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Review

Combinatorial Solid Phase Peptide Synthesis and Bioassays

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Solid phase peptide synthesis method, which was introduced by Merrifield in 1963, has spawned the concept of combinatorial chemistry. In this review, we summarize the present technologies of solid phase peptide synthesis (SPPS) that are related to combinatorial chemistry. The conventional methods of peptide library synthesis on polymer support are parallel synthesis, split and mix synthesis and reagent mixture synthesis. Combining surface chemistry with the recent technology of microelectronic semiconductor fabrication system, the peptide microarray synthesis methods on a planar solid support are developed, which leads to spatially addressable peptide library. There are two kinds of peptide microarray synthesis methodologies: pre-synthesized peptide immobilization onto a glass or membrane substrate and *in situ* peptide synthesis by a photolithography or the SPOT method. This review also discusses the application of peptide libraries for high-throughput bioassays, for example, peptide ligand screening for antibody or cell signaling, enzyme substrate and inhibitor screening as well as other applications.

Keywords: Bioassay, combinatorial chemistry, Microarray, Peptide library, Solid phase peptide synthesis

Introduction

Recent developments in the identification of novel drug targets based on the benefits of human genome projects as well as the continued improvements in peptide delivery technologies have created an increasing demand for highly effective synthetic peptide library systems. For the generation of synthetic peptide libraries, combinatorial chemistry has been utilized as an effective method. Combinatorial chemistry is a technology for creating a number of different compounds simultaneously and screening them rapidly for a useful compound. For example, billions of different heptapeptides can be synthesized at the same time through combinatorial chemistry. Such peptide libraries can be used for the screening enzymatic substrates and inhibitors or cell binding peptides. Unlike the conventional synthetic way of handling one molecule at a time, combinatorial chemistry has been regarded as an important tool for the discovery of new drug candidates, catalysts, and materials. In particular, solid phase peptide synthesis method, developed by R. B. Merrifield, has become a major breakthrough for the development of combinatorial chemistry (Merrifield, 1963). Furthermore, as scientists are now demanding for more efficient solid phase synthesis and screening methods, a variety of research fields such as solid-supports, linkers and peptide coupling chemistry, automated synthesis systems, and screening methods have been developed. Moreover, with recent developments in the microfabrication processes and the surface modification technologies, peptide library synthesis in a microarray format has become a common tool for high-throughput screening these days.

This review article presents a brief history and recent reports related to the peptide library synthesis using solid phase such as polymer supports and flat chip surface. The application of the prepared peptide libraries in a bioassay system will also be reviewed.

Peptide Library Synthesis on Polymer Support

Parallel synthesis Parallel synthesis of peptide libraries is based on a simple theory of combinatorial chemistry. Geysen *et al*. reported the first approach of peptide library synthesis by multi-pin technology (Geysen *et al*., 1984). Peptide libraries are synthesized in individual reaction chambers, and therefore, each product is pure, separated, well determined. However, this method generates only a small set of peptides library. Consequently, an automated synthesis system is required for the synthesis of large pool of peptide library.

Split and mix synthesis Split and mix synthesis can provide equimolar mixture of random and large peptide libraries. The split and mix synthesis method consists of three processes:

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O_1XXXXX
XO_2XXXX
XXO_3XXX
$XXXO_4XX$
$XXXXO_5X$
$XXXXXO_6$

Fig. 1. The six hexapeptide positional scanning libraries.

splitting, coupling and mixing. Firstly, polymer beads are split into multiple reaction chambers and coupled with individual compound units. After the reaction, the polymer beads are randomly mixed. Through the repetitive cycles of the procedures, peptide libraries can be obtained. This split and mix synthesis is a useful and relatively easier method for the synthesis of equimolar large peptide libraries than the parallel synthesis (Houghten, 1984; Lam *et al.*, 1991; Graven *et al.*, 2001; Christensen *et al.*, 2003). Using this method, the peptide libraries can be generated in such a way that each bead can display only one peptide. This one-bead one-compound (OBOC) combinatorial library can then be assayed for specific biological properties using either on-bead or a releasable solution phase assay.

Reagent mixture synthesis Reagent mixture synthesis method is a more convenient method than the split and mix synthesis method for the large peptide libraries. All the amino acid reagent mixture in one reaction chamber participates in the coupling reaction to make building blocks. To compensate for the different reaction rates of each amino acid reagent, isokinetic ratios are calculated and the corresponding amounts of each amino acid reagent are employed in the coupling reactions. Ostresh *et al.* performed the reagent mixture synthesis by calculating isokinetic ratios of *tert*-butyloxycarbonyl (Boc)-amino acid (Ostresh *et al.*, 1994). However, this method cannot be applied to one-bead one-compound combinatorial library synthesis since each single bead might contain a mixture of peptide products.

Nevertheless, the reagent mixture synthesis as well as split and mix synthesis is useful for positional scanning in the screening methods. Fig. 1 demonstrates hexapeptide libraries for positional scanning. 'O' is a residue that is known within the mixture as one of the monomers used to synthesize the library. 'X' is an equimolar mixture of all the amino acids used.

Peptide analysis Synthesized peptide libraries can be analyzed by using a variety of methods. If the product is pure, liquid chromatography or mass spectroscopy plays an important role in the analysis of the peptide after releasing the product from the polymer beads. If the peptide structure in one-bead one-compound library needs to be identified, the peptide sequences are generally analyzed by Edman degradation method (Lou *et al.*, 1996).

However, these methods can sometimes be time-consuming and expensive. In this regard, the ladder peptide synthesis method can be an efficient alternative method (Younquist *et al.*, 1995). During the synthesis of peptides on the polymer beads, a small portion of the peptides was *N*-terminally capped at each coupling cycle by inert compounds. Thereafter, peptide ladders were released and analyzed by mass spectroscopy. It should be noted that the ladder synthesis method has disadvantages in that all the ladder peptides are displayed together with the full-length library compound on the bead surface. Recently, Wang *et al.* attempted to establish a new ladder-synthesis method, which is a topologically segregated bilayer-bead concept, using allyloxycarbonyl (Alloc)-protected beads (Wang *et al.*, 2005). The segregated multilayer can be made by partial Alloc-deprotection under the biphasic condition (Fig. 2). This approach has some advantages in terms of being able to reduce the interference of coding tags during the biological screening of library compounds and enabling the determination of peptide sequence using Edman degradation method at the same time.

Peptide Microarray

The peptide array concept was first proposed by Southern

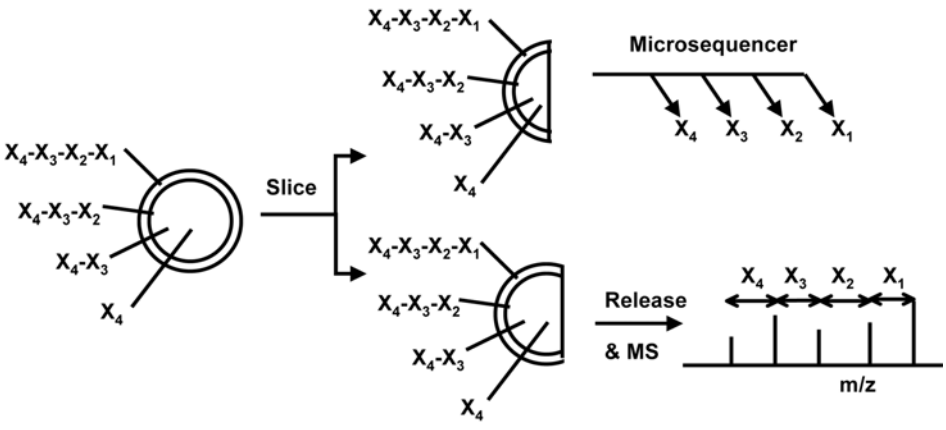

Fig. 2. Ladder synthesis of peptide libraries and determining peptide sequence by sequencer and mass spectroscopy (Wang *et al.*, 2005).

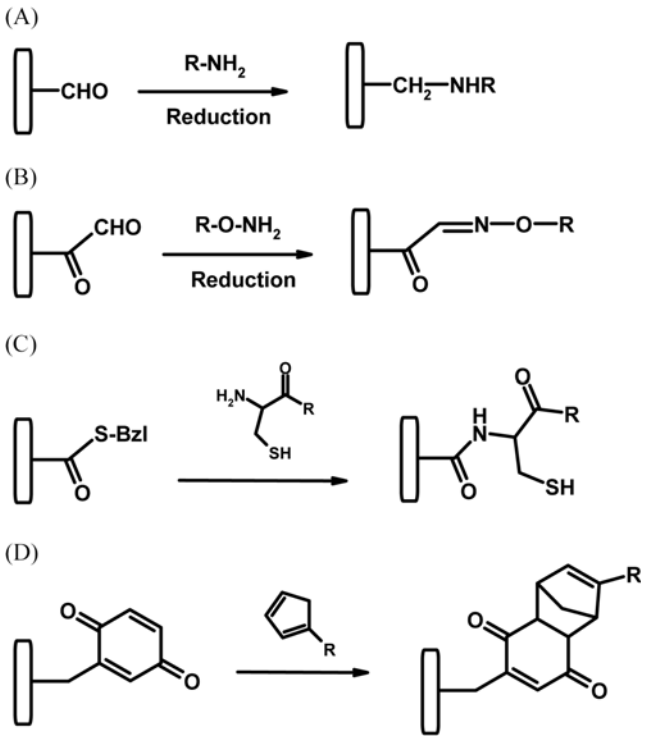

Fig. 3. Chemoselective immobilizations; (A) amino group to aldehyde linker, (B) aminooxyacetyl group to glyoxylyl linker, (C) cysteine residue to thioester linker and (D) cyclopentadiene residue to benzoquinone linker.

in 1988 (Southern, 1988). New microarray technologies such as photolithographic peptide synthesis on a glass surface (Fodor *et al.*, 1991) and the SPOT-synthesis of peptides on membrane supports (Frank, 1992), have opened great opportunities to the synthesis and subsequently screening of large arrays of the synthetic peptides on planar supports. There are two major classifications of peptide arrays according to their preparation methods: the immobilization of pre-synthesized peptide derivatives and *in situ* synthesis of peptides directly on the array surface.

Immobilization of pre-synthesized peptides The immobilization of pre-synthesized peptides is sometimes a more effective method than the *in situ* synthesis. Therefore, chemoselective immobilization methods have been widely used for the peptide microarray preparation (Fig. 3). Especially, they play an important role in the preparation of long-chain peptide arrays. Even though the pre-synthesized peptides are contaminated with their byproduct, only the peptide derivatives containing the chemoselective functional group can be attached to the appropriately modified chip surface. Moreover, the chemoselective immobilization can provide a useful method for controlling the orientation and the density of the immobilized peptides. However, the chemoselective immobilization methods have an intrinsic limitation in that it involves a laborious purification process of each peptide probes for the preparation of a high-density peptide array. Generally, chemoselective immobilization employs aldehyde or glyoxylyl functional linker on the chip/solid surface and the aminooxyacetyl groups in the peptide derivatives for the effective immobilization on the solid surface (Reimer *et al.*, 2002; Buss *et al.*, 2004; Diks *et al.*, 2004; Panse *et al.*, 2004; Rychelewski *et al.*, 2004; Schutkowski *et al.*, 2004). In addition, cystein residue can be used for the direct immobilization of the prepared peptide (Falsey *et al.*, 2001; Lesaicherre *et al.*, 2002a; Takahashi *et al.*, 2003). The native chemical ligation (Dawson *et al.*, 1994) can also be applied to immobilize *N*-terminal cysteine residue containing peptides to thioester-modified glass slides (Lesaicherre *et al.*, 2002b; Uttamchandani *et al.*, 2003; Uttamchandani *et al.*, 2004). Diels-Alder reaction (Houseman *et al.*, 2002) between benzoquinone groups on self-assembled monolayers and the cyclopentadiene-derivatized peptides can be applied for the oriented immobilization of peptide arrays (Fig. 3).

One of the most critical factors in bioassay using the surface-bound peptides is their accessibility to the target proteins or enzymes used in the screening process. To solve this potential problem and improve the efficiency of enzyme/substrate or antibody/peptide interactions on the surfaces, various kinds of spacers were introduced between the peptide and the chip surface such as 11-mercaptoundecanoic acid (Wegner *et al.*, 2002), hydrophilic polyethylene glycol chain (Falsey *et al.*, 2001), dextran (Luo *et al.*, 1995), bovine serum albumin (MacBeath *et al.*, 2000), human leptin (Lee *et al.*, 2004), and water-compatible supramolecular hydrogel (Kiyonaka *et al.*, 2004).

***In situ* synthesis** Compared with the chemoselective immobilization method, the *in situ* parallel synthesis method can provide miniaturized spatially addressed peptide arrays more rapidly and economically. However, this method sometimes brings about the problem in the quality of the resulting peptides, caused by the difficulty involved in monitoring the peptide coupling reaction.

This *in situ* synthesis can be classified into two methods according to their synthetic strategies: the SPOT synthesis (Frank, 1992) and the photolithographic synthesis (Fodor *et al.*, 1991).

In the SPOT synthesis, the peptides arrays are usually synthesized in a stepwise manner on a flat solid substrate such as functionalized cellulose membrane, polypropylene, and glass, following the standard Fmoc-based peptide chemistry (Frank, 1992; Min *et al.*, 2004). Firstly, small volumes of solutions containing activated amino acid are spotted on the solid substrate to make addressable array. Each spot can be considered as an independent microreactor composed of delivered droplet so that the functional surfaces react with the spotted amino acid to carry out solid-phase synthesis. In the SPOT synthesis, the employed solvent should be nonvolatile so as to maintain the wet state on the spot. The spot size is defined by the dispensed volume as well as the physical

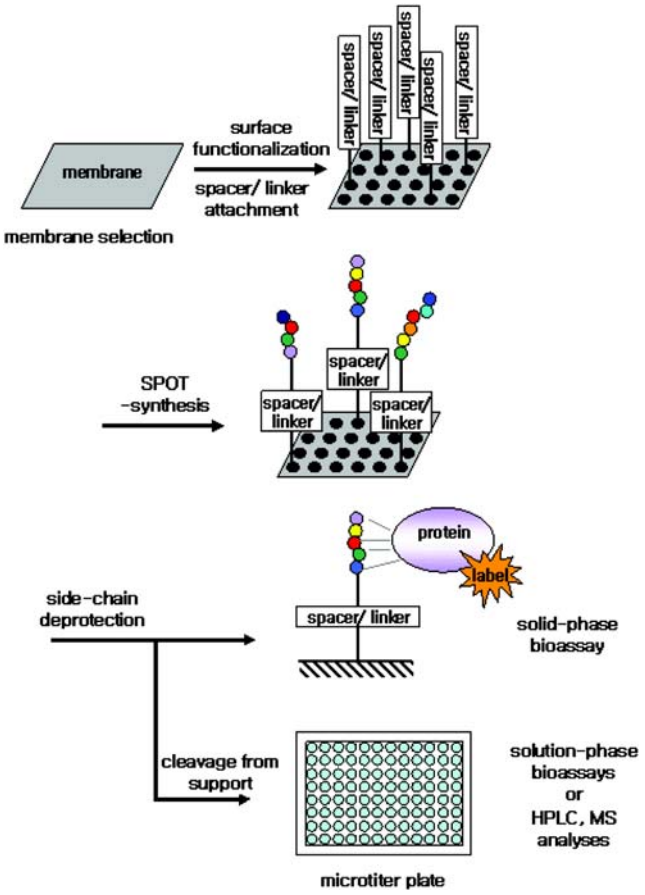

Fig. 4. Schematic view of the SPOT synthesis and their applications in binding and enzymatic assays.

properties of the surface. Although the resulting density of the peptide arrays from the SPOT synthesis (9-16 spots per cm^2) is not as great as photolithographic methods, it has several advantages such as relatively simpler experimental procedures and inexpensive equipment with highly flexible library formatting.

The general strategic steps for parallel peptide assembly on planar surfaces using SPOT synthesis is as follows: (i) select a suitable solid support meeting the chemical and the biological requirements and determine of the synthesis and screening method; (ii) functionalize the solid support for the selective attachment of activated amino acids; (iii) attach spacers and/or linkers in cases where solution-phase assays or analysis is required; (iv) conduct SPOT synthesis using activated amino acids; (v) select cleavage of side-chain protecting groups; (vi) screen support-bound or released peptides for subsequent bioassays/analysis (Frank *et al*., 1996; Kramer *et al*., 1998; Kramer *et al*., 1999; Wenschuh *et al*., 2000). The general scheme is shown in Fig. 4.

The practical Fmoc based peptide chemistry for simultaneous generation of peptide arrays can be applied on various kinds of planar supports (Wenschuh *et al*., 2000). These include ester-derivatized planar supports (Frank, 1992), CAPE (cellulose-amino-hydroxypropyl ether) membranes (Volkmer-Engert *et al*., 1997; Ast *et al*., 1999; Licha *et al*., 2000) and amino-functionalized polypropylene membranes (Wenschuh *et al*., 2000) and even amino-functionalized glass surface (Lesaicherre *et al*., 2002b).

Sometimes, SPOT synthesis can be accompanied with a cleavable liker system. In a case where the solution phase peptides need to be prepared for some specific bioassays and analyses, the linkages between the peptides and the surface should be stable during side chain deprotection with TFA solution. A glycolic acid type linker system which gives peptide free acids with sodium hydroxide solution (Baleux *et al*., 1986) or carboxamide by ammonia vapor (Bray *et al*., 1991) can fulfill the above-mentioned purposes. The safety-catch type linker such as the diketopiperazine-forming Lys-Pro linker (Bray *et al*., 1990) or the 2-(N^{Im}-Boc-imidazol-4-yl)-glycolic acid linker (Hoffmann *et al*., 1994) release the peptides in pH 7-8 buffer system so that the peptides can be directly applied into a bioassay.

Recent developments of novel modified surfaces, a new linker system and cleavage strategies as well as automated robot systems have extended the scope of the SPOT technology to the synthesis of peptide nucleic acids (PNA) (Weiler *et al*., 1997), and peptidomimetics such as peptomers or peptoids (Ast *et al*., 1999). With the developments of synthetic method accompanied by high-throughput solid and solution-phase screening methods, the SPOT technology will be the most convenient tools in biomedical research.

As the second *in situ* synthetic method of peptide microarrays, photolithographic synthesis has some characteristic merits. The conventional semiconductor fabrication system can be easily adapted and a large number of different peptides can be assembled on a unit area by combinatorial method. In this case, peptide microarray is generally synthesized using amino acid monomers with photolabile *N*-protecting group. Fodor *et al*. introduced the method of light-directed, spatially addressable peptide array synthesis by combining surface chemistry with microelectronic technology (Fodor *et al*., 1991). Fig. 5 shows the scheme of peptide array synthesis on a glass slide by photolithographic method. Firstly, amino groups on a glass slide are capped by photolabile protecting groups. The photolabile *N*-protecting group on the surface is site-specifically removed by the selective irradiation using a UV illuminator with a photomask. Thereafter, a monomer bearing the photolabile protecting group is coupled to the exposed amino group. Repetitive cycle of photo-deprotection and coupling steps generates the desired peptide microarray.

In fact, up to 40,000 compounds can be synthesized in $1cm^2$ using a $50 \times 50\ \mu m^2/1$ unit size checkerboard mask. The photolithographic method is more efficient for the fabrication of oligonucleotide microarrays (DNA chip). Because there are only four monomers for oligonucleotide library synthesis, the size of oligonucleotide libraries is much smaller than that of peptide libraries (i.e. for hexamer 6×10^7 peptides vs. 4096 nucleotides). The smaller chemical diversity of the nucleotides makes oligonucleotide synthesis much simpler than that for

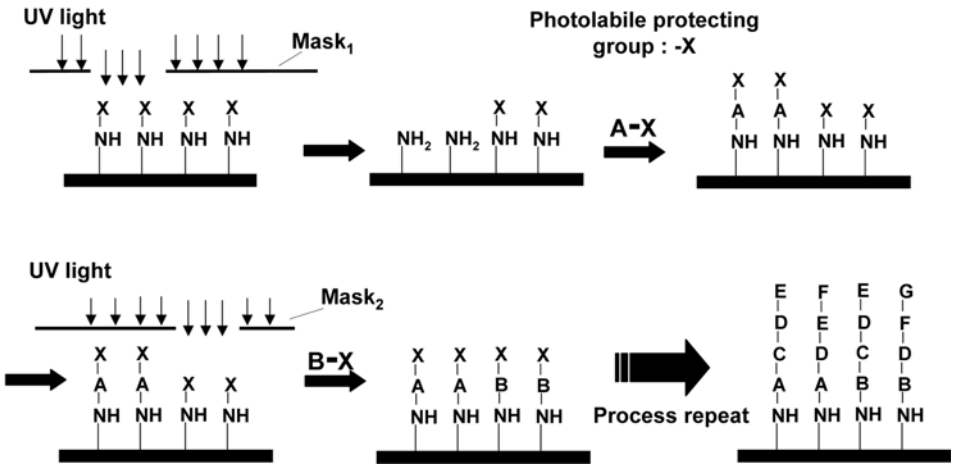

Fig. 5. Light-directed, spatially addressable peptide array synthesis (Fodor *et al*., 1991).

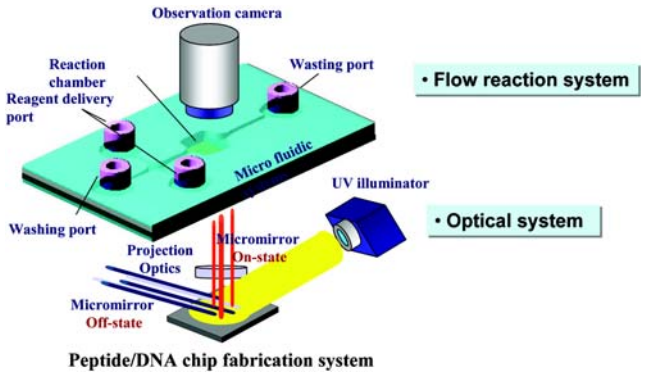

Fig. 6. Schematic view of the maskless array synthesis system.

peptides (Southern, 1996). Accordingly, many research groups have reported about the DNA chip fabrication by using photolithographic method (Pease *et al*., 1994).

However, this method requires a large quantity of photomasks for the selective illumination during the photolithography process. In fact, the oligonucleotide microarray of 25-mers requires 100 different photomasks, leading to high cost and long fabrication time. Recently, a few groups attempted to use digital micro mirror arrays as a virtual photomask instead of using a large quantity of photomasks. Singh-Gasson *et al*. have replaced these photomasks with commercially available digital micromirror array for the synthesis of DNA array chip (Singh-Gasson *et al*., 1999).

Fig. 6 illustrates a typical schematic view of maskless array synthesis system. The system consists of two parts; optical system and microfluidic system. The optical system consists of an illuminator and a micromirror array in which each mirror can be digitally controlled by a computer program (Lee *et al*., 2003). By controlling the deflection of the illuminated UV light on the micromirror arrays, the photolabile protecting group on the chip surface can be cleaved at the specified site. The microfluidic system part carries the monomer solutions to the chip surface, so that peptide or oligonucleotide libraries can be synthesized on the patterned surface.

On the other hand, Gao group presented a method of using photogenerated acid (PGA) in the maskless photolithography system to synthesize oligonucleotide and peptide microarrays (LeProust *et al*., 2000; Pellois *et al*., 2000; Pellois *et al*. 2002). Instead of preparing monomers containing photolabile protecting group, they utilized commercially available olignonucleotide and peptide monomers protected by 4,4'-dimethoxytrityl (DMT) and *tert*-butyloxycarbonyl (Boc) group, respectively. Triarylsulfonium hexafluoroantimonate, which is used as a PGA precursor, is decomposed to PGA upon exposure to the light (Gao *et al*., 1998). This, in turn, may cause the resultant PGA to remove acid labile protecting groups on the glass surface after UV irradiation on the specific site. In this manner, oligomers can be synthesized by the repetitive coupling and deprotection. It should be noted that in this method, micro-size physical or chemical barrier is needed to prevent the diffusion of PGA to the undesired area (Srivannavit *et al*., 2004).

Applications

Peptide libraries that were synthesized by the previously reviewed methods can be used as substrates for the biologically high-throughput screening in various biosystems.

Peptide ligand screening Since photolithographic *in situ* synthesis was first developed in 1991, light-directed combinatorial peptide arrays was applied to epitope mapping (Holmes *et al*., 1995). The binding affinity with antibody was measured by fluorescence intensity on a glass chip containing 1024 kinds of peptides.

Lam *et al*. first used OBOC approach and have found peptide ligands that bind with high affinity to anti-β-endorphin or streptavidin from peptide libraries synthesized by split and mix synthesis method (Lam *et al*., 1991). They performed an enzyme-linked colorimetric assay using a protein-phosphatase

conjugate, and detected the binding events of protein on the polymer beads. In addition, the same group reported on the cell-growth-on-bead assay to identify peptide ligands for the attachment and proliferation of lung cancer cells. They demonstrated that this method can be used as an efficient tool for isolating cancer cells for diagnosis and in cell biological studies (Lam *et al.*, 2002).

Zang *et al.* prepared cyclic peptide libraries using split and mix synthesis method to screen affinity ligands for streptavidin, and found that conformationally constrained cyclic peptide ligands bound 1000-fold more tightly than their linear peptides to streptavidin (Zang *et al*., 1998). In addition, one-bead one-peptide libraries have been used for the screening of affinity ligands for the proteins such as lime antibody (Yu *et al*., 1997), glycosylated haemoglobin (Chen *et al*., 1998), RNase S-Protein (Barnes *et al*., 2001), and immunoglubulin G (Verdoliva *et al*., 2005). On the other hand, Powell *et al.* described a protein-ligand binding assay method that is suitable for high-throughput screening applications (100,000 ligands per day) using MALDI-TOF instrument (Powell *et al*., 2004). As carbohydrates are of importance in a broad range of biological phenomena, glycopeptide libraries are emerging as α-galactosyl epitope mimetics and lectin ligands (Xian *et al*., 2004; Ying *et al*., 2005).

Enzyme substrate and inhibitor screening Phosphorylation of proteins by protein kinases is a crucial step in the regulation of cellular processes such as cell growth, differentiation and division. Lam *et al*. and Lou *et al*. reported solid phase assay systems for the detection of tyrosine kinase activity using radiolabelled beads with [^{32}P] ATP (Lam *et al*., 1995; Lou *et al*., 1996). Martin *et al*. reported an enzyme-linked solid-phase assay system that enabled the visual detection of peptide ligand and substrate from peptide libraries using alkaline phosphatase-conjugated secondary antibody (Martin *et al*., 2000). Peptide microarrays for on-chip screening of kinase substrate were fabricated by pre-synthesized peptide immobilization method and SPOT synthesis method. Pre-synthesized peptide libraries were immobilized covalently on the glass slides and reacted with protein kinases. The phosphorylation profile of substrate was analyzed by labeling with [^{32}P] ATP (MacBeath *et al*., 2000) or incubating in the solution of fluorescently-labeled antiphosphoamino acid antibodies (Uttamchandani *et al*., 2003). Buss *et al*. performed kinase substrate screening on the peptide arrays prepared by using SPOT synthesis method, and analyzed the specific peptide sequence containing phosphorylated serine by radiolabeling with [^{32}P] ATP method (Buss *et al*., 2004).

Meldal group synthesized peptide libraries on polymer beads to identify the inhibitors of cysteine protease and metalloproteinase (Graven *et al*., 2001; St. Hilaire *et al*., 2002; Christensen *et al*., 2003). The inhibitors were screened by fluorescence resonance energy transfer (FRET) assay on the basis of one-bead-two-compound approach using orthogonal protecting groups. Solid phase FRET assay was also performed in the peptide libraries to identify specific substrates of various proteases (Leon *et al*., 1998; Rosse *et al*., 2000).

The substrate specificity of protein phosphatases has been less explored compared with that of kinases. Recently, phosphotyrosyl peptide libraries were used for the identification of binding specificity and the substrate screening of protein tyrosine phosphatase (Pellegrini *et al*., 1998; Beebe *et al*., 2000; Wang *et al*., 2002). The phosphotyrosyl peptide libraries were released from the polymer beads and subsequently treated with phosphatase to remove phosphoryl group of the specific peptide sequence. Thereafter, the dephosphorylated peptides in the library were detected by the change of mass profile using mass spectrometry.

Peptide catalyst screening The high enantioselectivity and the massive diversity of libraries are the merits of peptide catalysts. There have been some reports on the discovery of catalytic peptides from peptide libraries; for example, an enantioselective acyl transfer catalyst (Copeland *et al*., 2001) and a Diels-Alder catalyst (Lingard *et al*., 2003).

Conclusion and Prospects

The recent growing demand for the high-throughput screening of peptide libraries has accelerated the development of the solid phase peptide synthesis. In particular, the strategy of one-bead one-compound (OBOC) library synthesis has provided a convenient tool for the preparation of large number of peptide libraries. Combined with ladder synthesis methods, the peptide library synthesis using polymeric beads has extended its application toward on-bead assay and on-bead analysis. On the other hand, the chemoselective immobilization technology has provided a useful method for the preparation of peptide arrays on flat surface by using appropriately modified pre-synthesized peptide. Furthermore, with the recent developments in the microfabrication process, the *in situ* parallel synthesis methods have became a rapid and economical tool for the preparation of the miniaturized spatially addressed peptide array. The peptide libraries from the polymeric beads and the peptide microarray have been successfully used for the screening of peptide ligand, enzyme substrate and inhibitor, and peptide catalyst. Due to these great achievements in the field of the solid phase peptide synthesis over the last decade, the scope of application of the synthesized peptide libraries and microarrays is expected to expand continuously.

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