



CRP determination based on a novel magnetic biosensor

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Abstract

The c-reactive protein (CRP) is a very significant human blood marker for inflammatory processes and is routinely determined for many clinical purposes. The widespread and well established detection method for this ~115 kDa hepatic protein is the high-sensitivity ELISA assay (hsCRP-ELISA) in blood serum. New approaches in medical CRP diagnosis (e.g. for CVD, inflammatory bowel disease) require rapid quantification in native matrices. A novel CRP determination method based on magnetic detection is described and tested for human blood serum, saliva and urine. The detection principle is based on two different anti-CRP antibodies (monoclonal, IgG) for CRP trapment and labelling. The linear detection range of this immunosensor ranged from 25 ng/ml to 2.5 µg/ml and is therefore much more sensitive than typical hsCRP-ELISA-assays. © 2006 Elsevier B.V. All rights reserved.

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1. Introduction

1.1. C-reactive protein

The c-reactive protein (CRP) has been known since the 1930s as an acute phase protein of the immune response in humans. The CRP is a protein of ~115 kDa, consisting of five identical subunits of ~23 kDa each. These subunits form a circular structure, called pentraxin. The CRP is nowadays routinely checked in blood counts and other medical diagnostics because of its relevance as a significant marker for inflammatory processes. The blood serum concentration of CRP can rise up to 1000-fold upon acute inflammatory stimulus (Black et al., 2004). An elevated CRP level is also discussed as a reliable indicator for cardiovascular disease (CVD) (Mazer and Rabbani, 2004; Li and Fang, 2004), and chronic inflammatory processes like inflammatory bowel disease (e.g. Crohn's disease) (Vermeire et

al., 2004). In general, CRP can stimulate phagocytosis, bind to immunoglobulin and activate the classical complement pathway. CRP is therefore a significant keystone of the human immune response. Normal blood serum concentrations of humans range from 1 to 5 mg of CRP/l. As the protein is expressed by hepatocytes only upon stimulus, CRP levels may also be significantly below 1 mg/l. Protein levels higher than 5 mg/l are an indication of inflammatory processes.

In routine clinical analysis, CRP levels are determined by ELISA-tests (Dominici et al., 2004; Clarke et al., 2005). These assays are well established and detection limits down to 0.2 mg/l are available (hsCRP-ELISA).

As new insights into immune response and diseases like inflammatory bowel disease (IBD, e.g. Crohn's disease, ulcerative colitis, Cabrera-Abreu et al., 2004) and colon cancer (Erlinger et al., 2004) show incidences of CRP occurrence and influence, new detection methods are under examination. In particular, other matrices like saliva (paradontosis, Christodoulides et al., 2005), faeces (Crohn's disease and colon cancer) and lachrymal (macula degeneration, Seddon et al., 2004) are not accessible for a CRP determination yet. In addition, difficult matrices may be analysed by a magnetic

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CRP separation step and a subsequent magnetic quantification measurement.

1.2. Immunosensors

Immunosensors use the high specificity and attraction of the antigen–antibody binding process as a measurement basis. The most common immunosensors involve optical detection, e.g. ELISA. Nevertheless, many other platforms have been used for immunosensors, e.g. radioactive markers, microcrystals, field effect transistors (FET) and surface plasmon resonance (SPR).

1.3. Measuring principle using magnetic detection

Although magnetic beads have been established in life sciences for over a decade now, the idea of using their paramagnetic character for detection in magnetic field sensors is quite new. Several authors describe the interaction of magnetic beads with a magnetic field and the ensuing visualisation of binding effects (Arakaki et al., 2004), or use the beads as a separation and magnetic immobilisation platform (Ruan et al., 2004; Rossi et al., 2004).

Magnetic beads are small, mainly globular, iron oxide containing particles. They are available with diameters ranging from the lower nanometer scale up to 100 μm . The normal magnetic bead structure consists of many iron oxide (magnetite) crystallites, which provide the paramagnetic attraction of the particles to a magnet. Typical magnetite contents are 50–60% of the bead volume. These magnetic nanoparticles are usually embedded into an organic polymer, e.g. polyvinylalcohol (PVA). The core shelling allows different surface modifications of the beads, of which many different types are already commercially available. A frequently used type of beads is the streptavidin-coated magnetic bead. Its main application area is the labelling and separation of biotinylated DNA.

The use of magnetic beads for the separation of DNA, proteins and even cells has been extensively analysed in the past decade and has led to many commercial applications (Olsvik et al., 1994). Labelling of analytes with magnetic beads and their use in combination with separate measurable molecules has also been described by Wang (2005). These approaches use the magnetic bead as a binding platform or linker between the analyte and label, which also allows an easy state of the art separation of analytes from a sample simply by using a magnet. This principle can be used not only for separation and subsequent detection by additional markers, as described by Kim and Park (2005); additionally, beads can also be used as an immobilisation platform and as a directly detectable element (Brzeska et al., 2004). Many of the assays described in this context base on the influence of magnetic beads on a magnetic field provided by a resonant coil. These can be used in batch (Lu et al., 2005) and flow-through systems (Choi et al., 2002).

Several magnetic detection techniques have been implemented in order to quantify target substances. Two magnetic detection techniques have been employed to measure the magnetic response of the particles with respect to a magnetic excitation field. Susceptometry (Kriz et al., 1998), i.e. lock-in detection

of the response to a magnetic excitation at a single frequency, is the most sensitive technique. Magnetic immunoassays based on this technique have been shown with a number of magnetic field sensors, namely giant magnetoresistance (GMR) sensors (Megens and Prins, 2005; Schotter et al., 2004; Baselt et al., 1998; Edelstein et al., 2000), spin valves (Ferreira et al., 2003; Graham et al., 2003), Hall probes (Ejsing et al., 2004), and induction coils (Kriz et al., 1998, 2005; Lu et al., 2005). The other technique, relaxometry (Matz et al., 1998), is based on recording the transient time of the magnetic response of the particles during the off-time of a pulsed excitation field. By analysing the relaxation time of the particle's magnetisation, a distinction between the Néel relaxation of bound particles and the Brownian relaxation of unbound carriers is feasible. Since a high magnetic field sensitivity is required in this case, a high magnetic field sensitivity is needed, as may be achieved with a superconducting quantum interference device (SQUID) (Matz et al., 1998; Chemla et al., 2000). Relaxometric measurements have also been demonstrated using a fluxgate (Ludwig et al., 2005) or an induction coil (Astalan et al., 2004).

Our approach is based on a new technique, the so-called frequency mixing. We use a two-frequency magnetic field excitation and detect the magnetic response at a third frequency which is a linear combination of the applied frequencies (Krause et al., 2003).

Using a magnetic bead not only for labelling and separation of an analyte, but also directly for quantification, provides an easy and simple way of detection. The magnetite core of the bead mentioned earlier cannot only be magnetically attracted, but will also influence the magnetic field when positioned in an excitation coil. In our case, the magnetic beads are exposed to the magnetic field of two excitation coils using different frequencies in one measurement head. The detection coil consists of a balanced arrangement of a measuring coil and a reference coil, balanced such that the directly induced signal vanishes in the case without beads. Then, the differences in the magnetic field between the measuring coil and the empty reference coil due to the presence of magnetic beads in the measurement coil are detectable and quantifiable in very low concentrations. A measurement head for the advanced and high sensitivity detection of magnetic beads has been developed by the Research Center, Juelich, Germany, and SENOVA GmbH, Jena, Germany.

The measuring system has been constructed for batch analysis of the samples. Therefore, a special plastic column (volume 0.75 ml), including a solid phase filter (sintered polyethylene, pore diameter 50 μm) as an easy-to-handle application platform, can be used. This system is patented by SENOVA as "ABICAP[®] columns". The surface of the ABICAP[®] sintered filter can be modified, analyte capture molecules such as antibodies can be bound and subsequently labelled by magnetic beads. The beads bound to the captured analyte can be detected and quantified.

2. Material and methods

2.1. Chemicals

The monoclonal antibodies C2 and C6 (both monoclonal, IgG) and the human CRP antigen were received from BioTrend

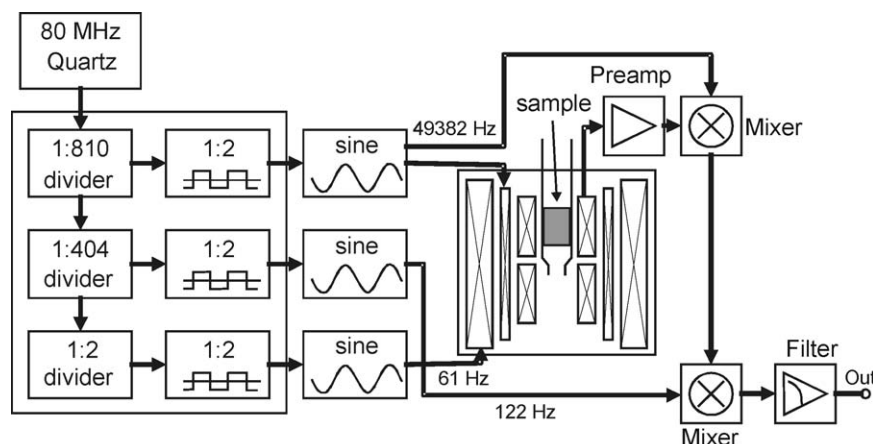


Fig. 1. Setup of the magnetic reader for the detection of magnetic nanoparticles using the frequency mixing technique. The frequency of an 80 MHz quartz oscillator is divided by three counters. The magnetic field B_1 of frequency $f_1 = 49382$ Hz is generated by a $1/1620$ division of the oscillator frequency, the magnetic field B_2 of frequency $f_2 = 61$ Hz by an additional $1/808$ division. After demodulation with f_1 , the frequency component $2f_2$ is demodulated in a second stage and used as the output signal.

(Cologne, Germany). Caseinbuffer (5.5%) was obtained from SDT (Baesweiler, Germany). Saliva (human, sterile filtrated, pooled) was received from the Center of Dento-Maxillo-Facial-Medicine, Department of Periodontology, Operative and Preventive Dentistry (Bonn, Germany). Blood serum (male human patients, pooled) was purchased from Sigma (Hannover, Germany). Urine samples were taken from a male test participant.

Water was obtained from a Millipore[®] unit. All other chemicals were purchased from standard commercial sources at analytical grade.

2.2. Magnetic beads

Magnetic beads were received from chemagen GmbH (Baesweiler, Germany). The beads were already coated with streptavidin. They were available at different particle sizes. The particles used are of type SAV1 (size 0.5–1 μm).

2.3. Columns

All measurements were performed using small ABICAP[®] plastic columns (volume 0.75 ml) which contained ABICAP[®] polyethylene (PE) sintered filters. These had a pore diameter of 50 μm . ABICAP[®] sintered filters and columns were received from SENOVA GmbH (Jena, Germany).

2.4. Magnetic reader

Recently, a novel detection technique for magnetic nanoparticles, based on frequency mixing at the nonlinear magnetisation curve of superparamagnets, was developed. This technique was filed for patent by Krause et al. (2003). Upon magnetic excitation at two distinct frequencies f_1 and f_2 incident on the sample, the response signal generated at a frequency representing a linear combination $mf_1 + nf_2$ is detected. The appearance of these components is highly specific to the nonlinearity of the magnetisation curve of the particles. The low-frequency field component is used to periodically drive the magnetic particles into saturation.

Therefore, its amplitude has to be sufficiently high, in the range of several millitesla. With low frequencies around 50 Hz, these field levels are easy to achieve. The component at low frequency, f_2 , serves as a periodic driver into the nonlinearity regime. Each time the low frequency field is close to its absolute maximum, the magnetic particles are magnetically saturated, whereas at the time of zero crossings, they are in their linear regime. Thus, the particles are switched between linear and nonlinear behaviour with a frequency $2 \times f_2$, equal to twice the driver frequency.

The high-frequency field component serves as a probe of the nonlinearity of the magnetisation curve. In order to achieve a high field sensitivity of the detection coil, a high frequency should be used. A frequency around 50 kHz constitutes a good compromise, yielding a high field sensitivity of the detection coil and an acceptable probing field amplitude of a few hundreds of microtesla. The response of the superparamagnetic particles to this probing field will differ depending on the respective state of the driving field. In case of saturation, the response will be lower than in the case of linearity. Thus, it is obvious that the simultaneous presence of both the driving field at frequency f_2 and the probing field at frequency f_1 leads to the appearance of a sum frequency (or beat frequency) at frequency $f_1 + 2 \times f_2$. In addition to this mixing component, other linear combinations also appear.

The experimental setup of our magnetic reader is depicted in Fig. 1. An 80 MHz quartz oscillator serves as a common reference. All frequencies are derived from this clock in order to ensure that they are phase-locked, thus eliminating problems from frequency drift. Coaxial coils provide magnetic excitation fields at two distinct frequencies $f_1 = 49.38$ kHz and $f_2 = 61$ Hz incident on the sample. By means of a differential pickup coil, the response signal of the sample inside the coil at a frequency $f_1 + 2f_2$ is detected. This mixing component was chosen since it is maximal for a vanishing static offset field. It is important to balance the differential pickup coil well against the middle excitation coil in order to minimize direct induction at $f_1 = 49.38$ kHz in the detection coil. Only if a balance level of about one part in 1000 is achieved, the preamplification factor can be chosen sufficiently high in order to overcome the input

noise of the mixer (MPY 100 from Burr-Brown). The signal is retrieved by first demodulating the probing frequency, f_1 , and subsequently demodulating at twice the driving frequency, $2f_2$. The total amplification factor of the detection stage exceeds 100,000. The output signal is low-pass filtered in order to suppress the carrier frequencies.

The magnetic reader system output was calibrated using different solutions of colloidal iron oxide particles. By serial dilution with PBS, 16 samples of an aqueous solution of magnetic nanoparticles with decreasing iron oxide concentration were prepared. A very large dynamic range of more than four orders of magnitude in iron concentration was observed. The output voltage was found to be linear in the range from 0.12 to 1300 mg/l iron concentration.

2.5. Antibody immobilisation

Biomolecules were immobilised adsorptively on polyethylene sintered filters. To enable optimal conditions, the ABICAP[®] columns were washed with degassed ethanol (96%). Afterwards, they were washed with ethanol–water (50/50) several times, followed by several washing steps with immobilisation buffer (carbonate-buffer, pH 9.5, 0.1 M). Capture antibody (C2) was immobilised for 1 h. Finally, ABICAP[®] columns were blocked using caseine 5.5 mg/ml in PBS-buffer (pH 7.3, 0.15 M) which were then ready-to-use. Columns could be prepared before use and stored for about 10 days at 4 °C without any loss of activity. All steps were performed as flow-through treatment.

2.6. Antibody modification

The anti-CRP antibody C6 was used as a detection antibody to allow binding of magnetic beads to antigen and sintered filters.

Since the magnetic beads were provided with a streptavidin surface, the antibodies were biotinylated at amino-residues using biotin-NHS (Strachan et al., 2004).

2.7. Measurements

Ready-to-use ABICAP[®] columns were washed using 0.5 ml PBS-buffer, and antigen (CRP in PBS-buffer, pH 7.3, 0.15 M) was supplied in different concentrations to the column (sample size 0.5 ml). After an additional washing step, pre-treated beads with detection antibody C6_{biotin} in PBS-buffer were flowed through the column (0.5 ml per column, bead content 125 µg), followed by two washing steps (each 0.5 ml PBS-buffer). Beads were incubated with detection antibody for 10 min.

The ABICAP[®] column was then analysed in the magnetic reader at room temperature (RT).

The saliva, urine and blood serum matrices were analysed both natively and spiked with different CRP-concentrations. For measurement, saliva and urine samples were buffered to pH 7.3. The human blood serum needed no additional buffering. All samples sizes were 0.5 ml.

3. Results

In order to enable a CRP detection using magnetic beads, appropriate antibodies had to be selected and established in a detection principle. For the purpose of fast and easy CRP-detection combined with a high specificity, the two anti-CRP antibodies clone C2 and clone C6 seemed to be most reasonable. One of them (C2) was used as capture antibody, adsorptively bound to the solid phase of the measurement column. The second antibody (C6) served as a detection label antibody, meaning

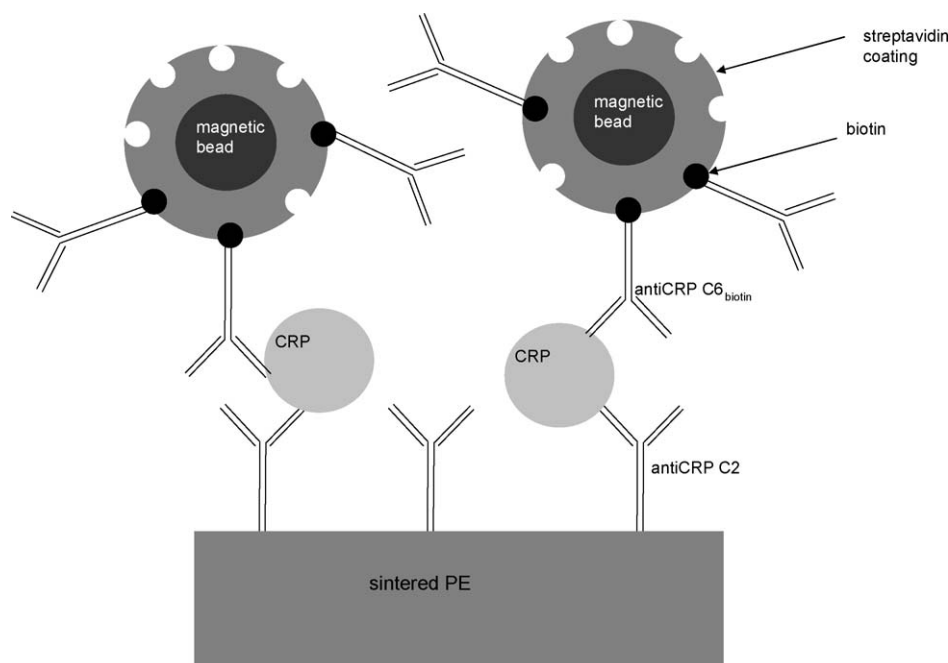


Fig. 2. Detection method using two different anti-CRP antibodies (C2 and C6) for CRP capturing and detection with magnetic beads. Magnetic bead binding results in a measurable alteration of the magnetic field.

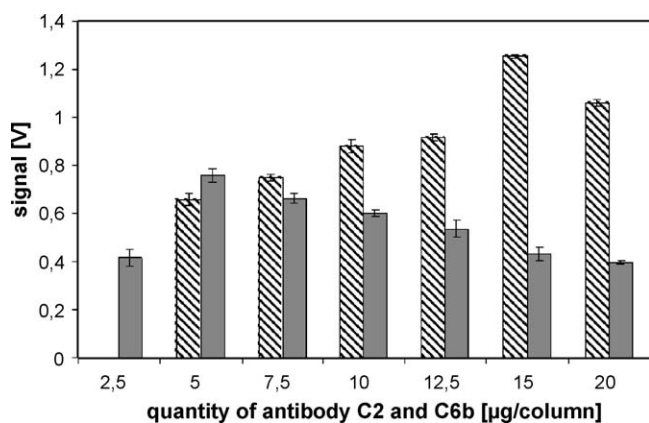


Fig. 3. Comparison of detection signals for 2.5 µg CRP/ml sample (sample size 0.5 ml) with different amounts of detection antibody (C6_{biotin}, grey) and capture antibody (C2, striped) attached to magnetic beads (final volume 0.5 ml). Only one antibody was varied, the corresponding partner antibody concentration was kept constant.

that it was biotinylated and attached to the streptavidin-coated magnetic beads. This antibody–magnetic bead complex interacts with the captured antigen (CRP, see Fig. 2) on the ABICAP[®] column and can be quantified by the magnetic reader.

For a detection method using magnetic beads, the most significant parameter is the bead type. The ABICAP[®] PE filters used had a pore diameter of 50 µm, but the use of beads with diameters higher than 1 µm resulted in increased unspecific binding on the column (tested diameters: 2 and 5 µm). The best results were obtained with beads of 0.5 µm in diameter. All beads had a pre-coating of streptavidin in order to allow the attachment of a biotinylated antibody.

Using an adsorptive immobilisation method, the amount of antibody provided has a very high influence on the final amount of bound antibodies. Therefore, the concentrations of capture and detection antibody were varied and the effects analysed. Fig. 3 shows the influence of different capture and detection antibody concentrations on single signals at samples with 2.5 µg CRP/ml. The given amount of detection antibody was attached to magnetic beads in a final volume of 0.5 ml. As a result of these optimisations, all further assays were performed with a quantity of 15 µg capture antibody (C2) and 5 µg detection antibody (C6_{biotin}) per ABICAP[®] column.

Another significant parameter for adsorptive antibody immobilisation is the pH value. Therefore, the influence of different buffer types and pH values was analysed. All buffers had a molarity of 0.15 M. A pH value of 9.5 in carbonate-buffer was found to yield the best condition for the immobilisation of the capture antibody (anti-CRP C2).

In order to analyse the optimal assay pH for antigen capturing and magnetic bead binding, several buffers at different pH values have been tested. All buffers were 0.15 M and phosphate, PBS or carbonate-based. A physiological pH value of 7.3 in PBS-buffer seems to be provide the best assay performance (see Fig. 4).

After the system optimisation, a standard calibration curve for the CRP detection based on magnetic beads was established. Measurements were performed at least three times (five times for low concentrations), all in pH 7.3 PBS-buffer (0.15 M). The mea-

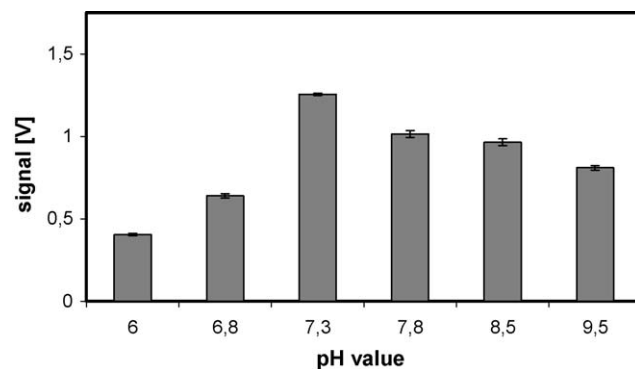


Fig. 4. Comparison of different assay pH values.

asurable dynamic range lasts is from 25.0 ng CRP/ml (28.5 pM) to 2.5 µg (2.9 nM) CRP/ml. Higher signals are obtainable, but leave the linear dynamic range (see Fig. 5). The detection limit of 10 times the technical background noise is located between 10.0 and 25.0 ng CRP/ml.

For the quantification of the assays liability and to determine the coefficient of variation (CV), an intra-assay was performed. Therefore, the assay was measured 12 times each for a blank, for a low (0.05 µg CRP/ml), medium (0.25 µg CRP/ml) and high (2.50 µg/ml) CRP-concentration sample. The resulting CVs were 3.57% for the blank, 5.64% for the low CRP-concentration, 3.71% for the medium CRP-concentration and 3.75% for the high CRP-concentration. The overall CV for the whole assay was 4.17%.

As this assay is aimed at CRP detection in different liquid matrices, an application for human saliva, urine and human blood serum was tested. The measurement of CRP spiked human saliva showed reasonable rates of recovery of typically 5% less CRP than spiked. No negative matrix induced effects, in comparison with the CRP determination in PBS-buffer, were noticeable. A native concentration of 38.5 ng CRP/ml was found in the pooled saliva.

To determine the CRP detectability, a simple calibration curve for human CRP in human blood serum was established (Fig. 6). The human blood serum contained about 1 mg CRP/ml on deliv-

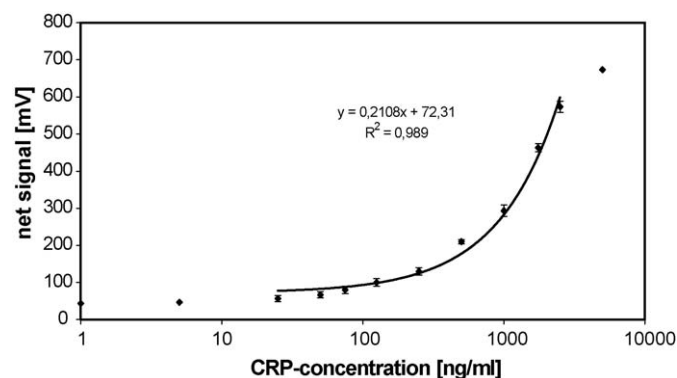


Fig. 5. Calibration curve of magnetic beads-based CRP biosensor assay in pH 7.3 PBS-buffer (0.15 M). Dynamic detection range is linear, but appears exponential due to the logarithmic concentration axis. Net signal denotes the signal without the blank value signal.

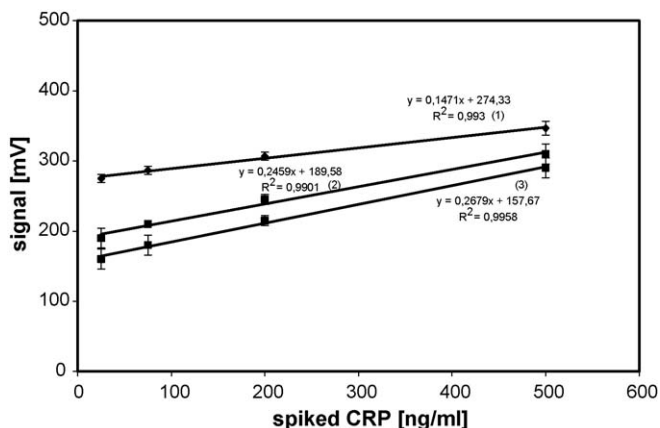


Fig. 6. Calibration curve of spiked CRP in human blood serum (1, pooled), human saliva (2, pooled) and human urine (3) samples.

ery, therefore the shown concentrations are spiked amounts of CRP. An analogous procedure was chosen for the CRP detection in urine and saliva, spiked amounts of CRP allowed to establish a calibration curve (Fig. 6).

4. Discussion

It has been shown that a CRP analysis using magnetic beads is possible. The detection of magnetic beads and the quantification in a special magnetic field reader offers a very sensitive and reliable detection method, and may be a platform for many other analytic assays in clinical, pharmaceutical and environmental research. The established sensing system is an immunosensor, and therefore offers the typical advantages of high specificity and sensitivity.

The optimal setup for this assay involves the use of 15 μg capture antibody (anti-CRP C2) on the ABICAP[®] column and 5 μg of biotinylated detection antibody (anti-CRP C6_{biotin}). The use of only 5 μg detection antibody results not only in the best signal response; it also decreases the necessary amount of modified (and therefore more expensive) antibody. Using higher amounts of detection antibody may result in an overcharge of the magnetic beads or may have negative steric effects on the antibody alignment on the bead surface. The optimal assay pH value of 7.3 is ideal for any physiological samples and measurements. This pH is not only most suitable for the capturing bio-component, but also for the use of, e.g. human blood serum as a matrix. Urine and saliva samples need to be buffered to this pH value.

A linear detection range for CRP of 25–2500 ng CRP/ml was found, which qualifies this assay system as a highly effective method for the CRP determination. Normal concentrations of CRP in human blood serum range up to 5 $\mu\text{g}/\text{ml}$ blood serum. Values higher than 5 $\mu\text{g}/\text{ml}$ indicate pathological findings such as inflammation. Typical values for a healthy person without any signs of inflammation are between 0 and 1 $\mu\text{g}/\text{ml}$ (Black et al., 2004). This range can be easily matched with the measurement principle described.

The established competitors for this type of CRP-biosensor are ELISA-tests. The newly available high sensitivity ELISA assays (hsCRP) exhibits a detection limit of 0.2 μg CRP/ml

(Dominici et al., 2004). The detection limits for normal ELISA CRP-assay are up to 2 μg CRP/ml. We conclude that the described CRP detection by magnetic beads is much more sensitive (detection limit 0.025 μg CRP/ml) and gives an advantage for measuring even very small CRP-concentrations. The overall CV of 4.17% is comparable to a typical ELISA assay (Wu, 2000) and perfectly acceptable.

The use of magnetic beads in CRP detection has also been described by other authors. Miao and Bard (2004) used the magnetic bead only as separation aid due to its magnetic attraction forces. The quantification of magnetic beads for the CRP detection has also been described by Kriz et al. (2005). In this publication, the authors describe the sedimentation of magnetic beads complexed with silica microparticles upon appearance of CRP in the sample. The content of magnetic beads in the sedimentated phase of the measurement vial is then analysed by a single magnetic resonant coil. The measurement needs only 11.5 min, but shows detection limits of 0.2 mg/l and a CV of 11%. The same research group published a rapid detection system based on the same technique (Ibraimi et al., 2005) which provides results after 5.5 min and has a detection limit of 3 mg/l and a CV of 10.5%. Our system is certainly more time consuming, but measurements can be performed within a total time period of 30 min. The most significant difference between both methods is the measurement head. Our system uses two different excitement coils in a frequency mixing mode. This enables the very low detection limit of 25 $\mu\text{g}/\text{l}$, accompanied by the low CV of 4.2%.

The first trials for CRP detection in human saliva and blood serum showed very positive results. The rates of spiked CRP recovery in saliva are very promising for an sensor application at dental practices for the detection of inflammatory processes in the mouth. The ability to calibrate the sensor in human blood serum for spiked CRP-concentrations in combination with already existing native CRP shows the independence of the analysis and the possibility of the application of this sensor system in clinical practice. CRP determinations in human blood serum are possible and reliable down to very small CRP-concentrations. The calibration curves in these matrices (Fig. 6) show minor differences between slopes and initial signals. These differences are caused by the matrix density and composition.

Disadvantages of this system are the sample size and the time for measurement. Sample sizes are currently 0.5 ml, which is quite high for blood serum analysis. In order to achieve a reduction of the required reagent amounts and thus the costs, future work will be directed towards a reduction of the sizes of the filter, of the column and of the measurement head of the magnetic reader. The required time for measurement (about 30 min) is quite fast, compared to an ELISA, but there are faster systems described. A significant feature of this sensor system is the possibility to extract CRP from difficult samples before measurement.

5. Conclusions

The development of a very sensitive and reliable immunosensor for the CRP detection based on magnetic beads and a special

measurement head has been shown. The linear detection range of 25 ng/ml to 2.5 µg/ml CRP covers the needs of CRP diagnostics. Further work is expected to lead to a reduction of the required sample volume and measurement time. The established method may be a useful detection platform for many other assays, especially in the time-critical field of point-of-care diagnostics. Therefore, the established CRP immunosensor using magnetic beads is a valuable and useful tool for significant diagnostic purposes, not only in the field of CRP detection in the context of cardiovascular and inflammatory bowel diseases.

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