the need for automated quantitative analysis of nucleic acids increased. In addition, problems with on-column detection are not envisioned, because nucleic acids exhibit very high extinction coefficients for UV detection at 260 nm. For a more sensitive laser induced fluorescence (LIF) detection, labeling with fluorescent nucleotide primers or intercalation for double-stranded DNA is readily available.

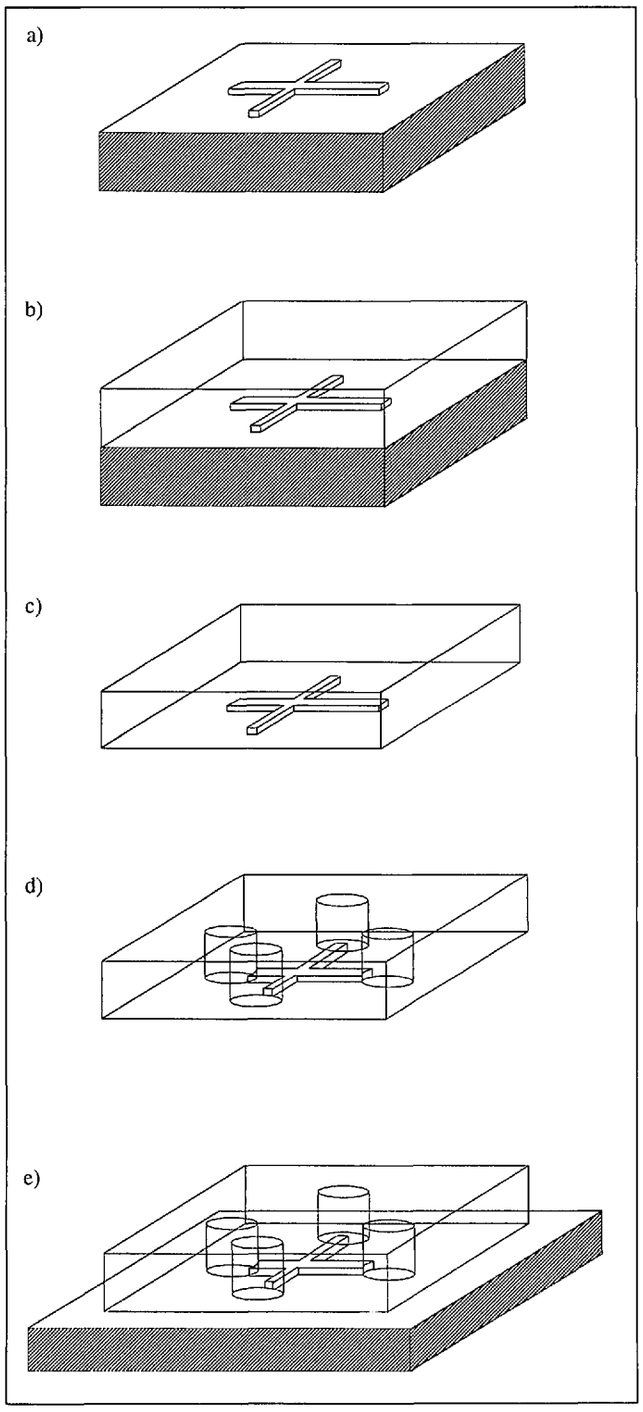

Fig. 9. *ICE Chip fabrication procedure:* a) *silicon master wafer with positive surface relief,* b) *premixed solution of Polydimethylsiloxane* (PDMS) *is poured over the master,* c) *cured PDMS slab is peeled from the master,* d) *liquid reservoirs are punched into the cured PDMS chip,* e) *ready to use device placed on a slab of PDMS* (reprinted with permission from [17], Copyright 1997 American Chemical Society)

Oligonucleotide Separations by CE

For the separation of oligonucleotides, at first, sieving media similar to slab gel formulations were developed using cross-linked polyacrylamide formulations. However, the difficulties associated with cross-linked polymer capillaries, in particular restricted capillary life time through 'bubble' formation, let to the exploration of hydrophilic replaceable polymer matrices. If the polymer concentration exceeds the entanglement threshold, a porous network similar to cross-linked gels is formed. Since the viscosity of the polymer solutions is low, the separation matrix can be pumped into a capillary prior to the separation and flushed out after the analysis. This approach is less error prone with better migration time and quantitation reproducibility and simpler to transfer into a routine environment. Also, a possible carry-over from previous injections is avoided since the capillary is replenished before each analysis.

Sieving matrices for the separation of small oligonucleotides with 10–200 bases have been developed. *Fig. 8* shows the separation of a phosphorothioate test mixture with a polyethylene based polymer matrix. Compared to phosphordiesters (data not shown), phosphorothioates show a different migration rate. Therefore, it can be suggested that the phosphorothioate solutes interact with the polymer in a chromatographic way. However, as demonstrated in *Fig. 8*, this interaction can be utilized to manipulate the separation by the addition of an organic modifier, *e.g.*, acetonitrile.

Integrated Capillary Electrophoresis

The formation of capillary channels on planar substrates allows for an arrangement of multiple parallel capillaries on one single substrate to achieve a high throughput. In addition, sample and fluid handling procedures can easily be integrated due to the ease of interfacing the channels without introducing extra band-broadening because of excessive mixing volumes. This approach, initiated in our department in 1989 [16] will have at least the separation power of capillary electrophoresis combined with the multiplexing capabilities of slab gel electrophoresis. For low cost fabrication of microchips for integrated capillary electrophoresis systems polydimethylsiloxane silicone elastomer was molded against a microfabricated master. Constrains of glass plate bonding at elevated temperature could thus be avoided. *Fig. 9* demonstrates the discrete steps of such a simple molding process.

The performance of the devices is demonstrated by separating X-174/Hae III DNA restriction fragments labeled with the intercalating dye YOYO-1 and shown in *Fig. 10.*

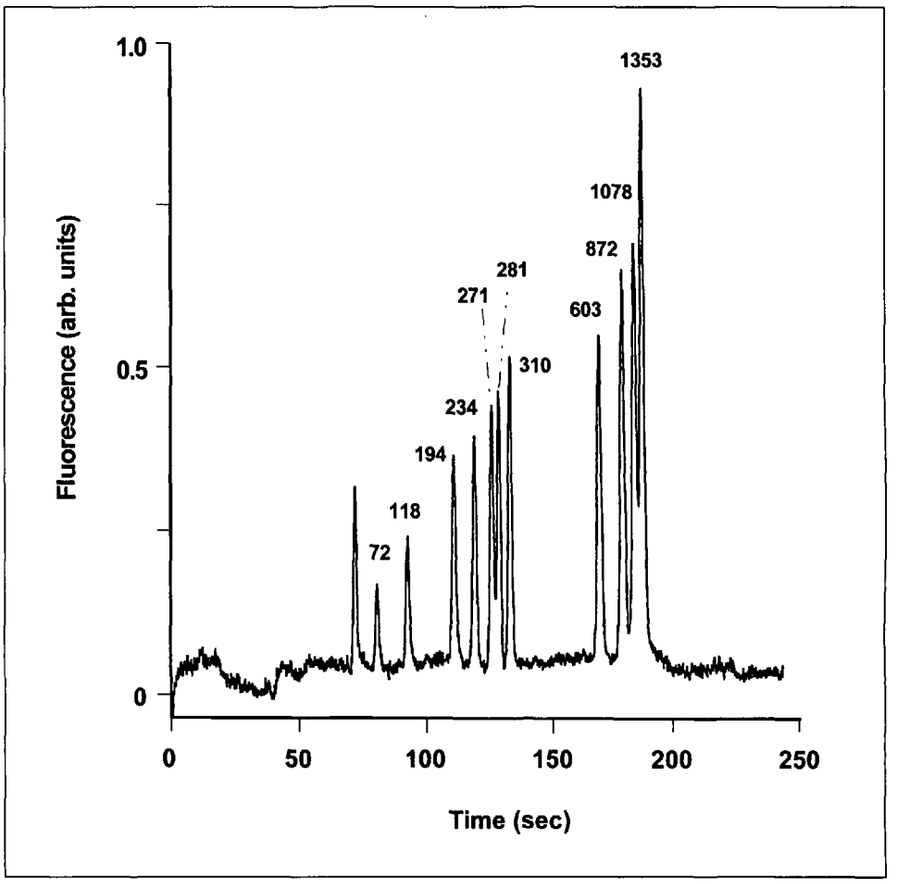

Fig. 10. *Separation of λ-DNA restriction fragments in free buffer solution.* Channel cross section: 50 μm × 20 μm. An electrical potential of 86 V/cm was applied and the fragments separated on a channel length of only 20 mm. 150 pl of a 41 pM DNA solution was injected resulting in an injected amount of 6.1 zmol (*ca.* 3700 molecules) (reprinted with permission from [17] Copyright 1997 American Chemical Society).

Capillary Affinity Gel Electrophoresis

The complexity of antisense samples in biological matrices demands for improvements in selectivity, resolution and sensitivity of the established separation methods. Based on our experiences with sieving media, we developed a novel approach to effectively combine the size separation with a biorecognition step, dubbed Capillary Affinity Gel Electrophoresis (CAGE). A single stranded oligonucleotide as recognition element was chemically bound to a polymer. Upon introduction of this polymer into the capillary and injection of a heterogeneous sample, all solutes with a complementary sequence would bind to the recognition element at low temperature. All non related oligonucleotides as well as non DNA related sample components would, at low temperature, migrate through the polymer network. Only upon raising the temperature above the melting temperature of the double-stranded complex, the bound solutes would dehybridize and start to migrate. In this way, a true two step-system is created, where selectivity can be controlled over the temperature as well as by the composition of the sieving matrix. *Fig. 11* shows an example with a model system, consisting of a 15-mer pdT chemically bound to a polymer. With this matrix, the complementary pdA_{15} oligonucleotide was hybridized at low temperature. Only upon raising the temperature above the melting point of the AT duplex, the pdA_{15}-mer could be eluted. Using a step temperature program single oligonucleotide mismatches with 1, 2, or 3 adenylic acids exchange for thymidylic acids, differing in their melting temperature as little as 1° could be separated.

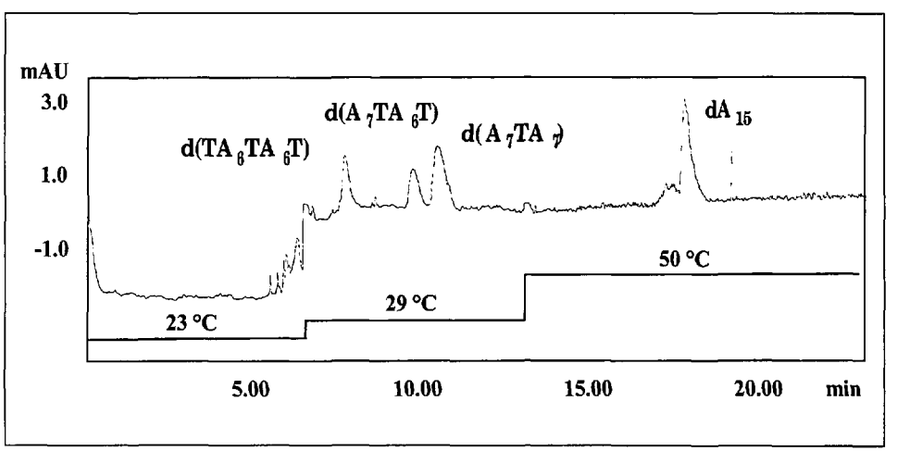

Fig. 11. *Electropherograms showing the separation of dA_{15} from oligonucleotides with 1,2, or 3 mismatches, using the CAGE approach.* CAGE Conditions: 3 %T of a dT_{15}-affinity polymer, dissolved in 100 mM TB. Concentration of each oligo was $5 \cdot 10^{-4}$ OD/ml. Co-injection of dA_{15} for 6 s at –6 kV and a mixture of $d(TA_6TA_6T)$, $d(A_7TA_6T)$ and $d(A_7TA_7)$ for 9 s at –8 kV.

MALDI-TOF Mass Spectrometry

Outline of Technology

In the last years the performance of non-scanning mass spectrometric instrumentation – with time-of-flight (TOF) mass spectrometry as the predominant representative – has dramatically improved and thus this type of mass spectrometers has successfully penetrated the analytical market. In contrast to classical magnetic and electrical field based mass spectrometry where – with the exception of some special modes – a mass range of ions is scanned over time, in non-scanning MS full spectral information is gathered without discarding ions produced in the ion source during the ionization process. This significantly higher transmission rate of the produced ions results in a significant increase in sensitivity when full spectrum data are compared.

In TOF mass spectrometry ions produced in an ion source are accelerated with the identical kinetic energy ($E = {}^1/_2mv^2$). In a field free region ion packages then 'fly' for a certain period of time according to their mass (m prop. $1/v^2$) and are detected when hitting a microchannel plate detector. In contrast to magnetic instruments the obtainable mass range theoretically is not limited.

Currently two main modes of ion production are established in combination with TOF: matrix assisted laser desorption and ionization (MALDI) and electrospray ionization (ESI). In MALDI-TOF-MS introduced in 1989 by *M. Karras* and *F. Hillenkamp* [18] (*Fig. 12*) samples are embedded and cocrystallized in an appropriate matrix like sinapinic acid. Subsequently, this matrix is exposed to pulsed laser light (typically 337 nm, 3 ns, 5 mJ). During this process the matrix is volatilized and the isolated sample molecules are gently ionized. Even large and labile biomolecules are desorbed and ionized without any fragmentation. This special be-

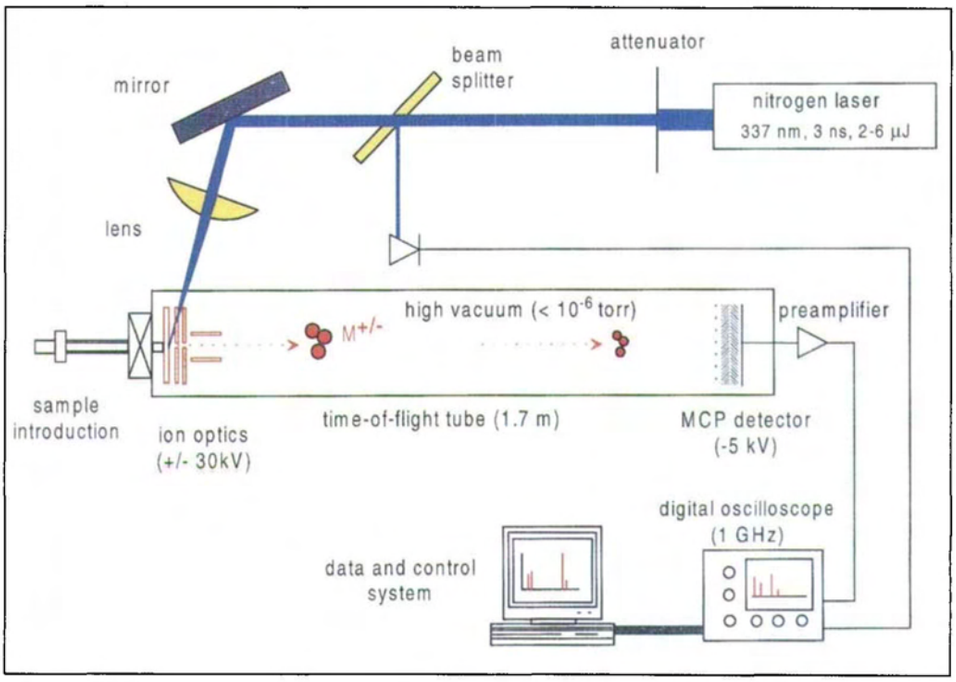

Fig. 12. *Schematic drawing of a MALDI-TOF MS*

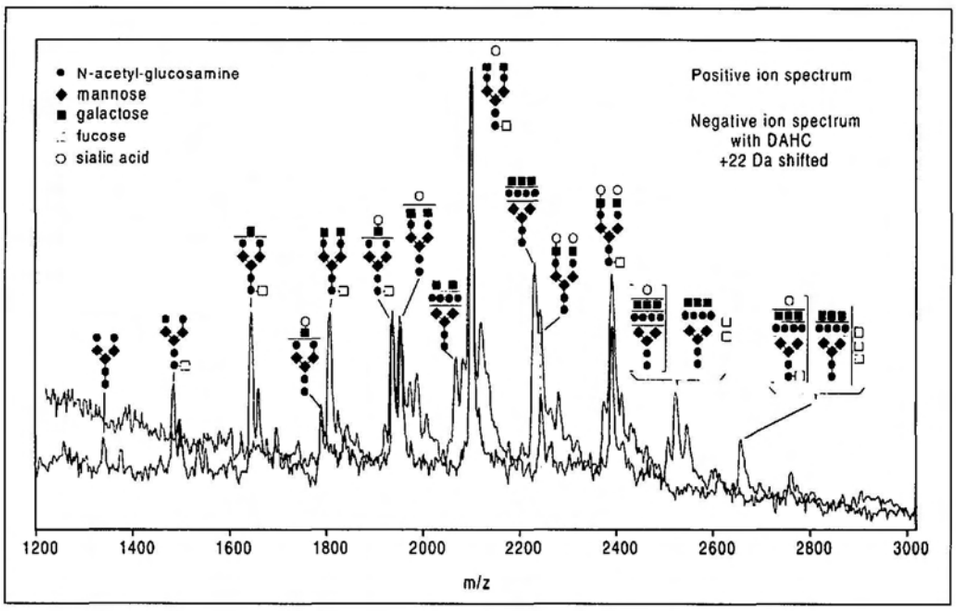

Fig. 13. *Analysis of an oligosaccharide library of human secretory component*

havior of the ionization process renders MALDI-TOF MS an ideal tool for a quick off-line analysis of mixtures of complex composition especially of biological origin. Current state of the art is the characterization of molecules from 300 to 250000 Da in amounts as low as 1 fmol. Due to recent improvements in source design and ion ejection MALDI-TOF MS offers now a resolution (M/DM) of up to 20000 (determined for Cytochrome C).

Its technical simplicity, ruggedness and nevertheless high information content of the data obtained made MALDI-TOF MS an instrument well accepted by researchers. It is used preferably in research and quality control laboratories as no complex fragmentation patterns have to be resolved. In the last several years MALDI-TOF MS has – especially due to the increase of spectral resolution as a consequence of improved ion extraction – found more and more acceptance in different areas of life science. Characterization of synthetic products, biopolymers, drugs, metabolic studies, investigation of oligosaccharides *etc.* were successfully performed. In the characterization of products of protein synthesis MALDI now is the method of choice. In the identification of isolated proteins MALDI based methods – in combination with established data banks – already has found wide acceptance.

An equally promising usage is expected from ESI-TOF MS as far as on-line characterization of biomolecules and bioprocesses are concerned. With this technology – whilst maintaining the key advantages of TOF mass spectrometry – effluents of HPLC, CE, and CEC can be subjected on-line to analysis *via* an electrospray or a nano electrospray interface. In contrast to MALDI, in ESI several charges are deposited on one molecule, the structural integrity, however, can be maintained. Mass resolution of state-of-the-art instrumentation is in the range of 5000 to 7000.

Analysis of Complex Oligosaccharides

Fig. 13 shows the result of an experiment where a complex carbohydrate mixture derived from human secretory component was analyzed. Due to the limited variation of monosaccharides present in this class of oligosaccharides a direct identification of the gross composition easily was possible. Presence or absence of sialic acid in isobaric compounds (*e.g.* two fucose residues have the same molecular weight as sialylic acid residues) could be detected by comparing the negative and positive ion profiles.

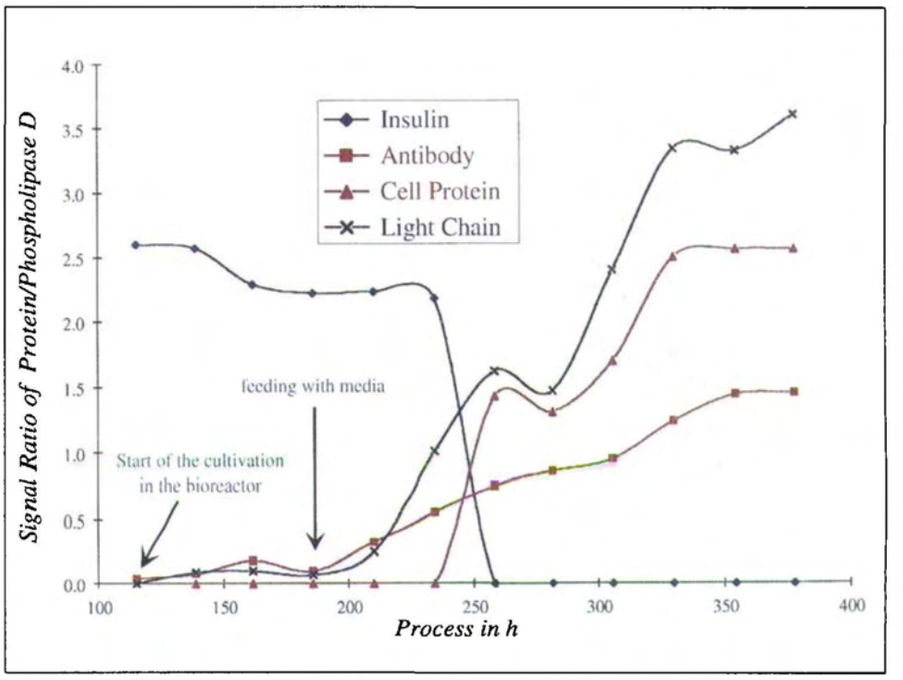

Fig. 14. *Monitoring of key proteins during the hybridoma cell cultivation process for antibody production*

Bioprocess Monitoring

Key requirement for a successful analysis of compounds in complex environments using MALDI-TOF is a careful selection of appropriate matrices and sample preparation methods [19][20]. Under optimized conditions then even quantitative results can be obtained. *Fig. 14* shows the utilization of MALDI-TOF MS for bioprocess monitoring in biotechnology. Profiles of insulin (cultivation additive), produced immunoglobulin G (IgG) and light chain antibody fragment as well as the fate of a selected cell protein was monitored. Samples were taken directly out of cultivation broth with drop dialysis as the only sample preparation step. Using phospholipase D as an internal standard the precision of the IgG determination was comparable to these obtained by affinity HPLC. Limit of detection in raw cultivation broth was found to be *ca.* 160 pmol absolute; limit of detection in pure solvents was in the fmol range.

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