technique that requires orthogonal labeling of analytes. For example, blue nanospheres and red nanoprisms could substitute for the Cy3 and Cy5 dyes that are commonly used to label the sample and control cDNA strands hybridized to a gene expression array. Even more importantly, these nanostructure labels could be visualized using an ordinary microscope camera rather than the expensive confocal imagers now used to scan fluorescently labeled gene chips. Methods for DNA sequencing, single nucleotide polymorphism analysis and protein array imaging would similarly benefit from multicolor labeling with tailored nanostructures.

Applying nanomaterials to bioanalysis, case by case

One element of biomolecular labeling will not be improved by the development of nanoparticle probes: the inherent complexity of matching the characteristics of a probe to the experiment being done. Just as a polar fluorophore wouldn't be used to probe the hydrophobic portion of a transmembrane protein, nanostructured probes will have to be designed to be compatible with particular experimental environments (Fig. 1; Table 1). This will involve not only designing the physical properties of the probe but also its surface chemistry and biocompatibility – a complex set of tasks for any application. Nevertheless, recent progress in applying nanoparticle probes to biological questions is promising. Work on using photostable, fluorescent quantum dots for imaging live cells, for example, has provided stunning hour-long movies of cellular internalization of the nanoparticles [13]. Organic fluorophores would photobleach within seconds under the same imaging conditions. The expectation of scientists working at the interface between nanostructured materials and biology is that, eventually, the catalogue of nanoparticle probes available for biology will be just as large as that for molecular probes – and might be even more useful.

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Techniques & Applications

Towards quantitative assays with peptide chips: a surface engineering approach

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The development of peptide and protein microarrays has created enormous opportunities in biomedical research. Current chip-based assays are well suited for identifying candidate protein or enzyme activities but still require conventional solution phase experiments to validate hits. Here, three surface-engineering strategies for microarray design are described and are illustrated in the development of a peptide chip for the quantitative analysis of kinase activity on solid support. These strategies promise to widen the application of microarrays by permitting the evaluation of hits in a chip-based format.

Published online: 10 May 2002

The development of novel microarray assay formats is transforming research programs in the biological sciences and accelerating discoveries in the pharmaceutical and diagnostics industries. Microarrays were first introduced nearly ten years ago as DNA or 'gene' chips, they have been commercialized and are now widely used for genetic analyses. The success of this immobilized format has motivated the development of peptide and protein biochips.

Recently, several groups have described peptide and protein arrays and applied these chips to a range of exciting studies. Spot synthesis on cellulose, for example, has been used to prepare peptide arrays for the identification of inhibitors of α-bungarotoxin [1] and for the investigation of structure–function relationships in the hYAP-WW domain [2]. In the field of protein chips, MacBeath and Schreiber immobilized a series of proteins on aldehyde-terminated glass slides and showed that they interact with other proteins and small

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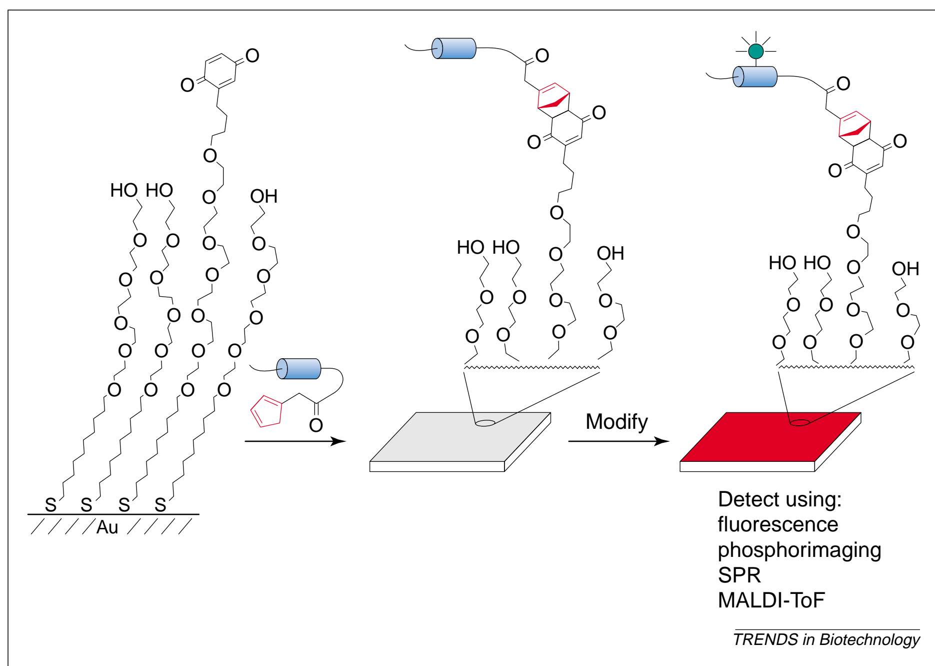

Fig. 1. Preparation of peptide chips for quantitative assays. The Diels-Alder reaction between a cyclopentadiene conjugate and benzoquinone groups on a self-assembled monolayer (left) affords rapid and selective immobilization of ligands with excellent control over density (middle). Modification of and protein binding to the substrates can be evaluated using phosphorimaging, fluorescence, surface plasmon resonance or matrix associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.

molecules in solution [3]. More recently, Snyder and coworkers described two classes of protein chips for the evaluation of the *Saccharomyces cerevisiae* proteome. The first chip used elastomeric microwells as a solid support for the immobilization and characterization of 119 kinases [4]. The second array was prepared by the immobilization of oligohistidine fusion proteins onto nickel(II)-coated glass slides. The authors then used the array to identify calmodulin- and phospholipid-binding motifs [5].

Although exciting, these peptide and protein chip strategies have two broad limitations. First, the unwanted adsorption of soluble proteins often competes with selective protein–substrate interactions, leading to background levels of signal and a loss of activity of the immobilized protein [6]. Under these conditions, the investigation of low affinity interactions is particularly difficult. Second, only a fraction of the immobilized proteins are competent to participate in binding interactions [7]. Many of the ligands are either immobilized in nonproductive orientations or are denatured to some extent, both of which compromise selective interactions with soluble proteins. A further confounding factor with nonspecific adsorption is that the distribution in protein orientations and the extent of protein denaturation can be highly variable [8].

A consequence of these limitations is that current protein and peptide arrays are not well suited for quantitative assays of protein–substrate interactions. Instead, the chips are used to identify candidate interactions and the ‘hits’ from these experiments are evaluated using solution phase assays to validate and further characterize binding events or enzymatic activities [1,2,4,5]. Our view is that chips can be engineered to perform as well as (or better than) solution assays, thereby enabling the parallel, quantitative measurement of binding affinities on the chip. Arrays that meet this standard would permit the rapid evaluation of candidates with minimal quantities of reagents. A sophisticated application of surface engineering strategies is necessary to achieve this level of performance, and here we outline three goals of surface engineering that promise to address current limitations in chip technology.

Well-defined substrates and immobilization chemistries

Many substrates used for the preparation of peptide and protein arrays (such as cellulose, glass and polymer substrates) are not structurally well defined and consequently do not allow surface properties to be engineered at the molecular scale [9]. Although these substrates have several benefits, including low cost and ease of preparation, they present immobilized ligands in a range of environments. Some ligands on the substrate will be accessible to interactions with soluble proteins, whereas others will be less active (or inactive) because they are buried or crowded at the interface. These limitations suggest a need for substrates that have well-defined and regular structures, are synthetically flexible and are compatible with the protocols and detection methods of microarray experiments.

Several methods have been used to immobilize peptides and proteins in microarray format. The reaction of surface-bound aldehydes with side chain amino groups [3] is convenient and applicable to most polypeptides but is limited by a lack of selectivity. The chemoselective ligation of aldehydes with aminooxy or *N*-terminal cysteine sulfhydryl groups [10], however, is selective but not general. Other immobilization chemistries, such as nickel(II)-oligohistidine complex formation, have excellent specificity but lack long-term stability [5]. These examples show that strategies are needed that immobilize a large family of molecules in a rapid, selective and stable manner without the need for extensive post-synthetic or post-translational modification of the ligand [11].

Inert surfaces

The most significant problem with current peptide and protein biochips is that they are not strictly inert to the nonspecific adsorption of protein. To minimize unwanted protein adsorption, chips are often treated with a blocking protein (such as serum albumin) before use or are used in the presence of detergents that prevent unwanted adsorption. Although these strategies are effective at eliminating the false positives, they do so at a price. The adsorption of blocking protein passivates sites for nonspecific adsorption but might also obscure the immobilized ligands, thereby interfering with desired interactions [6,8]. The presence of detergents, however, can disrupt selective

protein–substrate interactions or promote the denaturation of immobilized proteins [12]. It is clear that surfaces that are intrinsically inert to the nonspecific adsorption of protein would be of enormous value for the preparation of peptide and protein chips.

Several of the chips described to date have incorporated one or more of these features in their design but none has yet incorporated all three and performed quantitative assays. We developed a peptide chip that uses self-assembled monolayers of alkanethiolates on gold as a platform for the immobilization of ligands (Fig. 1) [13]. The strategy begins with monolayers that present benzoquinone and oligo(ethylene glycol) groups. The benzoquinone groups provide a handle for the selective, Diels-Alder-mediated immobilization of ligand–cyclopentadiene conjugates, and the oligo(ethylene glycol) groups resist the nonspecific adsorption of protein [9,14]. Three characteristics make these monolayers useful for the development of arrays for quantitative analysis. First, the oligo(ethylene glycol) groups reduce background signal to near zero by completely preventing the nonspecific adsorption of protein during an assay. Second, the Diels-Alder reaction is highly selective and compatible with the range of functional groups present in biological systems, ensuring chemoselective ligation of the analyte to the monolayer. Third, the immobilization reaction proceeds in high yield without side products, ensuring that all ligands in an array are presented at the same density, independent of the structure and properties of a particular biopolymer. A key feature of these substrates is that they present ligands in a regular, homogeneous microenvironment. Immobilized ligands have equal activities toward proteins and enzymes in solution, making the chips well suited for the quantitative analysis of protein binding and enzymatic modification [13,15,16].

Kinase activity assay

We used these monolayers for the evaluation tyrosine kinase activity. Solutions containing *c-src* and [γ-^{32}P]-ATP are spotted onto monolayers presenting an immobilized peptide substrate for the enzyme [13]. After incubation, phosphorylation of the immobilized peptides on the chip is analyzed using phosphorimaging. By including a soluble inhibitor at a range of concentrations, dose-dependent inhibition of phosphorylation can be determined for an inhibitor (or several inhibitors) on a single chip. Data obtained in these assays provide direct access to the inhibition constant (K_i) for each compound and because the determination of K_i values in solution-phase assays requires significant quantities of reagents, it is generally not calculated for most compounds identified in screens. This approach provides a route for determining inhibition constants in a single assay using 100-fold less enzyme and reagents.

These monolayer substrates are compatible with current array formats because they can be used with commercially available robotics to spot a large number of samples. Also, monolayers are compatible with a range of detection methods, including phosphorimaging, surface plasmon resonance, fluorescence and matrix-associated laser desorption ionization (MALDI) mass spectrometry. We used this approach to immobilize a series of peptide ligands and showed that these arrays are useful for the evaluation of protein binding and enzymatic modification [13,16]. We believe that these substrates will also be useful platforms for the preparation of related arrays that present carbohydrates and other small molecules.

This strategy for preparing peptide chips represents a general approach for the development of a broad range of biochips. First, the use of well-defined substrates ensures that ligands are presented in a homogeneous manner at the interface. Second, the use of inert surfaces significantly lowers background levels of activity and obviates the need for detergents or blocking procedures. Finally, the use of a well-defined surface chemistry to immobilize polypeptides ensures that each ligand is present in the same orientation, conformation and density. The application of these principles to the design of chips permits the quantitative evaluation of ligand activity on solid support. We believe that these features will enable screening and evaluation of targets in a single and efficient high-throughput format, allowing protein and peptide chips to achieve the widespread use of their DNA counterparts.

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