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Synthesis and Screening of a "One-Bead–One-Compound" Combinatorial Peptide Library

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By using a "split-synthesis" method in conjunction with standard solid-phase peptide synthesis, a library of peptide beads can be synthesized so there is only one unique peptide species on each bead—the "one-bead–one-compound" concept. With an appropriate screening method targeted for a specific biological, physical, or biochemical property, a unique peptide bead can be identified, physically isolated, and the peptide structure determined. The screening method may involve (1) on-bead assay such as direct binding or covalent modification or (2) releasable assay in which the peptides are released from the bead for biological or biochemical testing. Examples of these methods for various targets will be described. This one-bead–one-peptide library method enables one to synthesize and screen 10^7 unique peptides routinely and rapidly and has proved to be an invaluable tool for basic research and drug discovery.

INTRODUCTION

The combinatorial library field has grown enormously over the last 5 years. The initial focus was primarily on peptide and nucleic acid libraries (Gallop et al., 1994; Kenan et al., 1994; Lam, 1995). However, in the last 3 years, new methods in generating and screening nonpeptide or small organic libraries for drug discovery have rapidly become a main focus of many pharmaceutical companies (Gordon et al., 1994; Lebl et al., 1995). In this article, we shall restrict our discussion to peptide libraries. However, many of the concepts and methods described here can be applied equally well to small organic libraries.

There are four general methods of generating and screening huge (> 10^7 peptides) combinatorial peptide libraries: (1) biological libraries such as filamentous phage (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990), plasmid (Schatz 1993), or polysome (Kawasaki, 1991) libraries; (2) synthetic peptide library methods requiring deconvolution such as an iterative approach (Geysen et al., 1986; Houghten et al., 1991), positional scanning (Dooley and Houghten 1993); orthogonal partition approach (Déprez et al., 1995) and recursive deconvolution approach (Erb et al., 1994); (3) the one-bead–one-compound synthetic combinatorial library method or Selectide process (Lam et al., 1991; Lebl et al., 1995; Lam and Salmon, 1996; Lam and Lebl, 1996); and (4) synthetic peptide library using affinity column selection (Zuckermann et al., 1992). Enormous literature has been published on the first three general approaches for various biological targets. However,

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TABLE 1. Comparison Among Four Peptide Library Methods

	Biologic library[a]	Combinatorial library[b]	Selectide technology[c]	Affinity column selection method
Unnatural amino acids or small organic subunits	No	Yes	Yes	Yes
Constrained structure	Protein scaffold or simple disulfide	Simple to complex synthetic scaffold	Simple to complex synthetic scaffold	Simple to complex synthetic scaffold
Size of peptide	Small to very large	Small	Small	Small
Solution-phase assay	No	Yes	Yes	No
Binding assay	Yes	Yes	Yes	Yes
Screening approach	Parallel	Convergent	Parallel	Parallel
Ligand identification	Discrete	Deconvolution method	Discrete	Summation of many solutions
Biologic bias	Yes	No	No	No

[a]For example, filamentous phage.
[b]Iterative approach/positional scanning.
[c]One bead, one compound.

the affinity selection approach has only been applied successfully to limited targets (Songyang et al., 1993, 1994, 1995).

Comparison among many features of these four general peptide library methods is shown in Table 1. The main advantage of the biologic library method is that larger peptides can be made and specific protein folds can be incorporated into the generation of the peptide libraries. However, the main disadvantage of biologic libraries is that only natural amino acids can be used. In contrast, unnatural amino acids, small organic molecules, or complex synthetic scaffolding can be incorporated into the synthetic libraries.

The iterative approach and positional scanning approach require multiple synthesis and screening, and the structure of the active ligands are deduced by deconvolution methods, which is a convergent approach. However, the one-bead–one-compound combinatorial approach is a parallel approach in which each member of the library is synthesized concurrently and spatially separated, and the screening is performed concurrently. Discrete active compounds are isolated for structure determination. In the affinity column selection approach, the library is synthesized and screened in parallel. However, the isolated peptides are often sequenced concurrently, and the result is a summation of many possible solutions.

In this article, we shall focus on the detail methodology of the one-bead–one-compound combinatorial library method or the Selectide process. The one-bead–one-compound concept (Lam et al., 1991; Lam and Salmon, 1996) is based on our recognition that when a split-synthesis method (Furka et al., 1991; Lam et al., 1991; Houghton et al., 1991) is used in the synthesis of a random peptide library, each individual resin bead displays only one peptide entity (~10^{13} copies of the same peptide on a single bead). For the on-bead screening assays, up to 10^7 peptide beads can readily be synthesized and screened concurrently by one person within a week. Examples of various on-bead binding and solution-phase screening assays will be described.

METHODOLOGY

Preparation of Peptide Libraries

TentaGel Resin S Amino-NH_2 (Rapp Polymere, Tubingen, Germany) is used in the library synthesis (Lam and Lebl, 1994). Fmoc amino acids with standard side-chain protecting groups, benzotriazolyl-oxy-trisdimethylamino-phosphonium hexafluorophosphate (BOP), N-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), piperidine, and diisopropylcarbodiimide (DIC) were obtained from Advanced Chem Tech (Louisville, KY).

Standard solid-phase peptide synthesis chemistry (Fmoc chemistry) is used in the preparation of our libraries (Stewart and Young, 1984; Atherton and Sheppard, 1989). During the split synthesis,

10 g of TentaGel Resin S-amino-NH_2 beads are first swollen in dimethylformamide (DMF) for at least 2 hr and divided into 19 aliquots contained in 19 polypropylene vials. Threefold excess of 19 Fmoc-protected amino acids (all but cysteine; 0.3 M solution in 0.3 M HOBt) are added separately into each of the settled resin aliquots. Coupling is initiated by adding threefold excess of DIC or BOP and DIEA. In the case of DIC coupling, a trace amount of bromophenol blue may be added, and the vials are capped tightly and rocked gently for 30 min at room temperature or until all beads turn from blue to colorless (Krchnak et al., 1988). The bromophenol blue monitoring results are sometimes confirmed by a standard ninhydrin test (Kaiser et al., 1970). If coupling is incomplete, the supernatant is gently removed, and fresh Fmoc amino acids, BOP, DIEA, and HOBt are added for additional coupling. From our experience, double coupling is rarely needed. The resins are then mixed together in a siliconized cylindrical glass vessel fitted with a frit at the bottom where N_2 can be bubbled through for mixing of the resin or supernatant removed by N_2 under pressure from the top. After washing (8×) with DMF, 50 ml of 50% piperidine (in DMF) is added to cleave the Fmoc-protecting group. After 20 min in piperidine, the resins are washed (10×) with DMF. The resins are then divided into aliquots for another cycle of coupling. After all the randomization steps are completed and the Fmoc-protecting group is removed by piperidine, the resins are washed (8×) with DMF and mixed with 10 ml of modified reagent K [trifluoroacetic acid (TFA)-*p*-cresole-water-thioanisole-ethanedithiol, 82·5:5:5:5:2·5, v/w/v/v/v] (King et al., 1990) or a mixture of TFA-phenol-anisole-ethanedithiol (94:2:2:2, v/w/v/v) at room temperature for 3 hr. The resin is then thoroughly washed with DMF, neutralized with 10% DIEA (in DMF), thoroughly washed again, and stored in DMF at 4°C. For the synthesis of cyclic library, Fmoc-Cys(Trt) (Trt, trityl) is used to flank the random sequence. After deprotection, the linear library is cyclized with dimethylsulfoxide:anisole:trifluoroacetic acid (10:5:85) overnight and washed with DMF as described in Salmon et al. (1993).

Preparation of Doubly Releasable Peptide Libraries

Synthesis of doubly releasable library is performed in a manner similar to that described above. The only difference is the attachment of diaminocarboxylic acid in the first step and introduction of the Ida linker after selective deprotection of one of its amino groups (Kocis et al., 1993; Lebl et al., 1993; Salmon et al., 1993). The chemical structure of the Ida linker is shown in Figure 1.

Polymer carrier (TentaGel, Rapp Polymere, Tubingen, Germany; 5 g, 0.23 mmol/g, 130 μm average particle size) is swollen in DMF (swollen volume 25 ml) and Fmoc Lys(Boc) (3 equivalents) is coupled by DIC (three equivalents) in the presence of HOBt (three equivalents) in DMF. After 2 hr, the resin is washed (5× with DMF and 1× with DCM) and the Boc group is cleaved with 50% TFA in DCM (1 plus 20 min). After washing with DCM (5×) and DMF (4×), the resin is neutralized by washing with 2% DIEA in DMF (3 × 2 min), washed three times with DMF, and the Ida linker (three

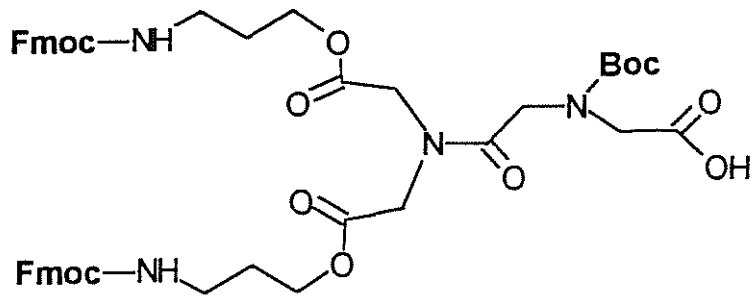

Figure 1. Chemical structure of the Ida linker (Kocis et al., 1993).

equivalents) is added, which is followed by the addition of DIC and HOBt (three equivalents each) in DMF (overnight). The resin is then washed five times with DMF, and the Fmoc group is cleaved by 50% piperidine in DMF (20 min). After additional washing, three times with DMF, and division of the resin into m reaction vessels, m individual Fmoc-protected amino acids are coupled to each part of the resin by using DIC and HOBt (three equivalents each) as described above. The coupling reaction is monitored by bromophenol blue method (Krchnak et al., 1988) and ninhydrin test (Kaiser et al., 1970) as described above. The resin is then combined, washed five times with DMF, and the Fmoc group is deprotected as above. This procedure (separated coupling and recombined deprotection) is repeated n times for a library of n-peptides. The library is then deprotected by application of mixture K (see above; King et al., 1990) for 2 hr, washed with TFA (3×), DCM (5×), DMF containing 0.1% HCl (4×), and 0.1% HCl in water (5×). The library has to be stored in an acidic solution to prevent premature loss of the synthesized peptides.

To verify the quality of the synthesized library, analytical release is performed (Lebl et al., 1994). The sample of the dried resin (5–10 mg) is shaken overnight with 2–5 ml of 0.1 M HEPES buffer (pH 8.5) in a polypropylene syringe equipped with a polypropylene or Teflon frit at the bottom and a polypropylene plunger. Absorbance of the solution (diluted if necessary) at 280 nm is measured, and the amount of the peptide released is calculated according to the formula

$$\text{release (mmol/g)} = (\text{absorbance} * \text{volume} * \text{dilution})/[(n * 1{,}197/x + m * 5{,}559/y) * \text{mass}],$$

where mass is amount of resin in g, x is number of amino acids used for randomization in positions where tyrosine was used, y is number of amino acids used for randomization in positions where tryptophan was used, n is the number of positions in the library in which tyrosine was used for randomization, and m is the number of positions in the library in which tryptophan was used for randomization. Tabulated values of absorbance coefficients for tyrosine (1,197) and tryptophan (5,559) are used in the calculations.

A solution of 0.2% NaOH (w/v) is introduced into the syringe with the test resin and the mixture is shaken for another 4 hr. The

solution is then expelled from the syringe, and absorbance is measured at 280 nm. The same calculation is performed by using the formula shown above with coefficients 1,507 and 5,377 instead of 1,197 and 5,559. Results of releases in both steps should not differ by more then 10% from the theoretical values, which can be calculated with the formula

$$\text{theoretical release (mmol/g)} = \text{subst.}/[1 + \text{subst.} * (3 * \text{MW} + 686)/1{,}000]$$

where subst. is the starting substitution of the resin (in mmol/g), MW is the average molecular weight of one library structure, and 686 is the molecular weight of Ida linker (without Fmoc groups) plus one Lys residue minus one molecule of water. In the case of peptide library, the average molecular weight of one natural amino acid is 119.7 (19 amino acids, Cys excluded). Average molecular weight of one structure in a pentapeptide library is therefore 598.5. For the resin substitution level of 0.2 mmol/g, we should expect 0.134 mmol of pentapeptide released from one arm of the linker per gram of library beads.

Peptide Library Screening by Enzyme-Linked Colorimetric Assay

The peptide library is gently mixed with an incremental increase of double-distilled water to remove all the DMF. After thoroughly washed with double-distilled water, the library is mixed with 0.1% gelatin (w/v) or bovine serum albumin (w/v) to block any nonspecific binding sites. The beads are then mixed with the acceptor–alkaline phosphatase complex in phosphate buffered saline (PBS) with 0.1% gelatin and 0.1% Tween 20 (v/v) with gentle mixing for 1–24 hr. The acceptor could be a receptor, an antibody, an enzyme, or another macromolecule. The concentration of acceptor–alkaline phosphatase complex should be as diluted as much as possible to minimize any nonspecific binding. The beads are then thoroughly washed with PBS with 0.1% Tween 20 followed by Tris buffered saline (TBS; 8 g NaCl, 0.2 g KCl, and 3 g Tris base in 1 L H_20, pH 8.0). BCIP substrate (33 ml of 5-bromo-4-chloro-3-indolyl-phosphate; 50 mg/ml DMF) in 10 ml of alkaline phosphatase buffer (5.85 g NaCl, 12.1 g Tris base, 0.476 g $MgCl_2$ in 1 L H_2O, pH 8.5) is then added. The beads are transferred to 10–20 polystyrene Petri dishes (100 × 20 mm). After 1–2 hr, some of the beads will turn turquoise, whereas the majority of the beads will remain colorless.

To confirm specificity, the color beads are carefully removed with the aid of manual micropipette fitted with a minipipette tip under a dissecting microscope, treated with 8 M guanidine hydrochloride, pH 1.0, for 20 min, and destained with DMF. The decolorized beads are then restained with this procedure but in the presence of a competing ligand. The color beads (false positive) are removed, and the remaining colorless beads are restained with the acceptor–alkaline phosphatase complex but in the absence of any competing ligands. At this stage, the stained beads (true positive) are then isolated, treated with 8 M guanidine hydrochloride, pH 1.0, for 20 min,

placed individually on each glass filter, and inserted into the cartridge of a microsequencer (e.g., ABI Model 477A, Applied Biosystem) for sequencing as described in Lam and Lebl (1994).

Instead of tagging the acceptor molecule directly with alkaline phosphatase, one may elect to use a secondary reagent to probe the acceptor. For example, the acceptor molecule is biotinylated and the bound acceptor detected by streptavidin-alkaline phosphatase. Alternatively, the acceptor molecule is unlabeled and an anti-acceptor antibody–alkaline phosphatase complex is used to probe the bead-bound acceptor (Lam et al., 1995a).

Peptide Library Screening for Protein Kinase Substrate Motif

A random penta- or heptapeptide library is thoroughly washed with double-distilled water followed by five rinses with MES buffer [30 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM magnesium chloride, 0.4 mg/ml bovine serum albumin, pH 6.8]. The phosphorylation reaction is conducted in MES buffer containing protein kinase (e.g., 1.8 μg/ml cAMP-dependent protein kinase, catalytic subunit from bovine heart; Sigma Chemical, St. Louis, MO), 0.1 μM [γ-^{32}P]ATP (specific activity 25 Ci/mmol, from ICN Biomedicals, Irvine, CA), and 1 ml settled beads in a final reaction volume of 2 ml. After incubation for 1–5 hr at room temperature, the beads are thoroughly washed with PBS-Tween (0.68 M NaCl, 13 mM KCl, 40 mM Na_2HPO_4, 7 mM KH_2PO_4, pH 7.0, with 0.05% Tween 20). The washed beads are then resuspended in 0.1 M HCl and heated to 100°C for 15 min. The peptide beads are then thoroughly washed with PBS-Tween, resuspended in a 1.5% agarose (SeaPlaque agarose; FMC BioProducts, Rockland, ME) solution of 70–75°C, carefully poured onto a glass plate (16 × 18 cm), and air dried overnight at room temperature. Glogos II autoradiogram markers (Stratagene, La Jolla, CA) are taped on each corner of the glass plate prior to exposure to align the immobilized beads with the film later on. After exposure for 20–30 hr at room temperature, the film is developed. The beads corresponding to the dark spots in the developed film are excised with a razor blade, added to 30 ml of molten 1.5% agarose solution (70–75°C) for 15 min, replated, dried, and exposed to X-ray films as described above. During this secondary screening, the beads are greatly diluted, and a single labeled peptide bead can be localized, dislodged by a 27-gauge needle, isolated, and microsequenced as described in Lam and Lebl (1994).

Peptide Library Screening for Peptides Specific for the Small Organic Dye Molecule

A random peptide library is thoroughly washed with double-distilled water and followed by five rinses with PBS-Tween. An organic dye (e.g., 10 μM indigo carmine; Aldrich Chemical, Milwaukee, WI) is dissolved in PBS-Tween and mixed with the bead library by gentle rocking overnight. The bead library is then poured into several Petri dishes (100 × 20 mm) and examined under a dissecting microscope. The beads stained with the color dyes are then isolated for microsequencing. To select for stronger binder, one may elect to

wash the stained library five times with PBS-Tween and resuspend the library in PBS-Tween with gentle rocking for 24–64 hr. The stained beads (with very slow dissociation rate) can then be isolated for microsequencing. Besides an aqueous condition, the whole experiment can be performed in an organic solvent such as DMF. However, be sure that glass rather than polystyrene Petri dishes are used if organic solvents are used.

Solution-Phase Releasable Screening Assay on a 96-Well Plate Format

A two-stage release assay is used for the solution-phase assay (Salmon et al., 1993). For the first-stage release, the releasable peptide library is first washed thoroughly with 0.1 M HCl, resuspended in 0.1 M HCl with 10% carboxymethylcellulose (w/v), mixed, and rapidly pipetted (approximately 500 beads/well) into the upper chambers of a 96-well filtration manifold (Model 09601, Millipore). After removal of the 0.1 M HCl by vacuum filtration, 100 µl of appropriate buffer or tissue culture medium (neutral pH) are added to each well with beads. The plates are then incubated overnight to cleave the peptide by diketopiperazine formation, and the peptide filtrates are vacuum filtered into a 96-well plate for subsequent biological assays.

After the first-stage assay, the beads contained in the "positive" wells are pipetted into a new 96-well filtration plate with a dissecting microscope at one bead per well. The second-stage release involves cleavage of the ester linker by alkali condition. This step can be accomplished either by adding 0.2% NaOH for an overnight incubation (followed by pH adjustment) or by overnight incubation with ammonia vapors. The filtrates of the second release are then used for biological testing. The individual peptide bead for each positive well in a second-stage assay is then recovered for microsequencing.

RESULTS AND DISCUSSION

Enzyme-Linked Colorimetric Assay

Applications of these screening methods have been published elsewhere. For example, by using an enzyme-linked colorimetric screening method, we identified ligands for various macromolecular targets: monoclonal antibodies with a continuous epitope (Lam et al., 1991, 1993a, 1993b) or discontinuous epitope (Lam et al., 1994a, 1996), cell-surface idiotypes of two B-cell lymphoma lines (Lam et al., 1995c), MHC-class I molecules (Smith et al., 1994), streptavidin (Lam et al., 1991; Lam and Lebl, 1992), and avidin (Lam and Lebl, 1992). Recently, we have modified our screening method by applying a dual-color detection scheme to eliminate many of the nonspecific binding beads (Lam et al., 1995a).

Table 2 shows the result of screening D-amino acid containing peptide and cyclic peptide libraries with streptavidin–alkaline phosphatase complex (Lam et al., 1994b). All the ligands shown in Table 2 are competitive with biotin, suggesting that they all bind to the biotin-binding pocket of streptavidin. Several investigators have established that linear HPQ and HPM are ligands specific for streptavidin (Devlin et al., 1990; Lam et al., 1991). Table 2 (under Linear) shows that when D-amino acids were incorporated into the random linear peptide library, some very different motifs (shown by parentheses in Table 2) emerged. For the all D-amino acid pentapeptide library (xxxxx), there was only one distinct motif: w(y/f)_(y/e/f)a. The peptide wyqea was identified on three different positive beads. When a combined L- and D-amino acid pentapeptide library (XxXxX) was screened, three motifs were discovered: _ _WpH, _w(F/Y)pH, and Y_ _fP. The first two motifs are somewhat related, but the last motif is distinctly different. This result illustrates that (1) D-amino acid can readily be used in the one-bead–one-compound library, (2) depending on the composition and design of the libraries, very different ligands

TABLE 2. Peptide Ligands for Streptavidin[a]

Linear		Cyclic (disulfide)	
XXXXX		CXXXXC	
HPQ		HHPM	
HPM		NHPM	
		QHPM	
		LHPM	
		RHPQ	
xxxxx		CXXXXXC	
wyqea (3)		HPQNN	LHPQN
wyhea	wfrya	HPQNV	WHPQN
wyefa	wymel	HPQQV	DHPQN
wyfya		HPMNP	(_HPQN)
wydya		HPMNA	
(wy_ya)		(HPQN_)	
f e		MQ	SHPQF
f			
XxXxX		CXXXXXXC	
YgWpH	DwFpH	HPQFAR	HPQNGG
LqWpH	RwYpH	HPQFAS	HPQNAQ
SyWpH	(_wFpH)	HPQFPQ	HPQVGI
WkWpH		HPQFPA	HPQSGM
AfWpH	Y	HPQAPK	
QrWpH		HPQAPY	
(_ _WpH)		HPQGPA	HHPQFP
	YvIfP	HPQGPG	
	YpFfP	(HPQFP_)	
	(Y_ _fP)	AA	
		G	

[a]X denotes all 18 L-amino acids plus glycine, except cysteine; x denotes all 18 D-amino acids plus glycine, except cysteine.

can be identified for the same target, and (3) with the parallel approach of the one-bead–one-compound library method, it is not uncommon to find different unrelated motifs when a specific library is screened with a specific target (e.g., the three motifs identified for streptavidin from the XxXxX library).

Table 2 (under Cyclic) shows the results of the screening of three cyclic peptide libraries with the streptavidin–alkaline phosphatase complex. A random tetra-, penta-, or hexapeptide sequence was flanked by two cysteines. After the library synthesis was completed, the library was cyclized by oxidation. Similar to those identified from a linear random L-amino acid peptide library, every ligand identified in this screen had either HPQ or HPM. However, closer examination showed that the HP(Q/M) tripeptide sequence resided in a specific position, depending on the ring size. For instance, in the case of the tetrameric cyclic library, HP(Q/M) resides adjacent to the cysteine at the carboxyl end. In contrast, all but one ligand from the hexameric cyclic library (12/13) reside adjacent to the cysteine at the amino terminus. In the case of the pentameric cyclic library, the HP(Q/M) tripeptide sequence resided at the amino terminus (five of the nine ligands) or in the middle of the sequence (four of the nine ligands) but not at the carboxyl terminus. This result indicates that the presentation of the HP(Q/M) tripeptide motif is dependent on the ring size and on the adjacent residues within the ring.

Posttranslation Modification Site Determination

The one-bead–one-compound library is an invaluable tool for the elucidation of posttranslational modification site(s) (Lam and Wu, 1994). For example, when the random peptide bead libraries were incubated with [γ-^{32}P]ATP and cAMP-dependent protein kinase, several [^{32}P]-labeled beads were isolated and sequenced. All beads had the _RR_S_ motif (Wu et al., 1994). Since then, we have applied the same method to other protein kinases such as p60$^{c\text{-}src}$ protein tyrosine kinase (PTK) and discovered that YIYGSFK and GIYWHHY are efficient and specific peptide substrates for this enzyme (Lam et al., 1995b; Lou et al., 1995, 1996). Similar work has also been done on p185$^{her/abl}$ PTK, c-abl PTK, and a plant-derived serine/threonine protein kinase. In principle, the same approach can also be applied to other posttranslational modifications other than phosphorylation provided that a labeled donor group is available. Alternatively, other nonradioactive detection schemes can also be used. For example, antiphosphotyrosine antibody rather than [γ-^{32}P]ATP can be used to detect tyrosine phosphorylation.

Ligand Interaction With a Small Molecule

In addition to macromolecular targets, we have also successfully applied the one-bead–one-compound library method to small molecular targets. This application is illustrated by the isolation of peptides that interact specifically with a small organic molecule, indigo carmine (MW 466.56; Lam et al., 1994c). Because indigo carmine is a symmetrical planar molecule, an identical motif has been identified from all D-amino-acid- or L-amino-acid-containing libraries: _ZOOOZ_, where Z is the basic residue such as Lys or Arg and O is the relatively hydrophobic amino acid. We have applied the same technique to identify peptides that interact very strongly with another small molecule, acridine orange (Lam et al., unpublished data).

Other On-Bead Binding Screening Assays

We reported that intact lymphoma cells (~10 μm in diameter) are able to rosette specifically around a peptide bead (~100 μm in diameter) provided that the peptide is specific for the cell surface receptor, in this case, the surface immunoglobulins of two murine B-cell lymphoma cell lines (Lam et al., 1995c). We have applied this whole-cell binding assay to screen for peptides that bind to other cancer cell lines. In principle, similar approaches could also be applied to discover ligands specific for intact viruses, bacteria, yeast, or other unicellular organisms.

Although we have used primarily enzyme-linked colorimetric assays in our screening efforts, other tagging techniques can also be used. We and others (Needels et al., 1993) have successfully applied fluorescent-labeled targets to screen library beads with a fluorescent activated cell sorter. In addition, radiolabeled (Kassarjian et al., 1993) and dye-labeled (Yoon and Still 1995) targets have also been used successfully to screen the one-bead–one-compound combinatorial libraries.

Solution-Phase Releasable Assay

On-bead screening methods are efficient, and more than 10^7 beads can be screened readily. However, there are biological endpoints for which this method is not applicable and a solution-phase assay is needed. In 1991, we proposed the use of a one-bead–one-compound releasable assay in which the ligands are covalently attached to each bead via cleavable linkers (single or dual-cleavable linker). The ligands can be released from individual bead for solution-phase assay (Lam et al., 1991; Lam and Salmon, 1992). The positive bead of origin can then be isolated for structure determination. The two general approaches that we had described (Lam and Salmon, 1996) were (1) in situ releasable assay, wherein the beads are immobilized in agar, and the biological endpoint is expressed as a signal surrounding an individual bead (e.g., a clear zone of inhibition for antibacterial or anticancer activity), and (2) a 96-well format, wherein a two-stage releasable assay is used (Salmon et al., 1993; Lebl et al., 1994), as described in detail in this article.

In some instances, combining the on-bead binding assay with the releasable solution-phase assay may be advantageous, so that beads positive for both orthogonal assays are more likely to be truly positive.

Structure Determination of Positive Beads

For peptide libraries with free N terminus, the positive beads are routinely sequenced by an automatic protein sequencer by using the standard Edman degradation procedure. For an N-terminally blocked library, one may still use the same sequencing method provided that a fraction (e.g., 20%) of the ligands in each bead has a free N terminus, which can easily be accomplished during the library synthesis step. For example, a mixture of N-α-Fmoc-amino acid (80%) and N-α-Boc-amino acid (20%) is used in the last cycle of library synthesis. After the Fmoc group is removed by piperidine, the N terminus may be blocked (e.g., by acetylation). During the side-chain deprotection step with TFA, the N-α-Boc group will be removed and the N terminus freed for Edman degradation. The same strategy can also be applied to other nonsequencible components such as a reduced peptide bond.

Application to Nonbiological System

Thus far, many of the applications of combinatorial chemistry have been to solve biological problems. In principle, the same approach can also be applied to physical sciences such as material science. With the one-bead–one-compound combinatorial approach, one might make thousands to millions of new compounds, either in the bead form or disc form, within a relatively short time. With an appropriate detection method, one can select for material with a desired physical, optical, electromagnetic, or chemical property. The combinatorial approach has been applied successfully for the discovery of new magnetoresistive materials (Briceno et al., 1995).

Perspectives

Combinatorial chemistry has become one of the most rapidly developing fields in the last few years. Pharmaceutical industries consider it one of the most important recent advances in the field of drug development. We believe that the impact of combinatorial chemistry on basic research is even more far reaching because it serves as extremely powerful tool in basic research in multiple disciplines of biological and physical sciences. The one-bead–one-compound library method is one of several combinatorial approaches available at this time.

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