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

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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, neoplasia 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 spectroscopy (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.

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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 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 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\(^2-9\). 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).

   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 on the mass to charge ratio: \( \frac{m}{Z} \) (\( m \) 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 be 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 \( \gamma \)-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 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).

![The Swiss-Pdb Viewer software tool](image)

**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)\textsuperscript{[11]}.

**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.

**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 on 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)\(^\text{[16]}\). The same is true for the molecular structure of the causative collagen IV as antigen in the Goodpasture syndrome\(^\text{[17]}\). This is shown in Figure 8.

**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)\(^\text{[18]}\). 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 \(C_n\)-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.

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