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Review

High density peptide microarrays. In situ synthesis and applications

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Summary

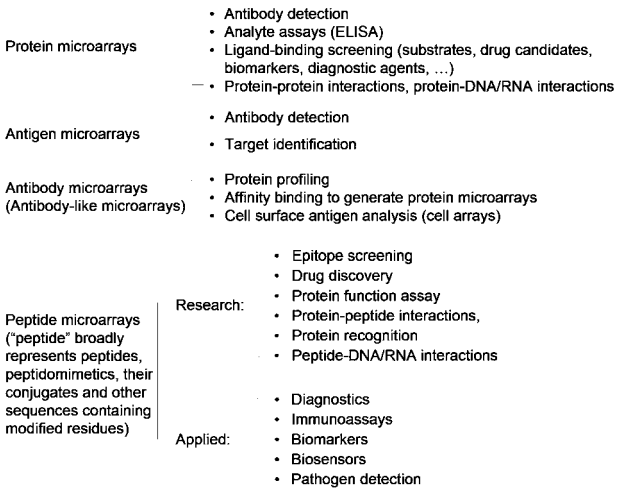
The technologies enabling the creation of large scale, miniaturized peptide or protein microarrays are emerging. The focuses of this review are the synthesis and applications of peptide and peptidomimetic microarrays, especially the light directed parallel synthesis of individually addressable high density peptide microarrays using a novel photogenerated reagent chemistry and digital photolithography (Gao et al., 1998, J. Am. Chem. Soc. 120, 12698; Pellois et al. 2002, Nat. Biotechnol. 20, 922). Concepts related to the synthesis are discussed, such as the reactions of photogenerated acids in the deprotection step of peptide synthesis or oligonucleotide synthesis, and the applications of high density peptide chips in antibody binding assays are discussed. Peptide chips provide versatile tools for probing antigen-antibody, protein-protein, peptide-ligand interactions and are basic components for miniaturization, automation, and system integration in research and clinical diagnosis applications.

Abbreviations: cm, centimeter; CPG, Controlled porous glass; DCM, dichloride methylene; DMT, 4,4'-dimethoxytrityl; Fmoc, 9-fluorenyl methoxycarbonyl; FR, fluorescein; ivDde, 4,4-dimethyl-2,6-dioxocyclohexylidene-)3-methyl-butyl; MeNPOC, (α-methyl-2-nitropiperonyl)oxycarbonyl; mRNA, message RNA; NPPOC-Hz, 2-(2-nitrophenyl)propoxycarbonyl hydrazine; NPPOC-pip, 2-(2-nitrophenyl)propoxycarbonyl piperidine; NVOC, nitroveratryloxycarbonyl; PGA, photogenerated acid; PGA-P, photogenerated acid precursor; PGB, photogenerated base; PGB-P, photogenerated base precursors; PGR, photogenerated reagents; PGR-P, photogenerated reagent precursor; PLPG, photo-labile protecting group; SPR, surface plasmon resonance; t-Boc, ter-butyloxycarbonyl; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

Introduction

The idea and the practice of using a large amount of molecules to simultaneously probe intermolecular interactions were first intensively pursued in the areas of peptides about two decades ago [1–8]. But the relative ease of DNA synthesis in a highly parallel fashion and the simplicity of DNA chemistry, such as complementary hybridization among nucleic acid sequences, have quickly led to widespread applications of DNA microarrays [9, 10], which now far outpace those of peptide microarrays. DNA chips have validated the format of addressable microarrays as a means of extremely efficient parallel data acquisition. When empowered with high speed data processing and statistical data analysis [11], microarray results, such as those of gene expression or single nucleotide polymorphism (SNP) analysis, offer insights into biological systems of individual and global components at an unprecedented comprehensive and detailed level [12–18].

Microarrays of protein [19–22], peptide or peptidomimetic molecules [23–35] are fundamentally different in their chemistry and use. In particular, there are 20 natural amino acids compared to only four natural DNA or RNA nucleotides. The chemistry of peptide synthesis is therefore much more complicated. Peptides and proteins have multifaceted properties, while nucleic acids are distinct in their backbone of negative charges. The interactions of peptides or proteins with other molecules are thus dominated with molecular forces of different nature. The diverse chemical properties of peptides and proteins permit their broad range of roles, for instance, as receptors, fusion mediators, structural stabilizers, and regulatory factors, implicated in, e.g., signaling, stimulation, catalysis, inhibition, and initiation of biological processes. Peptide or protein microarrays should find



Scheme 1. Illustration of the apllications of protein and peptide microarrays.

key applications for therapeutic target identification, protein function assay, drug discovery, and diagnostics (Scheme 1). Accordingly the applications involving peptides and proteins impose a number of requirements for the intended purposes (Scheme 1). It is desirable to develop peptide and protein microarrays that are easy to produce at minimal cost and time consumption, flexible to suit a broad range of needs, specific and sensitive for target detection, capable of large dynamic range detection, reliable in reproducibility, and stable under the conditions of assays and storage. To meet these requirements, technologies enabling the creation of useful peptide or protein microarrays are emerging. A number of reviews in this issue of the journal and elsewhere have thoroughly summarized the major topics in the field [19–22, 31–34]. The focuses of this review are the synthesis and applications of peptide and peptidomimetic microarrays (peptide microarrays), especially the light directed parallel synthesis of individually addressable high density peptide microarrays using a novel PGR chemistry [25, 36–41]. In this review, the term peptide chip refers more specifically to peptide microarrays of higher density (more than 1,000 features on a chip of about one square cm).

In situ synthesis of peptide microarrays

An overall review of peptide microarray synthesis

Both peptide microarrays and peptide libraries consist of a large number of sequences. The major difference between the two is that the specific sequence information can be obtained through the location within peptide microarrays [3–8, 25–29, 31–35]. Peptide microarrays refer to a format of flat surface on which peptide sequences are organized in rows and columns, and in most cases, according to a predetermined pattern. A peptide library is usually created by phage display [42–45] or combinatorial synthesis in solution or on certain types of supports, such as beads [3], based on a split-and-mix [46] strategy possibly to give one bead one compound (OBOC) [33]. Theis approach relies on special ways of tagging/coding the beads to register structural information on a bead. In these settings, up to 10^{12} different peptides (natural amino acids only for phage display) may be screened; the binding experiments are followed by 'decoding' on beads, using sequencing, chemical tracking, or other reading methods. The operations of such peptide library synthesis and binding assays tend to be laborious and time consuming. Recent developments in laboratory robots, synthesis instruments, and tagging methods, such as the direct sorting and radio frequency (RF) tracking instrument by Irori, have greatly reduced manual operations required [47]. In this process, milligram quantities of materials are prepared which can then be dissolved and the solution spotted onto a surface to form microarrays for screening assays [48, 49].

Peptide microarrays are made by several methods, including robotic deposition of pre-synthesized peptides [30], panning phage display peptides onto a flat surface [34], or *in situ* synthesis, which can be further divided into either direct synthesis or parallel synthesis [6, 25, 31–35, 41]. One of the clear advantages of peptide microarrays is that the binding results can be obtained immediately from the location of a specific sequence. The density of the sequences on a microarray ranges from a few to thousands in a square cm area for arrays of minute size, such as the size of 1×3 microscope slides, called peptide chips. Peptide and peptidomimetic microarrays made by synthetic methods should complement random peptide libraries in their capability of further refining the consensus binding sequences at a much higher resolution by discriminating at the level of a single amino acid and also expanding the sequence diversity due to their ease for incorporating synthetic building blocks.

There are additional challenges for peptide synthesis in a miniaturized format and scale. Unlike nucleotides of DNA which have four natural monomers formed by the same phosphorylated penta-saccharide and differing only in their base moieties, there are 20 amino acids containing side chains linked with a variety of functional groups, having different sizes or opposite polarities. Peptide solid phase synthesis using t-Boc (ter-butyloxycarbonyl) or fmoc (9-fluorenyl methoxycarbonyl) chemistry take much longer time per cycle ($\sim$ 2 h) and the stepwise yield ($\sim$ 97–98%) is not as ideal as oligonucleotide synthesis (> 98.5%) [50]. Since parallel synthesis, especially *in situ* parallel synthesis, is controlled by reaction cycles and, for instance, a pentapeptide array synthesis including all 20 amino acids would require 100 reaction cycles, a peptide chip synthesis potentially can take a few days and sequences synthesized are of moderate quality. Therefore, a useful synthesis method for peptide microarrays needs to provide solutions to overcome these barriers.

Direct synthesis on surface or *in situ* synthesis of microarrays is seen as an effective means of miniaturization of

traditional chemical reactions and assays. Several methods have been reported for *in situ* synthesis of peptide microarrays. A popular approach of direct synthesis on surface is SPOT [5, 31, 34], which uses a robotic instrument to deliver synthetic building blocks and reaction reagents, following standard chemical reaction conditions, onto a filter paper or a cellulose or nylon membrane sheet in an area of several square inches, and the solvent washing steps are manual. The SPOT peptide microarray is then incubated with the sample and the assays require milliliters of the sample. This method has gained wide acceptance to allow screening epitope sequences, enzymatic reaction substrates, and protein binding ligands at a low cost. SPOT synthesis also easily incorporates modified or non-natural amino acid building blocks, making possible the creation of diverse peptidomimetic sequences for binding assays. A recent application illustrates SPOT synthesis of a pair of peptide sequences at the same reaction site for the studies of protein-protein interactions potentially involving multiple weak contacts [51]. The synthesis used both fmoc-alanine and alloc-alanine at the first cycle to achieve selective deprotection and thus the synthesis of a peptide containing a Ser residue as a model for Elk-1 Ser383 phosphorylation by ERK-2 kinase and a peptide representing the Elk-1-docking domain. The results of the enzymatic experiments suggest that the Ser phosphorylation requires the presence of the Elk-1-docking domain. The synergic effects of a pair of discontinuous peptides were also observed for phosphatase activities of protein-tyrosine phosphatase (PTP) 1B on insulin receptor. Many examples shown in the literature have validated the peptide microarray platform as an effective tool for probing molecular recognition and interactions; this represents significant technological advancement for research in the fields of molecular immunology, signal transduction, drug discovery, and molecular diagnostics.

Another conceptually interesting method of *in situ* peptide microarray synthesis for producing high density peptide chips is light-directed parallel synthesis using nonconventional photo-labile protected amino acids as building blocks (Figure 1A) [6, 35]. The synthesis involves multi-cycles (Figure 1B) with each cycle using light irradiation through a photomask followed by adding one type of amino acid to specific locations where the photo-labile protecting group (PLPG) on the terminal of the growing chain has been removed upon light irradiation. The amino acids used in the synthesis are not conventional but protected at the amino position with a PLPG, such as nitroveratryloxycarbonyl (NVOC) or (α-methyl-2-nitropiperonyl)oxycarbonyl (MeNPOC). As demonstration of the technology, an endogenous opioid pentapeptide, leucine enkephalin YGGFL, and its sequence variations were synthesized with eight different PLPG-amino acids incorporated [6]. Although promising for high-density peptide chips, this process has practical limitations: photomasks are expensive and time consuming to produce and the operation requires expensive equipment and a high grade clean room environment; the preparation of

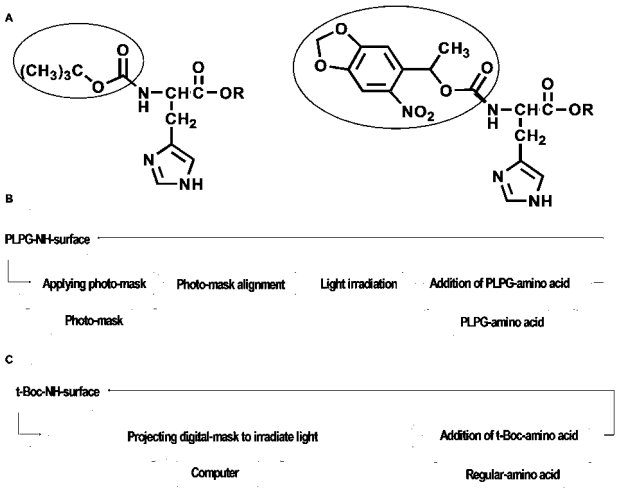


Figure 1. 1A. (left) Example of a conventional t-butyloxycarbonyl (t-Boc) protected amino acid and (right) a photo-labile protecting group (PLPG) protected amino acid. Here PLPG is (a-methyl-2-nitropiperonyl)oxycarbonyl (MeNPOC). 1B and 1C. Illustration of the process of light-directed *in situ* synthesis of peptide chips using 1B. photo-labile protected amino acids. Two key components are required: the microfabricated photo-mask and PLPC amino acids for each cycle of synthesis; and 1C. PGR chemistry and digital photolithography, requiring conventional amino acids.

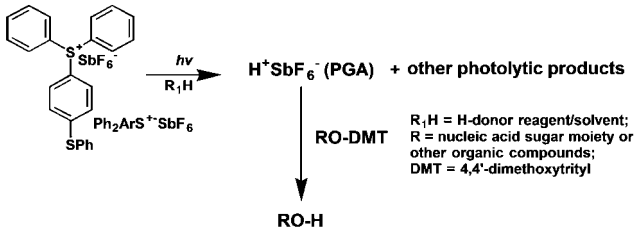


Figure 2. Reaction of PGA formation from triarylsulfonium hexafluoroantimonate. Other products include reduced aromatic compounds and phenylthioether compounds. PGA subsequently deprotects DMT as alcohol protecting group to release an active OH group.

the monomers containing PLPG is not a trivial task; and the photochemistry used is less efficient than conventional t-Boc or Fmoc chemistry [50]. These shortcomings cause poor synthesis and low sequence fidelity [52–54], rendering the method too difficult to be used in a regular laboratory setting and too costly to afford.

The reactions using photogenerated reagents

Basic concept

Parallel synthesis of microarrays containing libraries of molecules has distinct advantages in miniaturization. A key step in performing a large number of these reactions in parallel in a small area, e.g. cm^2, is being able to control at each site the reaction occurring or not occurring, or referring as gating of reactions. The incorporation of photo-labile group protection of functional groups, such as OH or NH_2, is a way of gating, which involves light irradiation for removal of the protecting group to allow subsequent reactions at

these deprotected reaction sites [6]. However, these photolytic reactions are not highly efficient, giving yield in low nineties. This limits the quality of the compounds that can be synthesized in multi-step syntheses. Another drawback is the requirement for photo-labile group protected monomers, which are not commercially available and their synthesis in many cases is practically impossible.

A breakthrough development in parallel synthesis is to control the reaction through the generation of reaction reagent [36]. The reactions as described below illustrate the use of solution *in situ* photogenerated reagents (PGR) to affect otherwise conventional reactions in a highly parallel fashion. The basic concept of the PGR chemistry is that a conventional chemical/biochemical reaction occurs between at least one reactant (generically denoted as 'A') and at least one reagent (generically denoted as 'R') intramolecularly or intermolecularly to give at least one product as depicted below:

$$\mathbf{A + R \rightarrow A' + R'}$$

A group of compounds that undergo photolytic reactions to produce R are photogenerated reagent precursors (PGR-Ps). The PGR thus produced then functions the same as a reagent conventionally used in a chemical reaction, and thus the reaction proceeds in an otherwise conventional way. The overall PGR chemistry reaction is depicted below.

$$\begin{gathered} \boldsymbol{hv} \\ \mathbf{(PGR\text{-}P) \rightarrow R} \\ \mathbf{A + R \rightarrow A' + R'} \end{gathered}$$

Although first proposed for using PGR-P to gate parallel synthesis in solution [36], there are a large number of PGA-P compounds available. These are cationic polymerization initiators or initiators of chemical amplification in photoresist processing of microelectronic devices [55–58]. These reactions occur within a thin film of polymer, in the presence of high concentrations of photo-acid generator and acid amplifiers. Photo-acid generator produces protic acid upon light irradiation; acid amplifiers are organic proton donors, sulfonate esters, and hindered acids, which undergo thermal decomposition under acidic catalytic conditions to give protons. The chemical amplification reaction has been used to remove the DMT (4,4'-dimethoxytrityl) group from the terminal OH of immobilized oligonucleotides embedded in a polymer coating in the synthesis of oligothymine sequences [59]. Novel applications of acid amplification also include a recent report on a silver-free, single-sheet imaging process, producing color images suitable for printing applications [60]. PGR chemistry differs from chemical amplification in that it does not require an amplifier and is applicable to a variety of different regents regularly used in chemical reactions, such as photogenerated bases (PGBs). However, besides PGAs, only few PGR compounds are available for general use. A limited few PGB precursor compounds exist, which fall into the structure family of photo-labile group protected amines [61, 62].

Photogenerated acid deprotection of DMT protected OH group

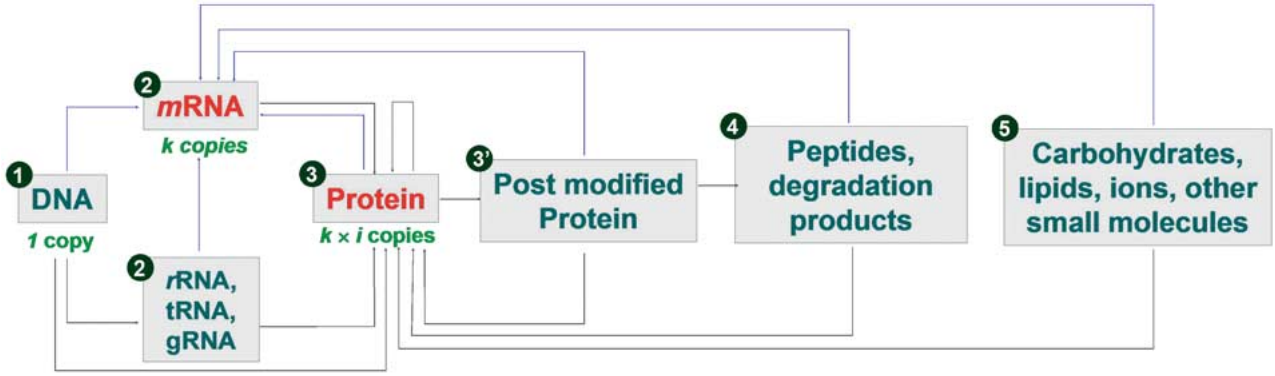
Novel solution PGR photochemistry was first reported for the deprotection of 5'-O-DMT of nucleosides using photogenerated acids (PGAs) and its application to the automated oligodeoxyribonucleotide synthesis [36]. The reaction of PGA formation and its subsequent deprotection of a protected alcohol molecule are shown in Figure 2. It was shown that protic PGA can be generated from a diazoketone sulfonate, sulfonium or iodium salts in an organic solution, such as dichloride methylene (DCM). The PGA solution at tens of mM concentration can effectively remove the DMT group to free the 5'-OH group of nucleosides or nucleotides as seen from HPLC profiles of the reactions, such as in deprotection of 5'-ODMT deoxynucleosides [36]. Oligonucleotides were synthesized at reasonable quality on CPG (Controlled Porous Glass) using PGA as deprotecting reagent. Recently, precursor for formation of photogenerated trichloroacetic acid (TCA) from 2-nitrobenzyl-trichloroacetate was reported to be used for oligonucleotide synthesis on a μmol scale [63]. The DMT deprotection reaction using PGA on a chip surface under optimal conditions requires only a few seconds and will be described in a separate review article [64].

Photogenerated acid deprotection of t-boc protected NH_2 group

The chemical principle of PGA reactions was extended to the deprotection reaction of the t-Boc group of amino acids [38]. Amino acids, such as Boc-Tyr(Bzl)-O- on HMBA-AM resin, were reacted with a sulfonium antimonate or an iodium antimonate at tens of mM concentration and in the presence of thioxanthone as sensitizer in DCM to give deprotected NH_2-Tyr(Bzl)-OH as the clean product, as examined by reverse phase HPLC. The PGA reactions were also performed on glass plates using fluorescein (FR) to monitor the deprotection results. High efficiency of deprotection would give higher reading of fluorescence, assuming the coupling efficiency of FR is proportional to the degree of deprotection by PGA. These results are of particular importance, since they opened up the possibilities of incorporation of hundreds of synthetic building blocks of amino acid analogs and provide a basis for combinatorial synthesis of individually addressable libraries of peptidomimetics on a microarray platform. The t-Boc deprotection reaction using PGA on a chip surface under optimal conditions requires much shorter time, i.e. 2–4 min, compared to conventional deprotection using trifluoroacetic acid (TFA, 10–15 min), and this shortening in reaction time is important for efficient parallel synthesis of peptide microarrays.

Photogenerated reagents

In a more general scheme, the deprotection of DMT or t-Boc group releases OH or NH_2 group to allow subsequently formation of phosphite triester or amide bonds. It is highly desirable that the PGR chemistry can find applications in other general types of organic reactions.



$$R(mRNA)_i = F[f_1(k_1DNA), f_{-2}(k_{-2}protein), f_{-3}(k_{-3}protein'), \ldots.]$$

$$R(protein)_i = F[f_2(k_2mRNA), f_i(k_iDNA), f_j(k_jprotein'), \ldots.]$$

Figure 6. Illustration of a conceptual biological system containing five major categories of components which modulate each other's formation, disappearance, and activities. The rate of formation of each species, such as R(mRNA) andR(protein) for molecule I, should be derived from a complex multiple component equation. The expression equation for the overall system will require parameters known for all steps. At present, only stable mRNA can be measured for their relative copies. There is a need for methods of measuring the presence of other types of molecules.

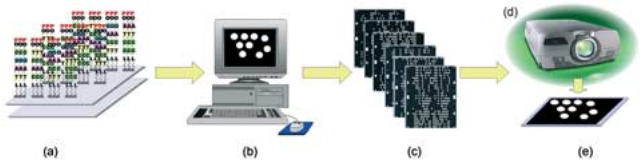


Figure 3. Illustration of combinatorial synthesis using the digital photolithographic approach. (a) Peptide microarray to be synthesized; (b) computer processing of the sequences; (c) light irradiation patterns generated according the peptide sequences to be synthesized; (d) and (e) light irradiation through a digital optical projection unit.

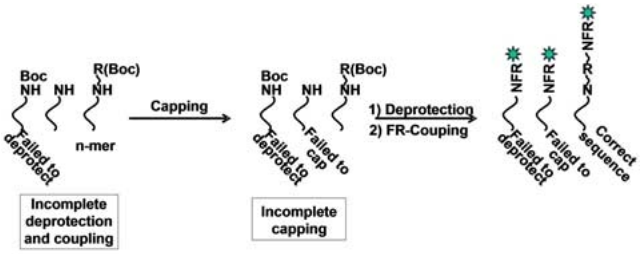


Figure 4. Illustration of some of the defects in *in situ* fluorescent monitoring of synthesis quality on chip.

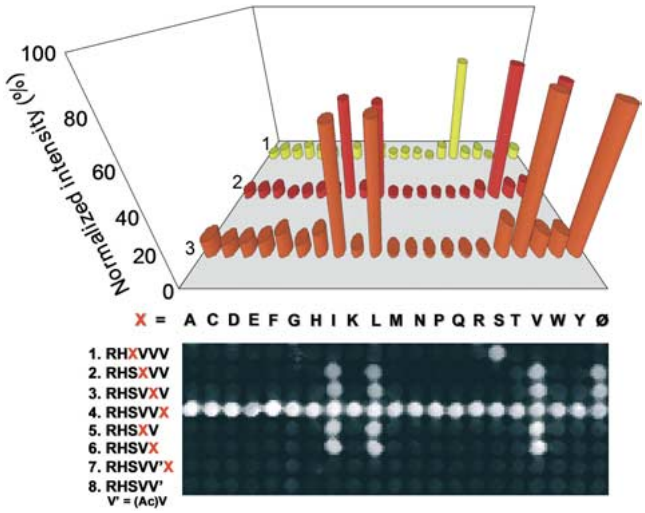


Figure 5. Peptide chip synthesized using PGA chemistry and digital photolithography for p53 antibody PAb240 (FR-labeled) epitope screening. The consensus sequence is RHSVV. X represents one of 20 amino acids. Line #4 validates the incorporation of 20 amino acids since coupling failure followed by capping would terminate the binding. Lines #7 and #8 validate the effective capping step, which terminates the synthesis and thus no binding was observed.

This in turn depends on the availability of diverse PGR reagents. Thus, new PGR reagents, such as a precursor for photogeneration of piperdine or hydrazine (2-(2-nitrophenyl)propoxycarbonyl piperidine (NPPOC-pip) or 2-(2-nitrophenyl)propoxycarbonyl hydrazine (NPPOC-Hz)) have been demonstrated and used in deprotection of fmoc-amino group and Lys side chain (i.e. removal of 4,4-dimethyl-2,6-dioxocyclohexylidene-)3-methyl-butyl, ivDde), respectively [62].

Nonlinear reaction correlations in PGA induced deprotection reactions

The PGA deprotection reactions studied on microchips follow a nonlinear relationship between light irradiation strength (i.e., the product of light intensity and irradiation time) and the reaction efficiency (Figure 2 in ref. [25] and Figure 4 in ref. [39]). In these experiments, light irradiation at different sites applies increasing or decreasing intensities or time (0–100% gray scale irradiation). After deprotection by PGA, FR was coupled to the reaction site and the resultant fluorescent signals were plotted as a function of light con-

ditions to give sigmoid curves. The nonlinear relationship shown is consistent with a possible general acid catalyzed reaction mechanism for removal of the t-Boc group from an amino group or the DMT group from a hydroxyl group. This correlation is preferred for minimal unwanted deprotection due to stray light [65] or acid diffusion, ensuring high quality synthesis. In contrast, photolytic reactions such as the cleavage of PLPG follows an undesirable linear relationship with the light intensity used [65].

Digital photolithography and combinatorial synthesis

A significant improvement in making photolithography a practical method for combinatorial chemical synthesis has been to introduce the use of a digital optical unit for light pattern projection onto the surface of a reaction device (Figure 3) [25, 36, 39, 66–68]. Light irradiation with selective patterns results in selectively photoactivating a reaction at certain sites, and thus permits parallel synthesis for the preparation of a large number of different molecules on the same reaction surface. For synthesis of biopolymers, such as oligonucleotides and peptides, this automated method provides greater flexibility since microarrays containing any desirable sequences can be obtained quickly through a computer controlled process (Figure 3). Specifically, a digital optical unit consists of a light source capable of generating enough energy for chemical reactions and a programmable light pattern formation and projection device conducting light irradiation. Texas Instruments' Digital Micromirror Device (DMD^{TM}) based on the Digital Light Processing (DLP^{TM}) technology [69] was first used for these applications. Light patterns can also be generated using a programmable laser source [41].

PGR chemistry is particularly suited for use in combination with digital photolithography for creation of microarrays [36]. To explore the best reaction conditions for microchip synthesis, such as the choice of PGA precursors, solvents, reaction time, light irradiation time or intensities, different PGR or PGA generation conditions can be screened in hours directly on the combinatorial synthesis platform [38]. For example, as shown in Figure 4, the chip synthesis begins with filling all reaction sites derivatized with t-Boc amino linker molecules with a PGA precursor solution; irradiating surface with a first light pattern to induce photolytic reactions in the first set of reaction sites; removal of the t-Boc group at these irradiated sites exposes a free NH_2 group. The second cycle of the synthesis uses a second set of reaction conditions; the irradiation uses a second light pattern to cause removal of the t-Boc group and formation of a free NH_2 group at a second set of selected reaction sites. The reactions are repeated multiple times for all reaction conditions to be tested. At the end of the reaction, the surface contains reaction sites in which the completion of the deprotection reactions is different due to different reaction conditions applied. The best reaction conditions should correlate with the efficient formation of the NH_2 groups each time the t-Boc group is removed. The last step of the chip synthesis is to couple with FR; the number of FR molecules coupled to the surface is proportional to the number of NH_2 groups. Therefore, the detection of the fluorescence intensity directly correlates with the efficiency of reaction conditions. This method allowed a quick search of optimal PGA conditions for oligonucleotide and peptide chip synthesis, and is applicable to the synthesis of microchips containing other types of molecules. The FR *in situ* assays are also used to optimize coupling and capping steps. As mentioned above, regular peptide synthesis requires about 2 hr per addition of monomer, but better reaction conditions for parallel synthesis found through optimization of t-Boc deprotection now require about 20 min [25], which is 6-fold reduction in reaction time per cycle, making it feasible to synthesize peptide microarrays of shorter sequences with molecular diversity in a matter of hours.

It is important to point out that the aforementioned *in situ* FR assays as a means of monitoring synthesis should be used with caution. This is because the reporter FR groups only reflect the total presence of the functional groups without relating to their history of formation [37]. Residual functional groups due to failed coupling and capping also contribute to coupling of FR. While it is a semi-quantitative comparison, the FR signal reading should be validated by cleavage of the synthesized sequences and their subsequent assays [37]. More careful design of an on-chip assay, such as inserting a repetitive step of coupling and then comparing the difference in the final FR signal readings, may help to reduce the influence of the artifacts in final fluorescence readings.

Peptide chip synthesis using PGA chemistry

The general synthesis of peptide chips using t-Boc chemistry involves steps of light controlled deprotection by PGA, coupling with a t-Boc protected amino acid, and capping (Figures 1C and 4). This is further illustrated using an example of the synthesis of peptide chips for p53 antibody PAb240 epitope screening based on the consensus sequence, RHSVV [25, 70, 71] Multiple subsets of peptides were designed and their sequences were compiled into a pseudo-sequence, which was used to direct the steps of amino acid addition by the synthesizer. At each synthesis cycle, a PGA solution was applied to the chip surface at the deprotection step; light irradiation from the digital optics unit at specific reaction sites was applied for duration of 2–4 min. The chip was then washed with solvent and subsequently allowed to react with an N-t-Boc protected amino acid. The amino acid coupled to where light irradiated and the unreacted reaction sites were capped with acetyl group. The final step was FR coupling and the fluorescent image was scanned after side chain deprotection. In the example shown in Figure 5, the combinatorial steps are at those positions marked with X, which represents the incorporation of natural 20 amino acids, eight synthetic peptidomimetics compounds, or acetylation, while the rest of the synthesis steps used a single amino acid. This was to freeze the sequence in all posi-

tions except one in order to identify sequence specificity associated with a particular position in a peptide sequence. The incorporation of all 20 natural amino acids was validated by the presence of fluorescent signals from the series of RHSVVX (X = one of the 20 amino acids) sequences. For this set of spots, if the synthesis of X failed, the site should be capped and the fluorescence tagging or the binding should not be observed. To test whether racemization occurred during synthesis, sequences containing the D-isomers of Ser and Val were synthesized. These sequences did not show binding to PAb240 antibody, providing evidence that racemization at C_α is negligible.

Peptide chip experimental design

Synthesis of peptide chips is challenging in its chemical requirement and experimental design for simultaneous large scale reactions. A synthesis without proper consideration of the chip design will not generate the results as hoped. In addition to have a clear goal prior to chip synthesis, no matter how perfectly a synthesis or an assay will be performed, there are always experimentally introduced errors and imperfection in chip results. Therefore, it is critical to incorporate on-chip controls and references to compensate for experimental errors and to establish bases for background correction and normalization of signal intensities. Although the chip designs depend on the type of chips, it is useful to pay attention to the follow points: (a) dividing a large data set into small subsets consisting of data points which contain systematic variations of one or few parameters and are easily compared; (b) replicating data points within a chip at different locations; (c) using a constant set of controls for *in situ* monitoring in synthesis and assays in each subset of data per chip; (d) incorporating background controls (no synthesis), known positive and negative controls at constant locations.

Applications of peptide chips

The following discussion is intended not to repeat topics covered by this special edition of protein and peptide microarray technologies, but to discuss a few examples representative of recent development of peptide microarrays as demonstration of the applications of high density peptide chips. There are also a number of comprehensive reviews of various peptide microarrays, their synthesis, fabrication, and applications [26–31, 33, 34, 42].

A brief overall review

Peptide microarrays have found important, diverse applications (Scheme 1). Peptide microarrays complement phage display well for discovery of binding sequences for protein profiling and studies of protein functions. Phage display [42–45] may represent a huge population of up to 10^{15} sequences, but its selection bias exists because only natural amino acids or certain types of amino acids and the related sequences are preferably expressed. Peptide microarrays usually contain hundreds to thousands of sequences, having residue-specific sequence definition and a large library of synthetic analogs of amino acids that can be incorporated into the synthesis. There have been hundreds of examples of SPOT synthesized peptide microarrays utilized for mapping binding sites and functional studies in proteins or other host molecules of biological and therapeutic importance as reviewed in this edition of the journal and literature [5, 31, 32]. Further, the combined use of phage display and peptide microarray through protein/peptide-peptide interactions has been successfully used for identification of unknown proteins [72, 73]; these selected target proteins may be further enriched and identified through hybridization of the phage sequences to a DNA microarray [34]. Another type of application of peptide microarrays is its combined use with combinatorial synthesis [33]. Novel applications for screening of inhibitors of protein kinases and peptide ligands for cell surface receptors have shown great promise in extending of peptide microarray applications from probing protein-protein interactions to cell signaling pathways and other high level complex interactions in biological systems. Low density peptide microarrays by spotting and using improved linker chemistry on gold-surface have been demonstrated for surface plasmon resonance (SPR) or SPR imaging measurements [26, 74]. In affinity tag screening [74], binding constants of the flag affinity peptides to its antibodies were obtained directly from SPR intensities. In the studies of kinase protein-substrate interactions, three types of measurements were demonstrated: SPR, fluorescence, and phosphorimaging, allowing activity assay and quantitative measurements for the inhibition of the enzymatic phosphorylation reactions [26]. Recently, a peptide microarray containing Pb(II) ion specific peptides has shown promising as a biosensor for metal ion detection [41].

High density peptide chips used in binding assays

High density peptide chips containing thousands or more features per cm^2 area are of tremendous potential for high throughput, automated binding and functional assays. The first example of epitope binding to an antibody using Y/P-GGFL pentapeptide chips made from eight different photolabile group protected monomers was demonstrated more than a decade ago [6]. The binding of YGGFL to anti-β-endorphin antibody (mAb 3E7) was detected using a FR labeled IgG secondary antibody. This photolithographic parallel synthesis method was also applied to the synthesis of biopolymers consisting of carbamade backbone from photolabile protected aminocarbonate monomers. High affinity binding was detected between the specific sequences and a carbamate specific antibody conjugated with FR [35]. Clearly, further development of these peptide chips using the prescribed approach [6, 35] is not feasible due to the requirement of at least 20 photo-labile protected monomers and less than satisfactory photolytic deprotection reactions.

Epitope screening using p53 antibodies

Recent development of the PGA chemistry for microarray synthesis has led to several reports on the applications of peptide chips, especially high density peptide chips, to epitope screening [25, 40, 41]. Peptide chips containing epitope sequences from tumor suppressor p53 and the related truncation, permutation, and amino acid analog modified sequences were constructed (*vide supra*). The p53 peptides are epitopes (residues 20–25 or 213–217) to antibody mAb DO1 (SDLHKL) [41] or PAb240 (RSHVV) [25], respectively. The binding to these antibodies has been used to detect p53 mutations or identify p53-like proteins. The binding to Pab240 used 0.04 μg of fluorescent dye labeled antibody in 40 μL solution and discrimination in binding specificity among the peptides was clearly detected. A representative image of p53 peptide chip binding to PAb240 is shown in Figure 5. From the point of view of practical applications, the photolithography based synthesis produces peptide chips of high spot uniformity and their data are easy to process due to regularly distributed spots in rows and columns. Therefore the binding data can be analyzed and the information can be obtained quickly. The p53 peptide chip binding studies relied on several hundreds of sequences of peptides and peptidomimetics incorporating 20 natural amino acids, D-isomers, and synthetic analogs of amino acids. These chips were made in replicates in a square cm area; these results unambiguously confirmed the RHSVV epitope sequence identified previously by phage display [70, 71] and found new peptidomimetic antibody recognition elements, such as RHS(Abu)(Abu/Nva/Nle) (2-aminobutyric acid, norvaline, norleucine) with moderate binding affinity to PAb240. These systematic variations of the sequence designs on peptide chips also permitted a better understanding of the specificity and selectivity of the epitope binding site of the antibody [25].

Affinity tag screening using antiflag antibody M2

Recently, there has been a rapidly increasing effort in large scale studies of proteome proteins [75–78]. Proteins are usually amplified in an expression system with fusion affinity tag and purified using affinity columns. Thus, as simultaneous protein expression and purification become necessary steps for achieving such large scale studies, the needs also increase for improved affinity tags and a larger pool selection of affinity sequences. High density peptide chips have been used to characterize the binding profile and map better binding sequences based on Flag peptide DYKDDDDK to anti-Flag antibody M2 (AFM2) [40]. The studies were divided into several subsets including those systematically mutating (10 amino acid variation at the first three positions of the sequence) and truncating (dimer to octamers) and a series of repeat sequences. After the initial binding assays, DYK and DYKDDDDK were found to have comparable binding and used as the positive controls and DYA as the negative controls. In each subset of designs on chip, the control sequences were included, making possible baseline subtraction, normalization and cross comparing the results from multiple subsets within and among chips. The results of these studies revealed selective dimers and trimers possible as AFM2 binding motifs and a number of stronger AFM2 affinity sequences compared to the original DYKDDDDK [40]. The high affinity binding to AFM2 by the repeating sequences was confirmed by on bead binding of the same set of sequences. In general, shorter sequences should be more selective in specific antibody binding but it remains to be seen if shorter sequences will provide sufficient binding affinity when incorporated into large proteins. The availability of these affinity peptides, nonetheless, provides possible choices for better matching of the fusion affinity tags and proteins. The finding of a number of high affinity sequences to AFM2 reveals possible undesirable cross-interactions of the N- or C-terminal of proteins to the affinity antibody, interfering with intended protein purification and analysis. There will be similar experiments performed for screening high affinity tags of different sequences specific to different antibodies.

Concluding Remarks

The growing interests in the studies on genomic or proteomic scales present unprecedented opportunities and daunting tasks. A clear trend is already in place to conduct experiments in a parallel fashion and down-sizing formats, which allow faster throughput, easy comparison of experimental results, and automation. In a way similar to the process of five decades for the improvement and adaptation of computers, we will see continuing shift in bio- and chemical technologies to miniaturization, system integration, parallelization, and automation. As discussed in this review, peptide chips have the promise as a key component for a miniaturized system that will allow not only synthesis, but also sample processing and interrogation and extrapolating information on genomic and proteomic scales.

This review emphasizes a technology platform of biochip synthesis and applications [25, 36–41]. The basis of the technology consists of three key components: (a) photogeneration of reagents for triggering conventional chemical reactions upon light irradiation, (b) digital photolithography, and (c) microchip for reactions in isolated sites. The combination of these elements creates a highly flexible, simple, efficient, low cost process for synthesis of peptide chips based on conventional peptide chemistry. Although still in an early stage, the peptide chips produced have been successfully used for rapid screening of specific binding sequences using epitope specific monoclonal antibodies. Peptide chips allow an important strategy for the effective use of a large number of data points, i.e., to systematically vary the sequences in a subset of data. By incorporating within each subset of data proper control sequences at multiple, designated positions on chip, rich information can be obtained

in a more reliable manner. Thus, the power of peptide chip applications is in massively parallel comparisons of the test points; quantitative analysis of absolute values is more challenging and still awaiting for method development [26, 74]. The advance in synthesis methods and instrument integration will explore the potential of peptide chips by increasing chemical structural diversity to peptide conjugates with carbohydrates, small molecules, nucleotides, and other types of molecules. These molecular chips will expand the applications from probing protein-substrate interactions and binding screening to screening of drug molecules of diverse structures [79] and receptor-substrate interactions. Biochips should be promising not only as powerful research tools but as a new generation of diagnostic devices that will accompany increasingly computer managed personal medical care.

The development of DNA chips, peptide chips, protein and antibody chips, and potentially many other types of biochips should pave the way to understanding systems biology. In this regard, we are still at the infancy of the development processes. In Figure 6 a conceptual network of a self-regulating biological system is shown. Presently, DNA chips, when sampled properly, allow a snapshot of the presence of stable mRNA. But our capability is very limited in detection and measurement of proteins and protein modifications, leaving many parameters in system's biological processes unknown. It is not surprising that the correlations of mRNA expression with protein expression are often non-existent. However, by investing intensive effort in high throughput parallel analysis of nucleic acids and proteins on a global scale, we are now more hopeful than ever to extrapolate the various parameters controlling the multiple stages of biological processes and to eventually understand the circuitry of the entire operation. In this process, *in situ* synthesized peptide and peptidomimetic microarrays will continue play an important role for detection and quantitation of proteins, identification of therapeutic protein/receptor targets, discovery of drug candidates, and many other applications.

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