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OPTICAL BIOSENSOR AS A TOOL FOR CHARACTERIZATION OF HUMAN IFN-GAMMA - RECEPTOR INTERACTION

ELENA KRACHMAROVA[1]*, DAMIEN MAURIN[2],
HUGUES LORTAT-JACOB[2] AND GENOVEVA NACHEVA[1]

1 – Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences, Sofia, Bulgaria
2 – Institut de Biologie Structurale, Grenoble, France
**Corresponding author: elenakrachmarova@bio21.bas.bg*

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Abstract: In the recent years, optical biosensors, such as Biacore™ system, have become increasingly popular for studying macromolecular interactions. The surface plasmon resonance (SPR) underlying the Biacore™ systems is a rapid method for analysing interactions between different molecules without the need for their pre-labelling. Biacore™ is based on a sensor chip with biospecific surface on which the ligand-analyte interaction takes place. Binding of molecules to the sensor surface generates a response, which is proportional to the bound mass, and can be detected down to changes of a few picograms per square millimetre, corresponding to concentrations of picomolar to nanomolar range in the bulk sample solution. An optical system is responsible for generating and reporting the SPR signal. The data generated by Biacore™ evaluation software gives information about the specificity of the binding, kinetics (rate of the association/disassociation) and affinity (strength) of the binding.

Human interferon-gamma (hIFNγ) exerts a wide range of immunoregulatory activities. The therapeutic applications of this cytokine challenged us to develop and optimize a highly efficient technology for its soluble production and purification. It is based on the fusion with solubility-enhancing SUMO-protein and expression in chaperon strain of *E. coli* BL21(DE3)/pG-KJE8. By using this methodology, we obtained highly pure and biologically active hIFNγ. SPR was used to evaluate its binding affinity to the extracellular part of the hIFNγ receptor (hIFNGR1). The resulting sensograms showed high affinity and establishment of strong hIFNγ/hIFNGR1 complex regardless of the analysed concentration of hIFNγ. These data are another confirmation for the effectiveness of the developed by us SUMO-methodology, since it yields recombinant protein with preserved biological characteristic. The obtained thermodynamic parameters

will be used for development and validation of *in silico* model of the hIFNγ/IFNGR1 interaction and further identification of the structural motifs important for the formation of stable complex.

INTRODUCTION

The optical system of the Biacore™ 3000 instrument is based on the optical phenomenon of surface plasmon resonance (SPR), which is used for detection of molecular interactions, that occur between two molecules (Schuck 1997). One of the molecules (usually the analyte) is in solution, which is passed over the sensor surface that carries the immobilized ligand on it. Binding of the target analyte to the ligand results in a detectable change in the angle of reflectivity due to the change of the mass on the sensor surface. This change is detected by the optical system of Biacore™ and results in the generation of a sensogram. A typical sensogram shows the change in the signal before and after addition of the target analyte. It also gives information about the rate of association of the analyte to the ligand, the stability of the complex and the rate of dissociation. Since the method detects only the change in mass, there is no need of labeling the interacting molecules, which is a big advantage of the method, since it does not require any changes in the molecular structure of the studied molecules.

Due to the high sensitivity of the method, SPR is used to study the antigen /antibody interactions (Ayela *et al.,* 2007), IgE recognition (Kim *et al.,* 2010) and recognition of enterotoxins in biological samples (Soelberg *et al.,* 2009). Studies on IFNγ performed with SPR include detection of the amount of secreted cytokine after mitogenic activation of CD4 T cells (Stybayeva *et al.,* 2010), direct determination of IFNγ in diluted blood plasma (Sipova *et al.,* 2012) and development of immune sensors (Chang *et al.,* 2012, Tuleuova *et al.,* 2010, Liu *et al.,* 2010).

Human interferon gamma (hIFNγ) is a pleiotropic cytokine, secreted by activated T-lymphocytes and natural killer cells (Boehm *et al.,* 1997) and has key role in the innate and adapted immunity. The molecule of hIFNγ represents a non-covalent homodimer in which the two subunits are in antiparallel orientation. Each monomer consists of 143 amino acids (aa) organized in six α-helices linked by unstructured loop regions. The biological activities of hIFNγ are mediated by a specific cell-surface receptor (IFNGR1), which is found on the surface of almost all cells (Schroder *et al.* 2004). The formation of the IFNγ/IFNGR1 complex results in activation of the Jak/STAT signaling pathway, which leads to establishment of immune response (Greenlund *et al.* 1993).

As a therapeutic and for experimental purposes, hIFNγ is expressed as recombinant protein in bacterial cells. Due to overexpression it aggregates in the form of inclusion bodies (IBs) (Ivanov *et al.* 1995). Its purification from IBs requires refolding step that often leads to loss of biological activity and low

yield. This challenged us to develop an efficient methodology for hIFNγ soluble expression and purification. It is based on fusion with solubility-enhancing SUMO-protein and results in obtaining highly pure and biologically active recombinant hIFNγ (Tileva *et al.* 2016).

The goal of this study was to characterize the affinity of hIFNγ, obtained by the developed by us methodology to the extracellular part of the IFNGR1 by using the high specificity of the optical phenomenon of SPR and thus to evaluate the efficiency of this SUMO approach. In addition, the obtained results will help us to validate *in silico* model of the hIFNγ/IFNGR1 interaction in order to identify the structural motifs responsible for the formation of stable complex.

MATERIAL AND METHODS

hIFNγ expression and purification

The hIFNγ gene was cloned in expression vector carrying the two chaperon system of E. coli. Its further expression and purification was performed as described in Tileva (Tileva *et al.,* 2016). In brief, the His6-SUMO-hIFNγ gene was cloned in chaperon strain of *E. coli* BL21(DE3)/pG-KJE8. Further, the fusion protein was expressed in the soluble fraction and purified by two-step chromatography procedure, i.e. affinity and ion-exchange chromatography The chromatography purification was performed on a ÄKTA™ purifier (GE Healthcare). In-between the two purification steps, the SUMO fusion partner was cleaved by using SUMO protease (Proteros Biostructures, GmbH) at protein/protease ratio 25/1. The proteolytic digestion was performed in parallel with dialysis for 12 h against 20 mM HEPES pH 8.0 and 40 mM NaCl at 4 °C. The complete removal of the fusion partner as well as the sequence of the purified target protein was confirmed by peptide mass finger-printing (ABSciex TOPLAB, Martinsried, Germany).

hIFNGR1 preparation and SPR Based-binding assay

The ectodomain of hIFNGR1 (residues 1 to 245, including the signal peptide to which a C-terminal His-tag was added) was cloned in pCDNA 3.3 vector and expressed in HEK 293 cells growing in suspension. The conditioned medium was collected every 4 to 5 days, pooled and the receptor was purified using Ni-NTA affinity chromatography using standard procedures. In order to oxidize glycans of the molecule, 270 μg/ml IFNGR1 (in 20mM phosphate buffer, pH 6), was reacted with 10 mM sodium periodate for 20 min in the dark at 4 °C. For quenching the reaction, 15 mM glycerol was used, and the sample was dialyzed against 20 mM phosphate buffer. Further, Biotin-LC-hydrazide was added to a concentration of 5 mM, and the mixture was incubated for 4h at 4 °C. Ethanolamine was added to the sample to a final concentration of 100 mM, which was then extensively dialyzed against phosphate-buffered saline, pH 7.2. The biotinylated receptor (40 μg/ml) was captured to a level of 1500 RU on a streptavidin surface prepared as

previously described (Sarrazin *et .al* 2005). In brief, for the activation of carboxyl groups of the dextran-coated chip CM4 sensor chip (GE Healthcare Life Sciences), it was reacted with 50 µl of 0.2 M N-ethyl-N′-(diethylaminopropyl)-carbodiimide and 0.05 M N-hydroxy-succinimide. After that, 50 µl of streptavidin (Sigma-Aldrich) at 0.2 mg/ml in 10 mM sodium acetate buffer, pH 4.2 was injected. 50 µl of 1 M ethanolamine pH 8.5 was used for blockage of remaining activated carboxyl groups. Reference surfaces were prepared by using the same procedure, except that all carboxyl groups were blocked and no ligand was added. Typically, this procedure allowed coupling 2000–2500 resonance units (RU) of streptavidin on both flow cells. Before use, the surface with the immobilized hIFNGR1 was conditioned with several injections of 10 mM HCl. For the binding assay, hIFNγ was diluted to the desired concentrations in HBS-EP buffer, injected at flow rate of 50 µl/min over the reference surface and the hIFNGR1 surface for 4 min at 25 °C. The surface of the chip was regenerated between each tested concentration with 1 min pulse of 10 Mm HCl. Biacore™ Biaevaluation software 3.1 was used for evaluation of the binding affinity of the analyte.

RESULTS AND DISCUSSION

The quality of the sample is crucial for the SPR technique in order to reduce the artefacts during the measurement. As a result from the developed by us SUMO-methodology (Tileva *et al.* 2016) the ligand (hIFNγ) was obtained with > 95 % purity, which is recommended for the SPR measurements.

hIFNGR1 was immobilized on the surface of CM4 sensor chip as described in *"Materials and Methods"*. It should be taken into account, that the immobilization of the ligand may restrict its motility and diffusion properties, which may lead to a change in the thermodynamics and kinetics of the reaction. However, since the golden surface of the CM4 chip is modified with dextran layer, this ensures preserved mobility of the ligand, which in turns leads to some diffusional freedom (Karlsson *et al.* 1994). The diffusion of the analyte from the bulk solution is an essential process that must occur quickly and evenly. Otherwise a concentration gradient may occur and affect the generated data (Day *et al.* 2002). The flow system used in Biacore™ is essential for the rapid delivery of a sufficient amount of analyte during the association phase, and analyte's rapid wash out in the dissociation process. This helps to minimize or even eliminate the concentration gradient on the surface of the chip, which would otherwise lead to incorrect data interpretation.

Applying the established experimental conditions, high affinity and strong interaction of the ligand hIFNγ with the receptor was observed at each of the tested concentrations (**Fig. 1**). This interaction was diffuse limited, which means

that shortly after the injection of the analyte over the surface of the sensor chip, the complex formation occurred faster than the diffusion of the analyte from the bulk solution. Therefore, the binding responses were fitted to an interaction model that included a mass transfer step.

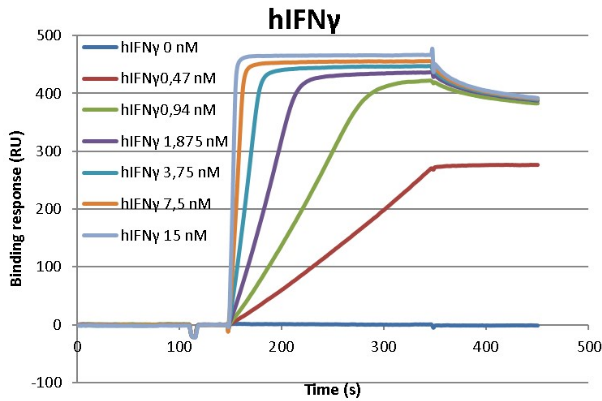

Fig. 1. SPR sensograms of the kinetics of the interaction of hIFNγ/hIFNGR1 at concentrations of 0.47; 0.94; 1.875; 3.75; 7.5 and 15 nM hIFNγ.

Based on the obtained sensograms k_{on}, k_{off} and the values of the equilibrium constants K_A and K_D were calculated (Table 1).

Table 1 Kinetic parameters of hIFNγ/IFNGR1 interaction

Protein	k_{on}, $M^{-1}s^{-1}$	k_{off}, s^{-1}	K_A, M^{-1}	K_D, nM
hIFNγ	$1{,}63 \times 10^7$	$4{,}02 \times 10^{-3}$	$4{,}04 \times 10^9$	0.247

The data presented in Table 1 shows that hIFNγ is characterized with high association rate constant (k_{on}) and upon binding with the cellular receptor it forms stable complex that is characterized with low dissociation rate constant (k_{off}).

There are only few data in the literature presenting the thermodynamic parameters of hIFNγ/IFNGR1 interaction obtained by SPR. Haelewyn and co-authors (Haelewyn *et al.,* 1997) measured the affinity of the full length hIFNγ to hIFNGR1 and demonstrated a marked decrease in the affinity (kon) of mutant IFNγ with 14 C-terminal amino acides truncated (IFNγ $_{\Delta}$14, Table 2).

Saesen and co-authors (Saesen *et al.* 2013) obtained similar data, when studying the effect of the C-terminus of IFNγ to its affinity to IFNGR1. While they obtained K_D=0,13 nM (0,247 nM in our case) for the wild type, they observed lower affinity (K_D = 0,31 nM) when mutating all three Arg to Ser in the D2 domain of the C-terminus (IFNγ $_{SD2}$, Table 2). This tendency was maintained also for the

second mutant (K_D = 0,39 nM), in which the D2 domain was completely removed ($IFN\gamma_{\Delta136}$, Table 2).

By using a reverse strategy, i.e. introduction of mutations into the binding domains of IFNGR1, Mikulesky and co-authors (Mikulesky *et al.* 2013) analyzed the affinity of mutant forms of IFNGR1 to a single-chain hIFNγ. Based on areas identified by computer protocol, the authors expressed and purified 14 receptor mutant analogues and examined their affinity to the single-chain IFNγ. They identified a mutant (N96W + H222R) that was characterized with five-fold increase in the affinity compared to the wild-type IFNGR1 as shown by the lower dissociation constant (k_{off}, Table 2).

Table 2 Reported thermodynamic parameters of hIFNγ/IFNGR1 interaction

Protein	k_{on}, $M^{-1}s^{-1}$	k_{off}, s^{-1}	K_A, M^{-1}	K_D, nM	Reference
hIFNγ	$6{,}1 \times 10^5$	$1{,}6 \times 10^{-3}$	$3{,}8 \times 10^8$	2,62	Haelewyn *et al.* , 1997
$IFN\gamma_{\Delta14}$	$9{,}3 \times 10^4$	$4{,}8 \times 10^{-3}$	$1{,}6 \times 10^7$	62,3	
hIFNγ	-	-	-	0,13	Saesen *et al.* , 2013
$IFN\gamma_{SD2}$	-	-	-	0,31	
$IFN\gamma_{\Delta136}$	-	-	-	0,39	
IFNGR1	$1{,}24 \times 10^6$	$3{,}78 \times 10^{-2}$	-	30,8	Mikulesky *et. al.* , 2013
N96W + H222R	$2{,}4 \times 10^6$	1×10^{-2}	-	4,16	

As seen from the reference data presented in Table 2, hIFNγ obtained by the SUMO methodology is characterized by more than 25 times higher association rate constant (Table 1, k_{on} data). Previously, we demonstrated that this preparation is characterized by completely preserved biological activity (Tileva *et al.* 2016). Taken together these data confirm that the developed by us methodology for expression and purification of recombinant proteins is effective and results in production of recombinant proteins with preserved biological properties.

CONCLUSIONS

The obtained in this study thermodynamic parameters will be used for validation of in silico model of the hIFNγ/IFNGR1 interaction and further identification of the structural motifs, important for the formation of stable complex. These data are also important prerequisite for the carried by us development of new drug candidates for treatment of autoimmune diseases, whose etiology is associated with overexpression of endogenous hIFNγ. These biotherapeutics will be based on inactive structural analogs of hIFNγ with preserved or increased affinity for IFNGR1 and will be used to regulate the activity of the overexpressed cytokine.

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AUTHORSHIP CONTRIBUTIONS: H. L-J. and G. N. were responsible for conception and design of the study, analysis and interpretation of the data and revising the manuscript; D. M. was responsible for the IFNGR1 expression and purification; E. K. was responsible for expression and purification of hIFNγ, performing the SPR experiments and drafting the manuscript.

REFERENCES

1. Ayela, C., Roquet, F., Valera, L., Granier, C., Nicu, L., Pugniere, M. 2007. Antibody–antigenic peptide interactions monitored by SPR and QCM-D: A model for SPR detection of IA-2 autoantibodies in human serum. *Biosensors and Bioelectronics* , 22 (12): 3113-3119.
2. Boehm, U., Klamp, T., Groot, M., Howard, J. C. 1997. Cellular responses to interferon-γ. *Annual review of immunology*, 15 (1): 749-795.
3. Chang, C. C., Lin, S., Lee, C. H., Chuang, T. L., Hsueh, P. R., Lai, H. C., Lin, C. W. 2012. Amplified surface plasmon resonance immunosensor for interferon-Gamma based on a streptavidin-incorporated aptamer. *Biosensors and Bioelectronics.,* 37 (1): 68-74.
4. Day, Y. S., Baird, C. L., Rich, R. L., Myszka, D. G. 2002. Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface-and solution-based biophysical methods. *Protein Science,* 11 (5): 1017-1025.
5. Greenlund, A. C., Farrar, M. A., Viviano, B. L., Schreiber, R. D. 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91) *The EMBO journal* 13 (7) :1591-1600
6. Haelewyn, J., Michiels, L., Verhaert, P., Hoylaerts, M. F., Witters, R., De Ley, M. 1997. Interaction of truncated human interferon gamma variants with the interferon gamma receptor: crucial importance of Arg-129. *Biochemical Journal*, 324 (2): 591-595.
7. I.G. Ivanov, V.P. Ivanov, V.S. Ivanova, L.N. Marekov, R.A. Vassileva, A.A. Sarafova, R.G. Tsanev, 1995. Method for producing of recombinant human cysteinless gamma-interferon free of methionine at N-terminal. European Patent 0 446 582 B1; C12N 15/23 (Date of publication: 04.01.1995; Appl. No 91100777.1; Date of filing: 22.01.91).
8. Karlsson, R., Roos, H., Faëgerstam, L., Persson, B. 1994. Kinetic and concentration analysis using BIA technology. *Methods* 6 (2): 99–110.
9. Kim, S., Lee, J., Lee, S. J., Lee, H. J. 2010. Ultra-sensitive detection of IgE using biofunctionalized nanoparticle-enhanced SPR. *Talanta*, 81 (4-5): 1755-1759.
10. Liu, Y., Tuleouva, N., Ramanculov, E., Revzin, A. 2010. Aptamer-based electrochemical biosensor for interferon gamma detection. *Analytical chemistry,* 82 (19): 8131-8136.

11. Mikulecký, P., Černý, J., Biedermannová, L., Petroková, H., Kuchař, M., Vondrášek, J., Malý, P., Šebo, P. & Schneider, B. 2013. Increasing Affinity of Interferon-Receptor 1 to Interferon-by Computer-Aided Design. *BioMed research international,* 2013
12. Saesen, E., Sarrazin, S., Laguri, C., Sadir, R., Maurin, D., Thomas, A., Imberty, A. Lortat-Jacob, H. 2013. Insights into the Mechanism by Which Interferon-γ Basic Amino Acid Clusters Mediate Protein Binding to Heparan Sulfate, J*ournal of the American Chemical Society,*135 (25): 9384-9390.
13. Sarrazin, S., Bonnaffé, D., Lubineau, A., Lortat-Jacob, H. 2005. Heparan Sulfate Mimicry A SYNTHETIC GLYCOCONJUGATE THAT RECOGNIZES THE HEPARIN BINDING DOMAIN OF INTERFERON-γ INHIBITS THE CYTOKINE ACTIVITY. *Journal of Biological Chemistry*, 280 (45): 37558-37564.
14. Schroder, K., Hertzog, P.J., Ravasi, T. and Hume, D.A. 2004. Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*, 75(2): 163-189.
15. Schuck P. 1997. Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules. *Annual review of biophysics and biomolecular structure,* 26 (1): 541–566.
16. Šípová, H., Ševců, V., Kuchař, M., Ahmad, J.N., Mikulecký, P., Osička, R., Malý, P., Homola, J. 2012. Surface plasmon resonance biosensor based on engineered proteins for direct detection of interferon-gamma in diluted blood plasma. Sensors and Actuators B: *Chemical*. 174(2012): 306-311.
17. Soelberg, S. D., Stevens, R. C., Limaye, A. P., Furlong, C. E. 2009. Surface plasmon resonance detection using antibody-linked magnetic nanoparticles for analyte capture, purification, concentration, and signal amplification. *Analytical chemistry.* 81 (6): 2357-2363.
18. Stybayeva, G., Kairova, M., Ramanculov, E., Simonian, A. L., Revzin, A. 2010. Detecting interferon-gamma release from human CD4 T-cells using surface plasmon resonance. Colloids and Surfaces B: *Biointerfaces*. 80 (2): 251-255.
19. Tileva, M., Krachmarova, E., Ivanov, I., Maskos, K., Nacheva, G. 2016. Production of aggregation prone human interferon gamma and its mutant in highly soluble and biologically active form by SUMO fusion technology, *Protein Expression and Purification* 117 (2016): 26-34.
20. Tuleuova, N., Jones, C. N., Yan, J., Ramanculov, E., Yokobayashi, Y., Revzin, A. 2010. Development of an aptamer beacon for detection of interferon-gamma. *Analytical chemistry,* 82 (5): 1851-1857.