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BIOSENSORS IN ANALYTICAL BIOTECHNOLOGY

Ruth Freitag

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Dedication

We would like to dedicate this book to Professor Schügerl.

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FOREWORD

The Good Manufacturing Praxis (GMP) requires better press control, safety and documentation than available today. The lack of instruments with suitable specificity and sensitivity is especially serious in biotechnological production presses with extremely complex and not well-defined cultivation media. The monoseptic operation of the bioreactors requires their wet steam sterilization, which restricts the suitable instruments used in situ to thermometer, pH- and pO_2-meters. The chemically specific analysis is performed outside of the reactor by aseptic on-line sampling and by Flow Injection Analysis (FIA), High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC). However, only the FIA system is quick enough to use it for process control. Good Laboratory Praxis (GLP) was introduced by many companies as a warrant for the high product quality of pharmaceutical products. However, due to the lack of quick aseptic sampling and fast on-line analysis of the medium components, the presently practiced GLP is far from perfect. The highly specific biosensors integrated in fast FIA systems are ideally suited for filling this gap, not only in the laboratory, but in the production press as well.

In this book aseptic sampling, FIA systems for monitoring the concentration of common substrates, different types of biosensors, and tools for the evaluation of the measured FIA-signals and the use of these systems in the biotechnological praxis are presented by authors who are working in institutes which have long-standing experience in these fields. They have successfully applied these biosensor-FIA systems for monitoring and control of several biotechnological processes. It is expected that the application of biosensors in combination with FIA systems will generally be used in future biotechnological laboratories and in other industries as well.

Professor Karl Schügerl
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CHAPTER 1

SAMPLING MODULES

Ruth Freitag

INTRODUCTION

A large number and variety of analytical procedures are involved in a typical biotechnological process.[1] There is monitoring and perhaps even control of the bioprocess proper and the downstream process. This calls for the surveillance of several parameters, including not only temperature and pH, but also various nutrients and metabolites, including the product itself. The metabolic state of the producing organisms is another parameter to consider. While temperature and pH control is more or less straightforwardly done using stable sterilizable in situ probes, biosensors have revolutionized the area of monitoring chemicals and biologicals. Analysis can be carried out in-line, in situ, on-line, at-line, off-line, or noninvasively (Fig. 1.1).[2] Among these types, the only one which requires no physical contact between the sensor and the bioprocess are the noninvasive systems. Noninvasive optical sensors (turbidimetric, nephelometric and fluorescence sensors) are, for example, used in biotechnology for the continuous monitoring of cell number and cell viability.[3,4]

In-line sensors must meet high demands for long-term stability and reliability. In addition, sterilization must be possible without biasing the sensor's output. Since the involvement of a biological molecule is inherent to biosensing, and sterilization procedures aim for the destruction of biological material, the number of biosensors suited for in-line application is exceedingly small. The arrangements for process interfacing and sample conditioning tend to be rather complicated and the number of in-line biosensors is not expected to increase in the near future in spite of efforts in that direction.[5,6]

Biosensors are mostly used in the on-line or at-line mode and are not themselves in contact with the reactor medium. Therefore a device, which concomitantly constitutes a sterile barrier is needed to provide

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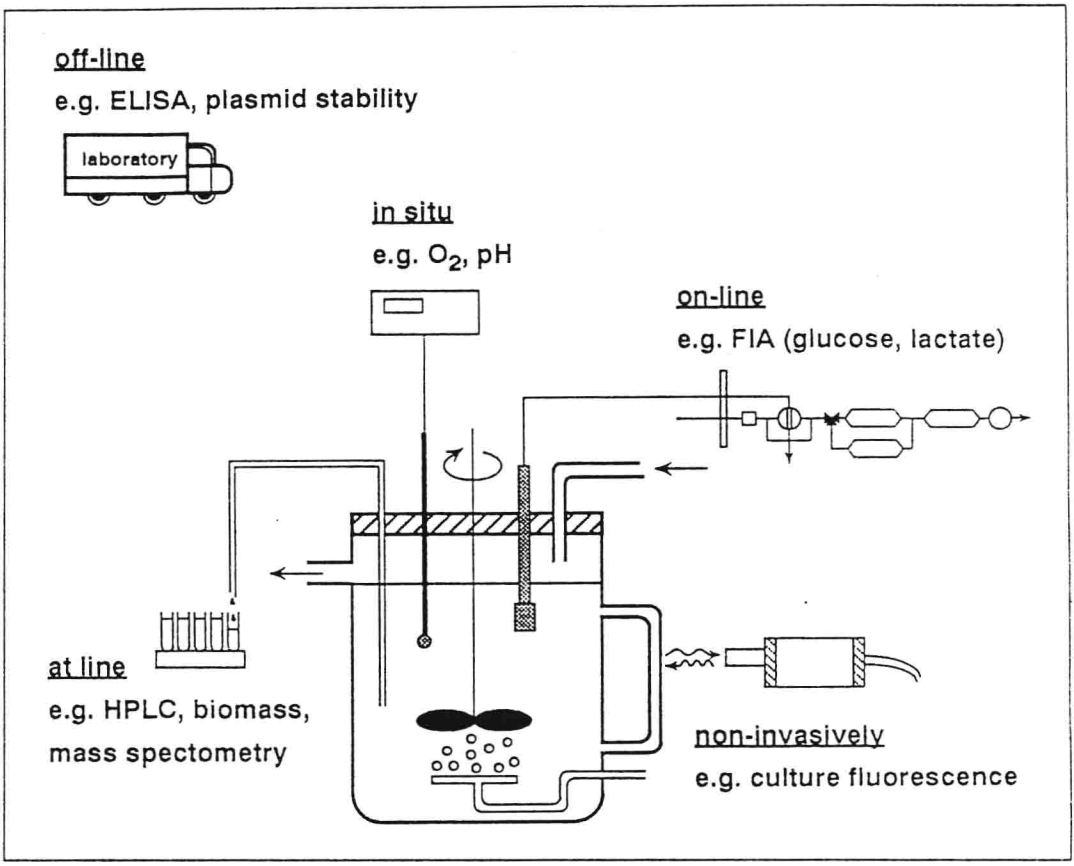

Fig. 1.1. Modes of process monitoring used in biotechnology.

a representative sample of the analyte to be considered. Both inclusive and exclusive sampling is possible. In the former mode, the sample represents the total composition of the liquid (or gaseous) phase of the bioreactor. In the latter case, a selection takes place, often according to size (i.e., by a filtration or dialysis step). The exact sampling procedure depends on the analytical mode chosen and the character of the analyte.

Until recently, off-line analysis was most commonly employed in biotechnology, where the sample could simply be withdrawn from the bioreactor using an inclusive module (e.g., a sterilizable valve or siphoning device). As demands for automated process monitoring and control increase, however, intermittent or continuous on-line monitoring is desired. In this case the sample needs to be provided automatically to the analyzer. The art of automatically and reproducibly withdrawing a representative sample by means of a continuous sam-

pling module has become highly important to biotechnological process analysis.[1,7-9]

In addition, the sample should be fully conditioned and ready for analysis, i.e., it should be diluted or concentrated, chemically modified or ridded of any substance which may bias the result in terms of properly monitoring the bioprocess. Sampling *cum* conditioning is often done by a so-called "autosampler" such as the GAT-BIOAS 3V,[10] a device which also provides the sensor with a calibration standard. Alternatively, a conditioning manifold may be part of the bioanalyzer. This approach is often taken in flow injection (FI-) systems.

The use of continuous sampling modules requires good knowledge of the physicochemical characteristics of the operation in question, otherwise a correlation between the measured results and the development of the bioprocess is difficult to establish. The time delay between sampling and the point at which the result of the analysis becomes available is crucial, for example, in the development of strategies for process control. While computerized models which allow us to deduce the state of the process from reproducibly obtained on-line and at-line data are becoming available,[11] real time analysis remains the ideal and the sampling procedure should help to approximate this situation as closely as possible.

On-line analysis is increasingly used for process monitoring in biotechnology.[1,12-15] While most of the work is still being done with yeast, fungi and bacteria cultures, applications in mammalian cell cultures are increasing. While mammalian cell cultures are rather vulnerable towards fungal and bacterial contamination, the fact that the creation of an optimal environment is more difficult for these organisms is an argument for close supervision of the critical parameters.

INCLUSIVE SAMPLING SYSTEMS

In spite of the recent advances in on-line monitoring, many process parameters in biotechnological processes are still established off-line. The samples are obtained manually using an inclusive sampling system. Typical sampling devices for analytes found in the liquid phase include sterilizable valves (e.g., made with Teflon instead of rubber as in the autoclavable system described in ref. 16) as well as hooded samplers and glass traps.[17-21] Cheap and efficient in situ steam (heat) sterilization is often carried out between samplings, however, chemical sterilization with alcohol, acid, peroxide or formaldehyde is also used. The dead volume of these sampling systems should be kept at minimum.

Mattiasson's group at the University of Lund in Sweden reported on the use of such a valve-based sampling system for on-line analysis of the glucose concentration in various yeast fermentations by an enzyme thermistor.[22,23] Romette[24] described an automated steam-sterilizable double valve system which also allowed the conditioning of the sample in terms of the pH-value and the ionic strength, in connection with

several enzyme electrodes for the automatic control of the glucose, lactate and glutamin concentration in mammalian cell cultures. If a valve-based system is used for automatic process analysis, all parts of the system, i.e. pumps, valves, the respective analyzer's fraction collector, are usually computer controlled.[16]

Mass spectrometry (MS) is the predominate analytical method for volatile metabolites.[25-27] Here sampling may simply be done by connecting the head space of the bioreactor with the vacuum-chamber of the mass spectrometer through a pervaporation module, (e.g., a capillary covered with a gas permeable membrane). Similar membrane covered probes can be used for sampling of the liquid phase.[9] As the range of molecular weights accessible to direct analysis by MS increases, the use of liquid sampling devices for MS-coupling should also increase.

EXCLUSIVE SAMPLING MODULES

Unless an analysis of the cell number or metabolic state is intended, the exclusion of the culture organisms and other solids helps to simplify the analysis. It is then not necessary, for example, to add a metabolic inhibitor to the sample stream or to include a precolumn in the High Performance Liquid Chromatographic (HPLC-)system. The tendency for the build up of a protein layer on the inner surface of the tubings of the respective analyzers is often also reduced in that case. The use of exclusive sampling systems, which produce a clear, cell-free, aseptic sample stream is therefore an important option in biotechnological process analysis.[7,9,28] The upper limit in membrane pore size suitable for aseptic sampling in a bioprocess is 0.2 μm. Only such a membrane will prevent the invasion of microorganisms from the outside; this constitutes a "sterile barrier." For the determination of low molecular weight substances, ultrafiltration or dialysis modules are used which exclude even biopolymers (proteins) from the sample. Perhaps the simplest way of interfacing a nonsterilizable biosensor (e.g. an enzyme electrode) with the monoseptic bioreaction mixture is to cover the sensor by such a sterile but analyte-permeable membrane and insert it into a standard sensor port.[29] If this is not possible, sterilizable sampling modules are often used to provide an external analyzer with the required sample stream.

Such modules may be placed inside the bioreactor (in situ/in-line sampling) or run in a bypass (external sampling). Each design has its particular advantages and disadvantages (Table 1.1). Their standing time will be limited due to membrane fouling and pore clogging. When the ensuing analytical data are used to characterize a bioprocess, the time lag and sample dispersion caused by the sampling process should be taken into consideration. In addition, transmembrane pressure and pore geometry are very important.[30]

Table 1.1. Advantages and disadvantages of internal and external sampling modules

Type	Comments
In Situ Modules	Advantages
	short answering times
	simple design
	less danger of culture contamination
	suited for coupling on On-line-Analyzers
	Disadvantages
	problem of long-term stability
	fouling
	adhesion of culture organisms (highly bioactive layer)
	not exchangeable
	positioning crucial
	size limitation
External Modules	Advantages
	exchangeable
	suited for coupling of On-line-Analyzers
	Disadvantages
	complex design (danger of breakdown, contamination)
	longer answering times
	fouling
	high tangential flow unless introduction of secondary flow (shear stress)
	bypass (change in sample composition and/or metabolic activity, e.g. due to oxygen depreviation)

The material of the filter or dialysis membrane should not invite the adhesion of the culture organisms. Membranes must therefore be, for example, smooth, otherwise a highly active biological layer is formed which may bias the sensor output. A case in point is the sucrose analysis in yeast cell fermentations carried out by Mandenius,[31] which failed to determine any sucrose despite the obvious metabolic activity within the fermenter. The metabolic activity of the layer of yeast cells that covered the sampling tubing was found to be the reason for this discrepancy. The number of suitable membrane materials is limited by the requirements of sterilizability and high mechanical, chemical and biological stability. Injury to the membrane may result in a contaminated culture or the escape of genetically modified organisms. Commonly used materials are: polypropylene, cellulose acetate, cellulose nitrate, nylon, acrylnitrile copolymers, polyvinylidene fluoride, polytetrafluoroethylene, polysulfone, silicone and certain asymmetric ceramic membranes. Underivatized cellulose is often less suitable, since a number of culture organisms are able to hydrolyze this material.

IN-LINE FILTRATION MODULES

In-line modules could be envisioned as dead end filter probes inside the bioreactor through which a continuous sample stream is forced by pump. They are characterized by short answering times and a comparatively simple design, since the number of connections, valves, and pumps—all well known troublemakers in terms of maintaining monosepsis—is small. Unless placed in a position of high turbulence within the reaction mixture, their standing time is rather limited due to pore clogging. If placed well, however, they show adequate long-term stability and cause little trouble in terms of membrane fouling.[28] The modules described below, have therefore been used mostly in stirred tank or air lift bioreactors. In addition, in-line modules often cannot be replaced without forsaking the culture. The provision of a sufficiently large filtration area is another aspect to consider.

Among the early successes in using an in-line sampling module for process monitoring is the steam-sterilizable disk-shaped module depicted in Figure 1.2.[28,32] The module may be fitted with micro- or ultrafiltration membranes. It has a dead volume of 1.35 ml and an interfacial area of 1350 mm^2. A response time of 60 minutes is given for the module at a flow rate of 0.5 ml/min.[28] Equipped with a polysulfone ultrafiltration membrane (100 kD) the module was first used in the on-line monitoring of the ammonium, sulfate, phosphate, urea and Penicillin V concentration during a 300 hour fermentation of *Penicillum chrysogenum* by a continuous flow analyzer. Its use in various other antibiotics production processes was later reported.[33-35] The disk-shaped module, however, can only be inserted into the bioreactor from the inside prior to sterilization. This renders it unsuitable for many large scale industrial applications and precludes the exchange without termination of the bioprocess.

A number of rod-shaped in-line filtration modules can be installed using a standard 19 mm sensor port (for examples, see refs. 36-38). The module by Marc et al[37] comes with a tubular alumina membrane whereas the other two may be fitted with various commercially available polymeric or ceramic tubular porous membranes. Due to their geometry, rod-shaped modules combine a large filtration area with a comparatively small internal volume and thus short answering times. The module originally developed by the University of Hannover, Germany[38,39] (Fig. 1.3) and currently distributed by ABC (Advanced Biotechnology Corporation, Puchheim-Munich, Germany) has been ascribed with a filtration area of 34.5 cm^2, a dead volume on the permeate side of 7 ml and an answering time of 9 minutes at a flow rate of 1 ml/min.[39]

Again, the position within the bioreactor and the standing time are decisive for the success of the rod-shaped in-line filtration modules as sampling modules. An application in microbial, fungal or yeast fermentations is fairly straightforward, since such cultures are able to

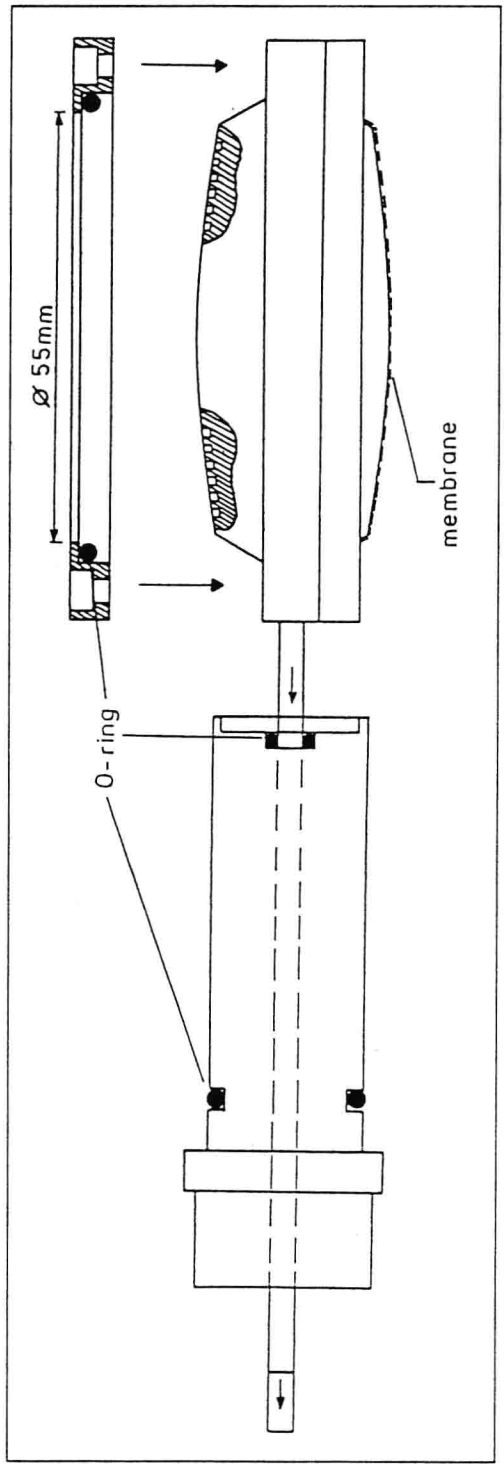

Fig. 1.2. Disk-shaped in line filtration module. Reproduced with permission from Lorenz TH et al, Chem Eng J 1987; 35:B15-B22.

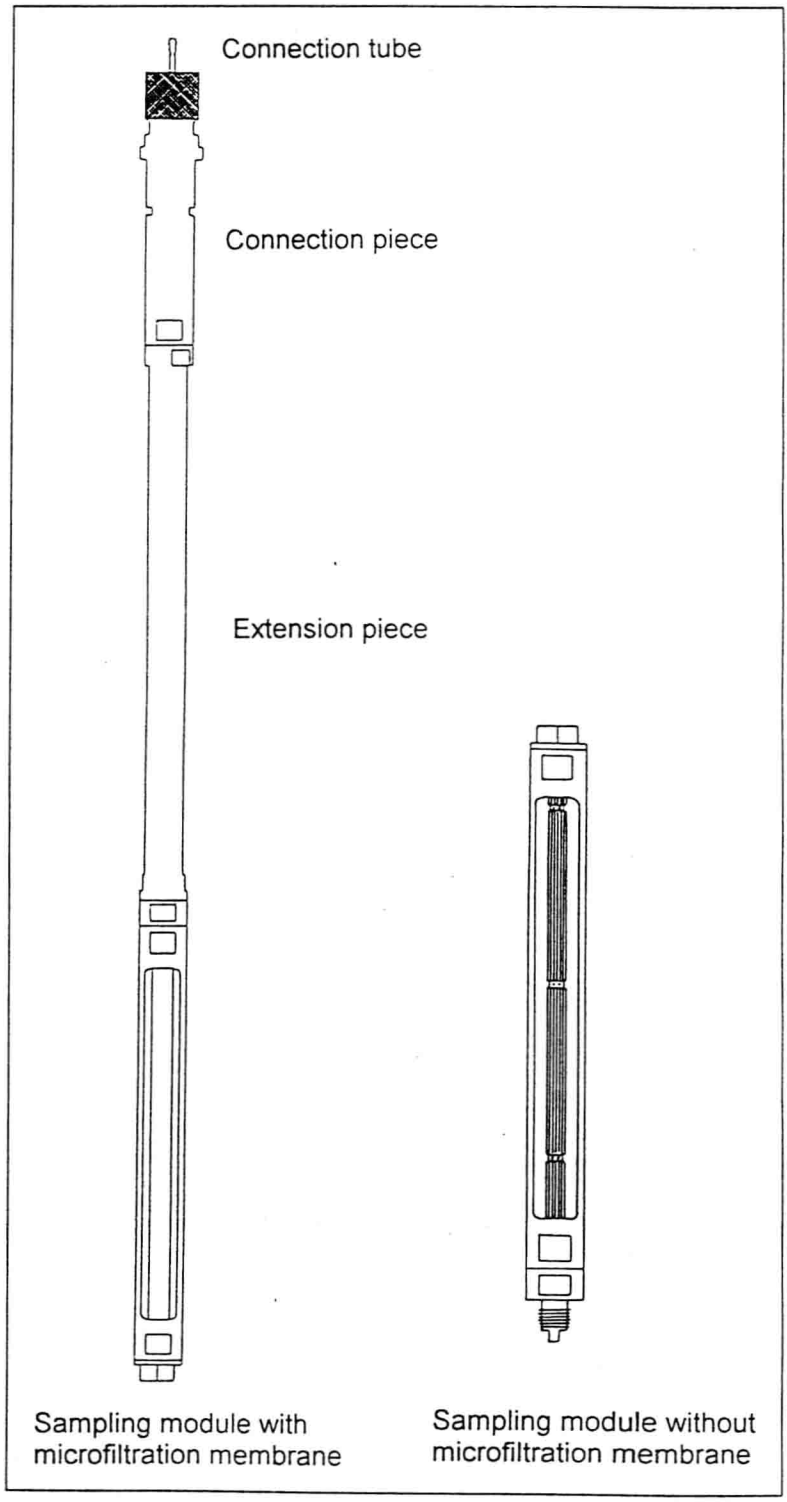

Fig. 1.3. Rod-shaped in line filtration modules (ABC/ University of Hannover Germany). Reproduced with permission from Graf H et al, Biotechnol Techniques 1991; 5:183-186.