Development of sampling and bioselective techniques for on-line clinical biosensors
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Chapter 7

On-line flow displacement immunoassay for fatty acid-binding protein

It would be possible to describe everything scientifically, but it would make no sense; it would be without meaning, as if you described a Beethoven symphony as a variation of wave pressure (Albert Einstein).

1 Kaptein, W.A., Korf, J., Cheng, W., Yang, M., Glatz, J.F.C. and Renneberg, R.
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Chapter 7

In standard displacement flow immunoassays analyte in the sample creates an active dissociation of labelled antigens (or antigen homologues) from an antigen binding site of an immobilized antibody, whereafter the labelled substance is measured downstream. Such systems have been described for molecules up to 1 kD. In this study, we demonstrate displacement in a flow system for the detection of a small protein, cytoplasmic heart-type fatty acid-binding protein (15 kD), a plasma marker for myocardial injury. The displacement system applies an inverse set-up: enzyme labelled monoclonal antibodies are associated to immobilized antigen, displaced by analyte in the sample. It allows detection of both physiological (2-12 µg l⁻¹) and pathological concentrations (12-2000 µg l⁻¹) of fatty acid-binding protein in an on-line flow system.

Introduction

Since the technique for preparation of monoclonal antibodies has been developed by Köhler and Milstein (1975), antigen-antibody interactions are widely used for detection of antigens in complex matrices, such as body fluids (Gosling, 1990). Following solid phase detection procedures, such as ELISA and RIA (Anderson et al., 1997), more complex systems like flow injection immunosystems (Gubitz and Shellum, 1993) and immunosensors (Aizawa, 1994; Morgan et al., 1996; Schreiber et al., 1997) have been developed. One of the approaches used in immunosensors is displacement, as described by our group (Kaptein et al., 1997b) and by others (Kusterbeck et al., 1990; Rabbany et al., 1994; Kronkvist et al., 1997; Narang et al., 1997). Displacement is a principle in which either antibodies or antigens are immobilized, whereas the respective interacting antigen (anologue) or antibody is labelled. After the antibody binding sites are saturated with antigens, the actual displacement consists of perfusing the sample along these antibody-antigen complexes, releasing labelled molecules from the immobilized site.

Flow displacement immunoassays can be divided into two different categories, one with immobilized antibodies and the other with immobilized antigens. In the most frequently used set-up, labelled antigens (or antigen analogues), associated to immobilized antibodies, are placed in a flow system. Some of the antigens in the sample, running through this flow system, will displace labelled analogues from the antibodies, thereby raising the concentration of the label downstream. This label is then analysed, directly or indirectly, by a colourimetrical (Warden et al., 1987), fluorometrical (Yu et al., 1996) or electrochemical (McNeil et al., 1997) detection. Such a flow displacement of antigen analogues has been described for small analytes like cortisol (Aizawa, 1994; Kronkvist et al., 1997), cocaine (Ogert et al., 1992) and trinitrotoluene (Whelán et al., 1993).

The second displacement set-up is performed by immobilizing an analyte, and displacing a labelled antibody, which was previously bound to the immobilized analyte. Freytag (1984) published a patent describing such a displacement system. Warden et al. (1987; 1990) described a displacement, denoted “repetitive hit-and-run immunoassay”, for T-2 toxin. In this assay, a 5-minute incubation was applied, and after analysis the column was regenerated.
So far, the application of both displacement set-ups has been demonstrated only for molecules with a molecular weight of less than 1000 Da. To explore the usefulness of the displacement technique for small proteins in an on-line flow system, we studied the displacement system for cytoplasmic heart-type fatty acid-binding protein (FABP, 15 kD) (Offner et al., 1988; Veerkamp et al., 1991; Glatz and van der Vusse, 1996). FABP is a novel plasma marker protein for acute myocardial infarction (AMI), which has a high sensitivity and specificity for early detection of AMI (Yoshimoto et al., 1995; Glatz et al., 1997). With an immobilized FABP and displacement of a labelled antibody, we were able to apply the displacement technique of labelled antibodies for the detection of physiological and pathological concentrations of FABP (2-2000 µg l\(^{-1}\)).

**Materials and Methods**

**Materials**

Protein-A Sepharose and N-hydroxysuccinimide (NHS) activated Sepharose 4 fast flow were obtained from Pharmacia, Biotech, Uppsala, Sweden. Anti-human heart-type FABP monoclonal antibodies 53E9, 67D3 and 66E2 were obtained as earlier described (Roos et al., 1995). Antibodies for both immobilization methods and for the catcher as well as the detection antibody were selected on their performance in ELISA’s and BIAcore experiments. The monoclonals 66E2 and 53E9 were labelled with horseradish peroxidase (with Sigma P8375, Sigma Chemical Co., St. Louis, MO). Recombinant human FABP (Schreiber et al., 1998) and recombinant rat FABP (Schaap et al., 1996) were obtained from T Börchers, Münster, Germany and F. Schaap, Maastricht, The Netherlands, respectively. Horse myoglobin, lysozyme and o-phenylene diamine dihydrochloride (OPD) for the detection of the horseradish peroxidase label were obtained from Sigma, St. Louis, MO.

**Buffers and substrates**

The perfusion buffer is a phosphate based buffer of pH 7.4, consisting of 5 mM glucose, 0.35 M NaCl, 2.5 mM KCl, 8 mM Na\(_2\)HPO\(_4\), 2.5 mM KH\(_2\)PO\(_4\) and 0.03 vol.% Tween-20. The substrate for the quantification of the HRP in the eluent contained 4 mg OPD dissolved in 10 ml distilled quarts water with 30 µl hydrogen peroxide. The enzyme reaction was stopped with 1.5 M H\(_2\)SO\(_4\).

**Equipment**

For the displacement, a multichannel peristaltic pump (Ismatec, IPC, Switzerland) was used with clear standard pump tubes (Skalar, Breda, The Netherlands). The columns were made in 25 µl Transferpettor-caps (Brand, Germany). Sampled fractions were measured
spectrophotometrically at 492 nm using a Dias ELISA reader and the BioLinx software (Dynatech Laboratories).

**Preparation of the displacement column**

**Immobilization of FABP by chemical linking**

For the chemical immobilization, the transferpettor-cap column was filled with NHS activated Sepharose. After washing it with at least 3 column volumes of propanol, the column was washed with 1 mM HCl. Coupling of rat or human FABP was performed by addition of an FABP-solution (0.3 mg ml\(^{-1}\) FABP in 0.2 M NaCO\(_3\), 0.5 M NaCl pH 8.3) to the column and incubation for 1 hour. Thereafter, the column was washed at least three times each with a sequence of 0.5 M ethanolamine, 0.5 M NaCl (pH 8.3) and 0.1 M acetate, 0.5 M NaCl (pH 5) for one hour. Then the column was equilibrated with buffer and 2 µg of the detection antibody was loaded. The column was subsequently washed for 16 hours to remove unbound and unspecifically bound label.

**Immobilization of FABP with antibodies**

For the immobilization of FABP with antibodies, 50 µl (0.2 mg ml\(^{-1}\) in buffer) catcher antibody was immobilized on the packed protein-A column material by perfusion. After this, the column was washed with at least two column volumes of buffer, followed by a perfusion of 30 µl (0.1 mg ml\(^{-1}\)) rat or human FABP in buffer, followed by another buffer wash of at least two column volumes. The detection antibody was subsequently bound to the FABP as described above.

**Description of the displacement experiments**

<table>
<thead>
<tr>
<th>Filling of the column with modified Sepharose</th>
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<td>↓  washing</td>
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<td>Loading of the column</td>
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<td>↓  washing for 16 hours</td>
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<td>Perfusing of the column with buffer, with or without FABP</td>
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<td>Collection of the fractions</td>
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<tr>
<td>Analysis of the 10 minutes fraction</td>
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After the preparation of the column, the actual experiment started. During the displacement experiments, the columns were perfused continuously. Eluates were collected
as 10-minute fractions. The column was continuously perfused with buffer or with buffer containing various concentrations of FABP. As a test for specificity, myoglobin (MW 18 kD), and lysozyme (MW 14.3 kD) were perfused. Displacement experiments were performed up to three days with the same columns. The flow rate was 4 µl min⁻¹.

**Analysis of the displacement column eluates**

From the displacement column described above, 5-20 µl aliquots of the 10-min fractions were taken and analysed by adding 100 µl substrate, and compared with a standard curve of the detection antibody. The colour reaction was stopped when OD₄₉₀,max = 0.5.

**Presentation and interpretation of the results**

For the interpretation of the semi-quantitative data, we compared columns with different characteristics. The concentrations of the labelled (detection) antibodies present in the 10-minute fractions are plotted against the time. Perfusions of different compounds are indicated in the graphs. The scale on the y-axis is arbitrary.

![Fig. 7.1. Two approaches for displacement of labelled antibodies. A. Displacement from chemically immobilized FABP; B. Displacement from antibody-based immobilized FABP.](image)

We applied a set-up displacing a labelled antibody from immobilized FABP. Two immobilization methods for the antigen FABP -- a direct chemical coupling and the use of a catcher antibody -- were evaluated. The actual displacement experiment (schematically represented in Fig. 7.1) is performed when these immobilized antibody-antigen complexes are placed in a flow system and exposed to FABP, which will partially displace the
antibodies. Evaluated are the two different immobilization procedures and antigens (human FABP and rat FABP), different antibodies as well as the sensitivity and specificity of the different systems.

**Results**

The sensitivity for FABP in displacement experiments for different catcher antibodies varied (Figs. 7.2A&B). The displacement after perfusion with 2000 ng ml$^{-1}$ FABP was not above the spontaneous dissociation for the 67D3 — FABP — 66E2-HRP complex, whereas the released amount of 66E2-HRP for the 53E9 — FABP — 66E2-HRP complex was clearly higher.

**Influence of different detection antibodies**

Displacement experiments with 53E9-HRP as detection antibody resulted in a constant decrease of the dissociation signal. Also, the displacement was more time-dependent than FABP-dependent for both the antibody-based and the chemical immobilization (data not shown). The dissociation was also large when compared to the displacement signal.

**Influence of method of FABP immobilization**

Two methods of immobilization – one antibody-based and the other chemical – were studied. For high concentrations of FABP, the antibody-based immobilization gave a concentration-related signal (Fig. 7.2A). However, the sandwich-system was not sensitive enough to detect physiological concentrations of FABP (2-5 ng ml$^{-1}$ (Wodzig et al., 1997)). The displacement signal for these FABP concentrations was not identifiable from the spontaneous dissociation signal. The chemically immobilized FABP displacement, where human FABP is coupled to Sepharose with an irreversible chemical reaction, enabled detection of sub-pathological (physiological) FABP concentrations (here 2-20 ng ml$^{-1}$; Fig. 7.2D).

To explore the specificity of the system, two proteins were used. Myoglobin (15 kD), a protein released at the same time after infarction (van Nieuwenhoven et al., 1995; Ishii et al., 1997) and roughly the same size, seemed to be the ideal test. However, using myoglobin (20 µg ml$^{-1}$) in a displacement experiment, we observed an increase of signal (Fig. 7.2C). When the perfused FABP concentrations were decreased to 2-20 ng ml$^{-1}$, the relative signal for myoglobin (20 µg ml$^{-1}$) was large (data not shown). This is most likely to be related to contamination of the (horse) myoglobin with (horse) FABP, because when myoglobin was tested with an ELISA for FABP (Wodzig et al., 1997), binding signals were observed. Similarly, human myoglobin (Fitzgerald Industries Int. Inc., Concord MA, USA), gave an even higher binding signal (around 0.1 percent), because of more contamination with FABP and/or higher binding affinities (data not shown). Lysozyme perfusion showed
hardly any increase of enzyme activity for the chemically immobilized FABP (Fig. 7.2D). The immobilization of FABP with antibodies showed more unspecific displacement, and chemical immobilization was therefore used for subsequent experiments (data not shown).

Fig. 7.2. Displacement experiments in continuous flow system after various FABP perfusions with different immobilization procedures. A. antibody-based immobilization; catcher antibody 67D3; B. antibody-based immobilization; catcher antibody 53E9; C, D. chemically immobilized FABP; E, F. chemically immobilized rat FABP. The drawn line indicates the amount of label detected, whereas the start of the FABP perfused are indicated with arrows, the length reflecting the concentration (in ng ml\(^{-1}\)) Myoglobin (in a concentration 20 µg ml\(^{-1}\)), and lysozyme (20 µg ml\(^{-1}\)) are indicated with the dotted arrows. For profile C and F the FABP perfusions are performed for 60 minutes, for the other 20 minutes. The 66E2-HRP units in the graphs are not equivalent.
Influence of species (rat or human) of immobilized FABP

We also studied immobilized rat FABP in an attempt to lower the affinity of some antibody-binding site - antigen complexes. It is known that the homology of rat FABP and human FABP is large (Londraville and Sidell, 1995). Cross-reaction of rat FABP with the human FABP monoclonal antibodies is therefore expected. Immobilization with catcher antibodies and rat FABP resulted in a high, fastly decreasing dissociation signal, of which no significant increase at FABP perfusions occurred (data not shown). The results for chemically immobilized rat FABP with 53E9-HRP were comparable (data not shown). For 66E2-HRP, the spontaneous dissociation was less. FABP concentrations of 200-2000 ng ml\(^{-1}\) showed concentration dependent displacement signals (Fig. 7.2E). However, for 2-20 ng ml\(^{-1}\) FABP, the spontaneous dissociation is more interfering than for immobilized human FABP (Fig. 7.2D compared to Fig. 7.2F). Interpretation of the signal is hampered because of simultaneous influence of dissociation, displacement and time effects on the amount of label released. Therefore, chemical immobilization of human FABP is preferred and used in subsequent experiments.

Influence of measurements on subsequent analysis

As described, the columns were used for up to 3 days. This enabled perfusions of different sequences through the column. For columns with chemically immobilized human FABP, the signals of subsequent days became lower than previous days (data not shown). However, because the dissociation signal decreased as much as the displacement signal, this decrease did not influence the relative amounts. For unstable, fast dissociation, such as when 53E9-HRP was used, the 53E9-HRP was washed out after one analysis day, leading to no release of label during perfusion after that (data not shown).

Fig. 7.3. Cumulative fractions per FABP perfusion with following buffer perfusate fractions. A. Low concentrations of FABP in perfusate; B. Medium concentrations of FABP in perfusate; C. High concentrations of FABP in perfusate. For Fig. A, the top values of Fig. 7.2H are used. For Fig. B&C, the surface under the curve of a response curve of 20-minute FABP perfusions is summed up. The 66E2-HRP units in the graphs are not equivalent.
Quantification of the signal

To explore the quantitative characteristics of the displacement for the chemically immobilized FABP, we compared the amount of label released at different FABP concentration perfusions. When the displacement signals per FABP perfusion are added, the displacement increases non-linear for the intervals 0-20, 0-200 and 0-2000 ng ml\(^{-1}\) FABP. The total amount of released detection antibodies increases when the FABP concentration increases, whereas the amount of detection antibodies per FABP molecule decreases on the complete FABP concentration tested (Figs. 7.3A-C).

Evaluation of the results

As illustrated above, displacement of antibodies can be used to detect an antigen in a flow system. The method of immobilization and the choice of antibody influence the sensitivity and selectivity. Because all monoclonal antibodies had approximately the same equilibrium constant, as well as the same association rate (Roos et al., 1995), the different sensitivity and selectivity can not simply be explained by differences in binding characteristics.

For the antibody-based immobilization, more blocking of the antibody-binding site of catcher antibody 53E9 in combination with 66E2-HRP compared to the 67D3 ― FABP ― 66E2-HRP might explain the difference, because FABP can reach the site more easily. Experiments with the detection antibody 53E9-HRP were also less successful than with 66E2-HRP in both antibody-based and chemically immobilized FABP. It is unclear whether this is a result of the labelling procedure or of the different epitope recognition.

The reason for a higher sensitivity for chemically immobilized FABP compared to antibody-based immobilization might be that less steric hinderance at the antibody binding site leads to an increase of the displacement. The explanation of a lower affinity of the antibody for the chemically immobilized FABP is less likely, because that would also increase the spontaneous dissociation signal and decrease the specificity.

The displacement signal was non-linearly related to the amount of FABP perfused. At higher FABP concentrations, the release of labelled antibody per molecule FABP was lower. This phenomenon has been described before for trinitrophenol in a displacement system where a labelled antigen homologue was displaced (Whelan et al., 1993a; Narang et al., 1997a). Displacement became linear at higher flow rates. This seems to indicate that at higher flow rates the displacement is diffusion-rate limited, whereas at lower flow rates, as used for the FABP detection, other effects, such as association-dissociation kinetics have an influence on the signal. It is unknown, for example, whether displacement is due to active pushing or preventing re-association with the immobilized antigen. However, the semi-quantitative data are not precise enough to set up an accurate model for the displacement phenomenon.
Discussion

The aim of this study is to investigate whether displacement can be used for the detection of FABP in a continuous sample and, if so, what influence some parameters have. A displacement selection system for fatty acid-binding protein is investigated, where labelled antibodies, associated to immobilized FABP, are displaced by FABP in the sample. These studies were successful, for both chemical and antibody-based immobilization of the FABP. Because the displacement detection is based on immunological recognition, this principle might be useful for more analytes, as previously shown, for the displacement of small, labelled analytes by analyte in the sample. Further research has to be performed to support this hypothesis.

The chemical immobilization of FABP is preferred because of the higher sensitivity and specificity. Different antibodies and antibody-combinations are studied, and they yield different results despite their similar binding characteristics. Semi-quantitative analysis of the displacement signal for the preferred set-up (chemically immobilized human FABP with 66E2-HRP associated) showed a non-linear FABP concentration-dependent displacement over the whole interval measured (0-2000 ng ml\(^{-1}\) FABP).

The data of this research demonstrate a non-linear displacement signal, which created a high sensitivity (concentrations of 2 ng ml\(^{-1}\) FABP give a signal above base level) and a large detection range (0-2000 ng ml\(^{-1}\) gave changes in signal). However, the quantification is rather complex. Whelan et al., showed that raising the flow creates a linear displacement efficiency (Whelan et al., 1993). However, increasing the flow rates decreases the sensitivity and lifespan of the system. A higher flow might be preferable for applications, where measurements are performed for a short time and the amounts of FABP are important, for example in the estimation of the size of an infarction after an AMI. For an alarm system, that detects an infarct after a major heart operation, a relatively stable system, which can be used for more than 24 hours, might be preferred. In respect to our findings, an exact configuration can not yet be described. The displacement column and the detection have to be made more homogeneous from one system to another. For example, using immobilization on fused silica in stead of Sepharose column material for trinitrotoluene, as described by Narang et al., (1997a), might lead to higher sensitivity and reproducibility. In general it can be concluded that, depending on the requirements of the assay, the set-up has to be chosen with consideration of linearity or sensitivity and lifespan extension.

The results described here demonstrate the displacement principle for FABP. However, additional experiments have to be performed to create reproducibility and quantitative measurements before clinical applications can be considered as an option. Additionally, a suitable on-line detection method has be to found, because the 10-minute fraction collection method is labour intensive, imprecise and impeding direct information about the concentration of the analyte. The development of a good detection method, especially in complex matrices such as body fluids, is a major issue in research for further quantification.
On-line flow displacement immunoassay for FABP

and following clinical evaluation. One of the methods presently under study is electrochemical detection, which is quite simple to use, relatively inexpensive and needs only small apparatus. Electrochemical detection enables the incorporation of the displacement technique with a small-size disposable immunosensor. This immunosensor can be applied for early detection of myocardial damage and reperfusion. For this, a low, constant flow of the system is required, which can be established with slow flow on-line sampling techniques such as microdialysis (Stjernstrom et al., 1993; Paez and Hernandez, 1997) or ultrafiltration (Moscone et al., 1996; Kaptein et al., 1997). For a combination with this type of sampling, the continuous signal of the flow displacement immunoassay is used to its full potential. To the best of our knowledge, no other assays based on immunological detection have been described for the creation of an on-line signal.

References

Chapter 7

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