Magnetic biosensor for the detection of *Yersinia pestis*

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Abstract

A novel type of magnetic-beads based magnetic biosensor is described for the detection of *Yersinia pestis*. Experiments were performed with the antigen fraction F1 of these bacteria. The magnetic sensor platform offers easy and reliable detection of *Y. pestis* by the use of magnetic beads for labelling and quantification in a single step due to their paramagnetic features. The system uses antiYPF1 antibodies as capture element on ABICAP® columns as core element of the magnetic sensor. Several immobilization methods for antibodies on polyethylene were exploited. The established biosensor has a linear detection range of 25–300 ng/ml *Y. pestis* antigen F1 and a detection limit of 2.5 ng/ml in buffer and human blood serum. The presented sensor system is small, simple, portable and therefore usable as off-lab detection unit for medical and warfare analytes.
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1. Introduction

Yersinia pestis is the causative agent of the plague, one of the oldest known infectious diseases (Mollaret et al., 1995). The so-called "Black Death" caused millions of victims in medieval and ancient times, but nowadays its occurrence is still reported in more than 20 countries around the world (Perry and Fetherston, 1997).

Y. pestis is a non-motile, Gram-negative bacterium discovered by Alexandre Yersin (Mollaret, 1995). The typical occurrence of a *Y. pestis* infection is the bubonic plague due to infectious flea bites; septicemic and pneumonic plague appear after aerosol contact and inhalation.

The use of *Y. pestis* in warfare as a biological weapon happened already in ancient centuries, but in times of increasing risk of bio-terrorism the deathly potential of this bacterium returns into the focus of governments and civilians. Especially the occurrence of infected patients in several countries around the world allows a very easy access to this highly dangerous bacterial species (Riedel, 2005).

The biosensor uses an immunosensoric detection method based on the *Y. pestis* YP19 antibody, which binds to the fraction 1 (F1) capsule protein. This capsule protein has antiphagocytic potential and is essential for the survival of the bacterium in mammalian hosts. The immuno-detection is therefore very reliable, because the F1 protein is unique to *Y. pestis* and present in living and dead cells (Benner et al., 1999).

This type of biosensors uses magnetic beads as detectable elements. The magnetic beads are detected in a magnetic field created in a special magnetic measurement head.

Several authors describe the influence of magnetic beads to a magnetic field and the accompanying visualisation of binding effects (Arakaki et al., 2004), or used the beads as separable and magnetic immobilizable platform (Ruan et al., 2004; Rossi et al., 2004). Splettstoesser et al. (2003) used magnetic beads with immobilized YPF1 antigen for the extraction of antibodies from serum and detected the beads by using a fluorescent label in a flow cytometry unit. A new approach is the use of magnetic beads as directly detectable labels by their paramagnetic properties.

Magnetic beads are small, mainly globular, iron oxide containing particles. They are available at diameter sizes of nanometers up to hundreds of micrometers. The normal magnetic bead organisation contains an iron oxide (magnetite) core, which provides the paramagnetic attraction of the particles to a magnet. Normal magnetite contents are 50–60% of the whole bead. This core is usually encased by an organic polymer, e.g. polyvinyl alcohol (PVA). The core shelling allows different surface modifications of the beads, from which many different types are already commercially available. A frequently used bead type is streptavidin-coated, their main application area is labelling and separation of biotinylated DNA.

The use of magnetic beads for the separation of DNA, proteins and even cells has been established for several years and has been utilized in many commercial applications (Olsvik et al., 1994). Labelling of analytes with magnetic beads and using these in combination with separate measurable molecules has also been described (Wang, 2005). These approaches use the magnetic bead as binding platform or linker between analyte and label, which also allows an easy but state of the art separation of analytes from a sample by simply using a magnet.

A new approach is the possibility to use a magnetic bead not only for labelling and separation of an analyte, but also directly for quantification. The magnetite core of the bead cannot only be magnetically attracted, it will also influence the magnetic field when positioned in a magnetic field coil. Our system uses two excitation coils and two detection coils in one measurement head. The measurement technique is based on applying two magnetic excitation fields of different frequency. Due to the nonlinearity of the magnetization curve of the superparamagnetic particles, frequency mixing components are generated which are detected by the differentially wound pickup coil (Krause et al., submitted for publication). The differences in the magnetic field between the measuring coil and the blank coil due to the presence of magnetic beads are then measurable and quantifiable (see Fig. 1). It was shown that this technique allows fast processing and a very large dynamic detection range. The measuring head for the detection of magnetic beads in small columns has been developed by the Research Center (Jülich, Germany), and SENOVA GmbH (Jena, Germany) and is described in Meyer et al. (2006) and in Krause et al. (submitted for publication).

The measuring system has been constructed for batch sample analysis. An ABICAP® column (volume 0.75 ml) including an ABICAP® filter (sintered polyethylene, pore diameter 50 μm) as cheap and easy to handle application platform was used. The polyethylene surface can be modified; analyte capture molecules like antibodies can be bound and further labelled by magnetic beads. The beads bound to the captured analyte can be detected and quantified.

2. Material and methods

Casein buffer (5.5%) was from SDT (Baesweiler, Germany). Amino-propyl-triethoxy-silane (APTES), succinic anhydride and N-hydroxy-succimide-biotin (NHS-Biotin) were from Fluka (Steinheim, Germany). N-hydroxy-succinimide (NHS), glutardialdehyde (GDA) and bovine serum albumin (BSA) were purchased from Merck (Darmstadt, Germany). Sulfo-HSAB was from Pierce (Rockford, USA). 4-Fluoro-3-nitrophenyl azide (FNPA) was from MP Biomedicals (Irvine, USA). Human blood serum (pooled) was purchased from Sigma-Aldrich (Steinheim, Germany).

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

The cell line G20 was obtained from SENOVA GmbH (Jena, Germany). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 5% heat-inactivated foetal bovine serum (FBS) (Gibco), glucose (4500 mg/l), L-glutamine (4 mM) and sodium pyruvate (110 mg/l) in T-flasks. Cultivation of cells was done at 37 °C with 5% CO_2 in a humidified incubator.

For the production of antibody, serum-free CD-Hybridoma Medium (Gibco) supplemented with GlutaMAX™ (Gibco) was used. The cells were grown for about 10 days, after which the supernatant was collected by centrifugation and kept at 4 °C until further purification.

One liter of the antiYPF1 antibody-containing supernatant was concentrated to 200 ml in a Quix Stand benchtop concentrator through a Xampler™ UFP-3-C-4A membrane with an exclusion limit of 3 kDa (A/G Technology Corporation, Needham, MA, USA). The average yield was 0.1–0.2 mg/ml of protein. The concentrated solution was filtered through a 0.2 μM cellulose acetate membrane (Sartorius, Goettingen, Germany) and thimerosal was added to a final concentration of 0.03%.

20 ml of the filtered solution were applied to a Sephadex G-25 superfine column (50 ml gel, XK 26/20, GE Healthcare, Amersham Biosciences) equilibrated with PBS buffer. The column was washed with 150 ml PBS at a flow rate of 2 ml/min. The antiYPF1 antibody-containing fraction was eluted between 20 and 50 ml and was concentrated up to 1 mg/ml with a 3 kDA MWCO Omega membrane (Pall Corporation, USA). The antibody-containing solution was sterile filtered through a 0.2 μM Minisart filter (Vivascience, Hannover, Germany) and stored at 4 °C. The average yield was 100–
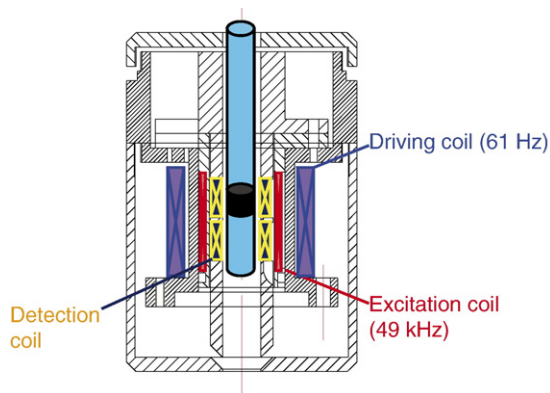

Fig. 1. Schematic drawing of the measurement system. The ABICAP® column is centered in the middle of the device. The polyethylene filter is shown in black.

150 mg pure antiYPF1 antibody per 1000 ml culture supernatant (Fig. 2).

Medium supernatant levels of antiYPF1 were assayed using an assay enzyme-linked immunosorbent assay (ELISA) with the F1 antigen (SENOVA GmbH, Jena, Germany). The assay was done as follows: Microlon 600, High binding ELISA plates (Greiner) were coated overnight at 4 °C with 100 μl of F1 antigen with a concentration of 1 μg/ml in 0.05 M carbonate–bicarbonate buffer, pH 9.6. Dilutions (100 μl) of the medium supernatant or the purified antiYPF1 in sample dilution buffer (0.15 M PBS+0.5% BSA, pH 7.4) were added and were incubated for 45 min at 37 °C. The plates were washed three times with 300 μl 0.15 M PBS+0.05% Tween 20 followed by an incubation of 100 μl ImmunoPure goat anti-mouse IgG (H+L), peroxidase conjugated (Pierce, Rockford, USA) at a dilution of 1/10,000 or 1/20,000 in sample dilution buffer. After the washing, the enzymatic activity was revealed with the SeramunBlau slow (ELISA) TMB/substrate solution (Seramun Diagnostics GmbH, Dolgenbrodt, Germany) and measured at 450 nm with a Dynatech MR 5000 microtiter plate reader. 18 pg and 250 pg antiYPF1 could be detected at dilutions of 1/10,000 and 1/20,000 of the anti-mouse IgG antibody respectively. The antibody showed no loss of reactivity during the purification (Fig. 3).

No cross-reactivity to other Yersinia species and non-Yersinia species was observed.

Magnetic beads were obtained from Chemagen GmbH (Baesweiler, Germany). The beads of type SAV1 (size 0.5–1 μm) were coated with streptavidin.

All sensor measurements were performed using small ABICAP® plastic columns (volume 0.75 ml) which contained ABICAP® polyethylene (PE) sintered filters. These had a pore
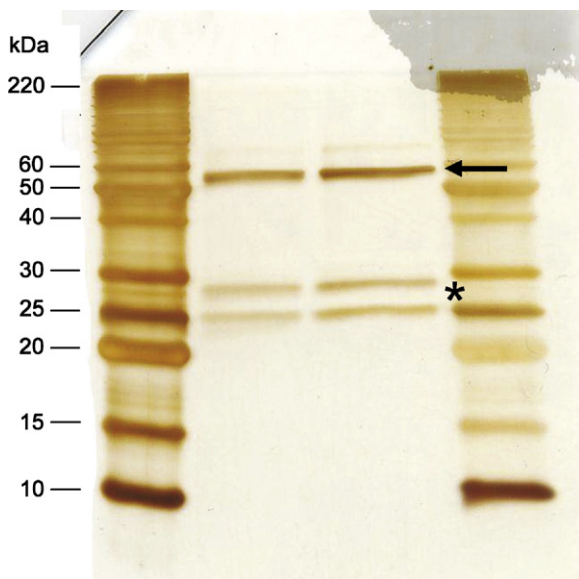

Fig. 2. Gel analysis of antiYPF1 antibody. Gel electrophoresis was done on a Coomassie stained 15% SDS-polyacrylamide gel. Lanes 1 and 4 contain Benchmark protein ladder (Invitrogen). Lane 2 contains antiYPF1 antibody from the cell line G20 culture supernatant. Lane 3 contains purified and concentrated antiYPF1 antibody. The heavy chains (55 kDa) are marked with an arrow and the light chains (25–30 kDa) are marked with a star.
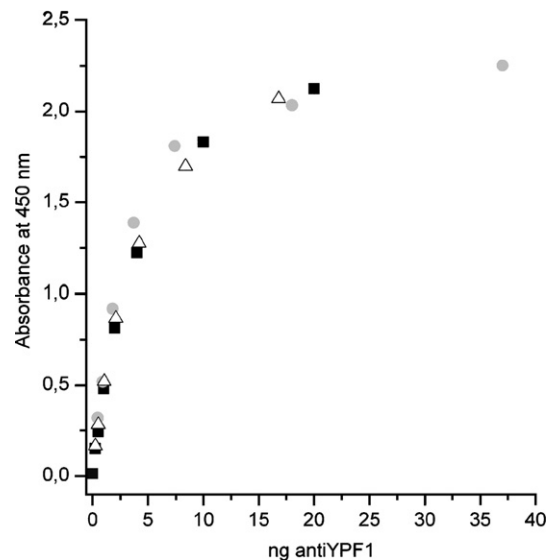

Fig. 3. ELISA for the detection of antiYPF1. The antibody was stable during purification. Grey circles, antibody from medium supernatant. Open triangles, antibody after buffer exchange on Sephadex G-25. Black squares, antibody after final concentration.

diameter of 50 μm. Sintered compacts and columns were obtained from SENOVA (Jena, Germany).

Biomolecules were immobilized on ABICAP® polyethylene sintered filters by different techniques. All immobilization steps were performed directly in the ABICAP® column.

2.1. Self-organised immobilization

ABICAP® PE sintered compacts were washed and degassed in ethanol (96%). Afterwards, PE sintered filters were washed in ethanol–water (50/50) for several times, followed by several washing steps in immobilization buffer (carbonate buffer, pH 9.5, 0.1 M). Antibody antiYPF1 was immobilized for 1 h using 10 μg of antibody per ABICAP® column. Finally, ABICAP® PE sintered filters were blocked using 5.5 mg/ml casein in PBS buffer (pH 7.3, 0.15 M).

2.2. NHS/DCC immobilization

ABICAP® PE sintered filters were treated with APTES to create an amino-surface for further immobilization steps. The use of succinic anhydride, DCC and NHS created an active ester residue for the covalent coupling of antibodies (carbonate buffer, 0.1 M, pH 8.3) at amino-residues. 10 μg of Y. pestis antiF1 antibody were used per column. Remaining active groups were blocked using BSA (2 mg/ml in same carbonate buffer).

2.3. GDA immobilization

For this immobilization method, sintered ABICAP® PE compacts with amino-surface were used (same method as in NHS/DCC) and incubated for 12 h in water (pH 8.0) containing 10 g/l GDA. After washing, antibodies (10 μg per sintered compact) were coupled in carbonate buffer (0.1 M, pH 8.3) and
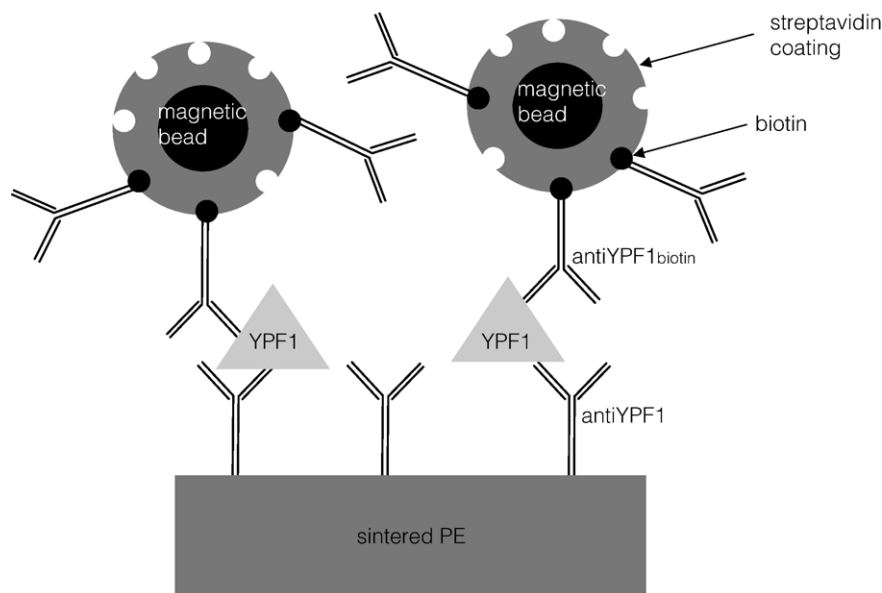
Fig. 4. Detection principle using antiYPF1 antibody for capturing of YPF1 antigen and detection with magnetic beads coupled to biotinylated antiYPF1 antibody. Magnetic beads binding results in an alteration of the induced magnetic field.

the remaining active groups blocked using BSA (2 mg/ml in same carbonate buffer).

2.4. Photolinkers

Photolinkers used were 4-Fluoro-3-nitrophenyl azide (FNPA, soluble in methanol) and Sulfo-HSAB (water-soluble). Antibodies were coupled in immobilization buffer (carbonate buffer, 0.1 M, pH 8.3) using 10 µg of antibody per sintered compact. Immobilization was finalised by blocking remaining reactive groups by using BSA (2 mg/ml) in immobilization buffer.

The antiYPF1 antibody was also used as detection antibody to allow binding of magnetic beads to antigen and sintered compacts. As magnetic beads were provided with a streptavidin surface, antibodies were biotinylated at amino-groups using biotin-NHS (Strachan et al., 2004).

To enable an optimisation process, several variables of this assay were analysed. Ready-to-use prepared columns (containing immobilized capture antibody) were washed using 0.5 ml PBS buffer 0.15 M, pH 7.3 and sample (YPF1 in 0.5 ml PBS buffer, pH 7.3, 0.15 M or in 0.5 ml human blood serum) was applied in different concentrations to the column. After an additional washing step (0.5 ml PBS), pre-treated beads with biotinylated detection antibody in PBS buffer (0.15 M, pH 7.3) were guided through the column, followed by two washing steps (0.5 ml PBS). The column was then analysed in the magnetic reader at room temperature (RT).

3. Results

The measuring principle is based on the capturing and labelling of the *Y. pestis* F1-antigen on the ABICAP® PE sintered filters inserted in small columns, which were placed in the central part of the magnetic sensor. The amount of magnetically labelled antigen can then be quantified in the magnetic detection system. For this purpose, biotinylated detection (label) antibody antiYPF1 and magnetic bead were previously coupled by a biotin–streptavidin system. The measuring principle is shown in Fig. 4.

In order to find an optimal surface modification and immobilization technique for the antibody immobilization on the ABICAP® PE filters, several methods were exploited as shown in Fig. 5.

The use of photolinkers resulted in lower assay signals. Best results were archived using covalent (GDA and NHS/DCC) methods and adsorptive binding.

For a better comparison of the best three immobilization methods, calibration curves for each method were determined (Fig. 6). The adsorptive immobilization method for antiYPF1 showed the highest signals and the highest calibration curve slope. The most suitable antibody immobilization was therefore
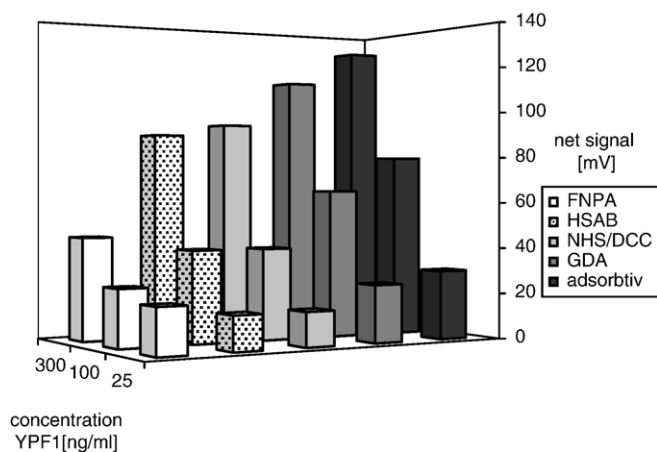

Fig. 5. Comparison of different antibody immobilization methods (FNPA = fluoro-nitrophenyl azide, HSAB = sulfo-HSAB, NHS/DCC = covalent method using active NHS-ester, GDA = glutardialdehyde).
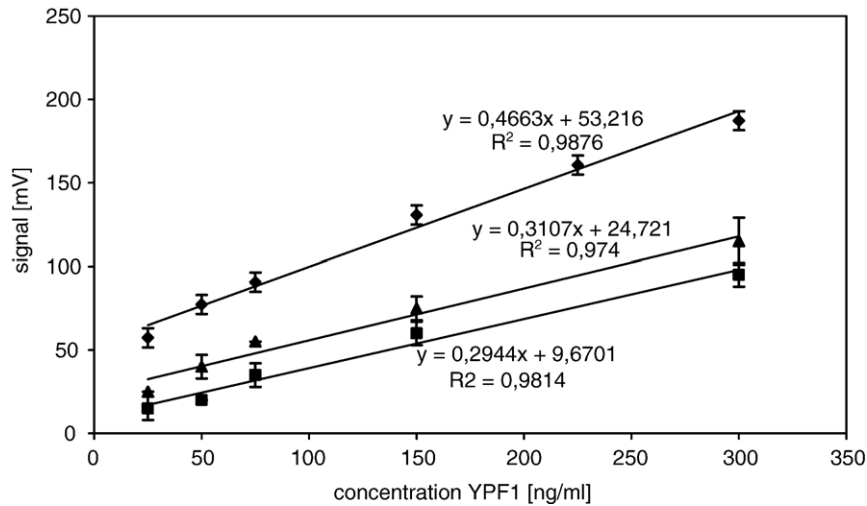
Fig. 6. Calibration curves for YPF1 by using the three best immobilization methods. (♦) Adsorptive immobilization, (▲) GDA immobilization method, (■) NHS/DCC immobilization.

the adsorption of the antibody on the hydrophobic surface of the PE-material. Because of its simplicity and very low standard deviations (see Fig. 6), this method was chosen for further developments. Further experiments also showed a good stability of the immobilized antibody on the polymer.

Furthermore, optimal conditions for the adsorptive antibody immobilization on the PE sintered filters were analysed. Testing of different buffer types, pH values and blocking agents (e.g. BSA) resulted in varying detection, and therefore, immobilization results. The best antibody immobilization was achieved with a 0.1 M carbonate buffer pH 9.5.

For further performance optimisation, the optimal antibody concentration coupled to the magnetic beads was determined. Antibody concentrations of 2.5 μg up to 20 μg per analytical volume for one column (which is 0.5 ml of 1:100 diluted magnetic beads) were analysed. The optimal antibody amount per column was 5 μg antibody per analytical volume.

Another important factor is the pH of the reaction buffers. Different buffer types, molarities and pH values were compared. The use of carbonate-based buffers of pH 8 up to 9.5 resulted in

quite low signals with low standard deviations. The use of phosphate buffers with pH lower than 6.5 showed extremely low signals. Best results were obtained with PBS buffers of pH 7.0–8.0. Testing different molarities showed that a 0.15 M PBS buffer pH 7.3 was most appropriate.

Finally, the optimised system was used for establishing a calibration curve for YPF1 antigen in PBS buffer (pH 7.3) and human blood serum (unbuffered) using 5 μg detection antibody YPF1 (biotinylated) per 0.5 ml diluted (1:100) magnetic bead suspension and in columns containing PE sintered filters (Abicap®) with adsorptively bound antiYPF1 capture antibody. The calibration curve is shown in Fig. 7.

In order to determine the assays liability and coefficient of variation (CV), an intra-assay for the YPF1 detection in PBS buffer and human blood serum was performed. Therefore, the assay has been measured 12 times for a blank, low (25 ng/ml), medium (100 ng/ml) and high (300 ng/ml) YPF1 concentration sample. The resulting CVs for measuring in PBS buffer were blank 8.2%, low 6.9%, medium 3.8% and high 5.3%. The overall CV for this assay was found to be 6.1%. The determined
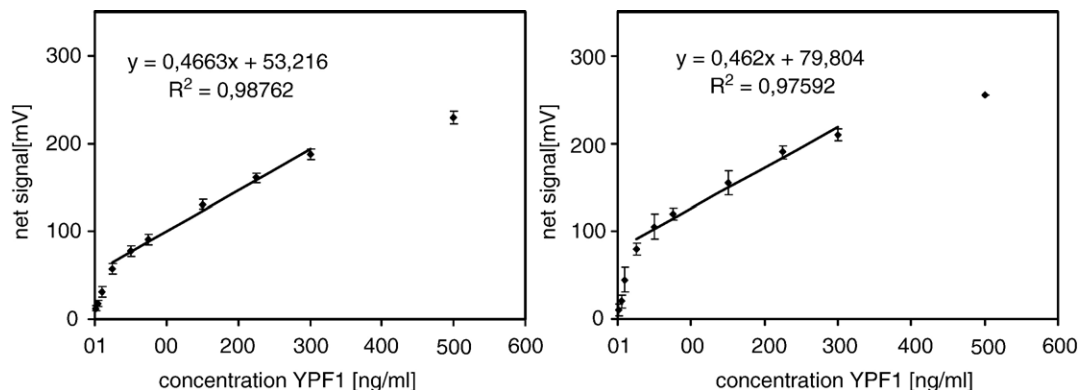

Fig. 7. Calibration curve for the detection of YPF1 antigen in PBS buffer (0.15 M, pH 7.3, left) and human blood serum (unbuffered, right) using optimised assay conditions.

CVs for measuring in human blood serum were blank 9.9%, low 8.4%, medium 6.1% and high 5.8% with an overall CV of 7.5%.

4. Discussion

Detection and quantification of *Y. pestis* antigen F1 by using magnetic particles has been successfully developed. The typical features of this unique detection system are its high specificity and sensitivity. Using only one monoclonal antibody against the YPF1 protein was possible because of the special protein characteristic. The YPF1 protein appears normally as 7-mer or 14-mer subunits (Tito et al., 2001) and therefore offers many epitopes.

The optimal setup for this biosensor assay included adsorptive antibody immobilization on PE-sintered compacts (ABICAP®). The higher performance of the adsorbance bound antibodies in comparison to other methods may be explained by the special material properties of PE surfaces. The hydrophobic surface allows unspecific adsorption of proteins with hydrophobic areas. Chemical immobilization methods tend to be complicated on this type of polymer matrix, but adsorptive immobilization on the cleaned and activated surface is a good alternative, especially because of the easy immobilization protocol.

The optimal assay pH value of 7.3 in physiological PBS buffer is most suitable for the detected antigen, but also offers advantages for measuring real samples. Possible samples are blood serum or bubonic ichor. The use of this magnetic bead-based sensor with difficult matrices like blood serum has been shown.

The linear detection range observed for YPF1 of 25 up to 300 ng/ml in buffer and human blood serum makes this assay system suitable for the detection and quantification of *Y. pestis*, which can be applied as cell lysate, inactivated or disrupted cells. A measurement of living cells extracted from tissue samples and applied in buffer is also possible. Suspect samples or typical warfare products as aerosols and dusts can be suspended in PBS buffer and also measured with this assay system. The detection limit of 2.5 ng/ml allows a reliable analysis of low concentrated *Y. pestis* F1 protein. Slight differences between the slopes of the linear detection ranges and higher standard deviations in the human blood serum calibration are caused by this complex and difficult matrix.

An additional major advantage of using magnetic beads is their possible separation from critical samples or difficult matrices by using their magnetic attraction forces. They can be easily extracted by using a magnet. Using this feature, samples can be pre-extracted and enriched.

Although no direct inter-assays between the described device and already established systems could be performed, a performance comparison to different systems is possible. Several reports have described detection systems for *Y. pestis* using PCR techniques (Chase et al., 2005; Hindson et al., 2005; Selvapandiyan et al., 2005). Advantages of these PCR based systems are certainly the very high sensitivity which can detect up to 50 cfu. Disadvantages are the highly complex systems and their time consuming measurement due to the PCR, but PCR

techniques will always be more sensitive than immuno-based detection systems. Rider et al. (2003) used a bioluminescence system based on B-cells for the detection of *Y. pestis* cells, the so-called CANARY sensor. This is a very sensitive (detection limit of 50 cfu), but also a very expensive, complex system which is certainly usable in laboratories but is unsuitable for field diagnosis. The magnetic bead-based system described here is small, simple and independent from any laboratory equipment. Therefore, measurements can be performed at the site of occurrence of the plague or where critical biohazardous materials are suspected (e.g. warfare bio-components or weapons).

Many presented detection systems for *Y. pestis* use ELISA tests against the F1 protein (Splettstoesser et al., 2004) or the LcrV antigen (Gomes-Solecki et al., 2005). Typical detection limits were 4 ng/ml for the F1 protein and 0.1 ng/ml for LcrV. Cao et al. (1995) report a detection limit for *Y. pestis* F1 of 5 ng/ml using a fiber optic biosensor; the linear detection range was found to be 50–400 ng/ml. A very sensitive system based on particle–antibody detection has been described by Biagini et al. (2005) with a detection limit of 0.3 ng/ml YPF1. A magnetic bead-based system has been described by Nikitin et al. (2006). Gomes-Solecki et al. (2005) discuss the advantage of an LcrV detection system for *Y. pestis* diagnostic because LcrV can be found in all *Y. pestis* strains, whereas some rare F1-deficient strains have already been found (Anisimov et al., 2004). But establishing a sensor system not only for medical diagnostics, but also for biological warfare, it has to be noticed that the LcrV protein is only secreted in the medium in growing cultures. When *Y. pestis* cells should be used as biological weapon, the use of stabilised (non-growing) cells is necessary, which cannot be detected by LcrV analysis.

A very interesting system has been described by Splettstoesser et al. (2003). This system uses a flow cytometry unit for the particle detection and quantification, which is quite complex and not easily transportable detection system and requires at least 1 h before any result is obtained. On the contrary, the magnetic detection system described here needs only a few minutes for sample preparation and entire measurement.

5. Conclusion

A new biosensor for the detection of *Y. pestis* F1-capsule protein has been established. The detection principle is based on the detection and quantification of magnetic beads by two frequency mixing resonant coils. The shown detection limits of 2.5 ng/ml in PBS buffer and human blood serum and the linear detection range of 25–300 ng/ml F1 are comparable to other high sensitivity and fast biosensor methods. The system offers the user an easy, transportable and highly sensitive measuring system.

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