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TECHNICAL NOTE

Production and analytical characterization of neopterin immunoreagents for biosensor developments

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Abstract Neopterin is a valuable biomarker of cellular immunity associated with various pathological situations such as viral and bacterial infections, autoimmune, cardiovascular, neurodegenerative and malignant disorders. To produce specific antibodies against neopterin for a rapid multi-biomarker-based diagnosis, a novel hapten derivative was synthesized and attached to carrier proteins. The thoroughly characterized conjugates were used for immunization of BALB/c mice and rabbits. The produced monoclonal antibody reached in both direct and indirect enzyme-linked immunosorbent assay (ELISA) format LoD of 0.18 and 0.45 $\mu g L^{-1}$, respectively, and was a superior immunoreagent for further biosensor developments with regard to assay sensitivity and material availability. The best polyclonal antibody was somewhat more sensitive in direct ELISA with LoD of 0.05 $\mu g L^{-1}$. The optimized ELISA method was evaluated with blood samples collected from patients with renal insufficiency, patients with sepsis, patients without confirmed clinical diagnosis, and healthy volunteers. In plasma samples, neopterin concentrations ranging from 3.2 to 103 $\mu g L^{-1}$ could be determined with the monoclonal ELISA whereas twofold lower results were obtained with the polyclonal ELISA. A satisfactory correlation of results was found between the polyclonal ELISA and IBL Neopterin ELISA kit within the concentration range 0.5–16 $\mu g L^{-1}$ (R=0.874; n=40), and slightly lower correlation was found for monoclonal-based ELISA (R=0.819; n=40). These data show that the generated antibodies may be used as functional analytical reagents for the integration into multianalyte biochip detection systems.

Keywords Neopterin · Hapten density · Monoclonal and polyclonal antibody · Real blood samples · ELISA evaluation · Multianalyte chip

Abbreviations

BALB/c	Laboratory-bred strain of the house mouse
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
DCC	*N, N′*-dicyclohexylcarbodiimide
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
EDC	*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
FIA	Fluoroimmunoassay
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IFA	Incomplete Freund's adjuvant
LoD	Limit of detection
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
NHS	*N*-Hydroxysuccinimide
OV	Ovalbumin

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pAb	Polyclonal antibody
PBS	Phosphate-buffered saline
RAM	Rabbit anti-mouse IgG
RIA	Radioimmunoassay
SwAR	Swine IgG against rabbit IgG labeled with HRP
TG	Thyroglobulin
TMB	3, 3′ 5, 5′-Tetramethylbenzidine
TOF	Time-of-flight mass spectrometer

Introduction

Neopterin, one of the compounds in the pteridine family, is an early and valuable biochemical marker of cellular immunity. Increased levels of neopterin can be detected in body fluids in various pathologies, such as viral and bacterial infections, autoimmune, cardiovascular, neurodegenerative, and malignant disorders [1]. Recent data show that serum neopterin is probably the best single predictor of death in healthy individuals with angiographic abnormalities [2, 3]. However, elevated neopterin concentrations are usually not indicative for one particular disease, and thus neopterin determinations are useful in recognizing a large spectrum of harmful disorders, including those which represent a risk for the recipients in the field of blood transfusion and organ transplantation [4, 5].

For the measurement of neopterin levels in biological samples, methods such as high-performance liquid chromatography (HPLC) [6–8], capillary electrophoresis [9] radioimmunoassay (RIA) [10, 11], fluoroimmunoassay (FIA) [12], and enzyme-linked immunosorbent assay (ELISA) [5] have been used. Instrumental methods are accurate and sensitive but they are less suitable for analysing a large number of samples within shorter time [6, 7, 9]. On the other hand, antibody-based methods such as ELISA or immunosensors represent an inexpensive, sensitive, and fast analytical alternative to the instrumental techniques.

Antibodies are specific recognition elements of the immunodetection system, which can be produced by immunization of animals with appropriate antigenic conjugates. Neopterin is a small molecule (hapten) which has to be covalently attached to a carrier protein in order to obtain a hapten-protein conjugate (immunogen) for the antibody preparation. Several conjugation approaches to the production of neopterin-protein conjugates have been reported. Nagatsu et al. [10] and Ogiwara et al. [13] introduced into the A-ring of neopterin molecule various spacers with reactive carboxylic group and subsequently prepared several immunogens for animal immunization. The obtained polyclonal antibodies exhibited a high specificity and sensitivity towards neopterin in both RIA and ELISA format. Barak et al. [14] used different proteins and carbodiimides in preparing neopterin conjugates and polyclonal antibodies for sensitive and specific test. The authors employed carbodiimides to cross-link the amino group of the pteridine structure with carboxylic group of the protein carriers. Monoclonal antibodies against neopterin were also prepared; however, their analytical characteristics do not allow the use of these antibodies in real neopterin analysis. The monoclonal antibody produced by Weyrer et al. [15] recognized specifically neopterin in RIA but the sensitivity of the assay was low when compared with polyclonal antibodies. Malakaneh et al. [16] used active succinimide ester of hapten for the conjugation reaction with bovine serum albumin. The affinity and assay sensitivity of the prepared monoclonal antibody was improved in ELISA when this reagent was combined with the enzyme conjugate prepared using one-step glutaraldehyde method, but this assay was still insufficient for real blood sample analysis.

The primary objective of this work was to produce functional monoclonal antibodies with a high degree of specificity and affinity towards neopterin marker and to use them in combination with other antibodies (raised against other important clinical biomarkers) for the development of a multianalyte biosensor detection in intelligent medical diagnostics systems. Because the methods reported by the above authors did not enable us to produce key immunoreagents in reasonable yields, a novel approach to the synthesis of the neopterin hapten was designed to obtain conjugates and antibodies in a sufficient amount and quality for the intended objectives. The produced antibodies and hapten conjugates were analytically characterized in conventional ELISA formats and their detection capabilities were preliminary verified in clinical samples.

Experimental

Reagents and chemicals

Neopterin derivatives 6-{[4-hydroxy-6-(D-erythro-1,2,3-trihydroxypropyl)-2-pteridinyl]-amino} hexanoic acid and *N*-[4-hydroxy-6-(D-erythro-1,2,3-trihydroxy-propyl)-2-pteridinyl glycine were synthesized at Vontor (Ing. T. Vontor, Pardubicka 628, Hradec Kralove IV, Czech Republic). Bovine serum albumin (BSA), thyroglobulin (TG), ovalbumin (OV), DMF, dimethylsulphoxide (DMSO), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), horseradish peroxidase (HRP), rabbit anti-mouse IgG (RAM), complete Freund's adjuvant

(CFA), incomplete Freund's adjuvant (IFA), Pristane, Sephadex G-25, Tween 20, and D-neopterin were purchased from Sigma-Aldrich Chemie (Steinheim, Germany), swine IgG against rabbit IgG labeled with HRP (SwAR) was purchased from Servaphrama (Prague, Czech Republic). Pteridin, 6-hydroxymethylpterin, xanthopterin, L-neopterin, L-biopterin, L-sepiapterin, 7, 8-dihydro-D-neopterin, 7, 8-dihydro-L-biopterin, (6R)-5, 6, 7, 8-tetrahydro-L-biopterin dihydrochloride, and folic acid were obtained from Schircks Laboratories (Jona, Switzerland); 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was from Serva (Heidelberg, Germany).

Instrumentation

Maxisorp microtitre plates were obtained from NUNC (Roskilde, Denmark). An automatic plate washer was from Tecan Columbus Pro (Austria). Absorbance was measured on ultra-microtitre plate reader EL 808 and processed by software KC^4 v. 3.1 (BIO-TEC Instruments, USA). Ultrasonic bath PS 04000A was purchased from Notus-Powersonic (Slovakia) and used for sonication of standard stock solutions. Neopterin ELISA kit was obtained from IBL International (Germany). Centrifuges CR3 22 and CR3i were from Juan (France). Vivapore 5 mL concentrators from Vivascience (Germany) and stirred ultrafiltration cell Amicon 8050 (Millipore, USA) were employed for the conjugate and antibody concentration. Protein A IgG purification kit and rapid ELISA mouse mAb isotyping kit were from Pierce (USA). The absorption spectra were measured by UVIKON XL spectrophotometer (Bio-Tec, Italy). Ultraflex III MALDI-TOF/TOF spectrometer and Flex Analysis 3.0 software (Bruker Daltonik, Germany) were used for mass spectra measurement and data processing.

Buffers and solutions

The following solutions were used: (1) 0.13 $molL^{-1}$ $NaHCO_3$ in water; (2) dialysis solution—0.1 $molL^{-1}$ $(NH_4)_2CO_3$ in water; (3) elution buffer—0.1–0.2 $molL^{-1}$ glycine-HCl (pH 2.5–3.0); (4) neutralization buffer (for antibody purification)—1 $molL^{-1}$ Tris–HCl (pH 8.5–9.0); (5) MALDI matrix solution—20 $mgmL^{-1}$ 2, 5-dihydroxybenzoic acid in water/acetonitrile/trifluoroacetic acid 79:20:1 (*v*/*v*/*v*); (6) coating buffer—50 $mmolL^{-1}$ carbonate buffer, pH 9.6; (7) phosphate-buffered saline (PBS) buffer—10 $mmolL^{-1}$ phosphate buffer with 145 $mmolL^{-1}$ NaCl (pH 7.2); (8) PBS–T_{20} buffer (washing and blocking buffer)—PBS buffer with 0.1% (*v*/*v*) Tween 20; (9) substrate solution—1 mL acetate buffer, 200 μL of TMB solution, and 20 μL of 6% H_2O_2 to 20 mL water; (10) acetate buffer—0.1 $molL^{-1}$ sodium acetate adjusted to pH 5.5 by 1 $molL^{-1}$citric acid; (11) TMB solution—10 mg mL^{-1} TMB in DMSO; and (12) stopping solution—2 mol L^{-1} H_2SO_4.

Calibration standards

Neopterin and other pteridine stock solutions (10 mgL^{-1}) were obtained by dissolving in hot milli-Q water and PBS buffer, respectively, and sonicated prior to dilution. The neopterin standards (0.1, 0.5, 2.0, 5.0, 10, 50, and 100 μgL^{-1}) were prepared by dilution of the neopterin stock solution with PBS buffer before the use in ELISA. Working solutions of pteridines were prepared by dilution of the stock solutions with PBS buffer with respect to the need for cross-reactivity testing.

Preparation of the hapten-protein conjugates

The synthesis of hapten, conjugation with carrier proteins and the characterization of the conjugates are described in the Electronic supplementary material.

Antibody production

Polyclonal antibodies

Immunogens (0.45 μg) were dissolved in 0.75 mL of PBS buffer and emulsified in 0.75 mL of CFA for the first immunization and in IFA for subsequent immunizations. The prepared emulsion (one immunization dose) was injected intradermally into 15 sites on the back side of the animal. Five booster injections were applied to New Zealand White rabbits at 30 day intervals. Antisera from animals were collected 10–12 days after each booster injection to monitor the immune response (antibody titre) during immunization. Ten to 12 days after the last immunization, blood samples were collected and the obtained antiserum was stored at −20 °C.

Monoclonal antibodies

BALB/c mice, aged 10–15 weeks, were injected intraperitoneally in 3–4-week intervals with 0.20 and 0.45 μg of immunogen dissolved in 100 μL of PBS buffer and then emulsified with 100 μL of CFA for the first immunization and with IFA for subsequent immunizations. Seven days after the fifth booster injection, spleens from mice were provided for hybridoma technology as described previously [17, 18]. The obtained clones grew as ascites tumors in BALB/c mice, which were injected with pristane 10 days before inoculation. The crude ascites pools were purified using protein A IgG purification kit. The eluted mAb was dialyzed against PBS buffer and lyophilized. Larger

amounts of the antibody were produced in vitro at EXBIO (Prague, Czech Republic).

Blood samples

The plasma and serum samples used for the evaluation of ELISA systems were collected at the Department of Internal Medicine, University Hospital Tübingen. The blood samples were obtained from patients with specific diagnosis, healthy volunteers and patients without confirmed clinical diagnosis. The samples were stored at −20 °C until analysis. Samples were diluted tenfold with PBS buffer prior to analysis.

ELISA performance

Direct competitive ELISA using polyclonal antibodies

Two hundred microliters of diluted antibody in coating buffer was added into each well on a microtitre plate and incubated at 4 °C overnight. The coated plate was washed three times with washing buffer and 100 μL of standard or sample and 100 μL of HRP conjugate in PBS-T_{20} buffer was added into wells. The plate was shaken for 1 min and then incubated at 4 °C for 1 h. Unbound compounds were removed by washing three times with washing buffer. Two hundred microliters of substrate solution was added into each well and the enzymatic reaction was stopped after 15 min of incubation at room temperature by addition of 100 μL of stopping reagent. The absorbance was measured at 450 nm.

Direct competitive ELISA using monoclonal antibodies

Two hundred microliters of RAM diluted with coating buffer in ratio of 1:5,000 was added into each well and incubated at 4 °C overnight. The coated plate was washed three times with washing buffer and 100 μL of monoclonal antibody was added into wells. The plate was incubated at 4 °C for 1 h and then washed three times with washing buffer. Following procedure (standard and sample addition, enzymatic reaction) was performed as described above.

Indirect competitive ELISA

A 0.2 gL^{-1} of neopterin-conjugate was diluted with coating buffer, and 200 μL of the solution was added into each well of a microtitre plate. The plate was incubated at 4 °C overnight and washed three times with washing buffer. Then, 100 μL of standard or sample and 100 μL of antibody in PBS-T_{20} buffer was added into wells. The plate was shaken for 1 min and incubated at 4 °C for 1 h and washed three times. One hundred microliter of SwAR in PBS-T_{20} buffer was added into wells. The plate was incubated at 4 °C for 1 h following the procedure described above.

Cross-reactivity and LoD

Percentage of cross-reactivity (CR) was calculated using the equation:

$$\mathrm{CR}(\%) = \frac{\mathrm{IC}_{50}(\mathrm{neopterin})}{\mathrm{IC}_{50}(\mathrm{cross-reactant})} \times 100,$$

where IC_{50} is the concentration of a competitor (cross-reactant) resulting in 50% reduction of HRP conjugate binding. The measured data were processed by KC^4 software version 3.1. ELISA curves were generated using Origin 7.5 software.

Limit of detection (LoD) was determined as follows: LoD=mean $A_0 - 3 \times$SD; where the mean A_0 is the mean value of absorbance for n zero standards and *SD* is standard deviation of the mean absorbance.

Results and discussion

Hapten design and preparation and characterization of neopterin-protein conjugates

Since the trihydroxypropyl moiety of the neopterin molecule is immunodominant, we aimed to introduce the spacer group with end carboxylic group into A-ring. However, efforts to accomplish this strategy failed particularly due to extremely low solubility of neopterin and due to low reactivity of NH_2−, NH− and O= groups in the A-ring. To overcome these problems, the neopterin hapten was prepared by a novel synthetic approach. With this approach a spacer arm with a reactive carboxylic group could be introduced into the A-ring of neopterin (for details see the Electronic supplementary material). The hapten was then conjugated to the carrier proteins BSA, OV, and TG and the obtained conjugates were characterized by absorption and MALDI-TOF/TOF mass spectra (Electronic supplementary materials (Figs. S2 and S3)). The hapten density for the BSA conjugates was from 0.7 to 6.7 mol of hapten groups per 1 mol of protein (Electronic supplementary material (Table S1)).

Antibody production and assay optimization

Groups of 3 rabbits were immunized with neopterin-BSA (6.7 mol hapten per mol protein), neopterin-TG and neopterin-OV, respectively, using conventional immunization scheme. The antibody formation during immunization was followed by both indirect and direct ELISA. The

rabbits produced mostly polyclonal antibodies (antisera) with titres of around 1:10,000. The detection capability of the antibodies was verified in optimized assay systems using immobilized antibodies and neopterin-HRP conjugate (direct ELISA) or using coating conjugates in combination with free antibodies (indirect ELISA). The best polyclonal antibody No. 3 (pAb No. 3) raised against neopterin-BSA exhibited in direct ELISA a 50% binding inhibition at 0.48 $\mu g L^{-1}$ whereas all other antibodies showed IC_{50} values in the range of 0.54–1.39 $\mu g L^{-1}$. The antibodies used in indirect ELISA exhibited the IC_{50} around 7.85 $\mu g L^{-1}$ which is significantly lower sensitivity in comparison to direct assay format and were not further characterized in this arrangement.

To produce monoclonal antibodies (mAbs), mice immunized with the above immunogens were employed for fusion experiments. A week after the fusion, the culture supernatants were screened by both ELISA formats. The best monoclonal antibody produced against neopterin-OV conjugate by clone 3E2 (mAb 3E2) was used for further characterization. The antibody being tested (rapid ELISA mouse mAb isotyping kit) proved to be IgG_1 with kappa light chains.

The optimized standard curves for neopterin in buffer are shown in Fig. 1. Importantly, the monoclonal antibody provided similar standard curves in both direct and indirect ELISA arrangement with the IC_{50} values 1.96 and 1.81 $\mu g L^{-1}$, and LoD of 0.45 and 0.18 $\mu g L^{-1}$, respectively. Thus, the antibody could be employed in optical biosensor setups based on hapten-conjugate (antigen) immobilization as well as in the systems working with the antibody immobilized on the sensing surface (unpublished results).

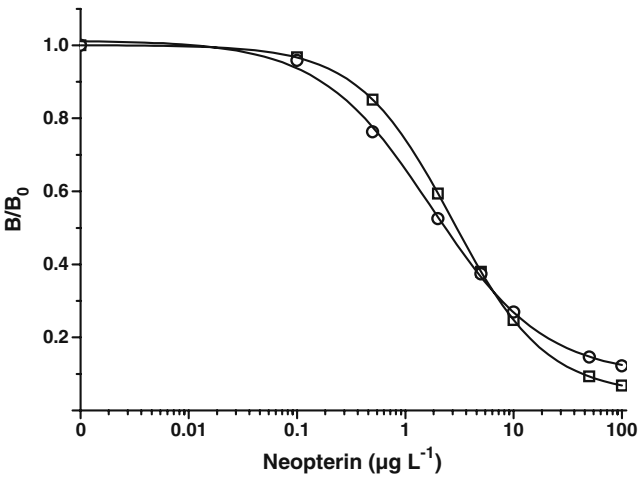

Fig. 1 Standard neopterin curves for monoclonal-based enzyme-linked immunosorbent assay (ELISA). Direct ELISA (*squares*), dilution/mAb 3E2 1:10,000 (starting concentration, 1.8 gL^{-1}), HRP conjugate 1:35,000. Indirect ELISA (*circles*), dilution/mAb 3E2 1:10,000 (starting concentration, 1.8 gL^{-1}), neopterin-BSA2 coating conjugate 1:10,000 (starting concentration, 0.2 gL^{-1})

It is common experience that antibodies can exhibit different detection properties when applied to various solid phase immunoassay systems. The mAb 3E2 offers good potential for utilization in different types of immunoassay techniques whereas the assay sensitivity is being kept approximately at the same level. The sensitivity of our mAb 3E2 is markedly higher in comparison to monoclonal antibodies produced by Weyrer et al. [15] and Malakaneh et al. [16]. The flat slope of the calibration curves made these monoclonal antibodies practically unusable in neopterin blood sample analysis.

Specificity of antibodies against neopterin

As expected, the antibodies exhibited high degree of specificity towards neopterin structure (Table 1). The specificity of the antibodies is expressed by the values of cross-reactivity between neopterin and the tested compounds. The assessment of the cross-reactivity values was carried out in optimized ELISA formats. Both polyclonal (pAb No. 3) and monoclonal (mAb 3E2) antibody exhibited low or negligible cross-reactivity with potential cross-reactants (neopterin=100%). The highest cross-reactivity of 9.1% and 4.8% was found for 7, 8-dihydro-D-neopterin in ELISA working with polyclonal and monoclonal antibody, respectively. The obtained data demonstrate that mAb 3E2 can be considered as a highly specific reagent for neopterin whereas the specificity of polyclonal antibody having a somewhat higher reactivity with 7, 8-dihydro-D-neopterin was satisfactory as well.

Production of coating neopterin conjugates for optical biosensors

In order to produce a sufficient amount of the conjugates for biosensor developments, several batches of the conjugates were prepared and tested for their immunoreactivity and assay sensitivity. The conjugation reactions with albumin carrier were conducted with respect to the reproducibility of the hapten density yields. Calculation of hapten density was carried out by MALDI-TOF/TOF mass spectrometry data (see the Electronic supplementary material). All neopterin-BSA conjugates presented in the Electronic supplementary material (Table S1) were prepared using NHS/DCC method apart from the conjugate BSA5, which was a product of the NHS/EDC procedure. To achieve a comparable hapten density to BSA2 (6.7 mol hapten per mol BSA), the NHS/DCC method was repeated under identical conjugation conditions using a newly prepared neopterin hapten derivative. Analysis of the hapten density in the conjugates showed that only 3.2 of the hapten groups were incorporated into albumin conjugates BSA6 and BSA7. Similar hapten density was obtained in the conjugate BSA5 when water soluble EDC was employed as

Table 1 Cross-reactivity pattern among neopterin and other pteridines

Compound	% cross-reactivity		
	Indirect mAb ELISA	Direct mAb ELISA	Direct pAb ELISA
D-Neopterin	100.0	100.0	100.0
7, 8-Dihydro-D-neopterin	4.4	4.8	9.1
L-Neopterin	0.3	0.4	0.2
L-Biopterin	0.5	0.6	<0.1
7, 8-Dihydro-L-biopterin	<0.1	0.1	<0.1
L-Sepiapterin	0.1	0.2	<0.1
5, 6, 7, 8-Tetrahydro-L-biopterin	<0.1	<0.1	<0.1
xanthopterin	<0.1	0.2	<0.1
6-Hydroxymethylpterin	0.5	0.7	<0.1
Pterin	<0.1	0.1	<0.1
Folic acid	<0.1	<0.1	<0.1

the conjugation reagent. When mass of the hapten derivative was reduced in the reaction mixture, the expected lower hapten densities in the conjugates BSA3 and BSA4 were found. It should be noted that incorporation of neopterin into conjugate proteins was improved when the hapten derivative (compound V, Electronic supplementary material (Fig. S1)) was recrystallized prior to conjugation procedure. Thus, stability and purity of this hapten material should be checked over longer time periods.

It appeared from our previous study with 2, 4-D-herbicide that lower ELISA sensitivity towards this analyte was found when conjugates with higher degree of hapten substitution were used for a microtitre plate coating [18]. In order to examine how neopterin density influenced the assay sensitivity of indirect ELISA system, the conjugates presented in the Electronic supplementary material (Table S1) were employed as coating agents and sensitivity of the optimized ELISAs was compared. Surprisingly, the IC_{50} values calculated for these optimized ELISAs were found to be similar (1.52–2.63 $\mu g L^{-1}$) whereas only a very small difference in the sensitivity and character of standard curves was observed (Fig. 2).

Assay working range for analysis of blood samples

The average basal concentrations of serum neopterin in healthy adults were reported to be 1.34±0.68 $\mu g L^{-1}$ [19] and 2.23–2.46 $\mu g L^{-1}$ [1] and a cut-off value for diagnostic ELISA tests was set at 3.04 $\mu g L^{-1}$ [4]. Neopterin concentrations higher than the cut-off value were considered to be elevated levels signalizing a possible risk in blood transfusion or organ transplantation [1, 4, 5]. The above cut-off value was considered as a reference parameter related to the sensitivity of assays developed in this work. To reduce matrix interferences in blood samples, a tenfold sample dilution with assay buffer is desired prior to analysis.

Analysis of blood samples for neopterin

The developed ELISAs based on the above polyclonal and monoclonal antibodies were evaluated using clinical samples collected from patients with sepsis, renal insufficiency, healthy volunteers and other patients without a confirmed clinical diagnosis. Direct ELISA was used due to rapid assay performance. As shown in Table 2, both ELISA methods allowed to measure increased levels in patient plasma samples (24.2–102.9 $\mu g L^{-1}$) versus healthy donors (1.1–4.2 $\mu g L^{-1}$), only patient P3 with sepsis showed a low neopterin level. The concentration of neopterin in samples is an average value obtained from two assay runs.

It is apparent from Table 2 that neopterin levels were about two times lower for pAb ELISA than the values

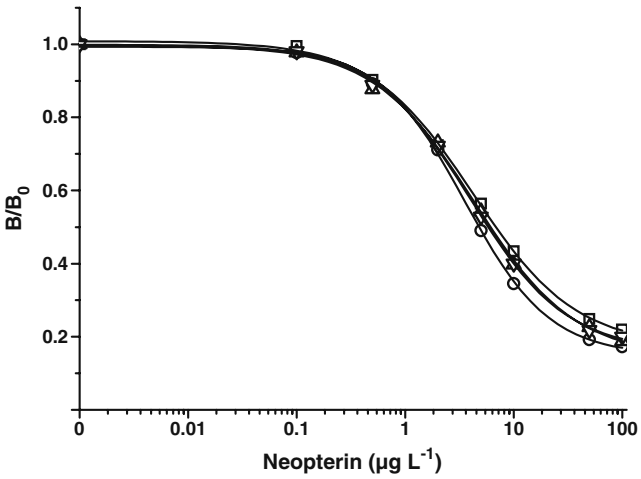

Fig. 2 Indirect ELISA curves for neopterin using mAb 3E2 and different coating conjugates: neopterin-BSA2 (*squares*), neopterin-BSA3 (*circles*), neopterin-BSA5 (*triangles*), and neopterin-BSA6 (*inverted triangles*). The respective concentrations are given in the Electronic supplementary material (Table S1)

Table 2 Neopterin in plasma samples measured by two enzyme-linked immunosorbent assays (ELISAs)

Sample	Neopterin ($\mu g L^{-1}$)	
	pAb ELISA	mAb ELISA
H1	2.4	4.2
H2	1.1	3.2
H3	1.9	3.3
P1	24.2	41.1
P2	53.0	102.9
P3	1.4	4.3
C1	24.3	43.1
C2	31.5	74.2
C3	44.4	85.1

H1–3 healthy volunteers, *P1–3* patients with sepsis, *C1–3* patients with renal insufficiency

obtained by mAb ELISA. A good correlation of the results obtained by these ELISAs within the concentration range of 1.1–102.9 $\mu g L^{-1}$ was found (y=1.956x+0.133; R=0.991; n=9). It can be noted that the measured concentrations of healthy individuals were slightly below the cut-off value for direct pAb ELISA whereas somewhat higher values were determined by mAb direct ELISA. Considering the fact that mAb ELISA measured twice higher concentration in all samples, then one can suppose that the sensitivity of this monoclonal-based assay was sufficient to distinguish basal from elevated neopterin levels. In another experiment, 40 serum samples were collected from individuals without confirmed clinical diagnosis. ELISA working with monoclonal antibody allowed to quantitate the neopterin levels in these samples in the range from 2.0 to 34.1 $\mu g L^{-1}$ whereas pAb ELISA exhibited again about two times lower results. There was found to be a good correlation of the results obtained by the two ELISAs (y=2.044x+1.159; R=0.961; n=40).

From the results, it follows that the antibodies exhibited different assay values when tenfold diluted blood plasma and serum samples were taken for analysis whereas both ELISA methods were calibrated using the same standards prepared in PBS buffer. Apparently, the cause of this phenomenon is a matrix effect having different influence on detection properties of the monoclonal and polyclonal antibody. Standard curves based on improved matrix buffer calibration could reduce this assay interference i.e. calibration should be performed in the presence of diluted serum or some equivalent medium.

Commercial neopterin ELISA kit (IBL, Germany) was employed as a reference immunoassay technique to our established ELISAs. The above serum samples were analyzed for neopterin determination using the IBL kit in parallel with ELISAs developed within this work. A satisfactory correlation of results was found between our pAb ELISA and IBL ELISA kit within the sample concentration range of 0.5–16 $\mu g L^{-1}$ (R=0.874; n=40) (Fig. 3a). A slightly lower correlation was found for mAb 3E2-based assay (R=0.819; n=40) (Fig. 3b).

Conclusions

The primary objective of the work was to prepare functional monoclonal antibodies with the required degree of specificity and affinity towards neopterin biomarker molecule and to use them together with other antibodies for the development of multianalyte biosensor detection in intelligent medical diagnostics systems. The produced monoclonal antibody mAb 3E2 enabled to measure elevated neopterin concentration in blood samples using both direct and indirect ELISA format. The LoD for these

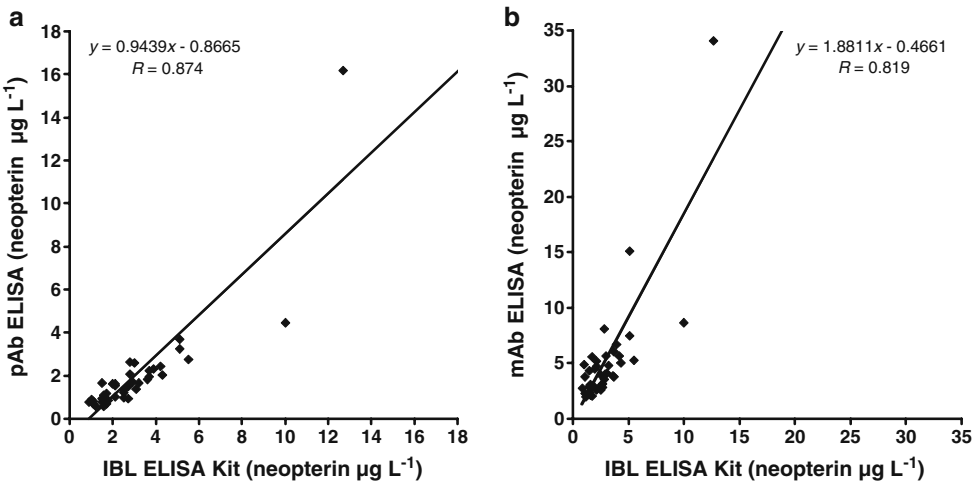

Fig. 3 Correlation of the results for the neopterin determination in serum samples **a** pAb ELISA versus IBL ELISA kit; **b** mAb ELISA versus IBL ELISA kit

monoclonal-based ELISAs was about 0.5 $\mu g L^{-1}$. The selected polyclonal rabbit antibody (pAb No. 3) was somewhat more sensitive in direct assay (LoD about 0.05 $\mu g L^{-1}$) but not sensitive enough in indirect ELISA arrangement working with coated conjugate. Thus, the mAb 3E2 was considered to be a superior immunoreagent for further biosensor developments with regard to assay sensitivity and material availability. Much attention was devoted to efforts to produce standard neopterin-protein conjugates for an immobilization on the microtitre plate or on the optical waveguide surfaces. Importantly, although some of these conjugate materials (prepared by the same or slightly modified conjugation procedure) differed in the hapten density, nearly the equal assay sensitivity was obtained and no Hook effect was observed in established ELISA systems. The presented data demonstrate that the prepared neopterin immunoreagents can be applicable together with other biological recognition elements for integration in multianalyte biochip systems. Taken together, our data indicate that the developed neopterin assay is suitable for the specific and sensitive quantification of neopterin plasma/serum levels in patients with various relevant pathological situations.

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