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Chemical Microarrays, Fragment Diversity, Label-Free Imaging by Plasmon Resonance—A Chemical Genomics Approach

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Abstract Chemical genomics aim to create synergy between synthetic small molecule chemistry and biosciences employing genomic tools and information. Central to chemical genomics is the discovery of bioactive compounds from novel targets for pharmaceutical lead development. The field is challenged both by the multitude and novelty of protein and other biomacromolecular targets to be studied. Affinity fingerprints, data sets of binding interactions between collections of chemicals and their macromolecular receptors, hold promise to guide drug design and study protein function for groups of related compounds and families of biomacromolecules. Despite their fundamental relevance, neither experimental protocols nor databases of quantitative and comprehensive description of binding interactions for small molecule ligands and biomacromolecular receptors are available. Chemical microarrays in combination with label-free imaging provide a novel route towards the systematic and standardized acquisition and application of such affinity fingerprint information. J. Cell. Biochem. Suppl. 39: 79–84, 2002.

Key words: affinity fingerprint; surface chemistry; self-assembled monolayer; drug discovery; SPR; ligand density; rational design; protein binding; optical biosensor; binding site

Chemical genomics is a highly interdisciplinary approach positioned to integrate chemistry and biology in the context of molecular medicine [Sehgal, 2002]. There are two distinct research activities in chemical genomics. The more traditional approach explores the serendipitous route of discovery by oberservation of cellular responses upon presence of a chemical entity. In this phenotype screening, the identification of the molecular target of the chemical compound is greatly facilitated by genomic sequence information. An overview on this area of research has been given recently [Zheng and Chan, 2002].

In parallel, chemical genomics is also perceived as an extension of rational approaches in drug discovery with the goal to improve on selection rules for chemical diversity in recognition of parameters depending upon the biological target class [Agrafiotis et al., 2002]. Under this regime, detailed knowledge of the physicochemical properties of gene products is assembled in a systematic fashion [Hochstrasser et al., 2002]. This body of knowledge is seen as the basis for downstream development of synthetic chemical regulators of protein activity.

Structure-based design plays an important role in rational approaches. High-throughput structure determination efforts are underway [Blundell et al., 2002]. Combined with novel virtual screening tools and enhanced computer power, this avenue is a straightforward extrapolation of the rational design paradigm. Specifically, the use of protein–ligand cocrystallization to verify virtual screening predictions will become an important addition to this toolbox. The shortcomings of structure-based approaches in de-novo ligand design can be compensated by feeding empirical data sets from high throughput screening into the system [Engels and Venkatarangan, 2001].

A severe limitation of both high throughput and virtual screening is the a priori requirement of binding site selection. In the case of high-throughput screening, a complementary ligand for displacement defines the locus of interest. Virtual screening is restricted to rigid

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protein models [Bailey et al., 2001]. Based on calculated or experimentally obtained biostructures, binding sites may be predicted [Glick et al., 2002]. But there remain issues with the validation of such predictions and therefore with the optimization of algorithms. Comprehensive mapping of a given protein's surface for small molecule binding sites and subsequent virtual screening against large in-silico compound collections is excluded for reasons of computer capacity.

The power of discovery of binding site conformational flexibility for allosteric modulation of protein activity has repeatedly been demonstrated [Pargellis et al., 2002]. Empirical methods for locus discovery and exploration would provide a useful addition to the currently available physicochemical protein datasets. Affinity fingerprints provide a global scan of the protein surface rather than the limited one-site investigation in competition experiments. Confusingly, data sets from competition screens were also referred to as affinity fingerprints although the readout related to site-specific ligand displacement [Kauvar et al., 1995].

A prerequisite for the empirical study of small molecule binding sites on protein surfaces apart from protein purity is the provision of a stable and correctly folded protein sample.

Affinity fingerprints need to be obtained under conditions similar to the native environment and should also allow for the study of protein complexes. In this respect, chemical microarrays are an attractive concept as the restraints imposed on the composition of the biological analyte and manipulation of the macromolecules are minimized.

Arrays can be defined as immobilized molecular diversity that is interrogated in a single binding or hybridization experiment. After the successful introduction of DNA microarrays to expression profiling [Schena et al., 1998], protein microarrays are being developed for the same application on the proteome level [Kodadek, 2001]. Unfortunately, microarray terminology is not consistent. DNA microarrays frequently are composed of assemblies of short single-strand oligonucleotides and serve the quantification of mRNA levels. Arrays presenting synthetic ligands for protein binding studies are sometimes referred to as protein arrays, nevertheless it has become more accepted to use the protein microarray label only for protein-containing arrays. Chemical microarrays contain synthetic small molecules libraries including peptide and carbohydrate collections. An overview of the field has recently been given [Lam and Renil, 2002].

Historically, peptide arrays were developed in parallel to oligonucleotide arrays using photolithography on glass [Fodor et al., 1991]. A technically less demanding approach is peptide library synthesis by liquid spotting on cellulose membranes [Frank, 2002]. This approach was extended to the creation of combinatorial compound libraries on polymer support using chemiluminescence imaging as detection method [Scharn et al., 2000]. Such arrays suffer from the compromise to function both as screening and in-situ synthesis platform. Each molecular entity remains at the site of assembly during the binding experiments. Apart from compromises in surface chemistry, this approach renders array production expensive and enhances array-to-array variations.

Alternatively, spotting of presynthesized chemical collections to functionalized surfaces provides for mass production of array copies. The overall experimental error is reduced, if fully characterized compound collections are immobilized using reliable pin tool technology. With the experiences made in high-throughput screening, greater emphasis is now being placed on quality control of chemical collections. Currently accepted standard for a library is that 90% of the compounds come in a purity greater than 70% [Barn et al., 2001]. This can only be achieved by cleave-and-characterize methodology or solution-phase synthesis and subsequent purification. On-array synthesis does not permit quality control at this rigorous level. Fourier-transformed infrared reflectance spectroscopy or other surface analysis methods are unsuitable for the assessment of side products and reaction yields.

The first off-line synthesized chemical arrays described in the literature displayed only three reference compounds, nevertheless the power of the principle was demonstrated by production and use of 10,800-spot slides [MacBeath et al., 1999]. In this work, control ligands were equipped with a reactive spacer moiety (tag) carrying a free thiol group. These tagged molecules were spotted onto silanated glass surface densely functionalized with maleimide spacer moieties. Thiol-maleimide conjugation chemistry is a mild and efficient method for the formation of covalent bonds. It was estimated that the

deposition of 1 nl aliquots from 5 μl of a 5 mM stock solution in a micro titer format source plate is sufficient for the production of 10,000 spots of 250 microns diameter. Fluorescently-labeled proteins were added, and the binding pattern was recorded by means of a commercial laser scanner. While addressing the issue of microarray mass production, this work revealed two additional challenges, namely surface architecture and binding detection.

It is understood that apart from general protein adsorption resistance properties, the most influential factor in support-sample interface design is ligand density. Experiences made with artefactual signals in solid phase binding experiments may be attributed to lack of control of ligand density. The lateral spacing of ligands on a flat surface is critical to reduce unspecific binding phenomena resulting from ligand aggregation and multivalent ligand–receptor interactions. In a typical array experiment, the protein of interest is interacting with numerous spatially segregated compounds in parallel. In order to create comparable data for each compound, care has to be taken to ensure the same conditions for all members of the array collection.

The high demands for ligand density control, minimized unspecific macromolecule binding, and robustness are almost exclusively met by self assembled monolayer (SAM) surfaces.

SAMs can be obtained on various support materials such as glass but are typically generated by chemisorption to a layer of gold or silver metal. Linear alkylchains that are alpha functionalized by means of a thiol group readily attach to a clean gold surface. Chemisorption is followed by packing of the hydrocarbon chains into a highly ordered arrangement. Such self-assembled monolayers on gold are among the best studied and best characterized interfaces in use for biomolecular interaction studies. Ethyleneglycol termination of self-assembled monolayers has been put forward as a highly protein adsorption-resistant modification [Prime and Whitesides, 1991].

Activated SAMs are monolayers exposing a reactive group such as an acid that was subsequently converted into a N-hydroxy succinimide active ester. Corresponding surfaces have been proposed as matrix for the immobilization of small molecule diversity [Lahiri et al., 1999]. Activation chemistries require the presence of a complementary reactive group and a spacer moiety on the ligand. Tagged ligand collections can be conveniently accessed through solid-phase chemistry starting from a linear spacer that is in alpha position conjugated through the reactive group to a complementary linker group on the resin and carries an orthogonally protected group in omega position. The resin linker protects the reactive group of the tag during synthesis steps taking place at the omega site, and under cleavage conditions releases the free tag–ligand conjugate into the supernatant for collection, analysis and spotting.

Commercial systems for the immobilization of biomolecules for binding studies employ a dextran hydrogel matrix on activated SAM. These surfaces are frequently used but carry the same inherent disadvantage as cellulose matrices for peptide arrays—there is little control over ligand density in such a flexible, three-dimensional interface architecture. Flat surfaces appear superior to hydrogels for small molecule display [Fischer et al., 2001].

The most popular use of dextran-SAMs are binding studies where the protein is immobilized and interrogated with soluble binding partners such as antibodies or chemical compounds. It is apparent though that protein immobilization creates problems such as slow and difficult to detect on-array denaturation [Ober and Ward, 2002].

A powerful feature of SAM chemistry is the use of two component mixtures for the lateral dilution of reactive groups. The reactive group-terminated SAM component is mixed with a second, inert, SAM-forming compound. If the two reagents are carefully designed, the mixture in solution is identical to the ratio of the two components in the immobilized monolayer. Subsequent conjugation of ligands takes place only at the reactive component, the inert component remains unconjugated and acts as a lateral spacer between the ligands. Optimization of the molecular structure of the SAM-components and mixture composition, SAM-formation conditions, conjugation chemistry, and spacer lengths can be highly effective in creating surfaces with little unspecific background binding and maximized ligand–receptor interaction.

The detection of a binding event between a biomacromolecule and an immobilized ligand is most easily accomplished by using enzyme-linked immunosorbent assay protocols or fluorescent or radioactive labels. For chemical

microarrays, fluorescence scanning and chemiluminescence imaging have been described. These methods suffer from various drawbacks. Not only is there a requirement for an antibody or the introduction of affinity tags or fluorophores into the biomacromolecule, but the read-out is biased by the antibody assay protocol, background binding of antibodies or detection enzymes, unspecific binding of the fluorophore to the surface and site-specific sterical hindrance of the affinity interaction by the fluorophore. Label-free imaging provides an alternative route to affinity fingerprints and circumvents abovementioned problems.

Label-free detection of biomacromolecule binding to immobilized partners can be achieved by a variety of optical methods, and the field has been reviewed recently [Cooper, 2002]. The most prominent commercial instruments employ dextran-SAM-surface chemistry with a thin layer of gold for detection of the effect of surface plasmon resonance [Jonsson et al., 1991]. The use of gold for both the formation of a well-defined biospecific surface as well as the basis for the optical detection system provides for a powerful combination. Plasmons are density waves of the electron gas of the metal structure that propagate along the surface of the metal layer. In the classical Kretschmann geometry for detection of surface plasmon resonance, the gold-glass slide is mounted on a prism using index matching fluid, and visible light or near infrared radiation is directed at the bottom of the slide [Kretschmann, 1968]. The intensity of the reflected light beam is measured. By variation of the angle or of the wavelength of the incident beam, a drop and regain in reflectance of the metal mirror can be recorded. Using gold of a thickness of ca. 50 nm, glass of a refractive index of 1.52 and analyte of 1.33 respectively, this reflectance dip occurs at an angle of 74° with full width half maximum (FWHM) of 7° or at a wavelength of 630 nm with a width of 70 nm (FWHM), respectively. The more narrow peak shape of the angular measurement explains for the greater sensitivity as compared to wavelength read-out. In biomolecular interaction analysis, the minimum of reflectance is measured with control buffer as the sample medium. If a macromolecule is added to the sample and binds to the surface, the dielectrical constant of the medium within the evanescent field increases and the resonance condition is affected, the minimum of reflectance is shifted to a higher wavelength or angle.

Surface plasmon resonance detection optics can be adapted to the requirements of array imaging. In a most straightforward set up, the array is illuminated homogeneously with monochromatic light tuned to a wavelength close to the resonance condition, and an image is taken by means of a charge-coupled device (CCD) [Nelson et al., 2001]. Upon binding of the macromolecule, the affected array spots lose reflectance, and a differential image reveals darkened spots. Prerequisite of this approach is that all array compounds and spot surfaces are of very similar physicochemical nature.

If the dielectric property of the buffer/gold interface varies in dependence on the chemical structure of the ligand, a one-wavelength SPR image will exhibit differing spot brightness across the array even in the presence of reference buffer and therefore not be amenable for further interpretation. This shortcoming can be avoided if stepwise variation of the wavelength is employed for the detection of the resonance dip. Individual reflectance minima can be obtained for each array compound and used as reference values.

In a different optical arrangement, the angular dependance of the SPR effect is recorded. Drawback of this approach is that the positional mapping of CCD pixels to array spots is lost as the prism is moved in the light path. Measures such as automated spot detection or multiple light paths can be implemented but limit this approach to low density arrays. An overview on current developments for two-dimensional SPR detection technology has been given, which also details a fourth imaging set-up that uses microstructure gratings instead of a prism for light coupling [Baird and Myszka, 2001].

Parallel reflectometric interference spectroscopy was used to observe binding of a panel of antibodies to an immobilized library of 36 compounds [Birkert et al., 2002]. Since interferometry is not compatible with self-assembled monolayers on noble metal films, the interface design of such devices is restricted to conventional glass silanation with the abovementioned drawback of lack over ligand density control.

It is notable, that the most significant advancement of the application of chemical microarrays to large scale protein affinity fingerprinting resides within the development of sophisticated surface chemistries. With a

growing body of basic research from various disciplines into the interface phenomena of biological samples and artificial solid phase structures, the translation of these fundamental findings into novel applications will provide fertile ground for future bioscience development such as cellular adhesion and recognition [Mrksich, 1998].

OUTLOOK

A major stepstone towards a comprehensive chemical genomics understanding is an affinity fingerprint database of proteins, protein complexes, and ultimately cells. Data sets of chemical compound libraries associated with parameters describing the respective binding strength of each individual member of the collection to a great variety of biomacromolecules will provide a strong empirical basis for the rational design of drugs or chemical tools. The technology elements for large scale affinity fingerprinting are currently being assembled. Central technical problems have been addressed, and robust platforms are being developed that will be able to operate in a high-throughput fashion and on every laboratory bench.

Great promise lies in a combination of prespotted chemical microarrays using self-assembled monolayer surface chemistries and label-free imaging based on surface plasmon resonance or interferometry. Such chemical microarrays can be used as disposables, and sub-milligram amounts of biomacromolecules will suffice to generate patterns of binding on such chips. Label-free detection of binding events across large collections of receptors and ligands is a central element towards standardized sets of data. Experimental bias from assay protocols as well as compound quality and solubility needs to be minimized. The use of label-free imaging on microarrays reduces the experimental complexity to only three components, namely the array surface including the displayed ligands, buffer medium, and the biomacromolecule. Focus on the fundamentals of affinity-based molecular recognition is the prerequisite for the collection of information that can be exchanged between different laboratories and managed in a centralized fashion.

Challenges remain in the selection of chemical diversity and the preparation of solubilized membrane proteins. The chemical universe is vast and selection rules for qualifying synthetic compounds for biological screening are both numerous and uncertain. It does not appear feasible and useful to represent current screening libraries on chemical microarrays since such libraries have been assembled in a random fashion over decades. As the immobilization of existing compounds is much more cumbersome than de-novo synthesis, the opportunity arises to apply most advanced design criteria in the construction of chemical microarrays.

Recent thinking in molecular design is approaching chemical diversity from the angle of fragments or pharmacophore substructures. In this concept, existing public or proprietary databases of bioactive compounds are interrogated and common motifs of minimal structural complexity are sought [Teague et al., 1999]. Minimal structural complexity roughly correlates with molecular weight. If a typical screening compound had a molecular mass of 500, a corresponding fragment would be of MW 250. To focus on fragments of compounds greatly diminishes the size of a chemical collection to be represented on a chemical microarray. The possible number of suitable organic compounds is much smaller if limited to a maximal molecular mass of 250. For this reason, a carefully selected collection of 100,000 fragments on a surface for affinity experiments provides better coverage of its respective chemical space than millions of synthetic molecules of varying complexity.

For a comprehensive scan of chemical versus biological diversity, not only large numbers of chemicals need to be assembled but also different proteins, protein complexes, and RNA need to be prepared in a purified solubilized form. For membrane-bound proteins such as G-protein coupled receptors and ion channnels this remains a formidable hurdle. Driven by biostructure initiatives, substantial progress is being made in this area [Muller, 2000].

Beyond these scientific and technical aspects lie the tasks of defining the scope, producing, distributing, and maintaining an emerging affinity fingerprint database. The corresponding logistical and political issues have recently been stated [MacBeath, 2001]. With the technological advances made in the development of robust tools for chemical genomics, adoption of the approach will spread and provide researchers with an unprecedented level of information at the interface of chemistry and biology.

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