Biosensors and Bioelectronics 25 (2010) 2302–2308

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A new assay design for clinical diagnostics based on alternative recognition elements

Christiane Albrecht[a,*], Peter Fechner[a], Dmytro Honcharenko[b], Lars Baltzer[b], Günter Gauglitz[a]

[a] Eberhard Karls University of Tuebingen, Institute of Physical and Theoretical Chemistry (IPTC), Auf der Morgenstelle 18, 72076 Tuebingen, Germany
[b] University of Uppsala, Department of Biochemistry and Organic Chemistry, Box 576, 75123 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 8 January 2010
Received in revised form 18 February 2010
Accepted 16 March 2010
Available online 24 March 2010

Keywords:
Immunoassays
(biological) Recognition elements
Kinetics
Clinical diagnostics

ABSTRACT

Herein, we present a new sandwich assay design containing a high affinity polypeptide scaffold as immobilized capture element and an antibody for detection.

These polypeptide scaffolds provide a good affinity towards one antigen and can be linked to biosensor surfaces without affecting their binding capabilities. Furthermore, the small peptides are very stable, which allows for regenerating the surface several hundreds of times and thus for reuse of the biosensor. Moreover, these receptors can be synthesized with different affinities towards one antigen, which has been proven by characterizing them using a label-free detection method RIfS (reflectometric interference spectroscopy) for collecting kinetic data. Polypeptide scaffolds with different affinities have been chosen and characterized.

Upon these results, sandwich-type assays have been set-up using a fluorescently labelled antibody as detection element. Thereby could be shown, that the working range of the assay can be shifted according to the affinity of the used capturing polypeptide scaffold. The scaffolds with a higher affinity towards the antigen can detect lower concentration, and in contrary, scaffolds with lower affinities can detect higher concentrations.

In consequence, using this new sandwich-type assay, we avoid the complex procedure to immobilize antibodies in correct orientation, but simultaneously keep this well-known recognition element in the assay for detection. Furthermore, in addition to all the acknowledged properties of immunoassays, we add the possibility of tuning the working range of assays in distinct manner according to request.

1. Introduction

During the last decades, immunoassays have become a more and more valuable tool in many areas of analytics (Borrebaeck, 2000). They take advantage of the positive abilities of antibodies, like their high affinities and their high selectivity for one antigen, but also suffer from their drawbacks e.g. the rather unstable structure. Antibodies can be made against many kinds of targets, against very small organic molecules as well as against huge proteins, which puts them up for a large area of applications e.g. water analytics and even more important clinical diagnostics (Ramirez et al., 2009).

For these immunoassay applications, the selection of the assay format has a significant impact on practical handling and the sensitivity of the testing results. Typically used assay formats are direct, competitive, displacement and sandwich-type formats (Sapsford et al., 2002). In clinical diagnostics, the sandwich-type assays are still the most commonly used ones due to lower limits of detection compared to other assay types, which are essential for a reliable analysis of many parameters (Jaras et al., 2007). The major drawback of this assay format is the necessity to immobilize the capture antibody on the surface. Antibodies are bio recognition elements, which have been evolving and have been specified over ages, whereby their tertiary structure is crucial for the detection of the antigen. Nevertheless, this highly specified tertiary structure of antibodies is rather susceptible and until now, many immobilization strategies have been developed (Barlen et al., 2009), but no ideal solution to immobilize antibodies without losing a big amount of the binding activity could be found yet. An additional difficulty is to guarantee the correct orientation of antibodies on the surface with the binding sites exposed to the antigen in solution. The most common immobilization procedures such as bonding via amino or carboxyl functions are not site-directed but result in statistically orientated immobilization. This leads to a loss in function and sensitivity as well as a loss in stability of the sensor surface. This reveals another difficulty in handling antibody surfaces. Due

* Corresponding author. Tel.: +49 0 7071 29 74668; fax: +49 0 7071 29 5772.
E-mail address: christiane.albrecht@uni-tuebingen.de (C. Albrecht).

0956-5663/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2010.03.022

to the delicate tertiary structure, antibodies have to be stabilized when spotted during the immobilization procedure as well when stored for longer time periods. There have been several attempts to overcome these problems, such as reducing antibodies down to their Fab fragments (Oshannessy and Hoffman, 1987) or building up artificial constructions like molecular imprinted polymers (MIPs) or plastic antibodies (Haupt and Mosbach, 1998). However, none of these attempts led to satisfactory results in sensitivity and stability.

To phrase the requirements of an ideal recognition element for immobilization, it needs a high affinity and specificity towards the antigen, which is not affected or reduced by being immobilized. Furthermore, the recognition element on the surface should be stable enough to be stored and not losing functionality within time. There are small-structured recognition molecules such as DNA which provide a high stability and represent a guide for new, rationally designed recognition elements. Here we present an immunoassay set-up using a small and stable peptide sequence as immobilized capture element (Baltzer, 2007). These are small helix-loop-helix motifs, which contain natural binders of the target analyte. These are easily accessible while concerted modifications made for immobilization at the artificial helices do not affect binding properties. Due to their small size, the peptides are very stable and can be stored without any further treatment.

With the availability of this stable capture element for immobilization, a new design for sandwich-type assays can be set up. In this paper, we present a completely new sandwich-type assay design for detection of C-reactive protein (CRP), using a tailored binder as capture element on the surface and an antibody as detection element. By this, disadvantages of immobilized antibodies are avoided by replacing them, but their long evolved abilities are still kept in the assay by keeping them as secondary detection element. This heterogeneous assembly in recognition elements is a new approach in assay development. Therefore, the new capture elements have been first characterized by determining their affinity constants towards CRP using reflectometric interference spectroscopy (RIfS), a label-free detection method.

Applications in clinical diagnostics are often using fluorescence based detection methods due to higher sensitivity and better elimination of background noise which is inevitable in complex matrices like human plasma or serum. Therefore, the heterogeneous sandwich assay was set upon a TIRF (total internal reflection fluorescence) based platform. Complete calibration curves were carried out for two different scaffold binders and it could be shown that the different affinities of the binders influence the working range of the assay.

2. Material and methods

2.1. Materials

Common chemicals of analytical grade were purchased from Sigma–Aldrich, Deisenhof, Germany, or Merck KGaA, Darmstadt, Germany. Aminodextran with 100 KDa molecular weight was purchased from Innovent e.V., Jena, Germany. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as a buffer solution (10 mM) containing 5 mM $CaCl_2$.For regeneration of the biosensor surface, a 10 mM glycine solution at a pH of 2.5 was used.

The C-reactive protein (CRP) and the two monoclonal antibodies from mouse (C5-clone M86005M) were purchased from Exbio, Prague, Czech Republic. The conjugation service of the detection antibody with DY647 was also kindly provided by Exbio, Prague, Czech Republic.

2.2. Conjugate binders

The design principles have been described in detail elsewhere (Baltzer, 2007). Briefly, all proteins which are detected in clinical diagnostics have a natural binder. In our case, it is a small organic molecule which binds to its target protein with high specificity. The conjugate binders consist of a stable polypeptide scaffold with 42 amino acid residues which have no affinity for the target protein. They were designed to form helix-loop-helix motifs based on amphiphilic helices because of their ability to present well-defined interaction surfaces in the folded state. The actual binder, the small organic molecule, can be attached to one of the amino acid residues. Furthermore, other linkers for labeling or immobilization can be attached at other positions of the protein scaffold without affecting the binding capability and affinity of the organic molecule. The mechanism of binding can be described as an adapted fit where the small molecule binds to its specific binding site and the polypeptide seeks the interaction of the lowest free energy that is compatible with small molecule complexation to its binding site and the spacer length. The binding energy comes most likely from hydrophobic interactions between polypeptide and protein and selectivity is provided by charge–charge interactions.

The obtained binders vary with regard to affinities due to the variable charge complementarity between target protein and polypeptide and with regard to the position to which the organic molecule is attached to.

The resulting binder molecules are robust and small with molecular weights of around 5 kDa while being capable of high affinities and specificities. Their chemical origin ensures that there is no prior relationship to any biomolecule and therefore, the probability of non-specific binding is low. They are prepared by chemical synthesis and modifications of amino acids are introduced specifically under full synthetic control. The batch-to-batch variation is negligible as they are prepared by chemical synthesis and found to be chromatographically pure.

The polypeptides were prepared by solid phase peptide synthesis using standard Fmoc protocols and PAL-PS as well as PEG-PS resins. They were purified by reversed phase HPLC and identified by MALDI-TOF MS. The small molecule warhead was prepared for conjugation by attaching an aminohexanoic residue and converting the carboxcylic acid to a p-nitrophenyl ester. The active ester was reacted with each polypeptide in pure DMSO solution to form an amide at the side chain of a lysine residue.

The binder molecules for CRP have been described in detail elsewhere (Tegler et al., in preparation and see supplementary material). In short, they were prepared based on the fact that phosphocholine binds CRP with 5 μM affinity. The phosphocholine group was linked by an aminohexanoic acid spacer to the side chain of a lysine residue. Here, one of the CRP binder molecules has been used in combinations with an antibody to form an assay for the identification and quantification of CRP (Fig. 1).

2.3. Set-ups

2.3.1. Reflectometric interference spectroscopy (RIfS)

For kinetic evaluation of the conjugate binders, a biosensor based on reflectometric interference spectroscopy (RIfS) was used. A detailed description of this technique can be found in Schmitt et al. (1997). In short, white light is guided to the transduction layer via an optical fiber and is partially reflected at each interface. The beams superimpose and build a characteristic interference spectrum which is detected using a diode-array spectrometer. Molecules bound to the surface change the physical thickness as well as the refractive index of the toggling layer, causing a shift in the reflectance spectrum. Thus, interaction between the antibody

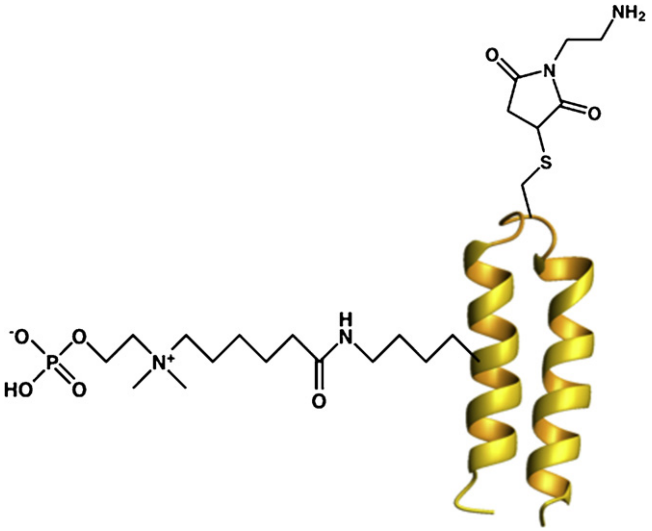

Fig. 1. Schematic structure of the CRP conjugate binder: the polypeptide backbone (in yellow) with attached phosphocholine (left) and amino linker for immobilization (on top). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

and the immobilized antigen can be detected time-resolved and label-free.

2.3.2. Total internal reflection fluorescence (TIRF)

The TIRF-based biosensor set-up RIANA was developed in a prior EU-project (RIver ANAlyser ENV4-CT95-0066). As described in detail by Barzen et al. (1998); Barzen et al. (2002), it consists of the following components: light emitted by a laser diode (Coherent Deutschland GmbH, Dieburg, Germany) with an intensity of 15 mW, operating at 635 nm, is coupled into the beveled edge of a bulk optical glass slide (KROMBACH Optische Werkstätte KG, Wetzlar-Naunheim, Germany). The laser beam is guided through the sensitive area of the glass transducer by total internal reflection. Fluorescent dyes close to the chip surface are excited by the evanescent field induced at the reflection spots. The emitted light is collected by polymer optical fibers, filtered by absorption filters with a cut-on at 690 nm (Omega® Optical Inc., Brattleboro, VT, USA) and detected by photodiodes using lock-in detection. For dilution series, sample preparations and transfer to the RIANA, an HTS PAL auto sampler with cycle composer software (CTC Analytics AG, Zwingen, Switzerland) is used. Liquid handling and data acquisition are fully automated and computer controlled. Using this set-up, the complete measurement cycle including washing steps, sample incubation and regeneration of the sensor surface takes about 12 min.

2.3.2.1. Immobilization protocol. To immobilize the conjugate binders at the sensor surface, the glass transducers were modified with hydrophilic polymer aminodextrane (AMD) as described by Piehler et al. (1996). The transducers were cleaned using a freshly prepared mixture of hydrogen peroxide and concentrated sulphuric acid (ratio 2:3) for up to 30 min and then rinsed with milli-Q water. After drying in a nitrogen flow, 25 μL of (3-glycidyloxypropyl)trimethoxysilane (GOPTS) were applied to the surface and left to react for up to 60 min. The silanised surface was rinsed with dry acetone and dried in a flow of nitrogen. The amino functionalized polymer AMD was dissolved in milli-Q water at a concentration of 0.2 mg μL^{-1}. 25 μL of this solution was instantaneously applied to the silanised surface and left to react for 24 h. Afterwards, the surface was rinsed with milli-Q water and dried in a nitrogen flow. To transform amino into carboxy functions, 25 μL of a solution of glutaric anhydride in N,N-dimethylformamide (DMF) at a concentration of 2.0 mg μL^{-1} were applied to the surface and left to react for 24 h. Then, the surface was rinsed with DMF and milli-Q water and subsequently dried. To activate carboxy functions, a solution of 1 M N-Hydroxysuccinimide (NHS) and 1.5 M diisopropylcarbodiimide (DIC) in DMF was prepared, applied to the surface and left to react for 4 h. The surface was then rinsed with DMF and acetone and dried with nitrogen. Then the binder (1-D37L34, and 3-D37L34 respectively) dissolved in HEPES buffer with $CaCl_2$ at a concentration of 1 mg/mL was applied to the surface and left to react for 24 h at room temperature.

2.3.2.2. Kinetic evaluation. Biomolecular recognition in between receptors and ligands is based on a variety of weak attractive forces, hydrogen bonding and hydrophobic interactions. In the binding pocket of a receptor, the spatial structure is optimized for the ligand and therefore the specific interaction is of high affinity and selectivity. Thermodynamic and kinetic characteristics provide insights to this interaction.

The equilibrium constant (also binding or affinity constant) K is given by the concentrations of ligand, receptor and ligand–receptor-complex in equilibrium in the law of mass action:

$$K = \frac{[RL]}{[R] \cdot [L]} \tag{1}$$

The binding constant K gives information about thermodynamic properties of the interaction, its value corresponds to the strength of the bonding. Typical values for antibody–antigen-interactions are in the range of 10^3–10^{15} L/mol. Nevertheless, K does not provide any information about the dynamic process of the interaction, velocity of association and dissociation. This is characterized by

$$\frac{d[RL]}{dt} = k_a \cdot [R] \cdot [L] - k_d \cdot [RL]$$
$$k_a : \text{association rate constant} \tag{2}$$
$$k_d : \text{dissociation rate constant}$$

The affinity constants K_{aff} and the dissociation constant K_{diss} are linked to k_a and k_d by (Atkins, 2006).

$$K_{aff} = \frac{k_a}{k_d} = \frac{1}{K_{diss}} \tag{3}$$

In this paper, affinity constants of different conjugate binders towards CRP were determined by evaluating the binding curves with three different methods. The conjugate binder was immobilized on the surface and the binding process of different concentrations of binding partner (CRP) in solution was monitored.

Assuming a constant concentration of the binding partner in solution, as assured by constant flow, kinetic of pseudo-first order can be applied for the association (Eddowes, 1987):

$$A(t) = A_{eq} \cdot (1 - e^{-k_s \cdot t})$$
$$A(t) : \text{surface loading} \tag{4}$$
$$A_{eq} : \text{surface loading in equilibrium}$$
$$k_s : \text{rate constant dependent on concentration}$$

$$k_s = k_a \cdot c + k_d$$
$$c : \text{concentration} \tag{5}$$

(A) So by plotting k_s against the concentration, k_a and k_d were determined by linear regression

(B) By approximation of the dissociation, k_d can be gained directly (Oshannessy et al., 1993):

$$A(t) = A_{eq} \cdot e^{-k_d \cdot t} \tag{6}$$

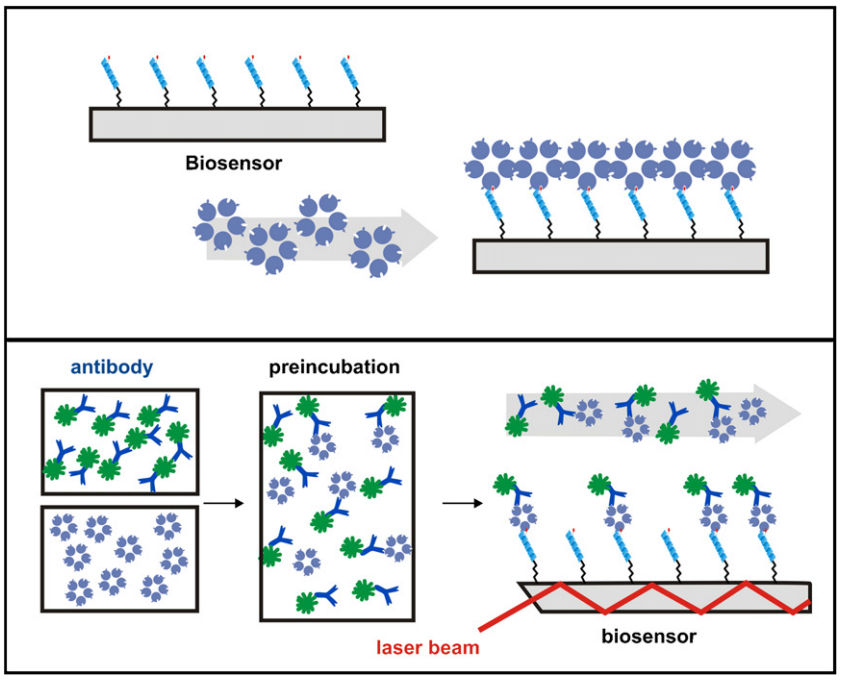

Fig. 2. Assay procedures: RIfS measurement (upper section): different concentrations of CRP (as indicated) were slowly flushed over the biosensor surface to be captured by the immobilized binders. TIRF measurement (lower section): indicated concentrations of CRP were pre-incubated with a constant amount of secondary detection antibody which is fluorescently labeled. After a precise incubation phase, the solution of CRP and antibody is slowly flushed over the biosensor surface; the antibody-CRP complex is captured by the immobilized binder.

k_a is again gained from plotting k_s against the concentration.

(C) An alternative method to calculate the affinity constants is in analogy to Langmuirĭs adsorption isotherm:

$$\frac{A_{eq}}{c} = K_{aff} \cdot A_{max} - K_{aff} \cdot A_{eq} \quad (7)$$

A_{max} : maximal surface loading

According to Scatchard (1949) A_{eq}/c is plotted against A_{eq} which reveals K_{aff} directly from the slope.

Regarding the different evaluation methods, small variations in the determination of the affinity constant K_{aff} are obtained within the different methods. The most common evaluation of K_{aff} is probably plotting A_{eq}/c versus A_{eq} since the fitting function is linear and the affinity constant K_{aff} is directly represented by the slope. Additionally, cooperativity can be detected by deviation of the linear behavior. The drawback of this plot is that no information about the rate-constants k_a and k_d can be obtained. This information can be gained either by plotting k_S against the concentration c or by fitting the dissociation phase separately. By plotting k_S against the concentration c, k_a can be determined by the slope while k_d can be determined by intercept of y-axis. But these determinations are always highly defected as small changes in slope cause high deviation in axis intercept. The strength of this evaluation compared to the Scatchard plot lies in a valid linearization for much higher concentrations of analyte. This is also reflected comparing the quality of the fitting parameter R of the two linearization methods. R is higher for plotting k_S versus the concentration c, because of the higher degree of linear behavior of k_S versus c, than A_{eq}/c versus A_{eq}. To overcome the weakness of high variation of k_d, it is advisable applying a non-linear fit during the dissociation phase of the binding curve to directly access this parameter. It is note worthy that this method is only valid if the binding curve reached its equilibrium and re-binding of the analyte to the surface is impossible, or at least negligible.

2.3.2.3. TIRF calibration. For calibration of the biosensors a dilution series is produced and each concentration step is measured threefold. The mean value and standard deviation (S.D.) for the replica are calculated and depicted in a semi logarithmic plot. To describe the sigmoid curve progression, a logistic fit function (Dudley et al., 1985) with four free parameters (A_1, A_2, x_0 and p) was used, according to

$$y = \frac{A_1 - A_2}{1 + \left(x/x_0\right)^p} + A_2 \quad (8)$$

A_1 is the lower and A_2 the upper asymptote. The range between A_1 and A_2 is defined as the dynamic signal range. The inflection point is given by the variable x_0 and represents the analyte concentration, which corresponds to a decrease of 50% of the dynamic signal range (IC_{50}). The slope of the tangent in this point is given by the parameter p. The working range (for this type of calibration curve) is often given by the 10–90% block of the dynamic signal range. In compliance with the IUPAC rules (Inczendy et al., 1998) the limit of detection (LOD) is calculated as three times the S.D. of the blank measurements ($S.D._{blanks}$). The limit of quantification (LOQ) is calculated as 10 times the S.D. of the blank measurements ($S.D._{blanks}$).

2.4. Assay procedure

For measurements by RIfS, the transducers have been modified according to the protocol described above and different concentrations of CRP were slowly flushed over the biosensor surface. After that, buffer was flushed over the surface followed by glycine solution to regenerate the surface and remove bound CRP molecules. Each concentration was measured threefold and the monitored averaged signal over time including standard deviation is shown. The assay procedure is schematically shown in Fig. 2 (upper section).

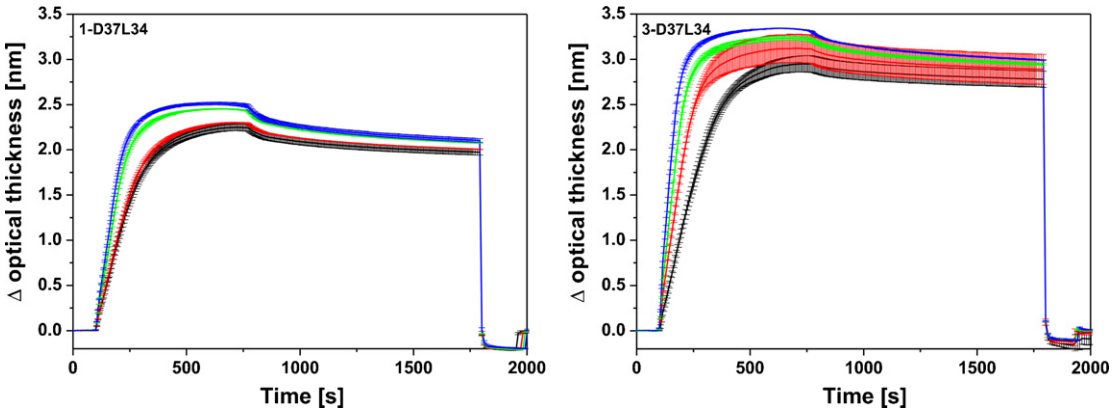

Fig. 3. Binding signal monitored by RIfS of applying different concentrations of CRP ($2.18 \cdot 10^{-8}$ M—black line, $3.27 \cdot 10^{-8}$ M—red line, $4.36 \cdot 10^{-8}$ M—green line, $5.45\ 10^{-8}$ M—blue line) to the biosensor surface with immobilized binder 1-D37L34 (left) and 3-D37L34 (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

For TIRF-based measurements, indicated concentrations of CRP were pre-incubated with a fixed amount of secondary detection antibody, which is fluorescently labeled. After a precise incubation phase, the solution of CRP and antibody is flushed over the biosensor surface to be captured by the immobilized binder. After reading out of the signal, regeneration solution is applied. The assay procedure is schematically shown in Fig. 2 (lower section).

3. Results and discussion

3.1. Characterization of conjugate binders

The affinity of a recognition element towards the analyte molecule influences the calibration curve in matters of calibration range and slope of the function. Therefore, the choice of recognition elements reveals a big potential in optimizing immunoassays for certain applications. Usually, properties of recognition elements cannot be influenced systematically as they evolve independently during immunization procedure (antibodies) or the structure is definitely given by the sequence of amino/nucleic acids (natural receptors/aptamers), which can hardly be modified. Concerning the artificial helix-loop-helix-binders, modifications influencing the affinity can easily be made. Thus, before applying them to immunoassays, they must be characterized carefully.

For the characterization of the two binders 1-D37L34 and 3-D37L34 the label-free detection method RIfS has been used. Transducers have been modified according to the protocol described above and experiments have been carried out as described before. Resulting binding curves are shown in Fig. 3. Both binders reveal a good binding of CRP (100–750 s) and a moderate dissociation of CRP while buffer is flushed over the surface (750–1800 s). Remaining CRP molecules can be removed from the surface by glycine solution at low pH without harming the immobilized capture elements on the surface, which ensures constant conditions and a high reproducibility of these measurements.

For the calculation of affinity constants, every binding curve was fitted by the functions described above. The associative part of the binding curve is fitted according to Eq. (4) in order to obtain parameters A_{eq} and k_s. The dissociative part of the curve is fitted according to Eq. (6) for obtaining parameter k_d. From these data, different mathematical procedures to calculate the affinity constant can be performed. As shown above, three different methods were followed; the necessary plotting is shown in Figure and calculated parameters are arranged in Table 1 (Figs. 3 and 4).

In principal, all three evaluation methods show the same trend. The affinity constant of 3-D37L34 is about half an order of magnitude higher than the one of 1-D37L34. Both binders reveal a quite high affinity which is comparable to antibodies. Common affinity constants of monoclonal antibodies are around 10^6–10^{10} L/mol whereby affinity constants of other man-made recognition structures like MIPs (molecular imprinted polymers) are in a lower range of 10^5–10^6 L/mol (Wei and Mizaikoff, 2007). More important for potential application is the difference in affinity of the capturing element. Carrying out calibrations on these biosensors should reveal systematically different calibration curves. The assay with the high affinity binder 3-D37L34 should be able to determine lower concentrations and therefore, the working range should be shifted towards lower analyte concentrations. Contrary, the assay with the low affinity binder should be able to determine higher concentrations and have a working range shifted towards higher concentrations.

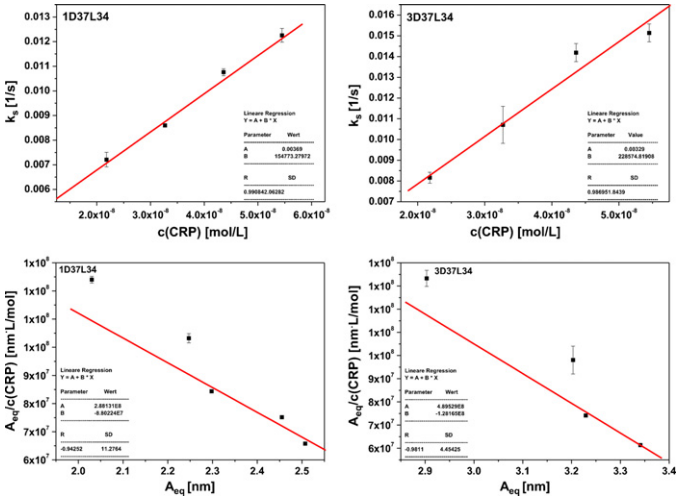

Fig. 4. Plotting schemes for different evaluation as described above. In the upper section, k_s is plotted against the concentration c and linearly fitted in order to receive k_a and k_d. In the lower section A_{eq}/c is plotted against A_{eq} and linearly plotted to receive K_{aff} according to Eq. (7).

3.2. Application of conjugate binders

Having characterized the affinities of the binders, their big potential for application is shown by setting them up in a fully automated biosensor system which has already been proven to be suitable for various immunoassay measurements (Albrecht et al., 2008; Brecht et al., 1998; Kappel et al., 2007). As discussed above, for different reasons the sandwich-type assay is most commonly used.

Table 1
Summary of the kinetic constants gained from RIfS measurements for the binders 1-D37L34 and 3-D37L34. The evaluation methods A, B and C are explained above.

Binder	Evaluation method	k_a [10^5 (L/mol·s)]	k_d [10^{-3} (1/s)]	K_{aff} [10^7 (L/mol)]	K_{diss} [10^{-8} (mol/L)]
1-D37L34	A	1.59	3.7	4.19	2.38
	B	1.59	2.7	5.82	1.72
	C	–	–	8.80	1.14
3-D37L34	A	2.29	3.3	6.95	1.44
	B	2.29	2.9	10	0.99
	C	–	–	12.8	0.78

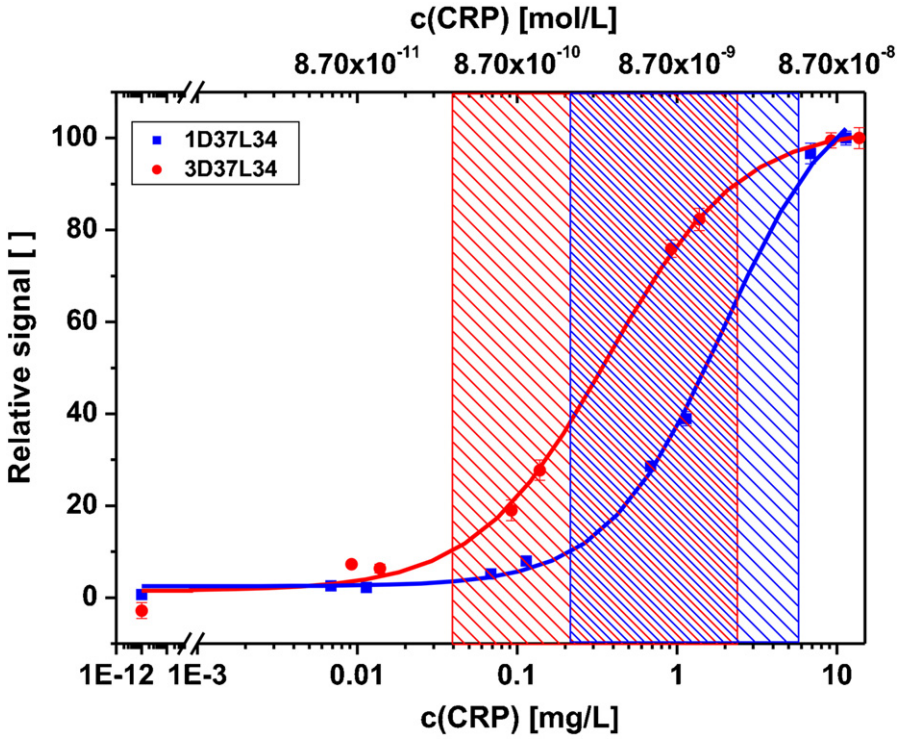

Fig. 5. Calibration curves for CRP with immobilized binders 1-D37L34 (blue curve) and 3-D37L34 (red curve), measured from 0.0075 to 15.1 mg/L. The detection antibody concentration was 0.1 mg/L in each sample. The working range of each curve is shaded in the corresponding color. ((For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)).

Therefore, a second recognition element, which identifies another epitope of the analyte is necessary. This secondary element is in no need of immobilization which qualifies antibodies to be best suited and advantage can be taken from their high affinity and specificity without drawbacks. As the detection is based on fluorescence, the antibody carries is dye-labeled.

A great benefit, which is taken from both, the sophisticated surface chemistry and fluorescence detection principal from the backside of the transducer, is the possibility to measure in demanding matrices such as milk or serum. For approaching real application measurements which should be carried out in human serum, these measurements use 0.2 mg/mL BSA (bovine serum albumin) which simulates a protein concentration of 5% serum sample. No drawback from these conditions could be observed, which makes this set-up most promising for real application measurements (Fig. 5).

The TIRF measurements were carried out to calibrate the biosensor for a quantitative determination of CRP according to the assay procedure describes before.

The obtained calibration curves are depicted in Fig. 5. As predicted in the section above, the calibration curve of the low affinity binder 1-D37L34 is shifted towards higher concentrations covering a working range from 0.22 to 5.75 mg/L (corresponding to $1.9{\cdot}10^{-9}$ to $5{\cdot}10^{-8}$ M). The assay with the high affinity binder covers a working range from 0.039 to 2.3 mg/L (corresponding to $3.4{\cdot}10^{-10}$ to $2{\cdot}10^{-8}$ M).

These results show impressively that the new small peptide binders offer the possibility to tune working ranges of assays. By combining several conjugate binders for the same target molecule but with different affinities on one chip, a huge working range can be covered and measured in one trial. Moreover, the binders can be combined with monoclonal antibodies in a sandwich-type format which enables a sensitive determination of low concentrations.

4. Conclusions

This work introduced the implementation of a new bio recognition element into a sandwich-type assay format in combination with a monoclonal antibody. The artificial binders replace the established immobilized antibody as capture element and therefore resulted in a big improvement to the assay. Furthermore, the binders were characterized regarding their affinity towards the analyte and their influence on assay properties could be shown by putting them up in automated biosensor platform. The potential to tune working ranges of assays is of high interest especially regarding analytes, which are monitored over a broad concentration range e.g. the used analyte CRP is a systemic marker of inflammation which needs to be determined in a great range from 2 to 500 mg/L depending on the type of inflammation, but it is also of great interest to monitor very low concentrations <1 mg/L as this so called high sensitivity CRP is an established indicator for risk of heart dis-

eases. This great range over several orders of magnitudes cannot be covered by one assay with adequate sensitivity. Therefore, we offer a possibility to put up assays, which work under the exactly same conditions, but cover different areas of interest. For a future application, it is a clear aim to have more conjugate binders with lower and higher affinities to cover the whole area of interest of CRP. Besides, due to a well-characterized surface chemistry, the assay is very stable and results are of great reproducibility.

Acknowledgement

This work was funded by the 'Health-CARE by biosensor Measurement And Networking' (CARE-MAN) (NMP4-CT-2006-017333) research project supported by the European Commission under the Sixth Framework Program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.03.022.

References

Albrecht, C., Kaeppel, N., Gauglitz, G., 2008. Two immunoassay formats for fully automated CRP detection in human serum. Analytical and Bioanalytical Chemistry 391 (5), 1845–1852.

Atkins, P.W., 2006. Atkins' Physical Chemistry Peter Atkins; Julio de Paula, eighth ed. Oxford University Press, Oxford [u.a.].

Baltzer, L., 2007. Polypeptide conjugate binders for protein recognition. Creative Chemical Sensor Systems 277, 89–106.

Barlen, B., Mazumdar, S.D., Keusgen, M., 2009. Immobilisation of biomolecules for biosensors. Physica Status Solidi A, 409–416, wiley Inmer Science.

Barzen, C., Brecht, A., Gauglitz, G., 2002. Optical multiple-analyte immunosensor for water pollution control. Biosensors & Bioelectronics 17 (4), 289–295.

Borrebaeck, C.A.K., 2000. Antibodies in diagnostics—from immunoassays to protein chips. Immunology Today 21 (8), 379–382.

Brecht, A., Klotz, A., Barzen, C., Gauglitz, G., Harris, R.D., Quigley, G.R., Wilkinson, J.S., Sztajnbok, P., Abuknesha, R., Gascon, J., Oubina, A., Barcelo, D., 1998. Optical Immunoprobe Development for Multiresidue Monitoring in Water. Elsevier Science Bv, pp. 69–79.

Dudley, R.A., Edwards, P., Ekins, R.P., Finney, D.J., McKenzie, I.G.M., Raab, G.M., Rodbard, D., Rodgers, R.P.C., 1985. Guidelines for immunoassay data-processing. Clinical Chemistry 31 (8), 1264–1271.

Eddowes, M.J., 1987. Direct immunochemical sensing—basic chemical principles and fundamental limitations. Biosensors 3 (1), 1–15.

Haupt, K., Mosbach, K., 1998. Plastic antibodies: developments and applications. Trends in Biotechnology 16 (11), 468–475.

Inczendy, J., Lengyel, T., Ure, A.M., 1998. Compendium of Analytical Nomenclature—The orange book, third ed. Blackwell Science.

Jaras, K., Ressine, A., Nilsson, E., Malm, J., Marko-Varga, G., Lilja, H., Laurell, T., 2007. Reverse-phase versus sandwich antibody microarray, technical comparison from a clinical perspective. Analytical Chemistry 79 (15), 5817–5825.

Kappel, N.D., Proll, F., Gauglitz, G., 2007. Development of a TIRF-based biosensor for sensitive detection of progesterone in bovine milk. Biosensors & Bioelectronics 22 (9–10), 2295–2300.

Oshannessy, D.J., Brighamburke, M., Soneson, K.K., Hensley, P., Brooks, I., 1993. Determination of rate and equilibrium binding constants for macromolecular interactions using surface-plasmon resonance—use of nonlinear least-squares analysis-methods. Analytical Biochemistry 212 (2), 457–468.

Oshannessy, D.J., Hoffman, W.L., 1987. Site-directed immobilization of glycoproteins on hydrazide-containing solid supports. Biotechnology and Applied Biochemistry 9 (6), 488–496.

Piehler, J., Brecht, A., Geckeler, K.E., Gauglitz, G., 1996. Surface modification for direct immunoprobes. Biosensensors & Bioelectronics 11, 579–590.

Ramirez, N.B., Salgado, A.M., Valdman, B., 2009. The evolution and developments of immunosensors for health and environmental monitoring: problems and perspectives. Brazilian Journal of Chemical Engineering 26 (2), 227–249.

Sapsford, K.E., Charles, P.T., Patterson, C.H., Ligler, F.S., 2002. Demonstration of four immunoassay formats using the array biosensor. Analytical Chemistry 74 (5), 1061–1068.

Scatchard, G., 1949. The attractions of proteins for small molecules and ions. Annals of the New York Academy of Sciences 51 (4), 660–672.

Schmitt, H.M., Brecht, A., Piehler, J., Gauglitz, G., 1997. An integrated system for optical biomolecular interaction analysis. Elsevier Advanced Technology, 809–816.

Tegler, L.T., Nonglaton, G., Büttner, F., Caldwell, K., Christopeit, T., Danielson, H., Fromell, K., Gossas, T., Larsson, A., Longati, P., Norberg, T., Ramapanicker, R., Rydberg, J., Baltzer, L., Efficient protein binders for the C-reactive protein from a designed chemically modified peptide library—a new concept for biomolecular recognition. manuscript in preparation.

Wei, S.T., Mizaikoff, B., 2007. Binding site characteristics of 17 beta-estradiol imprinted polymers. Biosensors & Bioelectronics 23 (2), 201–209.