Immobilization ofHis-taggedkinaseJAK2ontothesurfaceofa plasmon resonance gold disc modified with different copper (II) complexes

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Abstract

New surface plasmon resonance (SPR) sensing platform swhich consists of copper (II) complexes of a pentetic acid thiol ligand (DPTA-Cu(II)) and of a thiol derivative of dipyrromethene (DPM-Cu(II) created on the surface of gold SPR disc were applied to oriented immobilization of His-tagged Janus kinase 2 (GST-His_6-JAK2). This method is based on the covalent bond formation between histidine from a His-tag chain of a protein and Cu(II) centres from the complexes. The kinetic and thermodynamic parameters of the oriented immobilization of GST-His_6-JAK2 protein to DPTA-Cu(II) and DPM-Cu(II) complexes attached to the Au surface of a SPR disc were discussed.

Keywords: His-tagged protein immobilization, surface plasmon resonance biosensors, dipyrromethene-Cu(II), pentetic acid thiol ligand-Cu(II), Janus Kinase 2, kinetic and thermodynamic analysis

1. Introduction

Janus kinases (JAK) are non-receptor tyrosine kinases. The JAK family in mammals consists of four members: JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase 2) [1]. They are multidomain proteins with a molecular weight of about 120–140 kDa. Catalytic domains of these kinases, located at the C-terminus of the protein sequence are functional tyrosine kinases. JAKs have a so-called 'activation loop' that regulates kinase activity and is a major site of autophosphorylation [2]. The JAK kinases are involved in the JAK/STAT pathway, which is the major signaling cascade downstream from cytokine, chemokine and growth factor receptors. Stimulation of cells with a cytokine results in activation of JAKs, which phosphorylate and activate STATs, promoting their dimerization and nuclear translocation where they regulate transcription of STAT-dependent genes. Under normal physiological conditions the ligand-dependent activation of the JAK/STAT signaling is transient and tightly regulated [3]. Abnormal JAK/STAT signaling pathways may cause higher activation of JAK2 which can promote the expression of important oncogenes and has been implicated in tumorigenesis of the primary mediastinal B-cell lymphomas (PMBL) and other hematopoietic cancers. Therefore, kinases can be important targets of molecular therapy directed against these diseases.

The most commonly used method to determine Janus kinase activity are radioisotope methods [4], phosphor specific antibodies [5], surface plasmon resonance [6] and fluorescence resonance energy transfer-based systems [7].

In order to explore the kinase – potential drug interactions we have developed electrochemical biosensors to study the interaction between recombinant histidine-tagged Rio1 protein with small molecule inhibitors [8]. Recombinant rHis_6-Rio1 protein was covalently immobilized to the NAC-N(IDA-like)-Cu(II) complex on the surface of gold electrodes.

Here, we present the work on biosensors based on the principle of surface plasmon resonance (SPR), which have emerged among the most widely used label free detection tools for the study of biomolecular interactions such as protein–protein [9,10], antibody-antigen [11,12] and receptor–ligand [13,14]. SPR offers the ability for direct measuring of interactions in real time, which allows the quantitative determination of kinetic and thermodynamic parameters [15].

Oriented immobilization of proteins on a solid surface is a fundamental interest in the study of the interaction between receptors and ligands [16–19]. The His-tag chemistry causes the correct orientation of the protein. The His-tag typically consists of five or six consecutive His residues added to the C- or N-terminus of the protein [20]. The most popular systems for oriented immobilization of His-tagged proteins are complexes of nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) with transition metal ions (Ni^{2+}, Cu^{2+}) [20–28]. Nitrilotriacetic acid forms a tetradentatechelate with Ni^{2+} and Cu^{2+}, which is able to bind His-tagged proteins via coordinated bonds [29]. A coordinative complex with a binding affinity of K_D equal to 1 mM is simply formed between one molecule of NTA and two imidazole rings on His-tagged protein [20,27]. Dextran-based SPR chips modified with covalently linked NTA molecules are commercially available and have been used for the detection of the complex between Me^{2+}-NTA and His-tagged proteins [20,24,26,30].

Searching for new methods for oriented immobilization of His-tagged proteins, we developed a new systems based on copper(II) complexes with a pentetic acid thiol ligand (DPTA-Cu(II)) and a thiol derivative of dipyrromethene (DPM-Cu(II)). These two complexes were deposited on the surface of the gold electrode destined for electrochemical measurements [8,31–33]. The main goal of the electrochemical study was the development of the analytical tools suitable for determination of interactions between His-tagged proteins, immobilized on the surface copper(II) complexes, and their ligands/inhibitors [8,31–33]. The thiol derivative of DPM immobilized on the gold surface is suitable for Cu(II) complexation [34,35]. The complex between DPM-Cu(II) and histidine chains was described as of the distorted square-planar type [36]. The pentetic acid thiol ligand (DPTA) is a

derivative of diethylene triamine pentaacetic acid (H5DPTA). The molecule of H_5DPTA can provide eight potential binding/coordinating centres [37]. Siddiqi et al. studied the complexes of H_5DPTA with metal ions such as Cr^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+} [38]. The spectrophotometric data suggest a hexacoordinate distorted octahedral environment around the metal ion. The two available binding sites in Me^{2+}-H_3DPTA complexes can be used to form a bond with imidazole units from histidine tags.

In this work, a strategy toward oriented immobilization of the His-tagged protein (GST-His_6-JAK2) to the SPR sensing platforms based on new copper(II) complexes with the pentetic acid thiol ligand DPTA-Cu(II) and the thiol derivative of dipyrromethene DPM-Cu(II), as well as the kinetic and thermodynamic parameters of these processes are reported.

2. Experimental

2.1. Reagents and materials

The thiol derivative of pentetic acid (DPTA) was synthesized at the Chemistry Department of Leuven University, Belgium [39]. The thiol derivative of dipyrromethene (DPM) was synthesized by ProChimia Surfaces Company (Sopot, Poland). N-acetylcysteamine (NAC), copper(II)acetate, TRIS-hydrochloride, glycerol, sodium chloride and chloroform were obtained from Sigma-Aldrich (Poznań, Poland). Methanol and ethanol were purchased from POCH (Gliwice, Poland). Tween 20 was supplied by BioShop (LabEmpire, Rzeszów, Poland). All aqueous solutions were prepared with autoclaved and deionized water (resistivity of 18.2 MΩcm) obtained with a Milli-Q reagent grade water system (Millipore, Bedford, MA). All buffers used for SPR experiments were rigorously filtrated through 0.22 μm sterile PVDF filters (Roth, Germany).

2.2. Janus kinase 2 (GST-His_6-JAK2) preparation

The kinase domain of JAK2 was expressed in a baculovirus expression system Bac-to-Bac in HF insect cells (Invitrogen). The protein coding sequence was cloned into the plasmid pFastBacHT (Invitrogen) in frame with glutathione S-transferase (GST), S- and hexahistidine-tag (His_6) sequences. Subsequently, the whole cassette was introduced into the bacmid DNA. Transfection of Sf21 cells with recombinant bacmid, propagation of recombinant baculovirus in Sf21 cells and infection of HF cells were all carried out according to the Invitrogen's manual.

The recombinant JAK2 was purified from HF insect cells lysates applying two-step affinity chromatography on Ni-NTA-agarose and glutathione-agarose.

HF cells were lysed by freeze-thaw cycles in the lysis buffer: 50 mM Tris–HCl pH 8.0, 0.5 M NaCl, 1% Tween-20, 1 mM DTT, 10% glycerol, 10 mM imidazole, containing a proteinase inhibitor cocktail (Roche). The lysate was clarified (50000g, 60 min, 4 C) and the supernatant was adsorbed onto Ni-NTA agarose. The resin was washed with the buffer: 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 1% Tween 20, 10% glycerol, 10 mM imidazole and the proteins were eluted with the elution buffer (wash buffer containing 0.25 M imidazole). The eluent was diluted two-fold with the wash buffer and incubated with the GSH-agarose beads (Sigma-Aldrich) for 2 h at 4 °C. After washing out unadsorbed contaminants from the beads, the adsorbed protein was eluted with dialysis buffer containing 10 mM reduced glutathione (Sigma-Aldrich). Collected fractions were dialysed against 25 mM Tris–Cl pH

8.0, 0.1 M NaCl, 0.05% Tween 20, 1 mM DTT, 10% glycerol and then concentrated on Microcon filters (MWCO 50 kDa, Millipore), aliquoted and kept frozen at -80 °C.

The applied procedure allowed to obtain the recombinant JAK2 (GST-His_6-JAK2) of high purity as judged by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (Fig. S1) [40] and Western blotting using a mouse monoclonal [HIS-1] to 6xHis tag alkaline phosphatase-conjugated antibody (Abcam).

The protein concentration was determined using the Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard.

2.3. Successive steps of SPR platform modification

An Autolab Springle SPR system (Eco Chemie, TheNetherlands) equipped with a thermostatic water bath (Julabo Labortechnik, Germany) was used. The SPR sensing platform modification is illustrated in Fig. 1. Gold-coated SPR discs were supplied by Eco Chemie, The Netherlands. Prior to modification, discs were rinsed with Milli-Q water then with ethanol and dried thoroughly in a stream of nitrogen (ultra-pure 6.0, AirProducts, Poland). Golddiscs were treated with a UV–ozone chamber (Novascan, USA) for 20 min in order to remove any organic contaminations. Clean gold SPR discs were soaked in mixed solution of pentetic acid thiol ligand (DPTA) and N-acetylcysteamine (NAC) (molar ratio 1:100) ethanol/water mixture (80:20, v/v). The second monolayer was prepared by immersion of clean gold SPR disc into the mixed solution of thiol derivative of dipyrromethene (DPM) and NAC (molar ratio 1:100) in chloroform. In the next step, the modified gold SPR discs were dipped in $1.0x10^{-3}$ M copper acetate solution in chloroform/methanol mixture (1:1, v/v). Afterwards, discs were rinsed with a chloroform/methanol mixture, then with methanol, Milli-Q and gently dried in a stream of nitrogen before placing in SPR chamber. Each modification step was performed at room temperature for 3h.

2.4. Immobilization of GST-His_6-JAK2

GST-His_6-JAK2 immobilization was performed under static conditions at three different temperatures: 5 °C, 7 °C and 10 °C using Tris buffer (0.1 M NaCl, $5.0x10^{-2}$ M TRIS–HCl, 10% glycerol, 0.05% Tween-20, pH 8.0). Freshly modified discs with DPTA-Cu(II) or DPM-Cu(II) complexes were placed in the SPR chamber and conditioned in buffer for 1h. Every 20 min discs were washed with 2 mL of buffer. After conditioning, the SPR chamber was supplemented with 100 μL of buffer and conditioned for 30 min. GST-His_6-JAK2 probe was diluted with buffer to $1x10^{-6}$ M, briefly vortexed and injected on a modified SPR gold disc (6, 12, 26, and 44 μL) in order to obtain the following concentrations: 50, 100, 200, 300 nM. After 2h incubation, GST-His_6-JAK2 solution was rinsed off with 2 mL of the buffer and the value of the SPR angle shift was recorded 20 min after the end wash. Binding experiments were performed by increasing concentration of GST-His_6-JAK2 injected on the surface of the prepared SPR sensing platform.

2.5. Kinetic analysis

To compare the binding affinity of the GST-His_6-Jak2 to different Cu(II) complexes, the sensonograms from SPR experiments were processed with the kinetic evaluation software that was supplied with Autolab Springle SPR system.

3. Results and discussion

3.1. SPR binding responses of GST-His$_6$-JAK2 over DPTA-Cu(II) and DPM-Cu(II) complexes immobilized on the surface of a gold SPR disc

Measuring the binding reaction using a SPR sensing platform requires that one of the partners should be immobilized on the surface. In the current study, the pentetic acid thiol ligand (DPTA) and dipyrromethene (DPM) were immobilized onto the gold surface of a SPR gold disc. In our previous study, these two molecules have been already successfully used with His-tagged proteins in electrochemical biosensors [8,31–33]. It also has been proved that to prevent intermolecular hydrogen bonding between molecules of thiol-dipyrromethene, an increase of the distance between the DPM units is necessary. This was achieved by formation of mixed SAMs [34]. Here, N-acetylcysteamine (NAC) was selected for dilution of dipyrromethene (DPM) and pentetic acid thiol ligand (DPTA) molecules in a molar ratio 100:1 in order to get efficient complexation of Cu(II). The mixed monolayer with N-acetylcysteamine is more stable and better organized than the mixed monolayer based on the aliphatic thiols [34,35], because NAC molecules form a hydrogen bonding network via peptide bonds. NAC has been also used as spacer molecule during preparation of electrochemical biosensors [8,31–33]. Fig. 1 illustrates the process of the SPR sensing platform fabrication. Copper (II) ions were chelated by DPTA and DPM moieties on the SAMs upon incubation in $Cu(OOCCH_3)_2$ solution outside the SPR chamber. The necessity of using organic solvents (a mixture of chloroform and methanol) in this step might affect adversely the SPR chamber. Formations of DPTA-Cu(II) and DMP-Cu(II) complexes on the surface were verified by electrochemical methods [8,31–33].

The SPR sensing platform based on the DPTA-Cu(II) and DPM-Cu(II) complexes were used to measure the binding capacity of His-tagged Janus kinase 2 protein.

The best conditions such as the temperature of the measurement and the concentration range for the immobilization of GST-His$_6$-JAK2 proteins were found experimentally. The optimization of the protein immobilization temperature was performed at 25, 15, 10 and 5 °C. The immobilization of GST-His$_6$-JAK2 protein on the surface of the DPTA-Cu(II) complex at 25 and 15 °C was not observed (data not shown). The shift of the SPR angle was recorded at 10 and 5 °C (Fig. 2 and Fig. S2A). Therefore, low temperatures in the range from 5 to 10 °C were selected for the study of interactions between GST-His$_6$-JAK2 protein and the copper (II) complexes. The temperature of 5 °C was the most beneficial for immobilization of His-tagged kinase JAK2 on the surface of DPTA-Cu(II) and DPM-Cu(II)complexes.

The sensing of kinase GST-His$_6$-JAK2 using SPR gold discs modified with the DPTA-Cu(II) complex was performed in the concentration range from 50 nM to 300 nM. The typical sensonogram collected for the DPTA-Cu(II)/GST-His$_6$-JAK2 interaction is illustrated in Fig. 2A. At a concentration of 300 nM of His-tagged Janus kinase 2 protein, the DPTA-Cu(II) sensing platform showed a binding capacity in the range of 60.0±3.2 m° at 5 °C. The probe-immobilizing procedure resulted in around 0.50±0.09 ng of kinase GST-His$_6$-JAK2 attached per 1 mm^{-2} of disc surface assuming that 120 m° corresponds to 1ng mm^{-2}.

The sensonogram for DPTA-Cu(II)-GST-His$_6$-JAK2 was curve-fitted by using kinetic and equilibrium analysis methods. For kinetic analysis, data were globally fitted to a 1:1 interaction model that included a term for mass transport to obtain the binding parameters. For equilibrium analyses, the responses at equilibrium were plotted against the analyte concentration and fitted to a simple 1:1 binding isotherm. The calculated association constant (KA) describing the DPTA-Cu(II)/GST-His$_6$-JAK2 interactions were in range $6.91x10^6 \pm 0.52x10^6$ M^{-1} and $8.24x10^6 \pm 1.32x10^6$ M^{-1} at 5 °C, for the kinetic and equilibrium analysis method, respectively (Table1, Table2).

Fig. 2B shows the SPR sensonogram for the interaction between the kinase GST-His6-JAK2 and DPM-Cu(II) complex. Immobilization of His-tagged protein was carried out in the concentration range from 25 nM to 200 nM which was determined experimentally. For the highest concentration of GST-His_6-JAK2 (200nM) changing the SPR angle in the range of 202.0±6.2 m° at 5 °C was observed. The adsorbed protein concentration corresponded to 1.68±0.25 ng mm^{-2}. The calculated association constants were $14.90x10^6$±$0.10x10^6$ M^{-1} and $16.50x10^6$±$0.60x10^6$ M^{-1} for the kinetic and equilibrium analysis method, respectively (Table1 and Table2). During the immobilization of the same kinase GST-His_6-JAK2 concentration at the surface of a gold disk modified with DPTA-Cu(II) complex almost four time smaller changes of the SPR angle (43.6±3.4 m°) were observed. The association constant is twice as large for the interaction of DPM-Cu/GST-His_6-JAK2 in comparison to DPTA-Cu(II)/GST-His_6-JAK2. Thus, the proposed SPR sensing platform modified with the DPM-Cu(II) complex was more sensitive than the one containing the DPTA-Cu(II)complex.

In order to confirm the stability of His-tagged Janus kinase 2 binding to the dipyrromethene-Cu(II) complex have been made measurement during the time. After the immobilization the 200 mM GST-His_6-JAK2 from the SPR chamber, the value of the SPR angle shift was recorded during 6h. At this time, no changes were observed in SPR angle as shown in Fig. S3.

In the literature, various methods for NTA molecule immobilization on the surface of the SPR discs were described and the binding constants for these systems are very similar to the ones described by Nieba et al. (~$1.0x10^6$ M^{-1}) [20, 41–43]. Nakamura et al. have studied different anchors for immobilization of NTA molecules onto the gold surface which were chelated with nickel(II) [25]. Such sensing platforms have affinity to the His-tagged endoglucanase in the range of $1.0x10^9$ M^{-1} at 18 °C. The copper (II) ions were also used for the oriented immobilization of proteins with His-tags [8,27,44]. Maalouli et al. have developed a SPR sensing platform based on the NTA-Cu(II) complex designed for deposition of His-tagged proteins [27]. These authors have investigated two different peptides: Bradykinine and P6c. The binding affinity of these proteins to the NTA-Cu(II) complex were determined as $0.14x10^6$ M^{-1} and $4.0x10^6$ M^{-1}. The strength of the interaction between the His-tagged protein and copper (II) were determined for the complex of Cu(II) with DPTA. The association constant for DPTA-Cu(II)/rHis_6-Rio1 was $2.38x10^6$ M^{-1}.

The values of the association constants described in the literature are comparable with these obtained for the new SPR sensing platforms incorporating copper (II) complexes with DPTA and DPM. The new sensing platform incorporating DPTA-Cu(II) and DPM-Cu(II) complexes showed protein loading comparable to commercially available NTA chips based on dextran chemistry and can thus be regarded as an interesting alternative.

3.2. Temperature dependence of GST-His_6-JAK2 interactions with DPTA-Cu(II) and DPM-Cu(II)

The ability to monitor the binding reaction from 4 to 40 °C using surface plasmon resonance, allows to collect temperature-dependent binding constants of the sensing platform [45,46]. The binding data for the DPTA-Cu(II)/GST-His_6-JAK2 and DPM-Cu(II)/GST-His_6-JAK2 interactions were collected at 5, 7 and 10 °C on the SPR sensing platform, as shown in Fig. 2 and Fig. S2. Obtained data were analysed using two methods: kinetic (Table1) and equilibrium analyses (Table2). Both methods of SPR analysis revealed that the binding affinity of the GST-His_6-JAK2 to the SPR sensing platform with DPTA-Cu(II) and DPM-Cu(II) weakened as the temperature increased from 5 to 10 °C.

The association constant for DPTA-Cu(II)/GST-His_6-JAK2 interaction decreased three fold, from $6.91x10^6$ M^{-1} to $2.10x10^6$ M^{-1} for kinetic analysis (Table1) and from $8.24x10^6$ M^{-1} to $3.30x10^6$ M^{-}

^{1} for equilibrium analysis (Table2). At the same time the dissociation constant increased only two fold. This relationship is apparent for both methods of analysis, equilibrium as well as kinetic.

The association constant calculated for GST-His_6-JAK deposition onto the DPM-Cu(II) SPR sensing platform decreased only one and a half times as the temperature increased, from $14.90x10^6$ M^{-1} to $9.45x10^6$ M^{-1} (Table1) and $16.50x10^6$ M^{-1} to $9.67x10^6$ M^{-1} (Table2). The value of K_D in this case also increased two fold as in the case of interaction between GST-His_6-JAK2 and DPTA-Cu(II) complex. This dependence was observed in both methods used to analyse data. The SPR sensing platform based on DPM-Cu(II) has a greater affinity for the binding of His-tagged protein with increasing temperature of measurements than the layer containing DPTA-Cu(II).

3.3. Thermodynamic analysis

The dissociation constants (K_D) determined at each temperature were used to obtain thermodynamic parameters by plotting ln (K_D) versus 1/T [45]. The thermodynamic parameters such as enthalpy (ΔH) and entropy (ΔS) values were obtained using the nonintegrated form of the van't Hoff equation:

$$\ln K_D = \Delta H/RT - \Delta S/R$$

This equation is of the linear form y=ax+b. Plotting $y=\ln(K_D)$ versus x=1/T gives $a=\Delta H/R$ and $b=\Delta S/R$, where R is the universal gas constant, 8.314 J mol^{-1} K^{-1}.

The Gibbs free energy (ΔG) was calculated from the following equation:

$$\Delta G = \Delta H - T\Delta S$$

for the temperature of 25 °C as a reference temperature [46,47].

Since data were collected at three different temperatures, it is possible to perform a thermodynamic analysis. The dissociation constants (K_D) determined at each temperature by two methods, kinetic (Fig. 3A) and equilibrium (Fig. 3B) analysis, were used to obtain thermodynamic parameters by plotting ln (K_D) versus 1/T [45,48].

The van't Hoff plots were generated to determine the enthalpy (ΔH) and entropy (ΔS) of binding GST-His_6-JAK2 to the two types of sensing platforms, based on DPTA-Cu(II) or DPM-Cu(II) complexes (Fig. 3). The van't Hoff plots were linear for both DPTA-Cu(II)/GST-His_6-JAK2 and DPM-Cu(II)/GST-His_6-JAK2, which was consistent with an invariant binding mechanism across the temperature range studies. For binding of His-tagged Janus kinase 2 protein to DPTA-Cu(II), the ΔH calculated vales were -118.14 kJ mol^{-1} (from kinetic analysis) and -80.56 kJ mol^{-1} (from equilibrium analysis). The lower values of enthalpy, -57.76 kJ mol^{-1} and -71.68 kJ mol^{-1} from kinetic and equilibrium analysis respectively, were obtained for interaction of GST-His_6-JAK2 and DPM-Cu(II)complex. The values of enthalpy and the Gibbs free energy (ΔG) determined for DPTA-Cu(II)/GST-His_6-JAK2 and DPM-Cu(II)/GST-His_6-JAK2 calculated according to equilibrium analysis of SPR data were very similar to parameters measured by kinetic analysis (Table3). The largest ΔG value was obtained for the thiol derivative of dipyrromethene-Cu(II)surface. This suggests that His-tagged Janus kinase 2 is more easily and quickly bonded to this complex. When ΔH and ΔS are negative, the association is enthalpically driven, which should be supplied partially by the interaction between copper (II) ions and His-tag from the protein. According to the literature, if the value of ΔH and ΔS are less than zero, and where $|T\Delta S|$ is smaller than $|\Delta H|$, the process occurs spontaneously [49]. On the basis of the thermodynamic data obtained (Table3), it might be concluded that interactions between His-tagged Janus kinase 2 and copper (II) complexes with DPTA and DPM are spontaneous processes.

4. Conclusions

New strategies for oriented immobilization of His-tagged proteins to the SPR sensing platform were developed. Pentetic acid thiol ligand and dipyrromethene molecules were introduced onto the gold surface of a SPR disc to form a self-assembled monolayer using a gold-sulfur linkage. The immobilization of His-tagged Janus kinase 2 protein onto the DPTA and DPM SAMs through copper (II) centres was studied. The kinetic and thermodynamic parameters of binding GST-His_6-JAK2 to DPTA-Cu(II) and DPM-Cu(II) complexes were investigated using the surface plasmon resonance technique. The DPM-Cu(II) surface showed a larger binding affinity than the DPTA-Cu(II) surface. The binding capacity for these two new SPR sensing platforms are comparable to the Biacore NTA-chip based on NTA-modified dextran layers.

The proposed SPR sensing platform might also be used for investigations of interactions between other biological systems involving His-tagged proteins and their ligands.

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Table 1. The comparison of association (K_A) and dissociation (K_D) constants for GST-His_6-JAK2 binding to DPTA-Cu(II) and DPM-Cu(II) complexes calculated based on kinetic analysis at various temperatures. (n = 3)

	DPTA-Cu(II)			**DPM-Cu(II)**		
Temperature [°C]	5	7	10	5	7	10
K_A **[10^6 M^{-1}]**	6.9 ± 0.5	5.1 ± 1.1	2.1 ± 0.7	14.9 ± 0.1	10.8 ± 0.2	9.5 ± 0.6
K_D **[10^{-7} M]**	1.5 ± 0.1	2.0 ± 0.7	2.7 ± 0.8	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.1

Table 2. The comparison of association (K_A) and dissociation (K_D) constant for GST-His_6-JAK2 binding to DPTA-Cu(II) and DPM-Cu(II) complexes calculated based on equilibrium analysis at various temperatures. (n = 3)

	DPTA-Cu(II)			**DPM-Cu(II)**		
Temperature [°C]	5	7	10	5	7	10
K_A **[10^6 M^{-1}]**	8.2 ± 1.3	5.6 ± 0.6	3.3 ± 0.7	16.5 ± 0.6	10.7 ± 0.1	9.7 ± 0.3
K_D **[10^{-7} M]**	1.2 ± 0.1	1.8 ± 0.8	3.0 ± 0.9	0.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.5

Table 3. Summary of the thermodynamic parameters of interactions between GST-His_6-JAK2 and DPTA-Cu(II) or DPM-Cu(II) calculated based on kinetic and equilibrium analysis.

	Kinetic Analysis		***Equilibrium Analysis***	
	DPTA-Cu(II)	**DPM-Cu(II)**	**IDA-Cu(II)**	**DPM-Cu(II)**
ΔH [kJ mol^{-1}]	-118.14	-57.76	-80.56	-71.68
ΔS [kJ K^{-1} mol^{-1}]	-0.29	-0.07	-0.16	-0.12
ΔG [kJ mol^{-1}]	-33.18	-36.63	-30.83	-38.82

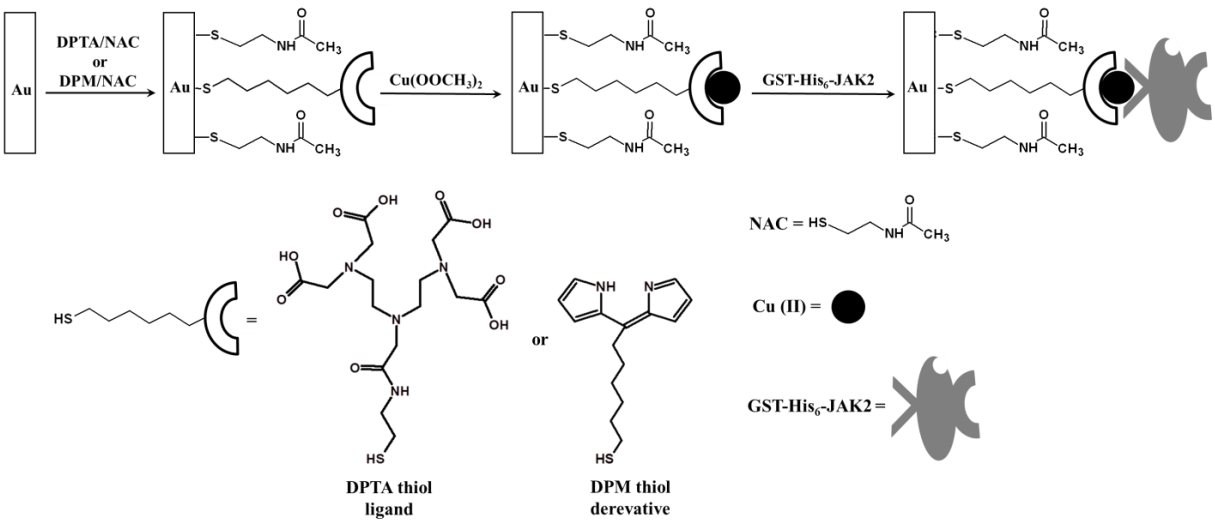

Fig. 1. Scheme of the SPR sensing platform based on DPTA-Cu(II) and DPM-Cu(II)complexes.

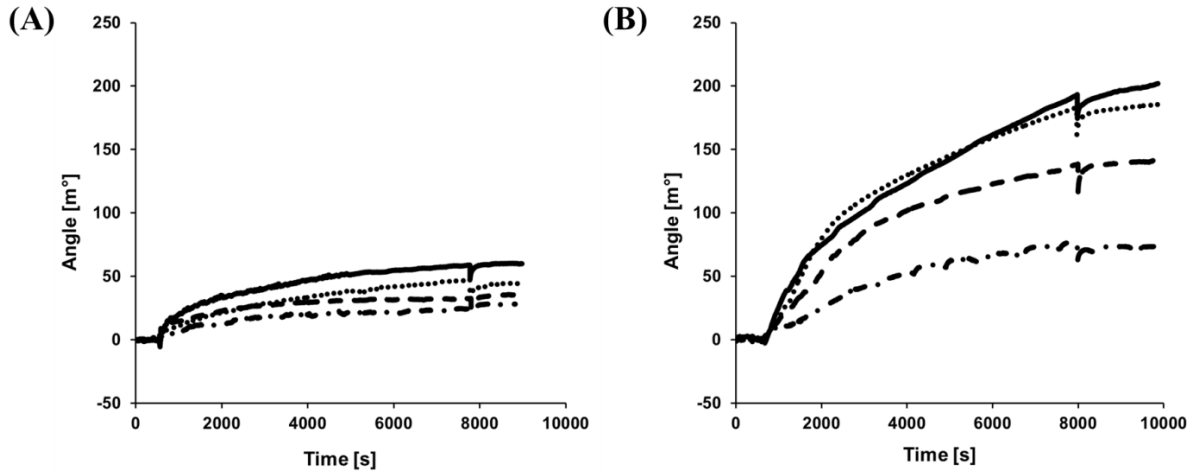

Fig. 2. Concentration dependence of the SPR sensing platforms binding response: (A) DPTA-Cu(II) for 300, 200, 100 and 50 nM of GST-His_6-JAK2; (B) DPM-Cu(II) for 200, 100, 50 and 25 nM of GST-His_6-JAK2. The measurements were performed at 5 °C.

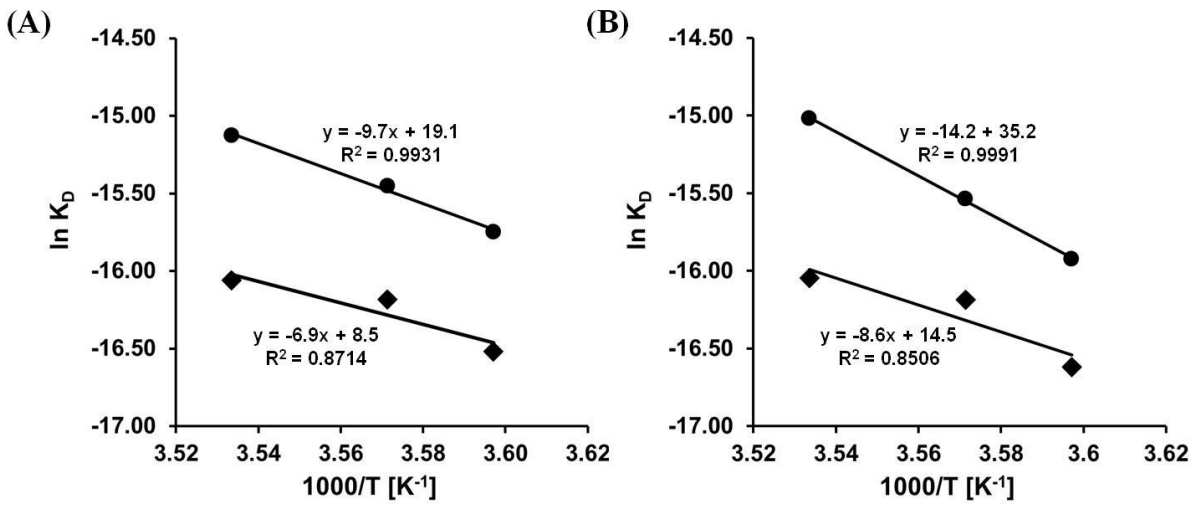

Fig. 3. The van't Hoff plots of the temperature dependence of GST-His_6-JAK2 binding to DPTM-Cu(II) (circles) and DPM-Cu(II) (diamond) calculated based on equilibrium (A) and kinetic (B) analysis.