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# Resonance Raman spectroscopic study of interaction of hemin chloride with polyclonal antibodies

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**Background**: Currently particular attention is given to structural analysis of heme-containing proteins by use of model systems. Antibodies (Abs) to hemes enable to obtain new oxidoreductase biomimetic systems, to determine the topology of Ab active site where heme binds with the antibody, and to define the functional role of various structural groups of hemes. Because of its high sensitivity and specificity, resonance Raman spectroscopy (RRS) became a powerful technique for studying the structure and function of metalloproteins and porphyrins. RRS should allow probing structural perturbations in the porphyrin macrocycle due to the binding of axial ligands, redox transformations of the central metal ion, or protein interaction. **The aim** of this study was to develop a new oxidoreductase biomimetic system by the use of polyclonal antibodies that specifically bind hemin chloride, to carry out a detailed analysis of the relationships that exist between the antibody and hapte structural groups.

**Materials and methods**. Hemin; hematoporphyrin; protoporphyrin IX; polyclonal Ab to hemin; indirect ELISA; competitive ELISA; RRS.

**Results**. Polyclonal antibodies against hemin have been obtained and characterised. Resonance Raman spectroscopy using 413.1 nm excitation was applied to probe the interaction between hemin chloride and polyclonal antibodies. It has been found that the RR spectra are dominated by the porphyrin in-plane totally symmetric vibrational modes. Positions of oxidation-state (1371 $cm^{-1}$) and spin-state (1490 and 1566 $cm^{-1}$) marker bands have indicated a high-spin 5-coordinated Fe(III) ion state. The binding of porphyrin with antibodies resulted in appearance of bands at 1361 and 1585 $cm^{-1}$, indicating a reduction of the central iron ion and spin-state transformation.

**Conclusions**. It has been demonstrated that the interaction between hemin chloride and polyclonal antibodies proceeds through axial ligation of the iron ion and pyrrole rings containing vinyl groups. The RR spectra show that iron ion complexation results in the transition from a 5-coordinate high spin Fe(III) toward a 6-coordinate low spin Fe(II) ion state.

**Key words**: hemin chloride, polyclonal antibodies, resonance Raman spectroscopy

## BACKGROUND

Various processes and technologies such as drug metabolism (1, 2), formation of antibiotic and drug resistance (3), secondary metabolism in microbes and plants, new bioprocesses and biocatalysts depend on heme-containing proteins (4). The design of biomimetic systems that are able to reproduce the reactions catalysed by heme-containing oxidoreductases and which are easier to handle than enzymes should provide interesting tools both for the development of new catalysts for oxidation reactions which are important in industrial and fine chemistry and for the study of the oxidative metabolism of new biologically active molecules (4).

Antibodies (Abs) to hemes and to the antigenic determinants of heme enzyme prostetic group enable to obtain new oxidoreductase biomimetic systems,

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to determine the topology of Ab active site where heme binds with the antibody, and to define the functional role of various structural groups of hemes (5).

Catalytic antibodies combine the exquisite specificity of antibody recognition with catalytic activity (5–7). Currently particular attention is being given to studies of catalytic mechanisms, kinetics, protein chemical and structural analysis of this group of proteins (8–10). For designing new catalytic antibodies it is important to understand the binding of antibodies to hemes at the molecular level. Among the techniques suitable for this task, vibrational spectroscopy, especially resonance Raman (RR) scattering, seems to be particularly attractive. The superior sensitivity coupled with high specificity make resonance Raman spectroscopy (RRS) a powerful technique for studying the structure and function of metalloproteins and porphyrins (11–14). RRS allows probing structural perturbations in porphyrin macrocycle due to the binding of axial ligands, redox transformations of the central metal ion, or protein interaction at the level of $10^{-1}$–$10^{-2}$ Å (14). Importantly, vibrations from the protein matrix or other molecules in solution do not perturb the spectrum arising form the porphyrin chromophore, because its vibrational lines are selectively enhanced by a factor of $10^3$–$10^5$ when the excitation wavelength of the laser beam falls into the electronic absorption band of porphyrin (11, 12).

Most investigations on catalytic Abs have been concerned with monoclonal catalytic antibodies (4, 10). Another field of investigation is concerned with analysis of polyclonal catalytic antibodies (PCAs) (5). The analysis of PCAs is of particular value – polyclonal Ab represents the entirity of the immune response, the production of polyclonal Abs as compared with that of monoclonal Abs is relatively simple, they are of potential value as catalysts for technological application. This application is supported by the growing awareness that PCAs might be much less heterogenic than it has been supposed – polyclonal catalytic immunoglobulins exibit functional homogeneity and their catalytic properties were shown to be substantially better than those of analogous reactions catalysed by monoclonal Ab (5, 15). The relative immunogenic capabilities of a series of haptens may be assessed more effectively by PCAs than by studies on a small selection of isolated monoclonal antibodies (7).

This study describes polyclonal Abs that specifically bind hemin chloride (Fig. 1). We utilized resonance Raman spectroscopy at 413.1 nm excitation to probe the interaction of hemin chloride with polyclonal Abs.

The aim of this study was to develop a new oxidoreductase biomimetic system by using polyclonal antibodies that specifically bind hemin chloride, to carry out a detailed analysis of the relationships that exist between the antibody and hapten structural groups.

## MATERIALS AND METHODS

**Chemicals**. Hemin chloride (chloro-protoporphyrin IX iron(III)), hematoporphyrin and hematoporphyrin IX disodium salt were obtained from Fluka (Fluka Chemie GMBH). For RRS due to its low solubility in water, hemin chloride was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 mM and subsequently diluted with the aqueous 10 mM phosphate buffer, pH 7.0, to the final concentration of 0.01 mM. The working solution also contained 1.40 M of DMSO.

**Animals**. Two female rabbits (weighing 2–3 kg), obtained from Laboratory Animal Center, Institute of Immunology, Vilnius, Lithuania, were used for the experiment. All procedures were carried out in accordance with the European Union guidelines and were approved by the Ethics Committee on Animal Experimentation, Institute of Immunology, Vilnius, Lithuania.

**Preparation of polyclonal antibodies**. Prior to activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), hemin chloride, hematoporphyrine and protoporphyrine IX were dissolved in DMSO and conjugated with carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) at a molar ratio 40:1 (40 ηmol of heme to 1 ηmol of carrier protein) according to Harlow & Lane (16). The conjugates were purified by ion-exchange chromatography on DEAE cellulose (50 mM Tris-HCl, pH 7.5; 1 M NaCl). Two one-month old rabbits were immunized with the hapten-KLH conjugate on the 0, 21st, 31st, 41st, 51st days and were bleeded on the 7th day after the fifth immunization. The antibodies from immune sera and control non-immune sera were purified on a column of protein A (HiTrap rProtein A FF, Amersham Biosciences) according to Heelan (17). The antibody titers were determined by ELISA for binding to the hapten-BSA conjugate using peroxidase-linked goat anti-rabbit antibodies as described below.

**Enzyme-linked immunosorbent assay**. Antibody activity and specificity to hapten were measured by indirect ELISA. 96 well microtiter plates were coated with hapten-BSA conjugate and BSA (control) at a concentration of 6 μg/ml in carbonate-bicarbonate buffer (50 mM; pH 9.5) and incubated for 1 h at 37 °C and 12 h at 4 °C, washed 5 times with PBS (0.05% Tween-20; pH 7.4); blocked with 0.1% BSA in carbonate-bicarbonate buffer (50 Mm; pH 9.5) for 1 h at 37 °C. Polyclonal rabbit antibodies against heme were applied (1:50; 1:100; 1:1000; 1: 2000; 1: 4000; 1: 10000 in PBS (0.05% Tween-20; pH 7.4)) and incubated at 4 °C overnight. Anti-heme antibodies were revealed after incubation for 1 h at 37 °C with peroxidase

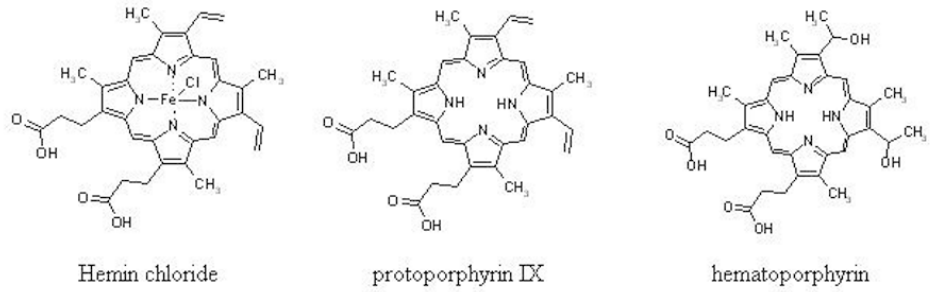


**Fig. 1.** Structural formulae of hemes

labeled goat anti-rabbit antibodies (Sigma) in PBS (0.05% Tween-20; pH 7.4) using TMB as a substrate. After each step up to pouring of the substrate, microtiter plate wells were washed 5 times with PBS (0.05% Tween-20; pH 7.4). Optical density (OD) was measured with a Sunrise type photometer (TECAN, Austria). The specificity of antibodies was expressed as the ratio S/N = $(OD_{BSA\text{-}heme} - OD_{PBS\text{-}Tw\text{-}20}) / (OD_{BSA} - OD_{PBS\text{-}Tw\text{-}20})$.

**Determination of binding constant**. The binding constants were determined by competitive ELISA as follows: mixtures of a given antibody (at a concentration equal to its titer) and increasing amounts of hemin chloride ($10^{-4}$–$10^{-12}$ M) were incubated overnight at 4 °C and them then poured in microtiter wells previously coated with a heme-BSA conjugate (6 μg/ml) in carbonate-bicarbonate buffer (50 mM, pH 9.5). After incubation at 4 °C overnight, anti-heme antibodies were stained with goat-antirabbit antibodies (Sigma) and labelled with peroxidase, using TMB as a substrate. The dissociation constants were then determined as the concentration of heme inhibiting by 50% the binding of the antibody to the immobilized antigen.

**Raman spectroscopy**. Raman measurements were carried out in 90° geometry. The 413.1 nm beam of the Kr-ion laser (Coherent, Model: Innova 90-K) was used as the excitation source. The laser power at the sample was typically 30–50 mW. The laser plasma lines were attenuated by means of an interference filter. The Raman scattering light was analyzed with a 400 mm focal length, f/2.5 spectrograph equipped with 1200 lines/mm grating by a thermoelectrically cooled (203 K) CCD camera (Princeton Instruments, Model: Spec-10:256E). A holographic filter (Kaiser Optical Systems, Model: HNPF 413.1–1.0) was placed in front of the entrance slit of the spectrograph to eliminate Rayleigh scattering from the sample. To remove the effects of the optical elements of the spectrometer on the intensities of polarized Raman spectra, a polarization scrambler was put in front of the entrance slit. The Raman frequencies were calibrated using the toluene spectrum. The integration time was 1 s. Each spectrum was recorded by accumulation of 200 scans. The overlapped bands were digitally decomposed into the components of a mixed Gaussian–Lorentzian shape. First, resonance Raman spectrum of 0.01 mM hemin chloride in 10 mM phosphate buffer solution (pH 7.0) containing 1.40 DMSO was recorded. Subsequently, specific anti-hemin chloride antibodies at a final 0.007 mM concentration were introduced and the RR spectra were collected.

## RESULTS

The polyclonal Ab obtained were characterized by determining their specificity to hapten and binding constants by indirect and competitive ELISA. Table 1 shows the specificity of polyclonal antibodies to hemin chloride and their cross-reactivity with the other cognate hemes, hematoporphyrin and protoporphyrin IX. The highest cross-reactivity was observed with protoporphyrine IX. Pyrrole rings containing vinyl groups are structural elements of both these hemes (Fig. 1). In contrast, hematoporphyrin containing hydroxyethyl groups instead of vinyl ones (Fig. 1) showed a lower cross-reactivity (Table 1). Since the other structural elements of the tetrapyrrole ring are the same for all three hemes, it might be concluded that vinyl groups themselves and/or their influence on tetrapyrrole ring conformation play a crucial role in the ligand–antibody interaction.

Table 1. **Specificity and crossreactivity of anti-hemin antibodies**

| Anti-hemin Ab (titer) | Hapten | | |
|---|---|---|---|
| | Hematoporphyrin (S/N) | Hemin chloride (S/N) | Protoporphyrin IX (S/N) |
| 1/2000 | 8.9 | 51.85 | 46.86 |

Table 2. **Binding parameters of the antibody–hemin chloride complexes**

| Antibodies | Dissociation constants Kd (M) | Average affinity $K_o$ ($M^{-1}$) |
|---|---|---|
| Anti-hemin Ab | $1.585 \times 10^{-7}$ | $6.60 \times 10^{6}$ |

Table 2 shows the dissociation and afinity constants determined by competitive ELISA of the anti-hemin chloride Ig and hapten complex. The hemin chloride–antibody complex showed a dissociation constant of $1.585 \times 10^{-7}$ (M). The average affinity of the pool of antibodies recognizing hemin chloride is $6.60 \times 10^{6}$ $M^{-1}$.

Further the model hemin chloride–antibody complex was analysed by resonance Raman spectroscopy. The structure of hemin chloride and the

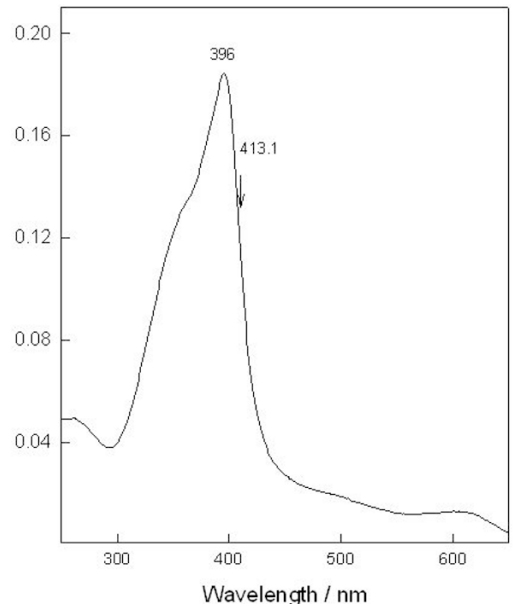


**Fig. 2.** Electron absorption spectrum of 0.01 mM hemin chloride in 20 mM aqueous phosphate buffer solution, pH 7.0, containing 1.40 M DMSO

electronic absorption spectrum are shown in Fig. 1 and 2, respectively. The electronic absorption spectrum of hemin chloride indicates that the 413.1-nm excitation wavelength is in resonance with the intense porphyrin Soret (B) band centered at 396 nm. Therefore, enhancement of Raman bands associated with totally symmetric in-plane vibrations ($A_{1g}$) is expected (11–14, 18–20). Splitting of the Soret band indicates that hemin chloride exists in the μ-oxo dimer form (20).

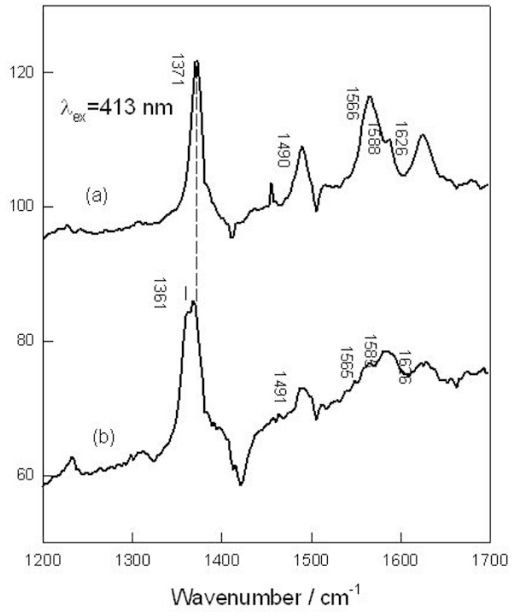


**Fig. 3.** Resonance Raman spectra of 0.01 mM hemin chloride in 10 mM phosphate buffer solution, pH 7.0, containing 1.40 M DMSO before (*a*) and after (*b*) antibody addition. Final antibody concentration in solution is 0.007 mM. Spectrum (*b*) was recorded at 30 min after addition of antibody. Excitation wavelength 413.1 nm

Resonance Raman spectra in the 1200–1700 $cm^{-1}$ spectral region for hemin chloride before and after the introduction of antibodies are shown in Fig. 3. The spectra are dominated by the porphyrin in-plane totally symmetric ($A_{1g}$) vibrational modes. The most prominent peak at 1371 $cm^{-1}$ belongs to $\nu_4$ ($A_{1g}$) mode, which is sensitive to electron density in the porphyrine macrocycle. This mode was used as an oxidation state of the central metal ion marker band (18–22). The position of the $\nu_4$ peak indicates that the iron ion is in the oxidized state ($Fe^{3+}$). The spin-state marker bands $\nu_3$ ($A_{1g}$) and $\nu_2$ ($A_{1g}$) are located at 1490 and 1566 $cm^{-1}$, respectively. The frequencies of these bands correspond to a high-spin 5-coordinated Fe(III) ion state (18–21). The highest frequency peak at 1626 $cm^{-1}$ is associated with two overlapped vibrations, non-totally symmetric depolarized $\nu_{10}$ ($B_{1g}$) mode and stretching ν(C=C) mode (18–20). Introduction of specific antibodies results in two major spectral perturbations (Fig. 3b). First, the intense $\nu_4$ band splits into two components. A new component appears at 1361 $cm^{-1}$. Secondly, the intensity of the $\nu_2$ band at 1566 $cm^{-1}$ considerably decreases, and a new peak arises at 1585 $cm^{-1}$. It should be noted that the relative intensity of the 1626-$cm^{-1}$ band considerably decreases. Figure 4 displays time-dependent spectral changes for the $\nu_4$ band contour in the frequency region of 1340–1400 $cm^{-1}$ after introduction of specific antibodies. The RR spectrum observed 30 min following the introduction of specific antibodies seems to consist of at least two bands. Therefore the complex contour of the $\nu_4$ band was digitally decomposed into two Gaussian–Lorentzian components. One can observe an increase in relative intensity of the lower frequency component at 1361 $cm^{-1}$ with time. More quantitatively this trend is demonstrated in Fig. 5, where the integrated intensity ratio, $A_{1361}/A_{1371}$, of 1361 and 1371 $cm^{-1}$ components is plotted against the time passed after the specific antibodies have been introduced into the solution containing hemin chloride. The integrated intensity ratio increases up to a time of 80 min and then levels off.

## DISCUSSION

The association of antibodies with metalloporphyrin cofactors represents a promising route to catalysts tailored for selective oxidation reactions analogous to those catalysed by hemoproteins such as peroxidases and cytochromes P-450 (23). The hapten hemin chloride–antibody complex was chosen for the RRS analysis due to its ability to generate a binding site in antibodies for an iron porphyrin and their ability to mimic coordinating ligand of iron in peroxidases (24).

The RR spectra show that an interaction between hemin chloride and specific polyclonal antibodies takes

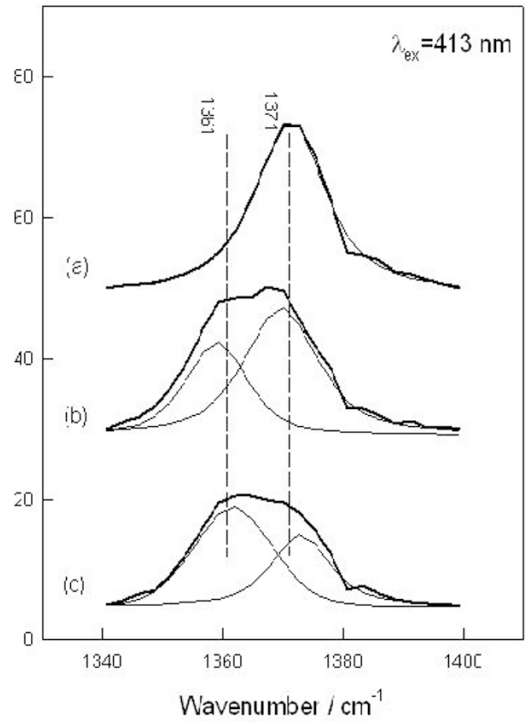


**Fig. 4.** Resonance Raman spectra in the $n_4$ mode region of 0.01 mM hemin chloride in 10 mM phosphate buffer solution (pH 7.0) containing 1.40 M DMSO at different times after addition of 0.007 mM of antibody: (*a*) 0 min, (*b*) 30 min, and (*c*) 120 min. Thinner lines represent fitted Gaussian–Lorentzian line shapes. Excitation wavelength 413.1 nm

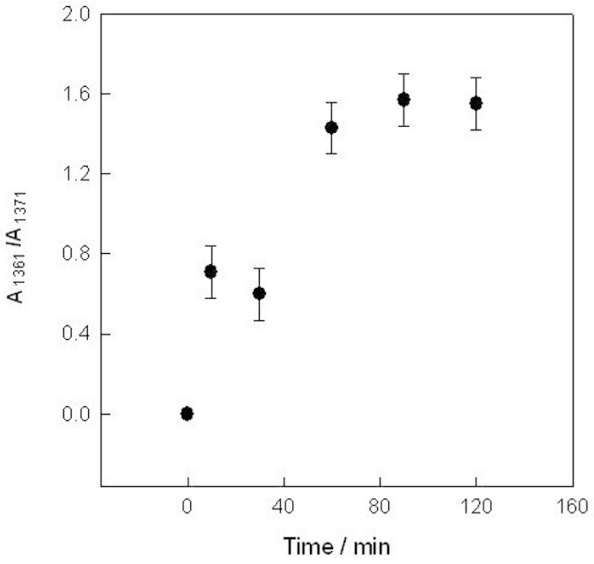


**Fig. 5.** Dependence of relative integrated intensity of 1361 and 1371 $cm^{-1}$ components on time after 0.007 mM of antibody was added into 10 mM phosphate buffer solution, pH 7.0, containing 0.01 mM hemin chloride and 1.40 M DMSO

place. Appearance of the oxidation state marker band ($\nu_4$) at lower frequencies (1361 $cm^{-1}$) upon introduction of specific antibodies points on reduction of the central iron ion from Fe(III) to Fe(II), because such frequency value is highly characteristic for the reduced iron–porphyrin complexes (18–21). Porphyrin macrocycle structure sensitive bands, $\nu_3$ and $\nu_4$, provide possibility to get an insight into the changes that occur in spin-state and coordination of the central metal ion (18–22). The $\nu_3$ and $\nu_4$ marker bands were found for the complex formed between hemin chloride and antibody at 1491 and 1585 $cm^{-1}$ (Fig. 3b), respectively, indicating that the complexation results in the transition from a 5-coordinate high spin Fe(III) toward a 6-coordinate low spin Fe(II) ion state. Thus, RR spectroscopy provides evidence that interaction between the antibody and hemin chloride takes place through the axial ligation of the iron ion. Spectroscopic data (Fig. 5) denote that the complexation reaction proceeds for approximately 80 min. Finally, it should be noted that the decrease in the relative intensity of 1626-$cm^{-1}$ band containing contribution from stretching C=C vibration might be associated with participitation of the vinyl groups in the complex formation.

The results obtained by RRS showed that in the model hemin–antibody complex the central iron atom of hemin is recognized by specific polyclonal antibodies. The anti-hemin polyclonal antibodies are a mixture of proteins exibiting different affinities for hemin among which a pool of antibodies able to bind the central iron atom exists. From the indirect ELISA results that showed high anti-hemin Ab cross-reactivity with protoporphyrine IX and hematoporphyrine, it might be proposed that pyrrole rings containing vinyl groups take place in an antibody–hapten binding too; the role of pyrrole rings with 1-hydroxy-ethyl groups in the Ab-hemine interaction is insignificant.

Further detailed analysis will be carried out to define active site topology by a comparison of the dissociation constants of various heme–antibody complexes, to detect the amino acids coordinating the iron atom in the antibody binding pocket, and to evaluate the influence of this complexation on fuctional/catalytic parameters of the model hemin-antibody molecules.

## CONCLUSIONS

Our results show that immunization of rabbits with hemin chloride-KLH conjugates leads to the production of highly specific antibodies to hemin chloride enabling to create model heme molecules that allow us to carry out a detailed analysis of the relationships that exist between the antibody and hapten structural groups. RRS has demonstrated that the interaction of hemin chloride and polyclonal antibodies proceeds through the axial ligation of the iron ion. The RR spectra showed that the complexation results in a transition from 5-coordinate high spin Fe(III) toward 6-coordinate low spin Fe(II) ion state. The results obtained from anti-hemin chloride Ab cross-reactivity with other cognate hemes, hematoporphyrine and protoporphyrine IX, showed that hemin-chloride recognition by Abs also

occurs via pyrrole rings containing vinyl groups. Further work is in progress to define the precise active site topology of hemin chloride-antibody complexes, to detect the amino acids coordinating the iron atom in the antibody binding pocket, and to evaluate the influence of this complexation on the fuctional parameters of the model molecules.

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**HEMINO CHLORIDO IR POLIKLONINIØ ANTIKÛNØ SÀVEIKOS TYRIMAS REZONANSINËS RAMANO SPEKTROSKOPIJOS METODU**

Santrauka

**Ávadas**. Pastaruoju metu ypatingas dëmesys skiriamas struktûrinei hemà turinèiø baltymø analizei. Tam daþnai pasitelkiamos modelinës sistemos. Specifiniø antikûnø prieð heminà

gavimas suteikia galimybæ sukurti naujas oksidoreduktaziø biomimetines sistemas, nustatyti antikûno jungimosi centro, kuriame hemas jungiasi su antikûnu, topologijà bei ávertinti skirtingø hemo struktûriniø grupiø vaidmená. Resonansinë Ramano spektroskopija (RRS) dël savo ypatingo jautrumo bei specifiðkumo tapo galingu árankiu tiriant metaloporfirinø bei porfirinø struktûrà ir funkcijas. RRS suteikia galimybæ tirti porfirino makrociklo struktûrinius pokyèius, atsirandanèius dël aksialiniø ligandø jungimosi, centrinio metalo jono oksidacijos-redukcijos transformacijø ar sàveikos su baltymu.

**Medþiagos ir metodai**. Heminas; hematoporfirinas; protoporfirinas IX; poliklonìniai antikûnai prieð heminà; netiesioginë ELISA; RRS.

**Rezultatai**. Buvo gauti ir apibûdinti poliklonìniai antikûnai prieð heminà. Hemino chlorido ir poliklonìniø antikûnø sàveika buvo tiriama rezonansinës Ramano spektroskopijos metodu naudojant 413.1 nm suþadinimà. RR spektre vyravo juostos, susijusios su plokðtumoje vykstanèiais visiðkai simetriniais porfirino virpesiais. Oksidacinei (1371 $cm^{-1}$) ir sukininei (1490 ir 1566 $cm^{-1}$) bûsenai jautriø juostø padëtis rodo, kad Fe(III) jonas yra aukðto sukinio bûsenos, koordinuotas 5-iais ligandais. Nustatyta, kad heminui jungiantis su antikûnais, RR spektre atsiranda 1361 ir 1585 $cm^{-1}$ juostos, bylojanèios geleþies jono redukcijà ir pasikeitusià jo sukininæ bûsenà.

**Iðvados**: Buvo nustatyta, kad hemino chlorido ir poliklonìniø antikûnø sàveikà nulemia geleþies jono aksialinis jungimas bei pirolo þiedø, turinèiø vinilo grupes, sàveika su antikûno aktyviuoju centru. RR spektrai rodo, kad geleþies jono kompleksacija susijusi su perëjimu ið aukðto sukinio 5-iø ligandø koordinuotos Fe(III) bûsenos á þemo sukino 6-iø ligandø koordinuotà Fe(II).

**Raktaþodþiai**: hemino chloridas, poliklonìniai antikûnai, rezonansinë Ramano spektroskopija