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Molecular Diagnostics

Springer

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Editors

Molecular Diagnostics

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Aims and Scope

This book series reviews current trends in modern biotechnology and biochemical engineering. Its aim is to cover all aspects of these interdisciplinary disciplines, where knowledge, methods and expertise are required from chemistry, biochemistry, microbiology, molecular biology, chemical engineering and computer science.

Volumes are organized topically and provide a comprehensive discussion of developments in the field over the past 3–5 years. The series also discusses new discoveries and applications. Special volumes are dedicated to selected topics which focus on new biotechnological products and new processes for their synthesis and purification.

In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

In references, Advances in Biochemical Engineering/Biotechnology is abbreviated as *Adv. Biochem. Engin./Biotechnol.* and cited as a journal.

Preface

Nearly 20 years ago the concept of molecular diagnostics moved into the focus of academic and industrial research. This shift was mainly driven by new techniques like DNA Microarrays which often result in miniaturization of assays and the automatization of processes. Since, then the field of molecular diagnostics has been expanded and clinical diagnostic laboratories function as a playing field for this expansion. Vast and dynamic changes in the test menus, instrumentation and clinical applications have been some of the impacts molecular diagnostics has had. Nevertheless, so far only a few promising techniques have become standard routine.

Human beings differ in their level of health. Even individuals with the same disease can have specific differences in their clinical picture. Therefore it is important to adjust therapy and medicaments individually. Today this approach is known as "personalized medicine". Molecular Diagnostics covers current molecular biological techniques used not only to identify the underlying molecular defects in inherited disease, but also to monitor therapies. Multiple studies in modern science have shown that changes on a molecular level are often directly linked to the origin of diseases. Consequently, new targets have been recognized and often used as potential biomarkers for intervention. These targets are able to act on different levels, like proteomics, genomics, or metabolomics.

The aim is to develop disease-specific biomarkers, which can be applied *in vivo* in the patient or *in vitro* by analyzing human samples like blood, tissues, or urine. Ideally, these markers allow not only a distinction between healthy and diseased people, but also, e.g., the classification of different types of cancer. Moreover, multiplexing multiparameter analyses often have a higher sensitivity, wide dynamic range, and need shorter incubation time. So the quite new field of "Molecular Diagnostics" is able to open new ways for detecting diseases, even at an early stage. This prevents severe harm for a patient, reduces costs and the effort of medical examinations, gives access to new methods in modern medicine, and is well-suited for point-of-care diagnostics.

Newly developed assays, devices for *in vitro* diagnostics and the corresponding imaging technologies should not only be available in specialized institutes or laboratories. Even common hospitals and surgeries, as well as general medicine, should gain access to these techniques.

The development of molecular diagnostic tools is an interdisciplinary task of clinicians, experimental and theoretical groups in universities, research institutes and industrial facilities. This multidisciplinarity gives the opportunity to use knowledge and resources of diverse institutes and researchers in an ideal way. Contributors of the chapters are well-known experts in their field, and come from a variety of disciplines, to ensure breadth and depth of coverage.

Therefore this volume contains contributions from scientists working in different fields and institutes.

We want to thank Springer-Verlag for giving us the opportunity to edit this volume. Furthermore for the constant help and support during the preparation of this volume, especially Ms. Karin Bartsch, Project Coordinator, and Ms. Elizabeth Hawkins, Chemistry Editor.

We also want to acknowledge the authors for distributing their chapters and spending their time preparing interesting articles.

Finally, we want to thank all colleagues who made this volume possible.

In conclusion, we hope that in this volume you will find inspiring literature and useful information about molecular diagnostics.

Sarah Schumacher
Harald Seitz

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Integration in Bioanalysis: Technologies for Point-of-Care Testing

Frank F. Bier and Soeren Schumacher

Abstract Biosensors, Lab-on-Chip technologies, and sensor-actor molecules are steps towards the integration of bioanalysis into small devices that will help in providing analysis where it is needed: the point-of-care. This article gives a brief overview of recent achievements and future prospects.

Contents

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1 Introduction

1.1 The Concept of Molecular Biomarkers

The last two decades have seen extensive effort put into genome and proteome research which has led to a deeper understanding of the molecular basis of diseases, their occurrence, development, and cure. As a consequence of this knowledge, more suitable therapies are on the horizon and are discussed widely as "personalized medicine". Molecular diagnostics will be an integrated part of this concept, since medication, success of treatment but also early occurrence of specific biomarkers for early detection of disease or even presymptomatic diagnosis will become the focus of medical treatments. Also genetic markers for risk screening and all aspects of companion diagnostics that define medication by the genetic constitution of a patient will help provide improved therapy.

Therefore, the molecular in vitro diagnostics market has good forecasts and is regarded to be a worldwide increasing market. Especially combined with point-of-care testing (sometimes better described as point-of-need) in vitro diagnostics might significantly improve the benefit obtained from molecular knowledge. Biochip- and Lab-on-Chip technologies designed for routine application open up the opportunity of performing complex analysis and multiparameter analysis on a small scale. Lab-on-Chip systems have the potential to transfer molecular diagnostics to the point-of-need.

A key component of the future development of diagnostics is the concept of biomarkers. In general biomarkers are all kinds of parameters that may be obtained from a patient and that are quantitative and correlate to a particular disease. Usually a biomarker qualifies to be called a surrogate marker when evidence has been gained from clinical studies that the biomarker represents a certain disease, a disease stage, or the patient's reaction towards a particular treatment or medication. While the general concept of a biomarker includes all kinds of physiological data such as heart beat and lung volume, molecular biomarkers concentrate on biochemical or genetic parameters and patterns thereof, sometimes named signatures.

In addition, diagnostics becomes more complex when a deeper look at these various biomarkers is necessary (Fig. 1). Within the Human Genome Project about 25,000 genes were identified. Considering the dogma of molecular biology these genes are transcribed to various forms of RNA, then translated into proteins and post-translationally modified. Going one step further, also variation in metabolism may be linked to diseases as well. In this regard, genomic, proteomic, glycomic, and metabolomic research has led to the need for detection and quantification of completely different types of analytes ranging from genes and proteins to small molecules and combinations thereof.

A multitude of detection methods are needed to match the requirements of each analyte. However, in many cases these methods are too sophisticated for routine diagnostics and in most cases too expensive as well. Hence, there is a need for new, more user-friendly technologies. This will lead to benefits for many patients and additionally may help to reduce costs caused by false and delayed treatments.

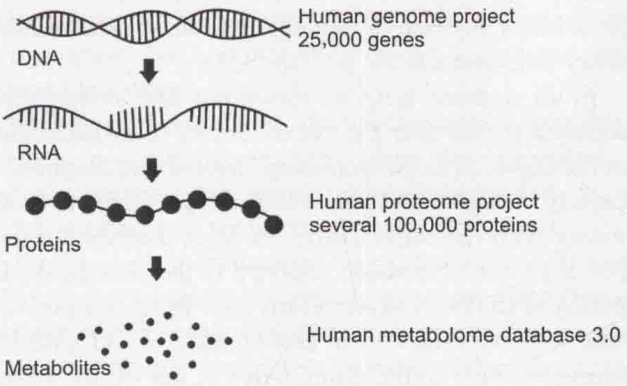

Fig. 1 Differentiation of biomarkers and number of possible diagnostic targets (data retrieved from corresponding homepages [30])

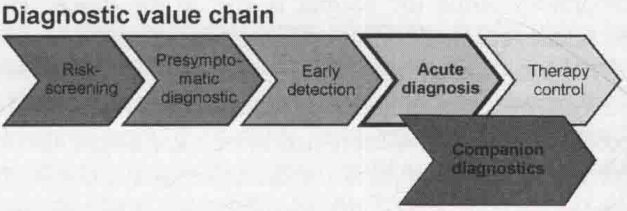

Fig. 2 Biomarkers will help to provide diagnostics at various stages of a disease even before a patient shows symptoms. Also during or after medical intervention biomarker based diagnostics will help to follow the success

1.2 Societal Needs

Having established this type of biomarker-related diagnosis, the accumulation of relevant data over a time period by electronic means will lead to increasing insight into long-term effects and later presymptomatic or even prognostic diagnosis may be achieved (Fig. 2).

Taking advantage of these new biomedical findings will provide an opportunity for improved patient-centered care. Hence, biomarker-based diagnostics will not only be used for curative purposes but also for prevention of diseases, enhancement of therapy success, and in general for increasing the quality of life.

From a more societal point of view diagnostics can effectively reduce costs within health-care systems. In terms of personalized medicine, collecting data over time will allow more rational access to the best therapy, and for large collectives of patients gathering data will lead to valuable information for a health-care system. Biomarker-based diagnostics will thus help to reduce health-care costs by reducing the number of second line therapies, reducing treatment costs, reducing the number of follow-up therapies, reducing nursing, reducing consequential costs, and reducing the period of sickness absence. In this regard, there are only very few economic studies about the impact of point-of-care testing. One example has been undertaken for emergency departments in the U.S. It was shown that fast measurement of the diagnostic marker Troponin directly at the patient can reduce costs

by a factor of four, by enabling faster patient management time and an overall better outcome for the patient [11].

From a more holistic viewpoint and considering the aging and increasing population, but also the ramifications of globalization, more versatile diagnostic technologies will be necessary. Moreover, diagnostics will have to be more frequently available and hence technologies have to be found that enable patient-near testing with the same quality as from the laboratory.

The scenario of bedside analysis is the first field of application of point-of-care testing (POCT). A short return time from sample to the location of the decision-maker, the so called "turn-around time" (TAT), is of great interest to the physician during his visit in the clinic. Also in the doctor's office it often might be of great help, if the doctor had access to the blood parameters or other results from the laboratory while the patient is still in the office. The decision for therapy could be better targeted and the patient would be pleased to be well informed about the physician's decision.

The role of biomarkers is to support the decision, which therapy might be most promising. A well-established biomarker might also be a guide for medication and for the appropriate dose for the individual patient. This scenario is called "companion diagnostics" and refers to the need of most medications to be appropriately adjusted to the physiological and genetic constitution of each patient individually to be most effective or sometimes even effective at all. Many drugs are known to be metabolized more or less effectively by different patients but up to now this information is only seldom available and useable by a doctor in his office. This information would be of help only if it were available immediately.

The utility and usefulness of biomarkers will increase, if samples other than blood, like saliva, urine, or other easily accessible body fluids are tested, which may help to make diagnosis less invasive.

Individual consultation and personalized therapy are the major trends of modern health care and both require diagnostics at the point-of-need and are what economists call the "market pull" for the development of Lab-on-Chip technologies for POCT [4].

1.3 Integration as a Key Parameter

Technologies that can be used for point-of-care testing have to match various requirements—especially because of the circumstances point-of-care testing is used in. Here, samples are not taken in a lab environment which enables users (medical personnel or physicians) to perform steps of sample preparation and its purification until results are obtained. More precisely, the technology has to provide user-friendly devices, that perform automatic processing of any sample of a body fluid and gives an interpretation of the measured results on a display. With this scenario in mind the following key features for technologies may be defined:

- User-friendliness: The devices have to be as easy as possible to use. This includes not only sample preparation, but also handling of the device and the small sample volumes required for testing.
- Miniaturization: In most application scenarios the device and also a possible base unit have to be as small as possible. By thinking about the assay itself, miniaturization of the assay will decrease the amount of sample needed for a particular analysis and will be of benefit in terms of faster reaction times.
- Parallelization: Because of the increasing knowledge in biomedicine in many cases a parallel analysis of different biomarkers can be beneficial. Therefore, technologies have to deliver not only a single parameter. Moreover, the possibility to determine a multiple of different parameters to make a diagnosis not only on the basis of one parameter can lead to faster therapeutic action and hence better patient outcome.
- Speed: Speed of analysis can be seen as crucial since nowadays applications in point-of-care testing are described as being linked to direct therapeutic action. For example, point-of-care testing for diabetes is directly linked to the injection of insulin, or a test for the determination of a cardiac infarction is directly linked to therapeutic action. These are two examples where patients directly benefit from a fast diagnosis and where speed especially in the second example is of great importance.
- Interdisciplinary: The key to obtaining such devices and fulfilling the above-mentioned criteria is the convergence of different technologies. Hence, an interdisciplinary approach has to be chosen which combines not only biochemistry, but also electroengineering, microfabrication, material sciences, and knowledge about production which all have to work together.

Taking these five key features into account, it is necessary to start as early as possible within the design process to think about a holistic system solution. In this process the concept of integration is essential since integration of steps, materials and processes may lead to the desired device features. In this regard, the following sections describe different degrees of integration and try to outline necessary design rules for implementation of interdisciplinary technologies for realizing systems for point-of-care testing.

2 Integration Steps

Point-of-care testing has to integrate laboratory-like procedures and guarantee laboratory standards. Moreover, POCT has to be connected to the data management system of the clinic or of the physician who is in charge of the patient. Figure 3 shows how integration of bioanalysis proceeds and which steps have to be taken during further development. It can be regarded as a road map for integration in POCT for the upcoming years.

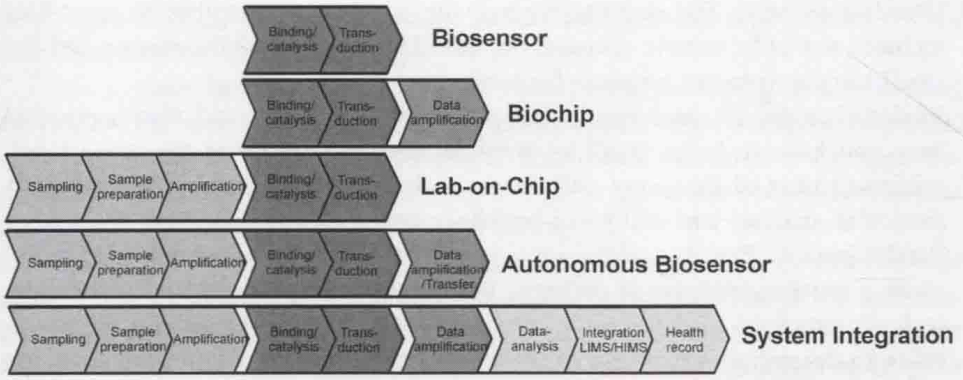

Fig. 3 Different steps of technological integration

2.1 Biosensors and Biochips

The concept of biosensors has a long history; usually Clark's glucose electrode proposed in 1962 is named as the birth of the technology [6]. Biosensors were defined by IUPAC in 1992; a biosensor is "a device that uses specific biochemical reactions mediated by isolated enzymes, immune systems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" [12]. By this definition classical biosensors are made from two components, the biological receptor molecule and the transducer, which are responsible for linking a biochemical reaction to a readout that may be quantified such as an optical or electronic signal.

Having a look at the technological side, in the early days of biosensors, there was a clear separation between the receptor molecules and the transducer which was made by physical entrapment of the receptor molecule within a membrane. The membrane itself was also used as a separation tool which only allows the analyte of interest to pass through. The next step in the process of integration was the generation of biosensors in which the membrane, the receptor molecule, and the transducer were all combined in one compartment [19]. Hence, the process of separation, binding, and transduction were located next to each other enabling faster electron transfer, better biosensor response, and higher sensitivities. These so-called membrane sensors where then replaced by second-generation biosensors in which the membrane was no longer necessary. This could be accomplished by new and more specific recognition elements which made the first separation step redundant. As fabrication technologies in microelectronics and microsystems progressed in the later 1980s smaller and affordable production of microelectronic and mechanical systems (MEMS) was achieved and thus the integration of receptor molecules, transducer, and the electronics necessary for data generation could be combined. The convergence of now three components led to the production of third-generation biosensors which were sometimes also termed "biochips" [19].

The literature contains a vast variety of attempts and concepts for biosensors and the number of publications is still increasing. Because of the impact of various technologies the improvements can be seen in all of these components with special emphasis on their interfaces. For example, the communication between enzymes and an electrode in an electrochemical sensor is of huge importance for its performance. In an amperometric detection mode electrons are measured which corresponds to the conversion of a substrate. To enhance the amount of electrons traveling from the enzyme to the electrode, two different methods can be chosen. One method is the possibility of using a sophisticated connecting layer in which the enzyme can be embedded. By adding a redox-mediator to this layer there is the possibility for an indirect electron transfer from the enzyme over the redox-mediator to the electrode. In a recent example, Nagel et al. showed the synthesis and application of a redox-polymer based on poly(*N*-isopropylacrylamide) (PNIPAM) with incorporated ferrocene moieties for an indirect electron transfer using NAD-dependent glucose dehydrogenase (NAD-GDH) or pyrroloquinoline quinone-dependent GDH (PQQ-GDH) and glucose as the analyte) [15]. The authors detected a heterogeneous electron transfer rate of 80 s^{-1}. This is twice as high compared to a normal self-assembled monolayer of a ferrocenepentanoate. Hence, by using hydrogels with incorporated mediators such as ferrocene a more effective electron transfer may be achieved. The other method is to modify the recognition element itself. To describe one example here, Demin and Hall modified a glucose oxidase (GOx) [10]. By different methods such as NMR spectroscopy and in silico calculations two considerations could be revealed: (i) oligosaccharide structures on the surface of the GOx are responsible for a larger space between the enzyme and the electrode; (ii) the path of the electron through the GOx could be shown hence the hemisphere of the enzyme could be determined through which the electron can pass to the electrode. From that, a genetically modified GOx was derived and produced bearing no oligosaccharide structures and a certain surface modification to facilitate direct immobilization. Hence, a better and direct electron transfer from the enzyme to the electrode could be accomplished.

This is a nice example of how the modification of biological recognition elements can lead to improved biosensor performance for applications such as glucose detection. Nevertheless, there is a trend to overcome the limitations of biological recognition elements such as stability problems under harsh conditions or batch-variations and to replace them with artificial receptor molecules. To obtain artificial receptors besides their chemical synthesis which is in most cases tedious and time-consuming, two approaches have been established in the last few decades. The first is the use of artificial DNA- or RNA-molecules called aptamers which may act as an antibody-like recognition element. For their synthesis a process called systematic evolution of ligands by exponential enrichment (SELEX), invented simultaneously by Gold and Szostak [9, 25], is used in which the tightest binding DNA- (or RNA-) strands are selected via a selection process. Through a generic approach aptamers against different molecules can be generated and used in biosensor applications [13]. Since the binding event is not directly linked to a signal generation most applications using aptamers are combined with an optical

transducer. One example is the detection of TNT by an aptamer within a fiber-optic biosensor. Because of the selectivity of the aptamer it was possible to discriminate TNT from other explosives [7].

The second is the concept of molecularly imprinted polymers (MIP). Here, a polymerization is carried out in the presence of the analyte which is also the template during the imprinting process. Within the polymerization mixture monomers, so-called functional monomers, are also used which can specifically interact with the template molecule by covalent or noncovalent means. After polymerization the template is extracted leaving an artificial binding site in which the analyte may (re-)bind. First adaptations of the MIP concept to biosensors can be traced back to the work of Mosbach [14]. This concept is also a generic approach and may be used for a great variety of different analytes. Using the noncovalent approach it was, for example, possible to obtain a binding polymer against nitrofurantion, an antibiotic frequently used in farming in former times, however nowadays prohibited due to toxic side effects. With these polymers it was possible to detect nitrofurantion directly from bird seed avoiding tedious mass analytical measurements [2]. One prominent example of a covalently imprinted polymer is the use of boronic acids as functional monomers for the detection of saccharides such as glucose, fructose, or saccharide derivatives such as fructosyl-valine [18, 20]. Since the binding event is also not linked to a direct detection in many cases the transducer chosen for molecularly imprinted polymers is either based on a mass change measured by quartz crystal microbalance (QCM) or cantilevers or based on the measurement of the latent heat of the binding using calorimetry. For a fructosyl-valine imprinted polymer it could be shown that the thermometric response of the binding event is about forty times higher compared to a control polymer without imprinted cavities [18].

Not only improvements on the recognition site are responsible for better biosensors. As already mentioned also improvements in the design and production of transducers may lead to great advancements in how biosensors will perform in various applications. Miniaturization of transducers is beneficial for cost reduction as well as user-friendliness. Because of the still ongoing race in miniaturizing electronics, especially electrochemical- and MEMS-based sensors may be miniaturized. A limitation of this trend will be, when problems arise from the small surface area with small possible surface loadings of enzymes leading to small signal amplitudes. In this regard different amplification methods have to be applied to gain signals with a high signal-to-noise ratio. Besides microelectronic devices also the manufacturing of micromechanical devices finds its way into the research field of biosensors. To give an example, the fabrication of a microcantilever enables the measurement of mass changes or changes in viscosity. In a biosensor for glucose detection Birkholz et al. used resonating microcantilevers to measure the change in viscosity of a hydrogel in which glucose was bound [5].

To summarize, improvements in recognition elements as well as in transducers are responsible for miniaturization and integration of biosensors and biochips. With smaller devices and more specific and direct biochemical reactions there is the potential for many more applications. Because of the specificity of the