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Applications of topologically segregated bilayer beads in 'one-bead one-compound' combinatorial libraries

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Abstract: We recently reported the use of a biphasic approach to generate topologically segregated bilayer beads. In generating 'one-bead one-compound' (OBOC) combinatorial libraries, novel encoding methods have been applied to these beads such as the testing library compound and the coding tags residing on the outer layer and inner core of each bead, respectively. In this report, we further exploit these bilayer beads by preparing target bead-libraries with low concentration of random peptides on the outer layer, and full substitution of coding peptides in the bead interior. The low concentration of peptide on the bead surface enables us to greatly increase the stringency of screening so that higher affinity ligands can easily be identified. Full substitution of the inner core of the beads enables us to have enough coding peptides inside the bead for direct microsequencing with Edman chemistry. The biphasic approach of preparing bilayer beads can be carried out at any point during the library construction. Therefore, the nonsequencable or fixed structures of the peptides can be bypassed in the coding tags. As a result, peptide libraries that otherwise cannot be sequenced can now be sequenced, and peptide segments with fixed residues within the libraries can be bypassed so that the microsequencing time can be significantly shortened. Furthermore, peptides with a branch of random sequence in the middle of a fixed peptide chain can be encoded with just the random sequence in the bead interior. We have successfully applied these novel OBOC library concepts in the optimization of cell-surface ligands for a human T-cell leukemia, Jurkat, cell line.

Abbreviations: One letter symbols for natural amino acids (D- or L-configurations) are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Nle, norleucine; DCM, dichloromethane; DMF,

N-N'-dimethylformamide; MeOH, methanol; EtOH, ethanol; Boc, tert-butoxycarbonyl; Fmoc-OSu, *N*-(9-fluorenylmethyloxy-carbonyloxy)-succinimide; Alloc-OSu, *N*-allyloxycarbonyloxy succinimide; HOBt, 1-hydroxybenzotriazole; DIC, 1,3-diisopropyl-carbodiimide; DIPEA, *N,N'*-diisopropylethylamine; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine) palladium (0); $PhSiH_3$, phenylsilane; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Ac, acetyl; Ar, argon; EDT, ethanedithiol; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; HCl, hydrochloric acid; OBOC, one-bead one-compound; PTH-amino acid, phenylthiohydantoin-amino acid; PBS-Tween, phosphate-buffer saline with 0.1% Tween-20 (v/v).

Introduction

In the 'one-bead one-compound' (OBOC) combinatorial library method (1), compound libraries are constructed with a 'split-mix' synthesis method (1–4). In such libraries, each bead displays only a single compound entity. Upon screening, individual positive compound-beads from the library can be physically isolated for structural determination. For peptide libraries, we routinely sequence individual beads with an automatic microsequencer using Edman chemistry. This necessitates that the peptide must have a free N-terminus and contains only α-amino acids. There is a need to develop methods to overcome such limitations. Once ligands against a specific target (e.g. protein, receptor, whole cell) have been identified, secondary libraries based on the motif of the primary library can be constructed and screened against the same target under more stringent conditions to identify higher affinity ligands. Common approaches to increase the screening stringency are to decrease the concentration of the target protein or to add a fixed concentration of a known competing ligand in the screening buffer (5). Another important approach is to lower the concentration of the ligands on the surface of each bead. However, a significant decrease in the substitution of the entire bead will lead to insufficient quantity of peptide for microsequencing. For this method to work, surface substitution of the bead must be low, but the peptide content in the bead interior needs to remain high. Recently, we reported on the development of novel encoding methods for OBOC small molecule or peptidomimetic libraries, where the library compound resides on the outer layer of the bead and the coding tag remains in the bead interior (6–8). Such topologically segregated bi-functional beads can easily be prepared with a biphasic method. In this method, TentaGel beads are first thoroughly swollen in water. After excess water is drained, a limiting amount of amino-protecting reagent (e.g. 0.25 Eq.), such as *N*-(9-fluorenylmethyloxycarbonyloxy)-succinimide (Fmoc-OSu) dissolved in an organic solvent mixture of dichloromethane (DCM)/ether (55 : 45, v/v), is added to the water-swollen beads. Under these conditions, only the outer layer of the TentaGel bead is derivatized. These bi-functional beads can be applied to OBOC peptide libraries with structures that cannot be sequenced with conventional methods. Furthermore, they can be used for the development of library beads with low substitution on the bead surface but normal substitution in the bead interior. In this report, the application of these novel combinatorial chemistry concepts in the optimization of cell surface ligands against the Jurkat cell line will be described.

Materials and Methods

All 19 N^{α}-Fmoc-D-amino acids (excluding cysteine), N^{α}-Fmoc-L-norleucine (Nle), L-Asp and L-Ile, N^{α}-acetyl (Ac)-Gly, Fmoc-OSu, *N*-allyloxycarbonyloxy succinimide (Alloc-Osu) were purchased from Chem-Impex International, Inc. (Wood Dale, IL, USA) or Advanced ChemTech (Louisville, KY, USA). All solvents, 1-hydroxybenzotriazole (HOBt), 1,3-diisopropylcarbodiimide (DIC) and *N,N'*-diisopropylethylamine (DIPEA) were acquired from commercial source and directly used without further purification unless otherwise noted. The tetrakis (triphenylphosphine) palladium (0) [$Pd(PPh_3)_4$] and phenylsilane ($PhSiH_3$) were purchased from Aldrich Chem. Co. TentaGel S NH_2 resins (capacity: 0.26 mmol/g, size: 90 μm) were purchased from Rapp Polymere GmbH, Tubingen, Germany. Jurkat cells (T lymphoblastic) were obtained from American Type Culture Collection (Rockville, MD, USA). Normal cells were obtained from healthy volunteers. The cells were maintained in RPMI 1640, 10% fetal bovine serum, 50 units/mL penicillin, and 50 mg/mL streptomycin (Life Technology, Inc., Rockville, MD, USA).

Peptide microsequencing was performed on an ABI protein sequencer following the manufacturer instructions. The equipment parameters are as follows: column, Spheri-5 C-18 phenylthiohydantoin (PTH) column; 5 μm, 2.1 × 220 mm; column temperature, 48 °C; flask temperature, 64 °C. Coupling and deprotection were monitored by Kaiser test.

Synthesis of different substitution beads (10 aliquots)

TentaGel S NH_2 beads were swollen in *N-N'*-dimethylformamide (DMF) overnight. The beads were split into 10

aliquots. Each aliquot was coupled with a homogeneous solution containing a predetermined ratio of Fmoc-Gly-OH and Ac-Gly-OH: [(molar concentration of Fmoc-Gly-OH) × (100%)/(molar concentration of Fmoc-Gly-OH + molar concentration of Ac-Gly-OH)] equals 0.5, 1, 2, 5, 10, 20, 40, 60, 80, and 100%.

Synthesis of p-L-D-I, H-P-Q-F, and biotin ligands at different substitutions on beads

Upon treatment with 25% piperidine for 10 min twice, each aliquot of the beads with different substitution of free amino groups was split into three equal portions. One portion of the 10 aliquots was successively coupled with N^{α}-Fmoc-L-Ile, L-Asp, L-Leu, and D-Pro in the presence of HOBt (3 Eq.) and DIC (3 Eq.) using Fmoc chemistry. After incubation with 25% piperidine for 10 min twice, the beads were deprotected with trifluoroacetic acid (TFA)/H_2O/tri-isopropylsilane (TIS) (95 : 2.5 : 2.5) for 2.5 h and thoroughly washed with DCM, methanol (MeOH), DMF, phosphate-buffered saline (PBS), and water. Using the same procedure, N^{α}-Fmoc-L-Phe, L-Gln, L-Pro, and L-His were successively assembled to another set of beads. The remaining set was then biotinylated with D-biotin in the presence of HBTU (3 Eq.) and DIPEA (6 Eq.).

Synthesis of model secondary libraries with low surface substitution and high surface substitution

For the preparation of a low surface substitution library, TentaGel beads (4.0 g) were first swollen in water for 24 h. After the water was drained, the beads were vigorously mixed with Fmoc-OSu (0.2 Eq.) and DIPEA (0.5 Eq.) in DCM/ether (110 : 90 mL) for 30 min. The beads were further treated with Boc_2O (3 Eq.)/DIPEA (6 Eq.) in DMF and then incubated with 25% piperidine for 10 min twice. After washing, the beads were mixed with a solution of Fmoc-Gly/Ac-Gly (1 : 4 Eq.) in the presence of HOBt (3 Eq.) and DIC (3 Eq.). After treatment with 55% TFA/DCM for 30 min and then with 25% piperidine for 10 min twice, the beads were assembled with a random tetrapeptide segment using 19 Fmoc-D-amino acids via 'split-mix' synthesis approach. After Fmoc-deprotection, the beads were swollen in water for 1 day (24 h). After the water was drained, the beads were vigorously mixed with Fmoc-OSu (0.04 Eq.)/DIPEA (0.1 Eq.) for 30 min and then with Alloc-OSu (3 Eq.)/DIPEA (6 Eq.) in DMF for 1 h. Upon Fmoc-deprotection, the beads were successively coupled with Fmoc-protected L-Ile, L-Asp, L-Nle, and D-Pro using peptide chemistry. The beads were then incubated with $Pd(PPh_3)_4$ (0.24 Eq.)/$PhSiH_3$ (20 Eq.)/DCM for 1 h under argon and 25% piperidine for 15 min, and then assembled with a second random tetrapeptide segment using 19 Fmoc-D-amino acids via 'split-mix' approach. After Fmoc-deprotection, the beads were treated with the TFA-based cleavage cocktail [TFA/phenol/ethanedithiol (EDT)/H_2O/thioanisole, 10 : 0.5 : 0.25 : 0.5 : 0.5, v/w/v/v/v] for 2.5 h and thoroughly washed with DCM, MeOH, DMF, PBS, and water. The library beads were stored in 70% ethanol (EtOH). The construction of the full surface substitution library was exactly the same as above except that the step of surface down-substitution with a mixture of Fmoc-Gly/Ac-Gly (1 : 4 Eq.) was eliminated.

Testing of p-Nle-D-I beads against Jurkat cell line

Ten aliquots of p-Nle-D-I beads with different substitutions were extensively washed with double-distilled water and PBS. About 100 μL of beads from each group were incubated with Jurkat cells in RPMI 1640 with 10% FBS. After 75 min of incubation, the relative number of cells bound to each bead was semiquantified under a dissecting microscope.

Testing of H-P-Q-F beads against steptavidin-alkaline phosphatase

Ten aliquots of H-P-Q-F beads were washed with double-distilled water and PBS-Tween. Streptavidin-alkaline phosphatase conjugate solution (1 : 100 000) was then added and incubated for 1 h. After thorough washing with TBS and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) buffer, BCIP solution (0.17 μg/mL) in BCIP buffer was added to the beads for color development. After an hour of incubation, the beads were washed, and the color intensity of the beads was semiquantified under a dissecting microscope.

Screening of the secondary libraries against Jurkat cell line and normal cells

About 0.2 mL of each of the surface down-substituted and full-substituted x_8-x_7-x_6-x_5-p-Nle-D-I-x_4-x_3-x_2-x_1 combinatorial libraries were thoroughly washed with PBS and incubated with Jurkat cells in RPMI 1640 with 10% FBS for 30 min. To remove the free cells, the beads were gently washed with PBS. The bead-library was then carefully

inspected under a dissecting microscope and the beads with tightly bound cells were carefully retrieved with a micropipette and collected into a small Petri dish containing PBS. Five drops of 8.0 M guanidine-hydrochloric acid (HCl) were then added to the dish to remove the bound cells from each bead. After incubation in guanidine-HCl for 1 h, the beads were thoroughly washed with PBS and incubated with normal peripheral mononuclear blood cells, using the same method outlined above. Beads that bound to normal cells were discarded, and the remaining beads were incubated with Jurkat cells again. Positive beads were isolated and submitted for microsequencing.

Results and Discussion

Effect of compound substitution on biologic screening

The OBOC combinatorial libraries with various substitutions can be easily constructed on beads via 'split-mix' synthesis approach such that a single compound entity is displayed on the bead surface (1,4). In principle, lowering the substitution of the beads will lead to higher stringency of screening because the concentration of compound in contact with the screening probe is lower. To demonstrate this effect, we synthesized beads with known ligands and evaluated the effect of ligand substitution on the binding to their respective receptor. The first receptor is $\alpha4\beta1$-integrin on the surface of Jurkat T-leukemia cell and its ligand is p-L-D-I (9). The second receptor is streptavidin and its ligands are biotin and H-P-Q-F (1). These ligands were individually synthesized on TentaGel beads at 10 different levels of substitution (0.5, 1, 2, 5, 10, 20, 40, 60, 80, and 100%). This can easily be accomplished by first derivatizing the entire bead with a mixture of Fmoc-Gly and Ac-Gly at pre-defined ratios prior to assembly of the ligands with Fmoc-chemistry. The p-L-D-I beads were screened against Jurkat cells using a whole cell-binding assay method (9,10), and the H-P-Q-F or biotin beads were tested against streptavidin-alkaline phosphatase conjugate with a standard enzyme-linked colorimetric assay method (11). The results are shown in Table 1. There was a gradual drop of Jurkat cell binding to p-L-D-I beads beginning at about 40% substitution. No cell binding was observed at 2% substitution. For streptavidin binding to H-P-Q-F beads, the drop in color intensity was more abrupt starting at 20% substitution. In contrast, streptavidin binding to beads containing the high affinity ligand biotin did not drop until the bead was down-substituted to 5%. These binding results demonstrated that compound substitution on beads could greatly affect bead-binding assays; however, if the bead ligand has high affinity for its receptor binding could occur even if it is at a very low concentration on the bead surface. For example, at 5% substitution, staining of biotin bead with streptavidin-alkaline phosphatase remained strong but staining of HPQF-bead was totally abolished (Table 1). This down-substitution concept could be readily applied to diverse OBOC combinatorial libraries to increase the stringency of screening, both for whole cell-binding assay and enzyme-linked colorimetric assay.

Generation of motif-based secondary libraries

The binding affinities of ligands identified through screening diverse random libraries are generally low, therefore these ligands need to be optimized. One approach is to design secondary or focused libraries, based on the motif of the ligands identified from the primary screen, and screen them under higher stringency (5). For example, in case of peptide libraries, residues that are critical for binding will be fixed and the adjacent residues will be randomized, with extension at either or both of the amino- and carboxyl-terminus. Alternatively, a nonessential residue at the middle of the peptide can be replaced with a L- or D-Lys where a random linear peptide can be branched from there.

Table 1. **Relative binding affinity of p-L-D-I, H-P-Q-F, and biotin beads at different substitutions against Jurkat cells[a] or streptavidin-alkaline phosphatase[b]**

Ligand substitution on beads (%)	0.5	1	2	5	10	20	40	60	80	100
Cell number on p-L-D-I beads[a]	–	–	–	+	+	++	++	+++	+++	+++
Color intensity on H-P-Q-F beads[b]	–	–	–	–	+	+	+++	+++	+++	+++
Color intensity on biotin beads[b]	+	+	+	+++	+++	+++	+++	+++	+++	+++

a. Semiquantitative whole cell binding assay: –, denotes average number of <5 cells bound per bead; +, 5–20 cells per beads; ++, 20–40 cells; +++, more than 40 cells.
b. Enzyme-linked colorimetric assay: –, denotes no color changed; +, slightly turquoise; ++, turquoise; +++, dark turquoise.

Common approaches to increase the screening stringency are (i) to lower the concentration of the target proteins used for screening, or (ii) to include a known competitive soluble ligand in the screening buffer. An alternative approach is to lower the concentration of ligands on the surface of the library beads. Under these conditions, the low affinity ligand-bead will remain negative and only those beads with high affinity will be positive. However, if the beads are down-substituted too much (e.g. <10% substitution), there will not be enough peptides on a single bead for reliable microsequencing. To overcome this problem, we have applied the bilayer bead approach to create OBOC combinatorial libraries with beads that are down-substituted on the outer layer, where ligand–receptor interaction occurs, but with full-substitution in the bead interior reserved for microsequencing.

We initially used proteases to generate topologically segregated bi-functional beads (12,13). However, this method is tedious and not very flexible. Subsequently, we developed a more simple and robust biphasic approach to derivatize bifunctional beads (6). In this approach, beads are thoroughly swollen in water and then reacted with a limiting amount of an amine-protecting reagent such as Fmoc-OSu to generate bilayer beads with protected outer layer and unprotected inner core. The bilayer beads allow the construction of the testing library compounds (e.g. small molecules, peptidomimetics, N-terminally blocked peptide) on the outer layer of beads and the coding tags or unprotected peptides in the bead interior.

To explore the applicability of the concepts of down-substitution and bilayer bead approaches for secondary libraries, we designed and synthesized a model secondary library based on p-Nle-D-I motif (Fig. 1). This motif is derived from the p-L-D-I motif that we have previously identified for the α4β1-integrin of Jurkat cell line (9). Our goal is to design a secondary library, with down-substituted outer layer (20%), so that higher affinity ligands to α4β1-integrin can be identified. Construction of this library began with the bilayer partition of TentaGel beads with Fmoc (0.2 Eq.) on the outer layer and butoxycarbonyl (Boc) (0.8 Eq.) in the interior using the biphasic approach (6). The outer layer of beads was further derivatized with a mixture of Fmoc-Gly (20%) and Ac-Gly (80%), so that only 20% of the amino-groups on the outer layer were free upon Fmoc-deprotection. At this point, the amount of free amino groups on the outer layer was approximately 4% (0.2 × 20%) of that of an underivatized bead. After Boc- and Fmoc-deprotection, standard 'split-mix' synthesis method was used to construct a tetrapeptide library segment (x_4-x_3-x_2-x_1-bead) on the down-substituted outer layer and the fully substituted bead interior using 19 D-amino acids as building blocks. The resulting beads were

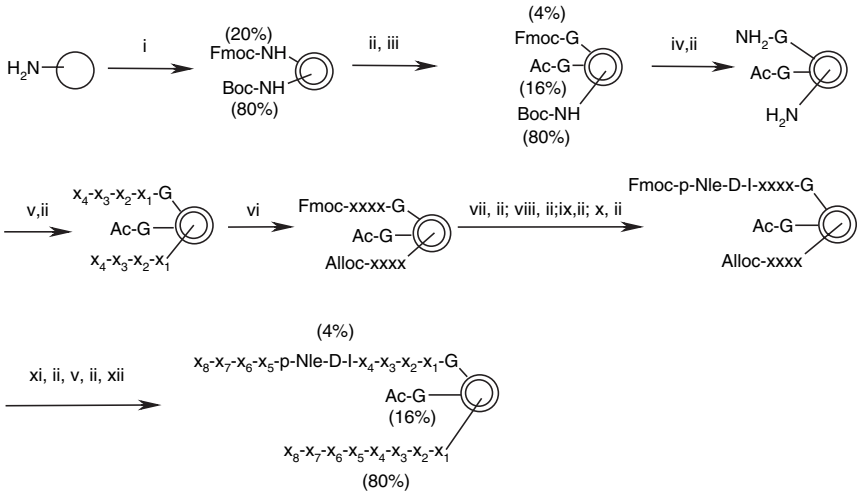

Figure 1. Synthetic route of surface down-substituted (p-Nle-D-I)-based secondary combinatorial peptide library. Reagents and conditions – (i) (a) water, 24 h; (b) *N*-(9-fluorenylmethyloxycarbonyloxy)-succinimide (Fmoc-OSu) (0.2 Eq.), *N,N*′-diisopropylethylamine (DIPEA), dichloromethane (DCM)/ether (55 : 45, v/v), 30 min; (c) Boc_2O (3 Eq.), DIPEA (6 Eq.); (ii) 25% piperidine in *N*-*N*′-dimethylformamide (DMF), 10 min; (iii) Fmoc-Gly-OH/Ac-Gly-OH (1 : 4), 1,3-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt); (iv) 55% trifluoroacetic acid (TFA), DCM, 30 min; (v) library assembly via 'split-mix' approach; (vi) (a) H_2O, 24 h; (b) Fmoc-Osu (0.04 Eq.), DIPEA, DCM/ether (55 : 45, v/v), 30 min; (c) *N*-allyloxycarbonyloxy succinimide (Alloc-OSu; 3 Eq.), DIPEA (6 Eq.); (vii) Fmoc-L-Ile-OH (3 Eq.), DIC (3 Eq.), HOBt (3 Eq.); (viii) Fmoc-L-Asp-OH (3 Eq.), DIC (3 Eq.), HOBt (3 Eq.); (ix) Fmoc-L-Nle-OH (3 Eq.), DIC (3 Eq.), HOBt (3 Eq.); (x) Fmoc-D-Pro-OH (3 Eq.), DIC (3 Eq.), HOBt (3 Eq.); (xi) tetrakis(triphenylphosphine) palladium (0) $[Pd(Ph_3P)_4]$ (0.24 Eq.), phenylsilane ($PhSiH_3$; 20 Eq.), DCM, argon (Ar), 1 h; (xii) TFA/phenol/water/thiaonisole/ethanedithiol (EDT) (10 : 0.5 : 0.5 : 0.5 : 0.25, v/w/v/v/v), 2.5 h.

then topologically segregated again with 0.04 Eq. of Fmoc-OSu to generate similar outer layer as the initial step. At this time, we purposely used Alloc-OSu to protect all the N-terminal amino groups in the bead interior, because Alloc-group can be removed later with palladium in the presence of Fmoc- and Boc-groups (14–16). Upon Fmoc-deprotection, p-Nle-D-I motif was assembled onto the random tetrapeptide segment on the outer layer of the bead with Fmoc-chemistry while the bead interior was Alloc-protected. After p-Nle-D-I motif was constructed on the outer layer, the Alloc-group in the bead interior was removed with palladium, and a second random peptide library segment (x_8-x_7-x_6-x_5-) was assembled to both layers simultaneously. As a result, a secondary library was generated, in which a motif-containing testing library compound (x_8-x_7-x_6-x_5-p-Nle-D-I-x_4-x_3-x_2-x_1) was displayed on the outer layer of beads at a low concentration (20% substitution) and a coding tag (x_8-x_7-x_6-x_5-x_4-x_3-x_2-x_1) that bypassed the p-Nle-D-I motif resided in the bead interior at full substitution.

It should be noted that the thickness of the outer layer generated by biphasic partition during the initial down-substitution step (step 1) and the subsequent library synthesis step (step 2) may not be identical (Fig. 2B,C). If the thickness of the outer layer generated in step 1 is larger than that generated in step 2, there will be a layer of peptide tag at the inner rim of the 20% substitution layer (Fig. 2B). Similarly, if the thickness of the outer layer generated in step 1 is smaller than that generated in step 2, there will be a layer of complete library compound at the outer rim of the 100% substitution core (Fig. 2C). However, this variability neither affects the compound displayed on the bead surface (screening) nor does it affect the majority of the coding peptide in the bead interior (sequencing).

Library screening

To validate the concept that screening stringency can be modulated by controlling the substitution of ligands on the bead surface, we screened two OBOC libraries against a T-cell leukemia (Jurkat) cell line using the whole cell-binding assay method (9,10). Although both of these two libraries have identical ligands (x_8-x_7-x_6-x_5-p-Nle-D-I-x_4-x_3-x_2-x_1) on the bead surface, one is fully substituted while the other is only 20% substituted on the outer layer. As expected, most of the beads in the 100% substituted library were coated by a monolayer of Jurkat cells (positive beads) because essentially every bead had a p-Nle-D-I motif (Fig. 3A). In contrast, there were significantly fewer positive beads in the down-substituted library (Fig. 3B), thus validating the low-substitution concept for high stringency screening. Of the 0.2 mL or 140 000 down-substituted library beads screened, 18 strongly positive beads were physically isolated and submitted for microsequencing.

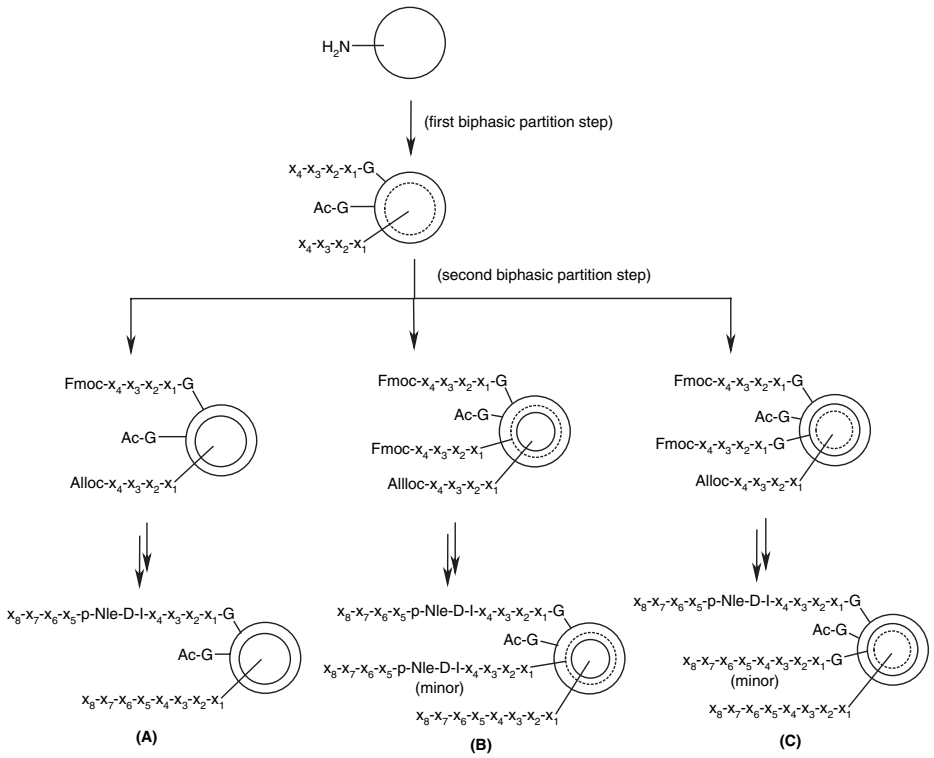

Figure 2. Variability of the chemical content at the bilayer interphase of the bead library after two steps of biphasic partition. Bead (A): ideal condition (the outer layer generated in the second bilayer-partition step exactly coincides with the outer layer generated by the preceding bilayer-partition step); beads (B and C) in practice, it is more likely that the two bilayer-partition steps do not exactly coincide with each other [bead (B) the second outer layer is bigger than the preceding one; bead (C) the second outer layer is smaller than the preceding one]. Nonetheless, such variation will neither affect the screening nor the sequencing.

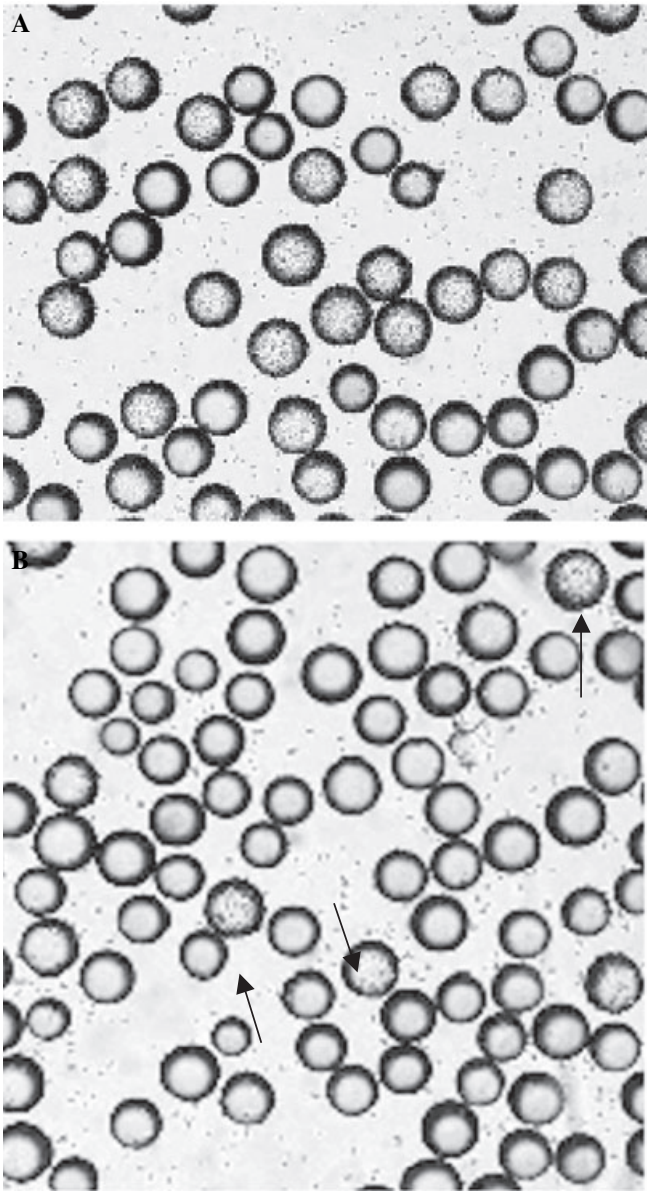

Figure 3. Photomicrographs of library screening demonstrating the concept of surface down-substitution leading to high screening stringency. Approximately same number of library beads (x_8-x_7-x_6-x_5-p-Nle-D-I-x_4-x_3-x_2-x_1-bead) were incubated with Jurkat T-leukemia cells at a ratio of 1 : 20 in the same volume of medium for 30 min, washed, and inspected under an inverted microscope for cell binding. (A) Fully substituted library with many strongly positive beads; (B) down-substituted library with only a few strongly positive beads.

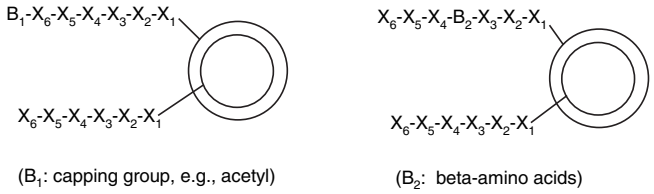

Figure 4. Diagram showing how one-bead one-compound (OBOC) combinatorial libraries containing N-terminally capped peptides (e.g. capped with B_1) or nonsequencable building blocks such as β-amino acids or β-turn mimetic building blocks (B_2) can be constructed and encoded with a sequencable peptide in the bead interior.

Bead sequencing

The bilayer bead library not only provides us with enough peptides in the inner core (100% substitution) for microsequencing, it also enables us to bypass any N-terminally capped residue or unsequencable residues such as β-amino acids or β-turn mimetics (Fig. 4). Furthermore, it allows us to bypass some of the fixed residues so that the microsequencing time can be shortened significantly as in the case of the x_8-x_7-x_6-x_5-p-Nle-D-I-x_4-x_3-x_2-x_1 library, in which only eight, instead of 12, amino acids were sequenced to obtained the final ligand structure. The decoding strategy is illustrated in Fig. 5. During the first four Edman cycles, the library residues (x_8, x_7, x_6, x_5) will be identified without any ambiguity because the compounds on the two layers have the same residue. In the subsequent Edman cycles, since the motif (p-Nle-D-I-) is also Edman-degradative, two PTH-amino acid peaks were generated, one from each layer. However, it is not difficult to determine the identity of these residues (x_4, x_3, x_2, and x_1) because PTH-amino acids generated from these residues have a much higher signal than those generated from the p-Nle-D-I motif.

This decoding strategy was validated by two random beads and 18 positive beads. Figure 6 illustrates the microsequencing profile of the first and the fifth cycle of a random bead. As expected, in the first cycle, x_8 was easily identified as G by the single peak. In the fifth cycle, x_4 was easily identified as lysine (K) by the higher peak. The smaller proline peak (P), derived from the outer layer, can be ignored. The additional small tyrosine peak (Y) in this cycle could also be ignored because it was due to incomplete Edman degradation of the preceding tyrosine residue at the x_5 position.

Using the above decoding strategy, the 18 ligand sequences were determined unambiguously (Table 2). Although there was no strong consensus among the high affinity ligands at the random positions x_8-x_7-x_6-x_5- or x_4-x_3-x_2-x_1, it is clear that there is a strong preference for one or more acidic residues at x_4-x_3-x_2-x_1, with 14 of 18 peptides having one or two D-Asp or D-Glu at the tetrapeptide segment at the carboxyl-end. When some of these ligands were resynthesized and printed on chemical microarrays (17,18), they bound Jurkat cells significantly stronger than the original p-L-D-I peptide.

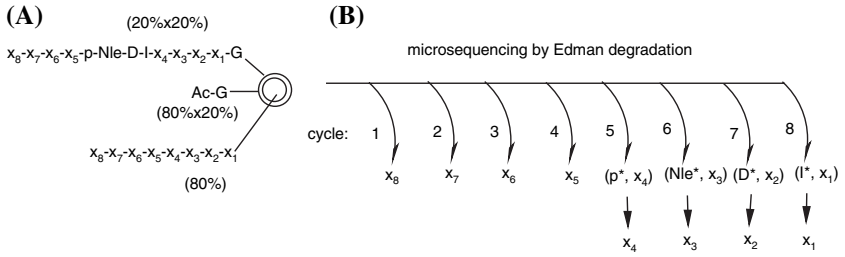

Figure 5. Decoding strategy of the surface down-substituted library by microsequencing. *Phenylthiohydantoin (PTH)-amino acid peaks of p, Nle, D and I are much smaller than those derived from x_4, x_3, x_2, and x_1, respectively.

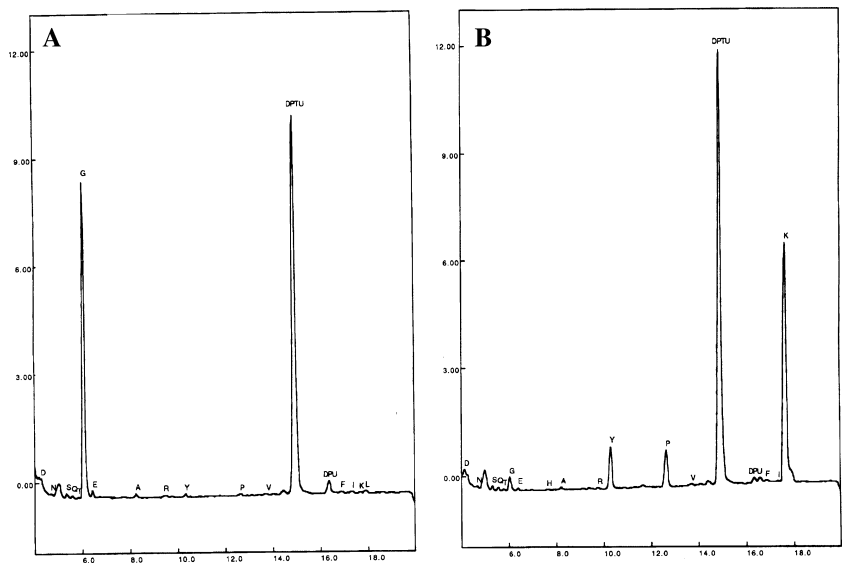

Figure 6. Microsequencing profile of one single peptide-bead (with surface down-substitution) in two Edman degradation cycles. (A) the first cycle; (B) the fifth cycle.

Table 2. **Amino acid sequence of strong binders for Jurkat cell line**[a]

Entry	Ligand sequence	Entry	Ligand sequence
1	fpqm-p-Nle-D-I-gggm	10	itga-p-Nle-D-I-kptp
2	gsly-p-Nle-D-I-kypg	11	idmv-p-Nle-D-I-lpdv
3	wvta-p-Nle-D-I-apwe	12	ssny-p-Nle-D-I-kese
4	ilti-p-Nle-D-I-deiv	13	yfhp-p-Nle-D-I-epeq
5	mqla-p-Nle-D-I-apdh	14	ihtp-p-Nle-D-I-aehe
6	lsgv-p-Nle-D-I-hpem	15	hhtw-p-Nle-D-I-qpie
7	hnkf-p-Nle-D-I-gype	16	htga-p-Nle-D-I-qedw
8	lehi-p-Nle-D-I-gydw	17	ehdi-p-Nle-D-I-kayr
9	pqqp-p-Nle-D-I-aewi	18	dhrt-p-Nle-D-I-faey

Nle, norleucine.
Fourteen of 18 peptides have at least a D-Asp or a D-Glu in the tetrapeptide segment at the carboxyl-side of the peptide.
a. In the sequences, lower case letters denote D-amino acids, capital letters denote L-amino acids.

Conclusion

The biphasic approach for the preparation of bilayer TentaGel beads before and/or during library synthesis enables us to greatly increase the versatility of the OBOC combinatorial library methods. We exploit the fact that on-bead screening often occurs on the bead surface and the bulk of the compounds inside the beads are for structure analysis. The topologically segregated beads generated by this approach allow us to develop encoded libraries with the testing compounds on the bead surface and the coding tags in the bead interior. These concepts, when applied to peptide libraries results in our ability to microsequence N-terminally blocked peptide libraries or peptide libraries with some nonsequencable building blocks along the peptide chain. We can also easily bypass the fixed sequence motif of the library compound so that only the variable residues are included in the coding peptide in the bead interior. This greatly increases the speed of microsequencing, often a rate-limiting step in the OBOC combinatorial peptide library method. Although not demonstrated in this report, one can easily envision that decoding of complex peptide libraries containing many nonsequencable moieties can be decoded by a combination of standard peptide-bead microsequencing and releasable coding tags that can be readily identified by mass spectroscopy (7,8). One limitation of the OBOC combinatorial library method is

that because the concentration of ligands on the bead surface is rather high, often it is difficult to differentiate between ligands with high affinity and those with moderate affinity. As clearly illustrated in this report, the stringency of whole cell-binding assay can be increased by decreasing the ligand concentration on the bead surface while keeping those in the bead interior at full substitution. As a result, ligands with higher affinity can be identified.

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