American Journal of Immunology 2 (1): 12-18, 2006
ISSN 1553-619X

Introduction of Three Complementary Technologies into Clinical Medicine: Development of New Diagnostic and Prognostic Parameters

[1]Gernot P. Tilz, [2]Marco Wiltgen, [1]Ulrike Demel, [3]Christian Faschinger, [1]Hans Schmiedinger and [1]Albin Hermetter
[1] Clinical Immunology and Jean Dausset Laboratory, Medical University of Graz, Auenbruggerplatz 8, A-8036 Graz, Austria
[2] Institute of Medical Informatics, Statistics and Documentation, Medical University of Graz, Auenbruggerplatz 8, A-8036 Graz, Austria
[3] Department of Ophthalmology, Medical University of Graz, Auenbruggerplatz 4, A-8036 Graz, Austria

Abstract : Molecular medicine leads us to understand some diseases at the molecular level. Examples are the analysis of immune complexes and receptor – anti receptor compounds used in clinical medicine. Structural changes of some serum proteins occur in inflammation, neoplasie and autoimmunity. The detection and analysis of such structural modifications may offer a new field for the diagnosis, prognosis and therapy of some diseases. Modern medicine requires new technologies with high sensitivity, specificity and applicability. For the first time in Austria we have combined fluorescence correlation spectroscope (FCS), Surface enhanced laser desorption ionisation – time of flight (SELDI-TOF) and the molecular modelling and visualization system according to the computer enhanced programs. Experimental and computational methods are combined in such a way, that clinical data can be interpreted by theoretical methods at a molecular level or vice versa the computational output delivers input for new investigations. We will test our combination of experimental and theoretical methods on well suited media such as: aqueous humour, sera and highly purified biologicals. These media contain albumin and other substances which might be modified structurally by tumour enzymes and inflammatory products, hence giving us a valuable indication of the underlying diseases. All the new methods quoted have their disadvantages which can be bypassed by multiplexing, what we have done. So we came to the detection of albumin in the aqueous humour and an increase of sensitivity for the detection of the Goodpasture-antigen. Results from structural analysis of albumin and collagen IV under normal conditions will serve as references for further investigations. Software for the structural analysis has been developed by us and results will be presented at this place. One method brings us single results. In view of the spectrum of parameters relevant to clinical entities, multiplexing is a new way of development. Since the technologies are new, the scientifically interested reader should be informed about the matters arising.

Key words: FCS, SELDI-TOF, Protein visualization and modelling, antibodies, 3D-structure

INTRODUCTION

Modern scientific medicine requires correlations between the etiopathogenesis, the diagnostic criteria, specific treatment and the clinical course. Examples are numerous, e.g. the Goodpasture syndrome.

High sophisticated methods bring us single results. In view of the spectrum of parameters relevant to clinical entities, multiplexing is a new way of development. Hence experimental methods such as fluorescence correlation spectroscopy (FCS), surface enhanced laser desorption ionisation – time of flight (SELDI-TOF) mass spectrometry are combined with computer methods in our laboratory and this for the first time.

Bioinformatics is the science relying on centralized data banks, connecting sequences to structural and functional information. Access to the databases is enabled via Internet. The growing amount of information in the databases enables new and powerfully perspectives for the interpretation and completion of actual experimental data by appropriate searches in the databases (e.g. comparing a new determined peptide sequence with known sequences annotated with structural and biological data). A growing section of bioinformatics deals with the computation and visualization of protein 3D structures.

Corresponding Author: Prof. Dr. Gernot P. Tilz, Clinical Immunology and Jean Dausset Laboratory, Medical University of Graz, Clinical Immunology, Auenbruggerplatz 8, A-8036 Graz, Austria

The data from the clinical experimental methods are used as input for the computational methods. Then the search in the databases for additional data linked to the experimental data and the modelling and analysis of protein structures enables an interpretation of the clinical data.

In this paper we present the scenario connecting the clinical laboratory (FCS, SELDI-TOF) and the computer laboratory (modelling, analysis and visualization of protein and protein complexes).

MATERIAL AND METHODS

1. FCS (Fluorescence correlation spectroscopy)

The FCS method consists of the direct measuring of molecules and the interaction in a volume element. Molecules have to be labelled by a fluorochrome and the Brownian movement of the fluorescent molecules is measured in a tiny virtual volume element, formed by a laser beam crossing a cuvette. Usually argon-ion lasers and helium-neon lasers are used for the excitation of the sample molecules. As fluorescent dyes rhodamine and

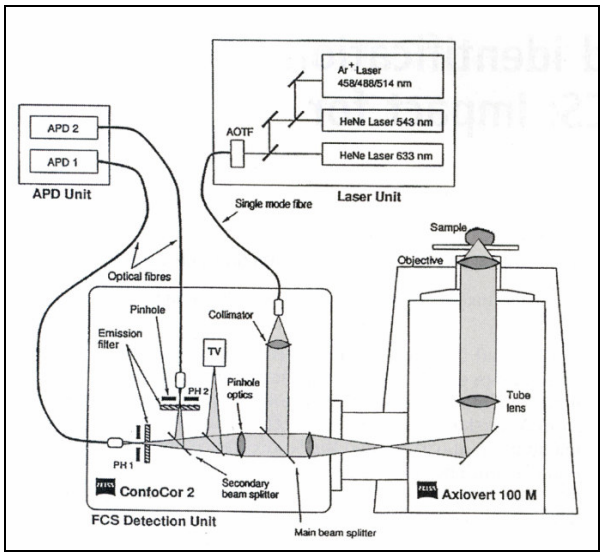

Fig. 1:Fluorescence Correlation Spectroscopy (FCS). The laser beams are focused to small spots in the sample solution. The fluorescence light is collected by the objective and passes through the beam splitter which reflects the excitation light into the objective and transmits the fluorescence light from the sample. Fluctuations in the fluorescence light are detected by photo diodes.

CY5 have been widely used and recently the Alexa dye was introduced. The optical aperture of the fluorescence correlation spectrometer ConfoCor 2 (Zeiss) is shown in Figure 1.

The sample molecules are excited by laser beams. To this purposes the laser beams are focused (with epi-illumination optics) to small spots in the sample solution. The fluorescence light emitted by the sample is collected by a high numerical aperture objective. The beam splitter, which reflects the excitation light to the objective, allows the separation of emitted fluorescence

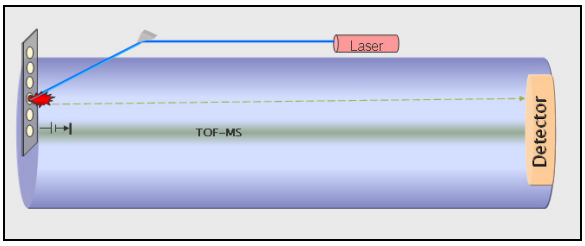

Fig. 2:Surface enhanced laser desorption ionisation time of flight (SELDI-TOF). The proteins are "eluted" from the matrix array by laser desorption. The masses of the ionized proteins are accurately determined by Time-of-Flight Mass Spectrometry.

light and excitation light by transmitting the fluorescence light. The transmitted fluorescence light passes then pinholes which block scattered excitation light and fluorescence light from outside the focal spot. The intensity of the filtered light is measured by photo diodes. The Brownian motion of the molecules in the sample is then detected as fluctuations of the emitted fluorescence intensity. The fluorescence intensity fluctuations are recorded over time and evaluated statistically by auto- and cross-correlation.

The behaviour of the proteins during the titration with potential ligands is observed. Antigen and antibody reaction can be characterised and we have used this system for the definition of Good pasture antibodies in the peripheral blood of patients suffering from Rapid Progressive Glomerulonephritis[1]. Other examples are the characterisation of the thyreo globulin in the peripheral blood of patients suffering from Hashimoto's disease. (Manuscript on the interaction between TNF and its receptor is in preparation).

2. SELDI – TOF (surface enhanced laser desorption ionisation – time of flight)

By mass spectrometry, starting from a small sample, the masses of various components in a mixture are determined and specific proteins can be identified by the characteristic peaks of the spectrogram [2-9].

SELDI–TOF consists on one part of an apparatus allowing the laser bombarding of molecules on a solid surface and on the other part on a system which measure the time of flight of the molecules (Figure 2). During the measurement the molecules are liberated from the matrix and carried to the detector.

The proteins are applied on the surface of a (SELDI)-matrix. Then the matrix is inserted into the vacuum chamber of the mass spectrometer. By the laser beam, the molecules embedded in the matrix are ionized (by absorption of the laser energy), liberated

from the matrix and accelerated in an electrical field. The velocity of the ionized molecules is depending of the mass to charge ratio: m/Z (m is the mass of the molecule and Z the electric charge). The time of flight (TOF) from the surface to the detector is therefore a function of the molecular mass and light ions are reaching the detector first, followed by the heavier. By measuring the time of flight the mass to charge ratio can by determined. The spectrum can be drawn and molecules characterized without additional labelling procedure, which is mandatory for FCS.

Examples are numerous in the literature ranging from low mass weight inflammatory peptide to split products of γ-globulines, even albumine.

3. Computer analysis of protein structures

Nowadays computer generated representations of protein structures, based on experimental data, are used. Once a protein structure has been determined (for example by crystallographic methods), it is deposited

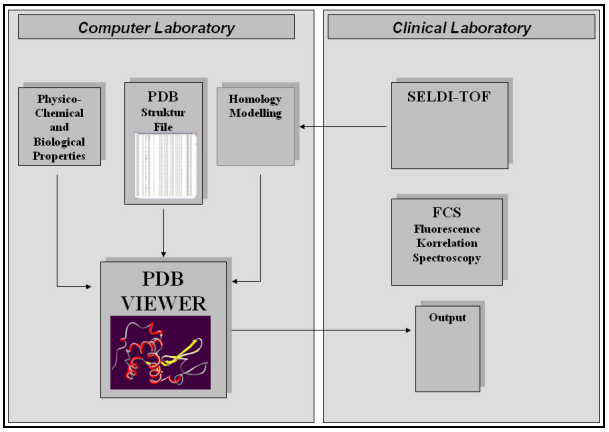

Fig. 3:The figure shows the connection between the computer laboratory and the clinical laboratory. Mass spectrometric methods (SELDI-TOF) deliver for a protein a characteristic peptide mass fingerprint allowing conclusions about the protein sequence. In homology modelling the protein sequence is aligned with sequences of known structures (PDB database) enabling a prediction of the structure. The structures are visualized (annotated with physicochemical properties) by the PDB viewer. The macromolecular interfaces of protein complexes, detected and characterized by FCS are analysed with special software tools.

into the protein database (PDB), an international repository for 3D structure files (http://www.rcsb.org/pdb)[10]. At the moment PDB contains more than 35.000 protein structures, which are stored as collections of Cartesian coordinates in the PDB files.

In our model a connection between the computer laboratory (bioinformatics) and the immunochemical set up is necessary, delivering each other with the necessary data (Figure 3).

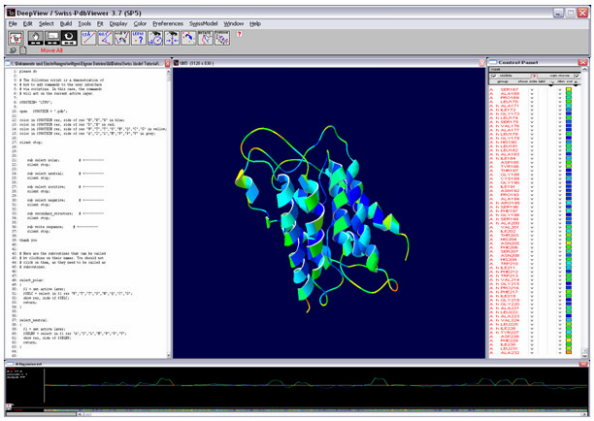

Fig. 4:The Swiss-Pdb Viewer software tool emphasis several windows. The main window (top) enables the interactive manipulation of the protein structure in the display window (middle). In the control panel window (right side) individual amino acid residues can be selected for display. The alignment window (bottom) displays the protein sequence. In the script window (left side) proprietary programs are initialized by mouse clicking, allowing special problem based visualizations.

Mutated proteins, involved in diseases, detected by SELDI are candidates for structural analysis on the computer. SELDI-TOF delivers for every protein a characteristic peptide mass fingerprint. From this fingerprint (the peptide mass spectrum) the fragments can be identified by data base aided methods (http://au.expasy.org/tools/) which allows conclusions about the peptide sequences leading to the identification of the protein. This is done by comparison of the mass spectra on the computer. When the sequence is determined and available, a structure prediction must be performed. The structural deviation between the genuine protein and the mutated one can give us an indication of the underlying disease at the molecular level.

3a) Homology modelling: For the mutated sequence, the structure can be predicted by homology modelling. Homology modelling relies on rules of spacing between atoms, bond lengths, bond angles etc., from observed values in known protein structures. In homology modelling a protein sequence with an unknown structure (the target) is aligned with one or more protein sequences with known structures (the templates). The necessary condition for successful homology modelling is a detectable similarity between the target sequence and the template sequence (more

than 30%) allowing the construction of a correct alignment. The alignment is used to determine the equivalent residues in the target and the template protein. Homology modelling is done by us with the program package "modeller" (release 7v7)[11].

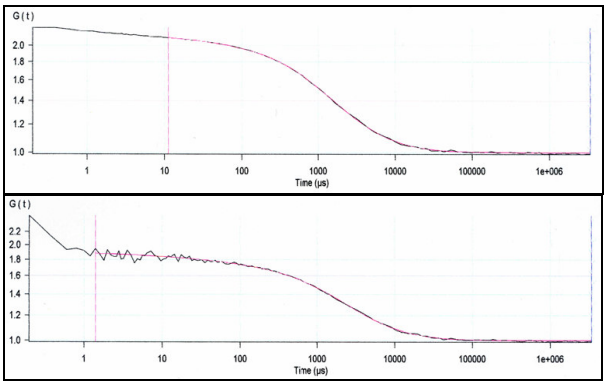

Fig. 5:Cross-correlation of the Goodpasture auto-Abs in serum. Evaluation of the upper curve shows a diffusion time of 1116 μsec and for the lower curve 1863 μsec as results.

3b) Structure visualization: Usually work with protein structures is performed on a workstation using a mouse as input device. The Swiss-Pdb Viewer is used by us [12]. This program converts the atomic coordinates into a view of the protein (Figure 4). The viewer provides ways for interactively manipulating the view of the protein molecule (rotations, zooming etc.) and enables features like distance measurements, calculation and display of hydrogen bonds etc. For special problem based representations, proprietary programs (written in a script language) can be read in, enabling complex and time consuming calculations on the protein structure [13].

To compare two protein structures and to discover structural similarities, the structures are superposed. The differences between two protein structures are expressed by the root mean squared deviation (RMS) of the respective atomic positions in the two structures and are visualized by colour encoding. Then significant structural deviations can be located.

3c) Analysis of macromolecular interfaces: For structure determination with x-ray crystallography, the protein molecules are often crystallized in complex with their ligands. The appropriate representation of the protein bounded with the ligand molecule offers insight into the dynamical behaviour and the active sites (binding sites) of the protein molecule. By using a PDB file of the complex, the interactions of the molecules can be visualized [14-15].

We use protein complexes (from the PDB database and/or detected and characterized by FCS or SELDI) to analyse the macromolecular interface, allowing a 3D visualization and making the involved residues easily detectable in the wealth of information provided by the complex structures. Calculations were done by a special developed computer program, where the results are used as input by the Swiss-Pdb viewer for visualization.

RESULTS

FCS technology: Results of the FCS show the application for the diagnosis of the Goodpasture syndrome. Sensitivity is about 200 times higher than traditional Elisa hence allowing us an early detection of autoimmunity in critical cases. Results are shown in Figure 5. indicating an approximately 200x higher sensitivity of FCS for the early detection of the ongoing disease. Results for titration curves in FCS are also shown for a different antigen allowing us a detection of antigen in the nanogram range.

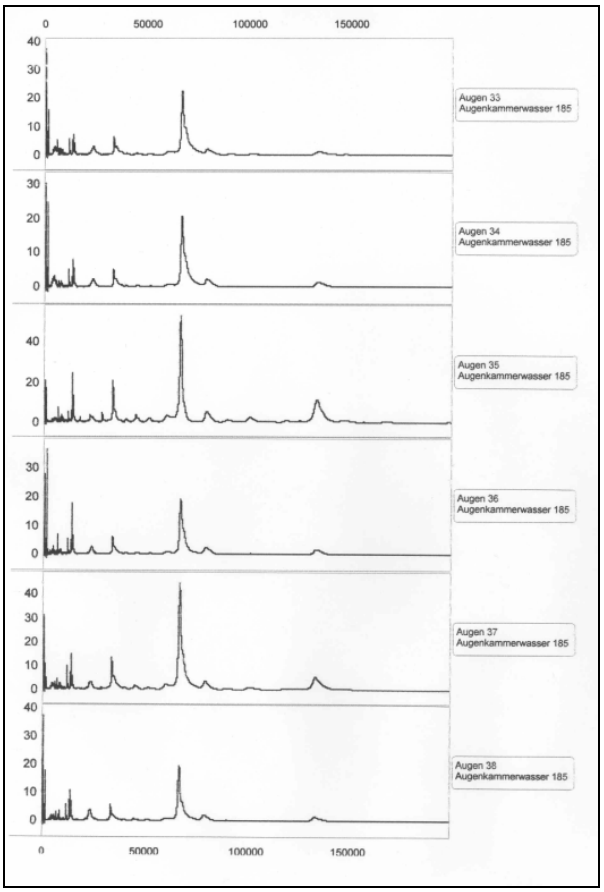

Fig. 6:SELDI-TO-MS mass spectrogram of albumin inaqueoue humour. The spectrum shows a main peak at 66465.2 Dalton, indicating albumin

SELDI technology: The spectrum of aqueous humour is shown, allowing us a clear characterisation of "Normal and Abnormal Constituency" (Figure 6). Further publications will be suitable, to find new

molecules relevant for tumours, autoimmunity and inflammatory situations.

Protein modelling: In SELDI technology we have discovered many molecules and consequently concentrated on albumin (binds water, cations as well as fatty acids, hormones, bilirubin and drugs), which is a major constituent in various fluids such as CSF and the aqueous humour. Based of these findings we developed software in our laboratory for the problem based visualization of the protein structure in complex with other molecules. Since it is expected that albumin should be modified in the case of inflammation, neoplasie and autoimmunity we will use the results from structural analysis of albumin under normal conditions as reference for further investigations (Figure 7)[16]. The same is true for the molecular structure of the causative collagen IV as antigen in the Goodpasture syndrome[17]. This is shown in Figure 8.

.

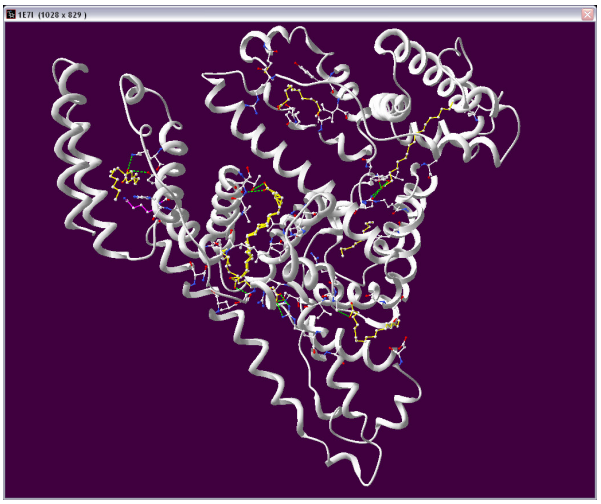

Fig. 7:Human serum albumin molecule in a complex with octadecanoic acid. The octadecanoic acid molecules are captured via hydrogen bonds (dotted lines) from the albumin molecule. The folding of the protein is visualized as backbone ribbon showing secondary structure elements, whereas the residues interacting with the acid molecules are represented as wire frame (atomic ball-and-stick model).

DISCUSSION

In this original contribution we show for the very first time the combination of 3 new technologies in medicine. The combination is necessary since the individual disadvantages can be neutralized and a potential complexing may result. FCS has a unique sensitivity coming down to one single molecule in clean systems. Fortunately the human situation is not as "clean" such as the purified Rhodamine solution (as it has been used by Rigler)[18]. Natural occurring fluorochromes, other proteins and contaminations may influence the results, so that the single molecule detection is illusive in biological systems (as opposed to the "clean theory").

A limiting disadvantage of FCS is the labelling of the reaction partners. And since the method is very sensitive, a background free labelling system must be established, which has been successfully built up in our laboratory. In doing this we were able to establish a sophisticated system for the coupling and purification of immunochemistry superior to any industrial standard.

The limitation of the labelling procedure can be avoided by mass spectroscopy, where two systems are available namely MALDI and SELDI. Whereas MALDI is complicated, SELDI can be build up within one week and bring us results in well-defined systems. Our example comes from the aqueous humour, showing for the very first time the application of SELDI. A disadvantage however is the occurrence of the "maxi parameter" spectrum of peaks, requiring a very potent computer technology for analyses. For this reason a network is necessary and we rely on the manufacturer or other data banks.

Additionally albumin, modified by cancer derived enzymes and degradation products may give us some indication about the underlying disorder. The same is true for the collagen IV, which in the case of the Goodpasture syndrome induces a detrimental autoimmune reaction destroying lung and renal tissue.

This brings us to the computer modelling of proteins according to the data banks. Being able to "see" the 3D structure of a protein and analyze its shape we can obtain crucial information for the understanding of protein properties and their interaction. In the world of protein molecules the application of visualization techniques is particularly rewarding, for it allows us to depict phenomena that cannot be seen by any other means. This is a new dimension of diagnostic and prognostic procedures since a modern aspect of diagnosis relies on the modification of well-known molecules by e. g. the side effect of tumours. Albumin can be modified by the enzymatic behaviour of tumours bringing us various sub splits of spectra relevant to various neoplastic and inflammatory conditions. Our result shows die analysis of the albumin and collagen IV under normal conditions as a standard for further developments. The 3D structure of mutated proteins can be predicted and superimposed to the protein in normal condition. The differences of the C_α -atom positions of the backbones in the two structures are measured. The distances in the two structures are colour encoded and mapped on one of the two superimposed structures. This allows the evaluation of which parts of the

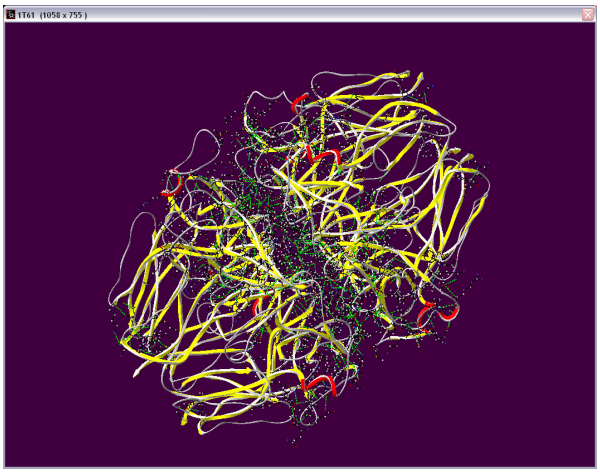

Fig. 8:Collagen IV molecule. The figure shows the crystal structure of collagen IV NC1 domain. 6 peptide chains are involved in the quaternary structure. The chains are represented by their secondary structure elements whereas the atoms from the groups close to another chain are represented as ball-and-stick. The hydrogen bonds between the chains are represented as dotted lines.

structures are showing a good or high similarity and where significant structural deviations are located. The molecular interface between proteins is of special importance for understanding their interactions and functions. The interactions are defined only by a subset of residues in proteins. Our software allows an easy detection of these residues annotated with physical-chemical properties. Additionally the identified residues are used to define the molecular surface at the interface. The resulting molecular surface (with projected physiochemical properties of the involved residues) can thereby aid exploration of molecular complementarities at the macromolecular interface.

CONCLUSION

In conclusion each of the new technologies (namely FCS, SELDI-TOF and computer based molecular modelling and visualization) shown has some advantages, however the disadvantages are considerable and only the combination with the other technologies will allow the break-through required for better diagnosis and hence evidence based medicine. Multiparameter analysis expanding to "maxi parameters" in SELDI renders the results difficult to interpret. High sensitivity in FCS makes us very dependent on the background noise due to natural contaminations or free dye. Coupling chemistry is difficult and much to superior to industrial standards.

With these pitfalls in mind computer modelling of suspect reactive partners may come to the rational diagnostic clue for evidence based medicine in the future.

ACKNOWLEDGEMENT

This study was supported by the „Jubiläumsfonds der Österreichischen Nationalbank" and the „Wissenschaftsfonds der Steiermärkischen Landesregierung".

REFERENCES

1. Földes-Papp, Z., Demel, U., Tilz, G.P., 2001. Ultrasensitive detection and identification of fluorescent molecules by FCS: Impact for immunobiology. PNAS, 98,20:11509-11514.
2. Domej, W., Földes-Papp, Z., Demel, U., Tilz, G.P., 2004. Clinical Applicability of Mass Spectrometry for Inhaled Carbon Compounds and the Characterization of Trace Element Patterns in Body Fluids. Current Pharmaceutical Biotechnology, 5, 1: 89 – 106.
3. Liu, J., Zheng, S., Yu, J., Zhang, J., Chen, Z., 2004. Serum protein fingerprinting coupled with artificial network distinguishes glioma from healthy population or brain benign tumour. J Zhjiang Univ SCI, 6B,1:4-10.
4. Clarke, C.H., Buckley, J.A., Fung, E.T., 2005. SELDI-TOF-MS proteomics of breast cancer. Clin Chem Lab Med, 43,12:1314-1320.
5. Boyce, E.A., Kohn, E.C., 2005. Ovarian cancer in the proteomics era: diagnosis, prognosis and therapeutics targets. Int J Gynecol cancer, 15:266-373.
6. Li, J., Orlandi, R., White, C.N., Rosenzweig, J., Zhao, J, Sergni, E., Morelli, D. et al., 2005. Independent Validation of Candidate Breast Cancer Serum Biomarkers Identified by Mass spectrometry. Clinical Chemistry, 51,12:2229-2235.
7. Fung, E.T., Yip, T., Lomas, L., Wang, Z., Yip, C., Meng, X. et al., 2005. Classification of cancer types by measuring variants of host response proteins using SELDI serum assays. Int J cancer, 115:783-789.
8. Vorderwülbecke, S., Cleverley, S., Weinberger, S.R., Wiesner, A., 2005. Protein quantification by the SELDI-TOF-MS-based ProteinChip System. Nature Methods, 2,5:393-395.
9. Tomosugi, N., Kitagawa, K., Takahashi, N., Sugai, S., Ishikawa, I., 2005. Diagnostic Potential of tear Proteomic Patterns in Sjögren's Syndrome. Journal of Proteome Research, 4:820-825.
10. Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, O.E., 2000. The Protein Data Bank. Nucleic Acids Research, 28: 235- 242.

11. Sali, A., Blundell, T.L., 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol., 234: 779-815.
12. Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modelling. Electrophoresis, 18: 2714-2723.
13. Wiltgen, M., Holzinger, A., 2005. Visualization in Bioinformatics: Protein Structures with Physicochemical and Biological Annotations. In: Zara J., Sloup J. (eds.) Central European Multimedia and Virtual Reality Conference CEMVRC 2005, : 69-74.
14. Gabdoulline, R.R., Wade, R.C., Walther, D., 2003. MolSurfer: A macromolecular interface navigator. Nucleid Acids Res., Jul 1;31 (13): 3349-51.
15. Finn, R.D., Marshall, M., Bateman, A., 2005. iPfam: visualization of protein-protein interactions in PDB at domain and amino acid resolutions. Bioinformatics, 21 (3): 410-412.
16. Battacharya, A.A., Grune, T., Curry, S., 2000. Crystallographic analysis reveals common modes of binding medium and long-chain fatty acids to human serum albumin. J. Mol. Biol. V303: 721-732.
17. Than, M.E., Henrich, S., Huber,R., Ries, A., Mann, K., Kuhn, K. et al., 2002. The 1.9-A crystal structure of the noncollagenous (NC1) domain of human placenta collagen IV shows stabilization via a novel type of covalent Met-Lys cross-link. Proc Natl Acad Sci USA., 14;99(10):6607-12.
18. Rigler, R., Mets, U., 1992. Int. Soc. Opt. eng. Laser Spectrosc. Biomol, 1921:239-248.