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Theoretical concepts of on-line liquid chromatographic–biochemical detection systems
II. Detection systems based on labelled affinity proteins

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Abstract

A theoretical concept for on-line liquid chromatography–biochemical detection (LC–BCD) using labelled affinity proteins as reporter molecules is presented. The BCD system is based on the post-column addition of labelled affinity proteins such as fluorescein-labelled streptavidin to the LC effluent. After a short reaction time, free and analyte-bound label are separated during passage through a column packed with an immobilised-ligand support. The bound fraction passes the column unretained and is measured downstream by means of a conventional HPLC detector. The theoretical model presented here relates the detector response to the most important instrumental and biochemical parameters such as dispersion, reaction time, concentration and affinity of the affinity protein and the number of binding sites. The theoretical concept is validated using fluorescein-labelled streptavidin and biotin as model system. © 1997 Elsevier Science B.V.

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1. Introduction

The goal of on-line coupling of biochemical detection (BCD) to liquid chromatography (LC) is to combine the high selectivity and sensitivity of bioassays with the separation power and ease of automation of HPLC [1–9]. Important application areas are bioanalysis and drug discovery, where active compounds have to be determined at low concentrations in highly complex matrices. Several on-line LC–BCD systems were described in the literature, differing in the way labels are used to monitor the biochemical reaction. Detection systems using both labelled affinity proteins [1,2,7–9] and labelled ligands [3–7] as reporter molecules were reported. Most of the BCD systems are similar to assay formats applied in microtiter plate assays. For example, continuous-flow immunodetection systems using labelled antibodies implement, similar to microtiter plate immunoassays, immobilised antigen supports to separate free and analyte-bound antibodies [1,2,11,12]. Unlike in microtiter plate assays, fast reaction times in the order of 1–2 min are obtained when the assay is carried out in the continuous-flow mode.

Depending on the objectives of the LC–BCD

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system, the various parameters that influence its performance such as concentrations of reactants, reaction time and design of reactor configuration need to be optimised. While the hardware set-up of the LC–BCD system is very similar to chemical post-column reaction detection systems, the implementation of biochemical equilibrium reactions adds more parameters to be optimised. Various models for chemical post-column reaction detection systems [12,13] and batch bioassays [14–16] have been reported. In this paper we present a theoretical concept for LC–BCD systems based on labelled affinity proteins which relates the detector response to the most important instrumental and biochemical parameters such as dispersion, reagent concentrations, reaction times, multivalency of the affinity protein, protein degradation and detectability of the labels. A similar concept for systems based on labelled ligands has been reported elsewhere [3,17]. The validity of the theoretical model is evaluated using the interaction of fluorescein-labelled streptavidin and biotin as a model system [18,19]. Streptavidin (M_r 60 000) is a protein from *Streptomyces avidinii* and possesses four high-affinity binding sites for biotin ($K_D = 1 \times 10^{-15}$ mol/l). The interaction between streptavidin and biotin is well documented and therefore very useful as a model for the evaluation of the post-column detection system.

2. Theory

2.1. Set-up

The flow injection (FI)-BCD system using labelled affinity proteins as reporter molecules is based on two steps (see Fig. 1). In a first step, a fixed amount of labelled affinity protein (P*) is added to the LC effluent to react with ligands (L) eluting from the LC column to form affinity protein–ligand complexes (P*L) in concentrations dependent on the ligand concentration. Assuming that the reactions that are used in the post-column reaction detection system obey simple mass action principles, the reaction of ligand and affinity protein can be described by:

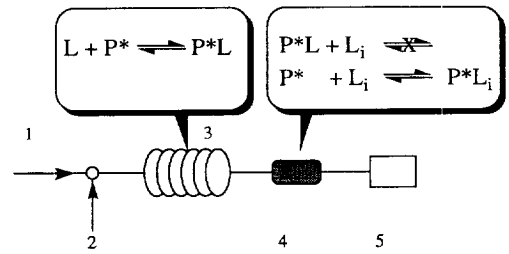

Fig. 1. Scheme of the BCD system. 1, eluate of the HPLC column; 2, reagent pump for labelled streptavidin solution; 3, reaction coil; 4, affinity column with the immobilized ligand; 5, fluorescence detector. P, affinity protein; L, ligand; L_i, immobilized ligand.

$$P^* + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} P^*L \qquad \text{reaction(1)}$$

in which k_{+1} is the association rate constant and k_{-1} the dissociation rate constant. In the second step, the excess of labelled affinity protein is removed by means of an affinity column on which the ligand (L_i) is immobilised. Again, an affinity reaction takes place:

$$P^* + L_i \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} P^*L_i \qquad \text{reaction (2)}$$

The bound affinity protein–ligand complex passes the affinity column unretained and is detected by means of a conventional detector.

Several parameters can be distinguished that determine the post-column BCD system based on labelled affinity proteins, i.e., dispersion, reaction kinetics, affinity separation and detectability of the labels. In this model we will not consider the effect of protein degradation caused by the presence of organic modifiers or denaturating surfaces, although these factors can have a significant influence on the detector response.

2.2. Dispersion

The influence of dispersion in a post-column reaction set-up has been discussed by Lillig and Engelhardt [13]. Dispersion caused by the BCD systems can be minimized by choosing appropriate reactor dimensions and generally is below 30% of

the total dispersion of the LC–BCD system [1–9]. Therefore, it can be assumed that the dispersion of the BCD system has only a small effect on the performance of the detection system.

2.3. Reaction efficiency

The reaction of the affinity protein with the ligand, as shown in reaction (1), is described by second-order kinetics, depending on two components, i.e., association rate (k_{+1}) and dissociation rate (k_{-1}):

$$\frac{d[P^*L]}{dt} = [P^*][L]k_{+1} - [P^*L]k_{-1} \quad (1)$$

The association rate constant is, in general, diffusion controlled and ranges from 10^7 to 10^8 l mol^{-1} s^{-1}. The dissociation rate constant, in contrast, is dependent on the affinity of the ligand and ranges from 10^{-5} s^{-1} for high-affinity ligands to 10^3 s^{-1} for low-affinity ligands [16,20]. Using only high-affinity ligands, in a first approximation it was assumed that dissociation is negligible in the time range of the post-column reaction detection (1–2 min), which is true for dissociation rate constants lower than 10^{-2} s^{-1}. Eq. (1) is integrated as described earlier [3]:

$$[P^*L]_{t=1} = [P^*]_0[L]_0 \frac{e^{-([L]_0-[P^*]_0)k_{+1}t} - 1}{[P^*]_0 e^{-([L]_0-[P^*]_0)k_{+1}t} - [L]_0} \quad (2)$$

in which $[P^*]_0$, $[L]_0$ represent the concentration of labelled affinity protein and injected ligand, respectively, and $[P^*L]_{t=1}$ the affinity protein–ligand complex formed. However, for low-affinity compounds with high dissociation rate constants, the above-made assumption is inaccurate. Integration of Eq. (1) when taking into account the dissociation rate constant can be done in a similar way as Weiland and Molinoff [14]:

$$k_{+1}t = \frac{[P^*L]_e}{[P^*]_0[L]_0 - [P^*L]_e^2} \times \left(\ln \frac{[P^* - L]_e([L]_0 - [P^*L][P^*L]_e/[P^*]_0)}{[L]_0([P^*L] - [P^*L]_e)} \right) \quad (3)$$

in which $[P^*L]_e$ is the concentration of affinity protein/ligand complex when the reaction reaches equilibrium. The equilibrium of the reaction is determined by the affinity of the affinity protein with its ligand ($K_D = k_{-1}/k_{+1}$). The concentration of $[P^*L]_e$ is calculated by [3]:

$$[P^*L]_e = \frac{[P^*]_0 + [L]_0 + K_D - \sqrt{([P^*]_0 + [L]_0 + K_D)^2 - 4[P^*]_0[L]_0}}{2} \quad (4)$$

Eq. (3) can be rewritten as

$$[P^*L]_{t=1} = [P^*L]_e[P^*][L] \cdot \left(\frac{1 - e^{-\alpha k_{+1}t}}{[P^*L]_e^2 - [P^*][L]\, e^{-\alpha k_{+1}t}} \right) \quad (5)$$

in which:

$$\alpha = [P^*L]_e - \frac{[P^*][L]}{[P^*L]_e} \quad (5a)$$

Eq. (5) relates the concentration of the affinity protein–ligand complex formed after reaction (1) to the concentration of injected ligand and its affinity.

2.4. Affinity column

Immobilized ligand supports should possess a high capacity to efficiently separate affinity protein–ligand complexes from free affinity proteins. Hage and co-workers [21,22] derived a theoretical equation for the binding efficiency of affinity columns, describing the binding affinity, f, as:

$$f = \exp\left[-\frac{k_{+2}m_L}{F} \right] \quad (6)$$

in which k_{+2} is the association rate constant of the affinity protein and the immobilized ligand, m_L the amount of immobilized ligand and F the flow-rate. For example, assuming $[P^*] = 1.5$ nmol/l, $m_L = 1000$ pmol, $k_{+2} = 10^7$ l mol^{-1} s^{-1} and $F = 10$ μl/s, the affinity protein will bind almost quantitatively (99.9%) to the affinity column during the passage. However, during long-term use, the column will slowly be saturated by the affinity protein. Thus, the concentration of free immobilized ligand will decrease resulting in a decreasing binding efficiency.

The breakthrough on affinity columns due to

dissociation of the bound affinity protein can be calculated according to Ohlson and Zopf [23]:

$$k = \frac{[P^*L_i] \cdot V_s}{[P^*] \cdot V_m} = \frac{\Phi \cdot Q_{max}}{K_i} \quad (7)$$

in which k' is the chromatographic retention factor, $[P^*L_i]$ the concentration of bound labelled affinity protein, $[P^*]$ the concentration of free labelled affinity protein, V_s the volume of the stationary phase, V_m the volume of the mobile phase, $F = V_s/V_m$, K_i the affinity constant for the binding of the affinity protein with the affinity column and Q_{max} the maximum accessible ligand sites.

From Eqs. (6) and (7) it can be concluded that for efficient trapping of the labelled affinity protein, the affinity column needs to possess sufficient specific capacity and length. Furthermore, a high affinity results in high breakthrough volumes for the labelled affinity protein.

Next to the efficient trapping of the labelled receptors by the affinity column, two other phenomena can be expected, i.e., dissociation of weak bound [P*L] complexes and dissociation of weak bound $[P^*L_i]$ complexes (Fig. 2).

In general, the strongly bound affinity protein–ligand complex will pass the affinity column unretained. For low-affinity interactions, however, the affinity protein–ligand complex formed in reaction (1) can dissociate during passage and, subsequently, the affinity protein is trapped on the affinity support (Fig. 2b), resulting in lower responses than predicted by the equation for reaction efficiency (Eq. (5)). The

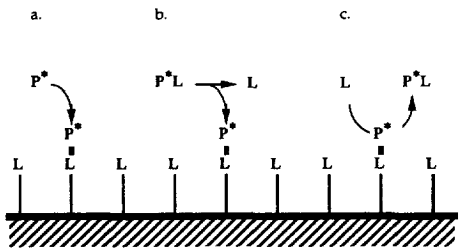

Fig. 2. Interactions of the affinity protein with the affinity column. (a) Direct reaction of the affinity protein. (b) Dissociation of weak affinity protein–ligand complexes and subsequent binding of the affinity protein to the affinity column. (c) Dissociation of weak affinity protein–immobilized ligand complex and subsequent bonding to passing ligands.

dissociation of the affinity protein–ligand complex during passage can be calculated by integrating the differential equations for the reaction of the affinity protein with the ligand (Eq. (1)) and the immobilized ligand:

$$\frac{d[P^*L_i]}{dt} = [P^*][L_i]k_{+2} - [P^*L_i]k_2 \quad (8)$$

in which, at $t=0$, the concentrations of affinity protein, ligand and affinity protein–ligand complex after reaction (1) are defined by Eq. (5).

The differential Eqs. (1) and (8) cannot be solved due to their complexity. If, however, low affinity ligands are used, the concentration of free ligand will not change significantly upon reaction with the labelled affinity protein [13]. Furthermore, the concentration of immobilized ligand is high compared to the concentration of labelled affinity protein (micromolar range vs. nanomolar range, respectively). Thus, the concentrations of free and immobilized ligand can be assumed constant during passage and the differential Eqs. (1) and (8) can be solved similar to Motulski and Mahan [15], with boundary condition that at $t=0$, $[P^*\text{-}L] = [P^*\text{-}L]_{t=1}$:

$$[P^*L]_{t=2} = \frac{[P^*]_0[L]_0k_1}{K_F - K_S} \cdot \left(\frac{k_2(K_F - K_S)}{K_F K_S} + \frac{k_2 - K_F \cdot \left(1 - \frac{[P^*L]_{t=1}}{[P^*]_0}\right)}{K_F} \cdot \exp^{-K_F t_2} - \frac{k_2 - K_S \cdot \left(1 - \frac{[P^*L]_{t=1}}{[P^*]_0}\right)}{K_S} \cdot \exp^{-K_S t_2} \right) \quad (9)$$

in which

$$K_A = k_1[L]_0 + k_{-1} \quad (9a)$$

$$K_B = k_2[L_I]_0 + k_{-2} \quad (9b)$$

$$K_F = 0.5K_A + K_B + \sqrt{(K_A - K_B)^2 + 4k_1k_2[L]_0[L_I]_0} \quad (9c)$$

$$K_S = 0.5K_A + K_B - \sqrt{(K_A - K_B)^2 + 4k_1k_2[L]_0[L_1]_0} \quad (9d)$$

in which t_2 is the time spent in the affinity column.

The above-made assumptions are not valid when the ligand concentration is affected by the reaction with the affinity protein, e.g., when compounds with intermediate affinities are used. In that case, the dissociation of affinity protein–ligand complexes during passage over the affinity column has to be calculated by numerical methods.

The affinity protein which is trapped by the affinity column can also react with the passing ligand (Fig. 2c). This principle has been used by Wortberg et al. [24], who developed a flow immunoassay using an affinity column which was loaded with fluorescence-labelled antibodies. Upon passing of the ligand, the bound antibodies were dissociated and measured by a fluorescence detector. In this assay detection limits of 1 ng/ml were obtained. However, long reaction times (>20 min) were essential to obtain sufficient sensitivity. In the presented BCD system, therefore, it can be expected that the trapped affinity protein bound to the immobilized ligand support will not dissociate when low concentrations of free ligands pass.

2.5. Multivalency

In some cases, the affinity proteins used in the BCD system possess more than one binding site for the ligand (e.g., antibodies) or form multimeric complexes in solution (e.g., streptavidin). If affinity columns are used, multivalent or multimeric affinity protein complexes pass the affinity column only when all binding sites are blocked by the ligands.

When ligands react with multivalent affinity proteins, multiple reactions take place. The formation of fully bound affinity proteins is dependent on the binding affinities of the individual binding sites and on possible cooperation between binding sites. If the affinity protein binding sites are fully equivalent, i.e., have equal affinity constants, the distribution of the affinity protein–ligand complexes can be calculated by normal chance calculations. For instance, streptavidin has four binding sites. Calculation of the concentration of 4:1 complexes of biotin and streptavidin show that even at low biotin concentrations

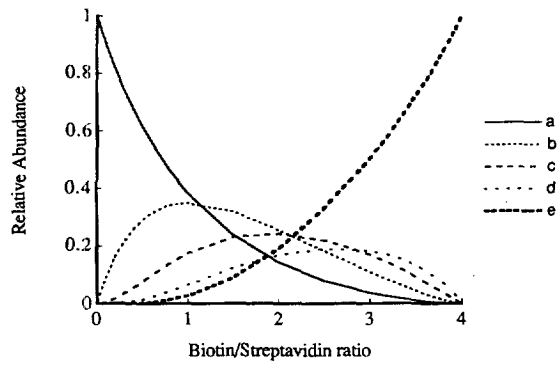

Fig. 3. Relative abundance of the biotin–streptavidin complexes at given biotin/streptavidin ratios. (a) Free streptavidin; (b) 1:1, (c) 2:1, (d) 3:1 and (e) 4:1 biotin/streptavidin ratio.

of ligands, a theoretical chance on the presence on 4:1 complexes exists (Fig. 3).

In contrast, when the four binding sites on a affinity protein show four different reaction rates, the binding sites will react sequentially. In that case, the theoretical model has to be adapted. Using the association rate constants of the consecutive binding sites, the amount of different complexes can be calculated by means of a numerical model.

2.6. Detection

The limit of detection (LOD) depends on the detector noise, N, which can be expressed as a function of the instrument noise in absence of any fluorescence signal (n_s, shot noise) and a variable part, which depends linearly on the background fluorescence (n_f, flicker noise) [25]:

$$N = n_f S + n_s \quad (10)$$

in which S is the background fluorescence signal.

In the labelled affinity protein set-up, the background signal is dependent on impurities in the affinity protein solution, for instance, denaturated fluorescent affinity proteins, labelled non-binding proteins or free low-molecular-mass fluorescence labels. By introducing a purity factor for the labelled affinity protein, p, the noise level can be correlated with the concentration of labelled affinity protein:

$$N = n_f[P^*]_0(1-p) + n_s \quad (11)$$

2.7. Combined model

The complexity of modelling of chemical reactions in flow-injection systems in one model has been discussed by Painton and Mottola [26]. It appeared that, by first approximation using first-order reaction kinetics, reaction kinetics and band-broadening are independent processes. Shih and Carr further evaluated this model by introducing a chromatographic system and came to the same conclusion [27].

Assuming independent band-broadening and reaction kinetics, the band-broadening was calculated as a dilution factor before reaction with the affinity protein (function f_d). Subsequently, the concentration of an affinity protein–ligand complex passing the detector ($[P^*L]_D$) can be determined by subsequent calculation of f_d as a function of the reaction efficiency (f_r), the performance of the affinity column (f_a) and the effect of multivalency on the affinity column (f_m):

$$[P^*L]_D = f_m(f_a(f_r(f_d([L]_0)))) \quad (12)$$

in which $[L]_0$ is the injected concentration of the ligand. The concentration of affinity protein–ligand complex is measured by means of a detector. The detector response (P) is dependent on the response factor (r) and the concentration of the affinity protein–ligand complex:

$$P = r[P^*L]_D \quad (13)$$

By simplifying the BCD system to these two equations, the detector response can be modelled.

3. Material and methods

3.1. Materials

Fluorescein-labelled streptavidin and Sepharose-immobilized biotin was purchased from Pierce (Rockford, IL, USA). Fluorescence-labelled avidin and biotin were purchased from Sigma (St. Louis, MO, USA). Sodium chloride and sodium phosphate were from Merck (Darmstadt, Germany). All other organic solvents came from Baker (Deventer, The Netherlands) and were of analytical grade. The binding buffer consisted of sodium phosphate (10 mmol/l, pH 8.0) containing 0.5 mol/l sodium chloride.

3.2. Batch experiments

The performance of the BCD system under equilibrium conditions was tested in batch by incubating 1 ml fluorescein-labelled streptavidin in binding buffer for 15 min with different biotin concentrations. Subsequently, the mixture was injected in a flow-injection (FI) set-up consisting of a Kratos-ABI (Ramsey, NJ, USA) Spectroflow 400 pump, a Gilson 231 autosampler equipped with a Rheodyne six-port injection valve (injection loop, 20 μl) and a Merck (Darmstadt, Germany) 1080 fluorescence detector (excitation wavelength, 486 nm; emission wavelength, 520 nm).

3.3. Flow-injection experiments

The BCD system was similar to the detection system as described in [1] with a few modifications. The FI-BCD system (scheme, see Fig. 1) consisted of a Kratos-ABI (Ramsey, NJ, USA) Spectroflow 400 pump and a Pharmacia (Uppsala, Sweden) P3500 pump used to deliver the mobile phase and the labelled streptavidin solution, respectively, a Gilson (Villiers-de-Bel, France) 231 autosampler equipped with a Rheodyne six-port injection valve (injection volume, 20 μl) and a Merck (Darmstadt, Germany) 1080 fluorescence detector (excitation wavelength, 486 nm; emission wavelength, 520 nm). The FI carrier solution consisted of binding buffer and was pumped at a flow-rate of 0.3 ml/min. The fluorescein-labelled streptavidin solutions were prepared in binding buffer and added to the LC carrier solution via inverted Y-type mixing unions. The fluorescein-labelled streptavidin solution was pumped at a flow-rate of 0.3 ml/min for FI-BCD systems. Knitted 0.30-mm I.D. PTFE reaction coils for reaction detection were used, with internal volumes depending on the reaction time needed. The reaction was performed at ambient temperature. Separation of free and bound streptavidin was performed using a 10×4.0 mm I.D. slurry-packed column consisting of Sepharose-bound biotin. The

system was controlled by Gilson 605 GSIOC driver software connected to a personal computer.

The influence of the reaction time on detection sensitivity was investigated by using different reaction coil volumes. Using reaction coils of 0.30 mm I.D., the influence of the coil length on the band-broadening can be neglected. The band-broadening of the total BCD system was investigated using potassium iodide as a dye.

3.4. Calculations

For all theoretical calculations the association rate constant of streptavidin was assumed to be 3.2×10^{6} l mol^{-1} s^{-1} [18]. The dissociation of the streptavidin–biotin complex was neglected due to the extremely small dissociation rate constant [18]. A dilution factor of 4 due to dispersion in the BCD set-up was determined by injection of potassium iodide. A constant dilution factor of 4 was therefore used throughout all theoretical calculations. The ratios of streptavidin–biotin complexes were calculated using a Runge–Kutta approximation assuming that all binding sites were kinetically equivalent.

The theoretical detector response was determined by first calculating the dispersion caused by the reaction detection system. Subsequently, the concentration of affinity protein and ligand were adjusted for the dilution due to the mixing of the ligand and the affinity protein solution. Finally, the concentration of affinity protein–ligand complex after passage through the affinity column was calculated using Eq. (10).

The detector noise (N) was calculated using Eq. (11). The signal (S) was calculated using the program for the theoretical detector response. The limit of detection (LOD) was determined by an iteration procedure in which the analyte concentration was varied by increments of 10^{-10} mol/l until a signal-to-noise ratio of 3 was obtained.

4. Results and discussion

4.1. Set-up

To check the validity of the developed model, a biochemical detection set-up (BCD) based on labelled affinity proteins was developed making use of fluorescein-labelled streptavidin. A similar method has been described by Przyjazny and co-workers [7,8] and Hentz and Bachas [19]. The solution of labelled streptavidin is continuously added to the carrier stream to react with biotin. After a given reaction time, the excess of unreacted streptavidin is trapped on an affinity column. The labelled streptavidin–biotin complex passes the affinity column unretained and is detected by means of a fluorescence detector (heterogeneous set-up). As discussed earlier, fluorescein-labelled streptavidin shows an enhancement in fluorescence activity when bound to biotin. Therefore, the separation step for bound and free streptavidin can be omitted (homogeneous set-up). By using the homogeneous set-up and the heterogeneous set-up the reaction kinetics and the performance of the affinity column, respectively, can be evaluated separately.

4.2. Equivalence

The fluorescence enhancement of the labelled streptavidin was measured using batch assays. Increasing amounts of biotin were added to a fixed concentration of fluorescent-labelled streptavidin and subsequently injected into a flow-injection (FI) system comprising a pump, an injector and a fluorescence detector. A linear relationship between the fluorescence enhancement and biotin concentration was found. Maximum fluorescence enhancement was obtained by a four-times excess of biotin compared to the streptavidin concentration (data not shown), indicating that the fluorescence enhancement for the four binding sites is equal.

To prove that the four binding sites have similar affinity, the theoretical concentration of 4:1 complexes of streptavidin at different biotin concentrations were calculated using a numerical model. The concentration of 4:1 complexes of streptavidin at different biotin concentrations was measured using a FI system comprising a biotin affinity column. Fig. 4 shows both the calculated and measured concentration of 4:1 complexes at different biotin concentrations assuming equal affinities for the binding sites. The dotted line shows the total concentration of streptavidin-bound biotin. Close resemblance exists

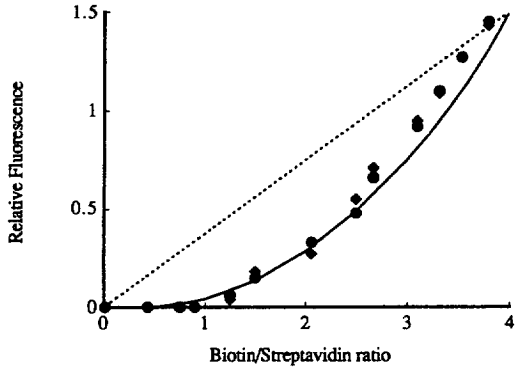

Fig. 4. Detector response vs. biotin/streptavidin ratio in a flow-injection (FI) set-up consisting of an affinity column. Dotted line, total concentration of streptavidin-bound biotin; solid line, calculated concentration of 4:1 biotin–streptavidin complexes; and ♦, measured detector response with FI system ($n=3$).

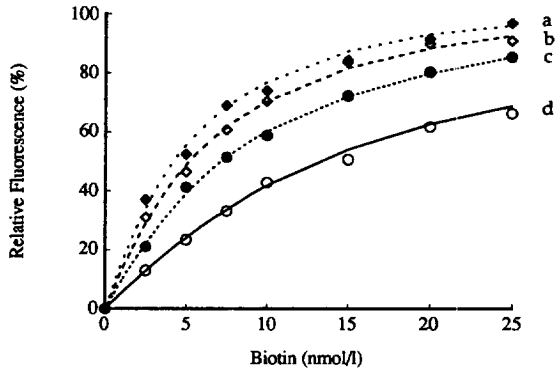

Fig. 5. Detector response vs. biotin concentration using the labelled streptavidin BCD system at (a) 15, (b) 30, (c) 60 and (d) 120 s reaction time. The points indicate measured data ($n=3$) and the lines calculated values. The dotted line represents the detector response under equilibrium conditions. Modelling conditions: dilution factor=4; $K_d=1$ pmol/l; $k_{+1}=1\times10^8$ l mol^{-1} s^{-1}; streptavidin concentration=0.5 nmol/l. Flow-rates: biotin=0.3 ml/min; labelled streptavidin=0.3 ml/min.

between theoretical and measured data, indicating equivalent binding sites.

4.3. Reaction kinetics

To understand the influence of reaction times on the detector response, the response of the BCD set-up was measured using fluorescence enhancement of labelled streptavidin by omitting the biotin affinity column (homogeneous set-up). The detector response was calculated using an association rate constant of $k_{+1}=3.2\times10^6$ l mol^{-1} s^{-1}. A similar association rate constant was used to model the association of biotin to avidin [3]. Fig. 5 depicts the measured and calculated detector response at a streptavidin concentration of 0.5 nmol/l using reaction times ranging from 15 to 120 s. By increasing the concentration of streptavidin, higher reaction recovery is obtained (Fig. 6). Thus, increasing the reaction time or the streptavidin concentration leads to an improved reaction efficiency.

The limit of detection (LOD) can be calculated by combining the signal, as calculated by the combined Eqs. (5) and (10), and the noise, as calculated by Eq. (13) (signal-to-noise ratio=3). Fig. 7a depicts the measured LOD vs. the concentration of streptavidin. At low streptavidin concentrations, the LOD is limited by the shot noise of the detector. At high streptavidin concentrations, the streptavidin purity becomes limiting. Fluorescent impurities that do not bind to the affinity column will result in an inherent background signal which results in a high flicker noise, thus increasing the detection limit. An optimal LOD is found at a streptavidin concentration of 10 nmol/l. Fig. 7b depicts the LOD vs. streptavidin concentration at 95% purity. Thus, by increasing the purity of the streptavidin, the detection limits can be decreased. By increasing the response factor, e.g., by using a more sensitive enzyme label, the detection limit can be further decreased (Fig. 7c; response

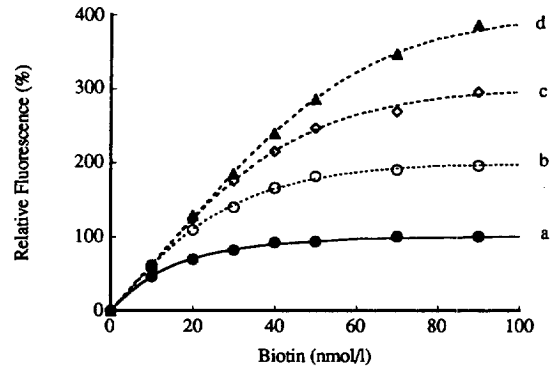

Fig. 6. Detector response vs. ligand concentration using the labelled streptavidin BCD system at four streptavidin concentrations, i.e., (a) 1, (b) 2, (c) 4 and (d) 16 nmol/l. Reaction time=30 s; for other conditions, see Fig. 4. The points indicate measured data ($n=3$) and the lines calculated values.

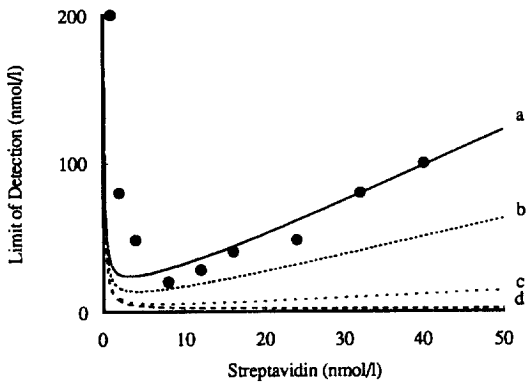

Fig. 7. LOD vs. streptavidin concentration using the labelled streptavidin BCD system. The affinity protein purity is (a) 75, (b) 95 and (d) 100%. Line (c) shows the LOD for streptavidin with a 10-fold higher response factor (r). Modelling conditions; $n_f=0.1$; $n_s=0.1$ nmol/l; for other conditions, see Fig. 4. The points indicate measured data ($n=3$) and the lines calculated values.

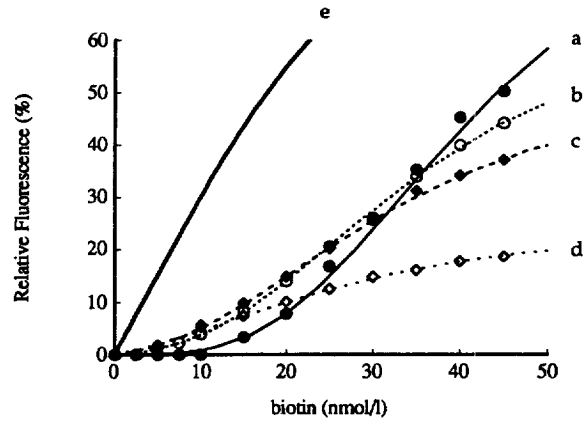

Fig. 8. (a) Detector response vs. biotin concentration using the labelled-streptavidin set-up, consisting of an affinity column. (b) As (a) only streptavidin is pre-titrated with biotin at a ratio of 1:1. (c) As (a) with a pre-titration ratio of 2:1; and (d) 3:1. Line (e) shows the response BCD set-up when the affinity column is omitted. The points indicate measured data ($n=3$) and the lines calculated values.

factor increased 10-fold). These results are in accordance with the measurements of Gunaratna and Wilson [10], who achieved detection limits in the 10 pmol/l range by using enzyme-labelled, highly purified antibodies.

4.4. Multivalency

The influence of the multivalency on the detection system was investigated with a BCD set-up comprising an affinity column (heterogeneous set-up). Due to initial breakthrough of labelled streptavidin, the heterogeneous set-up could only be used at low labelled streptavidin concentrations (data not shown). Fig. 8, line a, depicts a typical calibration plot of biotin using 0.5 nmol/l streptavidin in the heterogeneous set-up. The response of the BCD system is determined by the reaction efficiency (compare with Fig. 5) and the occurrence of 4:1 complexes of biotin–streptavidin (see Fig. 3). Due to non-linear occurrence of 4:1 complexes, only at concentrations higher than 20 nmol/l biotin is a detector response expected. It can be concluded that the multivalent affinity proteins are less suitable in combination with the present set-up. These findings are supported by Freytag et al. [11]. Using both monovalent Fab fragments and bivalent $(Fab)_2$ fragments in a flow-immunoassay, a higher response for the monovalent Fab fragments was observed.

By pre-titration of labelled streptavidin with biotin the ratios between the different biotin–streptavidin complexes can be altered, and thus, the detector response be shifted. Fig. 8 depicts the calculated and measured detector response at three pre-titration ratios of 1:1 (line b), 2:1 (line c) and 3:1 (line d), respectively. Pre-titration of streptavidin results in an increased chance of 4:1 complexes, thus both an increased sensitivity and noise due to the higher background concentration of 4:1 complexes can be expected. The influence of the pre-titration ration on the LOD is depicted in Fig. 9. An optimal limit of

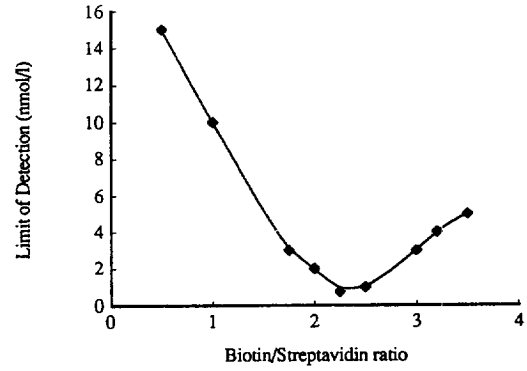

Fig. 9. LOD vs. biotin/streptavidin pre-titration ratio using the labelled-streptavidin BCD system ($n=3$).

detection is measured at a biotin/streptavidin ratio of 2.3:1.

5. Conclusions

A theoretical model has been developed which describes the parameters which influence the BCD set-up using labelled affinity proteins. Using fluorescein-labelled streptavidin, the developed model is tested for its validity. The obtained results suggest that the model describes accurately the response of the post-column detection system. The most important parameters which have to be optimized when developing on-line LC–BCD set-ups are the purity, the concentration of labelled affinity protein and the detector response.

A major obstacle in the development of labelled affinity protein LC–BCD systems has been the availability of pure and highly active affinity proteins. The advancements made in the area of molecular biology, however, will facilitate the availability of highly purified affinity proteins.

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