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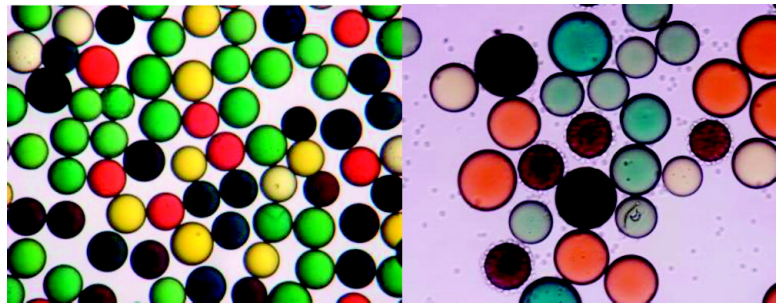

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Rainbow Beads: A Color Coding Method to Facilitate High-Throughput Screening and Optimization of One-Bead One-Compound Combinatorial Libraries

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We have developed a new color-encoding method that facilitates high-throughput screening of one-bead one-compound (OBOC) combinatorial libraries. Polymer beads displaying chemical compounds or families of compounds are stained with oil-based organic dyes that are used as coding tags. The color dyes do not affect cell binding to the compounds displayed on the surface of the beads. We have applied such rainbow beads in a multiplex manner to discover and profile ligands against cell surface receptors. In the first application, a series of OBOC libraries with different scaffolds or motifs are each color-coded; small samples of each library are then combined and screened concurrently against live cells for cell attachment. Preferred libraries can be rapidly identified and selected for subsequent large-scale screenings for cell surface binding ligands. In a second application, beads with a series of peptide analogues (e.g., alanine scan) are color-coded, combined, and tested for binding against a specific cell line in a single-tissue culture well; the critical residues required for binding can be easily determined. In a third application, ligands reacting against a series of integrins are color-coded and used as a readily applied research tool to determine the integrin profile of any cell type. One major advantage of this straightforward and yet powerful method is that only an ordinary inverted microscope is needed for the analysis, instead of sophisticated (and expensive) fluorescent microscopes or flow cytometers.

Introduction

The one-bead one-compound (OBOC) combinatorial strategy[1,2] has been proven to be a powerful tool for identification of synthetic ligands against specific biological targets.[3] With each bead displaying a single chemical entity, a large number of compounds can be rapidly screened with on-bead binding[3] or functional assays.[4,5] We have recently reviewed the use of OBOC combinatorial library methods to discover cell surface binding ligands that are suitable for targeted imaging and therapy.[1,2,6] In such experiments, OBOC bead libraries are incubated together with live cancer cells at 37 °C in culture medium. Beads coated by a monolayer of cells are isolated for structure determination. For peptide libraries with a free N-terminus, positive beads can be directly microsequenced with Edman chemistry. For N-terminally blocked peptides, peptides with β-amino acids, peptidomimetics, small molecules, or macrocyclic molecules, encoding strategies are needed. To achieve this, we have developed encoding systems using topographically segregated bilayer beads so that library compounds can be assembled on the outer layer of beads while coding tags are constructed in the bead interior.[7–10]

To screen for cell surface binding ligands, we often screen a single cell line against each OBOC library separately. Positive ligand beads are then sequenced, resynthesized, and evaluated for binding specificity to various cell lines. Once specific binding has been confirmed, analogues of the parent compound (e.g., alanine scan) will be synthesized and tested individually for cell binding. Although the OBOC combinatorial library method is highly efficient, finding the right library to screen and performing large number of cell binding assays on individual resynthesized ligand beads against a number of cell lines can be very time-consuming. The development of a method that enables us to perform cell binding assays in a multiplex fashion would greatly facilitate the discovery and optimization of cancer targeting ligands. Here we report a novel color-encoding method that allows us to tag individual beads or a library of compound beads. As a result, multiple different ligands or OBOC libraries can be incubated with cells in a single tissue culture well for cell adhesion studies and thus greatly enhance the efficiency of OBOC combinatorial library screening.

Results and Discussion

Cell Binding Properties of Color-Stained Beads. To apply rainbow beads in multiplex cell binding assays, the dye must not affect cell adhesion to the bead. To test this, we selected LLP2A, a high-affinity and highly specific ligand against malignant lymphoma,[3] as our model ligand. Portions of TentaGel beads displaying LLP2A were stained red, blue,

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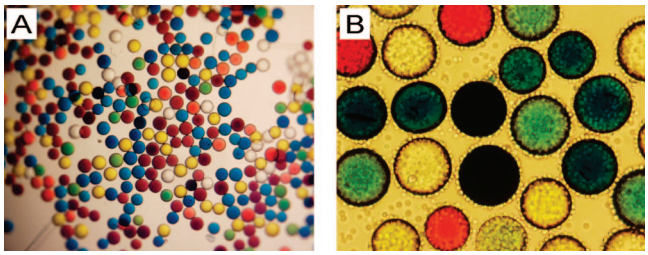

Figure 1. Photomicrograph of (A) mixture of colorless and the color stained LLP2A beads (90 μm) with different organic dyes and (B) the same mixture of color and colorless LLP2A beads incubated with Jurkat cells for 5 min. There was no difference in cell binding to colored or colorless LLP2A beads, indicating that the entrapped dyes inside the beads do not affect cell binding to the bead surface.

yellow, black, and green, respectively. The five-color coded LLP2A beads were mixed together with the blank unstained (colorless) LLP2A beads and incubated with Jurkat cells in culture medium (without phenol red) at 37 °C for 5 min and then inspected under an inverted microscope. Cell binding to these six color beads (including the colorless one) were essentially identical (Figure 1), indicating that ligands on the bead surface were available for cell binding without interference by the precipitated dyes in the bead interior.

Color-Coded OBOC Combinatorial Libraries. A preferred library for a specific cell line can be rapidly identified and selected using the rainbow-bead method. RGD (arginine-glycine-aspartic acid) based linear and cyclic peptides are well-known ligands against multiple different integrins present of the surface of many cell types. These include $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ integrins.[11] Many snake venoms contain constrained peptides or proteins with RGD motifs that target these receptors.[12,13] Conceivably, the binding affinities and specificity of these RGD containing cyclic peptides to different cell lines depend greatly on the neighboring amino acids as well as the ring size of these peptides. In our laboratory, we have already prepared six focused OBOC cyclic peptide libraries with RGD motif and various ring sizes (5-mer to 9-mer) to be screened by a series of cell lines: such as K562 (chronic myelocytic leukemia), MDA-MB-231 (breast cancer), and HUVEC (human umbilical vein endothelial cell). To separately screen six peptides libraries with three different cell lines (18 different screening experiments) is not only time-consuming but also makes it difficult for the investigator to compare directly one screening experiment with another. To overcome these problems, we converted these six colorless OBOC libraries to color-coded libraries by staining each library with a different color dye (Figure 2). The six color-coded bead libraries were then mixed together and screened against $\alpha_v\beta_3$-K562 cells (Figure 2) in a single dish. The majority of the strongly positive beads (completely coated by a monolayer of cells) were found to be red (8-mer library). Some less positive beads were yellow (9-mer library) and green (6-mer-A library). No positive beads were found to originate from beads with the remaining colors even after incubation at 37 °C for over 12 h. This indicates that $\alpha_v\beta_3$-K562 cells prefer the 8-mer disulfide bond containing cyclized RGD library more than the others. The positive beads covered with cells were retrieved and grouped together according to their color. Dye inside the beads was then removed with DMF prior to peptide microsequencing. This experiment allows us to interrogate multiple different chemical libraries in "one pot" with the same cells under identical condition and to quickly determine that the 8-mer cyclic library is the preferred library for the discovery of ligands against $\alpha_v\beta_3$ integrin. On the basis of this preliminary large-scale structure−activity relationship study (SAR study), we focused our effort on screening the 8-mer library and identified many ligands against $\alpha_v\beta_3$ integrin (manuscript in preparation).

Identification of Lead Compounds and SAR Studies. In OBOC combinatorial library screening, once the chemical structures of the positive beads are determined, the compounds are resynthesized on TentaGel resin for cell adhesion assays. This enables us to profile the binding specificities of these ligands against a large number of different cell lines. To facilitate this tedious process, we can apply the rainbow-bead concept to multiplex analysis. For example, beads displaying peptide sequences identified through screening an OBOC library with a cancer cell line are each colored with a different organic dye. The color-coded beads are then mixed together for cell adhesion studies, and peptide-beads that bind faster and stronger to the target cells can be easily visualized and identified under a light microscope. In principle, the whole process can be automatic and quantified with advanced image analysis software, which will greatly facilitate the determination of the binding characteristics of these ligands.

To optimize the initial lead compounds, we often perform simple structure−activity-relationship (SAR) experiments, such as alanine scan and truncation studies, so that the contribution of each residue to cell binding can be defined. In the alanine scan, each residue in the lead peptide is replaced by an alanine, one at a time. In truncation studies, amino acids from the N- and C-terminus are truncated one at a time. Binding studies with these analogues enable one to define the binding motif; based on that result, a focused library can be designed for further optimization of the lead compounds. The rainbow-bead method, again, can facilitate such analysis. For example, we have identified a bladder cancer binding heptapeptide (PLZ4) containing a DGR motif via screening an OBOC peptide library against bladder cancer cell lines (unpublished data). This peptide PLZ4 was found to bind to the 5637 bladder cancer cell line but not to normal bladder epithelial cells. To perform alanine scan, we synthesized seven peptide analogues on TentaGel beads by replacing residues X_1 to X_7 with alanine one at a time, and each was colored with a different dye (Figure 3A). The mixture of color beads and the parent peptide beads (PLZ4, white, uncolored) were then tested against the 5637 bladder cell line. As shown in Figure 3B, after incubation at 37 °C for 2 h, the cell binding to the blue (no. 1) and red (no. 5) beads were observed to be as strong as that to the white beads displaying the parent sequence, indicating that the amino acids on the X_1 and X_5 positions are not important for cell binding. In contrast, cell binding to both the black (no. 2) and green (no. 4) beads were completely negative (total lost of activities) demonstrating that aspartic acid at position 2 and arginine at position 4, constituting the DGR

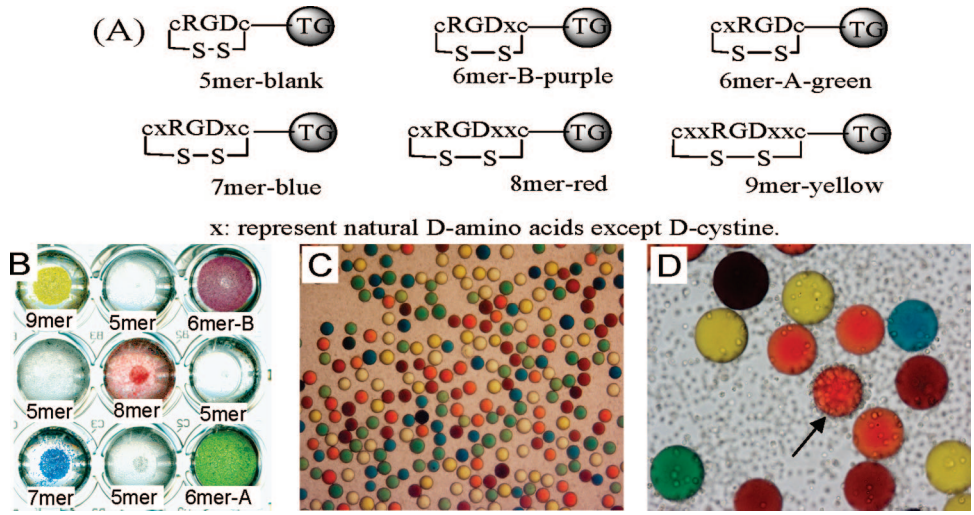

Figure 2. Photomicrographs of color-coded RGD related cyclic peptide libraries: (A) chemical structure of the six OBOC libraries, (B) mixture of bead samples from each of the color-coded cyclic peptide libraries, (C) low magnification, and (D) high magnification of color-coded cyclic peptide bead libraries screened with $\alpha_v\beta_3$-K562 cells; the red color-coded bead library (8-mer) was found to be the preferred OBOC library for cell binding.

A

NO.	sequences		binding
PLZ4	$X_1DGRX_5X_6X_7$	○	++++
#1	$ADGRX_5X_6X_7$	●	++++
#2	$X_1AGRX_5X_6X_7$	●	-
#3	$X_1DARX_5X_6X_7$	●	+
#4	$X_1DGAX_5X_6X_7$	●	-
#5	$X_1DGRAX_6X_7$	●	++++
#6	$X_1DGRX_5AX_7$	●	++
#7	$X_1DGRX_5X_6A$	●	-

Note: semi-quantitative relative binding activity: ++++ means very strong binding with 100% bead surface covered by cell; +++ means strong binding with 75% bead surface covered by cell; ++ means moderate binding with 50% bead surface covered by cell; + means weak binding with 25% bead surface covered by cell; - means no binding.

B

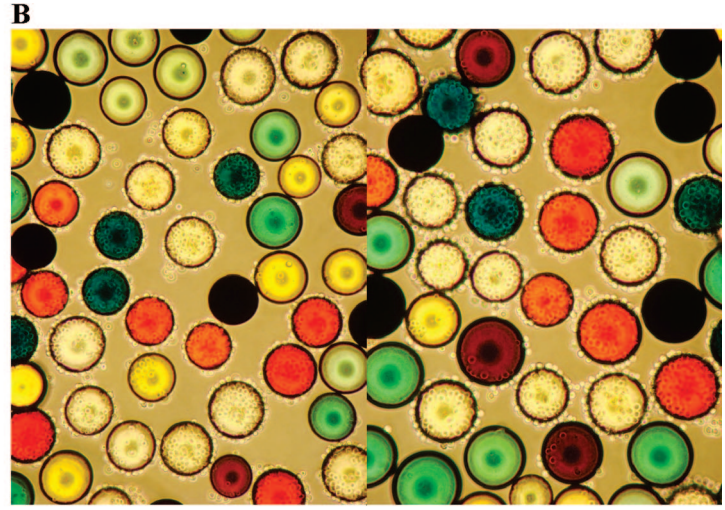

Figure 3. Multiplex alanine scan of peptide PLZ4 that was identified through screening a peptide library with 5637 bladder cancer cells. Each amino acid of peptide PLZ4 was replaced with alanine, respectively (A), and each peptide analogue was color-coded. The photomicrographs of the binding of 5637 bladder cancer cells with rainbow beads displaying PLZ4 analogues (B) showed similar strong bindings were observed on the white, blue, and red beads; weaker binding on yellow and brown beads; totally negative binding on black, green, and yellowish green beads.

motif, are critical in cell binding. Similarly, the yellowish green beads (no. 7) also lost their binding to the cells, indicating that X_7 also plays a very important role in the binding of 5637 cells to the PLZ4 ligand. The brown (no. 6) and yellow beads (no. 3) showed moderate cell binding activity, compared with the parent peptide, suggesting that position 3 and 6 of the parent peptide do play a role in cell binding. This rapid SAR study enables us to design focus libraries with identical or related amino acids fixed at those three crucial positions, while randomizing the building blocks in the remaining positions. Screening such highly focused libraries under higher stringency[6] will likely lead to the development of ligands with much higher affinity.

Cell Surface Receptor Profiling with Rainbow Beads. Through screening OBOC libraries, we have identified several different ligands with specific binding to different integrins on various cell lines. LLP2A is a peptidomimetic that binds activated $\alpha_4\beta_1$ integrin of malignant lymphoma cells.[3] Through screening disulfide bond-cyclized RGD libraries (Figure 2), we have identified D-amino acid-containing cyclic octapeptides (RGD1 and RGD2) that bind specifically to K562 myelocytic leukemia (with $\alpha_5\beta_1$) cell line and $\alpha_v\beta_3$-transfected K562 cell line. In addition, we have screened a focused cyclic octapeptide library (cXGXGXXc)[6] with U87MG human glioblastoma cell line, and identified LXY1, a ligand that has been shown to bind to $\alpha_3\beta_1$ integrin and to function as a highly specific in vivo imaging probe for glioblastoma xenograft.[14] We have also identified another peptide DGR1 which binds to a ovarian cancer cell line and the peptide LYK1 that bind to XPA-1 pancreatic cancer cell line. To evaluate the binding specificities of these six ligands to cell surface receptors, we prepared color-coded beads and tested them against six different cell lines: parent K562 cell ($\alpha_5\beta_1$ integrin), $\alpha_v\beta_3$-transfected K562 cell, α_3-transfected

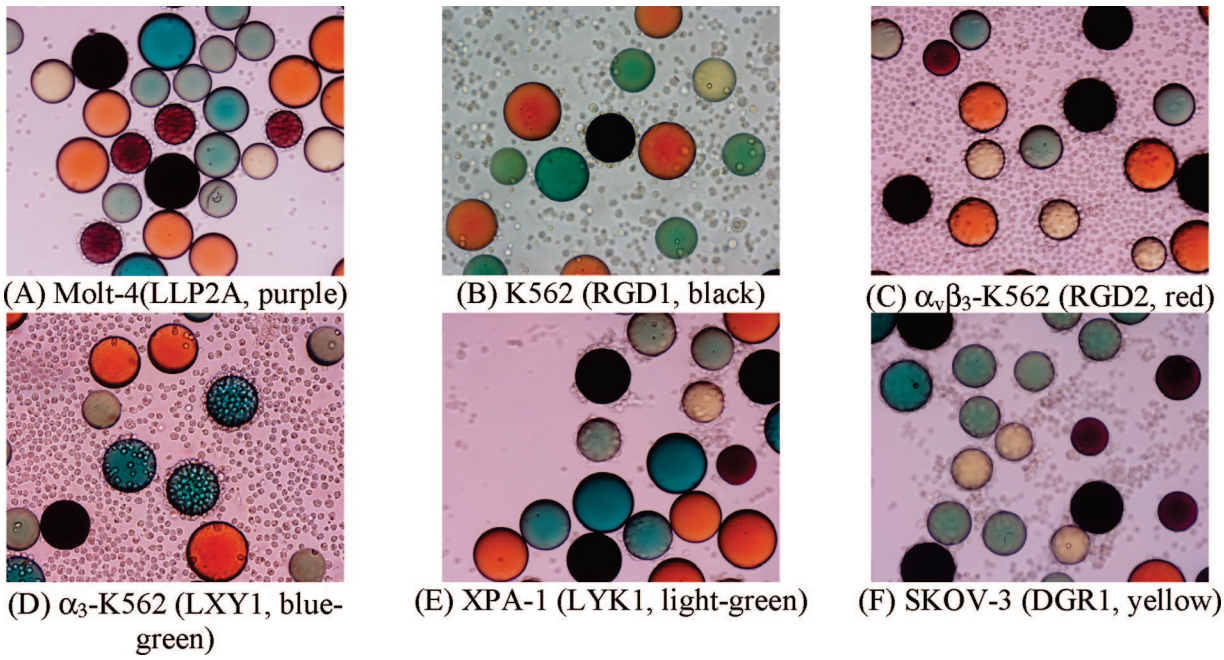

Figure 4. Photomicrographs showing multiplex cell adhesion assays on rainbow beads. In each photomicrograph, a rainbow bead mixture containing small samples of six different color-coded peptide-beads was incubated with one cell line. Six different experiments with six different cell lines (A−F) are shown. The color-coded beads displaying the peptides that were originally discovered through screening the corresponding cell lines (A−F) are indicated within the parenthesis.

Table 1. Specificity of the Cell Binding between Six Bead Ligands and Six Different Cancer Cell Lines[a]

bead color	ligand name	Molt-4($\alpha_4\beta_1$)	K562 ($\alpha_5\beta_1$)	$\alpha_v\beta_3$-K562 ($\alpha_v\beta_3/\alpha_5\beta_1$)	α_3-K562 ($\alpha_3\beta_1/\alpha_5\beta_1$)	XPA-1	SKOV-3 ($\alpha_3\beta_1$, $\alpha_v\beta_3/\alpha_v\beta_5$)
purple	LLP2A	++++	−	−	−	−	−
black	RGD1	−	++++	++++	+	+++	+++
red	RGD2	−	−	+++	−	−	+
blue-green	LXY1	−	−	+	++++	+	++++
light-green	LYK1	−	−	+	−	++++	++
yellow	DGR1	−	++	++++	+	+++	+++

[a] Semi-quantitative relative binding activity: ++++ means very strong binding with 100% bead surface covered by cell; +++ means strong binding with 75% bead surface covered by cell; ++ means moderate binding with 50% bead surface covered by cell; + means weak binding with 25% bead surface covered by cell; − means no binding.

K562 cell, Molt-4 T-leukemia cells ($\alpha_4\beta_1$ integrin), SKOV-3 ovarian cancer, and XPA-1 pancreatic cancer cell lines. Figure 4 shows the binding of these cells to the rainbow beads and Table 1 summarizes the semiquantitative result of such binding studies. As expected, LLP2A (purple bead) was found to only bind to Molt-4 cells that express activated $\alpha_4\beta_1$ integrin.[3] RGD1 peptide (black bead) was found to bind to several integrins ($\alpha_5\beta_1$, $\alpha_v\beta_3$, and possibly $\alpha_v\beta_5$) as indicated by its binding to a number of different cell lines. RGD2 peptide (red bead) on the other hand, was determined to be highly specific against $\alpha_v\beta_3$ integrin as shown by strong binding to $\alpha_v\beta_3$-transfected K562 cells and not to other cell lines. LXY1[6] peptide (blue-green bead) was found to bind strongly to α_3-transfected K562 cell and SKOV-3 ovarian cancer cell (known to express $\alpha_3\beta_1$ integrin). The LYK1 peptide (light-green bead) discovered through screening XPA-1 pancreatic cancer cell line, binds strongly to XPA-1 cells. The cell binding profile of DGR1 peptide (yellow bead) was found to be very similar to that of the RGD1 peptide, both of which were rather nonspecific and bound to a number of different receptors among different cell lines. Looking at each column in Table 1, it becomes apparent that Molt-4 cells (T-cell leukemia) probably has only one major integrin, whereas the XPA-1 (pancreatic cancer) and SKVO-3 cell lines (ovarian cancer) each have multiple integrins. This result is consistent with the reports that (i) α_6 integrin is upregulated and multi-β integrins (such as $\beta4$ and $\beta1$) exist in the early stage of pancreatic adenocarcinoma,[15,16] and (ii) the SKOV-3 cell line expresses $\alpha_3\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins.[17–20]

With the development of increasingly specific ligands against various integrins and cell surface receptors, it is conceivable that a rainbow cell surface ligand kit can be developed to rapidly profile the surface antigens of various cell lines. In addition, such cell surface binding kits can possibly be developed into a simple tool to profile a patient's cancer cells (e.g., collected from blood, ascites, and pleural fluid) in the clinic. This can also serve as a diagnostic tool for selection of effective cell surface targeting ligands for individualized patient therapy.

Conclusions

Beads displaying peptides or chemical molecules can be easily colored with organic dyes to form rainbow beads. The stain trapped in the bead's interior does not affect cell binding to the bead surface. The color coding method has been proven to be a powerful tool to facilitate the screening of OBOC libraries for cell-binding ligands. Color-coded OBOC libraries can also be used to rapidly profile the SAR of a

large number of chemical libraries against a specific cell type and possibly multiple cell types if the cells are fluorescent tagged as well. The comparison of the cell binding activities of different ligands, as well as the peptide analogues in SAR study (e.g., alanine scan) can be done in a "one-pot" assay with the rainbow beads and a simple inverted light microscope. Color-coded ligand beads against a series of different integrins can be used as a simple tool to rapidly determine the integrin profile of cells. A series of cell surface targeting ligands against cancer can be color-coded and used as a simple tool to profile patients' cancer cells (e.g., collected from blood, ascites, and pleural fluid) in the clinic. This can serve as diagnostic tool for selection of effective targeting ligands for individualized therapy.

Experimental Section

TentaGel resin (particle size = 90 μm) was purchased from Rapp Polymere Inc., Germany; Fmoc-protected amino acids for Fmoc peptides synthesis were purchased from Anaspec Inc., California. Coupling reagents, such as *N*,*N*-diisopropylcarboimide (DIC) and *N*-hydroxybenzotriazole (HOBt), were ordered from Sigma-Aldrich. Organic dyes, such as Sudan black B, Sudan blue II, scarlet red, disperse yellow, and purpurin, were ordered from Sigma-Aldrich. All chemicals were used as received; 5673 cells (a bladder cancer cell line), K562 cells (a chronic myelocytic leukemia cell line), SKOV-3 cells (a human ovarian adenocarcinoma cell line), Molt-4 cells (an acute lymphoblastic T-leukemia cell line), and Jurkat cells (an acute T cell leukemia line) were purchased from ATCC. XPA-1 cells (low-passage human pancreatic cancer cells) were established at the Johns Hopkins University, Baltimore, MD, and were kindly provided by Dr. Anirban Maitra; $\alpha_v\beta_3$-transfected K562 and α_3-transfected K562 cell lines were kindly provided by Dr. Yoshikazu Takada of University of California Davis School of Medicine.

Solid-Phase Peptide and Library Synthesis. Individual peptide or peptide libraries were synthesized on TentaGel resin using Fmoc chemistry. DIC/HOBt were used as coupling reagents and K reagents (TFA/phenol/H_2O/thioanisole/tripropylsilane = 10:0.75:0.5:0.5:0.25) as cleavage and side-chain deprotection reagents.[21] Peptide cyclization via disulfide bonds was achieved by mixing peptide beads overnight in a 0.2 M acetate buffer (pH 6) containing 20% of DMSO (v/v). The Ellman test[21] was used to monitor the completion of cyclization. Resins were washed thoroughly and sequentially with DMF, MeOH, water, and then 75% ethanol. The washing cycles were then repeated twice, and the final bead-libraries were stored in 75% ethanol at 4 °C.

General Procedure of Bead Staining. After the solid-phase synthesis of ligands or peptide libraries on TentaGel resin, the ligand beads were stained into different colors. Saturated solutions of organic dyes, such as Sudan black B, Sudan blue II, scarlet red, disperse yellow, and purpurin were prepared in DMF and centrifuged to collect clear supernatant. The green staining solution was prepared by mixing the saturated Sudan blue II and disperse yellow solution (1:5 v/v) in DMF. Peptide beads were mixed with the organic dye solution in DMF for 1 h. The beads were then transferred to disposable polypropylene column with polyethylene frits and washed twice with hexane, followed by three washes with water to remove DMF and to precipitate organic dye molecules inside the beads. The water-soaked beads were washed twice with hexane and lyophilized for long-term storage. Prior to cell binding studies, the dry beads were washed 3× (30 s each time) with hexane to remove the residual dye on the surface of beads, followed by swelling in water for 2 h. The beads were then ready for cell adhesion assays. Unused beads were stored in 0.1% sodium azide aqueous solution.

Cell Binding Assay. Ligand beads were washed sequentially three times with double-distilled water, three times with phosphate-buffer saline (PBS), and three times with cell culture media. The thoroughly washed beads were then incubated with cancer cells (3×10^5 mL^{-1}) in a 35 mm Petri dish or multiwell plate at 37 °C with gentle shaking (30 rpm) in humidified incubator with 5% CO_2. After incubation, beads were inspected under an ordinary inverted microscope. If needed, beads coated by a monolayer of cells were retrieved with hand-held micropipette, and the colored beads destained by DMF prior to structure determination.

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