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Implementation of affinity solid-phases in continuous-flow biochemical detection

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Abstract

A continuous-flow biochemical detection system is presented which allows the use of solid-phase immobilised affinity proteins. The biochemical detection is performed by mixing analyte with a labelled ligand followed by the addition of solid-phase immobilised affinity protein. After a reaction time of 85 s, free and bound label are separated by means of a hollow fibre module. Quantitation of the free label is performed with a conventional flow-through fluorescence detector. Total assay time amounts to less than 2 min. Biotin was chosen as the model compound using a range of streptavidin-coated solid-phases and an antibody-coated solid-phase as affinity material, and fluorescein–biotin as low-molecular-mass label. The relative standard deviation for twenty repetitive injections was 10.9%. A calibration curve was constructed in the concentration range between 20 and 400 nmol l^{-1} leading to a correlation coefficient of 0.994. A limit of detection of 8 nmol l^{-1} was obtained. © 1997 Elsevier Science B.V.

Keywords: Detection, LC; Affinity-based detection; Fluorescein; Biotin; Streptavidin

1. Introduction

Affinity recognition is a successful tool in detection techniques for various fields of application. Compounds of interest (ligands) are recognised through the reaction of affinity proteins, such as antibodies and receptors. The affinity reactions are monitored by labelling the affinity protein, or by using a labelled ligand which competes for the same binding site as the analyte. Assays based on this principle, often referred to as bioassays, are routinely applied in clinical assays allowing sensitive and selective detection in complex biological matrices [1]. Bioassays also offer the possibility to detect unknown compounds which interact with the affinity protein. This feature explains the use of bioassays in screening methods for drug discovery or for recognition of hormone abuse in doping [2]. Process monitoring requires fast analytical systems, which may be obtained by employing affinity-based detection, thus circumventing the need for complicated sample handling [3–6]. For the same reasons, affinity-based detection has also been used in bioanalysis [7,8].

Even though low sensitivities are achieved with bioassays, cross-reactive compounds can often not be distinguished from the analyte leading to inaccurate analytical data. In order to obtain accurate data, the analyte needs to be isolated from the interfering compounds. This is typically performed by collecting

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chromatographically separated fractions prior to the assay [2,9].

Bioassays are often performed as batch assays on microtitre plates. Despite the widespread use of batch assays, they exhibit some disadvantages. When performed manually, batch assays are very tedious. Automation, on the other hand, is only possible with sophisticated robotic systems. Furthermore, batch assays require long incubation times (>2 h) leading to long response times. Applications calling for fast response times only employ detection systems with short reaction times. In contrast to microtitre plate assays, flow assays offer this possibility with reaction times in the range of a couple of min. As an additional advantage, flow assays allow automation with conventional LC hardware [10].

Flow bioassays are, most of the time, carried out in sequential operations, analogous to reagent addition, washing and incubation steps performed in batch assays. Sequential addition immunoassays (SAIAs) are an example for this assay format [4,11–14]. Key element in SAIAs is the separation of free and bound label which is performed with an affinity column. Short response times are achieved through a high surface loading and a favourable flow pattern through the affinity column material. However, the affinity column needs to be regenerated after every analysis, eliminating the possibility to couple SAIAs on-line to processes in which continuous measurement is needed, e.g., liquid chromatography.

For on-line coupling of bioassays to liquid chromatography, real continuous-flow assays have been developed, which do not require washing, incubation and regeneration steps [15–17]. Some allow quantitation of the mixture of free and bound label. This is only possible when free and bound label differ in detection characteristics (homogeneous systems). A more general approach is the heterogeneous set-up, quantifying either the free or the bound label fraction. Continuous-flow heterogeneous systems require a continuous separation of free and bound label, which has been achieved with a restricted-access column [18], and with a hollow fibre module [19].

When employing a hollow fibre module for the separation of free and bound label, the system is operated in a way that free label passes the membrane, whereas high-molecular-mass compounds, including affinity-protein bound label, are retained. In this way, two streams are obtained, one containing pure free label (permeate), whereas the retentate contains the affinity-bound label. In principle, both streams are a measure for the original analyte concentration.

Preferably, the separation of free and bound label should be fast, efficient and stable for a prolonged time period. The previously described hollow fibre module caused a residence time of 6 s [19]. Here, a smaller hollow fibre module is used with a decreased residence time of only 0.5 s, which limits extra-column bandbroadening significantly. Robustness of the separation was investigated previously as stability of the flux and proved to be adequate [19].

So far, only soluble affinity proteins have been used in heterogeneous continuous-flow biochemical detection for analyte recognition [8,20]. The aim of this paper is to increase the range of affinity entities which can be used. Especially, the implementation of solid-phase immobilised affinity proteins (in the following referred to as affinity solid-phases) would open up the possibility to use the knowledge on batch solid-phase assays developed over the years [21]. In flow assays, affinity solid-phases have mainly been used as columns in the assay format of the above mentioned SAIAs [4,11–14]. An affinity solid-phase has also been used in a fluidized-bed reactor for the purification of soluble protein ligands [22]. The unique feature of fluidized-bed reactors is the compatibility with particulate matter. However, identical to affinity columns, regeneration in-between runs is still required. In order to circumvent problems caused by regeneration of affinity solid-phases, Ruzicka et al. described the use of stationary phases in flow-injection analysis as a consumable [23–25]. Fresh stationary phase is trapped for each experiment in a reactor and exchanged between runs. Thus, a fresh surface is offered for every new analysis. On the other hand, however, an adverse effect of introducing solid-phases into biochemical detection may be adsorption of the label as a consequence of the large increase in surface.

This paper describes biochemical detection based on affinity solid-phases using a hollow fibre module for the separation of free and bound label. The intent here is to develop a continuous-flow set-up which allows on-line coupling to LC. The hollow fibre module yields a particle-free permeate stream which

allows quantitation of free label with conventional flow-through detectors.

2. Materials and methods

2.1. Chemicals

D-(+)-Biotin was purchased from Merck (Darmstadt, Germany) and 5-(biotinamidocaproylamido)pentylthioureidylfluorescein (fluorescein-biotin) from Sigma (St. Louis, MO, USA). Streptavidin immobilised on macroporous metacrylate polymer beads was obtained from Boehringer Mannheim (Mannheim, Germany), streptavidin on 4% beaded agarose from Sigma, streptavidin-coated microspheres from Bangs Labs. (Carmel, IN, USA), immunopure goat anti-biotin from Pierce (Rockford, IL, USA) and CNBr-activated Sepharose from Pharmacia (Uppsala, Sweden). Tween 20, sodium chloride, sodium phosphate, potassium chloride and potassium phosphate were purchased from Merck. All chemicals used were of analytical grade. HPLC-grade water was produced in a Milli-Q system (Millipore, Bedford, MA, USA) and used throughout this work.

2.2. Instrumentation

The flow injection (FI) system consisted of a Gilson (Villiers-le-Bel, France) XL autosampler equipped with a Rheodyne (Cotati, CA, USA) six-port injection valve (injection loop 20 μl), a Pharmacia-LKB (Uppsala, Sweden) 2248 LC-pump which delivered the carrier and a Gilson Minipuls which delivered the bead suspension and the fluorescein-biotin solution at a flow-rate of 0.2 ml min^{-1} each, except where specified otherwise. A Jasco (Tokyo, Japan) fluorescence detector FP 920 (λ_{ex}= 486 nm, λ_{em}=516 nm) was used for detection. Data acquisition occurred with Gilson 715 software. The analytical system was controlled by Gilson 719 Turbo Pascal software.

Phosphate-buffered saline containing 0.5% Tween 20 (PBST) was used throughout as FI carrier. The bead suspension (166 nmol l^{-1}, except where specified otherwise) and fluorescein-biotin solution (1.66 nmol l^{-1}, except where specified otherwise) were prepared in PBST and added to the FI carrier via inverted Y-type mixing unions (Upchurch, Oak Harbor, WA, USA). The solid-phase was kept in suspension by continuous magnetic stirring. A knitted 0.5-mm I.D. PTFE reaction coil with a volume of 858 μl was used. The system was operated at room temperature.

2.3. Hollow fibre module

Separation of free and antibody-bound antigen was performed with a custom-made hollow-fibre module (HFM) consisting of one capillary cross-flow membrane (X-Flow, Almelo, Netherlands, type UFM, M5) of 2-cm length, 0.5-mm I.D. and M_r 50 000 cut-off. The HFM previously described [19] was adapted by reducing the length and channel width. Table 1 summarises the HFM's characteristics.

2.4. Ligand binding experiments

In order to investigate the influence of the ligand concentration on the percentage binding, solutions of fluorescein-biotin were injected into the FI-system described above, but substituting the continuous addition of label with PBST, i.e. two streams of PBST and the streptavidin bead suspension (166 nmol l^{-1} of binding sites) were delivered at a flow-rate of 0.2 ml min^{-1} each. The time for the affinity reaction was 85 s. Fluorescence detection occurred at λ_{ex}=486 nm and λ_{em}=516 nm. The percentage of binding was then determined by dividing the results thus obtained with signals for fluorescein-biotin obtained in the same FI-system when only pumping PBST.

For equilibrium conditions, the same solutions of fluorescein-biotin were used, diluted 3 times and incubated for 3 h with and without 55 nmol l^{-1} streptavidin beads. After centrifugation, free label

Table 1
Hollow fibre module characteristics

Internal fibre diameter	0.5 mm
Fibre wall thickness	0.25 mm
Nominal M_r cut-off	50 000
Fibre length	23 mm
Membrane surface area	36 mm^2
Retentate volume	4.5 μl

was quantified in a FI system consisting of a Gilson XL autosampler equipped with a Rheodyne six-port injection valve (injection loop 20 μl), a Pharmacia-LKB 2248 LC pump which delivered the carrier at a flow-rate of 0.6 ml min^{-1}, and a Jasco fluorescence FP 920 detector (λ_{ex}=486 nm, λ_{em}=516 nm) for detection.

2.5. SAIA

For the SAIA experiment, a single line FI system was used consisting of a Gilson XL autosampler equipped with a Rheodyne six-port injection valve (injection loop 20 μl), a Pharmacia-LKB 2248 LC pump which delivered the carrier at a flow-rate of 0.6 ml min^{-1}, a stainless-steel column (10×1 mm) containing 20 μl of solid-phase which is an estimate of the amount of solid-phase contacted by the analyte in the biochemical detection system, and a Jasco fluorescence detector FP 920 (λ_{ex}=486 nm, λ_{em}=516 nm) for detection. For each experiment, fresh solid-phase was used. Prior to injection, 400 nmol l^{-1} biotin was diluted 1:1 with the label solution (1.66 nmol l^{-1} fluorescein-biotin) also used in the biochemical detection system.

3. Results and discussion

3.1. Affinity solid-phases for detection

Solid-phases can be employed as consumable affinity materials in continuous-flow biochemical detection when using a recently presented hollow fibre module for separating free and bound label [19]. This is shown with a model system using biotin as analyte, fluorescein-biotin as label, and different solid-phases for affinity recognition of the analyte.

The characteristics of affinity solid-phases are determined by the support backbone, by the affinity protein, and by the chemistry of immobilisation. The support provides the backbone for immobilising the affinity protein and determines the achievable binding site density and accessibility [26]. In traditional affinity chromatography, ideal affinity supports should consist of a porous network allowing unimpaired mass transfer of the eluent, the particles should be uniform, rigid and spherical, have a protein non-interactive surface, and exhibit high mechanical and chemical stability [27]. For implementation into biochemical detection, affinity supports should exhibit the same characteristics. However, it may prove advantageous to use non-porous supports or supports with large, flow-through pores rather than traditional porous affinity supports in order to reduce diffusion-limited mass transfer. Well-defined surface characteristics, such as specific surface area, pore size, pore distribution, and particle size, are crucial for evaluating the influence of the affinity solid-phase on the detection system.

Affinity proteins may differ in selectivity and affinity. Appropriate choice of the affinity protein used in a certain application is therefore of great importance [28]. Here, two types of affinity proteins were used for the model compound biotin, namely streptavidin and anti-biotin antibodies.

Affinity proteins can be immobilised in various ways to solid-phases. The coupling chemistry affects kinetic parameters in biospecific binding [29] and will therefore influence the performance of the affinity solid-phase.

The characteristics of the affinity solid-phases used during this investigation are summarised in Table 2. Carbohydrate gels, such as beaded agarose, provide a hydrophilic surface with low nonspecific binding properties. For this reason, they have been used traditionally in affinity chromatography [26]. With particle sizes up to 165 μm, they are large in size and their surface properties are rather poorly defined. Popularity of agarose-phases is ensured by the ease of derivatisation with a variety of well-established methods and the nondenaturing protein-friendly surface of the material.

Nowadays also, synthetic polymeric affinity solid-phases are frequently used. Their surface characteristics are often better defined and they have been reported to offer favourable properties regarding nonspecific binding [26]. Both porous and non-porous materials exist and have been used here. The smallest particles used during this investigation were 18 μm, but particles sizes down to 0.1 μm are commercially available.

Optimisation of reaction conditions in this paper was carried out with streptavidin metacrylate polymer beads.

Table 2
Characteristics of affinity solid phases

Solid-phase affinity material	Backbone	Spacer	Particle size (μm)	Pore size	Binding capacity used in BCD (nmol l^{-1})	Bound label (%)	Signal for 20 μl injection of 400 nmol l^{-1} biotin (arb. units)	R.S.D. (%) (n=3)
Streptavidin beads	Metacrylate polymer beads	10 atoms	50	100 nm	55	20	286	4.9
Streptavidin microspheres	Polymeric	unknown	18	non-porous	5.5	20	445	3.7
Streptavidin agarose	4% Beaded	7 atoms	60–140	unknown	110	11	90	7.9
Antibody agarose	4% Beaded agarose	none	45–165	unknown	<44	19	137	3.0

3.2. Design of the biochemical detection system

3.2.1. Hardware considerations

Previously described biochemical detection systems used two HPLC piston pumps for pumping the two reagent streams [18,19]. By using a peristaltic pump for the reagent streams, one pump suffices for pumping both streams and compatibility with protein solutions as well as with solid-phase suspensions is ensured. The 0.5 mm I.D. PTFE-capillaries used previously [19] proved to be compatible with the solid-phases used during this investigation with diameters up to 165 μm. Back-pressure, necessary to create the driving force for the membrane separation, was previously controlled with a metering valve at the retentate outlet [19]. When introducing affinity solid-phase as a reagent, blocking of the metering valve rapidly occurred. As an alternative, restriction of the retentate flow occurred by pumping it after the retentate outlet with the peristaltic pump (see Fig. 1). The back-pressure created in this way was sufficient to cause a flux across the membrane of 0.05 ml min^{-1}, i.e. 8.3% of the total flow.

3.2.2. Assay format

The set-up of the model system described here differs from the previously described ones in that it is a competitive system (see Fig. 1). In the competitive set-up the biochemical reaction between analyte and binding site on the one hand and label and

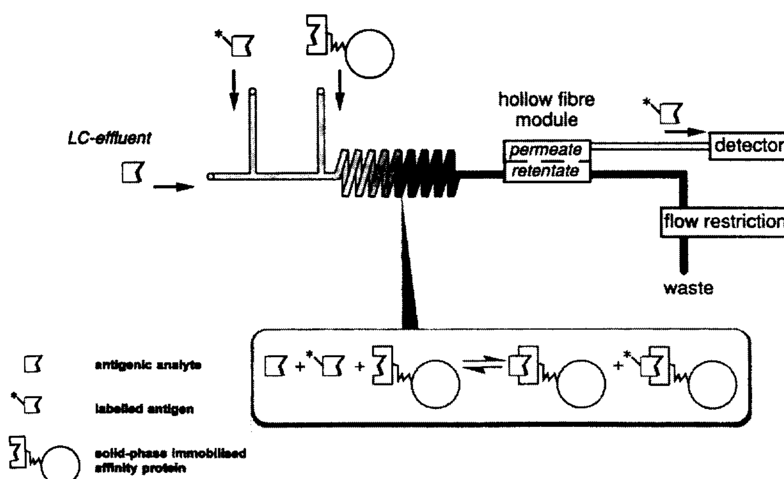

Fig. 1. Competitive biochemical detection system based on labelled antigens.

binding site on the other hand proceed simultaneously. Earlier described biochemical detection systems consist of a sequential, two-step biochemical reaction [18,19]. First, analyte reacts with the binding sites and then label is added to the reaction mixture in order to titrate the left-over binding sites. In contrast, in the competitive set-up used here the analyte is first mixed with label. Hereafter, the affinity protein is added and in the time allowed for reaction, the analyte and label compete for the constant amount of binding sites. The analyte can be mixed with the label by continuously adding the label to the carrier as shown in Fig. 1, or by adding the label to the sample prior to injection. Here, only the continuous addition of label is considered in order to allow future on-line coupling to liquid chromatography without further changes.

Since the biochemical reactions in the competitive assay format proceed simultaneously, optimal reaction times should be shorter than in the previously described sequential systems which require a two-step reaction. A shorter reaction time is favourable to short response times of the detection system and to limiting extra-column bandbroadening when coupling the biochemical detection system to liquid chromatography.

3.3. Optimisation of reaction conditions

3.3.1. Reaction time

The optimal reaction time in a biochemical detection system is determined on the one hand by the reaction kinetics and on the other hand by bandbroadening occurring as a consequence of dispersion in the open tubular reaction coils. Reaction kinetics depend on mass transfer of reactants to the site of the initial interaction and on the reaction rate of the interaction. The reaction rate is reflected in the association rate constant. For solid-phase batch assays, association rate constants of affinity proteins immobilised on solid-phases are generally two orders of magnitude lower than for soluble affinity proteins, since diffusion and mass transport limitations are particularly strong [21]. In biochemical detection, mass transfer of the reactants is enhanced by using knitted reaction coils. However, when this mixing is not sufficiently thorough, mass transfer will at least partly be diffusion-controlled. Analogous to the results found for batch assays, slower reaction kinetics may then be expected in biochemical detection employing solid-phase immobilised reactants in comparison to a system employing soluble reactants. As a consequence, optimal reaction times will be longer. However, increased reaction times will only lead to increased signals until counteracted by bandbroadening caused by dispersion in the open-tubular reactor. Therefore, the influence of the reaction time on the signal should exhibit an optimum.

The influence of the reaction time on detection sensitivity has been investigated in Fig. 2. The reaction time was changed by varying the flow-rate using a reaction coil of 858 μl. Increased reaction times resulted in larger peak heights as a consequence of a more complete affinity reaction. The expected optimum, however, is not yet reached even at a reaction time of 3 min. Bandbroadening in the open-tubular post-column reactor causes loss in resolution when coupling the system to liquid chromatography. In order to limit extra-column bandbroadening, a reaction time of 85 s was chosen. Under these circumstances, a peak width of $\sigma_v = 110$ μl was obtained, which is acceptable if the selectivity of the detection system is considered.

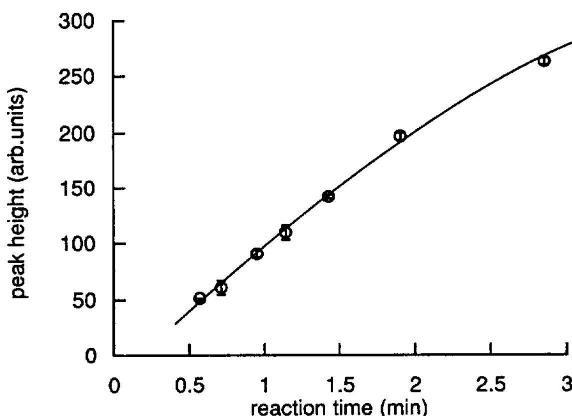

Fig. 2. Influence of reaction time. Signals for 20-μl injections of 200 nmol l^{-1} biotin (○). The error bars represent standard deviations (n=3). The carrier PBST, the label solution (1.66 nmol l^{-1} fluorescein-biotin) and the streptavidin bead suspension (166 nmol l^{-1} binding sites) were delivered at a flow-rate of 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and 0.5 ml min^{-1} each. A reaction coil of 858 μl was used. Fluorescence detection occurred at λ_{exc}=486 nm and λ_{em}=516 nm.

3.3.2. Affinity protein concentration

Increasing the affinity protein concentration favours fast reaction kinetics and increases the amount of bound ligand [21]. As a result of this increase, the higher binding site concentration results in a decrease of background signal and baseline noise when quantitating free label. Increased concentrations of binding sites should, therefore, improve the sensitivity of the system. In contrast, systems detecting bound label have increasing background and noise levels at higher binding site concentrations leading to a narrow optimal binding site concentration [18].

Fig. 3 represents signals for biotin at different binding site concentrations. The binding site concentration was calculated based on biotin binding capacity specified by the manufacturer. The binding site concentration in the biochemical detection system was varied by diluting the solid-phase. In the concentration range of 14–55 nmol l^{-1} the signal increases strongly with the binding site concentration. At higher concentrations, the signal increase levels off, indicating that the affinity reaction is not diffusion limited at this stage. Hence, reaction kinetics are not affected at higher binding site concentrations.

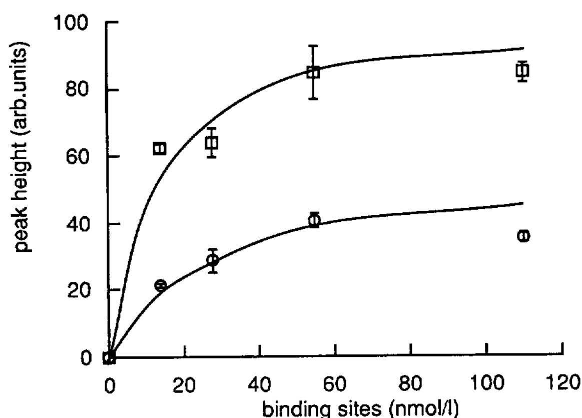

Fig. 3. Influence of binding site concentration. Signals for 20-μl injections of 8 (○) and 40 nmol l^{-1} biotin (□). The error bars represent standard deviations (n=3). The carrier PBST, the label solution (1.66 nmol l^{-1} fluorescein-biotin) and the streptavidin bead suspension were delivered at a flow-rate of 0.2 ml min^{-1} each. The time for the affinity reaction was 85 s. Fluorescence detection occurred at λ_{exc}=486 nm and λ_{em}=516 nm.

In contrast to previous systems, the binding sites are not distributed equally over the reaction volume. In a large part of the reaction volume, no binding sites are present, whereas locally a high binding site density exists. High binding site density is one of the reasons for the efficient reactions in affinity chromatography. On the other hand, ligands are allowed to be in contact with the affinity solid-phase for a fairly long period of time, whereas the residence time of the ligands in the affinity column of a SAIA is extremely short. We compared the binding efficiency of the present biochemical detection system with a SAIA set-up. In both cases, 20% of binding occurred indicating that the increased contact time between ligand and binding site in biochemical detection balanced the unequal distribution of binding sites over the reaction volume.

The high cost of biochemicals often limits the amount of affinity protein which may be consumed. Using solid-phases as affinity material, also compatibility with the flow system needs to be considered, since too dense suspensions will clog the system. During this investigation, a suspension of 250 μl of gel per 50 ml buffer was used, which leads to a binding site concentration of 55 nmol l^{-1} in the flow system.

3.3.3. Label concentration

The concentration of label is another important parameter which requires optimisation. In the competitive biochemical detection system the label competes with the analyte for the binding site. With the short reaction time used here, the amount of free label is measured kinetically. Under these conditions a percentage of the total amount of ligand is bound which is hardly affected by the total ligand concentration, as shown in Fig. 4. Experimental results were obtained by injecting different concentrations of fluorescein-biotin into the flow system described in Fig. 1, but substituting the continuous addition of label with buffer. Fig. 4 also shows the influence of the ligand concentration on the percentage of free ligand under equilibrium conditions. For this purpose, different label concentrations were incubated off-line with streptavidin beads. Separation of free and bound ligand was achieved by centrifugation and quantitation occurred by direct injections of free ligand in a flow injection system. In contrast to the

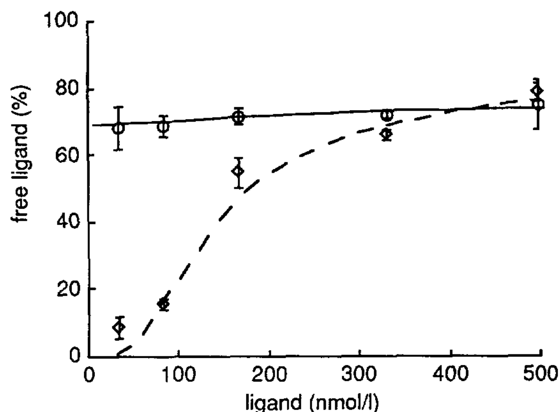

Fig. 4. Influence of ligand concentration on the percentage binding. Under biochemical detection conditions (○) and under equilibrium conditions (◇). The error bars represent standard deviations (n=3). For conditions see Section 2.

kinetic conditions of the biochemical detection, the percentage of free ligand is strongly influenced by the amount of total ligand added under equilibrium conditions.

In the complete set-up for biochemical detection, the signal for a certain analyte concentration increases with the label concentration added to the reaction mixture (see Fig. 5). On the other hand,

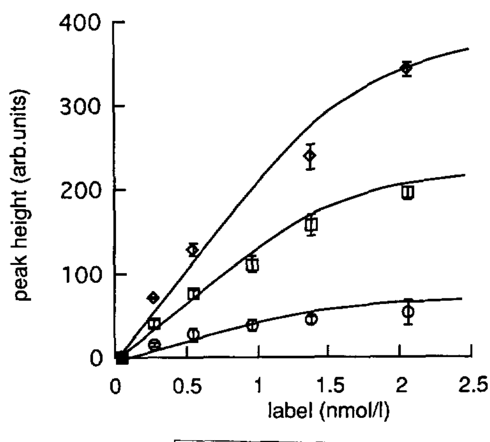

Fig. 5. Influence of label concentration. Signals for 20-μl injections of 8 (○) and 40 (□) and 100 nmol l^{-1} biotin (◇). The error bars represent standard deviations (n=3). The carrier PBST, the label-solution and the streptavidin bead suspension (166 nmol l^{-1} binding sites) were delivered at a flow-rate of 0.2 ml min^{-1} each. The time for the affinity reaction was 85 s. Fluorescence detection occurred at λ_{ex}=486 nm and λ_{em}=516 nm.

however, a low label concentration is preferred in order to limit the background and noise level. As described above, biochemical detection was operated under kinetic conditions. This allows the use of label concentrations which are significantly lower than the binding site concentration. For the experiments in this study, a label concentration of 0.55 nmol l^{-1} was chosen. In addition to a favourable background and noise level, such a limited amount of label reduces the cost of biochemical detection.

At the low label concentration used here, only a small part of the binding sites introduced into the flow system will be occupied after the biochemical reactions. This should allow the reuse of the affinity solid-phases without any further treatment, which has been shown with initial experiments.

3.4. Analytical data

A calibration curve was obtained with 20-μl injections of 20–400 nmol l^{-1} of biotin, which was linear in the range of 40–200 nmol l^{-1}. With non-linear curve-fitting, as commonly used for immunoassays [8], the operation range can be extended to 20–400 nmol l^{-1} (r=0.994). The detection limit estimated as a signal-to-noise ratio of 3 was 8 nmol l^{-1} for a 20-μl injection volume. This is in the same range as the detection limits reached with previous biochemical detection systems [18,19]. When injecting a 100 nmol l^{-1} solution of biotin for twenty times a correlation of variation of 10.9% is calculated, which is high in comparison to biochemical detection systems employing soluble affinity proteins [19]. A possible explanation for the result is inhomogeneity of the affinity solid-phase suspension and variation of the beads in density of bound streptavidin. Nevertheless, the correlation of variation is acceptable when considering that inaccuracies of biochemical assays commonly lie around 15% [30].

3.5. Influence of the solid-phase

Fig. 6 shows the flow injection peaks obtained with affinity solid-phases as summarised in Table 2. Continuous-flow experiments revealed a 20% binding of the label for streptavidin beads used throughout this paper. Due to limited availability of strep-

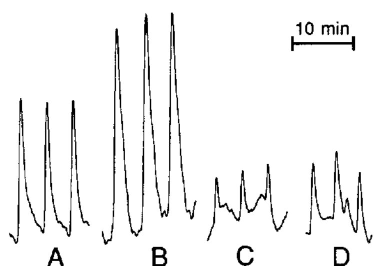

Fig. 6. Signals obtained with different affinity solid-phases. Flow injection peaks for 20-μl injections of 20 μl of biotin of 400 nmol l^{-1} using (A) streptavidin beads, (B) streptavidin agarose, (C) streptavidin microspheres and (D) antibody agarose as solid-phase. For characteristics of the materials and binding site concentrations used see Table 2. The carrier PBST, the label solution (1.66 nmol l^{-1} fluorescein-biotin) and the solid-phase suspension were delivered at a flow-rate of 0.2 ml min^{-1} each. The time for the affinity reaction was 85 s. Fluorescence detection occurred at λ_{ex}=486 nm and λ_{em}=516 nm.

tavidin–microspheres, the binding site concentration was decreased tenfold. Nevertheless, the same amount of label was bound, namely 20%. The smaller particle size entails a better suspension, thus the binding sites are more evenly distributed, which enhances reaction kinetics. Additionally, the material is non-porous, so that the binding sites are more exposed than when they are immobilised within pores, which is the case with the streptavidin beads. These advantageous characteristics were reflected in a 1.5 times higher response than for the streptavidin beads. Streptavidin agarose with the same amount of binding sites as the streptavidin beads lead to so little binding of the label, that the binding site concentration was doubled. Still, only 11% of the label was bound. This was reflected by the response for the analyte which was three times lower as compared to the response obtained with the streptavidin beads. Additionally to streptavidin, antibodies have been used as affinity proteins for biotin. The antibodies were immobilised onto the support according to manufacturers procedure. Using this antibody agarose, also 20% of binding of the label was achieved and a signal half that of the streptavidin beads.

Comparing the flow injection peaks in Fig. 6, different peak widths were observed for the different column materials. The different solid-phase materials lead to suspensions with different characteristics with respect to e.g. suspension density, homogeneity, and viscosity, which entail slightly different flux through the membrane of the hollow fibre module. As a consequence, the permeate flow, and accordingly the residence time of the sample plug in the detector, vary depending on the solid-phase used.

The beads used initially are a rather large and porous material. The use of non-porous materials is advantageous, since diffusion into the pores is not necessary, as was demonstrated by the signals obtained with streptavidin beads and with streptavidin–microspheres. Particle size also influences the system, since smaller particles form a suspension more easily and allow a larger surface with binding sites. These effects need to be evaluated in future. Nevertheless, the results show that a wide range of solid-phases can be implemented in biochemical detection.

4. Conclusions and future perspectives

The present work introduces the use of affinity solid-phases in continuous-flow biochemical detection. This has been made possible by employing a hollow fibre module for separating free and bound label. The main feature of the system is the increased choice of affinity materials which can be introduced into continuous-flow biochemical detection demonstrated by the use of affinity solid-phase suspensions. In this way, solid-phase immunoassays can be translated into continuous-flow immunochemical detection for on-line coupling to liquid chromatography. It is to be expected that other affinity suspensions may also be used in biochemical detection, such as membrane-bound receptors, whole cells, and imprinted polymers.

Additionally to previously described systems, continuous-flow biochemical detection can be performed in a kinetic, competitive set-up, which is advantageous with respect to speed. Instrumental requirements of the presented system allow easy implementation.

A similar set-up as the one presented in this paper should allow the analysis of solid-phase immobilised analytes using soluble affinity proteins for recognition and soluble labels for quantitation. This analytical challenge is found in the area of combinatorial chemistry where possible ligands are synthesised on a solid support.

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