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ORIGINAL PAPER

Development and characterization of new rat monoclonal antibodies for procalcitonin

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Abstract The development of selective and sensitive biological recognition elements, e.g., antibodies, for the detection of relevant blood markers is a great challenge in the field of biosensors. In this context, five new rat monoclonal antibodies (mAbs) for procalcitonin (PCT), a marker for bacterial infection and sepsis, were developed and characterized. One mAb, PROC1 3G3, was used as capture antibody. Four mAbs, PROC4 6C6, PROC4 6B2, PROC4 1G3, and PROC4 1D6, were used as detection mAbs, either as Protein G-purified or as biotinylated mAbs. A surface plasmon resonance (SPR) biosensor was used to characterize the antigen–antibody biomolecular interactions. The capture mAb (PROC1 3G3) has an equilibrium dissociation constant (K_D) of 3.42×10^{-8} M. All four detection mAbs (PROC4 6C6, PROC4 6B2, PROC4 1G3, and PROC4 1D6) are of high affinity (K_A=2.81–6.11 × 10^8 M^{-1}; K_D=1.64–3.56 $\times10^{-9}$ M) and have moderate dissociation rate constants (k_d=1.70–2.40$\times10^{-3}$ s^{-1}). Four different sandwich enzyme-linked immunosorbent assays (ELISAs) with standards of human recombinant (hr) PCT, using PROC1 3G3 as capture mAb and PROC4 mAbs as detection mAbs, respectively, led to highly specific determinations of PCT without cross-reactivities to calcitonin and katacalcin. The lower limits of quantification (LLOQ) for hrPCT (in 40 mM phosphate-buffered saline (PBS), pH 7.6) with these assays ranged from 2.3 to 12.8 μg L^{-1}. In addition, sandwich ELISAs were set up with biotinylated PROC4 mAbs, and with hrPCT in 4% human serum albumin (diluted 1:10 in 40 mM PBS, including 1:5 (v/v) LowCross Buffer®). The LLOQs of these sandwich assays ranged from 4.1 to 6.0 μg L^{-1} and were thus much closer together for the different assays. With the latter assay setup (PROC1 3G3 as capture mAb, PROC4 6C6–biotin as detection mAb) a first collection of five serum samples was determined (healthy volunteers, unspiked, and spiked). Recovery rates for the spiked samples ranged from 98.3 to 115.7%. The newly developed anti-PCT mAbs should find broad applications in immunosensors for point-of-care diagnostics of sepsis and systemic inflammation processes.

Keywords Procalcitonin · Katacalcin · Calcitonin · Monoclonal antibody · Sandwich ELISA · Surface plasmon resonance

Abbreviations

BSA	bovine serum albumin
D	dalton
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	enzyme-linked immunosorbent assay
HBS	HEPES-buffered solution
HRP	horseradish peroxidase
HSA	human serum albumin
IgG	immunoglobulin G
IC_{50}	inhibitory concentration 50%, test midpoint of the standard curve
K_A	association constant
K_D	dissociation constant
KLH	keyhole limpet hemocyanin
LLOQ	lower limit of quantification

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LOD	limit of detection
mAb	monoclonal antibody
NHS	*N*-hydroxysuccinimide
OVA	ovalbumin
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PCT	procalcitonin
POCT	point-of-care testing
RU	resonance units
SPR	surface plasmon resonance
TMB	3,3′,5,5′-tetramethylbenzidine

Introduction

Over the last decade, procalcitonin (PCT) became more and more popular as a specific and early marker for microbial infections and sepsis [1]. The detection of PCT levels in serum is decisive for early diagnostic reasons, but also for the monitoring of the effectiveness of the associated antibiotic therapy. PCT is a 116 amino acid polypeptide precursor of calcitonin with a molecular weight of 13 kDa. The existence of the prehormone was proven for the first time chromatographically in 1975 [2], while its amino acid sequence was resolved 10 years later [3]. In healthy persons, PCT is produced by the C-cells of the thyroid, and its concentration in serum is below 0.1 μg L^{-1} [4]. In the case of severe bacterial, parasitic, or fungal infections, the synthesis of PCT is induced by inflammatory cytokines (IL-1β and TNF-α) and is believed to take place in alternative sites such as liver [5] and peripheral mononuclear cells [6]. Depending on the severity of the infection, the concentration of PCT in the circulation increases up to several thousand-fold compared with physiological concentrations: PCT levels of 10 μg L^{-1} reflect systemic bacterial infections, whereas sepsis is suggested for concentrations ranging from 2 to 10 μg L^{-1} [1].

The first specific monoclonal antibodies (mAbs) for PCT were developed in 1988 and allowed its plasmatic detection without cross-reaction with the mature calcitonin [7]. Later, antibodies binding procalcitonin—but not free calcitonin, free katacalcin, and free N-procalcitonin—were patented [8]. The quantification of PCT as biomarker is roughly restricted to quantitative and qualitative chemiluminescence immunoassays or automated platforms available from BRAHMS AG, Hennigsdorf, Germany [9–12]. Recently, another clinical auto-analyzer for PCT measurements in serum or plasma was reported [13], but this system also uses antibodies from BRAHMS. Although rarely used, PCT can alternatively be measured using flow cytometry [14].

The aims of the present study were: (a) to raise specific and sensitive anti-PCT mAbs in rats (or mice) by immunization of immunogens with peptide sequences of the different domains of the prohormone; (b) to assess the interaction between anti-PCT mAb, immobilized on a chip surface, and its target analyte using a biosensor platform; (c) to define the kinetic parameters of the binding of the detection antibodies with procalcitonin; (d) to develop specific sandwich enzyme-linked immunosorbent assays (ELISAs) for measuring PCT; and (e) to have all immunochemical reagents (mAbs, labeled mAbs, etc.) available in-house for the development of new immunosensors for point-of-care analysis. Like for many other biomarkers, point-of-care testing (POCT) for PCT is still in its infancy, but should in the future provide an additional and/or alternative approach in medical diagnostics. The results reported here show the development and characterization (binding kinetics, sensitivity, and selectivity) of new rat mAbs for procalcitonin. In addition, the application with one selected and optimized sandwich ELISA is shown with a first set of five serum samples from healthy volunteers.

Materials and methods

Chemicals and proteins

Hydrogen peroxide, 30%, 3,3′,5,5′-tetramethylbenzidine (TMB), and dimethylsulfoxide, 99% (DMSO, Fluka), skimmed milk powder (Fluka), human serum albumin (HSA, 97–99%, lyophilized powder), and azide were purchased from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. Buffer salts (sodium hydrogen carbonate, sodium acetate, sodium chloride, sodium phosphate, disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate), and Tween 20 were from Merck (supplied through VWR International GmbH, Darmstadt, Germany). LowCross Buffer® medium, was purchased from CANDOR Bioscience GmbH, Weißensberg, Germany.

Mouse anti-rat IgG (TIB 172, κ-specific) and mouse anti-rat TIB 170 (IgG1-specific (Fc)) are commercially available through ATCC (American Type Culture Collection), Manassas, VA, USA). Mouse anti-rat IgG2c is an in-house clone (E. Kremmer, IMI, Helmholtz Center Munich, Germany).

Human recombinant procalcitonin (hrPCT; MW_{hrPCT}= 17,130 Da; 114 amino acids fragment (3–116) with an N-terminal hexahistidine tag, Prospec-Tany Technogene Ltd., Rehovot, Israel) was used as standard throughout all experiments. Synthetic human calcitonin (lyophilized) and synthetic human katacalcin (liquid) were purchased from MorphoSys AbD (Düsseldorf, Germany).

Preparation of immunogens

For the development of mAbs against procalcitonin, five different peptides with the following amino acid sequences

were chosen (Fig. 1): PROC1, FRSALESSPADPATL SEDE; PROC2, DSPRSKRCGNLST; PROC3, VGAPGKK RDMSS; PROC4, SDLERDHRPHV; and PROC5, YTQDFNKFHTFP.

These peptides were synthesized and conjugated to keyhole limpet hemocyanin (KLH) and ovalbumin (OVA) (PSL GmbH, Heidelberg, Germany).

Production of monoclonal antibodies

Approximately 50 μg of peptide–OVA or peptide–KLH was injected intraperitoneally (i.p.) and subcutaneously (s.c.) into LOU/C rats and into C57BL/6 mice, using incomplete Freund's adjuvant supplemented with 5 nmol CpG 2006 (rats) or CpG 1668 (mice), respectively (TIB MOLBIOL, Berlin, Germany). After a 2-month interval a final boost with CpG omitting incomplete Freund's adjuvant was given i.p. and s.c. 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat (or mouse) immune spleen cells was performed according to the standard procedure [15]. Hybridoma supernatants were tested in a solid-phase immunoassay using the corresponding peptide–KLH or peptide–OVA conjugate adsorbed to polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound mouse mAbs were detected using biotin-labeled rat anti-mouse IgG antibodies (H+L, Dianova, Hamburg, Germany), whereas bound rat mAbs were detected with a cocktail of biotinylated mouse mAbs against the rat IgG heavy chains, thus avoiding IgM mAbs (α-IgG1, α-IgG2a, α-IgG2b (ATCC, Manassas, VA, USA), and α-IgG2c (Ascenion, Munich, Germany)). The biotinylated mAbs were visualized with peroxidase-labeled avidin (Alexis, Grünberg, Germany) and *o*-phenylenediamine as chromogen in the peroxidase reaction.

Irrelevant peptide–KLH or peptide–OVA conjugates, which were prepared in the same way, were used as negative controls.

Several rats and mice were also immunized with peptides PROC2, PROC3, and PROC5. For these peptides

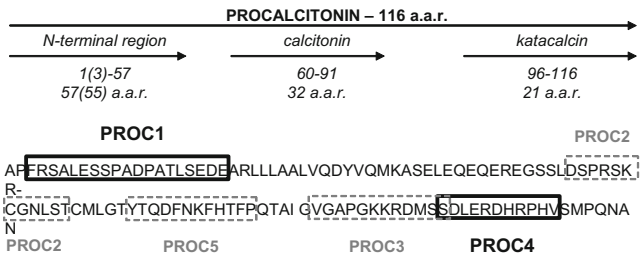

Fig. 1 Schematic representation of human procalcitonin (116 amino acids) and localization of the peptide sequences (*PROC1* to *PROC5*) used for immunizations. Sequences *PROC1* and *PROC4* were the basis for the newly developed antibodies

many mAbs against the peptide were screened, but none of them recognized procalcitonin.

The rat mAbs used in the final optimized sandwich ELISAs were: PROC1 3G3 (used as capture mAb, type IgG2a,κ); and PROC4 6C6, type IgG2c,κ; PROC4 6B2, type IgG2c,κ; PROC4 1G3, type IgG2c,κ; PROC4 1D6, type IgG1,κ (all used as detection mAbs).

Purification of monoclonal antibodies and determination of protein concentration

Purification of antibodies was performed via Protein G. Culture supernatants were added to Protein G-Sepharose 4 Fast Flow (GE Healthcare Europe GmbH, Munich, Germany). Elution of antibodies from the column was carried out with 0.1 M citrate buffer, pH 2.7. Dialysis was done three times against 40 mM PBS, pH 7.6, with 100 times of the antibody volume. Antibody solutions were filtered sterile with a 0.2-μm filter and stored at 4 °C until used; 0.1% (w/v) azide was added for long-term storage (4 °C, refrigerator).

The protein concentrations of the antibodies were determined spectrophotometrically, based on absorbance measurements at 235 and 280 nm [16].

Biotinylation of PROC4 mAbs

Protein G-purified mAbs PROC4 6C6, PROC4 1D6, PROC4 6B2, and PROC4 1G3 were biotinylated. A 100-μL aliquot of carbonate buffer, pH 9.5, was added to 1 mL of mAb solution (1.5–2.7 g L^{-1} in 40 mM PBS, pH 7.6). A 5-mg sample of Biotin-X-NHS (Calbiochem, Darmstadt, Germany) was dissolved in 1 mL of dimethylformamide (DMF), and 100 μL of this solution was added to 1.1 mL of mAb solution (see above) and incubated overnight. The solution was dialyzed three times against 40 mM PBS, pH 7.6. BSA (0.1% w/v) and 0.1% (w/v) azide were added to the solutions. Biotinylated mAbs were tested for PCT against an irrelevant peptide and optimum dilutions of all four biotinylated mAbs were determined. In general, biotinylated mAbs, which might react with the irrelevant peptide, will be discarded.

Sandwich ELISA for procalcitonin

Microtiter plates (NUNC MaxiSorp, Nunc, Wiesbaden, Germany) were coated overnight at 4 °C (refrigerator) with 100 μL per well of mAb PROC1 3G3 (4 mg L^{-1}) in 50 mM carbonate buffer, pH 9.6. The next day, plates were washed with 4 mM PBST, pH 7.4–7.6, in an automated microtiter plate washer (Bio-Tek ELX405R, Biotek Instruments GmbH, Bad Friedrichshall, Germany). Then, 200 μL per well of 1% (w/v) skimmed milk powder in 40 mM PBS, pH 7.6, were added and incubated at room temperature (RT) for 1–2 h. After a washing step (4 mM PBST, pH 7.4–7.6), 100 μL per

well of hrPCT, in 40 mM PBS, pH 7.6, were added in different concentrations (0–3,333.3 μg L^{-1}) and incubated for 1 h at RT. The plates were washed again (4 mM PBST, pH 7.4–7.6), and 100 μL per well of Protein G-purified mAb PROC4 6C6 (18.6 μg L^{-1}), PROC4 6B2 (29.8 μg L^{-1}), PROC4 1G3 (33.4 μg L^{-1}), or PROC4 1D6 (84 μg L^{-1}), respectively, in 40 mM PBS, pH 7.6, were added and incubated for 1 h at RT. After another washing step, 100 μL per well of a mixture (1:1) of biotinylated mouse anti-rat IgG1 (TIB 170) and biotinylated mouse anti-IgG2c mAb (1:2,000 in 40 mM PBS, pH 7.6) were added and incubated for 1 h at RT. Plates were washed again (4 mM PBST, pH 7.4–7.6), and 100 μL per well of streptavidin–HRP (1:30,000; Pierce/Perbio Science Germany, Bonn, Germany) in 40 mM PBS, pH 7.6, were added and incubated for 1 h at RT. The plates were washed and 100 μL per well of substrate for HRP were added (0.4 mM TMB, 1.3 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.5). The enzymatic reaction was stopped after 10 min with 50 μL per well of 2 M H_2SO_4, and absorbance was measured at 450 nm (reference 650 nm) (ThermoMax, Molecular Devices, Palo Alto, CA, USA). The experimental points of the standard curves for hrPCT, calcitonin, and katacalcin were fitted to the four-parameter logistic equation using the software SigmaPlot version 10 (Systat Software, Inc, San Jose, CA, USA):

$$y = \frac{A - D}{1 + \left(x/C\right)^{B}} + D$$

where A is the y-value corresponding to the asymptote at low values of the x-axis, and D is the y-value corresponding to the asymptote at high values of the x-axis. The coefficient C is the x-value corresponding to the midpoint between A and D (e.g., μg L^{-1}; IC_{50}). The coefficient B describes how rapidly the curve makes its transition from the asymptotes in the center of the curve.

For a better comparison of standard curves, the corresponding absorbance values (A) of standards were normalized to % control according to the formula:

$$\%\ \text{control} = \frac{A - A_{\text{background}}}{A_0 - A_{\text{background}}} \times 100\%,$$

where $A_{\text{background}}$ is the value of absorbance without analyte and detection mAb, and A_0 is the value of absorbance for the zero standard (e.g., 40 mM PBS).

From these normalized standard curves, the working range of the assays was determined from 10 to 90% control.

Sandwich ELISA for procalcitonin using biotinylated PROC4 mAbs

Sandwich ELISAs for procalcitonin were also carried out with biotinylated PROC4 mAbs. Here, instead of unbiotinylated PROC4 mAbs 100 μL per well of PROC4 6C6–biotin mAb PROC4 6B2–biotin, or PROC4 1G3–biotin diluted 1:10,000, and PROC4 1D6 diluted 1:20,000, respectively, in 40 mM PBS, pH 7.6, were used. This allowed the omission of one step (mixture of biotinylated mouse anti-rat IgG1 (TIB 170) and biotinylated mouse anti-IgG2c mAbs), and therefore the assay was faster. For these assays, hrPCT standards (concentrations from 0 to 3,660 μg L^{-1}) were set up in 4% (w/v) HSA, diluted 1:10 (v/v) in 40 mM PBS, pH 7.6, including 1:5 (v/v) LowCross Buffer® (medium). All other steps were identically carried out as described in Sandwich ELISA for procalcitonin.

Determination of cross-reactivity to calcitonin and katacalcin in sandwich ELISAs

Sandwich ELISAs for calcitonin and katacalcin were carried out identically as described for Sandwich ELISA for procalcitonin (in 40 mM PBS), and as described in Sandwich ELISA for procalcitonin using biotinylated PROC4 mAbs. Results of cross-reactants were always compared with results for hrPCT on the same microtiter plate. The cross-reactivity values were calculated as followed: $CR_{cal}(\%) = (IC_{50}\ \text{hrPCT} / IC_{50}\ \text{calcitonin}) \times 100\%$, or $CR_{katacal}(\%) = (IC_{50}\ \text{hrPCT} / IC_{50}\ \text{katacalcin}) \times 100\%$.

Analysis of serum samples for procalcitonin using sandwich ELISA (with biotinylated mAb PROC4 6C6)

Using the combination of PROC1 3G3 as capture mAb and PROC4 6C6–biotin as detection mAb (with streptavidin–HRP for the final detection), a collection of five serum samples (Sj07–Sj11) of healthy volunteers (University Hospital of Graz, Austria) was analyzed by this sandwich ELISA. Before shipment, the concentrations of procalcitonin were determined by BRAHMS LIA (luminescence immunoassay), BRAHMS AG, Hennigsdorf, Germany) and were about 0.1 μg L^{-1} (University Hospital of Graz, Austria, kindly provided by Mr. Werner Doll). For the analysis of these serum samples with our sandwich ELISA, hrPCT standards were set up in 4% (w/v) HSA, diluted 1:10 (v/v) in 40 mM PBS, pH 7.6, including 1:5 (v/v) LowCross Buffer® (medium). Samples were also diluted 1:10 (v/v) in the identical diluent. In addition, these five serum samples were spiked with 20 μg L^{-1} of hrPCT. Furthermore, as quality control of our assay, two control samples (original standards taken within the working range of the standard curve, 18.3 μg L^{-1} and 36.6 μg L^{-1}), were analyzed as unknowns.

Surface plasmon resonance measurements

A BIACORE 3000 biosensor (GE Healthcare Europe, Munich, Germany) was used to determine the binding

characteristics of the five antibodies with hrPCT. The SPR signal is expressed in resonance units (RU). The experiments were performed at 25 °C. CM5 chips modified with carboxydextran and SPR reagents were purchased from GE Healthcare Europe (Munich, Germany). A HEPES-buffered solution (HBS) was used, unless otherwise noted, as dilution and running buffer for the SPR measurements. The HBS buffer contained 0.05% (v/v) of surfactant P20 (GE Healthcare Europe), 10 mM HEPES (Sigma Aldrich GmbH, Steinheim, Germany), 3 mM EDTA (Sigma Aldrich GmbH, Steinheim, Germany), and 0.15 M NaCl (Merck KGaA, Darmstadt, Germany); the pH was adjusted to 7.4. The buffer solutions were filtered (Whatman polyamide membrane filter, pore size 0.2 μm, Schleicher and Schuell GmbH, Dassel, Germany).

The anti-PCT mAb PROC1 3G3 (IgG2a) was used as coating antibody for sandwich combinations and covalently coupled to CM5 sensor chips using the amine coupling kit supplied by the company [17]. Approximately 10,000 RU (corresponding to an antibody surface concentration of 10 ng mm^{-2}) was immobilized by passing a 50 mg L^{-1} of PROC1 3G3 mAb solution in 10 mM sodium acetate, pH 5.5, over the sensor surface previously activated by the EDC/NHS method. Underivatized active ester groups were deactivated with 1 M ethanolamine hydrochloride solution. In all assays, nonspecific binding to a reference flow cell coated with an irrelevant but similar mAb (i.e., it mimics the active flow cell in respect to antibody type and load) was subtracted to obtain corrected sensorgrams. The irrelevant mAb was coupled to sensor chips using the amine coupling kit as described for mAb PROC1 3G3. Approximately 10,000 RU was immobilized by passing a 50 mg L^{-1} mAb solution in 10 mM sodium acetate, pH 5.0.

A calibration curve with hrPCT standard concentrations from 0 to 20,000 μg L^{-1} in HBS (pH 7.4) was established by injecting for 1 min hrPCT aliquots at 30 μL min^{-1} over the sensor surface. The SPR response was recorded after a dissociation time of 2.5 min. The five injections were made for concentrations ranging from 0 to 5 mg L^{-1}. One experiment was carried out for the concentrations 10 and 20 mg L^{-1}.

In the experimental setup used (as an example see Fig. 2), a 2.5 mg L^{-1} solution of hrPCT in HBS was first injected at 30 μL min^{-1} for 1 min over the mAb PROC1 3G3-coated surface (Fig. 2, B) and allowed to dissociate for 2.5 min (Fig. 2, C). Subsequently, seven concentrations (0, 1, 2.5, 5, 10, 25, and 50 mg L^{-1}) of one of the four detection anti-PCT mAbs (PROC4 6C6, PROC4 6B2, PROC4 1G3, and PROC4 1D6) in HBS were passed over the sensor chip at a flow rate of 30 μL min^{-1} for 1 min (Fig. 2, D shows 10 mg L^{-1} of mAb PROC4 6C6). The same binding experiment was carried out in the case of mAb PROC4 1G3 using 40 mM PBS (pH 7.6) containing 5 mM NaH_2PO_4, 35 mM Na_2HPO_4, 100 mM NaCl, and 0.05% (v/v) P20. Dissociation was thereafter observed by passing the corresponding buffer solution over the chip surface for 6 min (Fig. 2, E). The coated mAb PROC1 3G3 surfaces were regenerated after each measurement by a 60-s injection of 10 mM glycine-HCl (pH 2.5) at 30 μL min^{-1} (Fig. 2, F). This was followed by the stabilization of the surface with running buffer (HBS, Fig. 2, G). The binding capacity of the sensor surface remained constant throughout 80 binding/regenerations cycles.

Association and dissociation rate constants, k_a and k_d respectively, were calculated using the BIAevaluation software version 4.1 (GE Healthcare); k_a and k_d were fitted from sensorgram plots of (detection) antibody amount bound to captured hrPCT on a coated mAb PROC1 3G3 sensor surface versus time, at a hrPCT concentration of 2.5 mg L^{-1} as mean of a range of seven concentrations of

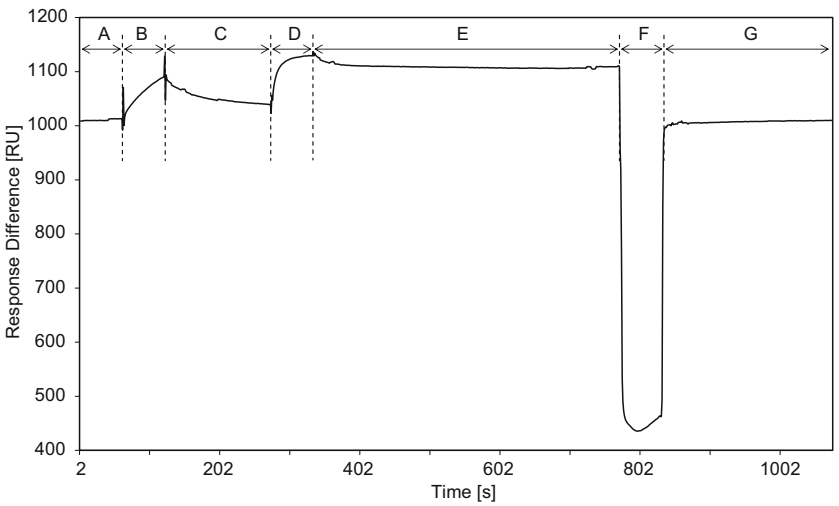

Fig. 2 Example of a typical subtracted sensorgram (difference curves of measurement flow cell minus reference flow cell) with mAb PROC1 3G3 as capture and PROC4 6C6 as detection mAb. *A–G* show the different steps of the experimental setup: *A* baseline, *B* hrPCT binding (2.5 mg L^{-1}), *C* hrPCT dissociation, *D* binding of mAb PROC4 6C6 (10 mg L^{-1}), *E* dissociation of mAb PROC4 6C6, *F* surface regeneration (glycine-HCl, pH 2.5), *G* signal stabilization

antibodies. The same portion of the binding curve was chosen for analysis at each mAb concentration. Molar values were determined assuming a molecular weight of 150 kDa for all the mAbs. The calculation of the kinetic parameters was performed using a one-to-one (Langmuir) binding model. Each sensorgram plot was corrected for linear baseline drift and nonspecific adsorption of the secondary anti-PCT mAb on the PROC1 3G3-coated surface in the absence of hrPCT. The equilibrium association (K_A) and dissociation (K_D) constants were calculated as k_d/k_a and k_a/k_d, respectively. The quality of the fitted data was evaluated by comparison between calculated and experimental curves and by the magnitude of the χ^2 parameter.

Results and discussion

From the results of the ELISA screening on microtiter plates, we selected hybridoma cell lines that secreted procalcitonin-specific mAbs. One rat mAb—PROC1 3G3—showing a specificity for the N-terminal region of the PCT, was gained and used as coating mAb to build up the sandwich immunoassay. Another four rat mAbs were selected as detection mAbs: PROC4 6C6 (IgG2c,κ), PROC4 6B2 (IgG2c,κ), PROC4 1G3 (IgG2c,κ), and PROC4 1D6 (IgG1,κ). Rats and mice, which were immunized with peptide–protein conjugates using the sequences of PROC2, PROC3, and PROC5, recognized the peptide itself, but none of them recognized procalcitonin.

High affinities of mAbs to their corresponding antigen are essential as they determine the feasibility or failure of highly sensitive immunochemical methods (e.g., immunoassays, immunosensors). The kinetic parameters of PCT–mAb interactions were first assessed using SPR (Biacore). The polysorbate surfactant P20 was included in the HBS buffer at a concentration normally used in the ELISA formats (0.05%) to diminish nonspecific interactions. The affinity of the anti-PCT mAb could not be calculated when the running buffer contained a lower amount of the surfactant (0.005%). Using this low amount of surfactant led to higher nonspecific binding to the reference flow cell, and therefore the substracted sensorgrams (measurement minus reference flow cell) were not suitable for binding calculations (χ^2 was too high).

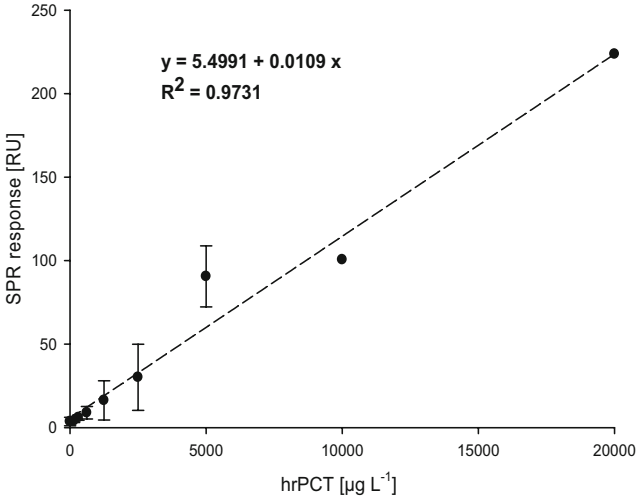

Fig. 3 Calibration curve of hrPCT in HBS (mAb PROC1 3G3) for standard concentrations ranging from 0 to 20,000 µg L^{-1}. A linear regression fit was used

The dissociation constant (K_D) of the capture mAb often defines the lower limit of assay detection, thus detection sensitivity increases with decreasing K_D. The kinetic parameters of the anti-PCT mAb PROC1 3G3 interaction were determined using a 1:1 Langmuir binding model corrected for linear baseline drift. A standard curve of hrPCT calibrators ranging from 0 to 20,000 µg L^{-1} is illustrated in Fig. 3. Over this concentration range, a linear regression fitted the dose–response relationship satisfactorily. The detection limit was determined from the mean measurement observed for the zero standard plus three times the standard deviation (SD) of the mean (mean+3SD). This value was established as 92.5 µg L^{-1} (5.4 nM; n=38 measurements of zero standard (HBS), and MW_{hrPCT}=17,130 Da). Such a detection value does not allow the detection of PCT in biological samples. However, to the best of our knowledge, we report for the first time the proof of principle of the label-free detection of procalcitonin using a specific mAb immobilized on the surface of a biosensing platform, i.e.,

Table 1 Association rate constant k_a, dissociation rate constant k_d, equilibrium association constant K_A, and equilibrium dissociation constant K_D of the four selected anti-PCT mAbs as measured by BIACORE 3000

Detection mAb	IgG subclass	$k_a\times10^5$ (M^{-1} s^{-1})	$k_d\times10^{-3}$ (s^{-1})	$K_A\times10^8$ (M^{-1})	$K_D\times10^{-9}$ (M)	χ^2
PROC4 6C6	IgG2c	14.6	2.40	6.11	1.64	1.41
PROC4 6B2	IgG2c	10.8	1.94	5.56	1.80	1.95
PROC4 1G3	IgG2c	7.25	1.84	3.94	2.54	0.52
PROC4 1D6	IgG1	4.77	1.70	2.81	3.56	2.76

The constant values were obtained from a nonlinear global regression analysis of the association and dissociation phases of the sensorgrams using a 1:1 Langmuir binding model with drifting baseline

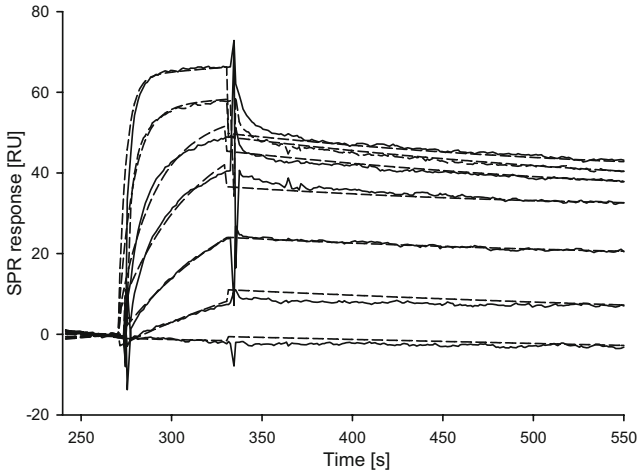

Fig. 4 Overlay plot of subtracted sensorgrams generated concentrations of 0, 1, 2.5, 5, 10, 25, and 50 mg L^{-1} of mAb PROC4 1G3 in HBS binding to PCT (2.5 mg L^{-1}) captured on a mAb PROC1 3G3-coated surface. The *dashed lines* show the results from global fitting to a 1:1 binding model corrected for a drifted baseline

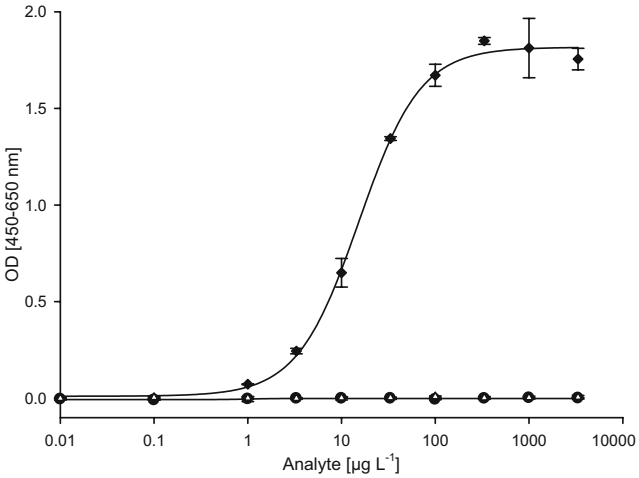

Fig. 5 Standard curves in sandwich ELISA for hrPCT (◆), calcitonin (△), and katacalcin (●) in 40 mM PBS, pH 7.6, using the combination of mAbs PROC1 3G3 and PROC4 6C6

the biological recognition element (here mAb PROC1 3G3) is directly attached to the sensing surface. All other automated platforms [9–13] are based on immunoassay formats, which use labels (e.g., an enzyme and chemiluminescence or fluorescence detection).

The association rate constant (k_a) and dissociation rate constant (k_d) of mAb PROC1 3G3 were 1.23×10^3 M^{-1} s^{-1} and 4.22×10^{-5} s^{-1}, respectively. The dissociation constant (K_D) was 3.42×10^{-8} M; this value fits with a typical antibody–antigen complex with a good affinity antibody (K_D from 10^{-8} to 10^{-10} M) [18].

The association (k_a) and dissociation (k_d) rate constants as well as the constants at equilibrium for the binding between hrPCT and the detection mAbs are summarized in Table 1. χ^2 values are also listed. χ^2 is a standard statistical measure of the convenience of the 1:1 Langmuir binding model corrected for linear baseline drift. For a good fitting χ^2 must be of the same order of magnitude as the noise in resonance units. The kinetics were measured at low procalcitonin surface density (between 15 and 35 RU) and six different PROC4 mAb concentration levels (Table 1). A representative global analysis of response data for mAb PROC4 1G3 is shown in Fig. 4. Under the experimental conditions employed, the affinity constants (K_A) of the detection antibodies ranged from 2.81 to 6.11×10^8 L mol^{-1}. The four detection mAbs displayed similar dissociation constants in the nanomolar range ($K_D=10^{-9}$ M). These very low values signify that the four detection mAbs have very high affinities for PCT. Slightly higher association rate constants and lower dissociation constants (better antibody–antigen affinities) were calculated in the case of the three mAbs having the IgG2c subclass (PROC4 6C6, 6B2, and 1G3) compared with mAb PROC4 1D6 (subclass IgG1). The kinetic parameters were also determined for mAb PROC4 1G3 using a PBS buffer containing 0.05% P20. The association rate constant (k_a) and

Table 2 Sandwich ELISAs with Protein G-purified PROC4 mAbs: summary of immunoassay parameters

Detection mAb	*B*	IC_{50} (µg L^{-1})	LOD[a] (µg L^{-1})	LLOQ[a] (µg L^{-1})	Working range[b] (µg L^{-1})		R^2
					10% control	90% control	
PROC4 6C6	1.32±0.09	15.30±0.96	1.4	2.3	2.7	80.3	0.999
PROC4 6B2	1.56±0.34	23.72±3.90	2.7	5.0	7.0[c]	72.7[c]	0.988
PROC4 1G3	1.92±0.19	16.19±1.08	2.5	3.5	4.5	48.9	0.998
PROC4 1D6	1.70±0.11	50.24±2.18	8.2	12.8	12.8	177.8	0.999

B slope, IC_{50} test midpoint, R^2 logistic fit correlation

[a] Validation parameters (*LOD* limit of detection and *LLOQ* lower limit of quantification); $SD_{zero\ standard}$ was determined with 12 measurements of a zero standard (40 mM PBS)

[b] Working range of sandwich ELISAs; basis is one standard curve; each standard determined with n=2–4

[c] 15% and 85% control was calculated as working range

Table 3 Sandwich ELISAs with biotinylated PROC4 mAbs: summary of immunoassay parameters

Detection mAb	*B*	IC_{50} (μg L^{-1})	LOD[a] (μg L^{-1})	LLOQ[a] (μg L^{-1})	Working range (μg L^{-1})	
					10% control	90% control
PROC4 6C6–biotin[b]	1.45±0.13	59.9±5.2	1.5	6.0	11.7	270.1
PROC4 6B2–biotin	1.52±0.13	46.3±6.0	2.1	4.1	10.1	191.7
PROC4 1G3–biotin	1.38±0.04	77.2±18.2	2.1	6.0	14.4	374.6
PROC4 1D6–biotin	1.41±0.16	46.0±3.3	1.0	4.2	9.0	220.8

All values given are mean values of three standard curves (for each standard curve standards were determined with n=2–4)
B slope, IC_{50} test midpoint
[a] *LOD* limit of detection, *LLOQ* lower limit of quantification
[b] All values given for PROC4 6C6–biotin are mean values of five standard curves (for each standard curve standards were determined with n=2–4)

dissociation rate constant (k_d) of mAb PROC4 1G3 measured in PBS were 3.85×10^5 M^{-1} s^{-1} and 2.0×10^{-3} s^{-1}, respectively. The dissociation constant (K_D) was 5.21×10^{-9} M. In this buffer the dissociation tendency of the mAb–PCT complex slightly increased compared with HBS due to a lower association rate constant (k_a).

The reactivity of the selected mAbs was further tested by sandwich ELISA with hrPCT as standard, and katacalcin and calcitonin as cross-reactants. Figure 5 shows as an example these standard curves with PROC1 3G3 as capture mAb and PROC4 6C6 as detection mAb using hrPCT, (synthetic human) katacalcin and (synthetic human) calcitonin as standards at concentrations ranging from 0.01 to 3,333.3 μg L^{-1} in 40 mM PBS (pH 7.6). The experimental points were fitted to a four-parameter logistic function. With this sandwich ELISA, no cross-reactivities were observed for katacalcin and calcitonin (Fig. 5).

In addition to the slope of standard curves, the sensitivity of immunochemical methods is typically described as the limit of detection (LOD) and the lower limit of quantification (LLOQ). In accordance with the IUPAC directives [19], LOD was calculated as signal of the zero concentration (n=12) plus three times the standard deviation ($SD_{zero\ standard}$), and the LLOQ plus ten times the $SD_{zero\ standard}$, respectively. As is usual for this kind of calibration curve, the dynamic (working) ranges of the assay were measured as the values were between 10 and 90% control of the maximal response, respectively. Immunoassay parameters (*B*, IC_{50}, LOD, LLOQ, 10% and 90% control, and R^2) for the four standard curves of hrPCT assays are listed in Table 2.

The $SD_{zero\ standard}$ values were determined for each detection antibody. LODs were between 1.4 and 8.2 μg L^{-1} and LLOQs between 2.3 and 12.8 μg L^{-1}. The test midpoints (IC_{50}) of these sandwich ELISAs were in the 15.3–50.2 μg L^{-1} range. The mAb PROC4 1D6 (IgG1 subclass) displayed the highest LOD and LLOQ values associated with a less favorable working range. Among the detection mAbs having the IgG2c subclass, the mAbs PROC1 3G3–PROC4 6C6 combination was found to be the best to build up a sensitive immunoassay for PCT having the lowest limit of quantification (2.3 μg L^{-1}). This result confirmed the SPR experiments in which mAb PROC4 6C6 was found to form

Table 4 Spike-recovery analysis of serum samples from healthy volunteers for procalcitonin (mAb PROC1 3G3 as capture and PROC4 6C6–biotin as detection mAb)

	Serum sample						
	Sj07	Sj08	Sj09	Sj10	Sj11	Control 1	Control 2
Concentration determined by reference analysis[a] (μg L^{-1})	0.10	0.11	0.12	<0.1	0.10	18.30	36.60
Concentration determined by ELISA[b] (μg L^{-1})	<LLOQ[c]	<LLOQ[c]	<LLOQ[c]	<LLOQ[c]	<LLOQ[c]	16.97±1.98	32.40±1.36
Concentration determined after spike with 20 μg $L^{-1}$of hrPCT (μg L^{-1})	20.26±0.19	20.84±1.15	19.68±1.35	21.14±2.74	19.69±1.24	–	–
Recovery (%)	101.2[d]	104.2[d]	98.3[d]	115.7[d]	98.4[d]	92.7	88.5

[a] Reference analysis was done by luminescence immunoassay (BRAHMS AG), University Hospital of Graz, Austria
[b] Serum samples were diluted 1:10 (1:10, v/v)) in 40 mM PBS (including LowCross Buffer® 1:5 (v/v))
[c] LLOQ for this standard curve was 8.6 μg L^{-1} (please note: LLOQ value given here refers to the single standard curve for the analysis of serum samples; LLOQ given in Table 3 is a mean value obtained from three standard curves)
[d] Recovery values given include the concentration of procalcitonin, which was already present in the samples

the strongest complex with PCT (K_D=1.64 ×10^{-9} M). The dynamic range of this assay (2.7–80.3 μg L^{-1}), when confirmed in the case of biological samples (serum, plasma), would allow the measurement of PCT in the case of systemic bacterial infection (≥10 μg L^{-1}) and sepsis (PCT level between 2 and 10 μg L^{-1}) [1].

As described before, the combination of mAbs PROC1 3G3–PROC4 6C6 did not show any cross-reactivity to synthetic human calcitonin and synthetic human katacalcin; no signal for both peptides was visible, even at concentrations higher than 3,000 μg L^{-1} (Fig. 5). The specificity of this assay was obtained with the capture mAb, which reacts with a peptide sequence within the N-terminal region of PCT, whereas the detection mAbs recognized a 14 amino acid motif of the katacalcin region. The same katacalcin region is recognized by all four detection mAbs. There were also no cross-reactivities for katacalcin and for calcitonin observed with all other combinations of mAbs (mAbs PROC1 3G3–PROC4 6B2; mAbs PROC1 3G3–PROC4 1G3; mAbs PROC1 3G3–PROC4 1D6, data not shown).

All four sandwich ELISAs were additionally carried out with biotinylated PROC4 mAbs. This system, also in connection with the setup of standards in 4% HSA (diluted 1:10 in 40 mM PBS, pH 7.6, with the addition of 1:5 (v/v) of LowCross Buffer®) was part of the optimization of the sandwich assay format (shorter assay time), and towards the analysis of serum samples. Generally, the usage of this 'diluent' for the hrPCT standards led to more stable assays and to better recovery values for PCT in serum samples.

In addition, all standard curves for hrPCT with the different biotinylated PROC4 detection mAbs were very similar and were slightly less sensitive than with unbiotinylated PROC4 mAbs (IC_{50} 46.0–77.2 μg L^{-1}, LLOQ 4.1–6.0 μg L^{-1}); PROC4 1D6–biotin performed better than without biotinylation (Table 3).

Regarding the cross-reactivities with katacalcin and calcitonin, the same results as before, i.e., no cross-reactivities, were obtained with all four sandwich ELISAs using biotinylated PROC4 mAbs (data not shown).

As proof of principle, a collection of five serum samples of healthy volunteers (provided by the University Hospital of Graz, Austria) was analyzed in the sandwich ELISA with the latter setup (biotinylated PROC4 6C6, hrPCT in 4% HSA (diluted 1:10 in 40 mM PBS, including 1:5 (v/v) LowCross Buffer®). In addition, a spike recovery (spiked concentration 20 μg L^{-1} of hrPCT) was determined for these samples. As expected, our assay could not quantify procalcitonin in the serum samples of healthy volunteers (concentration was about 0.1 μg L^{-1}); in all samples the result of our ELISA was below the limit of quantification (Table 4). Recovery values for the spiked samples ranged from 98.3 to 115.7% (Table 4). In addition, as a quality control of the assay, two standards (controls 1 and 2) were analyzed as unknowns, and the recovery values were about 90% (Table 4).

Conclusion

Five new rat mAbs for procalcitonin were developed and characterized. Their affinities for hrPCT were determined using SPR measurements. The five mAbs formed strong complexes with PCT with equilibrium dissociation constants (K_D) in the 10^{-8}–10^{-9} M range. Using appropriate combinations of one coating mAb, specific for the N-terminal region of PCT, and four detection mAbs, which recognize a peptide sequence in the katacalcin region, we could develop PCT-specific sandwich immunoassays, either with Protein G-purified or with biotinylated PROC4 mAbs, which did not display cross-reactivities for calcitonin and katacalcin. The combination of mAbs PROC1 3G3–PROC4 6C6 was found to be the best to build up the sandwich immunoassay with the highest sensitivity, when the detection mAbs were not biotinylated. With biotinylated detection PROC4 mAbs, all standard curves were comparable with one another and performed very similarly.

These newly developed mAbs are now ready to be formatted into new immunosensors for point-of-care diagnostics (e.g., [20]).

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