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ORIGINAL PAPER

Two immunoassay formats for fully automated CRP detection in human serum

Christiane Albrecht · Nina Kaeppel · Guenter Gauglitz

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Abstract Immunoassays are a proven approach towards fast, sensitive, cost-effective and easy-to-use analytical systems which are able to measure a variety of interesting analytes, especially in medical diagnostics. Herein, we report two assay formats, binding inhibition and sandwich assay format, for detection of C-reactive protein (CRP) in human serum. Both assays were characterised and compared with respect to their suitability and adaption into a complete sensor system. An automated, optical biosensor system, based on evanescent field technology, was used to carry out a full threefold calibration in each case. Owing to the resulting working ranges, 0.044–2.9 mg L^{-1} and 0.13–22.9 mg L^{-1}, respectively, the assays qualify for use in detecting high-sensitivity CRP (C-reactive protein).

Keywords Optical immunosensor · River analyser (RIANA) · C-reactive protein (CRP) · High-sensitivity CRP (hsCRP) · Healthcare · TIRF

Introduction

The C-reactive protein (CRP) is of great interest in the diagnostics of inflammations. Although CRP was originally crystallised in 1947 [1], suitable forms for further physiological structure analysis have only been obtained more recently. The human C-reactive protein belongs to the pentraxin family. It is composed of five identical non-glycosylated polypeptide subunits (molecular weight 23 kDa), each containing 206 amino acid residues. The protomers are noncovalently associated in an annular configuration with cyclic pentameric symmetry (Fig. 1) [2].

CRP was the first acute-phase protein to be discovered in 1930. It is a sensitive, systemic marker of inflammation, infection and tissue damage. The production of CRP is part of the nonspecific acute-phase response. Therefore, it was not considered to provide useful clinical information at first. Although the discrete CRP values can indeed never deliver complete diagnoses, they are still valuable supplementary hints in emergency diagnostics. Increased CRP values indicate acute inflammations, and the so-called high-sensitivity CRP (hsCRP) is an established indicator for risk of heart disease. The concentration of CRP in the blood of a young adult is about 1–10 mg L^{-1}, and can rise up to 20–40 mg L^{-1} in the case of a viral infection or even up to about 500 mg L^{-1} in the case of a bacterial infection. This crude classification and the long plasma half-life of about 19 h help one to monitor the progress of diagnosed diseases.

Regarding the role of high-sensitivity CRP in the field of cardiovascular risk assessment, extremely low concentrations have to be detected and monitored over longer periods. In general, concentrations of < 1 mg L^{-1} are classified as a low risk level and concentrations of > 3 mg L^{-1} as high risk level.

In clinical laboratories, CRP is determined routinely via enzyme-linked immunosorbent assay (ELISA) which is known to be a highly sensitive method [4, 5]. However, until now, it has not been possible to establish an automated routine procedure for CRP determination. So far, qualified technicians carry out these analyses, making single tests expensive and only available in well-equipped laboratories. Another critical aspect is the resulting long turn-around-time (TAT), which is the time period from sampling until

C. Albrecht (✉) · N. Kaeppel · G. Gauglitz
Institute of Physical and Theoretical Chemistry (IPTC),
Eberhard-Karls-University of Tuebingen,
Auf der Morgenstelle 8,
72076 Tuebingen, Germany
e-mail: christiane.albrecht@ipc.uni-tuebingen.de
URL: http://barolo.ipc.uni-tuebingen.de

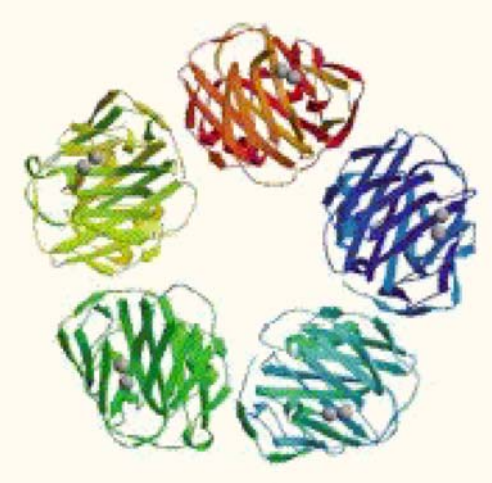

Fig. 1 Ribbon diagram of the crystal structure of CRP [3]

receiving the measuring results. For emergency diagnostics, test stripes are often used, promising quick results. However, they yield only semiquantitative results. The aim of the EU-funded project CARE-MAN (HealthCARE by biosensor Measurement And Networking, project no. NMP4-CT-2006–017333) is the development of a new automated medical instrument based on latest biosensor technologies, providing fully quantitative results in a short measurement time. Therefore, several immunoassays for a couple of different clinical parameters are developed. The presented immunoassays for CRP are based on evanescent field technology and were carried out on an instrument which was developed in a prior EU-funded project RIANA (RIver ANAlyser, project no. ENV4-CT95–0066).

In this article, two different immunoassay formats for CRP determination will be presented. As already mentioned, immunoassays play an important role in medical analytics owing to the high specificity of an antibody for its antigen. Additionally, a high affinity of the antibody is essential to reach the demanded low limits of detection. Hence, in both assays discussed, the detection antibody carries a fluorescent label as the detection of the fluorescent signal is very efficient.

The two presented assay formats are suitable for measuring different analytes in real human serum samples without any pretreatment. The first assay format discussed is a sandwich assay. Here, the antigen is bound to the immobilised capture antibody at one epitope, and is detected using the fluorescently labelled detection antibody binding to a second epitope. In medical diagnostics, the sandwich assay format is the most commonly one used as an ELISA assay. However, a relatively large antigen is needed containing at least two epitopes for antibody binding. In contrast, using the binding inhibition assay which will be discussed subsequently, the sample containing the analyte is preincubated with detection antibody and only antibodies with free binding sites can bind to the surface and can thus be detected. Therefore, the measurements must be fully automated ensuring constant time steps and thus the reproducibility of the obtained results. A considerable advantage of this format is that it is not restricted to large antigens, but can also be used for small analytes with only one epitope, such as hormones and other small organic molecules [6].

As the application of both assay types is highly promising, the sandwich and the binding inhibition assay were developed, and their results presented and compared regarding their role in inflammation and cardiovascular disease diagnostics.

Experimental

Materials

Common chemicals of analytical grade were purchased from Sigma Aldrich, Deisenhof, Germany, or Merck KGaA, Darmstadt, Germany. The protein CRP and the two monoclonal antibodies from mouse (C5-clone M86005M, C7-clone M86007M) were purchased from Exbio, Prague, Czech Republic. The conjugation service of the detection antibody (clone-C7) with DY647 was also kindly provided by Exbio, Prague, Czech Republic. The dye/protein (D/P) ratio was determined to be 4.1. The CRP-free serum (8CFS) was purchased from Hytest, Turku, Finland. The amino-dextran with 100-kDa molecular weight was purchased from Innovent e.V., Jena, Germany. Phosphate-buffered saline (PBS) was used as a buffer solution (tenfold PBS, pH 6.8, comprising 1,500 mM sodium chloride and 100 mM potassium phosphate monobasic). The spotting buffer was kindly supplied by Biotools, Madrid, Spain.

For regeneration of the biosensor surface using the binding inhibition assay, a solution of 0.5% sodium dodecyl sulfate (SDS) in milli-Q water was used. To regenerate the surface using the sandwich assay, a solution of 6 mM sodium hydroxide (NaOH) and 0.6% ethanol (EtOH) in milli-Q water was used.

Biosensor setup based on label-free detection (RIfS)

For the characterisation of surface chemistry stability a biosensor based on reflectometric interference spectroscopy (RIfS) was used. White light is guided to the transduction layer via an optical fibre and is partially reflected at each interface. The beams superimpose and build a characteristic interference spectrum which is detected using a diode array. Antibodies bound to the surface change the physical thickness and the refractive index of the toggling layer, causing a shift in the reflectance spectrum. Thus, interaction

between the antibody and the immobilised antigen can be detected time-resolved and label-free. A scheme of the detection method is shown in Fig. 2. A detailed description of the RIfS setup can be found elsewhere [7].

Biosensor setup based on fluorescence detection (TIRF)

The biosensor setup RIANA was developed in a prior EU-project (RIver ANAlyser ENV4-CT95–0066). As described in detail by Barzen et al. [8–10], it consists of the following components: Light emitted by a laser diode (Coherent Deutschland GmbH, Dieburg, Germany) with an intensity of 15 W, operating at 635 nm, is coupled into the bevelled 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 fibres, filtered by absorption filters with a cut-on at 690 m (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 autosampler with cycle composer software (CTC Analytics AG, Zwingen, Switzerland) is used. Liquid handling and data acquisition are fully automated and computer controlled. The whole measurement cycle including washing steps, sample incubation and regeneration of the sensor surface takes about 12 min.

Immobilisation protocols

To immobilise proteins at the sensor surface, the glass transducers were modified with the hydrophilic polymer AMD as described by Piehler et al. [11] The transducers were cleaned using a freshly prepared mixture of hydrogen peroxide and concentrated sulfuric 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) was 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-functionalised polymer AMD was dissolved in milli-Q water at a concentration of 0.2 mg μL^{-1}. A 25-µL aliquot of this solution was instantaneously applied to the silanised surface and left to react for up to 24 h. Afterwards, the surface was rinsed with milli-Q water and dried in a nitrogen flow. To transform amino groups 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} was applied to the surface and left to react for 24 h. The surface was then 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.

For the binding inhibition assay, 25 µL of a CRP solution at a concentration of 1 mg mL^{-1} in a spotting buffer was applied to the surface immediately. For the sandwich assay, 25 µL of a solution at a concentration of 1 mg mL^{-1} of the C5-clone antibody was applied to the surface, and both were left to react for about 24 h. Prior to the measurement, the transducer was rinsed with milli-Q water and dried with nitrogen.

Assay procedure

Two different assay types, binding inhibition and sandwich assay, are used to measure CRP. Both assays take advantage of well-established surface chemistry. In both cases, the immobilised aminodextran layer reduces the nonspecific binding of antibodies and other serum proteins to the surface. To further reduce unspecific binding and thus improve the binding of the specific partners to the surface, the serum samples were diluted with milli-Q water to a factor of 1:100.

For the sandwich assay, the capture antibody (C5-clone) is covalently immobilised at the sensor surface. To prevent unspecific binding to the modified surface, the sensor is incubated once for 20 min with a PBS solution containing 0.1% Tween-20 directly before the measurements. Next,

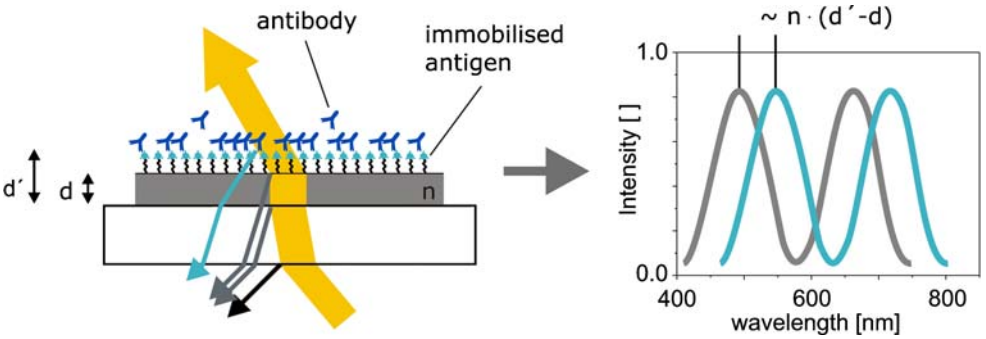

Fig. 2 Scheme of the RIfS detection method: *left* the reflected beams superimpose and the optical thickness of the transducer is changed by binding events on the surface; *right* the change of the interference spectrum is shown

400 μL diluted serum sample solution containing the analyte is slowly pumped over the sensor surface, followed by 400 μL buffer solution containing the labelled detection antibody. In this assay format, a high fluorescence signal indicates a high analyte concentration.

For the binding inhibition assay, the analyte CRP itself is covalently bound to the sensor surface. The total sample volume is 1,000 μL. Each measurement requires 900 μL serum, CRP-spiked and 1:100-diluted, which was mixed by the autosampler with 100 μL of an antibody stock solution containing the antibody in tenfold PBS and was then preincubated for approximately 5 min. The antibody binds the analyte during a defined incubation time. Whilst pumping the sample over the sensor surface, only antibodies with free binding sites can bind to the surface. This format leads to an inverse calibration curve: a high signal corresponds to a low analyte concentration, and vice versa.

To ensure the binding inhibition assay is quantitative, the binding of the antibody to the surface must be mass-transport-limited. This allows the signal to be dependent of the diffusion rate to the surface rather than dependent on the kinetics of the surface binding. The number of high-affinity binding sites at the surface has to be much higher than the number of antibodies used for the measurement. Therefore, small amounts of antibody are used and a huge excess of analyte is immobilised at the surface. This was demonstrated by additional reflectometric interference spectroscopy (RIfS) measurements as already described in the literature [12–14]. The general assay procedure for both formats is shown in Fig. 3.

For calibration of the biosensors, each concentration step is measured threefold, and the mean value and standard deviation (SD) for the replicates are calculated and depicted in a semilogarithmic plot. For the binding inhibition assay, all spiked sample measurements were normalised to the mean value of the blank measurements (nine measurements). For the sandwich assay, relative signals given by the RIANA are taken without normalisation. To fit the data set, a logistic fit function [14] with four free parameters (A_1, A_2, x_0 and p) was used, according to

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

where A_1 is the upper and A_2 the lower asymptote, respectively, for the binding inhibition assay; for the sandwich assay, A_1 is the lower asymptote and A_2 is the higher 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. The curve-fitting model used is based on an analytical expression, and this logistic function is used as a mathematical model to describe a sigmoid curve progression. This is a well-published and well-agreed approach to the quantitative evaluation of immunoassays in the scientific community [15].

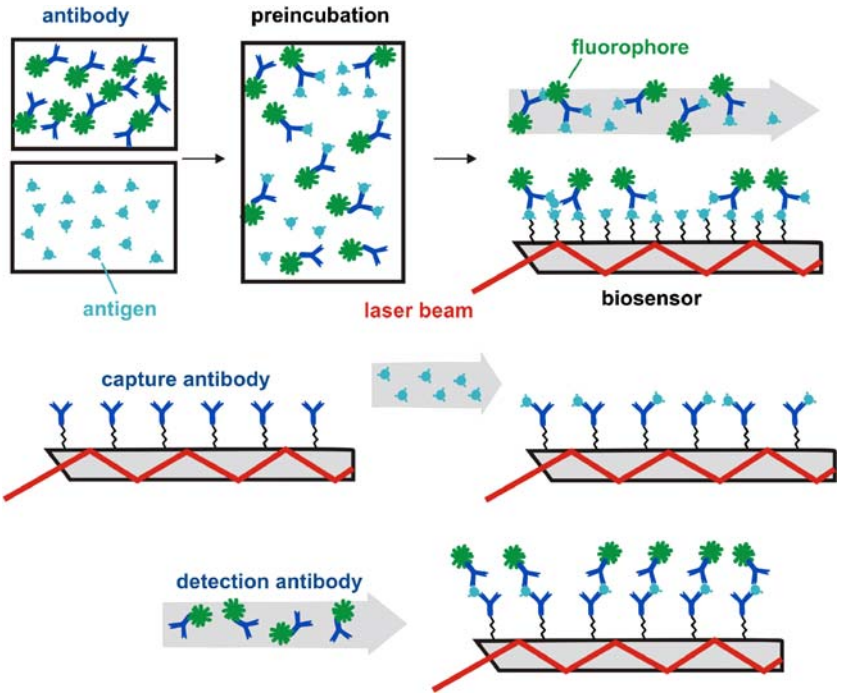

Fig. 3 Principle of the binding inhibition (*top*) and sandwich assay (*bottom*) procedures

In compliance with the IUPAC rules [16], the limit of detection (LOD) is calculated as three times the SD of the blank measurements (SD_{blanks}). The limit of quantification (LOQ) is calculated as ten times the SD of the blank measurements (SD_{blanks}).

Results and discussion

Development and optimisation of surface chemistry and assay conditions

A prerequisite for immunoassays is to establish a reliably working assay procedure, especially for reusable ones. Since the fluorescence-based detection technique which is used to carry out the quantitative calibrations cannot provide detailed information about the specificity of the binding procedure on the surface, the label-free detection method RIfS was used for preliminary investigations. The general approach towards the development of both assay formats is the same. First, the capture antibody (or antigen) has to be immobilised at the transducer surface, whereas the glass surface itself has to be protected to prevent unspecific binding. The binding of the antigen and the detection antibody (or detection antibody) then has to be verified. The last step includes the determination of a regeneration solution which ensures multiple measurements on the same chip. Several immobilisation techniques were tested, and for both assays the same dextran chemistry as described in detail in Assay procedure turned out to be suitable. This is due to the fact that dextran provides significantly more binding sites on the surface than chain-like polymers such as poly(ethylene glycol) (PEG), and thus a high surface loading with antibody is reached resulting in a high signal [10]. Together with its ability to reduce unspecific binding considerably, the dextran chemistry is suitable for both assay formats.

Next, different regeneration solutions were tested and a solution containing 6 mM NaOH and 0.6% EtOH qualified for regeneration of the sandwich assay and a 0.5% SDS solution for the binding inhibition assay, respectively. In both cases the regeneration process is complete when the signal of the RIfS measurements gets back to the baseline. Finding an ideal regeneration solution is a prerequisite for a reusable assay as it must be disruptive enough to remove binding partners and at the same time mild enough not to harm the binding sites at the surface.

RIfS measurements of the sandwich assay and the binding inhibition assay are depicted in Fig. 4. The curve of the sandwich assay shows 40-pm binding of the antigen which is injected at 100 s. Afterwards, the antibody is injected at 1,000 s resulting in the detection of an increase of the optical thickness of 100 pm. The subsequent

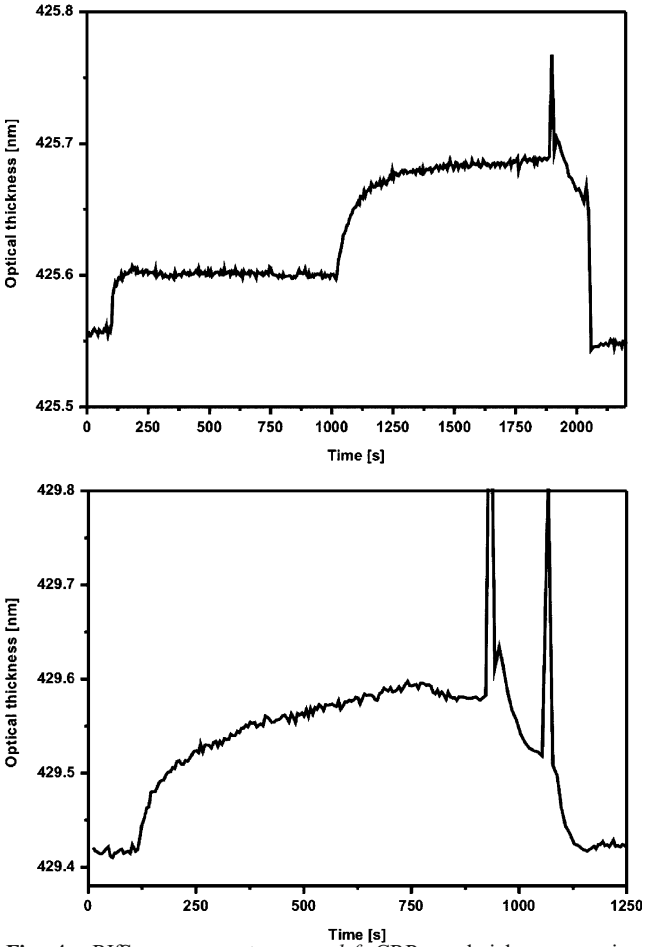

Fig. 4 RIfS measurement curves: *left* CRP sandwich assay using 10 mg L^{-1} CRP and 2 mg L^{-1} detection antibody; *right* binding inhibition assay using 30 mg L^{-1} detection antibody

regeneration at 1,900 s removes the binding partners completely, and the base level is reached again.

For the binding inhibition assay, antibody is injected at 110 s and increases the signal about 160 pm and

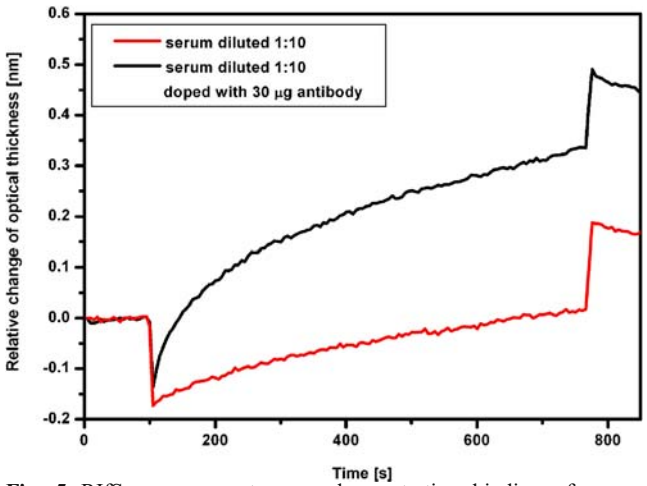

Fig. 5 RIfS measurement curve demonstrating binding of serum proportionally to antibody spiked serum (c(Ab) 30 mg L^{-1})

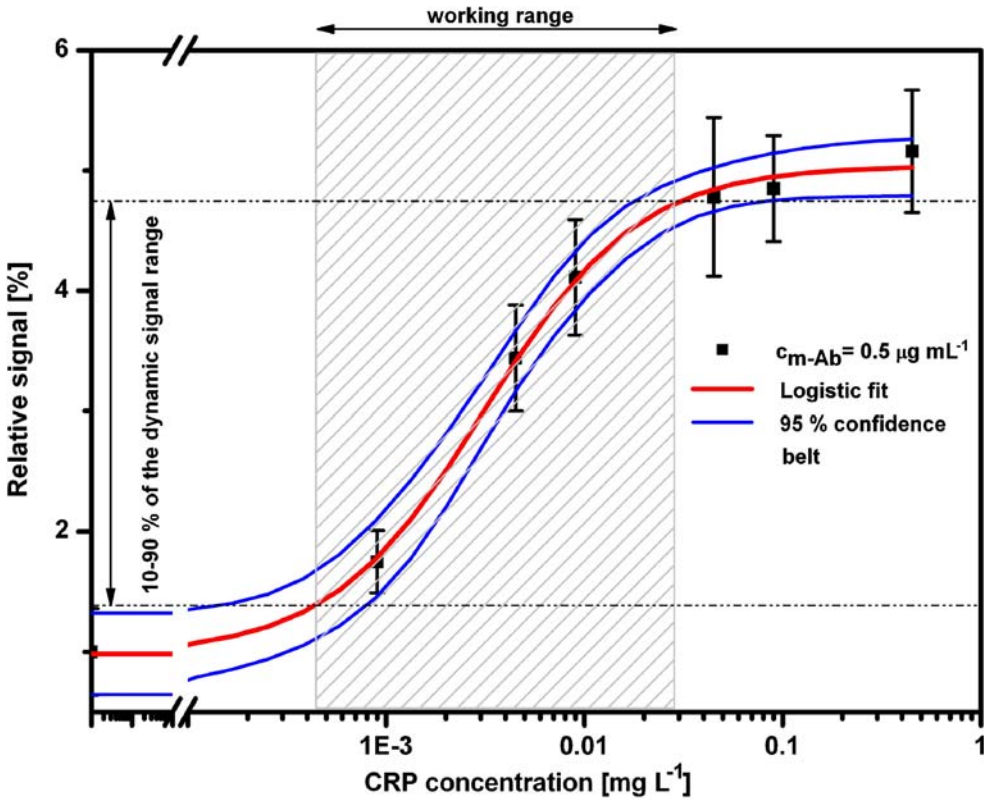

Fig. 6 Calibration curve and 95% confidence belt for CRP sandwich assay measured from 0.9 to 0.0009 mg L^{-1}. The detection antibody concentration (c_{mAb}) was 0.4 mg L^{-1} in each sample. The working range is shaded in *grey*

is completely reset by the regeneration solution injected at 900 s.

Concerning long-term stability, both assays allow up to 100 measurements (data not shown), which does not achieve the same stability conditions of assays used to determine endocrine disruptors [17] due to the lower stability of proteins in general compared with small organic molecules.

Based on this assay procedure, test measurements were carried out to determine the influence of serum. In principle, unspecific binding of serum to the surface cannot be prevented completely. However, RIfS measurements could prove that the specific antibody binding is highly superior to the unspecific serum protein adsorption. Two measurement curves with antigen immobilised at the surface are shown in Fig. 5. The first measurement (grey line) represents unspecific binding of serum to the surface. For the second measurement, the serum was spiked with 30 mg L^{-1} antibody. Due to the detection method, both curves show a leap of refractive index at the beginning and end of the sample injection because the setup is referenced to the PBS buffer system. Nevertheless, the antibody-spiked sample shows a factor-of-2.7 binding increase over the pure serum sample (460 pm vs. 170 pm). Comparing these results with the buffer measurements (Fig. 4, right), the antibody turns out to have better binding abilities in its physiological environment as the signal in serum is nearly doubled.

For these reasons, calibrations were carried out in 1:100-diluted serums as therein unspecific binding to the surface

Table 1 Summary of the measured relative signal values for the sandwich assay with corresponding errors (replicate measurements)

Concentration (mg L^{-1})	Calculated relative signals with corresponding error (%)
0	1±0.36
0.0009	1.75±0.26
0.0045	3.44±0.44
0.009	4.11±0.48
0.045	4.78±0.66
0.09	4.85±0.44
0.45	5.16±0.51

Table 2 Summary of the measured relative signal values for the binding inhibition assay with corresponding errors (replicate measurements)

Concentration (mg L^{-1})	Calculated relative signals with corresponding error (%)
0	100±2.19
0.000045	92.25±3.46
0.0045	79.03±1.15
0.045	48.17±8.8
0.09	14.28±7.11
0.45	3.06±0.51
0.9	0.8±3.54

Table 3 Summary of all parameters of the logistic function (A_1, A_2, x_0 and p) and validation parameters (LOD, LOQ) for both calibration curves

Parameter	Sandwich assay	Binding inhibition assay
A_1	0.98±0.11	97.01±1.19%
A_2	5.04±0.079	−6.85±2.62%
x_0 (mg L^{-1})	0.0031±0.0003	0.03±0.004
p	1.12±0.11	0.83±0.06
c_{mAb} (μg mL^{-1})	0.4	0.05
LOD (mg L^{-1})	0.13	0.055
LOQ (mg L^{-1})	2.30	0.5
Working range (mg L^{-1})	0.044–2.9	0.13–22.9

Parameter x_0 of the logistic function is equivalent to the test midpoint IC_{50}

should be negligible but also a suitable environment for the antibodies is provided.

The antibody concentrations have to be optimised directly on the TIRF setup as the RIfS setup does not allow for the amplifying factor of the fluorescence signal with TIRF. Test measurements recommended concentrations of 0.4 mg L^{-1} for the sandwich assay, and 0.05 mg L^{-1} for the binding inhibition assay (data not shown).

Calibration of the sandwich assay

As mentioned above, the sandwich format is only applicable for large analytes with at least two binding sites for antibodies. In the case of CRP, this requirement is met and a full threefold calibration could be carried out.

Figure 6 shows the results of the calibration of CRP with the sandwich assay. The dilution series starts with a 450 μg L^{-1} stock solution, and all lower concentrations were automatically diluted by the PAL HTS autosampler via concentration steps. These steps were used as stocks for the subsequent lower concentrations. The IC_{50} (parameter x_0 of the logistic function) could be obtained at 0.0031± 0.0003 mg L^{-1}. For the SD_{blanks}, we obtained a value of 0.11 and using the logistic function we calculated an LOD of 0.0013 mg L^{-1} and an LOQ of 0.023 mg L^{-1} for the diluted calibration series. Taking into account the dilution factor of 100, the LOD and LOQ for real samples is 0.13 mg L^{-1} and 2.30 mg L^{-1}, respectively. The relative signal values of the calibration curve are summarised in Tables 1 and 2, whereas the parameters of the corresponding logistic fits are shown in Table 3.

Calibration of the binding inhibition assay

Regarding the binding inhibition assay, only one binding site for the antibody is required. Nevertheless, a certain size

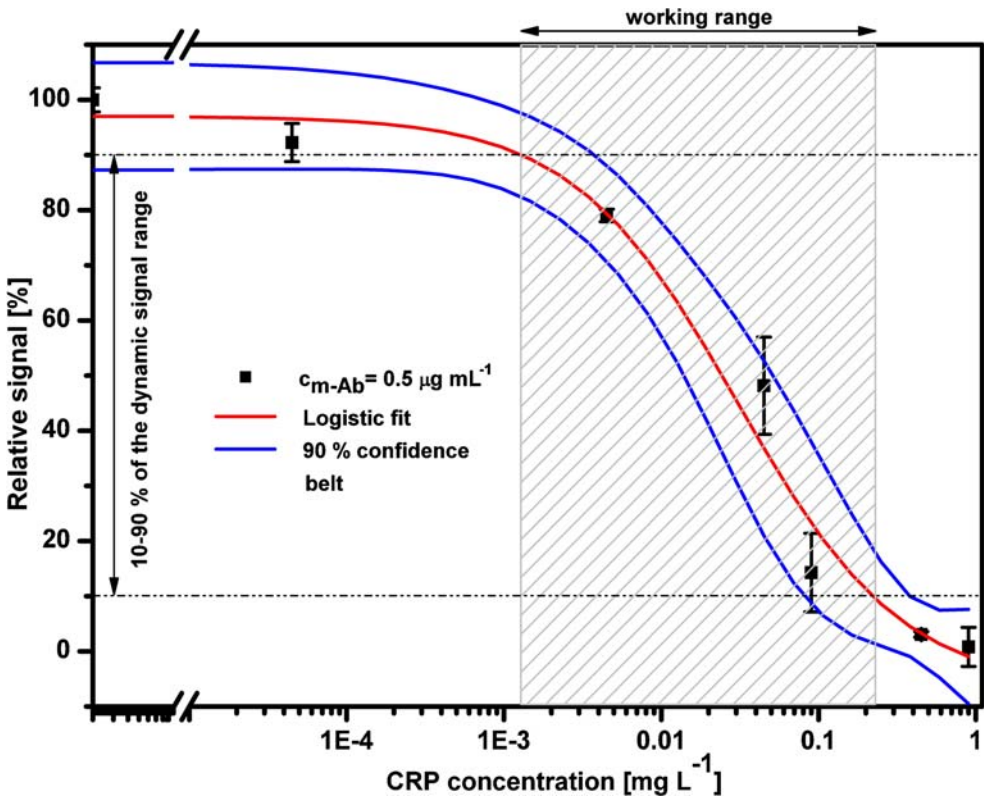

Fig. 7 Calibration curve and 90% confidence belt for CRP binding inhibition assay measured from 0.9 to 0.000045 mg L^{-1}. The detection antibody concentration (c_{mAb}) was 0.05 mg L^{-1} in each sample. The working range is shaded in *grey*

of the protein is necessary for immobilisation. Figure 7 shows the results of the calibration of CRP in the binding inhibition assay. The dilution series was accomplished according to the procedure discussed in Development and optimisation of surface chemistry and assayconditions. The IC_{50} (parameter x_0 of the logistic function) could be obtained at 0.03±0.004 mg L^{-1}. For the SD_{blanks}, a value of 2.19% was obtained, and with the logistic function an LOD of 0.00055 mg L^{-1} and an LOQ of 0.005 mg L^{-1} were calculated for the diluted calibration series. Taking into account the dilution factor of 100, the LOD and LOQ for real samples is 0.055 mg L^{-1} and 0.5 mg L^{-1}, respectively.

Comparison of the used assay formats

To rank these results, we first need to clarify the specification to a CRP immunoassay. CRP is an analyte which is always present in the human body. Compared with other inflammation markers such as the cytokines occurring only in the nanogram per litre range, CRP concentration in blood of young adults is always measurable in the range of about 1 mg L^{-1}. In the case of an actual serious bacterial inflammation, the CRP level can rise up to 500 mg L^{-1}. In between these two critical values, no exact statement can be given without information on more specific parameters. Thus, not just low limits of detection are required for the assessment of inflammations, but a reliable crude classification which is primarily quick, easily manageable and cheap.

The actual method to determine CRP in human serum is the ELISA. This method is usually carried out on nanotiter well plates. Therefore, every working step, including prearrangement, pipetting and washing, is manually accomplished by a skilled worker. This manual procedure is time-consuming, expensive and error-prone.

Our fully automated measuring procedure proved that both assay formats can detect CRP in the required range. Both assays show a high level of reliability which is assured by the given confidence belts and the low slope of sigmoid curves. These results provide a rather wide working range over approximately two orders of magnitude. Nevertheless, the working ranges of the two assays do not cover the same area. Using the sandwich format, the working area reaches from 0.044 to 2.9 mg L^{-1}, the working range of the binding inhibition assay from 0.13 to 22.9 mg L^{-1}. Considering the classification of CRP concentration mentioned above, a definite decision about a present inflammation can be made with both assay formats. In addition, the binding inhibition assay allows one to distinguish not only between samples from infected and healthy persons, but also between viral and bacterial infections.

However, the far more important fact is that our developed assays can measure high-sensitivity CRP (hsCRP) quantitatively in the required range between 1 and 3 mg L^{-1}.

Conclusion

It was possible to establish assay procedures for two different assay formats to measure CRP in human serum using a fully automated immunoassay system. A threefold calibration was carried out with the sandwich and the binding inhibition assays. Although, the samples were diluted, sufficient limits of detection could be reached, showing values of 0.13 mg L^{-1} for the sandwich assay and 0.055 mg L^{-1} for the binding inhibition assay. If the dilution factor is taken into account, curves cover an appropriate area to distinguish in principle between ill and healthy persons; moreover, both assays can detect hsCRP for cardiovascular risk assessment. There are some differences between the results of the two formats concerning standard deviations and working range. Nevertheless, for both assays the reliability is high enough to provide a valuable contribution to the diagnostic procedure. Thus, both assay formats are suitable for further development such as improvement of the surface stability which will also improve the precision of the assays. Furthermore, these approaches will lead to the development of a multianalyte assay which represents the next step towards a fully automated high-throughput analyser.

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