Development of sampling and bioselective techniques for on-line clinical biosensors
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 11-03-2019
Chapter 8

Summary and concluding remarks

Some are wise, some otherwise.
Chapter 8

This thesis describes the development of techniques designed for continuous on-line bedside and ambulant biosensor devices. Two aspects for continuous on-line analysis are studied: the creation of contact with the analysis site and the development of bioselective methods. The contact with the analysis site was acquired with an on-line sampling device. The selection was created with an enzymatic detection for two metabolites, glucose and lactate and an immuno displacement method for the detection of the hormone cortisol and the early infarction marker fatty acid-binding protein (FABP).

These two aspects are described in subsequent sections in this thesis. The two sections are introduced by an overview of research in this area. A general introduction is given to contextualize the research in the broader perspective of clinical biosensor development.

Chapter 1 presents a general introduction of biosensors that are currently available for in vivo, ex vivo and in vitro (bio)chemical monitoring in clinical medicine. At present, most biosensors are not able to perform in vivo, but are utilized with discrete (blood) samples. The original goal to implant biosensors in ways similar to the implantation of pacemakers is risky because of stability, reliability and biocompatibility problems. In addition, it is difficult to calibrate an implanted sensor. Realistic on-line monitoring might be achieved by combining biosensors with an on-line microdialysis or ultrafiltration sampling device. Techniques which might be implemented in such a device, are also introduced.

The first section (chapter 2, chapter 3 and chapter 4) of the thesis focuses on on-line sampling techniques and the bioselective detection of two metabolites, glucose and lactate. Chapter 2 surveys the microdialysis (MD) and ultrafiltration (UF) literature. MD is a dynamic sampling method based on analyte diffusion across a semi-permeable membrane due to a concentration gradient. UF is a technique during which endogenous fluid is withdrawn from the sampling site by means of an underpressure. These are the only two on-line sampling techniques available besides undiluted blood sampling. Our group (in Groningen) recently introduced on-line UF. It relies on the constant withdrawal of fluid with a sampling probe, using underpressure as the driving force. Different sources of sampling are described. Special attention is given to subcutaneous and transcutaneous sampling for estimation of blood (glucose) concentrations. In our opinion, the blood compartment and subcutaneous compartment are definitely two clearly distinguished sections of the body, whereas the differences between the blood and the transcutaneous compartment are even more distinct. Even for small molecules like glucose, changes in the blood compartment can be observed in the subcutaneous tissue only with a large delay. The sampling of body fluids is therefore not only a technical challenge, but also requires a detailed knowledge of the pathophysiology.

Chapter 3 describes ultrafiltration (UF) experiments in anaesthesized rats. Contrary to MD, UF does not cause drainage of analytes (depletion of the analyte being removed by the dialysis fluid), and may therefore give better insight into the real “undisturbed tissue” physiology. Probes were implemented into the subcutaneous (s.c.) tissue and into the jugular vein (i.v.). Sampling was performed with an ultraslow, continuous flow of 100 nl min⁻¹, created with a disposable syringe and controlled by a fluid restriction in the
outlet of this syringe. This syringe (a “monovette”) enabled pulsefree pumping of these ultraslow flow rates, using very inexpensive material. In both s.c. and i.v., the samples were analysed for the glucose concentration with a bioselective enzyme reactor, using electrochemical detection. These concentrations were compared with discrete blood samples. Reported for the first time here, the small hollow fiber probes enabled i.v. sampling, and therefore created a new field of UF sampling. Batch-wise sampling in s.c. tissue with large probes has been described before. However, continuous UF sampling experiments for on-line monitoring of analytes in the s.c. compartment has never been described before. The outcomes of the experiments of chapter 3 demonstrate that UF sampling is a successful continuous sampling system for both the s.c. and i.v. compartments. The correlation between the glucose concentrations found with the UF i.v. samples and the discrete samples was very high ($r>0.995; p<0.001$). After a mathematical correction for the difference between whole blood and UF (for the presence or absence of cells), the concentrations of glucose in the discrete blood samples and the UF were nearly the same, indicating that the barrier (if any) of the membrane for glucose molecules is the same as for water molecules. Obviously, because both samples are taken from the same body compartment, there are no physiological differences. With s.c. sampling, a clear difference in the glucose concentrations between subcutaneous fluid and blood was observed. This was not only a delay in changes of the glucose concentrations, but also the shape of the curve of the glucose concentration over time after a glucose load was different. This supports our statement that blood glucose control performed on subcutaneous analysis may create large artefacts. The development of probes for intravenous sampling is a good way to prevent these complicated problems.

A new ultraslow sampling technique is described in chapter 4. Whereas normally MD is performed with flow rates of 1-20 $\mu l\ min^{-1}$, we describe sampling with an ultraslow MD technique, usMD, with flow rates as low as 100-300 $\mu l\ min^{-1}$. By this, artefacts caused by drainage are reduced. We compared this usMD with UF by alternating between UF and usMD and changing flow rates. A dual bi-enzymatic system combined with electrochemical detection for glucose and lactate was applied to study both sampling techniques. The probe was inserted into the s.c. tissue of the rat. Under the conditions of the experiments, there was no difference in the sampled fluid (dialysate or ultrafiltrate). In contrast to state-of-the-art MD, with at least one order of magnitude higher flow rates ($\mu l\ min^{-1}$), the recovery of this usMD was 100 percent. This means that the actual concentrations in the dialysate are the same as in the extracellular s.c. tissue fluid, and therefore, complicated calculations for absolute concentrations can be avoided. For UF, the fluid influx from the tissue to the probe through the membrane was not hindered in the applied flows. This is in contrast to findings in our group concerning UF sampling in s.c. tissue in humans, where obstruction of the fluid to enter the probe was observed. This means that UF is a suitable sampling technique for on-line sampling in s.c. tissue in the rat for flow rates up to (at least) 300 $\mu l\ min^{-1}$.

A careful evaluation of the sampling site, the (expected) recovery, the sensitivity of the analysis method and the conditions in which the measurements are taken all have to be made before choosing between the UF or MD sampling method. In the described
application, there was no preference for one sampling method above the other. However, in general, the UF sampling may be preferable, because the probe configuration is less complex and sterilization can be achieved more easily. Also, when the probe will be blocked during the experiment, this can be checked immediately with UF (especially when, in the future, flow meters are accessible to measure flow rates this low), whereas the recovery of the usMD might decrease without awareness of the experimentalist.

Both MD and UF have the potential to be used in in vivo or ex vivo monitoring systems. Also, they can be used for analysis of time profiles by continuous collection and off-line analysis afterwards. Biosensors might function better in combination with UF/usMD sampling devices than in direct contact with the body, because of the relatively "clean" sample. Due to the approximate 100% recovery, calibration is easier than in conventional, fast-flow MD sampling.

The second section (chapter 5, chapter 6 and chapter 7) investigates the immuno-displacement method as a bioselective step for continuous sampling to monitor the hormone cortisol and the early heart infarction marker FABP. Displacement happens when the complementary binding between an antigen binding site (ABS) of the antibody (Ab) and the corresponding antigen (Ag) or antigen analogue is actively broken after free antigen, the analyte, is added. Displacement immunoassays have been used for the analysis of discrete samples. Chapter 5 discusses the literature on displacement. On the one hand, compared to conventional competition and sandwich immunoassays, displacement assays have a lower sensitivity. On the other hand, displacement is the only way to analyze samples continuously.

Chapter 6 describes the experiments on displacement for cortisol in a continuous flow system. Cortisol-recognising antibodies were immobilized on sepharose protein-A columns; cortisol labelled with horseradish peroxidase is associated with this. When the columns are subsequently perfused with cortisol, displacement can be obtained. To detect concentrations of cortisol above 100 nM, high-affinity antibodies were found to be best suited, because the spontaneous dissociation was low, and the displacement was dependent on the cortisol concentration. When low-affinity antibodies were used, the spontaneous dissociation was too high, and the displacement was dependent only on the time of perfusion and not on the cortisol amount. For lower concentrations of cortisol (10-100 nM), the displacement signal of the high-affinity antibody did not exceed the background dissociation signal. Here, the low-affinity antibodies were a better option, because they released a concentration-dependent signal.

Chapter 7 describes the displacement for the protein cytoplasmic heart fatty acid-binding protein (FABP). The approach of these displacement experiments was different from the experiments with cortisol: the displacement assay applied to a high-molecular protein instead of a low-molecular hormone, and instead of displacement of a labelled antigen (analogue), the antibody was labelled and released in solution at displacement. Displacement of labelled antibodies has been observed in non-flow systems only after hours or even days of incubation. All the literature on displacement in flow
systems so far has described displacement for small analytes up to 500 Da. No experiments to date have reported a displacement signal for proteins. In our experiments, we have shown that for physiological (2-6 µg l\(^{-1}\)) and pathophysiological (6-2000 µg l\(^{-1}\)) concentrations of FABP, the displacement is detectable from the background signal of spontaneous dissociation. We immobilized the FABP on Sepharose beads in a column following two different methods: by direct chemical linkage to the carrier and by binding it with another antibody (“sandwich” format). Of these methods, the chemical linkage gave by far the best results, both for sensitivity as well as selectivity. It showed a displacement signal up to 2 µg l\(^{-1}\), whereas 20 µg ml\(^{-1}\) lysozyme (used as an unspecific control) did not show any increase of displacement signal.

A very important advantage of the application of labelled antibodies instead of labelled antigens is that labelling of antibodies can be done following a standard method, leaving the binding sites unaffected. Labelling of the antigen itself has to be adapted to every single analyte, and may harm the binding site.

The on-line sampling and bioselecting system developed for FABP may be the first step for a future bedside alarm system that detects a reinfarction as well as an infarction after a major heart operation. It might be useful for more analytes as well.

**Future outlook: on-line clinical biosensors.**

Future biosensors combining the ultraslow microdialysis or ultrafiltration with enzymatic metabolite conversion or displacement immunoselection will enable on-line biomedical monitoring systems for major metabolites and risk factors (e.g. a sensor as schematically represented in fig. 8.1). Harmful fluids to the tissue do not contact the body fluid, and potential toxic elements can be kept outside the body. Moreover, the proposed underpressure pump is not only pulse-free, but also very cheap (below 1 US$).
To establish the proposed devices, some technical challenges have to be overcome. The major next task that lies ahead, consists in developing a suitable on-line detection system. The creation of a detection system is an underestimated problem. Detection in physiological samples such as blood is extremely complex. The problem is merely not the detection limit, but the interference from other compounds in the matrix with the signal. We have not been able to solve the problem concerning the interference-free measurement of the displacement signal within the framework of this thesis.

After establishing the detection system, the different parts for sampling, bioselection and detection have to be combined. Flow rates, sensitivities and fluid handling all have to be carefully considered. As for the choice between an usMD or UF device, the effects of removing the fluid from the tissue have to be studied more in detail to make a good decision.

Clinically relevant applications of biosensors can be found across the entire field of medicine. On-line monitoring in combination with a feedback mechanism would be an especially important new application for biosensors. At present, the research on this specific type of biosensor aims at controlling blood glucose for diabetics. However, other applications can be found in the monitoring and controlling of drugs. In particular, biosensors will be useful in the control of drugs with small therapeutic intervals, such as digoxine and cytostatics. The proposed UF/MD displacement immunosensor system may create devices for this type of application. Other application fields can be found in on-line monitoring of cardial protein markers, not only for detection of (recurrent) infarctions, but also for visualising reperfusion of the blockage and estimation of the infarct size.

The ideal biosensor should be small, fast, safe, easy to handle and cheap. Furthermore, the reproducibility of the biosensor should be high and the shelf life long. It should create a maximum of information with minimal human intervention. The possibilities of biosensors seem to be unlimited. Research all over the world focuses on such devices. After the release of the first successful biosensors, future release will cascade because of the similarities of many designs. In an enzyme sensor, for example, an enzyme can be replaced by a similarly acting enzyme (e.g., glucose oxidase by lactate oxidase or glutamate oxidase) and the sensor design can be maintained. Analogously, replacing an antibody recognising one analyte by an antibody selective for another analyte will increase the diversity of immunosensors and reduce the development time.

Essential for this future work is good interaction between researchers in (bio)electronics, chemists and biologists on one hand and medical doctors, the future end-users, on the other hand. When this can be achieved, on-line clinical biosensors will have a major impact on the future of medical care.